PSEUDOMONAS, ALGINATE BIOSYNTHESIS AND CYSTIC FIBROSIS

by

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To Mum, Dad, Nicola, Leonie
and last, but not least, Fiona

Pathogenicity is but an accident
in the life of a parasite

Theobold Smith 1911

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ABSTRACT

This thesis documents the author's research on the pathogenesis and epidemiology of the pseudomonads in particular the association of *Pseudomonas aeruginosa* and *Pseudomonas cepacia* with pulmonary colonisation and progressive lung damage in patients with cystic fibrosis.

One of the key virulence determinants in pulmonary colonisation of cystic fibrosis patients by *P. aeruginosa* is biosynthesis of alginate. This unusual bacterial polysaccharide confers a strikingly mucoid colonial phenotype and *in vivo* is associated with the formation of microbial biofilms. During the last two decades, my research has focused on the genetic and molecular regulation of alginate biosynthesis. The origins of these studies derive from my PhD research on pyocins which led to the development of an internationally recognised typing system for *P. aeruginosa*. In the early 1970s, pyocin typing data suggested that mucoid *P. aeruginosa* arise *in vivo*, by mutation or environmental stimulation following primary asymptomatic colonisation with a nonmucoid parent strain. My genetic studies of alginate regulation were developed in the mid 1970s during an MRC Travelling Fellowship at Monash University, Melbourne and produced the first evidence for chromosomal genes controlling the mucoid phenotype. These embryonic studies and the *muc* mutations which were identified were to play an important part in the subsequent molecular unravelling of the sensory regulation of alginate biosynthesis in collaboration with colleagues in San Antonio. From 1980, my research on the microbiology of pulmonary infections in cystic fibrosis patients has included studies of antibiotic therapy, the epidemiology of pseudomonads and the use of animal models of chronic respiratory infection. Currently, in collaboration with colleagues at the MRC Human Genetics Unit in Edinburgh, I am involved in microbiological studies of the newly developed cystic fibrosis mouse.

The most common microbial pathogens in cystic fibrosis patients are *Staphylococcus aureus, Haemophilus influenzae* and *P. aeruginosa*. From the mid 1980s, however, transmissible and potentially fatal pulmonary infection by the phytopathogen *P. cepacia* has caused increasing concern to patients and those involved in patient care. A major aim of my current research is to clarify the epidemiology of *P. cepacia* and to identify the host and bacterial factors associated with transmission and pathogenesis.

DECLARATION

I declare that this thesis is my own composition. With the exception of paper 1 which describes work carried out as part of PhD studies at Edinburgh, no part of this thesis has previously been submitted towards a higher degree. In papers 13, 14, 18, 28, 29, 35, 37, 39, 41, 42, 43, 47, 49, 54 and 56 I was a collaborator and not the senior author. In all the remaining papers, I was the senior author and main progenitor of the work described.

ACKNOWLEDGEMENTS

It is a great pleasure to acknowledge the help and interest of friends, colleagues who have become friends, and many individuals who have inspired me to probe ever deeper into the role of pseudomonads as opportunistic pathogens. As a keen but naive young PhD student fascinated by the discoveries of Semmelweis and Pasteur, I was promoted from the highly infectious depths of *Shigella* dysentery to the Jekyll and Hyde world of pseudomonads. A gift of 400 strains of *Pseudomonas aeruginosa* supplied by my supervisor Professor RR Gillies sent me off on a fascinating voyage of discovery with visits to foreign shores and the importation of ideas and techniques from many fields of microbiology. I owe an enormous debt to my laboratory colleagues over the years in particular to Cathy Doherty, Janet Fyfe, Gillian Harris, Rosamund Williams, James Nelson, Sarah Butler, numerous Honours students and to the microbiologists, clinicians and patients who have ensured that the original collection of pseudomonads has multiplied many times over.

INTRODUCTION

This thesis concerns the role of *Pseudomonas aeruginosa* as an opportunistic pathogen, in particular, the manner in which the microbe undergoes unusual adaptation enabling it to colonise and cause pulmonary damage in patients with cystic fibrosis (CF). One of the key bacterial virulence determinants in this pathogenic process is biosynthesis of alginate an unusual bacterial exopolysaccharide which is responsible for a strikingly mucoid colonial phenotype. The association of mucoid alginate-producing variants of *P. aeruginosa* (MPA) and colonisation of the lungs of patients with CF has been described as "one of the most bizarre and intractable bacterial infections known to modern science" (Deretic *et al.*, 1987a). During the last two decades, the author has directed and participated in research focused on attempts to unravel this biological jigsaw puzzle. In pursuit of these aims, basic research has been integrated with practical improvements in the diagnosis and treatment of pulmonary infection in CF patients. Because of the complex regulation of alginate biosynthesis, and the multifactorial nature of the pathophysiology of pulmonary infection in CF patients, various aspects of antimicrobial therapy, epidemiology, biochemistry, and molecular biology have featured in these studies.

The origins of the research recorded here from 1968 derive from the author's research towards the degree of PhD under the supervision of the late Professor RR Gillies, Department of Medical Microbiology, University of Edinburgh. These early studies of the antibiotic-like bacteriocins of *P. aeruginosa* led to the development of an internationally recognised typing system, termed pyocin typing, for epidemiological investigations of pseudomonas infections. Early pyocin typing studies suggested that the MPA associated with chronic, debilitating pulmonary infections in CF patients, arise *in vivo* by mutation or induction following asymptomatic colonisation with a typical nonmucoid *P. aeruginosa* (NMPA). Subsequently, my research became increasingly focused on the genetic basis for alginate regulation and biosynthesis. In 1974, I was awarded a MRC Travelling Fellowship to develop this research at the University of Monash, Melbourne with Professor Bruce Holloway, the leading authority on pseudomonas genetics. At the time, pseudomonas genetics was in an embryonic stage; most of the gene mapping data was concerned with auxotrophic markers and very little was known of the genetic basis of pathogenic determinants. This visit was to prove very rewarding. As knowledge of pseudomonas genetics accelerated in the

1980's, the mutant strains isolated at Monash proved to be valuable tools in the gradual unravelling of the molecular basis of alginate biosynthesis and regulation.

On the author's return to Edinburgh in 1975, research on the genetics of alginate biosynthesis was continued and integrated with attempts to clarify the in-vivo role of alginate and other putative virulence factors in the pathophysiology of CF pulmonary infections. As a consequence of these initiatives, the team which developed, initially with Cathy Doherty and Janet Fyfe, became increasingly involved in the diagnosis, epidemiology and treatment of pulmonary infections in CF patients caused by *P. aeruginosa* and other pulmonary pathogens.

In 1975, a significant problem facing research on the pathophysiology of pseudomonas pulmonary infection in CF was the absence of an animal model of chronic infection; existing models including a burned mouse and guinea pig pneumonia model involved acute infections. In 1979, in an attempt to overcome this problem, a sabbatical was spent in the laboratory of Professor JW Costerton, Department of Biology, University of Calgary, Alberta. During this time, under the tuition of Kan Lam, experience was gained in the use of a newly developed rat lung model of chronic lung infection based on transtracheal instillation of *P. aeruginosa* embedded in agar beads into the distal bronchi of anaesthetised animals. Exchange visits were subsequently arranged with Kan Lam and Dr Neil Baker, The Ohio State University, to continue the rat lung studies in Edinburgh and to develop tracheal culture systems to study the adhesive mechanisms and antibiotic susceptibility of bacteria on ciliated respiratory surfaces. Complementary studies on the microscopy of CF sputa demonstrated that MPA growing *in vivo* formed microcolonies and biofilms adhering to the mucosal surface. In this way, pulmonary clearance is significantly reduced and the adherent bacteria protected from both phagocytosis and the action of antibiotics. Further collaborative research was carried out with Professor Mike Brown at the University of Aston on the isolation of alginate-producing mutants from wild-type NMPA to investigate the effects of growth limiting factors on alginate biosynthesis in cultures grown under strictly controlled conditions within chemostats. In addition, detailed chemical analysis of pseudomonas alginate became available through the use of NMR spectroscopy in association with Drs Nick Russell and Peter Gacesa at the University of Cardiff.

Concurrent with these basic studies on the pathophysiology and biochemistry of *P. aeruginosa*, the author's increasing involvement in clinical aspects of pulmonary infection led to

collaboration with clinical colleagues in Edinburgh and attempts to improve the management and assessment of therapy in CF patients. Accumulated evidence, suggested that much of the pulmonary damage observed in CF patients was due to an over stimulation of immune responses. The concept of a "frustrated phagocyte" was introduced and developed by the author (16) to describe the struggle between pulmonary phagocytes and biofilm-protected bacteria. Accordingly, in a somewhat paradoxical stratagem to combat bacterial infection, the innovative use of anti-inflammatory agents in the treatment of bacterial exacerbations in CF patients by Aurbach *et al.*, (1985) led to further clinical trials of steroid therapy in Edinburgh under the direction of Drs Sandy Raeburn and Andy Greening in which the group continues to participate. Whilst the results of these trials remain to be summarised, another aspect of the CF patients' inflammatory response was to prove useful in the diagnosis of pulmonary exacerbations and the assessment of efficacy of antibiotic therapy. In collaboration with Dr Sharon Glass, it was shown that measurement of the acute-phase reactant, C-reactive protein, in sera was a useful indicator of pulmonary exacerbations and in forecasting the outcome of antimicrobial therapy against infections in which bacteria are seldom eradicated.

By 1986, these multidisciplinary approaches to the problems posed by *P. aeruginosa* as an opportunistic pathogen had made significant progress and attracted interest from colleagues in a range of medical and scientific fields. In the 1980s, the author received over 70 invitations to present the group's research at scientific symposia. In 1987, this research was recognised by a silver award in the British Medical Association Video and Film Competition for a film made in association with Janet Fyfe entitled "Pseudomonas, a pathogen of medical progress". This international competition is a premier event for health and medical education films and in 1987 attracted 160 entries.

In the 1980s, *P. aeruginosa* colonisation of CF patients approached 100% in some centres and was accepted to be a major factor in morbidity and mortality. However, in North America reports appeared of a rising incidence in colonisation with another opportunistic pseudomonad, the phytopathogen *P. cepacia*. In anticipation of future problems in the UK, and in view of the lack of suitable typing methods for epidemiological studies, a visit was arranged through the British Council to Dr H Monteil, University of Strasbourg, to study the biology and epidemiology of this relatively unknown pathogen. Subsequently in Edinburgh, a typing technique for *P. cepacia*, based on bacteriocin production and susceptibility, was developed. In 1990, the value of this technique was confirmed in a large multicentre study

of *P. cepacia* typing methods organised by the Communicable Disease Center, Atlanta (35). In 1991, increasing concern with *P. cepacia* colonisation was recognised by an invitation from the CF Trust to join a *P. cepacia* working party and an invitation to provide a Lancet editorial on the subject. At the present time, under the auspices of the CF Trust, UK isolates of *P. cepacia* are referred to our laboratory for characterisation and the author's group is involved in a multicentred study on immunological responses to *P. cepacia* colonisation.

Meanwhile, epidemiological studies of *P. aeruginosa* pulmonary infections with Dr Mike Tredgett suggested that a particular subpopulation of *P. aeruginosa* strains was responsible for chronic pulmonary colonisation in CF patients and interestingly in non-CF patients with other chronic obstructive airways diseases. At that time, the nature of the bacterial colonising factors responsible was unknown. Preliminary evidence pointed to the involvement of enhanced mucinophilic and chemotactic properties in colonising strains. To investigate this working hypothesis, a collaborative study was set up with Dr John Sheehan, Department of Molecular Biology, University of Manchester, utilising highly purified mucin from CF patients and an elegant new method for producing mucin monolayers for electron microscopy. These studies suggested that the motile pseudomonas were attracted by chemotaxis to the mucin-rich surface of the CF lung thereby utilising a classic lung defence system in the early stages of colonisation. In a healthy lung, bacteria attracted to the mucosal surface are removed by mucociliary clearance; in CF patients, such removal is reduced by the viscous , dehydrated nature of the pulmonary mucosal blanket. To further compromise lung defences the emergence of alginate-producing *P. aeruginosa* enclosed in a protective biofilm thwart phagocytosis and antimicrobial therapy.

In parallel with studies on the pathophysiology of CF lung infection, ongoing attempts to understand the complex regulation of alginate biosynthesis in *P. aeruginosa* received a major boost in 1989. Following informal contacts with Dr Vojo Deretic, the author was awarded a visiting professorship at the University of Texas in San Antonio. This led to a very successful collaboration with Deretic's group on molecular and genetic aspects of alginate biosynthesis. In 1989, it was known that *alg*R, a regulatory gene involved in alginate biosynthesis was required for transcription of the *alg*D gene which encodes GDPmannose dehydrogenase, the pivotal enzyme in alginate biosynthesis. In 1988, Deretic's group had published the important observation that the product of *alg*R shared homology with regulatory elements of the important new class of environmentally responsive two-component systems

controlling a wide range of pathogenic and physiological processes including the osmotically-regulated porins of *Escherichia coli* and the virulence factors of *Bordetella pertussis* (Deretic *et al.*, 1989a; Miller *et al.*, 1989). As a result of these collaborations with Vojo Deretic, the *muc* mutations first mapped during the earlier sabbatical in Melbourne were shown to be important environmentally-responsive regulators of *alg*R and *alg*D. Hence, not only were important clues revealed of the regulation of alginate in the CF lung, but alginate biosynthesis was shown to be a valuable experimental model for study of an important new class of environmentally activated gene regulation including virulence genes. In 1992, this collaboration led the development of a genomic library for *P. cepacia* during a visit by James Nelson to San Antonio and a review of the global role of *muc* mutations in the regulation of alginate and other pseudomonas virulence determinants for the 100th volume of FEMS Letters (53).

Accumulated research on the pathophysiology of CF lung infections had indicated that immune-mediated tissue damage in response to pseudomonas antigens contributed significantly to progressive pulmonary deterioration in CF patients. Thus, it was felt that the future prophylactic use of pseudomonas vaccines, would benefit from the development of sensitive screening systems to detect asymptomatic pulmonary colonisation with *P. aeruginosa*. Two distinct approaches were attempted, the first based on monoclonal antibodies (Mabs), and the second on the polymerase chain reaction (PCR).

The use of Mab antibody probes and ELISA-based assays were developed in collaboration with Drs Keith James, James Nelson, Robin Barclay and the late Lisel Micklem. Lipopolysaccaride core mutants of *P. aeruginosa* were used as antigens to develop a sensitive sandwich ELISA system, presently undergoing field trials. Concurrently, a DNA-based system employing PCR has been developed in collaboration with Professor David Brock and Dr Iain McIntosh. This system uses synthetically prepared oligonucleotides based on DNA sequences from the *alg*D gene and was one of the early applications of PCR to the detection of bacteria in clinical specimens (49).

In September 1992, just three years after identification of the CF gene, the "arrival" of the CF mouse was announced simultaneously by groups in North Carolina, Cambridge and Edinburgh. Following an invitation from Drs David Porteous and Julia Dorin of the MRC

Human Genetics Unit, the author is presently collaborating on very promising microbiological studies of the "Edinburgh CF mouse".

The discussion which follows provides a more detailed account of the author's research and sets his selected publications (numbered throughout the text) in the context of contemporary knowledge. Key additional references are included in a separate general reference list.

Section A provides an introduction to the role of *P. aeruginosa* and other pseudomonads as highly adaptable microbes with a propensity to infect individuals compromised by underlying disease or immunosuppressive therapy. Section B discusses results of early studies on the bacteriocins of *P. aeruginosa* and the development of a typing technique based on pyocin production for epidemiological studies of a wide range of pseudomonas infections leading to a major interest in chronic pulmonary infections in patients with CF. Section C focuses on the pathophysiology and treatment of pulmonary infections in CF patients and the roles of the alginate exopolysaccharide in pulmonary colonisation. Section D deals with the gradual unravelling of the genetic and molecular regulation of alginate biosynthesis and its emergence as a valuable model for studies of an important new family of environmentally controlled virulence genes. Section E describes the emergence and challenge of *P. cepacia* as a highly transmissible pulmonary pathogen in CF patients.

DISCUSSION

SECTION A: THE GENUS *PSEUDOMONAS*

During the last decade, much new and important information has emerged on diverse aspects of the important group of Gram-negative bacilli collectively gathered under the taxonomic umbrella of the genus *Pseudomonas*. The pseudomonads are basically saprophytes inhabiting soil and aqueous environments. Major features of the genus, including taxonomy, identification, epidemiology and pathogenesis have been described by the author (29,33,46). The importance of the pseudomonads is based on their role as opportunistic pathogens for humans, animal and plants, as environmental scavengers in nature resulting in biodegradation and removal of a large number of natural and synthetic compounds and their usefulness as model systems to study metabolic pathways and the regulation of gene expression. The pseudomonads are also well-known for their ability to produce interesting industrial products from simple carbon sources (for example, alginates) for their innate resistance to many antimicrobial agents and for their extreme nutritional versatility. *Pseudomonas cepacia*, discussed in detail in section E, is even capable of utilising penicillin G and the potent synthetic herbicide "agent orange" as nutrients! Several species, including *P. fluorescens* and *P. cepacia* also act as agents of biological control by producing antimicrobial compounds which protect plants against bacterial and fungal infections.

SECTION B: BACTERIOCINS: PYOCINS OF *P. AERUGINOSA*

Bacteriocins are antibiotic-like agents produced by many bacterial species and differ from classic antibiotics in that their inhibitory activity is characteristically directed towards other members of the same, or closely related, species. The bacteriocins of *P. aeruginosa*, designated pyocins after the original species epithet *pyocyanea*, were first described in 1954 by Francois Jacob -- later to be awarded a Nobel laureate with his colleague Jacques Monod for their pioneering research in the new science of molecular biology. Few subsequent studies on pyocins were reported until those describing the production, detection and mode of action of pyocins formed the basis of my PhD thesis in 1968. Publications 1-5 and 9 described the heterogeneity and mode of action of pyocins. R pyocins, similar to those described by Jacob, are produced by the majority of *P. aeruginosa* and resemble the tail components of retractile

T-even bacteriophages. Other pyocins first described in publications 5 were designated flexuous or F pyocins and resemble the tail components of non-retractile bacteriophages. Electron microscopy (4,5) showed that R pyocins attach to lipopolysaccharide receptors on the surface of susceptible bacteria and that killing occurs following contraction of the pyocin particles. Although F pyocins also attach to lipopolysaccharide receptors, killing activity is not accompanied by visible contraction. Later publications revealed additional classes of pyocins including non-particulate, trypsin-sensitive and trypsin-resistant S pyocins which share receptors with iron-associated outer membrane proteins (9,24,54; Ohkawa *et al.*, 1980). Sharing of receptors between S pyocins and iron siderophores might explain the apparent suicidal nature of bacteriocin receptors. In Darwinian terms, production of bacteriocins is understandable, for example as a means of protecting the bacterial colony; in contrast, conservation of receptors which result in cell death appears suicidal. In the 1980s, however, the author's research, together with that of Kageyama and colleagues and a recent collaborative study with Smith and colleagues (54) showed: first, that bacteriocin receptors are associated with iron-chelating siderophores and the functioning of a complex high affinity iron uptake system in *P. aeruginosa*, secondly, that regulation and biosynthesis of all known classes of pyocins are encoded by chromosomal genes and not by plasmids as occurs in colicins, the best known and most studied group of bacteriocins.

In 1968, the author and RR Gillies introduced a typing system for *P. aeruginosa* based on pyocin production. Pyocin typing was described and evaluated in publications 1-3 and, incorporating later modifications (9,20), came to be internationally recognised as a major typing system for epidemiological studies of *P. aeruginosa*. As well as acting as a reference centre for typing of *P. aeruginosa*, the author has supplied typing kits to hundreds of laboratories throughout the world. Important modifications to the original method included improvements to the speed and discriminating power, the ability to type MPA isolated from CF patients and the introduction of a bacteriocin typing system for *P. cepacia* in 1986 (23).

In 1987/88 the group was invited by the Communicable Disease Centre, Atlanta to participate in a large multicentred study on typing systems for *P. cepacia* which showed bacteriocin typing to be the most discriminating method for characterisation of individual strains (35). A similar multicentered investigation of phenotypic and genotypic typing systems for typing of *P. aeruginosa* has also been completed and the results submitted for publication.

As pyocin typing became used routinely for surveillance of *P. aeruginosa* cross-infection, many interesting epidemiological studies were encountered. One such study involved collaboration with Dr Steve Alcock at the University of Aberdeen on the epidemiology of painful debilitating ear infections in North Sea divers. This study demonstrated strikingly the ability of *P. aeruginosa* survive in the helium-rich atmosphere associated with saturation diving. A surprising result was that despite the relatively cramped environmental conditions of the diving chambers, each containing several different strains of *P. aeruginosa*, within individual chambers only a single strain was responsible for cross-infection among a group of divers. This enhanced ability of particular strains to colonise the ear was to be paralleled in subsequent pulmonary studies in CF patients.

From accumulated evidence, it was suspected that patients with CF and other chronic obstructive lung diseases are most susceptible to a relatively small subpopulation of *P. aeruginosa* with enhanced colonising ability. A longitudinal study utilising computerised pyocin typing results from several thousand strains indicated that isolates from chronic pulmonary infections contained a significant proportion of relatively rare pyocin types (40). This evidence, obtained from a large retrospective study, was subsequently substantiated by prospective epidemiological studies employing pyocin typing and, in collaboration with Drs Ogle and Vasil, University of Colorado, a new DNA-based genomic system based on Southern blot hybridisation and DNA fragments derived from the pseudomonas exotoxin A gene (Ogle *et al.*, 1987). A later study suggested that chemotaxis and enhanced mucinophilic properties in a subpopulation of *P. aeruginosa* might be the key factors associated with pulmonary colonisation (41).

In the 1970s, the author's application of pyocin typing to investigate the clonal relationship between NMPA and MPA from individual CF patients suggested that the mucoid variants were derived from the original colonising strain rather than as *de facto* secondary colonisers (3). Although, this *in vivo* conversion hypothesis has come to be generally accepted, convincing evidence remained scanty until the development of genomic fingerprinting (Ogle *et al.*, 1987). Subsequently, an epidemiological study using pyocin typing and genomic fingerprinting confirmed the relationship in an investigation of transformation to the mucoid phenotype following water-borne acquisition of NMPA by two CF patients (52).

SECTION C: CYSTIC FIBROSIS AND MUCOID *P. AERUGINOSA*

In 1973, pyocin typing studies (3) in collaboration with Rosamund Williams, suggested that MPA emerge *in vivo* as a result of initial colonisation by clonally-related NMPA; this observation led to a major interest in these variants of *P. aeruginosa* as the major cause of morbidity and mortality in patients with CF.

Since 1973, I have directed a research group and instigated wide-ranging research initiatives and collaborations to solve the biological jigsaw puzzle posed by the association of MPA and the lungs of CF patients. In this thesis, major features of CF will be summarised before proceeding to a discussion of the microbiology and pathogenesis of pseudomonas infections in CF patients.

Cystic fibrosis:

CF is the most fatal inherited disease affecting caucasian populations with an incidence of approximately 1 in 2000 live births and a carrier rate of 1 in 20. The disease is inherited as an autosomal, recessive trait and usually presents at birth or early childhood as a triad of symptoms consisting of pancreatic insufficiency, raised sweat electrolytes and impaired mucociliary clearance in the lungs leading to repeated infections. CF is a major cause of morbidity and mortality in the paediatric and adolescent age groups. After ten years of intensive research, the race to identify the CF gene and the gene product was won in 1989 by North American teams led by Collins, Riordan and Tsui (Riordan *et al.*, 1989; Rommens *et al.*, 1989). The achievement, approximately five years after the first linkage data placed the CF gene on the long arm of chromosome 7, was described as one the most significant modern scientific and medical breakthroughs. The CF gene encodes a relatively large 250kd protein which has been called the CF Transmembrane Regulator (CFTR). By 1992, more than 200 different mutations had been identified in the CF gene, the most common and the first to be discovered being Δ-F-508 resulting in a three base pair deletion and the absence of phenylalanine. The basic biochemical abnormality caused by Δ-F-508 has not been clearly identified but is thought to involve faulty translocation of CFTR and a defective pump or channel involved in the regulation of chloride ion transport across epithelia. Of considerable biological significance was the discovery that CFTR is a member of a family of similar transmembrane proteins responsible for a wide range of eucaryotic and prokaryotic functions

-- for example, the P glycoprotein responsible for multiple drug resistance in humans and the transmembrane protein required for haemolysin transport in *Escherichia coli.*

Since 1989, the rate of advance in CF research has been exciting and unexpectedly fast. Achievements include novel functions for CFTR besides chloride secretion (Bradbury *et al.*, 1992), prenatal screening for CF (Mennie *et al.*, 1992) and *in vitro* fertilization and preimplantation diagnosis for couples carrying the CF gene (Handyside *et al.*, 1992). A viable mouse model of CF has been achieved by targeted insertional mutagenesis (Dorin *et al., 1992*) and the mice shown to possess enhanced susceptibility to pulmonary infection (Dorin, Porteous and Govan, unpublished results). In addition, a comprehensive mutational analysis of 267 CF patients has indicated that risk of chronic airways' colonisation with *P. aeruginosa* is predisposed by the CFTR genotype. Δ-F-508 heterozygotes with the second CF allele in the NBF-encoding exons have an increased risk of acquiring *P. aeruginosa* early in life (Kubisch *et al.*, 1993). Other recent developments in CF include approval to carry out pioneering gene therapy in CF patients and controversial demands for royalty payments from the institutions which discovered the CF gene.

Pathophysiology of pulmonary infection in CF:

Progressive lung damage caused by intermittent infective pulmonary exacerbations is a major complication of CF. The pathophysiology of lung disease in CF patients is complex but during the last decade, major progress has been achieved in understanding the mechanisms involved in the gradual changes which develop in the lung from birth through the stages of early bacterial colonisation to the later onset of progressive deterioration in lung function.

Bacterial colonisation frequently occurs at an early stage and sets in motion a sequence of complications that are secondary to inflammatory obstruction and non-distribution of ventilation, which lead to chronic bronchitis and bronchiolitis. A further response to this pathology is an increase in secretions of pulmonary mucus. Because of the basic CF defect, these secretions are dehydrated, highly viscous and associated with further airway obstruction, micro-abscess formation, local areas of over inflation, airway trapping and a vicious, unrelenting cycle of pulmonary exacerbations (Tomashefski *et al.*, 1983).

General Bacteriology of CF:

The pathological changes which occur in the CF lung are exacerbated by repeated episodes of bacterial infection. Surprisingly few bacterial pathogens are involved, the most frequent being *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa*. The role of *S. aureus* in the induction of lung damage was considered important, particularly in the first twenty years after the description of CF. However, in the last two decades there has been a relative decrease in the incidence of *S. aureus* while the rate of isolation of *P. aeruginosa* has increased (27, Hoiby, 1982). Currently, colonisation of the lungs of CF patients by MPA is the major milestone in the progress of the disease (52) and has been referred to as a "harbinger of death" (Pier, 1986).

The mucoid phenotype of *P. aeruginosa* results from copious production of the exopolysaccharide alginate. MPA are most commonly isolated from the tracheobronchial secretions of CF patients, to a lesser extent from the lungs of non-CF patients with chronic obstructive lung disease and occasionally from urinary tract infections (38). No natural environmental niche for MPA has been described. Recently, isolation of MPA from an equine pharynx has confirmed the predilection of the alginate-producing phenotype for the respiratory tract and provides an interesting parallel between veterinary and human microbiology (48).

Initial pulmonary colonisation of CF patients with NMPA is typically asymptomatic. Gradually, however, MPA emerge and come to predominate. This transition is associated with a pronounced humoral immune response to various pseudomonas antigens, including alginate, inflammatory-mediated tissue damage and repeated episodes of pulmonary exacerbation. In 1973, it was unclear whether the MPA were related to the original colonising strain of *P. aeruginosa* or represented secondary infection with pre-existing mucoid variants. Epidemiological studies to clarify the relationship were hampered by the difficulty in typing MPA because of the viscous nature of the bacterial alginate. This problem was overcome in a collaboration with Rosamund Williams; the original pyocin typing technique was modified and used to show that NMPA and MPA from the same patient belonged to the same pyocin type (3); based on these results we proposed that MPA do not colonise CF patients *de novo* but emerge as mutations from the original NMPA. In 1975, however, Zierdt and Williams proposed the alternative hypothesis that "the mucoid *P. aeruginosa* strain is

probably spread from patient to patient, rather than acquiring its mucoid characteristic *de novo* in the CF patient." Subsequently, further modification of pyocin typing was developed with Janet Fyfe and Gillian Harris (20) and used to "fingerprint" multiple colonies of *P. aeruginosa* isolated from individual sputum from 32 CF patients on a longitudinal basis. The results (30) clearly indicated that the majority of CF patients remain colonised with a single strain of *P. aeruginosa* which eventually exhibits the mucoid phenotype and secondly that cross-infection with MPA is rare and usually restricted to CF siblings. Ogle *et al.*(1987) reported similar results in 15 patients using a genomic typing system based on DNA probes derived from the exotoxin A gene and surrounding sequences.

The results of these early typing studies formed a watershed in the author's research. From the mid 1970s, major projects and collaborations were focused on the regulation of alginate biosynthesis *in vitro* and *in vivo* and on attempts to improve the diagnosis and management of CF lung infections through improved understanding of the underlying pathophysiology and the *in vivo* action of antibiotics. The following sections describe the various approaches employed in pursuit of these aims.

Structure and properties of alginate:

Bacterial pathogens present to the external environment a cell surface comprised of a complex mixture of macromolecules whose functions are important for survival *in vitro* and *in vivo*. These functions include presentation of bacterial antigens and mechanisms involved in colonisation and avoidance of host immune responses. Cell surface macromolecules include peptidoglycans, lipopolysaccharides, porin proteins and exopolysaccharides. The latter may form part of a discrete capsule or exist as a less organised slime layer. Bacteria that produce large amounts of exopolysaccharide typically have a gelatinous mucoid colonial morphology. Alginates are an unusual but important class of exopolysaccharide produced by *Azotobacter vinelandi* (17) and some species of *Pseudomonas* (12,14); production of alginate is recognised as the major virulence factor in the pathophysiology of *P. aeruginosa* infection in patients with CF (7,30).

Alginate was first isolated from brown algae, hence the name (Stanford, 1883). Although the mucoid form of *P. aeruginosa* had been described by Sonnenschein as early as 1927, the fact that mucoidy was due to alginate biosynthesis was not established until 1964 (Linker and

Jones, 1964). At the same time, the first report appeared of the association of MPA and pulmonary colonisation of CF patients (Doggett *et al.*, 1964).

Alginates from *P. aeruginosa* are acetylated unbranched (1-4) linked polysaccharides comprised of β-D-mannuronate and its C5-epimer α-l-guluronate. The relative proportion of the two uronic acids varies and is a major factor determining the properties of the polysaccharide; in general, alginate from a particular strain of *P. aeruginosa* has a constant mannuronate:guluronate ratio, regardless of growth conditions (Gacesa and Russell, 1990).

Alginate biosynthesis and hypersusceptibility:

In addition to the transition to the mucoid phenotype, *P. aeruginosa* strains isolated from CF patients are associated with several other phenotypic changes including loss of O-antigen specificity and unusual hypersusceptibility to a range of antimicrobial agents. Hypersusceptibility is frequently associated with the mucoid phenotype (13,19) and explains some of the early difficulties in attempts to generalise on antibiotic susceptibility of MPA and in particular the influence of alginate on susceptibility. In association with Janet Fyfe, hypersusceptibility was shown to be independent of alginate biosynthesis and loss of O-antigen and encoded by at least two chromosomal genes which we designated *bls* and *tps* (19).

P. aeruginosa is normally associated with intrinsic resistance to antimicrobial agents, indeed, resistance is generally held responsible for the emergence of *P. aeruginosa* as a significant opportunistic pathogen following the introduction of antibiotics. Thus, the apparent survival of hypersusceptible strains within the CF lung required explanation. The first report of hypersusceptible *P. aeruginosa* appeared in a paper by May and Ingold in 1972 and described respiratory isolates with enhanced susceptibility to carbenicillin. The author's studies (13,19, 22, 27) revealed that in addition to carbenicillin, hypersusceptibility extended to other β-lactams, and to other types of antibiotics including tetracyclines, trimethoprim, and quinolones. In addition, despite the ubiquitous nature of *P. aeruginosa*, hypersusceptible isolates were found to be confined to chronic respiratory infections. We also observed that in CF sputum, hypersusceptible strains were usually present in association with pseudomonads of normal susceptibility belonging to the same pyocin type indicating a close clonal relationship. Paradoxically, hypersusceptible strains are not eliminated during antibiotic therapy and intriguingly may even increase as a proportion of the total pseudomonas

population. Mutations encoding hypersusceptibility were transferred from a CF strain into the genetic strain PAO by plasmid transfer. Transductional analysis indicated the location of two loci (*bls* and *tps*) on the PAO chromosome linked to a *met* marker at 18 min. The *tps* mutation which enhances susceptibility to trimethoprim but not to β-lactams was also found in non-hypersusceptible strains isolated from the same CF patient. Based on the hypothesis that hypersusceptibility could arise from a single-step mutation at the *bls* locus, we postulated that it should be possible to detect similar mutations in *P. aeruginosa* PAO; such mutations were subsequently demonstrated following mutagenesis and the mutant strain PAO6511 shown to exhibit the same antibiogram as PAO recombinants from the original inter-strain cross. The molecular basis of hypersusceptibility is unclear though the range of different antibiotics involved suggests some form of relatively non-specific increase in permeability perhaps due to conformational changes in lipopolysaccharide.

The clinical relevance of hypersusceptibility in patients with CF can be considered in two ways. In clinical practice, considerable heterogeneity is observed in antibiotic susceptibility when multiple colonies of *P. aeruginosa* are examined from single CF sputum (15,27); thus examination of a single colonial representative from sputum culture may not be representative of the bacterial population *in vivo*. The paradoxical emergence and survival of hypersusceptible strains in the face of aggressive antipseudomonal therapy will be discussed later in considering the biofilm mode of growth *in vivo*. It was shown, however, that hypersusceptibility confers a considerable growth rate advantage for MPA growing under conditions of limited nutrients, for example when tracheobronchial mucin is used as the sole nutrient (30). The function of the *bls* and *tps* genes *in vivo* might be to enhance uptake of nutrients and compensate for the metabolic drain associated with alginate biosynthesis.

Mucoid *P. aeruginosa*:

In contrast to their predominance and stability in CF lungs, most MPA rapidly lose the mucoid phenotype when cultured *in vitro*. Factors influencing loss of mucoidy were clarified in a series of studies (6,10). Reversion was reduced by aeration and in a dose-dependent manner by the presence of surfactants including the major lung surfactant phosphotidylcholine (6). Fluctuation experiments suggested that instability results from the emergence of spontaneous nonmucoid mutants which in the absence of alginate biosynthesis have a growth rate advantage in conditions of poor aeration (10). Boyce and Miller (1982) later confirmed

that oxygen limitation rather than lack of aeration *per se* is selective for the nonmucoid phenotype.

Isolation of mucoid pseudomonads *in vitro*:

The first report of the isolation of MPA *in vitro* was associated with the presence of phage (Martin, 1973). Martin postulated that the mucoid variants were phage-dependent and that mucoidy was due to a form of pseudolysogeny. In contrast, the author's genetic studies in Melbourne showed that mucoidy was independent of phage activity and encoded by chromosomal genes which were given the named *muc* (11). This genetic evidence was a major observation with respect to the regulation of alginate biosynthesis and suggested that mucoid variants arise spontaneously and are phenotypically phage resistant because their phage receptors are masked by alginate. The mutational nature of MPA was later confirmed by the development of a selection technique for isolation of mucoid variants *in vitro* based on enhanced resistance to carbenicillin and aminoglycosides (8,12). Selection of mucoid variants in PAO strains containing conjugative plasmids allowed the first genetic analysis of alginate biosynthesis and the location of a cluster of *muc* mutations conferring mucoidy in a region between 66 and 71 min (11,17,30,37,43). The antibiotic-based selection technique was also used to demonstrate for the first time the existence of alginate-producing variants in other pseudomonads including *P. fluorescens, P. putida* and *P. mendocina* (12,14).

A second experimental approach demonstrated that the mucoid phenotype could be medium-dependent and induced by environmental factors. It must be stressed that such induction was not observed in NMPA and required the presence of *muc* mutations (37,43, 53).

Later studies showed that the colonial appearance of MPA was also affected by the environment, in particular in a change from a watery appearance to gelatinous in the presence of electrolytes such as Ca^2 (16,26,30,38). The degree of gelling which occurred in the presence of Ca^2 was shown to be strain dependent, a finding subsequently confirmed by analysis of the constituent mannuronic and guluronic acids and the observation that pseudomonas alginate lacked guluronic blocks and hence produced pliable rather than brittle gels (Gacesa and Russel, 1990).

The pathophysiology of *P. aeruginosa* in CF:

One of the most striking observations in the pathogenesis of *P. aeruginosa* is that NMPA which produce a range of virulence factors and are associated with localised and systemic infections with significant morbidity and mortality in non-CF patients cause only asymptomatic colonisation in CF patients. Thus, in an early review(7), which commented on the Jekyll and Hyde character of *P. aeruginosa* in CF, it was suggested that alginate biosynthesis and the properties of this unusual polysaccharide are major factors in bacterial survival and direct or indirect tissue damage in the CF lung.

These observation led to a series of studies whose aim was to explain the behaviour of MPA in CF lungs (16,26,30,38,44,52,57). When MPA were cultured *in vitro* and examined by phase contrast microscopy, the alginate exopolysaccharide appeared as an extracellular slime. The presence of Ca^2 at concentrations as low as 3mM resulted in the rapid formation of bacteria-containing microgels. By electronmicroscopy, microgels appeared as cotton-wool-like matrices enclosing bacterial cells.

In acute pulmonary infections in non-CF patients, *P. aeruginosa* is usually observed by microscopy to be dispersed throughout the sputum. In contrast, mucoid strains in CF sputum (16,26,30,38,44,52,57; Hoiby, 1982) and in CF post-mortem lung tissue (Lam *et al.*, 1980) often appear as microcolonies adhering to the bronchial mucosa. Individual microcolonies reach 60 um in diameter and thus are considerably larger than host phagocytic cells, including pulmonary neutrophils. A clear selective advantage of MPA in adherence to ciliated cells was also shown (Baker, 1990). The concept of bacterial biofilms (extended forms of microcolony) was originally proposed in a wider environmental context by Costerton *et al.* (1978). Subsequently, this was extended to include phenotypic plasticity whereby bacteria within the biofilm modify their cell surface components to operate optimally without the necessity for mutation (Costerton *et al.*, 1990). In the CF lung, the microcolony mode together with association of bacterial alginate with bronchial mucin (36) and the CF environment provide a plausible model to explain the various unusual phenotypic changes in *P. aeruginosa* isolated from CF patients, the pathophysiology of the pulmonary exacerbations and the resistance of the organisms to aggressive antimicrobial therapy with potent antipseudomonal agents.

In vitro studies (22) indicated that pseudomonas elastase, an important virulence factor during the early stages of colonisation by NMPA (Hoiby *et al.*, 1986) is produced to a lesser extent by MPA and led to speculation the alginate biofilm acts as an immunological adjuvant releasing elastase locally and concentrating its activity on the bronchial epithelium.

The author's research supported the use of quantitative sputum bacteriology to determine the bacterial load within CF lungs during exacerbations and to assist in the assessment of antimicrobial therapy (18,21,27,28). In long-established colonisation, however, the bacterial population remains relatively stable even after aggressive antibiotic therapy and quantitative bacteriology is not helpful. This problem was solved by our observation that serum levels of the acute-phase protein, C-reactive protein, provide a reliable index of exacerbation and effective antimicrobial therapy (31,32). Demonstration of reduced C-reactive protein concentrations and clinical improvement in the absence of significant reduction in bacterial load was partially explained by the suppression of host inflammatory response (18) and bacterial virulence determinants including elastase (21,30) and alginate (36, Morris and Brown (1988) by subinhibitory concentrations of antibiotics. In 1992, the author reported the use of an *alg*D-*xyl*E transcriptional fusion to provide evidence that ciprofloxacin could "switch off" transcription of the key alginate enzyme GDPmannose dehydrogenase at concentrations as low as 20% of the MIC (53).

The significance of early diagnosis of *P. aeruginosa*:

Epidemiological and genetic evidence suggests that NMPA play a key role as a microbial reservoir for selection of MPA and other phenotypes associated with chronic colonisation and progressive lung disease. Surveillance of faecal flora in CF patients provided additional evidence that the gastrointestinal tract is not a significant reservoir of *P. aeruginosa* prior to pulmonary colonisation (34). Thus, the author suggested that stratagems designed to prevent or minimise early pulmonary colonisation by NMPA might be fruitful (30). Such a strategy has recently been supported by the results of a controlled prospective study which indicated that chronic colonisation with *P. aeruginosa* can be prevented in CF patients by early institution of antibiotic therapy at the first isolation of NMPA from routine sputum cultures (Valerius *et al.*, 1991). Since early detection of NMPA would benefit such preventive therapy attempts were made to develop rapid and specific methods for detection of *P.aeruginosa* colonisation.

Immunological methodology:

CF patients mount a specific antibody response against cellular and extracellular antigens of *P. aeruginosa*. Thus detection of an increasing antibody response could be useful to diagnose progression from asymptomatic colonisation to invasive infection. For this purpose, in association with James Nelson and other colleagues, a monoclonal-antibody based ELISA system capable of rapid and specific detection of *P. aeruginosa* was developed utilising the mutant *P. aeruginosa* PAC605 which expresses core lipopolysaccharide common to all serotypes (39,42,47).

PCR methodology:

A DNA amplification procedure using heat stable Taq polymerase and the polymerase chain reaction was also developed for early detection of *P. aeruginosa* in collaboration with Dr Iain McIntosh and Professor David Brock (49). Selection of the oligonucleotide primers was based on the nucleotide sequence of the *alg*D gene encoding GDPmannose dehydrogenase, a major enzyme in the biosynthesis of alginate by *P. aeruginosa* (see Discussion, section D). Evaluation studies indicate that *P. aeruginosa* can be detected with a sensitivity approximating 10 bacteria in sputum harbouring large numbers of other respiratory pathogens. During development of the PCR technique it was interesting and unexpected to observe that strains of *P. putida*, *P. fluorescens* and *P. mendocina* did not produce a signal with the *alg*D primers despite our previous observations (12,14) that these strains contain alginate biosynthetic genes. The negative PCR result from these pseudomonads provided a reassuring specificity for the technique to identify *P. aeruginosa*; of interest, it also suggested differences in *alg*D sequences between MPA and other alginate-producing pseudomonads.

SECTION D: REGULATION OF ALGINATE BIOSYNTHEIS

During the 1980s, major advances were made in understanding the association of *P. aeruginosa* and CF, the clonal relationship between MPA and the original nonmucoid colonisers and the regulation of alginate biosynthesis.

In the early 1970s, bacterial genetics was almost synonymous with *Escherichia coli* genetics; interest and knowledge of the genetics of other organisms has developed more recently. The genus *Pseudomonas* attracted the attention of geneticists because of its biochemical diversity and clinical importance as an opportunist pathogen. In the early 1970s, an embryonic chromosomal map of *P. aeruginosa* PAO existed based on genetic recombination and the use of transducing phages (Holloway, 1955; Holloway *et al.*, 1960); however, no virulence determinants had been located nor had genetic circularity of the PAO chromosome been confirmed.

Genetic studies of alginate biosynthesis began in 1975 when the author was awarded a MRC Travelling Fellowship to work with Professor Bruce Holloway in the Department of Genetics at Monash University, Melbourne. Despite the successful transfer of the mucoid phenotype in *P. aeruginosa* PAO from mucoid donors to nonmucoid recipients, these early efforts were frustrated by anomalies in linkage data of *muc* mutations to adjoining auxotrophic markers, in particular to *his*-1, at that time located at 10 min. FP2, the sex factor used in these experiments was thought to transfer pieces of chromosome unidirectionally from a single origin. Consequently, transfer of markers located beyond 40 min from the origin of transfer produced few recombinants and relatively inaccurate mapping. In 1981, circularity of the PAO chromosome was confirmed and several markers, including *his*-1 relocated (Royle *et al.*, 1981). Even then, fate was unkind. *his*-1 and the *muc* mutations responsible for the mucoid phenotype were relocated around 70 min in an almost inaccessible region of the PAO chromosome akin to the "dark side of the moon".

In the late 1970s, other researchers failed to demonstrate a link between the mucoid phenotype and plasmid content (Markowitz *et al.*, 1978). Thus, we proceeded on the working hypothesis that all *P. aeruginosa* have the necessary chromosomal information for alginate biosynthesis but that expression of the exopolysaccharide is normally repressed or activated at levels too low to produce a mucoid phenotype. If this hypothesis is correct, mucoid variants could arise following spontaneous mutation in one of an unknown number of chromosomal regulator genes. An alternative hypothesis was that wild type *P. aeruginosa* have a defective enzyme in the alginate pathway and mucoid strains have a normal one. Enzyme analysis, however, had shown that NMPA expressed most alginate synthesising enzymes, albeit at low activity (Piggott *et al.*, 1981). Based on the genetic methodology available, Janet Fyfe

and I attempted to determine the number and location of genes responsible for the regulation of alginate biosynthesis.

The author's development of a selection technique (8) for isolation of alginate-producing variants *in vitro* allowed the isolation of *muc* mutations in various genetic backgrounds. *P. aeruginosa* PAO 381 was chosen as the principal genetic background for mapping of *muc* loci, as it contained a useful auxotrophic marker (*leu*-38) and the sex factor FP2. It should be emphasised that unlike auxotrophic or catabolic markers, *muc* mutations could not be selected directly in conjugation or transduction experiments. Hence, early attempts to map *muc* loci were dependent on the demonstration of linkage to known selectable markers.

On the basis that one or more *muc* mutations would be located somewhere on the PAO chromosome, mucoid PAO derivatives were used as donors in conjugation experiments with nonmucoid recipients and selection made for co-inheritance of auxotrophic markers and the mucoid phenotype in different regions of the chromosome. A significant early observation from these matings with mucoid donor strains was that the recombination frequencies for all markers were 10 to 50 fold lower than those obtained using isogenic nonmucoid donors suggesting that alginate was acting as a barrier to conjugation.

From the results of these early mating studies it was possible to identify a series of *muc* mutations (*muc*-2, *muc*-22, *muc*-23) located in two clusters on either side of *his*-1 located at 69 min following recalibration of the PAO chromosome (O'Hoy and Krishnapillai (1987) and the production of a combined physical and genetic map of PAO (Ratnaninsangsih *et al.*, 1990). Using transductional analysis and catabolic markers located in the region around *his*-1, *muc*-2 and *muc*-22 were more precisely located to a region close to *oru*-292 and *pru*-354 (35).

More accurate mapping by transductional analysis then became possible following the author's discovery of a particular type of nonmucoid revertant. Previously, it had been difficult to propagate transducing phage on mucoid variants, probably because phage receptors on the bacterial surface are blocked or the phage immobilised in the viscid alginate environment surrounding the cells. Thus, before *muc*-2 and *muc*-22 could be mapped by transduction, it was necessary to isolate nonmucoid derivatives which had arisen due to mutations in other parts of the alginate regulatory or structural pathway. Although phenotypically nonmucoid, these "suppressed" revertants are still able to transfer the *muc* allele, giving rise to mucoid

recombinants on selection for appropriate markers. In addition to extending knowledge of alginate genetics and instability of MPA, these suppressed mutants were to prove invaluable in mapping studies, and a decade later in analysing the wider regulatory role of *muc* mutations and alginate biosynthesis as a model for environmentally-controlled gene regulation (37,43,53). By 1983, we could conclude that at least two gene clusters, located at 70 min on either side of *his*-1, are involved in regulation of alginate biosynthesis in MPA.

The nature of the repression of alginate biosynthesis in wild type (nonmucoid) strains remained unknown. In the case of colanic acid responsible for the mucoid phenotype of *Escherichia coli*, indirect evidence for the protein nature of the capR cytoplasmic repressor was obtained by growing wild type strains on amino acid analogues, for example para-fluorophenylalanine (FPA; Kang and Markovitz, 1967). If grown on FPA at 37°C, a temperature normally associated with an active repressor and a nonmucoid phenotype, *Escherichia coli* produce an inactive repressor and a mucoid phenotype following incorporation of the analogue into the *cap*R product. The author's attempts to demonstrate a similar influence of FPA on the regulation of alginate biosynthesis were unsuccessful (unpublished data).

In 1981, a different approach to the genetic analysis of alginate production was pioneered in the United States. Ohman and Chakrabarty (1981) began to develop a genetic system using *P. aeruginosa* FRD1, a MPA isolated from a CF patient and stable nonmucoid derivatives of FRD1. Ohman and Chakrabarty mapped a mutation designated *alg* which was shown to be responsible for instability in FRD1 and independent of the *muc* mutations. Two additional gene clusters associated with alginate regulation and biosynthesis were then revealed. These comprised a cluster of regulatory genes represented by *alg*R located at 10 min and a second cluster at 20 min containing the genes for alginate synthetic enzymes, in particular *alg*D encoding GDPmannose dehydrogenase the key enzyme controlling alginate biosynthesis in MPA (Deretic *et al.*, 1987b; Tatnell *et al.*, 1993).

The author's earlier fluctuation experiments (10) had suggested that instability of the mucoid phenotype results from spontaneous mutations leading to loss of the mucoid phenotype. A variety of mutations distinct from those in the *muc* cluster and capable of "switching off" alginate biosynthesis would be expected and, historically, had been indicated by isolation of

the suppressed revertants used in transductional analysis (17). It now appeared that these mutations might be associated with *alg*R or the structural gene cluster at 20 min.

The approach taken by Chakrabarty, Ohman and coworkers combined with improvements in enzyme analysis have continued to prove valuable in understanding the complex regulation of alginate biosynthesis. At the time, however, no comparison could be made between the chromosomal markers employed by Chakrabarty and Ohman and those used in our PAO studies. The relationship between the alginate genes being studied in the two systems remained obscure and it was not possible to compare results from MPA isolated from CF patients with those in *P. aeruginosa* PAO. The possibility that PAO mucoid derivatives might be genetically distinct from mucoid isolates from CF patients was dispelled however by our observation that a *muc* locus in a CF isolate *P. aeruginosa* 492c, transferred by a R68.45-mediated interstrain cross, was located close to *muc*-2 and *muc*-22 at 70 min. In 1985, the relationship between the PAO mutants and *muc* determinants in clinical isolates was finally confirmed by construction of R′ plasmids and complementation data (53, Fyfe, 1985) and later by plasmid pM060-mediated conjugation (MacGeorge *et al.*, 1986).

Alginate regulation *in vivo*:

By the early 1990s, considerable advances had been made in understanding the pathophysiology of MPA in CF lungs (38,41,44,52,57; Costerton *et al.*, 1990). Important observations included evidence that MPA grow in the CF lung under iron-depleted conditions (Brown *et al.*, 1984) and that an overexhuberant antibody response to pseudomonas antigens, including alginate and iron-associated membrane proteins, play major roles in immune-mediated lung damage (Hoiby *et al.*, 1986; Pedersen *et al.*, 1990). In addition, after a relatively long hiatus following the initial mapping of *muc* mutations by the author and Janet Fyfe in 1980 (11), significant attention was directed to the genetics of alginate biosynthesis and many previously unsuspected details of the regulation of alginate biosyntheis unravelled (37,43, 53; Deretic *et al.*, 1991; May *et al.*, 1991). From these different perspectives it became possible to speculate on the regulation of alginate *in vivo*.

From the time of the early studies on MPA, there been debate as to whether mucoidy arose as a result of mutation and selection or from induction of exopolysaccharide synthesis in the whole bacterial population by some unknown environmental stimulus. By the 1990s, there was

evidence to suggest that both options are correct; namely, that mucoidy arises from mutation but that the degree of alginate biosynthesis can be influenced in both a positive and negative manner by environmental stimuli (37,43,53).

The possibility that environmental factors within CF bronchial secretions might induce or "switch on" alginate biosynthesis has long been an attractive hypothesis and was first postulated by Henriksen (1948). Early studies in the author's laboratory had included unsuccessful attempts to induce the mucoid phenotype in NMPA by repeated subculture of labelled strains in CF bronchial secretions. Whilst these attempts to transform wild-type strains to the mucoid phenotype had been unsuccessful , the induction of mucoidy in strains already containing *muc* mutations had been clearly demonstrated (15,38). The challenge was now to determine the exact mechanism(s) by which environmental stimuli influence alginate biosynthesis, the molecular basis of the *muc* master switch, and the interplay between *muc* mutations and other regulatory and structural genes responsible for alginate biosynthesis.

By the mid 1980s, the application of molecular studies had led to a rapid expansion in knowledge of alginate regulation. In 1987, Deretic *et al.* (1987a,b) proposed that the *alg*R gene encodes a positive-acting regulatory protein which mediates alginate production by activation of the *alg*D promoter; this in turn initiates production of GDPmannose dehydrogenase, a key enzyme in alginate biosynthesis. Recently, the *alg*R gene has been sequenced (Deretic *et al.*, 1989a) and found to have significant homology with several genes known to encode regulatory proteins that respond to environmental stimuli, including nitrogen starvation (*ntr*C), osmolarity (*omp*R), sporulation (*spo*OA) and bacterial chemotaxis (*che*B). These important observations suggested that *alg*R may be involved in a two component regulatory system for signal transduction from the environment into transcriptional activation of alginate genes. At this time the relationship between *alg*R (9 min), *alg*D (20 min) and the closely linked *muc* (53) and *alg*S/T genes (Flynn and Ohman, 1988) located at 70 min was unknown. From accumulated evidence, however, it seemed reasonable to speculate that alginate biosynthesis *in vivo* and the mucoid phenotype *in vitro* resulted from the influence of *muc* mutations superimposed on normal levels of alginate gene regulation. To pursue this working hypothesis we investigated cultural conditions responsible for "switching on" the mucoid phenotype in mucoid PAO mutants containing different *muc* alleles and in CF isolates expressing the same medium-dependent mucoid phenotype. We observed that group 2 mucoid variants (15), represented by *P. aeruginosa* PAO568 (*muc*-2) produced a nonmucoid

colonial phenotype on minimal agar with ammonium ions as the nitrogen source; in contrast, when nitrate was used as the nitrogen source most group 2 variants produced a mucoid phenotype under both aerobic and anaerobic conditions. Concurrently, Berry *et al.* (1989) investigated the affect of increased osmolarity on alginate biosynthesis in wild-type PAO. Although *alg*D expression was increased in the presence of raised osmolarity the mucoid phenotype was not expressed suggesting that other factors are required for full activation of alginate biosynthetic genes. The role of *muc* mutations in alginate biosynthesis and their connection with *alg*R and *alg*D was finally clarified in collaboration with Vojo Deretic and his group using an *alg*D-*xyl*E transcriptional fusion to measure the influence of environmental stimuli on strains of PAO containing different *muc* mutations (37,43,53). Individual *muc* alleles were shown to modify transcription of *alg*R and *alg*D either positively or negatively in the presence of elevated osmolarity or nitrogen limitation. The key role of *muc* mutations in the environmental regulation of mucoidy explains previous observations which indicated that although increased osmolarity was associated with increased alginate production in some MPA this stimulus alone was not sufficient to induce mucoidy in NMPA.

Further studies revealed a wider role for *muc* mutations in the control of other pseudomonas virulence factors. Use of PAO 568 (*muc*-2) which displays increased *alg*D transcription and mucoidy when stimulated by elevated osmolarity showed that *muc*-2 is involved in a see-saw expression pattern of *alg*D and the elastase structural gene *las*B (Mohr *et al.*, 1990). It was concluded that the *las*B gene and the alginate system are co-ordinately regulated with *muc*-2 joining the expanding family of global regulators (for example, the *Bvg* gene of *Bordetella pertussis*) which in response to environmental stimuli modify cassettes of virulence determinants and have been given the evocative title "virulons" (Deretic *et al*, 1989b).

Evidence that a variety of *muc* mutations affect regulation of alginate biosynthesis in response to environmental stimuli presents a clear indication of the complexity of the alginate regulatory system and the hazards of relying on a single MPA strain to generalise on the nature of the mucoid phenotype. For example, the extensive use of *P. aeruginosa* 8821 and its derivatives (Berry *et al*, 1989; May *et al*, 1991) has contributed significantly to a molecular understanding of alginate biosynthesis. Strain 8821 exhibits enhanced mucoidy in the presence of elevated osmolarity and hence supports the attractive hypothesis (Berry *et al.*, 1989) that elevated salt concentrations in CF lungs favours alginate biosynthesis. In reality, however, this

response is found in only a minority of CF isolates; a more common response is reduction of mucoidy in conditions of elevated osmolarity (37,53).

Recent studies have emphasised the complexity of alginate biosynthesis and indicate that control of mucoidy includes not only bacterial signal transduction systems, but also histone-like elements controlling nucleoid structure (Deretic *et al.*, 1991) and possibly factors affecting superhelicity (53). In particular, the influence of *muc* and other loci in the 70 min region of *P. aeruginosa* PAO continues to reveal the unexpected. Martin *et al.* (1993) have recently characterised a locus, *alg*U which shows sequence similarities with a sigma factor, SpoOH involved in control of sporulation and competence in *Bacillus subtilis*. *Alg*U was shown to be located close to *muc*-2 and *muc*-22 and may even be allelic with these determinants.

The involvement of such a sigma factor in alginate regulation is of particular interest. in *Azotobacter vinelandii*, the only bacterial species other than pseudomonads known to produce alginate, the exopolysaccharide participates in encystment, a developmental process associated with resistance to dehydration (23). Based on the observation that MPA are more resistant to dehydration than isogenic NMPA, the author and colleagues had previously suggested that alginate biosynthesis in MPA might represent a development process in *P. aeruginosa* (17,23). Exopolysaccharide synthesis in *P. aeruginosa* outside the CF lung is associated with the formation of microcolonies and biofilms, a process which represents differentiation from a planktonic (mobile) to a sessile (immobilised, biofilm-embedded) mode (Costerton *et al.*, 1990). It is possible that the emergence of MPA in the CF lung represents a variation of this process.

In conclusion, after a tortuous and eventful journey, control of mucoidy in *P. aeruginosa,* once considered to be a highly unique and specialised aspect of pseudomonas pathogenesis has become one of the focal systems for analysis of how bacterial pathogens adapt to host environment.

SECTION E: EMERGENCE OF *P. CEPACIA* AS A CF PATHOGEN

In 1992, the author was invited by the Lancet to comment on the growing anxiety amongst CF patients and their carers concerning transmission of *P. cepacia* and the clinical outcome of colonisation (51).

The issue of *P. cepacia* emerged in the early 1980s, when a North American clinic reported a disturbing increase in the isolation of *P. cepacia* from CF patients (Isles *et al.*, 1984). Young CF adults appear to be particularly at risk of acquiring the organism and three clinical sequelae of colonisation documented: 1) chronic asymptomatic carriage, 2) progressive deterioration over many months, and 3) "*P. cepacia* syndrome", characterised by rapidly fatal deterioration which may include septicemia, a rare occurrence in CF patients.

In 1986, the author and Sharon Glass documented the first death of a CF patient in the United Kingdom associated with *P. cepacia* (25). In this young 9-year-old female, the rapid terminal course of the infection contrasted with the typical prolonged deterioration seen in other CF pulmonary infections and emphasised the need for vigilance in monitoring and accurately identifying *P. cepacia* and determining the bacterial and host factors involved in its pathogenesis.

Prior to its notoriety in CF, *P. cepacia* was a relatively little known phytopathogen first described as the cause of bulb rot in onions (Burkholder, 1950). Human infections with *P. cepacia* are rare, and are generally restricted to hospitalised patients with reduced immunity who acquire the organism from contaminated equipment or disinfectant solutions (Holmes, 1986). *P. cepacia* is naturally resistant to most antibiotics and even when *in vitro* susceptibility is demonstrated there is little clinical response. Within the genus *Pseudomonas, P. cepacia* is only distantly related to *P. aeruginosa*. Alarmingly, its nearest neighbour based on the degree of DNA and RNA homology is *P. pseudomallei*, the causative agent of melioidosis and the most pathogenic of all pseudomonads (46).

With the exception of the fatal case referred to earlier, longitudinal surveillance of *P. cepacia* colonisation within the Edinburgh clinics between 1980 and 1989 gave little cause for concern. In 1990, however, the emergence a particularly transmissible strain within the Edinburgh CF

population and the spread of this strain to other regional CF centres in the UK heralded a major new challenge.

Evidence for person-to-person transmission of *P. cepacia* (LiPuma *et al.*, 1990), contamination of environmental surfaces (50) and for spread of the Edinburgh epidemic strain both within and between clinics by social contact (publication submitted) has led to much debate and controversy on the clinical significance of *P. cepacia* and whether colonised patients should be segregated within hospital and social contacts outside of hospital avoided (51,52,57). Some clinicians and microbiologists believe that the organism is a marker rather than a cause of morbidity. The author has addressed these important and controversial issues in a number of papers which include 1. The development of a typing technique for *P. cepacia* based on production of and sensitivity to bacteriocins (23, 35); *in vitro* evaluation of new antimicrobial agents against *P. cepacia* (55), diagnosis of *P. cepacia* colonisation by detection of antibodies against *P. cepacia* core lipopolysaccharide (56), updates on the microbiology of pulmonary infections in CF (45,52,57) and in a Lancet editorial (51).

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TYPING OF *PSEUDOMONAS PYOCYANEA* BY PYOCINE PRODUCTION

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PLATES LXXXIV–LXXXVI

PYOCINES are naturally occurring antibiotic substances produced by many strains of *Pseudomonas pyocyanea* (*Ps. aeruginosa*) and active mainly against other strains of this species.

Such activity, analogous to that of colicines, was first reported by Jacob (1954). Holloway (1960) studied the lysogenicity and pyocinogenicity of pseudomonas organisms and suggested that pyocine production might be useful as an epidemiological marker. In the past, bacteriophage typing and serological typing have been employed to differentiate strains of pseudomonas, and Gould and McLeod (1960) applied these methods simultaneously; they concluded that such double identification of strains would be valuable in tracing sources of infection. Their procedure, however, requires both the preparation of antisera and the maintenance of a collection of phages. The report of Darrell and Wahba (1964) on the use of pyocine production to classify strains has marked a step forward in the study of the epidemiology of pseudomonas infections, since the pyocine method is simpler than either the phage or serological method.

This paper records our experience of pyocine typing and specifies our standardised technique.

MATERIALS AND METHODS

Producer strains. The strains of *Pseudomonas pyocyanea* to be typed by their ability to produce pyocines included 2688 strains acquired from two hospital laboratories as subcultures of single colonies that had been isolated from a wide variety of lesions and 539 strains cultured from fomites, dust, sewer swabs and swabs taken from surgical instruments.

Identification of strains. Each isolate was examined for Gram reaction, oxidase production (Kovacs, 1956), motility in semi-solid agar, pigment production, utilisation of glucose and ability to liquefy gelatin (Kohn, 1953).

Indicator strains. A set of 8 indicator strains was selected from more than 300 strains tested for such a role. The strains tested were mainly of *Ps. pyocyanea*, but they included 50 strains of *Escherichia coli* and the set of indicator strains used in the colicine typing of *Shigella sonnei* by Gillies (1964). The 8 strains finally selected were all of *Ps. pyocyanea*; this set has been in use for more than 2 yr.

Culture media. Tryptone soya agar (Oxoid) was prepared according to the maker's instructions and 5 per cent. of horse blood was incorporated. We now use this tryptone soya blood agar (TSBA) as a routine for growing producer strains. Other media tested in developing the technique were nutrient agar (Cruickshank *et al.*, 1960, p. 195), MacConkey's agar, and Wahba's (1963) medium. Infusion broth (Cruickshank *et al.*, p. 190) was used to grow the indicator strains.

Pyocine typing technique

The strain to be typed (a potential pyocine producer strain) is streaked diametrically across a plate of TSBA in such a way that the width of the inoculum is *c.* 1 cm. The plate is then incubated at 32° C for 14 hr. The macroscopic growth is removed with a glass slide, 3–5 ml. $CHCl_3$ is placed in the lid of the petri dish and

TABLE I

Patterns of inhibition of pyocine types of Pseudomonas pyocyanea

Pyocine type of producer strain	Inhibition of indicator strain no.							
	1	2	3	4	5	6	7	8
1	+	+	+	+	+	−	+	+
2	−	+	−	−	−	−	−	−
3	+	+	+	−	+	−	+	−
4	+	+	+	+	+	−	−	+
5	−	−	−	−	+	−	−	−
6	+	+	+	+	+	−	+	−
7	+	+	+	−	−	−	+	+
8	−	+	+	+	−	−	+	−
9	−	−	−	−	+	−	+	−
10	+	+	+	+	+	+	+	+
11	+	+	+	−	−	−	+	−
12	+	+	−	+	+	−	−	+
13	−	−	−	+	−	−	−	+
14	−	−	+	−	+	−	+	−
15	−	+	−	−	+	−	+	−
16	+	−	+	+	−	−	+	+
17	−	−	+	−	−	−	+	−
18	+	−	+	+	+	−	+	+
19	−	−	+	+	−	−	+	−
20	−	−	−	−	+	+	−	−
21	−	+	−	+	+	−	−	−
22	+	+	+	−	+	+	+	−
23	+	−	−	−	+	−	+	−
24	−	−	+	+	+	−	+	+
25	+	−	+	−	−	−	+	−
26	+	−	−	−	−	−	+	−
27	+	−	+	−	+	−	+	−
28	−	−	−	+	−	−	+	−
29	−	+	−	−	+	−	−	−
30	−	+	+	−	−	−	−	−
31	−	−	−	−	−	−	+	−
32	−	−	−	+	+	−	−	+
33	+	+	+	+	+	+	+	−
34	−	−	−	−	−	−	−	+
35	+	+	−	−	+	−	+	−
36	−	+	−	+	−	−	−	+

+ = inhibition, − = no inhibition.

the dish with the medium is replaced on the lid for 15 min. so that microscopic remnants of the culture are killed.

The plate is then opened and the residual $CHCl_3$ is decanted and retrieved for future use by filtering through filter paper. Traces of $CHCl_3$ vapour are eliminated from the culture plate by exposing it to air for a few minutes.

Cultures of the 8 indicator strains grown in infusion broth for 4–6 hr at 37° C are streaked on to the $CHCl_3$-treated medium at right-angles to the line of the original inoculum; 5 strains are applied on the left side of the line and the other 3 strains on the right side. The plate is then incubated at 37° C for 8–18 hr.

Any pyocines produced by the original inoculum diffuse into the medium during the first period of incubation and then exert their inhibitory action on the indicator strains during the second. The pyocine types of the strains under test are recognised from the patterns of inhibition they produce on the indicator strains (figs. 1–4 and table I).

RESULTS

The technique has been used to type 3227 strains of *Pseudomonas pyocyanea* isolated since 1961; 88·4 per cent. of the strains were found to be typable and we have so far recognised 36 pyocine types in the species (table I). Fifteen of the types were regularly encountered in the Edinburgh area. This experience compares favourably with that of Wahba (1965), who differentiated only 10 types by using 12 indicator strains.

Influence of temperature and period of incubation

In an attempt to explain this difference we tested 180 strains in parallel by Wahba's method and our own, i.e. each producer strain was grown on Wahba's medium and TSBA at 32° C for 14 hr and on the same media at 37° C for 24 hr (Wahba's method) before clearing the plates and applying the 8 indicator strains. An example of these tests is shown in fig. 5 and it will be noted that with our method of primary incubation (32° C for 14 hr) inhibition of 4 indicator strains was obtained with both media, whereas no inhibition of any indicator strain was obtained on either medium when the producer strain was incubated at 37° C for 24 hr.

In tests of the 180 strains grown on TSBA medium, 160 (89·4 per cent.) of the strains were typable by our method of incubation, but 85 of these gave no inhibition of the indicators under Wahba's conditions. Forty-five strains (34 typed and 11 untypable) gave identical results with the two methods of incubation and 50 strains gave different inhibition patterns by the two methods. Forty-four of the strains showing a difference in patterns gave characteristic patterns on the plates incubated under our conditions but only unrecognisable and atypical patterns on the plates incubated under Wahba's conditions.

Another advantage of incubation at the lower temperature is that fewer strains are adherent to the medium and the removal of the growth is accordingly more easy.

Pyocine production in other media

Tests with nutrient agar and MacConkey's agar as substrates for growth of producer strains revealed that neither was as good as TSBA whatever the temperature and time of incubation; strains either appeared as pyocine non-producing or gave aberrant patterns.

Validity of the technique

Many of our strains were retested after storage on nutrient agar for periods varying from 3 mth to 2 yr and 6 strains were retested at least

twice weekly for more than 1 yr. In no instance was any quantitative or qualitative alteration in pyocine production noted.

TABLE II

Constancy of pyocine type in replicate isolates of Pseudomonas pyocyanea obtained on different occasions from the same site in the same patient; results from 133 patients

No. of replicate isolates obtained from patient *	No. of patients whose isolates were	
	all of one type	of more than one type
1	52	16
2	25	4
3	9	1
4	4	1
5	8	0
>5 †	10	3

* Excluding the first strain isolated from the patient.

† Number of isolates from patients in whom all were of the same type: 6, 7, 10, 11, 12, 12, 15, 15, 23, 24; number of isolates from patients who yielded strains of more than one type: 8, 10, 17.

TABLE III

Distribution of pyocine types in 575 strains of Pseudomonas pyocyanea isolated in two Scottish hospitals

Pyocine type	Percentage of strains belonging to stated pyocine type in	
	Hospital 1 (463 strains)*	Hospital 2 (112 strains)*
1	38·9	15·2
3	28·3	28·6
5	5·2	8·9
6	2·4	0·9
9	0	16·9
u/t	4·8	10·7
u/c	8·2	9·8
Other types	12·3 (16 types)	8·9 (8 types)

* Only one strain from each patient is represented; we have not included cases where more than one type was isolated.

u/t = untypable strains, i.e. not producing pyocines detectable with our indicators.

u/c = unclassifiable strains, i.e. solitary strains giving patterns other than those shown in table I.

To assess the stability of pyocine production in strains *in vivo*, we noted whether replicate isolates obtained on different days from the same site in a patient were of the same, or of a different type from the strain originally obtained from that patient. The results are summarised in table II and it will be noted that not all isolates from the same patient

PYOCINE TYPING OF *PSEUDOMONAS PYOCYANEA*

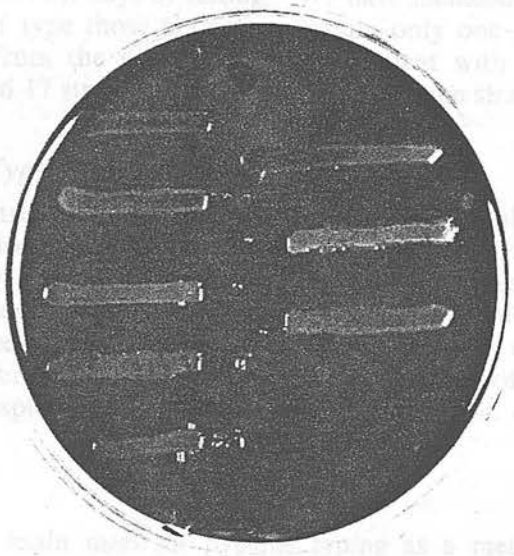

FIG. 1.—Inhibition pattern given by pyocine type-1 strains of *Pseudomonas pyocyanea*; all the indicator strains, except no. 6 (top right), are inhibited.

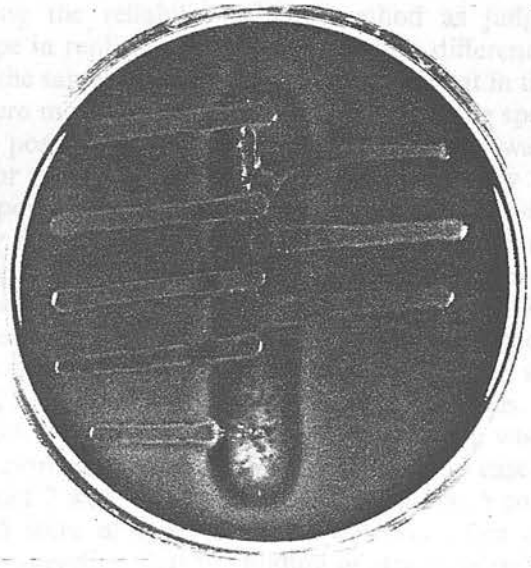

FIG. 2.—Type-5 strains have restricted activity and inhibit only the indicator strain no. 5 (bottom left).

were identical in type. In 108 patients the replicate isolates were all of the same type, but in 25 patients strains of different types were encountered on different days of testing. We have included as instances of differences of type those patients in whom only one strain in the series differed from the majority, e.g. the patient with 17 replicate isolations yielded 17 strains of pyocine type 1 and one strain of type 3.

Type distribution in two Scottish hospitals

The type distribution in these hospitals is shown in table III. The strains were collected between Aug. 1961 and Oct. 1964. It should be noted that only one strain from each patient is represented in this material and also that we have excluded all cases where more than one type was isolated. It will be seen that the distribution of types was significantly different in the two hospitals; thus, strains of type 9 were absent from Hospital 1, but accounted for 16·9 per cent. of all strains in Hospital 2.

Discussion

Perhaps the main merit of pyocine typing as a method for the epidemiological marking of strains of *Pseudomonas pyocyanea* is that it can be used in the humblest of diagnostic units since, unlike serological and bacteriophage methods, it requires no special materials for its performance.

In considering the reliability of the method as judged by the constancy of type in replicate isolates obtained on different days from the same site in the same patient, it should be noted that in the majority of instances where more than one type was isolated, the specimen was of faeces. It is possible that in these cases the patient was excreting strains of two or more types simultaneously. Originally we had no evidence to support this hypothesis, since in most cases we examined only one colony from each primary diagnostic plate, but recently we have had the opportunity of testing multiple colonies from such plates.

So far, we have examined 368 strains obtained from 45 diagnostic plates; 11 of the plates, from which 104 colonies were typed, had been seeded with specimens of urine, 7 (47 colonies) had been seeded with ear swabs and 27 (217 colonies) with specimens of faeces. Only two of the plates, both inoculated with specimens of faeces, were found to bear strains of more than one pyocine type; in one case 6 colonies were of type 3 and 2 were untypable and in the other, 5 colonies were of type 1 and 5 were of type 3. Thus we have some evidence to substantiate the suggestion that the finding of strains of different types in different specimens of faeces from the same patient may be the result of two different strains colonising the intestine and not that of one strain varying in its pyocine type.

Another index of reliability, which we would have liked to demonstrate, is the degree of uniformity of pyocine type in strains from the

same epidemic focus. However, many of our strains were acquired long after their isolation and had been harvested for another purpose, so that the relevant epidemiological information was often incomplete. It is, nevertheless, worth noting (table III) that the strains of type 9 were all isolated in one surgical unit in Hospital 2, where this type was endemic for some months and was isolated from many situations in the patients' environment. A similar state of affairs was found in another hospital where strains of type 8 were isolated from 5 cases of post-operative urinary tract infection in a male surgical ward in Feb. 1964 and from 2 similar cases in May 1964; again, strains of the same type were isolated from many objects in the environment. No further cases of infection with *Ps. pyocyanea* were noted in this ward until Oct. 1964, when 3 cases of post-operative urinary tract infection were discovered and again these were due to type-8 organisms; we are now investigating the situation in a prospective fashion.

The period and, more particularly, the temperature employed for primary incubation of the producer strain are critical in pyocine typing. Wahba (1963) experienced difficulties with certain strains that produced pyocine-inactivating substances as well as pyocines. These substances allowed confluent growth of the indicator strains when one would have expected to obtain zones of inhibition. Wahba overcame much of the difficulty by incorporating a triad of chemicals in his medium. We have had little difficulty with this problem and attribute our success to the conditions of incubation of the producer strain. As may be seen in fig. 5, inhibition was clearcut when the producer strain was grown at 32° C for 14 hr (our method), whereas on plates incubated at 37° C for 24 hr (Wahba's method) there was no inhibition of the indicator organisms.

Originally we incubated the producer strain at temperatures between 32° and 35° C, but we now adhere to the lower temperature since with certain strains, particularly those of type 5, the conditions of pyocine production are very critical and many strains of this type appear to be non-productive when grown at 35° C; on the other hand, the period of incubation is less critical since, with all types, this can be extended to 18 hr without interfering with the resultant patterns of inhibition. Thus the method is readily applicable in laboratories where an 18-hr period of incubation is more convenient than the shorter period we normally use. Such critical differences in incubation conditions in the detection of bacteriocines are not without precedent, for Abbott and Shannon (1958) and Gillies (1964) reported similar findings in the colicine typing of *Shigella sonnei*.

It might be thought that the differences between our findings and those of Wahba were associated with the difference in the set of indicator strains used, but this is only partly the case. We have recently had the opportunity of testing a modified set of Wahba's indicator strains obtained by the courtesy of Dr M. T. Parker, Cross-infection Laboratory, Colindale, London. With this set, we typed 90 strains in

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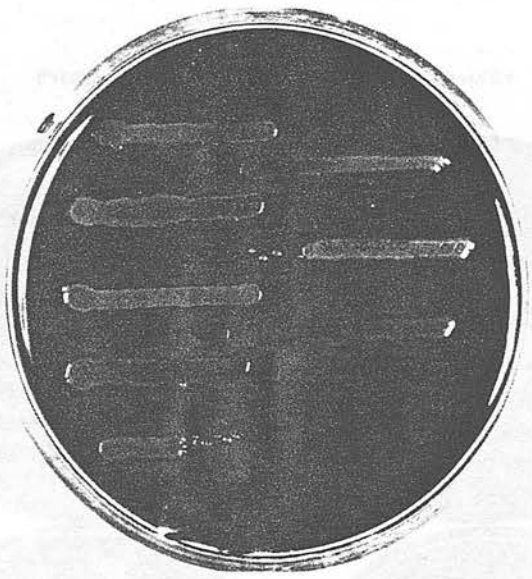

FIG. 3.—Pyocine type-9 strains inhibit indicator strains no. 5 (bottom left) and 7 (middle right).

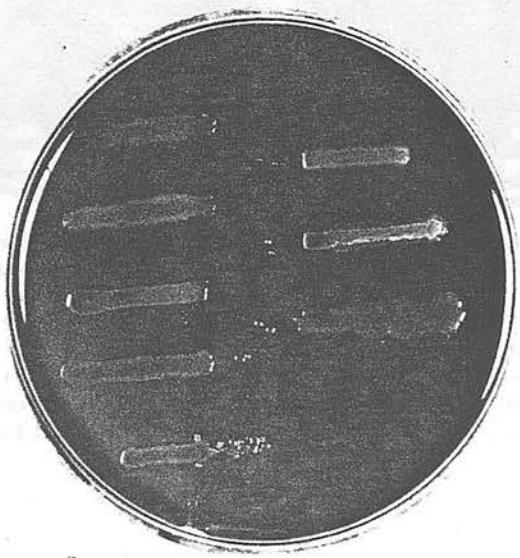

FIG. 4.—Type-10 strains inhibit all the indicator strains. The presence of resistant variant cells in some of the indicator cultures is clearly shown by the development of a few colonies in the pyocine zone.

PYOCINE TYPING OF *PSEUDOMONAS PYOCYANEA*

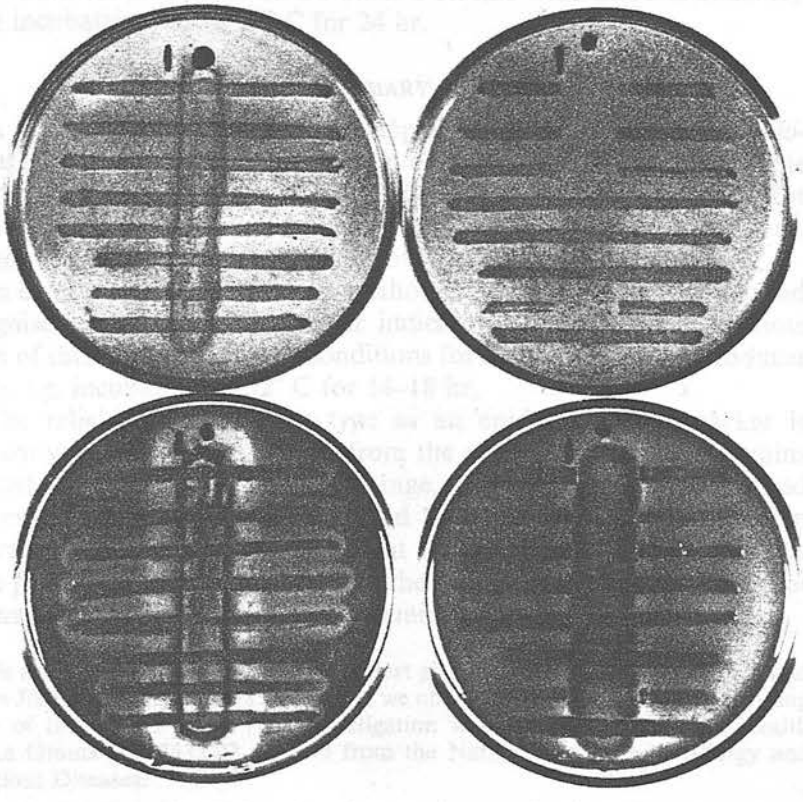

FIG. 5.—Patterns of inhibition given by a producer strain of pyocine type 11 tested in parallel on two plates of Wahba's medium (above) and two plates of TSBA (below) incubated at 37°C for 24 hr (plates on left) or at 32°C for 14 hr (plates on right) during growth of the producer strain. The producer strain was streaked vertically and the same set of 8 indicator strains were streaked horizontally across the whole width of each plate. No inhibition has occurred on the left-hand plates (primary incubation at 37°C) whereas clear-cut inhibition of the same 4 indicator strains has occurred on the right-hand plates (primary incubation at 32°C).

duplicate by the two methods of incubation on TSBA medium. Thirty-one strains gave inhibition patterns when primary incubation was at 32° C for 14 hr, but were without inhibitory activity when incubation was at 37° C for 24 hr. Eleven strains gave identical patterns of inhibition under both conditions of incubation and 4 strains were untypable under both conditions. The inhibition patterns of 36 of the remaining 44 strains were more clearcut when incubation was under our conditions and those of the other 8 strains were more satisfactory when incubation was at 37° C for 24 hr.

SUMMARY

A simple method has been developed for typing strains of *Pseudomonas pyocyanea* by their production of pyocines. Its use will enable diagnostic laboratories to characterise strains without the need for maintaining a collection of bacteriophages or a collection of antisera, neither of which is readily available outside reference laboratories.

In comparison with Wahba's method of pyocine typing our method recognises more types with fewer indicator strains and we attribute much of this difference to our conditions for incubation of the producer strain, i.e. incubation at 32° C for 14–18 hr.

The reliability of pyocine type as an epidemiological marker is eminently satisfactory as judged from the constancy of type in strains retested after varying periods of storage. Further studies are required to confirm its reliability as estimated from the constancy of type in replicate isolates obtained at different times from the same site in the same patient and as estimated from the uniformity of type in different strains obtained from the same epidemic focus.

We acknowledge the ever-willing support given to us by Mr D. O. Brown, and to Drs Joan McWilliam and J. Stevenson we offer our sincere thanks for providing many of the strains used. The investigation was supported by Public Health Service Grants AI-04833-02 and -03 from the National Institute of Allergy and Infectious Diseases.

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Further Studies in the Pyocine Typing of *Pseudomonas pyocyanea*

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FURTHER STUDIES IN THE PYOCINE TYPING OF *PSEUDOMONAS PYOCYANEA*

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PLATE III

WE have previously reported our method of typing strains of *Pseudomonas pyocyanea* (*Ps. aeruginosa*) by their ability to produce pyocines that give characteristic patterns of inhibition on a set of eight indicator strains (Gillies and Govan, 1966). We also noted that unless incubation of the strain to be typed was undertaken with fairly strict limits on the temperature and period of incubation, a significant proportion of strains gave aberrant results. Our method, although employing fewer indicator strains, was more sensitive than that of Darrell and Wahba (1964) and allowed the recognition of a greater number of pyocine types of *Ps. pyocyanea*.

In the present paper we report more fully on the main typing procedure and outline a method of subdividing strains of pyocine type 1, the commonest type in our series. We also present evidence in support of a hypothesis made in our earlier paper, namely that the finding of strains of more than one type in a patient's lesion is due to infection with strains of different types, and not to instability and variation of type in a single infecting strain.

MATERIALS AND METHODS

Producer strains. During the period 1961–1967, 5690 strains of *Pseudomonas pyocyanea* were typed by their pyocine-producing abilities. These strains were received from numerous diagnostic laboratories either as subcultures of single colonies or as primary diagnostic plate cultures, from which several colonies could be typed. The majority, 91 per cent., of the strains had been isolated from a wide variety of lesions: 4844 strains from lesions in patients being treated in hospital and 349 strains from lesions in patients being treated at home. The remaining 497 strains had been isolated from fomites, dust, surgical instruments, respirators and other apparatus employed in the care of patients in hospitals.

Indicator strains. The indicator strains used in the main pyocine typing procedure were the eight strains (no. 1–8) employed in our previous study. Five additional strains (A–E) of *Ps. pyocyanea* were used in the subtyping of pyocine type-1 strains. These two sets of indicator strains have been in use for more than 4 yr and 2 yr respectively.

Culture media. Tryptone soya agar (Oxoid) was prepared in accordance with the maker's instructions and 5 per cent. of horse blood was added. This tryptone soya blood agar (TSBA) was used for growing producer strains. Infusion broth (Cruickshank, 1965) was used to grow the indicator strains for 4 hr at 37°C before applying them to the processed test plate.

Pyocine typing techniques. The main typing procedure was unchanged from that previously reported (Gillies and Govan) and may be summarised as follows. The strain of

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Ps. pyocyanea to be typed (a potential producer strain) is streaked across the surface of a plate of TSBA so that the width of the inoculum is about 1 cm. The plate is then incubated at 32°C for 14 hr. Thereafter the macroscopic growth is removed with a glass slide and microscopic remnants of growth are killed by pouring 3–5 ml $CHCl_3$ into the lid of the petri dish and replacing the medium-containing portion.

TABLE I

Patterns of inhibition produced by the 37 different pyocine types of Pseudomonas pyocyanea

Pyocine type of producer strain no.	Inhibition of indicator strain no.							
	1	2	3	4	5	6	7	8
1	+	+	+	+	+	−	+	+
2	−	+	−	−	−	−	−	−
3	+	+	+	−	+	−	+	−
4	+	+	+	+	+	−	−	+
5	−	−	−	−	+	−	−	−
6	+	+	+	+	+	−	+	−
7	+	+	+	−	−	−	+	+
8	−	+	+	+	−	−	+	−
9	−	−	−	−	+	−	+	−
10	+	+	+	+	+	+	+	+
11	+	+	+	−	−	−	+	−
12	+	+	−	+	+	−	−	+
13	−	−	−	+	−	−	−	+
14	−	−	+	−	+	−	+	−
15	−	+	−	−	+	−	+	−
16	+	−	+	+	−	−	+	+
17	−	−	+	−	−	−	+	−
18	+	−	+	+	+	−	+	+
19	−	−	+	+	−	−	+	−
20	−	−	−	−	+	+	−	−
21	−	+	−	+	+	−	−	−
22	+	+	+	−	+	+	+	−
23	+	−	−	−	+	−	+	−
24	−	−	+	+	+	−	+	+
25	+	−	+	−	−	−	+	−
26	+	−	−	−	−	−	+	−
27	+	−	+	−	+	−	+	−
28	−	−	−	+	−	−	+	−
29	−	+	−	−	+	−	−	−
30	−	+	+	−	−	−	−	−
31	−	−	−	−	−	−	+	−
32	−	−	−	+	+	−	−	+
33	+	+	+	+	+	+	+	−
34	−	−	−	−	−	−	−	+
35	+	+	−	−	+	−	+	−
36	−	+	−	+	−	−	−	+
37	−	+	+	+	+	−	+	−

+ = Inhibition; − = no inhibition.

After 15 min. the plate is opened and residual $CHCl_3$ is decanted. Traces of $CHCl_3$ vapour are eliminated from the culture plate by exposing it to the air for a few minutes. The eight indicator strains are then applied at right-angles to the original inoculum line. Any pyocines produced by the original inoculum will have diffused into the medium and can then exert their inhibitory activity on the indicator strains during the second period of incubation, which is at 37°C for 8–18 hr. From the patterns of inhibition on the indicator strains, 37 pyocine types of *Ps. pyocyanea* are recognised (table I).

The technique for subdividing pyocine type-1 strains is identical to that in the main typing procedure except that five additional indicator strains are used to recognise eight subtype patterns (table II).

TABLE II

Patterns of inhibition produced by the 8 subtypes of pyocine type 1 of Pseudomonas pyocyanea

Pyocine subtype of type-1 producer strain *	Inhibition of indicator strain				
	A	B	C	D	E
a	+	+	+	+	+
b	−	+	+	+	+
c	−	−	+	+	+
d	+	−	+	+	+
e	−	+	+	−	+
f	−	−	−	−	−
g	−	−	+	−	+
h	−	+	−	+	+

* Strains in these subtypes are designated as " type 1a ", " type 1b ", etc.

TABLE III

Constancy of pyocine type in sets of replicate isolates of Pseudomonas pyocyanea obtained on different occasions from the same site in the same patient. Results from 626 sets of isolates from 511 patients

Number of replicate isolates obtained from site *	Number of sets where the isolates were	
	all of one type	of more than one type
1	292	33
2	98	19
3	53	2
4	24	5
5	22	1
6	13	1
7	11	2
8	10	0
9	2	1
10	6	0
11	4	1
>11†	22	4

* Excluding the first strain isolated from the site.

† Number of isolates from sites where all isolates were of the same type were 13, 15, 15, 16, 16, 16, 17, 17, 18, 19, 20, 21, 25, 25, 27, 29, 29, 30, 37, 43, 44 and 54. Number of isolates from sites that yielded strains of more than one type were 13, 14, 17 and 18.

RESULTS

Results obtained with the main typing method

We have continued to collect evidence on the validity of the typing method by checking the stability of pyocine production in strains of *Pseudomonas pyocyanea* stored on nutrient agar at room temperature (*c.* 21°C) for periods varying from 3 mth to 4 yr. Tests were made on 260 strains in 22 pyocine types, and 15 (5·7 per cent.) of the strains were found to produce patterns of inhibition

TABLE IV

*Distribution of pyocine types in 2396 strains of Pseudomonas pyocyanea isolated during the years 1961–1967 from patients in hospital and patients in the open community**

Pyocine type	Percentage of strains belonging to stated pyocine type in patients † in		
	hospital (1945 strains)	the open community (451 strains)	either hospital or the open community (2396 strains)
1	32·6	34·4	34·2
3	28·0	18·8	25·2
5	5·3	7·1	5·7
10	2·5	3·1	2·9
35	2·1	2·7	2·9
29	1·6	2·0	2·1
27	3·3	0·9	2·0
16	0·9	3·8	1·7
11	2·2	1·3	1·6
9	2·7	0·4	1·3
u/t	6·9	10·2	7·6
u/c	6·2	3·1	5·5
Other types	5·7 (17 types)	12·2 (14 types)	7·3 (24 types)

* The data include those given in table III of our previous paper (Gillies and Govan).
† Only one strain from each patient is represented. Strains from cases from which more than one type was isolated are not included. Only one strain from each epidemic is represented.
u/t = Untypable strains, i.e., not producing pyocines detectable with our indicators.
u/c = Unclassifiable strains, i.e., solitary strains giving patterns other than those shown in table I.

that differed from those given on initial typing. Thirteen of the 15 strains had been stored for more than 6 mth and the remaining two strains showed loss of activity to only one of the eight indicator strains. Six producer strains, used as controls, have been tested at least twice weekly for more than 4 yr and another two producer strains have been tested similarly for more than 2 yr. These eight producer strains have been entirely consistent in their production of pyocines as judged by the patterns of inhibition they produced on the set of indicator strains.

To determine the stability of pyocine production in bacteria living in their natural habitats we have noted whether there is uniformity of pyocine type

PROGRESS IN PYOCINE TYPING

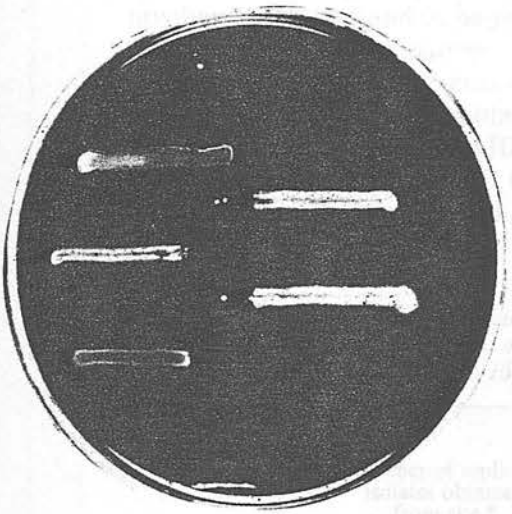

FIG. 1.—Pattern of inhibition given by a producer strain of pyocine type 1b incubated for 14 hr at 32°C during growth of the producer strain. All the indicator strains except A (top left) are inhibited.

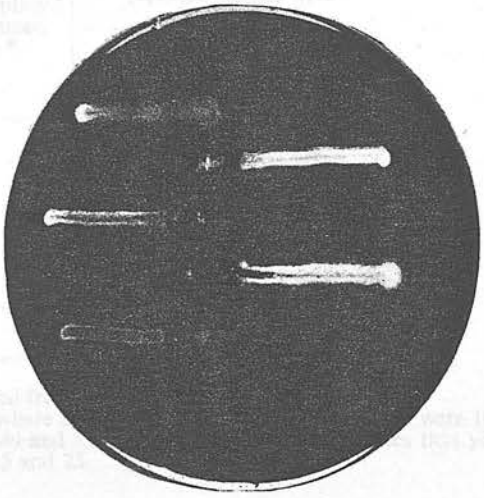

FIG. 2.—The pattern of inhibition obtained in a test identical to that outlined in fig. 1 except that the producer strain was incubated for 14 hr at 35°C. Although the same pattern of inhibition is discernible, the amount of resistant growth in the zones of inhibition has increased.

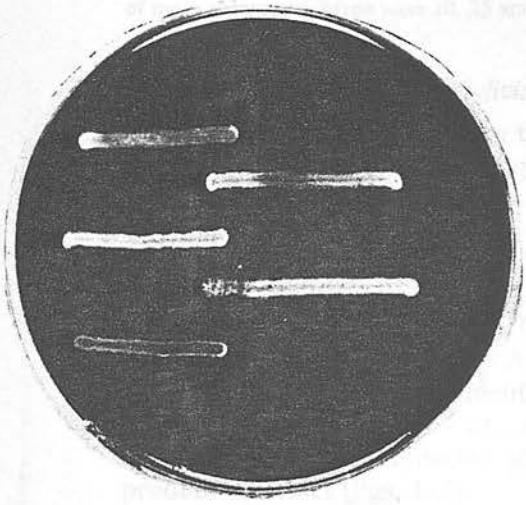

FIG. 3.—Pattern of inhibition obtained in a test identical to those in figs. 1 and 2, except that the producer strain was incubated for 14 hr at 37°C. None of the indicator strains was inhibited. The difficulty in removing all the growth of the producer strain after incubation at 37°C is apparent.

among strains of *Ps. pyocyanea* isolated from different cases in the same epidemic. During the last 4 yr we investigated 20 different epidemics, each involving between 5 and 90 patients, and found that every strain in each epidemic gave the same typing pattern. As another index of the stability of pyocine production *in vivo* we determined the constancy of pyocine type in replicate isolates of *Ps. pyocyanea* obtained from the same site in the same patient. The results are given in table III; they show that not all replicate isolates were of the same pyocine type as the original isolate from the same site. The explanation of this finding will be discussed below.

TABLE V

Constancy of subtype in sets of replicate isolates of pyocine type-1 strains of Pseudomonas pyocyanea obtained on different occasions from the same site in the same patient. Results from 190 sets of isolates from 162 patients

Number of replicate isolates obtained from site *	Number of sets where the replicate isolates were	
	all of one subtype	of more than one subtype
1	95	4
2	23	0
3	12	5
4	10	0
5	8	3
6	4	0
7	3	0
8	3	1
>8 †	16	3

* Excluding the first strain isolated from the site.

† Number of isolates from sites where all isolates were of the same subtype were 10, 10, 10, 11, 11, 11, 13, 14, 15, 16, 17, 17, 19, 22, 44 and 55. Number of isolates from sites that yielded strains of more than one subtype were 10, 25 and 25.

Subdivision of pyocine type-1

The distribution of pyocine types of *Ps. pyocyanea* collected between 1961 and 1967 is given in table IV. Type-1 strains were the commonest and accounted for more than one-third of all strains examined. As already stated, the method of subdividing pyocine type-1 strains is identical with that employed in the main typing procedure except that five additional indicator strains are used. By this modified method we have so far recognised eight distinct subtypes (a–h) of pyocine type 1 (table II). Strains in these subtypes are designated " type 1a ", " type 1b ", etc. As in the main pyocine typing procedure, subtyping is temperature-dependent, and the incubation of the producer strain must be done at 32°C for 14 hr, and certainly for not more than 18 hr, otherwise aberrant patterns of inhibition are obtained and some strains do not appear to produce pyocines (figs. 1–3).

The eight subtypes of pyocine type 1 are apparently stable in their pyocine-producing abilities when they are cultured and stored *in vitro*, since there was no alteration, either quantitative or qualitative, in the patterns of inhibition when strains were retested after several months' storage on nutrient agar at room temperature (*c.* 21°C). The in-vivo stability of pyocine production in the eight subtypes was determined by observation of the constancy of pyocine subtype in replicate isolates obtained from the same site in the same patient. The results are summarised in table V. As in the observations on the main series of types (table III), not all replicate isolates were found to be of the same pyocine subtype as the original isolate from the same site.

The distribution of subtypes of pyocine type-1 strains is given in table VI.

TABLE VI

Distribution of subtypes in 795 pyocine type-1 strains of Pseudomonas pyocyanea isolated from patients in hospital and patients in the open community *

Pyocine subtype of type-1 strains	Percentage of strains belonging to stated pyocine subtype in patients † in		
	hospital (662 strains)	the open community (133 strains)	either hospital or the open community (795 strains)
a	5·7	2·3	5·7
b	29·6	36·2	30·3
c	30·2	17·3	26·0
d	18·1	15·0	17·4
e	2·7	1·5	2·6
f	4·4	12·0	5·7
g	2·4	2·3	2·9
h	4·8	6·7	5·9
u/c	2·1	6·7	3·5

* Strains collected during the period 1961–1967.

† Only one strain from each patient is represented. Strains from cases where more than one subtype was isolated are not included. Only one strain from each epidemic is represented.

u/c = Unclassifiable strains, i.e., solitary strains giving patterns other than those shown in table II.

Occurrence of more than one pyocine type or subtype of Pseudomonas pyocyanea in the same lesion

We have previously suggested (Gillies and Govan) that when a series of replicate isolates of *Ps. pyocyanea* from the same site in the same patient includes one or more of the isolates differing in pyocine type from that of the original isolate, it is possible that the patient is yielding different strains with which he had been separately infected. This seems as likely an explanation of the findings as is the supposition that strains are variable in their pattern of pyocine production.

We have attempted to collect evidence in support of our hypothesis by typing numerous colonies of *Ps. pyocyanea* from individual diagnostic plates. The results are given in table VII. An average of six (range 2–33) colonies per plate were typed and the results are given separately for strains isolated from

patients sick at home (home series) and those in hospital (hospital series). It was found that the frequency with which more than one pyocine type or subtype was present in a plating was more than twice as great in platings from specimens in the hospital series (1 in 9·8) as in platings from specimens in the home series (1 in 22·1).

TABLE VII

Frequency with which more than one pyocine type of Pseudomonas pyocyanea was found in specimens from patients at home and patients in hospital

Nature of specimen	Number of specimens from patients				Frequency ratio * in	
	at home		in hospital		home cases	hospital cases
	with more than one type	Total	with more than one type	Total		
Faeces	10	158	1	18	1/15·8	1/18
Urine	2 †	77	10	76	1/38·5	1/7·6
Others ‡	1	53	12	131	1/53	1/10·9
Total	13	288	23	225	1/22·1	1/9·8

* Total number of specimens in category divided by the number of these specimens from which strains of more than one pyocine type were isolated.
† These two patients had recently returned home from hospital.
‡ Swabs from ears, throats, burns and bedsores; blood cultures, cerebrospinal fluid, and sputum.

DISCUSSION

The continuation of our studies on the pyocine typing of strains of *Pseudomonas pyocyanea* has confirmed the validity of the method for epidemiological studies. Our technique has been used successfully by workers in Britain (Brumfitt, Percival and Leigh, 1967; Tinne *et al.*, 1967) and abroad (Fierer, Taylor and Gezon, 1967), and a new pyocine type, type 37, has been added after strains with a distinctive pattern of inhibition (table I) had been isolated from patients in several centres on different occasions during the last 2 yr.

The sensitivity and usefulness of pyocine typing are enhanced by the fact that it is now possible to recognise eight subtypes within type 1, which is the type most commonly encountered in our series. Workers in other countries tell us that pyocine type-1 strains are also the most prevalent strains in their areas. The critical nature of the conditions under which the pyocine-producing strains should be incubated in typing tests has been emphasised previously (Gillies and Govan, 1966) and these conditions have been found equally important in the subtyping of pyocine type-1 strains. In the experiment shown in figs. 1–3 the producer strain of *Ps. pyocyanea* (pyocine type 1, subtype b; i.e., type 1b) was identical on all three plates, but the plates were incubated at different temperatures, namely 32°, 35° and 37°C, for 14 hr during pyocine production. The

clear-cut inhibition of all indicator strains except strain A when the producer strain was incubated at 32°C (fig. 1) contrasts strongly with the lack of inhibition of any of the indicator strains when incubation of the producer strain was at 37°C (fig. 3). When incubation of the producer strain was at 35°C (fig. 2) areas of central growth occurred in the inhibition zones of indicator strains C, D and E. This latter phenomenon was noted by Wahba (1963), who attempted to eliminate it by adding a triad of chemicals to his medium. However, we have shown that by adhering to the lower temperature for incubation of the producer strain and by restricting the period of incubation to not more than 18 hr it is possible to obtain clear-cut patterns of inhibition of the indicator strains in the subtyping of pyocine type-1 strains. Among others, Dixon (personal communication) and Phillips *et al.* (1968) have found our method of subdividing strains of pyocine type 1 to be reliable and epidemiologically valuable.

It has already been noted that not all replicate isolates of *Ps. pyocyanea* from any one site in a patient may belong to the same pyocine type or subtype as the strain originally isolated from that site (tables III and V). Two possible reasons for this lack of constancy of pyocine type or subtype are (1) a variability of pyocine production in a single strain and (2) the infection of a patient with two or more strains originally different in type. While collecting the information summarised in tables III and V we noted that strains of more than one pyocine type were isolated from the same lesion more frequently in patients being treated in hospital than in patients being treated at home. It seems unlikely that the patient's environment *per se* would dictate instability of pyocine production. On the other hand, patients in the hospital environment are more liable to become infected with different strains of *Ps. pyocyanea* than are patients being treated at home. Another significant finding in our prospective survey (table VII) is that the frequency with which more than one pyocine type is found in the same lesion varies between the different categories of specimens more greatly in the home series than in the hospital series. The only similarity between the two series is in the results for faecal specimens. These specimens were from patients suffering from gastro-intestinal upset and most of them yielded bacteria of one or other species belonging to the family Enterobacteriaceae; the isolation of *Ps. pyocyanea* from them was therefore considered to be incidental.

Although in table VII two patients have been allocated to the home series as cases from whose urine more than one pyocine type of *Ps. pyocyanea* had been isolated, it must be stated that both patients had recently returned from hospital, one after prostatectomy and the other, a woman, after investigation of genito-urinary disease. Thus the great frequency with which more than one pyocine type of *Ps. pyocyanea* was found in the hospital series, coupled with the fact that there was relatively little difference in such frequency among the various types of specimens in that series, reflects the great risk of patients becoming infected with new strains of *Ps. pyocyanea* when they are treated in the hospital environment. If it were postulated that inconstancy of pyocine type was due to instability of pyocine production *in vivo* it would be very difficult to explain the contrasting findings in the two series of patients.

SUMMARY

Further experience of the typing of strains of *Pseudomonas pyocyanea* (*Ps. aeruginosa*) by their production of pyocines has confirmed the value of pyocine typing for epidemiological studies.

The introduction of five new indicator strains has made it possible to distinguish eight subtypes of the commonly occurring pyocine type 1. The subtyping of the type-1 strains shows the same dependence on use of the correct temperature and duration of incubation of the producer strain as does the main typing procedure.

In studies of the reliability of the typing and subtyping methods for epidemiological purposes it was noted that not all strains of *Ps. pyocyanea* isolated from the same site in the same patient were of the same pyocine type or subtype. When several colonies of *Ps. pyocyanea* from individual diagnostic plates were typed it was found that more than one pyocine type or subtype was more frequently present in the same lesion in patients in hospital than in patients being treated at home. This finding suggests that the occurrence of more than one pyocine type or subtype of *Ps. pyocyanea* in a patient is generally due to his having been separately infected with strains of more than one type, rather than to instability of pyocine production in a single infecting strain.

The number of colleagues who have supplied us with strains of *Pseudomonas pyocyanea* is too great for us to thank them individually, but we wish particularly to thank Dr J. H. Bowie and other colleagues in the Bacteriology Department, Royal Infirmary, Edinburgh. Mr D. O. Brown has given us unstinted technical help. These investigations were made possible by Public Health Service Grants AI-04833-02 and -03 from the National Institute of Allergy and Infectious Diseases and by a grant from the Scottish Hospitals Endowments Research Trust.

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PYOCINE TYPING OF MUCOID STRAINS OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CHILDREN WITH CYSTIC FIBROSIS

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CHILDREN suffering from cystic fibrosis (CF) are very susceptible to pulmonary infections especially with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Huang, Van Loon and Sheng, 1961; Iacocca, Sibinga and Barbero, 1963), and strains of the latter organism isolated from such infections are unusual in that many of them form very mucoid colonies on solid media (Doggett, 1969; Doggett, Harrison and Carter, 1971). Mucoid and non-mucoid strains of *P. aeruginosa* are often found in the same specimen, and as the infection progresses the mucoid strain usually comes to predominate with a concomitant decline in the condition of the patient (Doggett *et al.*, 1966). The relationship between mucoid and non-mucoid colonial forms has not been firmly established, but some workers believe them to be variants of the same strain (Feigelson and Pecau, 1966). Characterisation of the mucoid strains is difficult, but Diaz, Mosovich and Neter (1970) reported that, when serological typing was possible, mucoid and non-mucoid isolates from the same specimen belonged to the same serotype. Other authors have suggested that the mucoid organism is another and super-infecting strain that finds the environment of the cystic fibrotic lung particularly suited to its growth (Doggett, Harrison and Wallis, 1964).

This paper reports a preliminary investigation in which pyocine typing (Gillies and Govan, 1966; Govan and Gillies, 1969) was used to examine the relationship between mucoid and non-mucoid strains.

MATERIALS AND METHODS

Isolation of strains. Sputum specimens were obtained from patients attending the CF Clinic at the Royal Hospital for Sick Children, Edinburgh, or strains of *P. aeruginosa* isolated from the same patients were sent to us from the Central Microbiological Laboratories, Edinburgh. Specimens were plated on horse-blood agar, MacConkey's medium and cetrimide agar (0·03 per cent. cetrimide in nutrient agar), and incubated aerobically at 37°C for 18 hr. Colonies from cetrimide plates were picked and their identification confirmed as *P. aeruginosa* by the oxidase test (Kovács, 1956) and by their pigment production on King's media (King, Ward and Raney, 1954). The MacConkey plates were also examined for *P. aeruginosa*, particularly when there was little or no growth on the cetrimide agar.

Pyocine typing. Strains were typed within a few days of isolation, and sub-cultures were stored on nutrient agar slopes in screw-capped bottles at room temperature (*c.* 22°C) in the dark. The method of pyocine typing described by Gillies and Govan was used. In addition to the tryptone soya blood agar medium used by these authors, various other media were tested—a minimal medium (Clarkson and Meadow, 1971) incorporating 0·1 per cent. of glucose, sodium glutamate or sodium succinate as sole carbon source, and also a very simple medium containing L-asparagine (Georgia and Poe, 1931).

Production of pyocines in liquid culture. A 100-ml volume of tryptone soya broth in a 2-l flask was inoculated with 2 ml of an overnight broth culture of the strain to be examined and then incubated in an orbital incubator at 37°C and 80 r.p.m. for 4 hr. Mitomycin C (1·5 μg per ml final concentration) was added to some cultures as an inducing agent. For

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both induced and uninduced cultures, incubation was continued for a further 4 hr and then the lysates were centrifuged to remove the cells; the supernates containing the pyocines were sterilised with 5 per cent. (v/v) chloroform for 15 min. and the chloroform was then allowed to settle and the supernates were decanted.

Detection of pyocine in liquid cultures. Four-hour broth cultures of the indicator strains, nos. 1–8 (Gillies and Govan), were used to make lawns on nutrient agar plates containing 9 ml of agar in plastic petri dishes (diameter 9 cm). Doubling dilutions of the pyocine extract were prepared in sterile physiological saline and 0·02-ml drops of each dilution were spotted on to the well-dried indicator lawns. The drops were allowed to dry and the plates were incubated aerobically at 37°C overnight. The presence of pyocine activity was indicated by zones of inhibition on the indicator lawns.

Detection of bacteriophage. When bacteriophages that attacked the indicator strains were present in the pyocine extracts, they were recognised on the pyocine-titration plates by their plaque-forming ability.

TABLE

Pyocine typing of mucoid and non-mucoid P. aeruginosa strains isolated from the sputum of children with cystic fibrosis

Patient number	Character of *P. aeruginosa*					
	Colony form		Pyocine type on TSBA*		Pyocine type after induction in broth	
	Mucoid	Non-mucoid	Mucoid	Non-mucoid	Mucoid	Non-mucoid
1	+	+	1/c	1/c	1/c	1/c
2	+	+	u/t	13	13	13
6	+	+	u/t	3	3	3
11	+	+	(3)†	3	3	3
33	+	+	u/t	1/h	1/h	1/h
34	+	+	(1/h)†	1/h	1/h	1/h
5	+	−	(29)†	−	29	−
10	+	−	u/t	−	1/b	−
42	+	−	u/t	−	5	−

+ = Strain isolated; − = strain not isolated; u/t = untypable.
* TSBA = Tryptone Soya Agar with 5 per cent. horse blood.
† Indicator strains weakly inhibited.

RESULTS

Pseudomonas aeruginosa was isolated from the sputum of nine of the 25 patients examined and from six of these nine specimens mucoid and non-mucoid strains were isolated simultaneously. The non-mucoid strains were all typable by the standard typing technique, whereas five of the six mucoid strains were untypable or gave very indistinct patterns (the table).

When the typing method was modified by the use of different growth media, all the strains grew on each of the substrates tested but there was little reduction in the amount of slime produced by the mucoid strains. When sodium succinate was used as the sole carbon source, it was possible to show some inhibition of the indicator strains by the mucoid strains but the results were neither clear nor reproducible.

Growth of mucoid strains in broth culture at 32°C resulted in the spontaneous production of pyocines in low concentration; higher concentrations and clearer results were obtained when such broth cultures were induced with Mitomycin C. Identical inhibition patterns corresponding to those of recognised pyocine types were obtained with induced and un-

induced cultures. In each of the six patients, the mucoid and non-mucoid strains isolated from the same specimen were found to belong to the same pyocine type (the table). When replicate specimens were obtained (2–9 specimens per patient), five of the six patients were found to carry the same pyocine type of *P. aeruginosa* for 2–8 mth.

In addition, three patients produced only mucoid strains of *P. aeruginosa*, and when these strains were characterised by extraction and assay of their pyocines, they were found to belong to recognised pyocine types (the table).

To confirm that the typing of strains after induction with Mitomycin C did not inevitably result in pyocine production, attempts were made to induce pyocine production in 12 wild strains of *P. aeruginosa* that were untypable by the standard technique. No inhibition of the indicator strains due to pyocine activity was observed with any of these strains.

Lysogeny is very common in *P. aeruginosa* (Holloway, 1969), and bacteriophages attacking the indicator strains were found in many of the cystic fibrosis strains examined. The similar range of activity of the phages from mucoid and non-mucoid strains from the same patient was taken as further evidence that these strains were related. It was possible to distinguish between inhibition due to pyocine and due to phage at higher dilutions of the extract where distinct phage plaques were observable, in contrast to the gradual thinning of the indicator lawn due to pyocine inhibition.

Discussion

The origin of the mucoid variant of *P. aeruginosa* in patients with CF has been a subject of controversy, and although most workers agree that the mucoid and non-mucoid colonial forms are variants of the same strain, this has not been conclusively demonstrated (Feigelson and Pecau, 1966); the present study provides evidence that they are.

Strains of *P. aeruginosa* may be typed serologically, by their sensitivity to bacteriophages and by pyocine typing. The first two of these methods may be used in combination and depend on properties of the outer surface of the cell, respectively of the O antigens and of the receptor sites for bacteriophages. Mucoid strains are not easily typed by these methods because of the mucus surrounding the cell surface. Pyocine typing depends on the release of pyocines from the cell into the surrounding medium and the subsequent action of these pyocines on the indicator strains.

Gillies and Govan (1966) found that 90–95 per cent. of *P. aeruginosa* strains were typable by their technique. In this study the non-mucoid strains of CF origin were all typable, but only a small number of the mucoid isolates could be typed by the standard method. Rosenstein and Drachman (1968) were able to type 79 per cent. of *P. aeruginosa* strains from CF patients, but they did not state the number of mucoid strains examined. Schwarzmann and Boring (1971) suggested that mucoid strains may be apyocinogenic. We consider it more likely that the mucoid strains produce pyocines and that these are prevented from diffusing into the medium by the mucus surrounding the cells. Some of the mucoid strains in this series were found to be typable by the standard method, and this may be related to the size and nature of the pyocines produced, to the amount of slime elaborated by the organisms or to both. When simple synthetic media were used for typing, some pyocine-inhibition patterns were observed, suggesting that the mucoid strains were not apyocinogenic. In shaken broth cultures of mucoid strains the slime was dispersed and the pyocines produced by the cells were released into the medium. The presence of the pyocines was demonstrated by the assay of the culture supernates against the standard indicator strains and the inhibition patterns produced could be recognised as those of standard pyocine types.

Summary

The relationship between mucoid and non-mucoid strains of *Pseudomonas aeruginosa* isolated from the sputum of children with cystic fibrosis was investigated by typing the strains by pyocine production. The mucoid strains were usually untypable by the standard technique, but extraction and assay of the pyocines produced in liquid cultures showed

that the strains belonged to recognised pyocine types. When mucoid and non-mucoid strains occurred simultaneously, they were of the same pyocine type; it is suggested that they are variants of the same strain.

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Studies on the Pyocins of *Pseudomonas aeruginosa*: Morphology and Mode of Action of Contractile Pyocins

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SUMMARY

The adsorption and inhibitory action of pyocin 21 on sensitive cells of *Pseudomonas aeruginosa* (*P. pyocyanea*) were observed by fluorescence microscopy and by using agar cultures. By electron microscopy, preparations from four pyocinogenic strains of *P. aeruginosa* were seen to include contracted and uncontracted particles, isolated tail cores and sheaths. The importance of adsorption and contraction of the particles on the surface of sensitive bacteria for the inhibitory action of these pyocins is suggested by the following evidence: (1) Particles adsorbed to sensitive bacteria at 0 °C, but contraction of particles was not then observed and the bacteria were not disrupted. (2) Pyocin particles did not adsorb to pyocin-resistant bacteria. (3) The ability to adsorb to sensitive bacteria and to kill them was absent after removal of the contractile sheath with sodium dodecyl sulphate. (4) When treated with homologous antiserum, pyocin activity was neutralized and the agglutinated particles remained unadsorbed and uncontracted. (5) Bacterial receptors for contractile pyocins contain lipopolysaccharide; such extracts from sensitive, but not from resistant, cells of *P. aeruginosa* inactivated pyocins, and contracted particles were seen adsorbed to lipopolysaccharide fragments.

INTRODUCTION

Bacteriocins are antibiotic substances produced by certain strains of bacteria and differ from classical antibiotics in that their lethal activity is restricted to other strains of the same or closely related species. Jacob (1954) described a bacteriocin from a strain of *Pseudomonas aeruginosa* and called the substance pyocin; synthesis of the pyocin, C10, was inducible with u.v. irradiation. Kageyama (1964) reported that mitomycin C could induce the synthesis of pyocin R and by electron microscopy he demonstrated rod-like particles in a purified pyocin preparation. Further electron micrographs of pyocins (Ishii, Nishi & Egami, 1965; Higerd, Baechler & Berk, 1967; Govan, 1968) have revealed that one type of pyocin resembles headless contractile bacteriophages, e.g. the T-even coliphages, consisting of a contractile sheath surrounding an inner core. Higerd, Baechler & Berk (1969) demonstrated attachment of pyocin C9 to bacterial wall fragments and reported an apparent correlation between uncontracted particles and lethal activity. Yui, Ishii & Egami (1969) reported that the lethality of pyocin R was lost after treatment with *p*-chloromercuribenzoate and restored by addition of β-mercaptoethanol; electron microscopy revealed a corresponding change in the proportion of contracted to uncontracted particles.

The importance of *Pseudomonas aeruginosa* as an opportunist pathogen has become increasingly obvious in recent years and the incidence of Pseudomonas infections has also risen considerably (Asay & Koch, 1960). Holloway (1960) reported that pyocinogeny occurred frequently in strains of *P. aeruginosa* and suggested that pyocin production might

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be a suitable epidemiological marker for this species. Such a method of characterizing strains of *P. aeruginosa*, pyocin typing, employs a standard set of strains of *P. aeruginosa* to detect pyocinogeny in a test strain (Gillies & Govan, 1966; Govan & Gillies, 1969); the method is now used in many centres (Phillips, Lobo, Fernandes & Gundara, 1968; Csiszar & Lanyi, 1970; Ziv, Mushin & Tagg, 1971; Neussel, 1971).

This paper describes the morphology and mode of action of several contractile pyocins obtained from strains of *Pseudomonas aeruginosa* belonging to different pyocin types.

METHODS

The pyocinogenic strains used were *Pseudomonas aeruginosa* 21, 355, 149 and 430; these belong to pyocin types 1, 3, 9 and 10 respectively (Gillies & Govan, 1966). The eight indicator strains of *P. aeruginosa* (1 to 8) used in the pyocin-typing technique (Gillies & Govan, 1966) were used to assay pyocin activity.

Extraction of non-induced pyocin by freezing and thawing. The pyocinogenic strain of *Pseudomonas aeruginosa* was inoculated over the entire surfaces of several Petri dishes containing tryptone soya agar (Oxoid) and the plates were incubated for 18 h at 32 °C; thereafter they were frozen at −70 °C for 1 h and then allowed to thaw at room temperature before the expressed fluid was removed with a sterile Pasteur pipette and centrifuged at 1200 *g*. The supernatant, containing pyocin activity, was treated with 5 % (v/v) chloroform to kill any remaining bacteria.

Induction of pyocin in fluid media. The medium used was tryptone soya broth (Oxoid) or sodium glutamate broth (Kageyama & Egami, 1962). A 2 ml portion of a static overnight culture of the pyocinogenic strain was added to 200 ml of sterile broth and incubated with agitation for 2 to 3 h at 32 °C. Mitomycin C was added to a final concentration of 1 μg/ml and incubation continued until lysis of the culture occurred, normally after a 3 to 4 h incubation period. The lysate was centrifuged at 2400 *g* for 30 min to remove bacterial debris and the supernate treated with 5 % (v/v) chloroform. The supernatant fraction was designated crude pyocin. Control cultures containing no mitomycin C were also investigated.

Purification of pyocins. Crude pyocin lysates were purified by a modification of the method of Kageyama & Egami (1962).

After treatment with 5 % (v/v) chloroform, one litre of lysate was treated with 60 ml of M-$MnCl_2.4H_2O$ added slowly during agitation and the pH adjusted to 7·5 with N-sodium hydroxide. The precipitate containing viscous material was removed by centrifugation at 2400 *g* for 15 min. The supernatant fraction was designated 'partially purified pyocin'. Further purification was carried out by adding ammonium sulphate to 70 % saturation and allowing it to stand overnight at 4 °C. The precipitate, containing pyocin activity, was collected by centrifugation for 30 min at 2400 *g* at 4 °C. The precipitate was dissolved in 50 ml of 0·01 M-tris (hydroxymethyl) methylamine buffer (pH 7·5) containing 0·01 M-$MgCl_2.6H_2O$ and 0·01 M-$MgSO_4.7H_2O$, and dialysed against 2 l of the same buffer overnight at 4 °C. If necessary the resulting preparation was clarified by centrifugation (2400 *g* for 15 min at 4 °C).

The pyocin preparation was then centrifuged for 90 min at 100000 *g* under refrigeration (Spinco, type 40 rotor). The gelatinous sediment was gently dissolved in 20 ml of buffer and chromatographed on DEAE-cellulose (Pharmacia, Uppsala, Sweden) previously washed and equilibrated with the same buffer. An 8 ml sample of pyocin was applied to the column (28 × 1·5 cm) and allowed to adsorb for 1 h; 200 ml of buffer were passed

through the column to remove material not adhering to the cellulose and the sample was then eluted with 800 ml of a sodium chloride gradient (0 to 1·0 M in 0·01 M-buffer). The u.v. absorbancy of fractions at 280 nm was measured with a Unicam SP 500 spectrophotometer and, after assay for pyocin activity, appropriate fractions were dialysed against tris buffer to remove sodium chloride. This purified pyocin was then concentrated by a further cycle of ultracentrifugation.

Assay of pyocin activity. A 4 h nutrient broth culture at 37 °C of a pyocin-sensitive strain of *Pseudomonas aeruginosa* (containing approximately 10^8 organisms/ml) was used to flood the surface of a nutrient agar plate (9 ml of medium contained in an 8·5 cm plastic Petri dish), excess broth culture was removed with a sterile Pasteur pipette and the bacterial lawn allowed to dry at room temperature. Doubling dilutions of the pyocin preparation were made in sterile saline (0·85 %) and a drop of each dilution applied to the surface of the plate by means of a calibrated platinum-tipped pipette delivering 0·02 ml per drop. When the drops had dried the plates were incubated overnight at 37 °C. The titre of pyocin activity (units/ml) was read as 50 times the reciprocal of the highest dilution causing complete inhibition of the sensitive strain.

Agar-slide technique. Sterile nutrient agar was poured into a channel, measuring 8·0 × 2·2 × 0·1 cm, in a mould. Before the agar gelled, glass microscope slides were placed at right-angles across the agar channel, taking care that no air bubbles formed. After 15 min when the agar had gelled, the slides were inverted bearing a uniform block of nutrient agar, and excess agar was cut away.

A drop of fluid taken from a 4 h nutrient broth culture of *Pseudomonas aeruginosa* strain 6, 8 or 21 was placed on the agar block together with a drop of pyocin 21 preparation (3200 units/ml) obtained by the freezing and thawing technique. After absorption, a cover glass was applied to the agar surface, excess agar removed with a scalpel and the edges of the block sealed with molten paraffin wax. The preparation was incubated at 37 °C in a moist chamber and examined at regular intervals by phase-contrast microscopy in a Leitz Ortholux microscope, Vernier readings being used to locate fields of view.

Preparation of pyocin antisera. Rabbits were inoculated subcutaneously with 1 ml volumes, containing 0·5 ml of purified pyocin 21, titre 200000 units/ml, mixed with an equal volume of Freund's complete adjuvant; another rabbit received the same amount of pyocin subcutaneously, without adjuvant, and a similar dose after seven days. Rabbits were bled before immunization and at various intervals afterwards.

Effect of antiserum on pyocin activity. Doubling dilutions of antiserum were prepared in 0·25 ml volumes of sterile saline. To each dilution was added 0·25 ml of pyocin 21, twice as much as caused confluent lysis of the sensitive strain 8. The mixtures were incubated at 37 °C in a water bath for 1 h and then assayed for pyocin activity.

Effect of lipopolysaccharide on pyocin activity. Ether-extracted lipopolysaccharide (LPS) from the pyocin-sensitive strains 1 and 8 was dissolved to a concentration of 4 mg/ml in 1 M-ammonium acetate. Then 100000 units of partially purified pyocin (21 or 430) in 0·5 ml of 0·1 M-ammonium acetate were mixed with 0·5 ml of LPS and incubated in a water bath at 37 °C for 1 h. Doubling dilutions were made in sterile saline and the titre of pyocin activity measured. Pyocin 21 was also examined after treatment with LPS from the resistant strain 6.

Effect of sodium dodecyl sulphate on pyocin activity. Doubling dilutions of 2 % sodium dodecyl sulphate (SDS) were prepared in 0·5 ml volumes of distilled water and 100000 units of partially purified pyocin 21 in 0·5 ml volumes were added to each dilution. The mixtures were incubated at 37 °C in a water bath for 1 h and assayed for pyocin activity.

Fluorescent antibody studies. A 0·5 ml volume of purified pyocin 21 containing 100000 units/ml was added to 2 ml of a 4 h peptone–water culture of strains 6, 8 or 21. After 10 min at 37 °C the preparation was centrifuged for 45 min at 2850 *g* and the bacterial cells resuspended in 2 ml of 0·01 M-tris buffer. Then 0·5 ml of the gamma globulin component of pyocin antiserum (neutralization titre of 4000), purified according to the method of Campbell, Garvey, Cremer & Sussdorf (1963), was added; the contents were incubated for a further 45 min at 37 °C, recentrifuged, and the pellet suspended in 1 ml of 0·01 M-phosphate buffer (pH 7·1). A heat-fixed smear was prepared on a glass slide and covered with anti-rabbit gamma globulin labelled with fluorescein isothiocyanate (FITC, Hoechst, Marburg) for 30 min. The smear was washed twice (5 min) in phosphate buffer and finally in distilled water. A Leitz Ortholux microscope, fitted with an HBO 200 lamp and a UG I filter, was used.

Electron microscopy. A negative-staining technique (Brenner & Horne, 1959) was used. Pyocin preparations were centrifuged for 90 min at 100000 *g* under refrigeration (Spinco, type 40 rotor). The sediment was resuspended in 1 M-ammonium acetate, pH 7·0. Equal volumes of this suspension and 2 % potassium phosphotungstate at pH 7·0 were mixed on the surface of a clean glass slide and a drop of the mixture transferred to the surface of a grid. After 30 s, excess fluid was removed and the preparation allowed to dry.

In order to observe the effects of contractile pyocin on sensitive and resistant strains of *Pseudomonas aeruginosa*, 0·5 ml of induced partially purified pyocin 21 (400000 units/ml) was added to 3·5 ml of a 4 h peptone–water culture of strains 8 or 6. After incubation at 37 or 0 °C for various durations the suspension was fixed in 4 % formaldehyde, centrifuged at 2850 *g* for 45 min and resuspended in 1 M-ammonium acetate. When the resistant strain 6 was treated with pyocin, centrifugation was also carried out at 100000 *g* for 90 min to precipitate both cells and pyocin particles.

To observe the interaction of pyocin and antisera, 0·5 ml of pyocin 21 antiserum (neutralization titre 1:500) and 0·5 ml of partially purified pyocin 21 (50000 units/ml) were mixed and incubated in a water bath for 1 h at 37 °C. To this was added a 3·5 ml vol. of a 4 h peptone–water culture of the sensitive strain 8 (10^8 organisms/ml) and the mixture held for a further 15 min at 37 °C before fixation with 4 % formaldehyde. After centrifugation for 90 min at 100000 *g* the sediment was resuspended in 1 M-ammonium acetate.

To observe the interaction of pyocins and LPS, 0·5 ml samples of pyocin and LPS (described earlier) were mixed and held for 1 h at 37 °C. Each suspension was centrifuged at 41000 *g* for 45 min and the sediment resuspended in ammonium acetate.

Electron micrographs were made at 50 kV using Ilford Special Contrasty plates.

RESULTS

Non-induced production of pyocin. Non-induced pyocins, extracted as described earlier from agar cultures of *Pseudomonas aeruginosa* strains 21, 355, 149 and 430, were tested for inhibitory activity against the eight indicator strains of *P. aeruginosa* used in the standard pyocin-typing technique. The patterns of inhibition which resulted were identical to those obtained with the same pyocinogenic strains using the standard pyocin-typing method. Similar results were obtained when the pyocinogenic strains were grown for 18 h in tryptone soya broth or sodium glutamate broth and the cell-free extracts tested for pyocin activity. When assayed quantitatively the production of pyocin by the four strains in agar or broth cultures reached a maximum of approximately 3200 units/ml after 12 h growth at 32 °C.

Induction of pyocin with mitomycin C. The addition of mitomycin C to give a final concen-

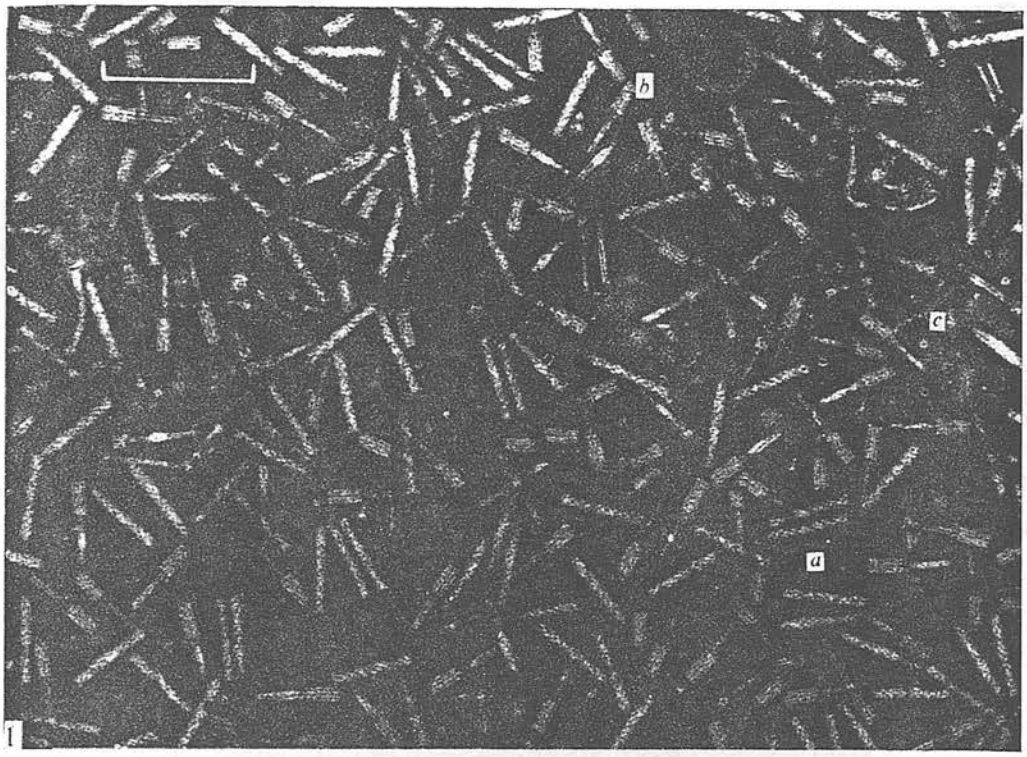

Fig. 1. Pyocin induced from *Pseudomonas aeruginosa* 355. Uncontracted, bullet-like particles possessing a base-plate (*a*), and contracted particles (*b*) consisting of a hollow or partially-filled core surrounded by a sheath can be seen. Short fibres extend from the base of the contracted sheath and the other end is sealed or partially open. Ringlets resembling minute cogwheels are also visible (*c*). Bar marker = 200 nm.

tration of 1 μg/ml in cultures of the pyocinogenic strains in the logarithmic phase led to a considerable increase in pyocin production. After bacterial lysis, crude pyocin preparations were regularly obtained with a titre of 400000 units/ml. After purification and ultracentrifugation, pyocin titres of $2{\cdot}5 \times 10^7$ units/ml could be obtained. The high-strength pyocin preparations obtained from these four pyocinogenic strains after induction with mitomycin C gave the same inhibition patterns against the eight indicator strains as did pyocin preparations obtained from non-induced cultures of the organisms.

Serological response to pyocin. After a single subcutaneous injection of purified pyocin 21 in Freund's complete adjuvant, the neutralization titre of sera from rabbits against the homologous pyocin rose from 1000 after nine days to a maximum of 32000 after two months. When a subcutaneous inoculation of pyocin was given without adjuvant and a second administered seven days later, a maximum titre of neutralizing antibodies of 1000 was reached 14 days after the second injection.

Pyocin and agar slide cultures of Pseudomonas aeruginosa. The inhibitory activity of non-induced pyocin 21, obtained by the freezing and thawing technique, was examined on agar slide cultures of *P. aeruginosa* indicator strains 6 and 8 which are respectively resistant and sensitive to this pyocin. Cells of indicator strain 8 failed to multiply in the presence of pyocin 21. In contrast, no activity was noted against cells belonging to indicator strain 6 or to the pyocinogenic strain 21.

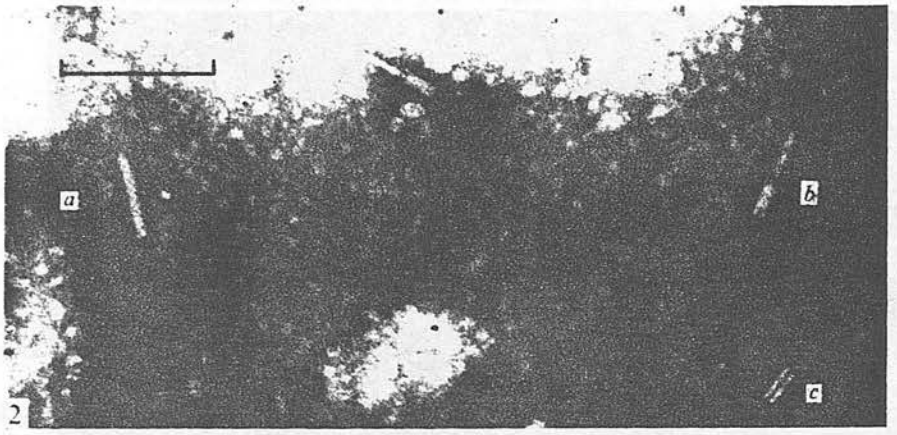

Fig. 2. Pyocin induced from *Pseudomonas aeruginosa* 21. A helical substructure is suggested in the uncontracted particle (*a*). The contracted particle (*b*) shows a hollow tail core enclosed in a sheath closed at one end and possessing hook-like fibres at the other. A discarded empty sheath is also visible (*c*). Bar marker = 200 nm.

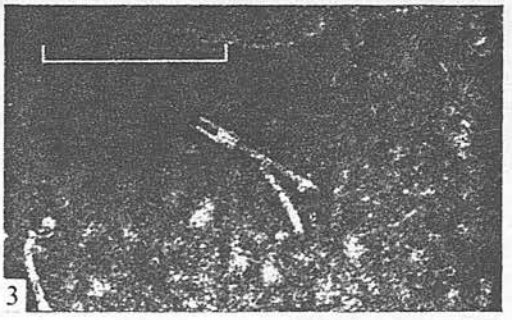

Fig. 3. Contracted pyocin particle in induced preparation from *Pseudomonas aeruginosa* 149; the partial disengagement of a tail core from its sheath is visible. Bar marker = 200 nm.

Detection of pyocin adsorption to sensitive bacteria by using a fluorescent-antibody technique. Cells of indicator strain 8 after reaction with pyocin 21, fluoresced when treated with a gamma globulin component of homologous antiserum followed by anti-rabbit gamma globulin conjugated with FITC. No fluorescence of bacteria was observed (i) when indicator strain 6, or the pyocinogenic strain 21, was used, (ii) in the absence of pyocin, or (iii) when pre-immune gamma globulin was used.

Electron microscopy of pyocin preparations

Electron microscopy of induced, partially purified pyocin preparations from the four pyocinogenic strains of *Pseudomonas aeruginosa* revealed the presence of numerous structures resembling the tail components of contractile bacteriophages. Both uncontracted and contracted forms were visible (Fig. 1). Contracted particles consisted of a core partially surrounded by a sheath. In most particles the core appeared empty but occasionally cores were seen to contain material. Uncontracted particles resembled bullets and a base-plate was visible at the broader end. Hook-like pins extended from the lower ends of contracted sheaths and occasionally several fibres were seen attached to the base-plate. Isolated

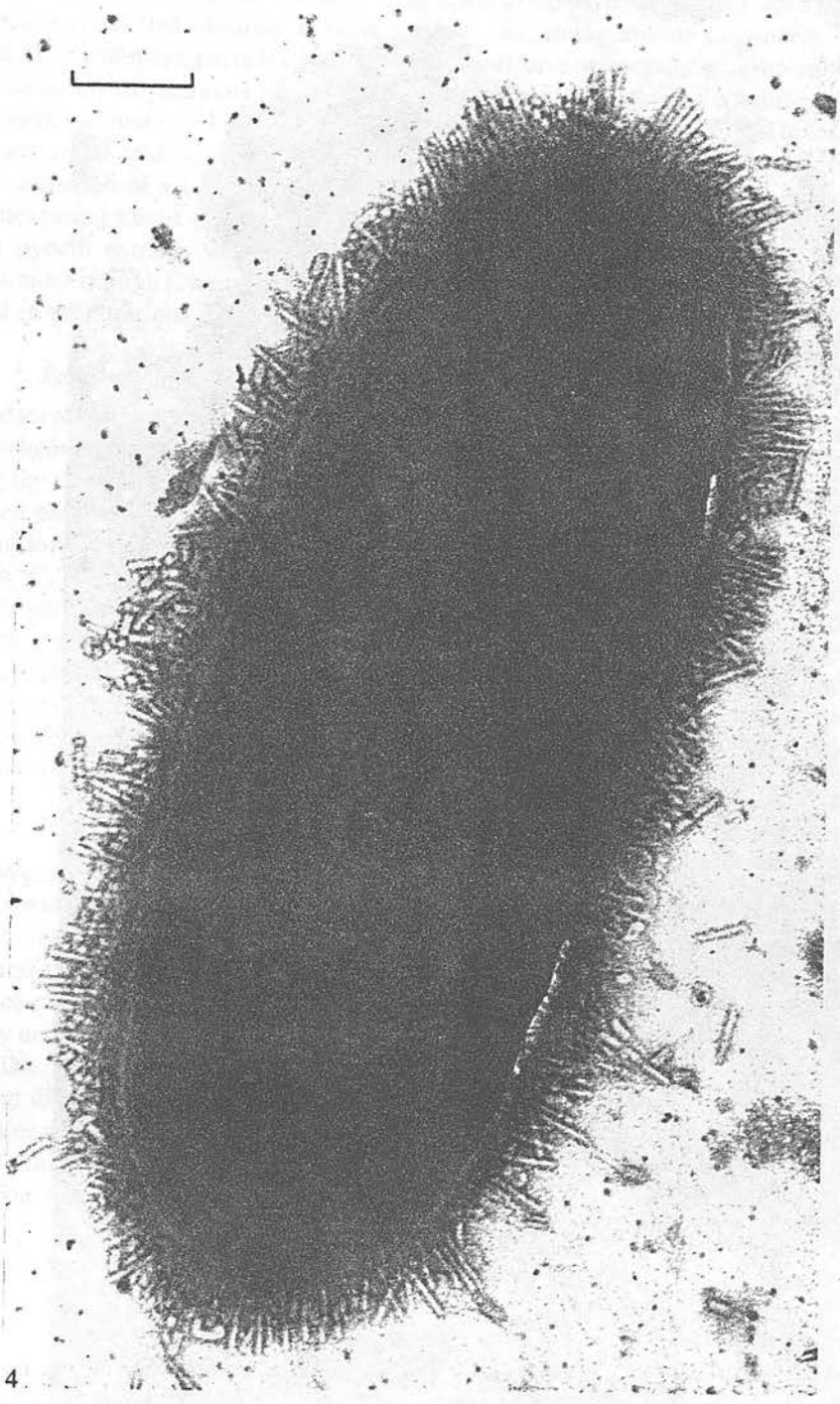

Fig. 4. *Pseudomonas aeruginosa* indicator strain 8 after 1 min contact (37 °C) with induced pyocin 21. The bacterial surface is surrounded by uncontracted pyocin particles. Bar marker = 200 nm.

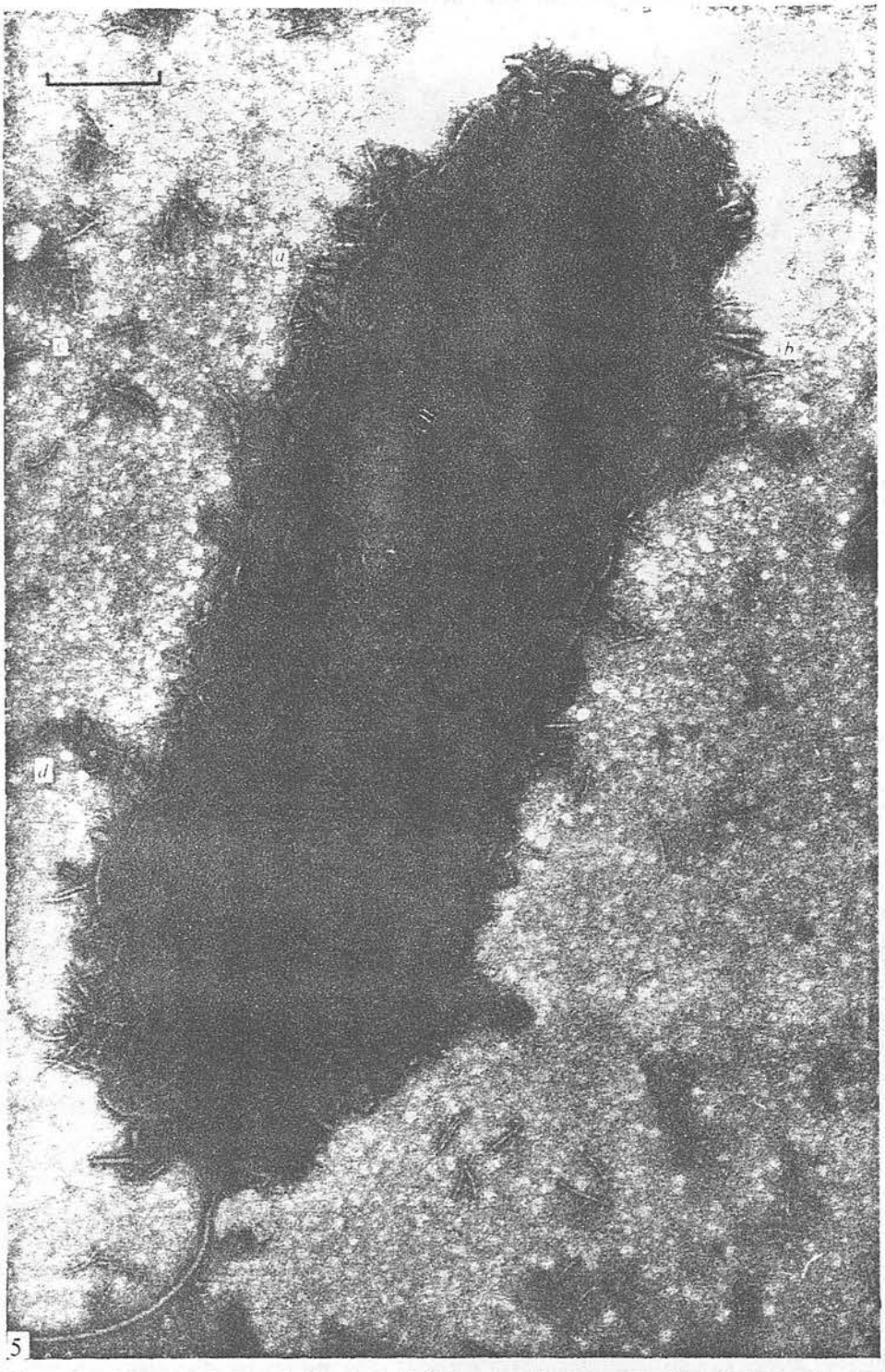

Fig. 5. *Pseudomonas aeruginosa* indicator strain 8 after 20 min contact at 37 °C with induced pyocin 21. The bacterial surface appears convoluted and covered with many pyocin particles. Contracted particles are visible (*a*) attached to the bacterial surface, and several uncontracted particles (*b*) can be seen close to the bacterial surface. Many discarded cores (*c*) and empty sheaths (*d*) are present in the vicinity of the bacterium. Bar marker = 200 nm.

contracted sheaths were observed and long sheath-like structures were seen composed of numerous connected sheaths. Hollow ringlets resembling minute cogwheels were also observed. The extended particles possessed a helical or horizontally striated substructure of approximately 20 subunits (Fig. 2). The partial disengagement of a tailpiece from its collar sheath is shown in Fig. 3. The majority of particles, regardless of the pyocinogenic strain used, measured approximately 100×15 nm in the uncontracted state; contracted particles consisted of a hollow tailpiece, approximately 100×7 nm, partially enclosed in a sheath measuring about 45×17 nm.

When pyocin extracts were prepared from non-induced cultures of the pyocinogenic strains, similar though fewer particles were observed. Intact bacteriophage particles were not observed in preparations from any of the strains examined.

Relationship between lethal activity and the nature of pyocin particles

The adsorption and inhibitory activity of pyocin 21 towards sensitive strains of *Pseudomonas aeruginosa* was investigated by fluorescence microscopy and the agar-slide technique; by using the electron microscope, additional features of this activity could be seen: (1) Uncontracted particles attached rapidly by the broader or base-plate end to sensitive bacteria. (2) If fixation was carried out immediately after addition of pyocin or if the system was held at 0 °C, no contraction of adsorbed particles was observed (Fig. 4). Fig. 5 shows a cell of *P. aeruginosa* indicator strain 8, 20 min after the addition of pyocin; the entire surface of the bacterium appears smothered with rod-like particles. A number of empty sheaths and detached tailpieces can be detected in the vicinity of the bacterium. The surface of the bacterium is grossly irregular as if disruption were imminent and, indeed, after 2 h incubation with the pyocin preparation, more than 90 % of the bacteria were seen to be completely disrupted.

The importance of contraction in pyocin activity

The pyocin produced by *Pseudomonas aeruginosa* strain 21 had no inhibitory effect on indicator strain 6 when tested by the standard pyocin-typing method or at higher concentrations after induction and purification. When pyocin 21 was mixed with indicator strain 6 (Fig. 6) and the system examined in the electron microscope, no attachment of particles to the surface of bacteria was observed and most of the particles remained uncontracted. The inhibitory activity of pyocin 21 against indicator strain 8 was destroyed after treatment with 0·02 % SDS. Electron microscopy showed that the particles had lost the outer contractile sheath and did not adsorb to the surfaces of sensitive bacteria. No change in lethal activity of pyocin or morphological alteration was observed after treatment with SDS at concentrations less than 0·01 %.

Homologous antiserum neutralized the activity of pyocin 21, and in the electron microscope (Fig. 7) agglutinated particles appeared in the uncontracted state and no particles were found adsorbed to sensitive bacteria.

The role of bacterial LPS as a receptor for contractile pyocins

After adding contractile pyocins 21 or 430 to sensitive cells of *Pseudomonas aeruginosa*, rosette-like formations of contracted particles were often observed attached either to the bacteria (Fig. 8) or to cell debris (Fig. 9). When a pyocin preparation was mixed with LPS from the sensitive strains of *P. aeruginosa* the pyocin was inactivated and electron microscopy (Fig. 10) revealed the attachment of contracted particles to LPS fragments. No loss of

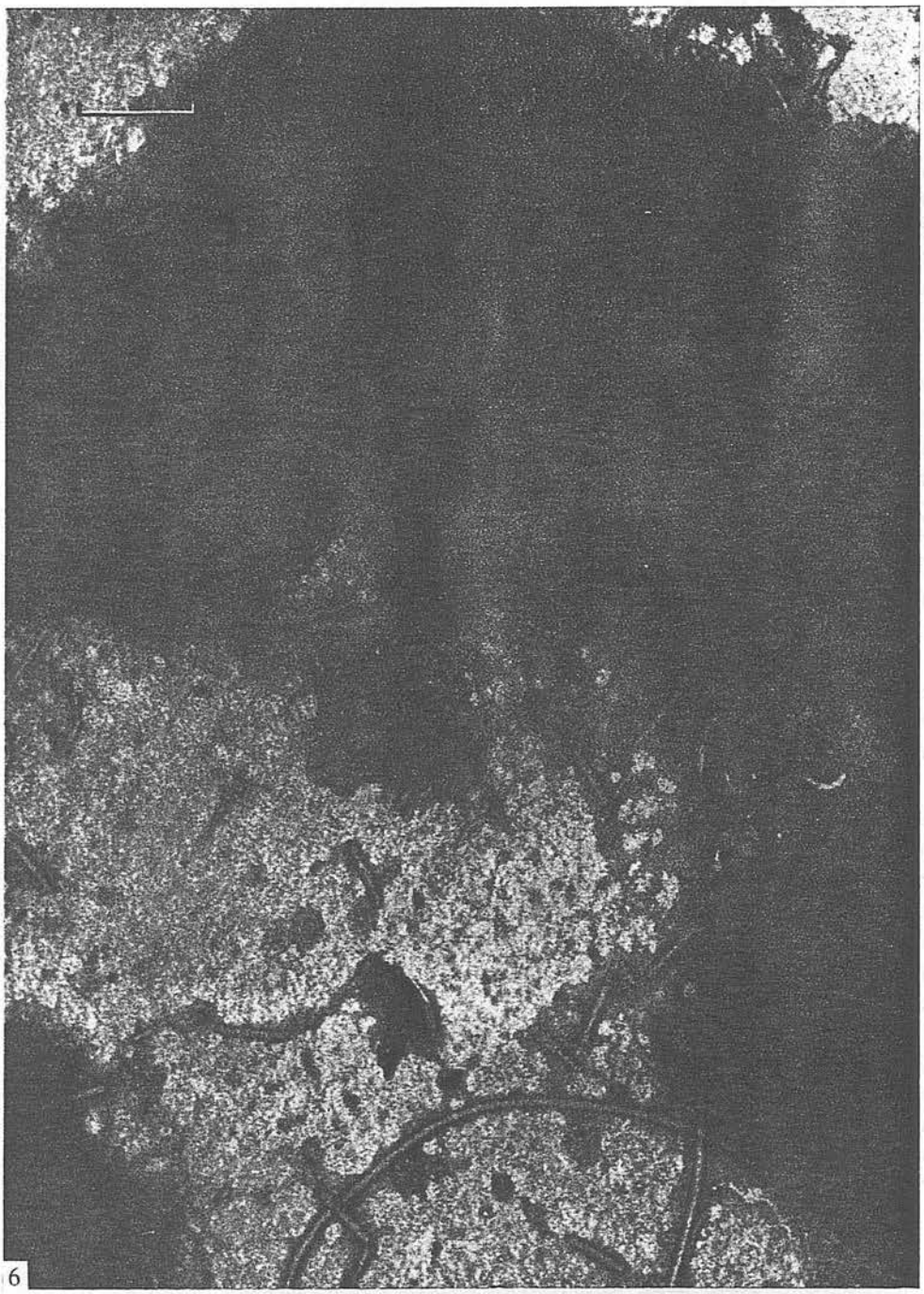

Fig. 6. *Pseudomonas aeruginosa* indicator strain 6 in the presence of induced pyocin 21. Indicator strain 6 is resistant to pyocin 21; the pyocin particles did not adsorb to the bacterial surface and most particles remained uncontracted. Bar marker = 200 nm.

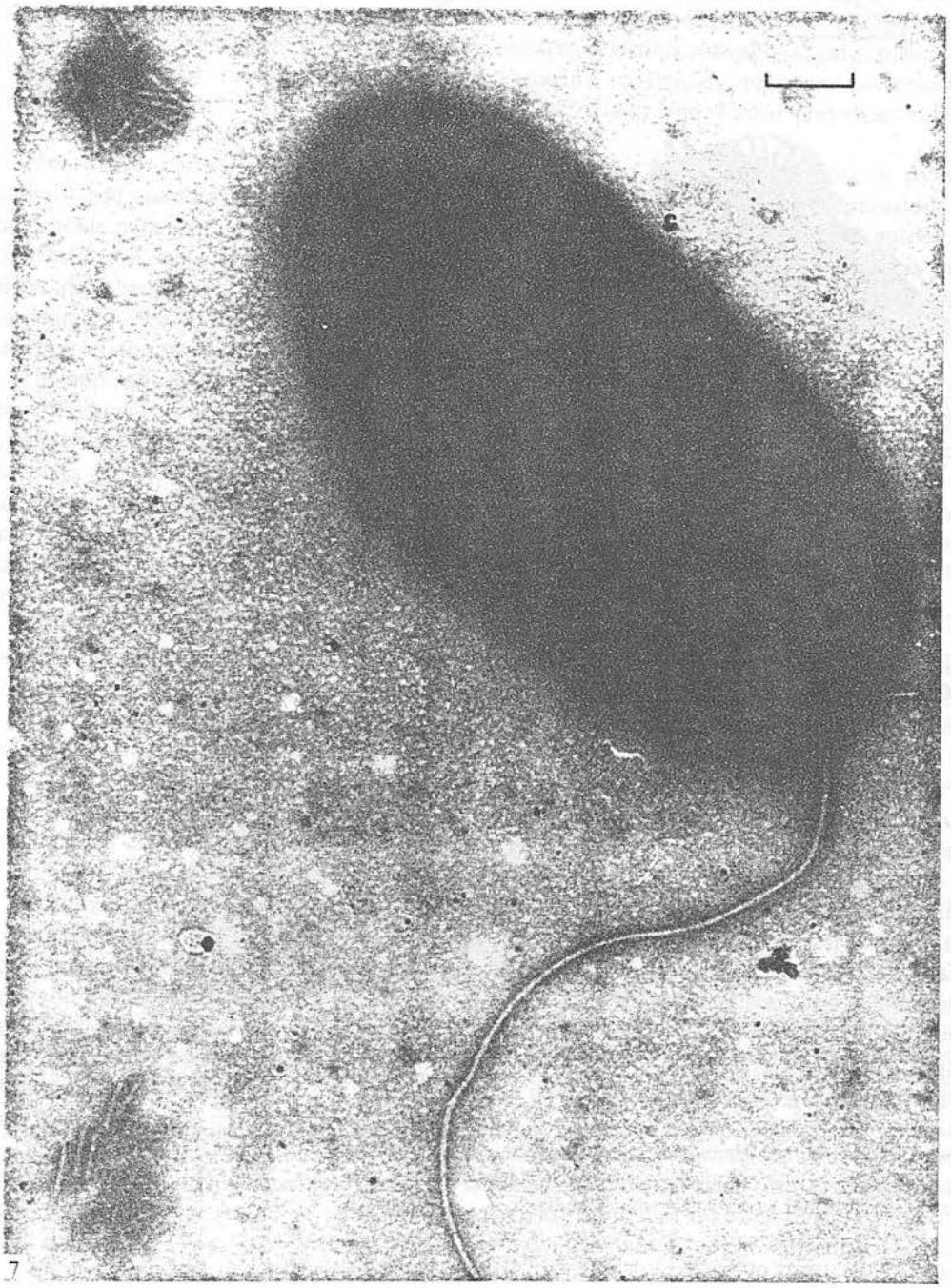

Fig. 7. *Pseudomonas aeruginosa* indicator strain 8 in the presence of induced pyocin 21 which had previously been treated with specific antiserum. Indicator strain 8 is sensitive to pyocin 21; however, no attachment of pyocin to the bacterial surface occurred and the agglutinated particles remained uncontracted. Bar marker = 200 nm.

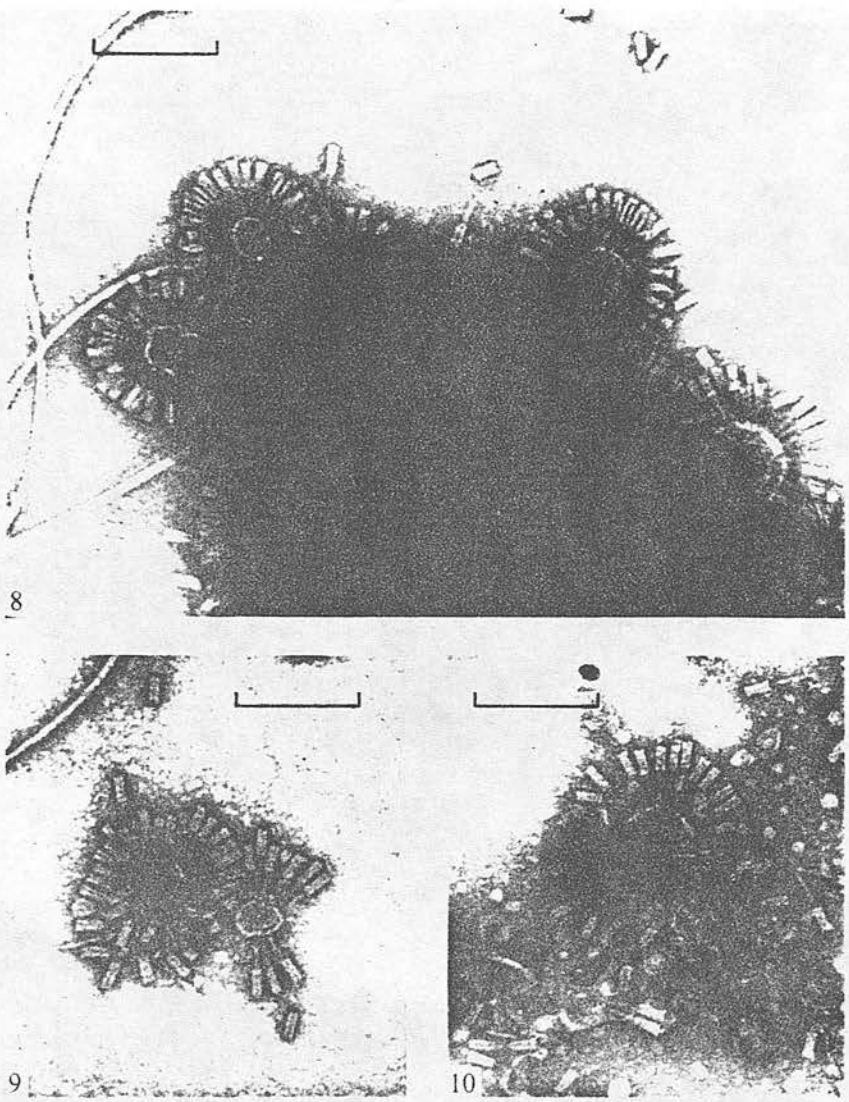

Fig. 8. *Pseudomonas aeruginosa* indicator strain 8 after treatment with induced contractile pyocin 430. Several rosettes of contracted pyocin particles are formed around rounded fragments at the bacterial surface. Bar marker = 200 nm.

Fig. 9. Rosettes of contracted, induced pyocin 430 surrounding cell material from *Pseudomonas aeruginosa* indicator strain 8. Bar marker = 200 nm.

Fig. 10. Contracted particles of induced pyocin 430 adsorbed to lipopolysaccharide from *Pseudomonas aeruginosa* indicator strain 1. Bar marker = 200 nm.

pyocin 21 activity occurred nor was attachment of the particles observed when this pyocin was mixed with LPS from the resistant strain.

DISCUSSION

The adsorption of pyocin to sensitive cells of *Pseudomonas aeruginosa* can be demonstrated in the light microscope by using a fluorescent antibody technique and the inhibitory

activity demonstrated by the agar-slide technique. Electron microscopy, however, reveals in greater detail the nature of these pyocins and their possible mode of action. The contractile pyocins of *P. aeruginosa* strains 21, 355, 149 and 430 resemble morphologically pyocin R (Ishii *et al.* 1965), pyocin C9 (Higerd *et al.* 1967), pyocin C10 (Bradley, 1967) and bacteriocins from *Proteus* (Coetzee, de Klerk, Coetzee & Smit, 1968) and *Vibrio* (Jayawardene & Farkas-Himsley, 1969).

The electron micrographs reproduced here suggest the sequence of events in pyocin activity and the significance of the various particles already described in preparations of contractile pyocins. Uncontracted particles rapidly attach by the base-plate to receptors on the surface of sensitive cells of *Pseudomonas aeruginosa*. Once established, contact is firm since absorbed particles are sedimented at 2850 *g* whereas a much higher gravitational force is required to sediment unadsorbed particles. After adsorption, there is a rapid contraction of the particles followed by disruption of the bacterium.

The importance of adsorption and contraction for pyocin activity is emphasised by several experimental findings: (1) At 0 °C particles adsorb to sensitive bacteria but do not contract and lysis of the bacteria is not observed. (2) In the presence of resistant bacteria pyocin particles do not adsorb to the cell surface but can be observed in the uncontracted state in the vicinity of the bacterium. (3) Lethality and the ability to absorb to sensitive bacteria are lost after removal of the contractile sheath by SDS. (4) When treated with homologous antiserum, pyocins no longer adsorb to sensitive bacteria and the agglutinated particles remain uncontracted.

The receptors on the bacterial surface to which contractile pyocins adsorb appear to be in the LPS fraction. Ikeda & Egami (1969) reported that LPS extracted from pyocin-sensitive but not resistant cells of *Pseudomonas aeruginosa* inactivated pyocin R. Similarly LPS extracts have been shown to inactivate and adsorb a contractile bacteriocin from *Proteus vulgaris* (Smit, Hugo & de Klerk, 1969). The inability of bacteria of indicator strain 1 to adsorb pyocin 21 after treatment with EDTA is consistent with the view that the pyocin receptor contains LPS (Stewart & Young, 1971). Electron microscopy indicates that inactivation of contractile pyocins from *P. aeruginosa* strains 21 and 430 by LPS extracted from indicator strain 1 is due to the adsorption of pyocin particles to the LPS fragments.

Although they resemble contractile bacteriophages in many properties, the contractile pyocins do not replicate in sensitive host bacteria. In morphological terms, their lack of any structure resembling a bacteriophage head indicates a concomitant lack of the main reservoir of nucleic acid. Jacob (1954) reported that pyocin C10 was resistant to ribonuclease and deoxyribonuclease. By electron microscopy, Bradley (1967) observed that some core particles of this contractile pyocin contained material, possibly nucleic acid, and similar observations were made with the pyocins studied in this investigation (Fig. 1). The nature and function of this material remains unknown.

The mode of action of contractile pyocins after adsorption to the bacterial surface requires further investigation. Kinetic studies on the antibacterial activity of pyocin R (Kageyama, Ikeda & Egami, 1964) and pyocin 21 (Young, 1970) suggested that cell death can result from adsorption of a single pyocin particle. Kaziro & Tanaka (1965*a*, *b*) reported that pyocin R inhibited macro-molecular synthesis by inactivation of bacterial ribosomes. The relationship between pyocin 21 and other contractile pyocins requires investigation and further studies may reveal more than one mechanism for the activity of contractile pyocins.

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Studies on the Pyocins of *Pseudomonas aeruginosa*: Production of Contractile and Flexuous Pyocins in *Pseudomonas aeruginosa*

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SUMMARY

Strains of *Pseudomonas aeruginosa* (*P. pyocyanea*) were examined for pyocin production by means of a standard pyocin-typing technique and also a modified method which depends on the ability of some pyocins to pass through a cellulose acetate membrane. The results suggested that strains could produce (1) pyocins incapable of spontaneous passage through the membrane, (2) pyocins capable of passage through the membrane, (3) both kinds of pyocin simultaneously. Electron microscopy revealed that pyocins in the first circumstance included contractile pyocins and those in the second included flexuous, rod-like particles. *P. aeruginosa* 430 produced both contractile and flexuous pyocins simultaneously. The pyocins could be separated by absorption with sensitive bacteria or by gel filtration. The two pyocins differed serologically and in their range of inhibitory activity against strains of *P. aeruginosa*.

INTRODUCTION

In studies of *Pseudomonas aeruginosa* by means of the standard pyocin-typing technique (Gillies & Govan, 1966) differences in the extent of inhibition zones of the eight indicator strains suggested that some strains were capable of producing simultaneously pyocins of differing diffusibility. These observations led to the development of a method of subdividing type 1 strains into eight subtypes by means of five additional indicator strains (Govan & Gillies, 1969). The use of a cellulose acetate membrane in pyocin typing was suggested to facilitate removal of macroscopic growth of the pyocinogenic strain (Kohn, 1966). However, applying the membrane modification to the standard typing technique has not proved satisfactory because many pyocins appear incapable of spontaneous passage through the membrane (Govan, 1968; MacPherson & Gillies, 1969).

In addition to contractile pyocins such as R (Ishii, Nishi & Egami, 1965), C9 (Higerd, Baechler & Berk, 1967) and several others (Govan, 1968; 1974), a flexous, non-contractile type, pyocin 28, has been described (Takeya, Minamishima, Amako & Ohnishi, 1967) and also a non-particulate type designated pyocin S (Ito, Kageyama & Egami, 1970). Amako, Yasunaka & Takeya (1970) observed contractile and flexuous bacteriocins in mitomycin C-induced lysates of *Pseudomonas fluorescens*. Ito *et al.* (1970) reported the production of contractile and S-type pyocins together in *P. aeruginosa* strains M11 and P28 after induction with mitomycin C or ultraviolet irradiation. The simultaneous production of contractile and flexuous pyocins by *P. aeruginosa* 430 is described in this paper.

METHODS

Bacteria. The following pyocinogenic strains of *Pseudomonas aeruginosa* were investigated: 21, 355, 450, 318, 295, 430 and 485. The indicator strains of *P. aeruginosa* used to detect pyocin activity were those of Gillies & Govan (1966), numbered 1 to 8.

Pyocin typing. Pyocin typing was performed as previously described (Gillies & Govan, 1966).

Pyocin typing with cellulose acetate strips. Pyocin typing was also carried out with cellulose acetate electrophoresis strips (Oxoid, pore size approx. 0·45 μm) and a modification of the method of Kohn (1966). A strip of cellulose acetate was placed on the surface of a tryptone soya agar plate incorporating 7 % (v/v) defibrinated horse blood and the pyocinogenic strain of *Pseudomonas aeruginosa* was inoculated directly on to the acetate surface with a sterile swab. After 18 h incubation at 32 °C the strip and macroscopic growth were removed and the surface of the medium exposed to chloroform vapour for 15 min. The eight indicator strains of *P. aeruginosa* (4 h nutrient broth cultures at 37 °C) were then streaked at right-angles over the growth area of the pyocinogenic strain and the plates reincubated overnight at 37 °C.

Production and purification of pyocins. Extraction, purification and assay of pyocins from induced and non-induced cultures of pyocinogenic strains were carried out as described earlier (Govan, 1974).

Separation of pyocins by absorption. Cells of *Pseudomonas aeruginosa* were grown overnight at 37 °C on nutrient agar plates. The growth from four plates was harvested, washed in sterile saline (0·85 %) and centrifuged at 1200 *g* for 20 min. Ten ml of pyocin preparation were added to the pellet of bacteria and the contents were mixed. The mixture was left at room temperature for 20 min and the bacteria removed by centrifugation. The supernatant was treated with 5 % (v/v) chloroform to kill any remaining viable bacteria and assayed for residual pyocin activity.

Centrifugation of pyocins through a sucrose density gradient. Partially purified induced pyocin preparations from *Pseudomonas aeruginosa* 485 (0·2 ml volumes) were centrifuged for 2 h at 130000 *g* through a linear sucrose gradient (10 to 40 %) in a refrigerated Spinco SW 50 rotor. Fractions (0·25 ml) of the gradient were assayed for pyocin activity against indicator strains 1 and 5.

Separation of pyocins by gel filtration. Attempts were made to separate induced pyocins from *Pseudomonas aeruginosa* 430 by means of agarose gels (Pharmacia, Uppsala, Sweden). Columns were prepared with either Sepharose 2B (exclusion limit mol. wt 25×10^6) or 4B (exclusion limit 3×10^6). Both gels were provided in liquid phase; 0·02 % sodium azide was incorporated. The gels were washed several times in 0·01 M-tris (hydroxymethyl) methylamine buffer (pH 7·5) containing 0·01 M-$MgCl_2.6H_2O$ and 0·01 M-$MgSO_4.7H_2O$, after which the slurry was used to prepare a column (35 × 2·5 cm) incorporating a sample applicator. After 300 ml of buffer had been passed through the column, 3 ml of partially purified, induced but unseparated pyocin 430 (400000 units/ml) were pipetted on to the surface of the sample applicator. After allowing adsorption for 20 min, the column was eluted with the same buffer at a pressure head of 7 cm. Fractions were collected in 5 ml volumes and assayed for pyocin activity.

Treatment of pyocin with antiserum or lipopolysaccharide. Antiserum to pyocin was prepared and the actions of antiserum and lipopolysaccharide (LPS) on pyocin activity were investigated as described in the previous paper (Govan, 1974).

Immunodiffusion. Immunodiffusion experiments were carried out by using a modification

Table 1. *Inhibition patterns produced by pyocinogenic strains of Pseudomonas aeruginosa against indicator strains*

Pyocinogenic strain	Pyocin-typing technique	Indicator strains 1	2	3	4	5	6	7	8
21	S	+	+	+	+	+	−	+	+
	M	−	−	−	−	−	−	−	−
355	S	+	+	+	−	+	−	+	−
	M	−	−	−	−	−	−	−	−
450	S	+	−	+	+	−	−	+	+
	M	+	−	+	+	−	−	+	+
318	S	−	−	−	−	+	−	−	−
	M	−	−	−	−	+	−	−	−
295	S	−	−	+	−	−	−	+	−
	M	−	−	+	−	−	−	+	−
430	S	+	+	+	+	+	+	+	+
	M	−	−	−	−	−	+	−	−
485	S	+	+	+	−	+	−	+	−
	M	−	−	−	−	+	−	−	−

S, Standard typing technique (Gillies & Govan, 1966).
M, Modified technique using cellulose acetate (see text for details).
+, Inhibition of indicator strain; −, no inhibition.

of the method of Ouchterlony (1949). A solution of 2 % Oxoid Ionagar no. 2 in 0·9 % saline and containing 0·2 % sodium azide was steamed for 1 h. A mixture of 9 ml of 0·1 M-citric acid and 41 ml of 0·2 M-Na_2HPO_4 was heated to 90 °C and mixed with 50 ml of agar solution. The molten agar was then dispensed in 10 ml amounts in plastic Petri dishes 8·5 cm in diameter. After the agar had set, a central well and six equidistant lateral wells were cut from the agar with a gel cutter (Shandon, London); the central well was filled with antiserum and various antigens were distributed in the lateral wells. The plates were placed in a moist container at 4 °C and photographed after seven days.

Electron microscopy. Electron microscopy was carried out by using techniques described earlier (Govan, 1974).

RESULTS

Pyocin typing by the standard method and the modified cellulose acetate technique

The results of typing *Pseudomonas aeruginosa* strains 21, 355, 450, 318, 295, 430 and 485 by the standard technique (Gillies & Govan, 1966) and a modified technique using cellulose acetate (Kohn, 1966; Govan, 1968) are shown in Table 1.

By using the standard pyocin-typing technique the patterns of inhibition against the eight indicator strains of *Pseudomonas aeruginosa* showed that the strains belonged to pyocin types 1, 3, 16, 5, 17, 10 and 3 respectively. When using the cellulose acetate technique, pyocins of strains 21 and 355 no longer inhibited any indicator strain whilst strains 450, 318 and 295 showed no change in the patterns of inhibition. Strain 430 inhibited all eight indicator strains in the standard typing technique (Fig. 1) whereas only indicator strain 6 was inhibited in the modified method (Fig. 2). Strain 485 inhibited strains 1, 2, 3, 5 and 7 by the standard method but only indicator strain 5 when cellulose acetate was used.

Strains 21, 355 and 430 produced the contractile pyocins described previously (Govan, 1974). Non-induced cultures of strains 450, 318 and 295 produced low titres of pyocin

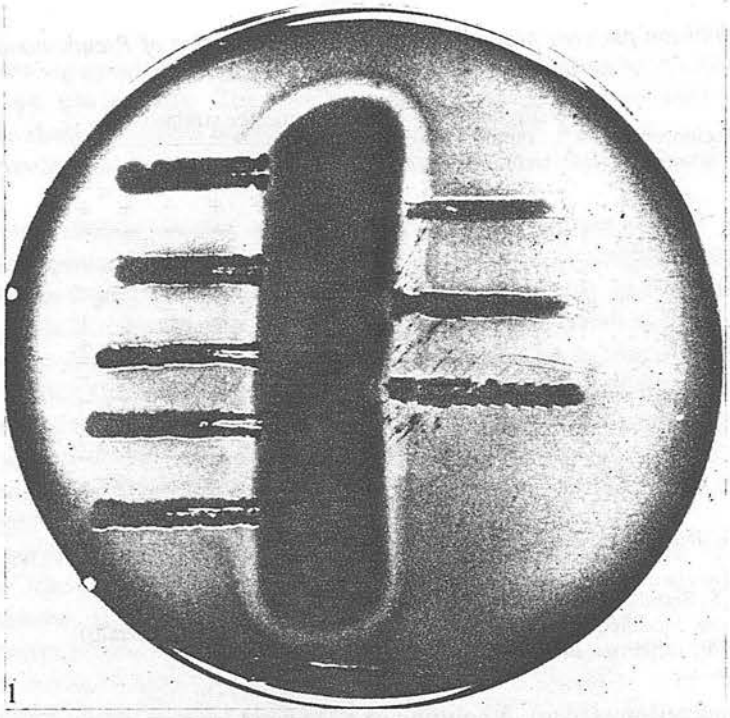

Fig. 1. Pyocin-typing plate of *Pseudomonas aeruginosa* strain 430 by the standard pyocin-typing technique, showing inhibition of all eight indicator strains.

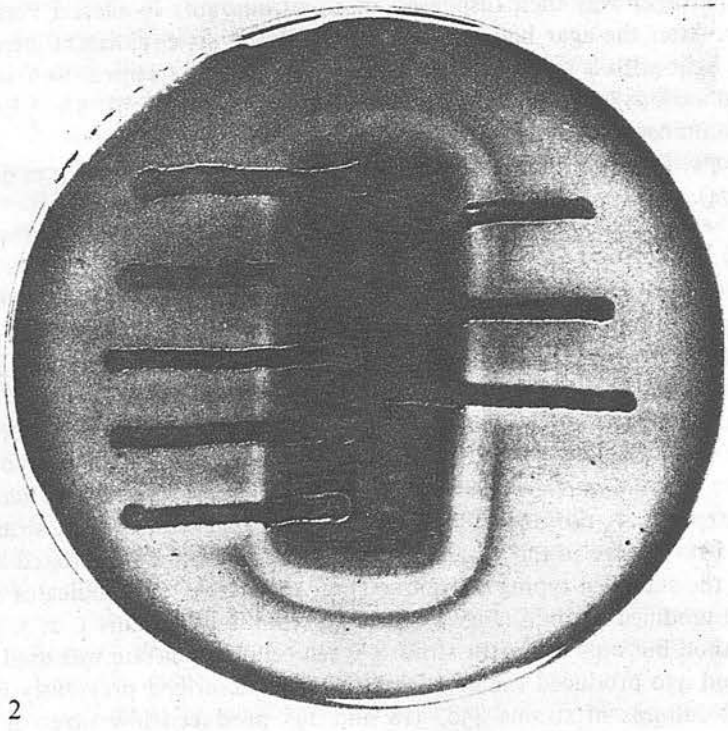

Fig. 2. Pyocin-typing plate of *Pseudomonas aeruginosa* strain 430 with the cellulose acetate modification; only indicator strain 6 is inhibited with this method.

Table 2. *Inhibitory activity of induced unseparated pyocins* 430 *and* 485 *after absorption with bacteria of Pseudomonas aeruginosa*

Pyocin	Absorbed with bacteria of indicator strain	Pyocin activity (units/ml) against indicator strain 1	5	6	8
430	6	100000	100000	0	100000
430	8	0	0	100000	0
430	C	100000	100000	100000	100000
485	1	0	1600	0	0
485	5	0	0	0	0
485	C	100000	100000	0	0

C, control: no absorption carried out.

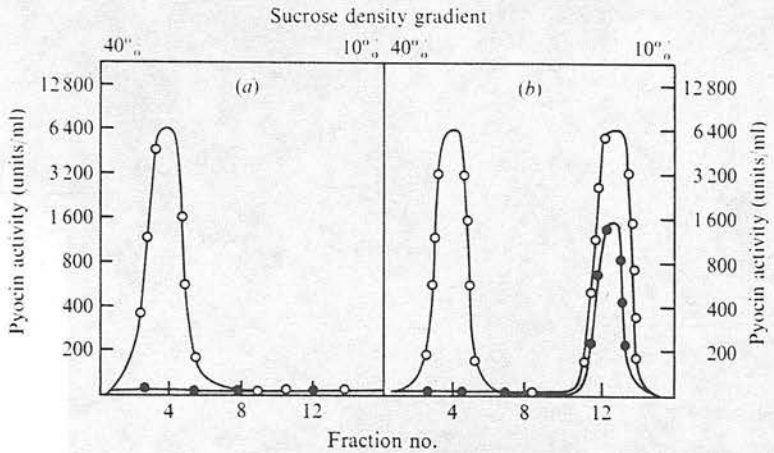

Fig. 3. Assay of induced pyocin preparations from *Pseudomonas aeruginosa* strain 485 after centrifugation through a sucrose density gradient. Fractions were assayed for pyocin activity against indicator strain 1 (*a*) and indicator strain 5 (*b*): ○—○ unseparated pyocin 485; ●—● pyocin 485 after absorption with bacteria of indicator strain 1.

activity (approximately 3200 units/ml) after growth in liquid or solid media, but after induction with mitomycin C crude lysates of each strain had titres of 100000 to 400000 units/ml. Electron microscopy of partially purified lysates revealed only a single type of particle; this was a flexuous rod approximately 10 nm wide and 100 to 200 nm long. No change in morphology of the pyocin particles was observed after their attachment to the surface of a sensitive bacterium.

Absorption of pyocin preparations derived from strains 430 *and* 485 *by the eight indicator strains*

Unabsorbed pyocin 430 inhibited all eight indicator strains, but after absorption with washed cells of indicator strain 6 pyocin activity remained towards the seven other strains, i.e. 1, 2, 3, 4, 5, 7 and 8. After absorption with each of these strains activity towards indicator strain 6 remained.

Unabsorbed pyocin 485 inhibited indicator strains 1, 2, 3, 5 and 7 but did not inhibit strains 4, 6 and 8 and absorption of this pyocin with strains 4, 6 or 8 produced no alteration

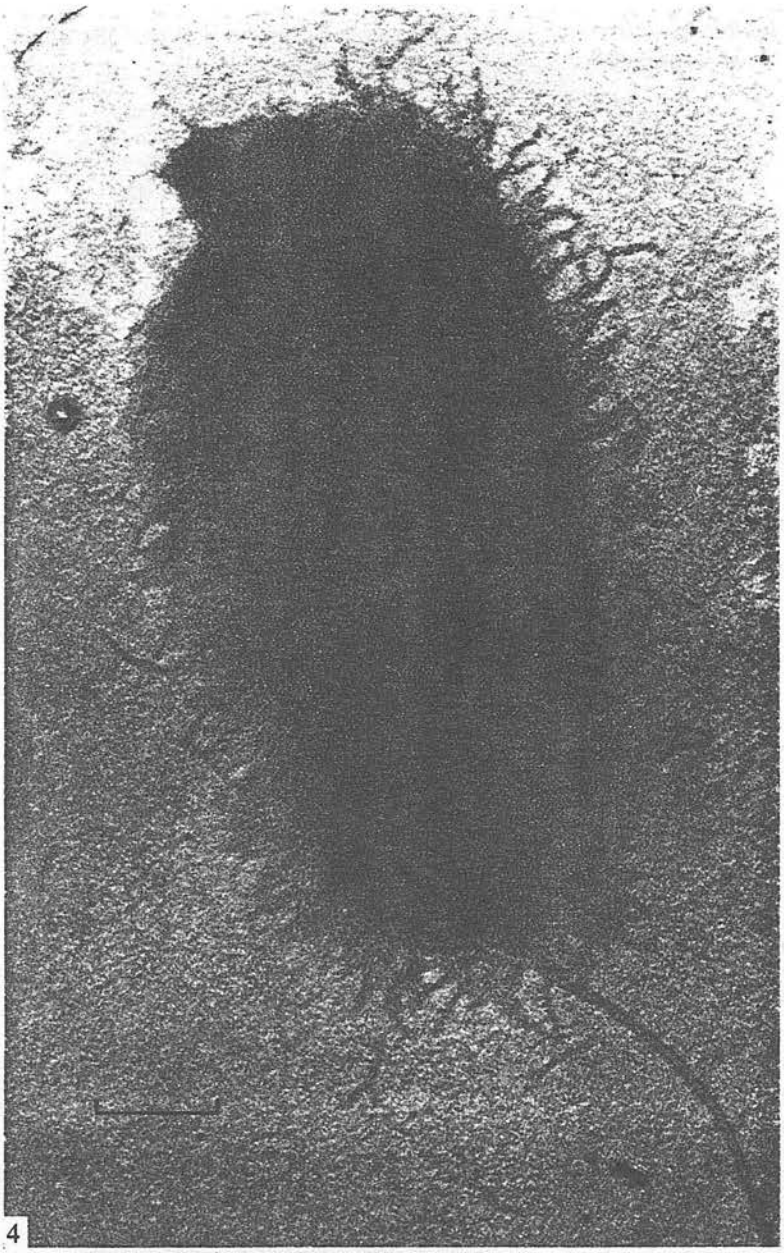

Fig. 4. *Pseudomonas aeruginosa* indicator strain 6 after treatment with induced flexuous pyocin 430f. The bacterial surface is surrounded with flexuous, rod-like pyocin particles. Bar marker = 200 nm.

in pyocin activity. Absorption with indicator strains 1, 2, 3 or 7 did not remove pyocin activity towards indicator strain 5 but absorption with indicator strain 5 removed pyocin activity to all the indicator strains.

Partially purified induced pyocin preparations (100000 units/ml) were obtained from strains 430 and 485 and a quantitative assay of pyocin activity was carried out after absorption with washed bacteria of indicator strains 6 and 8 or 1 and 5 respectively (Table 2).

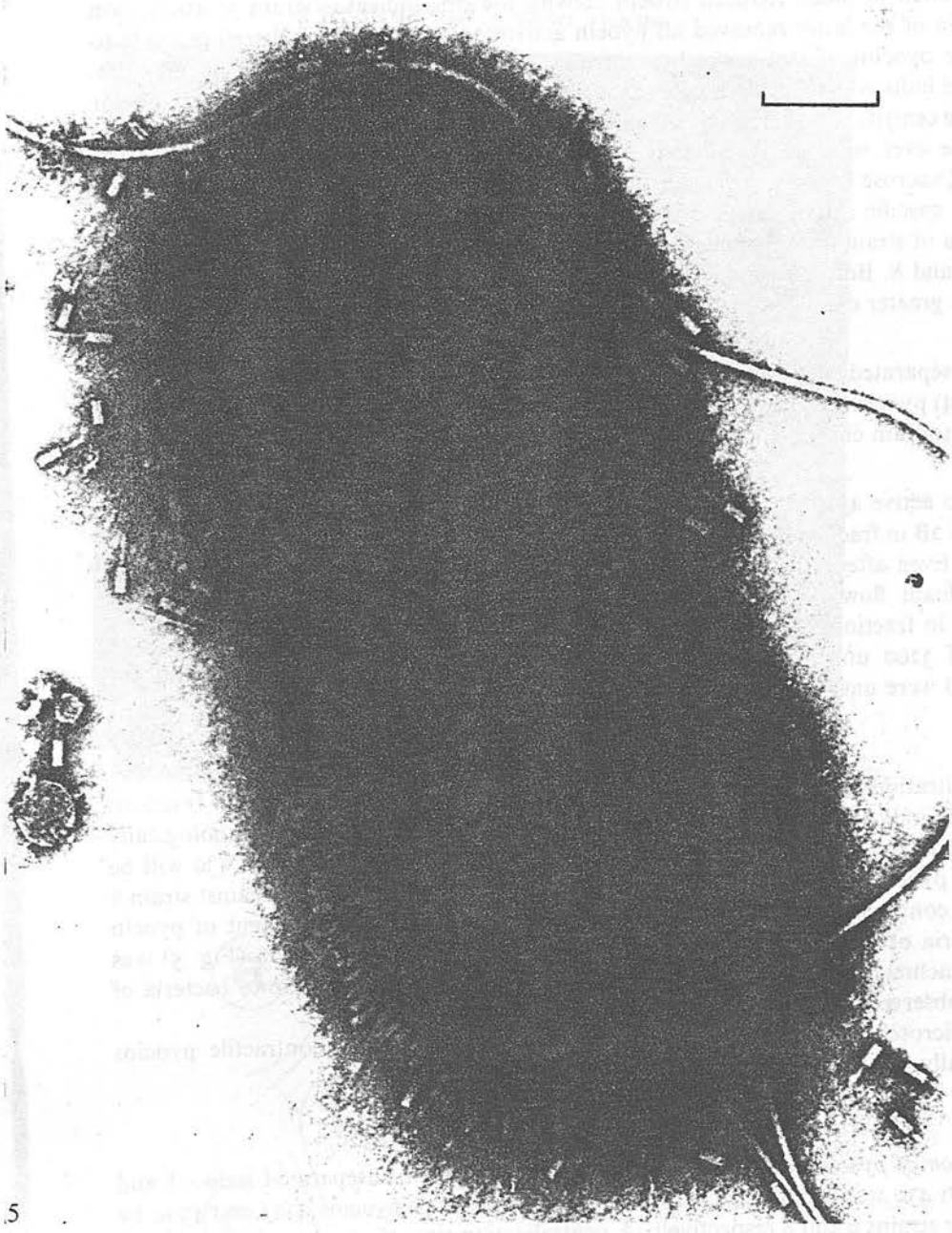

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Fig. 5. *Pseudomonas aeruginosa* indicator strain 8 after treatment with induced contractile pyocin 430c. Many contracted pyocin particles can be seen adsorbed to the bacterial surface and in the vicinity of the bacterium. Bar marker = 200 nm.

Absorption failed to separate discrete pyocins from pyocin 485. Absorption with indicator strain 1 resulted in much reduced pyocin activity towards indicator strain 5; absorption with bacteria of the latter removed all pyocin activity. An attempt was therefore made to separate the pyocins of strain 485 by centrifugation through a sucrose density gradient. Unseparated induced pyocin 485 and a preparation obtained after absorption with indicator strain 1 were centrifuged. A fraction which inhibited indicator strain 1 was found only at the 30 % sucrose level, whereas pyocin activity against indicator strain 5 was found at both the 10 and 30 % sucrose levels (Fig. 3). Absorption experiments with pyocin 430 showed that a pyocin with specific activity against indicator strain 6 could be obtained after absorption with bacteria of strain 8; absorption with strain 6 left undiminished pyocin activity towards strains 1, 5 and 8. Both pyocins of strain 430 were inducible with mitomycin C and were examined in greater detail.

Ion-exchange chromatography

When unseparated induced pyocin 430 was chromatographed on DEAE-cellulose (Govan, 1974) pyocin activity against indicator strains 6 and 8 was eluted as a single peak at 0·2 to 0·3 M-sodium chloride.

Gel filtration

Pyocin 430 active against indicator strain 6 but not strain 8, was eluted from a column of Sepharose 2B in fraction 16 (80 ml) and reached a maximum (50000 units/ml) at fraction 20 (100 ml). Even after elution with 250 ml buffer, no activity against strain 8 was noted. When the eluant flow through the column was reversed, pyocin activity was detected commencing in fraction 6. This activity was directed against strain 8 only and reached a maximum of 3200 units/ml. Attempts to separate the pyocins of strain 430 by using Sepharose 4B were unsuccessful.

Electron microscopy

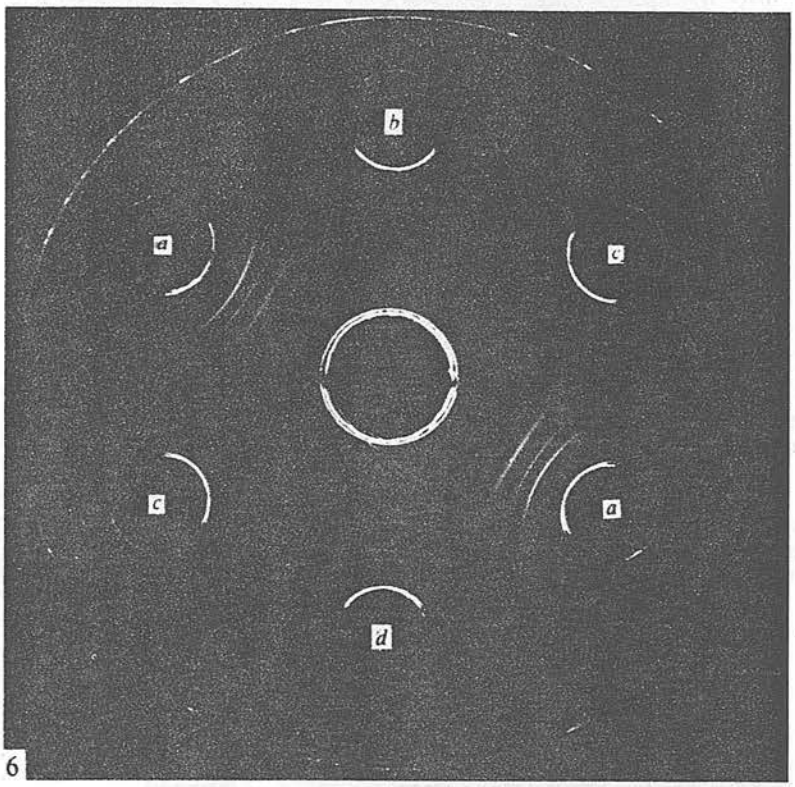
After gel filtration of unseparated, induced pyocin 430, electron microscopy of fractions active towards indicator strain 6 revealed only flexuous pyocin particles morphologically similar to those of strains 450, 318 and 295; this flexuous pyocin from strain 430 will be referred to as pyocin 430f. The fractions containing pyocin activity directed against strain 8 showed only contractile pyocin particles; designated pyocin 430c. Attachment of pyocin 430f to bacteria of strain 6 (Fig. 4) and pyocin 430c to bacteria of strain 8 (Fig. 5) was noted. No attachment of pyocin 430f to bacteria of strain 8 or pyocin 430c to bacteria of strain 6 was observed.

Electron microscopy of unseparated induced pyocin 485 revealed contractile pyocins and occasionally flexuous rods; these were not investigated further.

Serological investigations of pyocins 430f and 430c

Neutralization of pyocin activity. Antiserum prepared against unseparated induced and purified pyocin 430 was examined for neutralizing antibodies to pyocins 430f and 430c by using indicator strains 6 and 8 respectively. A neutralization titre of 2000 was found against both pyocins. No neutralization of pyocin activity was observed when using pre-immune sera.

Gel diffusion. By means of a double diffusion technique, antiserum against unseparated induced and purified pyocin 430 was examined against pyocins 430f and 430c. Pyocin 430f gave a single precipitin line whereas 430c produced three distinct lines. When unseparated pyocin 430, pyocin 430f and pyocin 430c were placed in adjacent wells (Fig. 6) the single



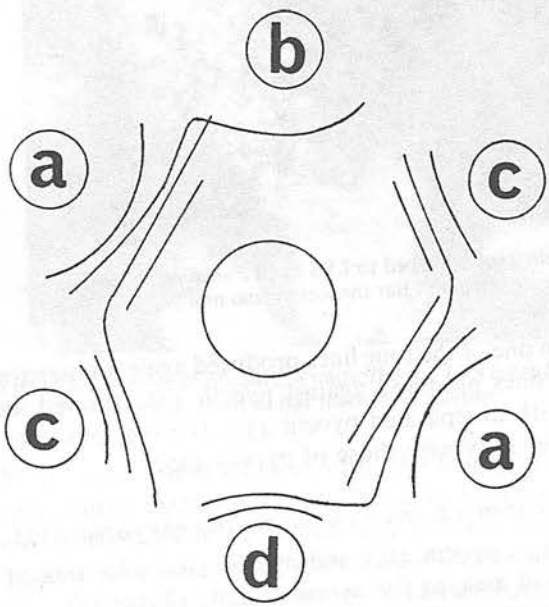


Fig. 6. Antigen–antibody precipitation reaction of induced pyocins in agar gel. The central well contains antiserum to unseparated pyocin 430, and (*a*) unseparated pyocin 430, (*b*) flexuous pyocin 430f, (*c*) contractile pyocin 430c, and (*d*) unseparated pyocin 485.

Table 3. *Inhibitory activity of induced pyocins from Pseudomonas aeruginosa* 430 *after treatment with LPS from indicator strains* 6 *and* 8

Pyocin	Treated with LPS from indicator strain	Pyocin activity (units/ml) against indicator strain 6	8
430*	6	50000	50000
430*	8	50000	0
430*	C	50000	50000
430f	6	50000	0
430f	8	50000	0
430f	C	50000	0
430c	6	0	50000
430c	8	0	0
430c	C	0	50000

* Unseparated pyocin 430.
C, control: pyocin preparation treated with portion of 0·1 M-ammonium acetate.

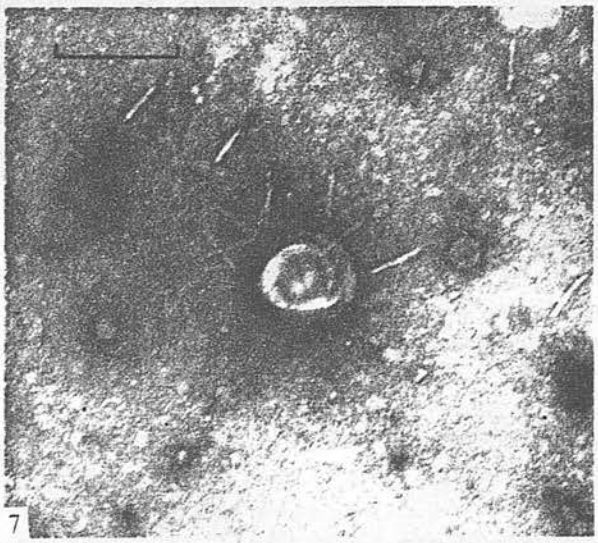


Fig. 7. Induced pyocin 430f adsorbed to LPS from *Pseudomonas aeruginosa* indicator strain 6. Bar marker = 200 nm.

line of 430f showed identity with one of the four lines produced against unseparated pyocin 430; similarly one of the three lines produced against pyocin 430c showed identity with another of the lines obtained with unseparated pyocin 430. The precipitin line of pyocin 430f showed a reaction of non-identity with those of pyocin 430c.

The effect of LPS from indicator strains 6 *and* 8 *on the activity of pyocins* 430*f and* 430*c*

Unseparated induced pyocin 430, pyocin 430f and pyocin 430c were treated with LPS from indicator strains 6 and 8 and assayed for pyocin activity (Table 3).

Pyocin 430c, when present in unseparated induced pyocin 430 preparations, or as a separated fraction, was completely inactivated by LPS from indicator strain 8; no inactiva-

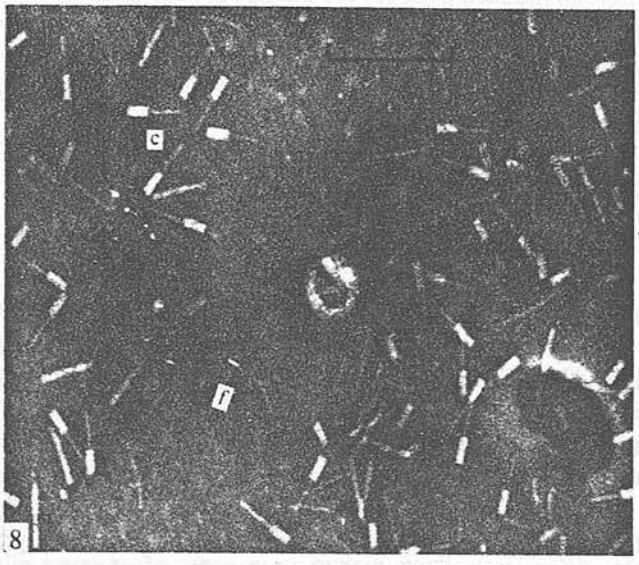


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Fig. 8. Rounded fragment of LPS from *Pseudomonas aeruginosa* indicator strain 6 in the presence of unseparated induced pyocin 430 containing both flexuous (f) and contractile (c) pyocins. The selective adsorption of flexuous pyocin particles to the LPS is visible. Bar marker = 200 nm.

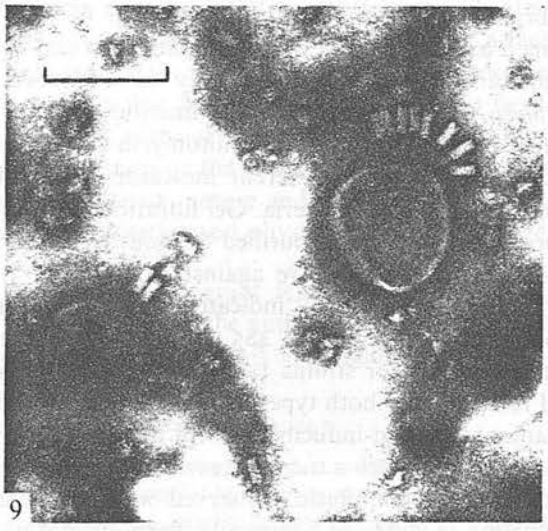

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Fig. 9. Contracted particles of induced pyocin 2285 adsorbed to LPS from *Pseudomonas aeruginosa* indicator strain 6. Bar marker = 200 nm.

tion of pyocin 430c was noted after treatment with LPS from strain 6. In contrast, pyocin 430f which specifically inhibited strain 6 was unaffected by LPS extracts from strain 6 or 8; the biological activity of the LPS fraction from strain 6 was confirmed by its ability to inactivate the lethal activity of a contractile pyocin from *Pseudomonas aeruginosa* strain 2285.

Electron microscopy of pyocin – LPS interaction. When unseparated induced pyocin 430 or induced pyocin 430c was treated with LPS extracted from strain 8 and examined in the electron microscope, contracted pyocin particles were found attached to the surface of the

LPS fragments. Although induced pyocin 430f did not appear to be inactivated by treatment with LPS from strain 6 the flexuous particles were found attached to LPS fragments (Fig. 7), and indeed when unseparated induced pyocin 430 was treated with LPS from strain 6, selective attachment of the flexuous 430f pyocin particles appeared to take place (Fig. 8). The biological activity of LPS from indicator strain 6 was confirmed in the electron microscope by its ability to absorb a contractile pyocin 2285 (Fig. 9). Pyocin 430c or 430f particles did not absorb to LPS from strains 6 or 8 respectively.

DISCUSSION

These results suggest that a modified typing technique employing a cellulose acetate membrane might prove a useful preliminary step in screening pyocinogenic strains of *Pseudomonas aeruginosa* for the production of contractile or flexuous pyocins.

Strains 21 and 355 show pyocin activity when examined by the standard typing technique but not when examined by the modified typing technique. Electron micrographs have shown (Govan, 1968; 1974) that these strains produce contractile pyocins. This type of particle may absorb to or be inactivated by the cellulose acetate membrane, because the dimensions of the particles (100 × 15 nm) should otherwise have allowed their passage through a membrane with a pore size of approximately 0·45 μm. Flexuous pyocins were produced by pyocinogenic strains 450, 318 and 295 and the inhibition pattern produced by these strains was unaffected by cellulose acetate. Recent investigations indicate that, in addition to flexuous pyocins, non-particulate S-type pyocins can also pass spontaneously through these cellulose acetate membranes (J. R. W. Govan, unpublished).

The use of the modified technique suggested that strains 430 and 485 produce more than one type of pyocin simultaneously and this possibility was examined.

Pseudomonas aeruginosa 430 produced both contractile and flexuous pyocins whose production could be increased by induction with mitomycin C. Since the inhibitory activity of these pyocins was directed towards different indicator strains the pyocins could be separated by absorption with sensitive bacteria. Gel filtration with Sepharose 2B, however, provided a method for the preparation of purified flexuous pyocin. *P. aeruginosa* 485 produced an inducible contractile pyocin active against strains 1, 2, 3, 5 and 7 and also a non-inducible pyocin which inhibited only indicator strain 5. The non-inducible pyocin could be isolated from unseparated pyocin 485 by removing the contractile pyocin by absorption with bacteria of indicator strains 1, 2, 3 or 7. Indicator strain 5 could not be used since it possessed receptors for both types of pyocin. Gel filtration was not attempted because of the small amount of non-inducible pyocin present, but the two pyocins could be separated by centrifugation through a sucrose density gradient.

The many different patterns of inhibition observed with the pyocin-typing techniques must result from production of different contractile, flexuous and non-particulate pyocins or from combinations of these. A study of these different pyocins may allow interpretation of the inhibition patterns in terms of production of individual pyocins. Subdivision of strains of *Pseudomonas aeruginosa* belonging to pyocin type 1 has already been reported (Govan & Gillies, 1969). Current investigations (J. R. W. Govan, unpublished) suggest that many other pyocin types of *P. aeruginosa* are capable of subdivision when the individual pyocins responsible are examined more closely.

It seemed worthwhile to investigate the possibility that flexuous pyocin particles are precursors of the more complex contractile pyocins especially when both pyocins are produced by the same strain of *Pseudomonas aeruginosa* as in the case of strain 430.

Morphologically, flexuous pyocin particles can often be distinguished from the tail cores of contractile pyocins by their variable length and less rigid appearance. Further evidence that these are two quite different entities is suggested by the following observations. (i) The two pyocins have different spectra of activity and in strain 430 there was not even a sharing of inhibitory activity against any one indicator strain. (ii) The flexuous pyocin could adhere to and kill sensitive bacteria whereas the isolated tail core of the contractile pyocin lost the ability to adsorb to such bacteria (Govan, 1974). (iii) No serological relationship could be demonstrated between the two pyocins of strain 430 by using the gel diffusion technique.

The determination of a chromosomal locus for control of synthesis of the contractile pyocin R2 (Kageyama, 1970*a*, *b*) suggests that contractile pyocins are the result of prophage alteration. A similar location for factors determining flexuous pyocins would suggest that they originate from prophage elements controlling synthesis of non-contractile bacteriophages of group B (Bradley, 1967). Such bacteriophages are known to exist in Pseudomonas (Slayter, Holloway & Hall, 1964; Bradley, 1966) but normally possess a knob-like tip to the tail extremity which is absent in flexuous pyocins.

LPS extracted from sensitive bacteria of *Pseudomonas aeruginosa* neutralizes the inhibitory activity of contractile pyocins by absorbing pyocin particles (Govan, 1974). LPS from indicator strain 6 absorbed and inactivated a contractile pyocin 2285 and had no effect on the inhibitory activity of the flexuous pyocin 430f; this suggests that there was a different chemical receptor for this flexuous pyocin. However, electron micrographs show the apparent attachment of pyocin 430f to LPS fragments from indicator strain 6. Since the contractile pyocin 430c did not inhibit this strain and did not attach to intact bacteria or LPS fragments from indicator strain 6, the adsorbed particles could not be the sheathless remains of pyocin 430c. Perhaps the bacterial receptors for the flexuous pyocin contain LPS but attachment is reversible without concomitant loss of inhibitory activity. Further investigations are required to determine the nature of these bacterial receptors.

A continued study of the incidence, nature and mode of action of pyocins is important in studies of the epidemiology, genetics and physiology of *Pseudomonas aeruginosa*.

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MUCOID STRAINS OF *PSEUDOMONAS AERUGINOSA*: THE INFLUENCE OF CULTURE MEDIUM ON THE STABILITY OF MUCUS PRODUCTION

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IN general, mucoid strains of *Pseudomonas aeruginosa* are not commonly isolated from patients (Doggett, 1969), but children with the disease cystic fibrosis (CF) are very susceptible to pulmonary infection with *P. aeruginosa* and the infecting strain then characteristically appears to change to a mucoid form (Iacocca, Sibinga and Barbero, 1963; Doggett *et al.*, 1966). The transition to the mucoid state is associated with an increase in the severity of the pulmonary condition (Burns and May, 1968; Diaz, Mosovich and Neter, 1970), and infection with mucoid *P. aeruginosa* is a very common cause of death of patients with CF (Doggett and Harrison, 1969).

The factors responsible for the predominance of mucoid *P. aeruginosa* in the CF patient remain obscure. A non-mucoid strain of the organism always precedes the mucoid form in the patient (Doggett *et al.*, 1966) and when the two forms are found in the same specimen they belong to the same serotype (Diaz *et al.*, 1970) and pyocine type (Williams and Govan, 1973). These observations from patients suggest that the mucoid variant arises from the non-mucoid form *in vivo*. Martin (1973) observed the appearance of rings of mucoid growth immediately around plaques of phage lysis, and obtained mucoid variants by subculture from these. She found that neither phage-induced nor naturally occurring mucoid strains are stable *in vitro*. Reversion to the non-mucoid form *in vitro* occurs within a primary culture on solid (agar) medium, as outgrowths from the edge of a mucoid colony. Zierdt and Schmidt (1964) noted that the mucoid characteristic is rapidly lost by serial transfer on solid medium. So far there is no generally accepted explanation provided by these studies for the mechanism determining the emergence of the mucoid form *in vivo* and reversion to the non-mucoid state *in vitro*.

A means of stabilising mucus production in *P. aeruginosa* might now be of use in the study of this organism in relation to CF and might lead to the identification of factors responsible for the predominance and stability of mucoid strains in the CF patient. The present paper describes the influence of various substances on the stability of mucus production *in vitro* by naturally occurring mucoid strains and in those obtained *in vitro* by the action of phage.

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Materials and methods

Strains of P. aeruginosa

The following strains were examined in detail; the suffix (M) indicates a mucoid strain. Strains 156(M), 157(M) and 158(M) were isolated from the sputum of CF patients. Two other naturally occurring mucoid strains, 2324(M) and 6944(M), were isolated from the sputum of non-CF patients. Mucoid variants were isolated by the method to be described from the following non-mucoid strains of *P. aeruginosa*: no. 2285, a stock pyocinogenic strain isolated in this laboratory; no. P14, a mouse-virulent strain obtained from Dr R. J. Jones, Birmingham Accident Hospital; PTO13, a strain carrying the fertility factor FP2 and obtained from Professor B. W. Holloway, Department of Genetics, Monash University, Australia.

Isolation of mucoid variants by a phage procedure

Plates of nutrient agar (Oxoid, Colombia Agar, CM331) were separately flooded with overnight nutrient-broth (Oxoid no. 2, CM67) cultures of strains 2285, P14 or PTO13 and the excess fluid was removed. The agar surface was allowed to dry at room temperature (*c.* 18°C) by removing the lids of the petri dishes for 30 min. The virulent *P. aeruginosa* phage E79 obtained from Professor B. W. Holloway, was propagated on his *P. aeruginosa* strain PAO1. The lawns of strains 2285, P14 and PTO13 were flooded with phage E79 at approximately the routine test dilution (RTD); excess fluid was removed and the plates were incubated overnight at 37°C. Mucoid variants could be distinguished from other phage-resistant colonies by their large size and watery appearance after incubation for 24 h at 37°C followed by a further period of 24 h at room temperature.

Pyocine typing

Pyocine typing was carried out as described by Williams and Govan (1973).

Precipitation of extracellular material

Growth from cultures on nutrient agar was suspended in physiological saline (NaCl, 0·85% w/v) and centrifuged to remove cells. Nine volumes of a mixture of ethanol and benzene (1:1 v/v) was added to 1 volume of supernate at 4°C (Doggett, Harrison and Wallis, 1964), and a precipitate formed.

Studies of mucus production on solid media

Media. Blood agar was nutrient agar (Oxoid Columbia) with defibrinated horse blood (Oxoid, 5% v/v). Chocolate blood agar was prepared by adding blood to melted nutrient agar at 75°C. Other media used were MacConkey Agar (Oxoid, CM7), Desoxycholate Citrate Agar (DCA; Oxoid, CM227), cetrimide agar (Cetavlon, ICI, 0·03% w/v in nutrient agar) and nitrogen-deficient agar (Sutherland and Wilkinson, 1965).

Subculture. Mucoid colonies were selected and subcultured every 48 h on the above media contained in vented plastic petri dishes. Incubation was aerobic and for 24 h at 37°C with no additional gas and then at room temperature for a further 24 h.

Studies of mucus production in liquid media

Media. Nutrient broth (Oxoid no. 2) contained Lab-Lemco (Oxoid, L30) 10 g, peptone (Oxoid, L37) 10 g and NaCl 5 g per litre. Deoxycholate-citrate broth (DCB) contained Lab-Lemco (Oxoid, L29) 5 g, peptone (Oxoid, L37) 5 g, sodium deoxycholate (BDH) 5 g, sodium thiosulphate (anhydrous) 5·4 g, sodium citrate 8·5 g, ferric citrate 1 g, lactose 10 g, and neutral red 0·02 g per litre. Minimal broth (Kelly and Clarke, 1962) was supplemented with tryptophan 25 μg per ml.

Basal broth, used to assess the influence of various substances on stabilisation of mucus production, consisted of Lab-Lemco 5 g and peptone (Oxoid, L37) 5 g per litre. The test substances were prepared as follows: aqueous solutions (10%) of sodium deoxycholate (w/v), sodium thiosulphate (w/v), Teepol (Shell Chemicals Ltd; v/v), Tween 80 (Hopkin and Williams Ltd; v/v) and sodium dodecyl sulphate (Koch-Light Laboratories Ltd; w/v) were prepared and heated to 60°C for 1 h. N-acetyl-L-cysteine (BDH) was dissolved in physiological saline, the *p*H adjusted to 7·0 with M NaOH and the solution passed through a membrane filter (Millipore, 0·45 μm). Egg-yolk solution was prepared by mixing a fresh egg yolk in 225 ml of borate-buffered saline (NaCl 8·5 g, boric acid 10·94 g, borax 1·9 g per litre). After filtration through muslin and Cellosene Wadding, Hyflo Super Cel (Johns-Manville, USA) 10 g was added and the preparation left at 4°C for 1 h. After filtration through Whatman no. 1 paper, the solution was passed through a Seitz filter and stored at 4°C. Egg-yolk lecithin (BDH) 100 mg and synthetic dipalmitoyl-L-3-lecithin (Koch-Light Laboratories Ltd) 100 mg were dissolved in 1-ml volumes of absolute alcohol at 50°C.

Inoculum. A single mucoid colony picked from an overnight nutrient-agar culture was suspended in 2 ml of physiological saline, and 0·1 ml of a 10-fold dilution of this suspension in saline was used to seed 10 ml of broth. For experiments involving the simultaneous growth of strains PTO13 and PTO13(M), inocula of 0·05 ml were used.

Determination of frequency of mucoid and non-mucoid colony-forming units

Portions (0·1-ml volume) were removed from broth cultures grown at 37°C (without aeration) and 10-fold serial dilutions prepared in physiological saline; 0·1-ml volumes of suitable dilutions were spread over the surface of nutrient-agar plates with sterile glass spreaders. The plates were examined by transmitted light after incubation for 24 h at 37°C. The number of mucoid and non-mucoid colonies was counted on plates containing 100 to 300 discrete colonies if possible. Mucoid colonies became large and tended to coalesce when the time of incubation exceeded 24 h or if the number of colonies was greater than 300 per plate. An average count was calculated from three plates spread from the same dilution. The inoculum suspension was treated in a similar manner to ensure that all cells were in the mucus-producing form.

RESULTS

Isolation of mucoid variants with phage E79

In studies with phage E79 and *P. aeruginosa* strains 2285, P14 and PTO13, mucoid variants designated 2285(M), P14(M) and PTO13(M) and non-mucoid resistant variants were obtained in a ratio of approximately 1 to 100. The mucoid variants were of the same pyocine type as their parent strains, namely type 22 (no. 2285), type 16 (no. P14) and type 1/b (no. PTO13). The addition of an ethanol-benzene mixture to culture supernates of the mucoid variants (see *Methods*) produced a white fibrillar, water-soluble precipitate similar to that obtained with the naturally occurring mucoid strains 156(M), 157(M) and 158(M) isolated from CF patients. No such precipitate was observed when culture supernates of the parent strains were similarly treated.

Stability of mucus production on solid media

When mucoid strains 156(M), 157(M) and 158(M), freshly isolated from CF patients, and the mucoid variants 2285(M), P14(M) and PTO13(M) were grown on a range of solid media including nutrient agar, horse-blood agar,

chocolate-blood agar, MacConkey's agar, and cetrimide agar, they showed reversion to a non-mucoid colony form. It was noted that the use of a nitrogen-deficient medium (Sutherland and Wilkinson, 1965), used to enhance bacterial polysaccharide synthesis, did not lead to greater stability of mucus production. Instability was observed after growth for several days on a single plate as a rough, spreading outgrowth from a mucoid colony; after several serial subcultures this was accompanied by the appearance of both mucoid and non-mucoid colonies on the same plate. Even though totally mucoid colonies were selected, complete loss of mucoid growth occurred in most instances within 15 serial subcultures. In contrast, mucus production was found to be stable on DCA after many subcultures. The least stable strain was 158(M), which reverted to the non-mucoid form on DCA, but only after 50 subcultures. Each of the other five mucoid strains remained completely stable even after several hundred subcultures.

Stability of mucus production on DCA and instability on nutrient agar was examined in a larger series of 23 mucoid strains that included 18 strains isolated from nine CF patients, two strains obtained from non-CF patients and the variants 2285(M), P14(M) and PTO13(M). On DCA medium, mucus production remained stable with all strains after 15 subcultures. On nutrient agar, all but one strain showed instability of mucus production after several subcultures; six strains had reverted completely to the non-mucoid form within five subcultures and a further eight strains showed complete reversion by the 15th subculture. In addition to instability of mucus production, all strains, when grown on nutrient agar, showed a " collapse " (flattening) of mucoid growth after incubation for several days; often but not invariably this preceded the

TABLE I

Stability of mucus production by Pseudomonas aeruginosa strain PTO13(M) grown in liquid media

Duration of incubation (h)	Ratio of mucoid to non-mucoid colonies (at the stated dilution) on plating from liquid cultures* in		
	nutrient broth	minimal broth	sodium deoxycholate broth
0	53/0 (10^2)	61/0 (10^2)	55/0 (10^2)
7	147/0 (10^4)	16/0 (10^3)	28/0 (10^4)
24	181/152 (10^5)	44/0 (10^5)	55/0 (10^4)
48	54/69 (10^6)	149/0 (10^5)	83/0 (10^4)
72	32/155 (10^5)	18/0 (10^5)	39/0 (10^5)
96	8/159 (10^5)	187/0 (10^5)	209/0 (10^4)
120	0/97 (10^5)	154/74 (10^5)	403/0 (10^3)
144	...	175/407 (10^5)	114/0 (10^4)

* Incubated for the stated time and then subcultured to nutrient-agar plates. The ratios given are of average counts of mucoid and non-mucoid colonies obtained from the lowest dilution that yielded discrete colonies.

appearance of non-mucoid outgrowths from mucoid colonies. The phenomenon was never observed on DCA medium. The " collapsed " mucoid growth could be easily distinguished in appearance from the non-mucoid outgrowths and the penomenon was distinct from instability of mucus production *per se*, because subculture from a " collapsed " growth produced mucoid colonies. Replicate isolates from a CF patient did not show greater stability than the original isolate, and reversion to the non-mucoid form occurred regardless of the origin of the mucoid strains.

Stability of mucus production by strain PTO13(M) during growth in liquid media

When subcultures to solid media were made from cultures of the mucoid *P. aeruginosa* strain PTO13(M) grown in nutrient broth, both mucoid and non-mucoid colonies were observed within 24 h. As incubation was continued in this medium, the proportion of non-mucoid colonies increased until eventually no mucoid colonies were observed (table I). Instability of mucus production proceeded more rapidly in nutrient broth than in minimal broth. In contrast, when the same organism was grown in DCB and subcultures were made on solid media, only mucoid colonies were ever observed even in cultures that had been incubated for 40 days at 37°C.

When strain PTO13(M) and the non-mucoid parent strain PTO13 were inoculated simultaneously and in approximately equal concentrations into the same flask of DCB, the non-mucoid strain gradually came to predominate (table II).

The ability of each of the constituents of DCB to stabilise mucus production in strain PTO13(M) was assessed by omitting each in turn from the complete medium. When lactose, neutral red, sodium citrate and ferric citrate were

TABLE II

Combined growth of Pseudomonas aeruginosa strains PTO13(M) and PTO13 in deoxycholate-citrate broth (DCB)

Duration of incubation (h)	Ratio of mucoid to non-mucoid colonies (at the stated dilution) on plating from liquid cultures* of strain or strains		
	PTO13(M) and PTO13 together	PTO13(M) alone	PTO13 alone
0	24/25 (10^2)	108/0 (10^1)	0/180 (10^1)
2·5	84/90 (10^2)	75/0 (10^2)	0/67 (10^2)
5	160/255 (10^3)	176/0 (10^3)	0/378 (10^3)
8	133/400 (10^3)	258/0 (10^3)	0/414 (10^3)
11	27/114 (10^4)	128/0 (10^4)	0/149 (10^4)
24	57/329 (10^4)	103/0 (10^4)	0/219 (10^4)
31	27/176 (10^4)	145/0 (10^4)	0/227 (10^4)
50	30/253 (10^4)	147/0 (10^4)	0/226 (10^4)
72	20/623 (10^4)	140/0 (10^4)	0/220 (10^4)

* See footnote to table I.

omitted, stabilisation of mucus production remained complete when subcultures were made to solid media after incubation for 24 h, whereas growth in a medium without sodium deoxycholate or sodium thiosulphate resulted in reversion to the non-mucoid form. When sodium deoxycholate and sodium thiosulphate were added to basal broth at the concentrations present in the complete DCB medium (0·5% and 0·54% respectively) considerable stabilisation of mucus production occurred. After incubation for 48 h in this medium, subcultures to solid media showed a ratio of 20 mucoid colonies to one non-mucoid colony, whereas growth for a similar period in the basal medium resulted in a ratio of one mucoid colony to four non-mucoid colonies. When the concentration of sodium thiosulphate was held at 0·54% and sodium deoxycholate employed at concentrations between 0·1 and 0·6%, maximum stabilisation was observed at 0·5%.

Stability of mucus production by strain PTO13(M) during growth in surfactant media

In view of the physiological importance of surfactants in the human respiratory tract, the ability of surfactants other than sodium deoxycholate to stabilise mucus production was investigated. Strain PTO13(M) was grown in basal medium supplemented with various surfactants and subcultures made to solid media at various intervals to assay the number of mucoid and non-mucoid colony-forming units.

Teepol and Tween 80 at 0·5% and sodium lauryl sulphate at 1·0% stabilised mucus production completely in strain PTO13(M) when incubation was continued for 7 days at 37°C. As the concentration of each surfactant was reduced, however, the observed number of non-mucoid colonies increased and the mucoid forms decreased. Stabilisation of mucus production by strain PTO13(M) was also observed in the presence of a specific concentration of cetrimide: the minimum inhibitory concentration (MIC) of cetrimide for the mucoid strain PTO13(M) and the non-mucoid parent strain PTO13 was 0·15%. When strain PTO13(M) was grown in basal broth containing various lower concentrations of cetrimide, complete stabilisation over 48 h occurred only at 0·0018% (table III). N-acetyl-L-cysteine, a mucolytic surfactant used therapeutically to liquefy tracheobronchial secretions, failed to stabilise mucus production at 2% but showed a limited ability at lower concentrations (table IV). Lecithin, the major surfactant found in human lungs, was found to be a very effective stabiliser of mucus production. The data given in table V indicate the stability of mucus production in basal broth containing 0·5% egg-yolk solution as a source of lecithin (see *Methods*) and the ability of a non-mucoid revertant of strain PTO13(M) to grow in this medium either alone or in the presence of the mucoid organism. At concentrations lower than 0·5%, the ability of this egg-yolk solution to stabilise mucus production gradually decreased. Purified egg-yolk lecithin stabilised mucus production completely over 48 h at all concentrations examined between 0·1 and 0·006%. Over a similar concentration range, synthetic dipalmitoyl lecithin effected complete stabilisation only at

the lower concentrations. These findings with dipalmitoyl lecithin were confirmed when the experiment was repeated with an extended range of concentrations and a longer incubation period (table VI). Maximum stabilisation of mucus production by strain PTO13(M) over a 96-h period occurred in the presence of very low concentrations (*c.* 0·0015%) of dipalmitoyl lecithin.

Discussion

These studies were undertaken to investigate the stability of mucus production in both naturally occurring mucoid strains of *Pseudomonas aeruginosa* and in mucoid variants obtained *in vitro* by the action of phage.

The ability to maintain mucoid *P. aeruginosa* indefinitely by subculture on DCA will be a useful aid in the study of these organisms. Present knowledge of

Table III

Effect of cetrimide on stabilisation of mucus production by Pseudomonas aeruginosa strain PTO13(M)

Percentage concentration (w/v) of cetrimide in basal broth	Ratio of mucoid to non-mucoid colonies (at the stated dilution) on plating from liquid cultures*
0·007	160/152 (10^5)
0·003	95/33 (10^5)
0·0018	57/0 (10^6)
0·0009	94/17 (10^6)
0	270/390 (10^5)

* See footnote to table I.

Table IV

Effect of acetyl cysteine on stabilisation of mucus production by Pseudomonas aeruginosa PTO13(M)

Percentage concentration (w/v) of acetyl cysteine in basal broth	Ratio of muciod to non-mucoid colonies (at the stated dilution) on plating from liquid cultures*
2	150/350 (10^5)
1	118/28 (10^6)
0·8	158/6 (10^6)
0·4	126/34 (10^6)
0·2	65/65 (10^6)
0†	35/50 (10^6)

* See footnote to table I.

† Basal broth containing 0·08% NaCl was used as solvent control.

TABLE V

Combined growth of Pseudomonas aeruginosa strain PTO13(M) and a non-mucoid variant of strain PTO13(M) in basal broth containing 0·5% egg-yolk solution

Duration of incubation (h)	Ratio of mucoid to non-mucoid colonies (at the stated dilution) on plating from liquid cultures* of strain or strains		
	PTO13(M) and PTO13 together	PTO13(M) alone	PTO13 alone
0	66/109 (...)	81/0 (...)	0/112 (...)
24	129/28 (10^5)	245/0 (10^5)	0/65 (10^5)
48	234/108 (10^5)	295/0 (10^5)	0/250 (10^5)
72	152/107 (10^5)	149/0 (10^5)	0/151 (10^5)
144	78/184 (10^4)	192/3 (10^4)	0/148 (10^5)

* See footnote to table I.

TABLE VI

Effect of dipalmitoyl lecithin on the stabilisation of mucus production by Pseudomonas aeruginosa strain PTO13(M)

Percentage concentration (w/v) of dipalmitoyl lecithin in basal broth	Ratio of mucoid to non-mucoid colonies (at the stated dilution) on plating from liquid cultures*
0·05	29/50 (10^5)
0·025	36/288 (10^5)
0·012	28/55 (10^5)
0·006	20/273 (10^5)
0·003	25/16 (10^5)
0·0015	102/0 (10^5)
0·0007	28/195 (10^5)
0·0003	15/416 (10^5)
0·00015	8/182 (10^5)
0†	19/38 (10^5)

* See footnote to table I.
† Basal broth containing 0·95% alcohol was used as solvent control.

the association of mucoid *P. aeruginosa* and CF suggests that the pulmonary system of the CF patient has the biological ability either to induce mucus production in *P. aeruginosa* or to select naturally occurring mucoid variants. Martin (1973) suggested that phage might be responsible for the induction of the mucoid form *in vivo*, inducing phage being provided by successive infections with different strains of *P. aeruginosa*. Prolonged chemotherapy has been discounted as the selective factor (Doggett, 1969), but other possibilities include the antiphagocytic effect of the bacterial mucus demonstrated *in vitro* by

Schwarzmann and Boring (1971) and inhibition by the mucus of the immune response of the patient to mucoid strains, i.e., the opsonising effect of the antibodies on mucoid bacterial cells might be less than on non-mucoid cells (Høiby and Axelsen, 1973).

The ability of various surfactants to stabilise mucus production *in vitro* suggests that such substances, e.g., dipalmitoyl lecithin, might contribute to the stability of mucoid strains *in vivo*. Dipalmitoyl lecithin is the major surfactant found in the human lung (Brown, 1964) and although the concentration at which maximum stabilisation occurred appears low it is approximately the same concentration as that found in normal lungs and shown *in vitro* to produce surfactant activity similar to that of extracts of normal lungs (Thannhauser, Bennotti and Boncoddo, 1946; Kuenzig, Hamilton and Peltier, 1965). It is perhaps significant that after pulmonary infections in CF patients the next highest incidence of mucoid *P. aeruginosa* also occurs in the lung in cases of chronic bronchitis (Burns and May, 1968).

The mechanism by which mucus production is established in the presence of surfactants requires further investigation. The results presented here suggest that it is not simply the preferential inhibition of non-mucoid cells, e.g., strain PTO13 outgrew the mucoid form when both were introduced into the same flask of DCB or lecithin medium. Stability of mucus production was maintained for at least 40 days in a primary DCB culture and was not accompanied by a reduction in the rate of bacterial growth when dipalmitoyl lecithin was present. Thus it would appear that the phenomenon is not due merely to a slower rate of reversion to the non-mucoid form. It was noted that mucoid colonies never appeared when cultures of the non-mucoid strain PTO13, grown in the presence of surfactants, were subcultured to nutrient agar.

Emphasis has been placed in these experiments on mucus production in *P. aeruginosa* strain PTO13(M). This strain was chosen because the genetic background of the parent strain PTO13 is already well known (Stanisich and Holloway, 1969*a* and *b*). Strain PTO13 contains the fertility factor FP2. Preliminary studies (Govan, unpublished) by conjugation techniques with PTO13(M) as the donor strain have indicated the transfer of the mucoid characteristic to a non-mucoid strain of *P. aeruginosa*. It is hoped that studies on the stability of mucus production in the presence of surfactants and on genetic aspects of mucus production may lead to a greater understanding of mucoid *P. aeruginosa* and the role of these organisms in CF disease.

Summary

Mucoid strains of *Pseudomonas aeruginosa* isolated from the respiratory tract of patients with cystic fibrosis (CF), those obtained from non-CF patients, and those obtained *in vitro* by the action of phage, were found to be stable in their mucoid colonial form when serially subcultured on deoxycholate-citrate agar. The ability of anionic, cationic and neutral surfactants to stabilise mucus production is described. The possible importance of dipalmitoyl lecithin as a stabilising agent for mucus production *in vivo* is considered, with particular reference to the role of mucoid *P. aeruginosa* in CF disease.

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MUCOID PSEUDOMONAS AERUGINOSA & RESPIRATORY INFECTION IN CYSTIC FIBROSIS

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Patients with cystic fibrosis (CF) are very susceptible to respiratory infections due to *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa;* the last named often present in a mucoid form, especially in severely ill patients. This statement could initiate much discussion on a number of points which remain the subject of controversy: 1. The relative importance of Staph. aureus as the main pathogen and the necessity of prior infection with Staph. aureus before the appearance of the predatory P. aeruginosa, 2. The significance of P. aeruginosa in either the non-mucoid or mucoid form to the clinical prognosis for the patient: is the presence of mucoid P. aeruginosa in the lung of the patient an insignificant consequence of pre-existing lung damage or does it contribute to it? In writing this article the author has taken cogniscance of the published literature and presumes that infection with mucoid P. aeruginosa is detrimental to the CF patient and that an understanding of the factors responsible for the association between mucoid P. aeruginosa and respiratory infection in CF is desirable.

Before discussing current research a brief outline will be given (1) regarding the manner in which the CF patient is rendered susceptible to respiratory infection and (2) the relevant biological properties of P. aeruginosa.

Susceptibility of the CF lung to bacterial infection.

In the healthy lung invading bacteria which settle in the upper respiratory tract are removed upwards by the wave-like movement of the ciliated mucus-coated epithelium lining the bronchial passages. Bacteria which escape this muco-ciliary escalator and penetrate the narrower non-ciliated alveolar passages are eliminated by an alternate defence mechanism namely ingestion and destruction by alveolar macrophages (phagocytosis). In the CF patient the presence of abnormally viscid bronchial secretions reduces the efficiency of the muco-ciliary escalator. Additional factors predisposing to infection in the CF lung are pre-existing lung damage due to previous bacterial infection or resulting from stasis caused by the viscid nature of pulmonary secretions. Prolonged antibiotic therapy may interfere with phagocytosis and also predispose towards infection with resistant micro-organisms such as P. aeruginosa.

P. aeruginosa: its role as a pathogen.

P. aeruginosa is the most troublesome member of a group of bacteria known in medical microbiology as opportunist pathogens, i.e. bacterial species which do not possess the invasiveness and virulence characteristic of many classical disease-producing bacteria but nevertheless cause infections due to a combination of factors. Such factors are normally a susceptible host whose natural defences against infection have been reduced by underlying disease and chemotherapy (e.g. in a patient with leukemia) and the ability of the opportunist pathogen to adapt and survive in environments hostile to many other bacteria, e.g. in the presence of antibiotics and disinfectants or in conditions of limited nutrition.

P. aeruginosa is a bacillus approximately 1 x 3 μ (1 mm = 1000 μ) in size. It is ubiquitous and can be isolated from a wide variety of habitats especially moist areas such as sinks and drains. It is one of the most adaptable of all micro-organisms and can utilise a large range of organic material for growth (from simple amino acids to human, and plant tissue and aviation fuels). It causes troublesome and sometimes fatal infections in man, animals, insects and plants. In man, almost no part of the body is sacrosanct. Infections caused by P. aeruginosa include meningitis, corneal ulcers, respiratory infections, urinary tract infections, infections of the nails and surface wounds such as burns. Despite this catalogue of crime, the organism is by nature a free-living saprophyte and harmless to the healthy human. It does, however, take advantage of any patient whose natural immunity has been compromised by disease or chemotherapy or when physical defences against bacterial infection such as skin or mucus membranes have been breached by contaminated equipment or impaired through tissue damage, e.g. in the case of corneal ulceration following minor eye injury. P. aeruginosa is naturally resistant to many antiseptics and disinfectants and to many commonly used antibiotics, the exceptions being carbenicillin (a penicillin derivative produced to combat P. aeruginosa, in particular) and the aminoglycoside antibiotics, gentamicin, tobramycin and amikacin. Infections with P. aeruginosa are often chronic because of the reduced immune state of the host, the antibiotic resistance of the organism and the difficulty in transporting antibiotics to the site of infection at high enough concentrations to be lethal for the bacteria and in the case of the aminoglycosides, non-toxic to the patient. P. aeruginosa does not produce a potent lethal toxin as does Clostridium tetani but instead owes its pathogenicity to the production of a barrage of relatively

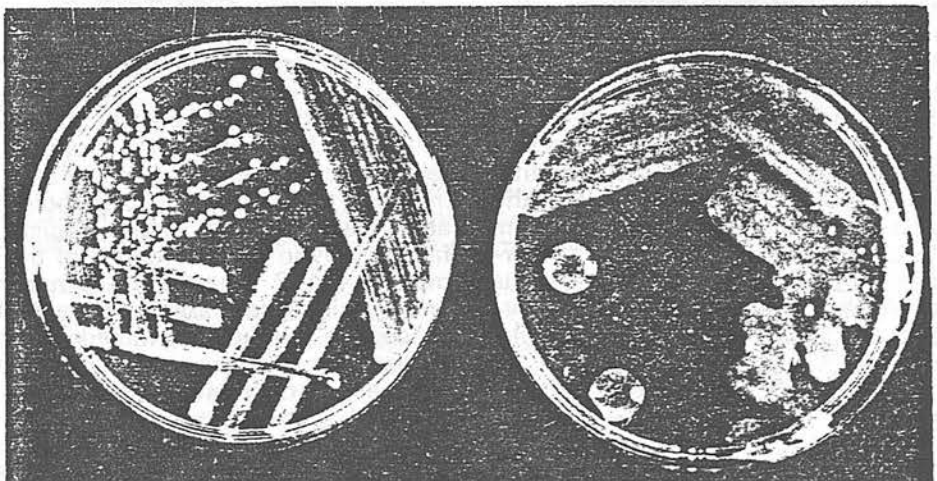

Figure 1. Contrasting cultural growth of a typical non-mucoid **P. aeruginosa** *(left) and a mucoid variant (right).*

weak toxins whose individual importance depends upon the site of infection. One of the most striking observations made in relation to P. aeruginosa is the association of the unusual mucoid form of the organism and respiratory infection in CF patients. Despite the widespread occurrence of P. aeruginosa in nature and in human and animal infections the mucoid form is rarely encountered and yet accounts for more than 75% of P. aeruginosa isolated from CF patients (Figure 1).

The notorious ability of P. aeruginosa to adapt to and survive in a very wide range of environments and to infect virtually any part of the human anatomy has earned it the title 'opportunist pathogen par excellence'. The reader may consider however that the association of the mucoid form of P. aeruginosa and debilitating respiratory infection in the CF patient justifiably merits more unflattering descriptions such as a microbial hyena or bacterial Jekyll and Hyde.

Nature of the mucus produced by mucoid P. aeruginosa.

Not only is the mucoid form of P. aeruginosa rarely encountered in the non CF patient, but in addition, the bacterial mucus itself is unusual. It is secreted from the bacterial cell as an exopolysaccharide, a polymer of mannuronic and guluronic acids and closely resembles the alginates obtained from brown seaweed. It chares with the seaweed alginates the ability to form viscid aqueous solutions which gel readily in the presence of Ca $^{--}$ ions. It is not produced by typical non-mucoid strains of P. aeruginosa and differs chemically from the bronchial mucus of the lung. There is little convincing evidence to suggest that the mucus produced by P. aeruginosa is toxic per se; it seems more probable that the pathogenic significance of the bacterial mucus is related to its high viscosity (Figure 2) in a lung already under stress due to the presence of viscid bronchial secretions and in addition the protection it could afford the bacterial cell in the presence of antibiotics and macrophages (Figure 3).

Mucoid P. aeruginosa and CF.

It has been observed in isolates of P. aeruginosa from CF patients that primary infection is usually with the non-mucoid form of the organism. Subsequently a mucoid variant emerges, comes to predominate and is often associated with a concomittant decline in the condition of the patient.

Pyocin typing (bacterial 'fingerprinting') of P. aeruginosa indicates that when mucoid and non-mucoid P. aeruginosa are isolated from the same CF patient they usually belong to the same pyocin type (Williams & Govan, 1973); in addition, continued studies have shown that the pyocin types encountered in mucoid isolates are not unusual but similar to those found in non-mucoid strains of P. aeruginosa isolated from the environment and from other pseudomonas infections. The hypothesis follows that the appearance of mucoid P. aeruginosa in the CF lung is not the result of secondary infection with naturally occurring mucoid strains of the organism but rather that the environment of the CF lung induces or switches on mucus production in the organism, or alternatively, selects out mucoid variants which have appeared as the result of spontaneous mutation. In either case the influence of the lung environment must be considerable since, in general, mucoid P. aeruginosa rapidly revert to the non-mucoid form when cultured in vitro. Mucus production can be stabilised in vitro by growth in the presence of surfactants similar to those found in the normal lung e.g. dipalmitoyl lecithin (Govan, 1975). To date, however, no sputum component obtained from a CF patient has been found to be capable of inducing mucus production in non-mucoid strains. Martin (1975) obtained mucoid variants of P. aeruginosa from non-mucoid strains after growth in the presence of bacterial viruses and suggested that such viruses or bacteriophage might be responsible for the induction of mucus production in P. aeruginosa in the CF patient. Little is known of the incidence and survival of such viruses in the human lung and their significance in the emergence of the mucoid P. aeruginosa remains unknown.

More recently with the financial support of the Trust (Project 113) we have made progress in understanding the nature of mucoid P. aeruginosa and the association with the CF patient by seeking answers to the following questions: Does the emergence of mucoid P. aeruginosa in the CF patient result from the selection of a rare mutation and can such a mutation be demonstrated? What advantages would such a mutation confer on the bacterium and could these be relevant in the CF lung? Is the bacterial exopolysaccharide antigenic i.e. capable of inducing the formation of antibodies and what role might immunological factors play in the emergence of the mucoid form? What is the genetic and physiological basis for exopolysaccharide production in P. aeruginosa?

Increased antibotic resistance of mucoid P. aeruginosa.

On the basis that mucoid variants of P. aeruginosa might be more resistant to antibiotics than non-mucoid strains, antibiotic therapy has been suggested as a plausible reason for the emergence and high incidence of mucoid P. aeruginosa in CF patients. The arguments against such a mechanism are that previous studies had detected no difference in the sensitivity of mucoid and non-mucoid strains towards anti-pseudomonas antibiotics (e.g. Carbenicillin), that mucoid strains were seldom isolated from the many other types of chronic infections due to P. aeruginosa and that, on occasion, mucoid P. aeruginosa had been isolated from patients who had not been given antibiotics. Our recent studies however, suggest that it would be wrong to ignore the possible influence of antibiotic therapy.

We examined the sensitivity of mucoid and non-mucoid strains of P. aeruginosa isolated from ten CF patients to the antipseudomonas agents carbenicillin and tobramycin and the antistaphylococcal agent flucloxacillin. The technique used to measure sensitivity was more precise than that used in previous studies. The results indicated that in each of the ten patients the mucoid form of P. aeruginosa was approximately 75% more resistant to each of the antibiotics than the non-mucoid form; the increased resistance was not observed in non-mucoid revertants.

The increase in resistance associated with the mucoid form is small compared to other mechanisms of resistance e.g. that mediated by

Figure 2. The highly viscid exopolysaccharide produce by mucoid variants of **P. aeruginosa.**

bacterial plasmids. It would be easily missed using conventional methods of assessing antibiotic sensitivity. Its discovery however is significant because it has provided the first convincing evidence that spontaneous mucoid variants exist in non-mucoid P. aeruginosa isolated from CF patients.

Isolation of mucoid variants.

Mucoid variants were isolated from the non-mucoid strains of P. aeruginosa isolated from each of the ten patients and from other non-mucoid P. aeruginosa not associated with CF. The technique used to isolate the mucoid variants was based on exposing approximately 10^7 cells of P. aeruginosa to a concentration of antibiotic slightly greater than that necesary to kill the non-mucoid population (Govan & Fyfe, 1978). In addition, analysis of the exopolusaccharide produced by the antibiotic-selected mucoid variants showed it to possess the same chemical structure and viscosity as the exopolysaccharide from clinical isolates.

Antibiotic concentrations in body fluids.

In assessing the possible role of antibiotic therapy in the selection of mucoid variants in the CF patient we must explain why mucoid strains are seldom isolated in the many other kinds of infections due to P. aeruginosa. A possible explanation might be provided by a closer look at one of the basic principles of antibiotic therapy namely the transportation of antibiotic to the site of infection and to the bacterial cell in particular.

In vitro, the sensitivity of a bacterial strain to an antibiotic can be measured in the presence of predetermined concentrations of antibiotic and the effect of chemical interference reduced or eliminated by careful attention to the experimental conditions. In clinical practice, however, the situation is quite different. The transportation of an antibiotic to the site of infection and in sufficient concentration to inhibit or prevent bacterial growth is dependent upon many factors e.g. the dosage of antibiotic used and route of administration, the stability of the antibiotic in the presence of body fluids and the nature of the target site e.g. the urinary tract or lungs. The human lung, and the CF lung in particular, is one of the most difficult sites to penetrate. Marks et al. (1971), in a pharmacologic study of carbenicillin in CF patients, observed that whilst satisfactory levels of antibiotic could be achieved in serum and urine the maximum levels achieved in the lung, even with six times the recommended dosage, never reached the minimum inhibitory concentration of carbenicillin for most strains of P. aeruginosa. Thus the CF lung could be considered a specialised environment in which a small increase in resistance would be of considerable selective advantage, an advantage of little consequence in other body tissues where the level of antibiotic can exceed that necessary to inhibit both mucoid and non-mucoid forms.

It would be wrong to conclude from these results that carbenicillin, or antibiotic therapy in general, is the actual or only reason for the emergence of mucoid P.aeruginosa in the CF patient. Nevertheless, these in vitro studies provide new evidence which supports the theoretical basis of this hypothesis and answers some of the previous arguments against such a mechanism. These results also emphasise the value of adapting standard techniques to detect subtle differences in antibiotic resistance and similarly, the necessity for continuous and careful attention to the use of antibiotics and in particular to the concentrations which can be achieved in the lung. Although mucoid strains are more resistant to tobramycin, the level of this antibiotic which can be achieved, at high dosage, can exceed the inhibitory concentration for mucoid and non-mucoid strains of P. aeruginosa and might explain the successful use of this antibiotic in eradicating some strains of mucoid P. aeruginosa from CF patients (McCrae, Raeburn & Hanson, 1976).

Our studies also revealed that mucoid strains are not invariably more resistant to antibiotics and in fact are slightly more sensitive than non-mucoid variants to tetracycline. Although tetracycline cannot inhibit P. aeruginosa at the concentrations obtainable in the lung, this result is significant. Is this increased sensitivity restricted to tetracycline? What is the mechanism for this increased sensitivity and could other more suitable antibiotics be found with similar properties?

Antibiotic therapy plays a vital part in the management and well-being of the CF patient. We must continue to probe the weaknesses of bacteria in order to develop better antibiotics and in the case of the present generation of antibiotics, to seek the means of achieving safe but effective concentrations in the lungs of the CF patient.

The reasons for the emergence of the mucoid form in the CF patient could be multifactorial and our research programme also includes immunological, genetic and epidemiological studies.

The immunological basis for selection.

There are several ways in which the production of the bacterial mucus could result in a selective immunological advantage for a mucoid variant, e.g. there is some evidence to suggest that the bacterial mucus may prevent killing by macrophages. More convincing evidence however does exist that following the emergence of the mucoid form in the patient, antibodies are produced against the non-mucoid components of the cell. This finding has been taken as evidence that only in the mucoid state is P. aeruginosa an invasive pathogen in the lung rather than a transient contaminant. If the bacterial mucus is a relatively weak antigen, or is non-antigenic, then the antibodies produced against the strongly antigenic non-mucoid cell components could lead to the selective phagocytosis of non-mucoid cells. Previous reports describing the antigenicity of the mucus are not convincing and further investigations must be made

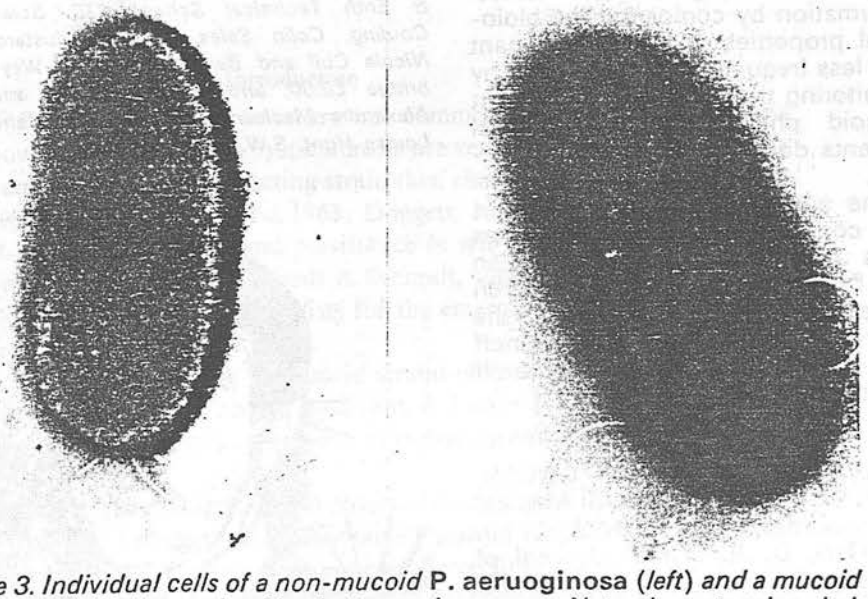

Figure 3. Individual cells of a non-mucoid **P. aeruoginosa** *(left) and a mucoid variant (right) observed with electron-microscopy. Note the extensive dark region of exopolysaccharide surrounding the mucoid variant.*

including those to surmount the technical problems associated with the viscid nature of the material. Another reason for continued immunological studies is to assess the effect of the bacterial mucus on the possible efficacy of vaccines against P. aeruginosa, presently under investigation.

The diverse population of mucoid P. aeruginosa within individual patients.

In the course of genetic studies into the nature of the bacterial mutation responsible for mucus production it was observed that mucoid variants isolated in vitro are not identical but can be divided into at least five phenotypic groups based on their cultural requirements for exopolysaccharide production and their degree of sensitivity to chemicals such as sodium deoxycholate. Subsequently an intensive examination of the population of mucoid P. aeruginosa in 20 patients revealed the same phenotypes and the observation made that, whilst an individual patient invariably harbours several phenotypes of mucoid P. aeruginosa simultaneously, the same two phenotypes predominate in all patients. Could this observation provide a clue to the reasons for the emergence of the mucoid form in the patient? It is hoped to gain further useful information by comparing the biological properties of the predominant and less frequent phenotypes and by monitoring the incidence of different mucoid phenotypes in individual patients during the course of infection.

The author readily acknowledges the contribution of his colleagues Miss Janet Fyfe, Miss C. McMillan and Dr K. C. Watson in this research project and financial support of the Jeffrey Charitable Trust of Crieff and the Cystic Fibrosis Research Trust.

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Christmas Cheer!

Another Christmas has passed us by and as usual the carol singers have been out in force and our most grateful thanks must be given to the following youngsters who raised such fantastic amounts: Ruislip Gardens Junior Church £12, The Afterthought, Ruislip Methodist Church YPG £25.50, St. Margarets R.C. Youth Club Twickenham £35, Sarah Corner. Alice Miller and Sarah Golton of Twickenham Girls School £3.15, Dawn Adams and her friends Rosemary Matthews, Steven Allen & Diana Hiller of Minchinhapmton £6.50, 1st Staincliffe (Christ Church) Guide Company Batley £15.50, the Hunt Family, High Wycombe £32 (including donation from Mrs. A. Smith, a local playgroup organiser), Suzanne Richards and Samantha Fortune £2, Miss Jane Williams (daughter Dr. Williams, Booth Hall) £4.54, 1st Copley Guides & Brownies, Huddersfield £4.80, the Choir at Christ Church, Shipley £33.50, Robinwood School, Todmorden £20, Jaqueline & Carol Jowitt and Alison & Hilary Webster, Otley £3.54. Miss Susan Hayward, Stamford Bridge £1.20, Goodmayes Primary School, Ilford £14.50, the Joint Churches in Epping £50.05, Miss Debra Colvin & friends, Benfleet £40, the two Simon Langton Schools, Canterbury £113.90, Ministry of Agriculture Staff at the Wye Sub-Centre £18, Brian & Cynthia Highbee and members of the Sunday School, Boughton Monchelsea £13, Bexley & Erith Technical School £30. Sarah Couling, Colin Sales, Stewart Mustard, Nicola Cull and Beverley Bristow, Weybridge £6.50, and Margaret, Toby and Alexandra Maclean, Rachel Howard, and Louise Hunt, S.W.18, £16.

£25 was received from the proceeds of Westone Lower Schools' Christmas Concerts and £8 was sent as part proceeds from two days of Xmas entertainments and sales at St. Davids School Purley. St. Pauls Hook C of E Primary School raised £50 during their Christmas concerts and Miss Karen Jones of Dudley made £3 by making and selling Christmas tags from old cards. £18 was collected at an afternoon Xmas presentation for parents at the Infants Dept., of Etwall CP School and £23.60 was sent as the proceeds from the Tower J.M.I. Schools' (Ware) performance of "A Christmas Carol".

£17.10 was collected at the 8th Pinner Scout Groups' Xmas Show and £14 at the Christmas Entertainment at Cannon Lane First School Pinner. £50 was collected at the Lovelace Junior School Xmas Concert at Chessington, and £25 at the carol service held by the Twickenham 7th & 7thA (Methodist) Brownie Packs. £27.50 was collected at the Christmas Play held by Coupe Green CP School, Preston and £40 by the Roman Hill Residents Association.

The following amounts were also the result of collections at carol services: Milton Regis Infants School £11.50. Sittingbourne & Tonge Division Girl Guides £10, Mills Hill CP School £20, Friesland School, Sandiacre £10, Dauridge Bay Middle School £9, Grindon Infant School, Sunderland £54, Brunswich Infants School Cambridge £4, Kilmacalm Primary School, Fenfrewshire £17, Ryton-on-Tyne County Junior School £13.75, Thaxted CP School, Dunmow £55.

A very proud Emma Lambourne is seen above handing to Deputy Executive Director, Bill Dower, a cheque for £530 being the amount raised for the Trust from the Annual Oxshott Christmas Fair held in Oxshott Village, Surrey. Emma is the daughter of new Walton and Weybridge Group Hon. Secretary Brenda Lambourne.

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Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: resistance of the mucoid form to carbenicillin, flucloxacillin and tobramycin and the isolation of mucoid variants *in vitro*

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Mucoid strains of *Pseudomonas aeruginosa* isolated from the sputa of patients with cystic fibrosis or *in vitro* through selection with phage were more resistant to carbenicillin, flucloxacillin and tobramycin and more sensitive to tetracycline than related non-mucoid strains. The observations led to the development of a technique for the isolation of mucoid variants *in vitro* based on enhanced resistance to carbenicillin. Mucoid variants were isolated from various strains of *Ps. aeruginosa* and the exopolysaccharide found to be similar to that obtained from mucoid *Ps. aeruginosa* isolated from patients with cystic fibrosis.

Introduction

Mucoid strains of *Pseudomonas aeruginosa* are not commonly isolated from patients (Doggett, 1969), however, children with cystic fibrosis are very susceptible to pulmonary infection with *Ps. aeruginosa* and the infecting strain then characteristically changes to a mucoid form (Iacocca, Sibinga & Barbero, 1963; Doggett, Harrison, Stillwell & Wallis, 1966). In contrast to their emergence and persistance *in vivo* mucoid strains generally revert to the non-mucoid form *in vitro* (Zierdt & Schmidt, 1964; Martin, 1973; Govan, 1975). No generally accepted explanation exists for the emergence of the mucoid form *in vivo* and reversion to non-mucoid *in vitro*.

The exopolysaccharides produced by 10 mucoid strains of *Ps. aeruginosa* isolated from 10 patients with cystic fibrosis were shown by Evans & Linker (1973) to be similar to each other and to consist of an acetylated polymer of D-mannuronic acid and L-guluronic acid.

In the course of phage typing, Martin (1973) observed mucoid growth of *Ps. aeruginosa* immediately around areas of phage lysis, and obtained mucoid variants after subculture from such areas. These mucoid variants were not considered to originate spontaneously, but to depend on the presence of phage in the lytic cycle for their initiation and continued existence.

Prolonged antibiotic therapy has been suggested as a causative factor in the emergence of mucoid *Ps. aeruginosa* in patients with cystic fibrosis (Iacocca, Sibinga & Barbero,

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1963; Doggett & Harrison, 1969). This paper follows a preliminary report (Govan, 1976) and describes the increased resistance of the mucoid form to 3 antibiotics used in the treatment of respiratory infection in the patient with cystic fibrosis and the isolation of spontaneous mucoid variants *in vitro* using these same antibiotics.

Materials and methods

Isolates of *Ps. aeruginosa* were obtained from the sputa of patients with cystic fibrosis and included non-mucoid and mucoid strains; these are prefixed CF, the suffix M indicates the mucoid form. The mucoid variant PAO 568 was derived from the non-mucoid strain PAO 381 (Stanisich & Holloway, 1969) after incubation with the virulent phage E79 (Govan, 1975). For the purpose of this study the term *mucoid* indicates variants which produce copious mucoid growth on agar medium within 24 h incubation at 37°C and resemble *Ps. aeruginosa* colonial type 5 (Phillips, 1969). Spontaneous non-mucoid revertants were isolated from PAO 568 and from the mucoid clinical isolates by subculture from cultures grown in nutrient broth (NB) at 37°C for 72 h without agitation to nutrient agar (NA). NB consisted of Nutrient Broth No. 2 (Oxoid) with 0·5% yeast extract (Oxoid) and NA was Columbia Agar Base (Oxoid).

The antibiotics were incorporated into Diagnostic Sensitivity Test Agar (DSTA, Oxoid CM 261), held at 45°C, dispensed in 20 ml volumes in plastic Petri dishes and used within 24 h. Calibrated platinum-tipped pipettes (Astell Laboratories, Catford, London) were used to apply a drop containing approximately 10^4 organisms from a logarithmic phase NB culture grown at 37°C in an Orbital incubator (Gallenkamp) at 140 rev/min. After the plates had been incubated at 37°C for 18 h the minimum inhibitory concentration (MIC) was calculated as the minimum concentration of antibiotic which inhibited growth of the organism.

In the single cell technique the culture was diluted in physiological saline such that an inoculum of 0·1 ml spread over the entire surface of the medium and incubated overnight at 37°C would give rise to approximately 10^2 bacterial colonies. The inoculum and the number of surviving colony forming units at different concentrations of antibiotic were recorded as the average colony count obtained from 3 culture plates and the MIC determined as before.

Organisms were grown in NB to the logarithmic phase, 0·05 ml ethyl methanesulphonate (Koch-Light, Colnbrook, Bucks) added to 10 ml culture and the mixture vortexed vigorously for 30 s. After incubation at 37°C for 1 h, without aeration, 0·1 ml of mutagenised culture was added to 10 ml NB and incubated at 37°C for 18 h.

The pyocine typing technique used was that described previously by Williams & Govan (1973) and incorporated the revised scheme of Govan (1978).

The method used for production and extraction of exopolysaccharide was based on that of Evans & Linker (1973). Freshly grown colonies of the mucoid variant on Pseudomonas Isolation Agar (PIA, Difco) were used to inoculate several plates of PIA. The plates were incubated for 24 h at 37°C followed by 24 h at room temperature (approximately 21°C); the surface growth was scraped off into 25 ml of sterile saline and vortexed vigorously until uniform. The suspension was centrifuged at 20,000 × g for 2 h and the supernatant retained. Three volumes of 95% ethanol were added slowly to the supernatant fraction with stirring. The gelatinous precipitate which formed was removed by means of a glass rod, washed twice in 95% ethanol and once with absolute alcohol, and finally dried *in vacuo* over P_2O_5.

Results

Association of the mucoid form of Ps. aeruginosa *with increased resistance to carbenicillin, flucloxacillin and tobramycin*

The antibiotic resistance of mucoid strains of *Ps. aeruginosa* isolated from 7 patients with cystic fibrosis was compared to that of non-mucoid *Ps. aeruginosa* isolated simultaneously from the same sputa. Using the standard agar dilution technique, the mucoid forms were consistently more resistant to carbenicillin, flucloxacillin and tobramycin (Table I). The close relationship between mucoid and non-mucoid *Ps. aeruginosa* isolated

Table I. Minimum inhibitory concentration of carbenicillin, flucloxacillin and tobramycin for mucoid and non-mucoid strains of *Ps. aeruginosa* isolated from 7 patients with cystic fibrosis. Standard agar dilution technique*

Strain	MIC (μg/ml) of:		
	Carbenicillin	Flucloxacillin	Tobramycin
CF 132	20	1600	0·30
CF 132M†	30	3000	0·45
CF 158	30	2000	0·25
CF 158M	50	4000	0·35
CF 176	70	4000	0·25
CF 176M	140	7000	0·50
CF 179	10	1800	0·25
CF 179M	40	3600	0·45
CF 400	80	4000	0·25
CF 400M	140	7000	0·45
CF 402	20	1800	0·50
CF 402M	30	3400	0·75
CF 404	20	1600	0·30
CF 404M	30	3000	0·45

*Antibiotic concentrations were raised in the following increments—carbenicillin 10 μg/ml, flucloxacillin 200 μg/ml and tobramycin 0·05 μg/ml.
†M = Mucoid.

from the same patient was indicated by pyocin typing which showed that both forms belonged to the same pyocin type. In each patient the MIC for a non-mucoid revertant. isolated *in vitro* from the mucoid form, was identical to that of the related non-mucoid clinical isolate. Since the increased resistance associated with the mucoid form was slight, further evidence was sought using the more sensitive single cell dilution technique. Investigation of the mucoid and non-mucoid isolates from all 7 patients and also non-mucoid revertants confirmed the increased resistance of the mucoid form to carbenicillin. A similar result was obtained using flucloxacillin and tobramycin and with all 3 antibiotics when the resistance of the mucoid variant PAO 568, isolated from PAO 381 *in vitro*, was compared to PAO 381 and the non-mucoid revertant PAO 575. The phenomenon of increased resistance associated with the mucoid form was not applicable to all other antibiotics; the mucoid strains were found to be more sensitive to tetracycline than related non-mucoid strains. Table II contains typical results obtained with carbenicillin and tetracycline when used against the mucoid strains CF 158M and PAO 568 and their

non-mucoid related forms. The MIC of carbenicillin for CF 158M was 50 μg/ml compared to 30 μg/ml for the non-mucoid strains CF 158 and CF 158MR. Similarly, the MIC of carbenicillin for the mucoid variant PAO 568 was 40 μg/ml compared to 20 μg/ml for the non-mucoid strains PAO 381 and PAO 575. In contrast using tetracycline, the MIC's for the mucoid strains was 20 μg/ml compared to 30 μg/ml for the related non-mucoid strains.

Table II. Sensitivity of mucoid and non-mucoid strains of *Ps. aeruginosa* to carbenicillin and tetracycline using the single cell agar dilution technique

		Colony count obtained with								
		No antibiotic	Carbenicillin (μg/ml)				Tetracycline (μg/ml)			
Strain	Description of strain	(Inoculum)	10	20	30	40	50	10	20	30
PAO 381	Non-mucoid parent	145	160	0	0	0	0	123	66	0
PAO 568	Mucoid variant of PAO 381	136	132	139	93	0	0	41	0	0
PAO 575	Non-mucoid revertant of PAO 568	118	29	0	0	0	0	108	63	0
CF 158	Non-mucoid isolate from patient with cystic fibrosis	119	116	104	0	0	0	114	121	0
CF 158M†	Mucoid variant isolated from same sputum as CF 158	115	119	109	100	105	0	54	0	0
CF 158MR	Non-mucoid revertant of CF 158M	240	157	130	0	0	0	193	193	0

†M = Mucoid.

Isolation of mucoid variants in vitro *by selection for antibiotic resistance*

Previously no mucoid variants of *Ps. aeruginosa* have been isolated other than from clinical material, generally sputum from patients with cystic fibrosis, or *in vitro* after incubation with phage. On the basis of the observed increased resistance of the mucoid strains to carbenicillin, flucloxacillin and tobramycin, these antibiotics were used successfully as selective agents to obtain mucoid variants from non-mucoid strains of *Ps. aeruginosa.*

The following procedure for the isolation of mucoid variants using carbenicillin was developed (Figure 1). Approximately 10^7 cells from an 18 h NB culture of *Ps. aeruginosa* were seeded onto the surface of DSTA or NA containing carbenicillin. The concentration of carbenicillin generally used was 1·5 times the MIC for the strain. After 18 h incubation at 37°C, the resistant colonies on the antibiotic medium were transferred by replica plating to freshly prepared PIA medium containing no antibiotic. After 18 h incubation at 37°C mucoid variants could be recognized as distinctive watery colonies amongst a background of non-mucoid growth.

Using this technique, mucoid variants were obtained from the 7 non-mucoid strains of *Ps. aeruginosa* tested in Table I and also from 4 non-mucoid strains isolated from 4 patients with cystic fibrosis from whom the mucoid form had not been isolated. Mucoid variants were also isolated from derivatives of *Ps. aeruginosa* strain PAO 1 (Holloway, 1969) using carbenicillin, flucloxacillin and tobramycin as the selective agent, e.g. having ascertained the MIC of tobramycin for PAO 381 to be 0·2 μg/ml mucoid

variants were isolated employing primary isolation plates containing 0·3 μg/ml tobramycin.

The frequency of isolation of mucoid variants observed with this technique was approximately 1 in 10^7 cells. In the case of PAO 381 this frequency was increased 40-fold following mutagenesis with ethyl methanesulphonate. The mucoid variants isolated through antibiotic selection did not require the continued presence of antibiotic for mucus production. Similarly, the phage-derived mucoid variant PAO 568 did not depend upon the continued presence of phage for mucus production. No phage activity directed towards the non-mucoid parent strain nor towards the strain used to propagate the phage was found in culture supernates after the mucoid variant had been subcultured several times on solid medium to remove residual phage acquired from the original selection plate.

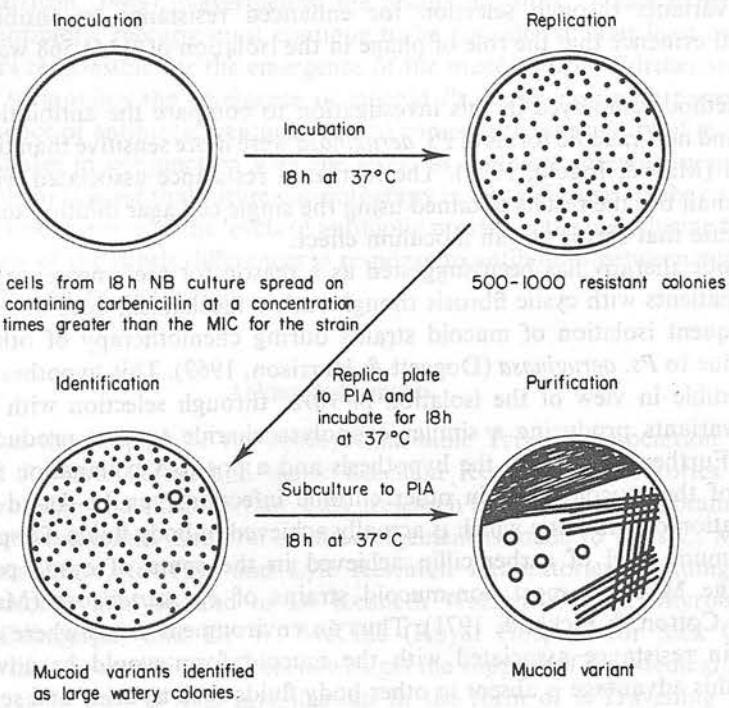

Figure 1. Technique for the isolation of mucoid variants of *Ps. aeruginosa in vitro* by selection for resistance to carbenicillin.

Physico-chemical properties of the exopolysaccharide produced by mucoid variants isolated in vitro

The mucoid variants obtained through selection with phage, carbenicillin, flucloxacillin or tobramycin produced exopolysaccharide which precipitated from cell-free extracts (see methods) on addition of three volumes of 95% (vol/vol) ethanol and redissolved in water to form a viscid solution. Similar precipitates were obtained from cell-free extracts obtained from each of the naturally occurring mucoid strains, CF 158M etc. but not from any of the non-mucoid strains investigated in this study. Infrared spectroscopy of the exopolysaccharide (Evans & Linker, 1973) from PAO 568 and from PAO 579

(mucoid variant of PAO 381 isolated using carbenicillin selection) indicated an acetylated polymer composed of mannuronic and guluronic acids.

Discussion

The physico-chemical properties of the exopolysaccharide from the mucoid variants isolated *in vitro* in this study are similar to those of the exopolysaccharides from mucoid strains isolated from patients with cystic fibrosis and described by Evans & Linker (1973). The ability to isolate mucoid variants *in vitro* from well characterised strains of *Ps. aeruginosa* is of considerable benefit in physiological and genetic studies of exopolysaccharide synthesis in mucoid *Ps. aeruginosa*. In contrast to the results of Martin (1973) the mucoid variant obtained in this study using phage was not dependent upon the continued presence of the phage for production of exopolysaccharide. The isolation of mucoid variants through selection for enhanced resistance to antibiotics provides additional evidence that the role of phage in the isolation of PAO 568 was as a selective agent.

The methods employed in this investigation to compare the antibiotic sensitivity of mucoid and non-mucoid forms of *Ps. aeruginosa* were more sensitive than those previously described (May & Ingold, 1973). The increased resistance associated with the mucoid form is small but the results obtained using the single cell agar dilution technique clearly demonstrate that this is not an inoculum effect.

Antibiotic therapy has been suggested as a reason for the emergence of the mucoid form in patients with cystic fibrosis though such a mechanism does not readily explain the infrequent isolation of mucoid strains during chemotherapy of other chronic infections due to *Ps. aeruginosa* (Doggett & Harrison, 1969). This hypothesis now appears more credible in view of the isolation *in vitro*, through selection with antibiotics, of mucoid variants producing a similar exopolysaccharide to that produced by clinical isolates. Further support for the hypothesis and a possible explanation for the relative absence of the mucoid form in other chronic infections can be found by noting the concentration of antibiotic which is actually achieved in body fluids. Despite high doses, the maximum level of carbenicillin achieved in the sputa of cystic patients seldom reaches the MIC for most non-mucoid strains of *Ps. aeruginosa* (Marks, Prentice, Swarson, Cotton & Eickhoff, 1971). Thus an environment exists where even the slight increase in resistance associated with the mucoid form would be advantageous. In contrast this advantage is absent in other body fluids such as urine and serum where the levels of carbenicillin achieved greatly exceed the MIC required for most non-mucoid and mucoid forms of *Ps. aeruginosa*.

The relevance of the increased resistance of the mucoid form of *Ps. aeruginosa* to the antistaphylococcal antibiotic flucloxacillin and the greater sensitivity to the broad spectrum agent tetracycline, may not be immediately apparent, since neither antibiotic is used in the treatment of infections due to *Ps. aeruginosa*. Continuous, life-long treatment with antistaphylococcal antibiotics has been recommended in patients with cystic fibrosis because of the belief that such patients are especially susceptible to this organism and that *Staphylococcus aureus* is the main respiratory pathogen (Lawson, 1970; Brumfitt & Hamilton-Miller, 1975). These opinions are not held unanimously, however, and to the arguments against antibiotic prophylaxis proposed by Raeburn (1976), e.g. the increased risk of infection with *Ps. aeruginosa*, must now be added the potential significance of antistaphylococcal antibiotics such as flucloxacillin in the emergence of

mucoid *Ps. aeruginosa*. The greater sensitivity of the mucoid form to tetracycline confirms quantitatively the result obtained by Zierdt & Schmidt (1964) using disc sensitivity tests but in addition offers the hope that other more appropriate antipseudomonas antibiotics might be developed with enhanced activity towards the mucoid form.

Although mucoid strains are more resistant to tobramycin and can be isolated using selection with this aminoglycoside the levels of this antibiotic achieved in the sputa of patients with cystic fibrosis are greater than the MICs for all the mucoid strains reported here and may explain the eradication of mucoid *Ps. aeruginosa* from some patients following treatment with this antibiotic (McCrae, Raeburn & Hanson, 1976).

The reasons for the emergence of the mucoid form of *Ps. aeruginosa* in patients with cystic fibrosis may be as protean and multifactorial as the disease itself. Mucoid *Ps. aeruginosa* has been isolated before the advent of antibiotics (Sonnenschein, 1927) and more recently, on occasion, from patients reported to have had no antibiotic treatment (Doggett & Harrison, 1969). Nevertheless, the results presented in this paper provide evidence that antibiotic therapy must continue to be considered as at least one of the potential factors responsible for the emergence of the mucoid form. Further studies are now necessary to monitor the emergence of mucoid *Ps. aeruginosa* with respect to the presence or absence of antibiotic treatment and to compare the sensitivity of mucoid and non-mucoid isolates in conjunction with the levels of antibiotic present in sputum. It seems reasonable to suggest that future chemotherapy in cystic fibrosis might continue to benefit from a close scrutiny of the levels of antibiotic present in the lung during treatment and an awareness of the subtle differences in response to antibiotics between mucoid and non-mucoid forms of *Ps. aeruginosa*.

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CHAPTER III

Pyocin Typing of *Pseudomonas aeruginosa*

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I. INTRODUCTION

The emergence of *Pseudomonas aeruginosa* in the last two decades as an important opportunist pathogen in nosocomial infections can be attributed to factors relating to both the organism and the human host. First, *Ps. aeruginosa* has a widespread distribution in nature, physiological adaptability and innate resistance to many antimicrobial agents. Second, there is

an increasing number of patients who are susceptible to infection with such an organism because of age, debilitation, or predisposing therapy, such as treatment with antibiotics, immunosuppressive agents or antimetabolites. The range of *Pseudomonas* infections is extensive and virtually no part of the human body is sacrosanct. No less extensive is the variety of sources in the hospital from which *Ps. aeruginosa* can be isolated. These factors and the difficulty encountered in successfully treating established infections make it essential constantly to monitor the different strains of *Ps. aeruginosa* present in the hospital environment in order to determine the mode of spread of a particular strain in an outbreak.

Three methods have been used to type *Ps. aeruginosa*, serotyping, phage typing and pyocin typing. The aim of this Chapter is to describe the techniques of pyocin typing and to comment on the value of this method for epidemiological studies.

II. PYOCIN TYPING

A. Pyocins (aeruginocins)

Pyocins are the bacteriocins of *Ps. aeruginosa*, i.e. antibiotic substances produced by strains of the species which have the characteristic property of being lethal only for other strains of *Ps. aeruginosa* or closely related species.

Pyocinogeny (the ability to produce pyocin) was first described by Jacob (1954) and is a stable characteristic which is normally repressed in most bacterial cells since synthesis and release of pyocin is lethal. The number of cells in which synthesis is initiated, can be increased by induction with ultra-violet light or Mitomycin C.

Several distinct categories of pyocin are now recognized (Holloway and Krishnapillai, 1974). R-type pyocins resemble the tail of contractile phage (Ishii *et al.*, 1965; Higerd *et al.*, 1967; Govan, 1968, 1974a, b) whilst morphologically distinct rod-shaped, flexuous or F-type pyocins resemble the tail of non-contractile *Pseudomonas* phage (Takeya *et al.*, 1969; Govan, 1974b). R-type pyocins may show immunological cross-reactivity with contractile phages (Ito and Kageyama, 1970) and synthesis of pyocin R is directed from a chromosomal locus (Kageyama, 1970a, b). Low molecular weight pyocins have also been described, pyocin A2 (Homma and Suzuki, 1966) associated with endotoxin from the bacterial cell wall and A3 derived from protoplasm. S-type pyocins, also of low molecular weight (approximately 10^5 Daltons), have been isolated by Ito *et al.* (1970).

Strains of *Ps. aeruginosa* are immune to their own pyocin, but sensitive cells are killed following attachment of pyocin to specific receptors on the cell surface. In the case of R-type pyocins, the receptors are lipopolysaccharide (Ikeda and Egami, 1969); contraction occurs following adsorp-

tion to the cell surface and is also observed following attachment of the pyocins to purified lipopolysaccharide from sensitive cells (Govan, 1974a). The nature of the receptor for F-type and low molecular weight pyocins remains unknown. Kaziro and Tanaka (1965a, b) have shown that in the case of pyocin R, cells are most sensitive in the logarithmic growth phase, synthesis of RNA, DNA and protein is interrupted at the ribosomal level without direct contact with ribosomes. The activity of F-type and S-type pyocins has not received much attention. When produced on solid medium,

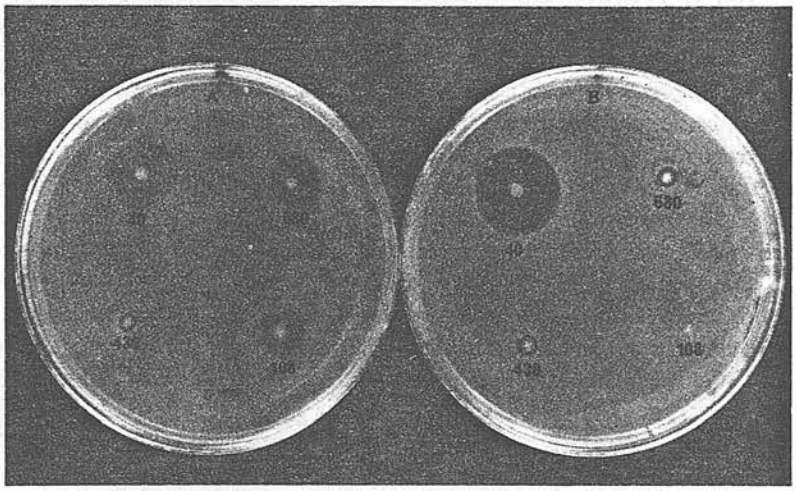

FIG. 1. Inhibition zones produced by four pyocinogenic strains of *Pseudomonas aeruginosa* against indicator strain 5. Plate A. Medium is Tryptone Soya Agar. Strains 40, 680 and 108 produce S-type pyocin activity; R-type pyocin activity is produced by strain 430. Plate B. Medium is Tryptone Soya Agar containing 0·05% trypsin. Strain 40 produces trypsin-resistant S-type activity; strain 680 now shows R-type activity—previously masked by S-type pyocin; strain 430 demonstrates the trypsin-resistance of R-type pyocin whilst the S-type pyocin of strain 108 is trypsin sensitive.

R- and F-type pyocins do not diffuse readily and their inhibitory action is restricted to an area beneath or closely surrounding the producer strain growth; in contrast S-type pyocins produce a wide zone of inhibition on solid media. S-type pyocins are generally trypsin sensitive but a trypsin resistant category has been observed (Govan, unpublished). These distinct types of pyocin can be produced alone or in combination (Ito *et al.*, 1970; Govan, 1974b; Figs. 1, 3 and 5) and, on the basis of biochemical studies, spectrum of activity and lack of serological cross-reactivity, represent distinct bacteriocins and not integrated forms or precursors.

B. Development of the method

Holloway (1960) reported that pyocinogeny was common in strains of *Ps. aeruginosa* and suggested that pyocin production might prove a useful epidemiological marker of this species. Darrell and Wahba (1964) described a cross-streaking technique based on the earlier colicin typing method for *Escherichia coli* of Abbott and Shannon (1958). The cross-streaking technique depends on the ability of a set of indicator strains of *Ps. aeruginosa* to provide patterns of inhibition when inoculated by "cross-streaking" over an area of medium which has previously supported the growth of the test or producer strain. According to the particular pattern of inhibition produced, the test strain is given a type designation. Strains producing no inhibition on any indicator strain are designated untypable (UT).

Darrell and Wahba used a set of 12 indicator strains and identified 11 types, labelled A-P, in 494 strains from 219 patients. Of these strains 91% were typable by this method and its reliability was supported by evidence of type stability in replicate isolates from the same patient. Wahba (1963) had attributed occasional heavy resistant growth in the area of inhibition to the production of extracellular enzymes which destroyed pyocin activity. Darrell and Wahba employed Oxoid Tryptone Soya Agar supplemented with iodoacetic acid, sodium citrate and dipotassium hydrogen phosphate to suppress the action of the pyocin-inactivating substances. Growth of the producer strain during the primary period of incubation was for 24 h at 37°C.

In 1966, Gillies and Govan described a cross-streaking technique which had been developed over several years, during which time the optimum conditions for production and detection of pyocins had been investigated and a set of indicator strains selected. Using eight indicator strains and growing the producer strain for 14–18 h at 32°C on Oxoid Tryptone Soya Agar incorporating 5% defibrinated blood, 88% of 5690 strains were typable and allocated to 37 pyocin types. The reliability of the technique for epidemiological purposes was satisfactorily validated in an extensive study (Gillies and Govan, 1966; Govan, 1968).

C. Method for pyocin typing (Gillies and Govan, 1966)

The strain to be typed is streaked diametrically across the surface of Oxoid Tryptone Soya Agar supplemented with 5% defibrinated horse blood to give an inoculum width of approximately 1 cm. After incubation for 14–18 h at 32°C the growth is removed with a microscope slide which has been dipped in chloroform and the remaining viable growth is killed by pouring approximately 3 ml chloroform into the lid of the Petri dish and replacing the medium-containing portion for 15 min. The plate is then opened and traces of chloroform vapour eliminated by exposing the medium

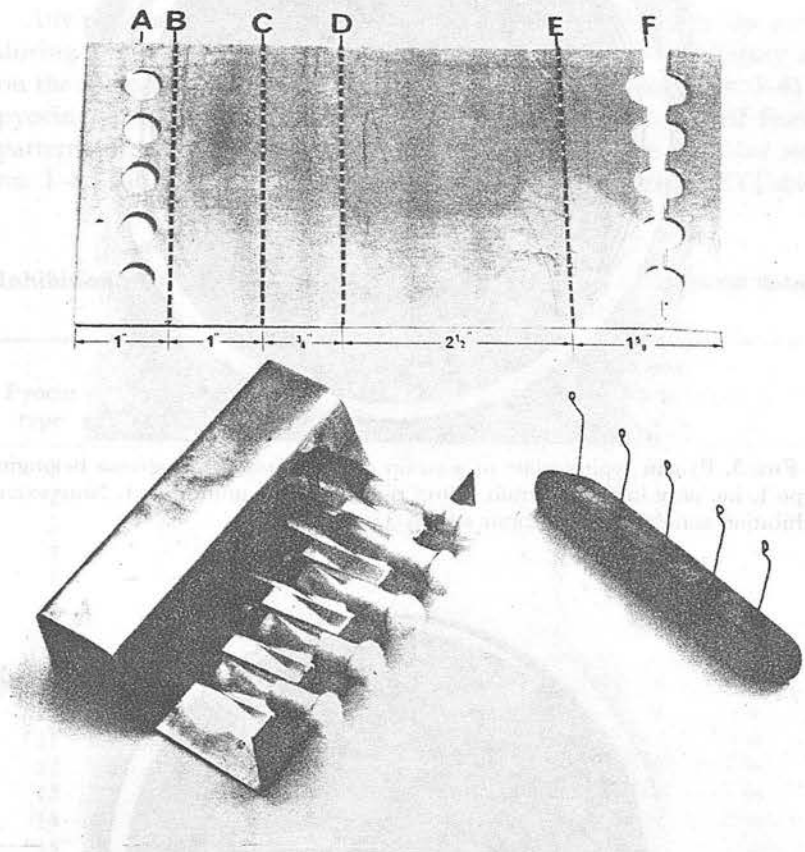

FIG. 2. Simple apparatus for application of indicator strains (Brown, 1973a). Reproduced by kind permission of *Medical Laboratory Technology*.

to the air for a few minutes. Plastic Petri dishes are not recommended but can be used provided that liquid chloroform is not allowed to come into direct contact with the plastic. The medium-containing portion of the dish can however be inverted over a small filter paper that has previously been saturated with chloroform.

Cultures of the indicator strains, grown under static conditions in Oxoid Nutrient Broth No. 2 for 4 h at 37°C, are streaked on to the medium by means of a multiple loop inoculator (Fig. 2) at right angles to the line of the original inoculum, starting from the original test strain growth area; indicator strains no. 1–5 are applied on the left side of the plate and no. 6–8 on the right side. When further indicator strains, A–E, are used, these

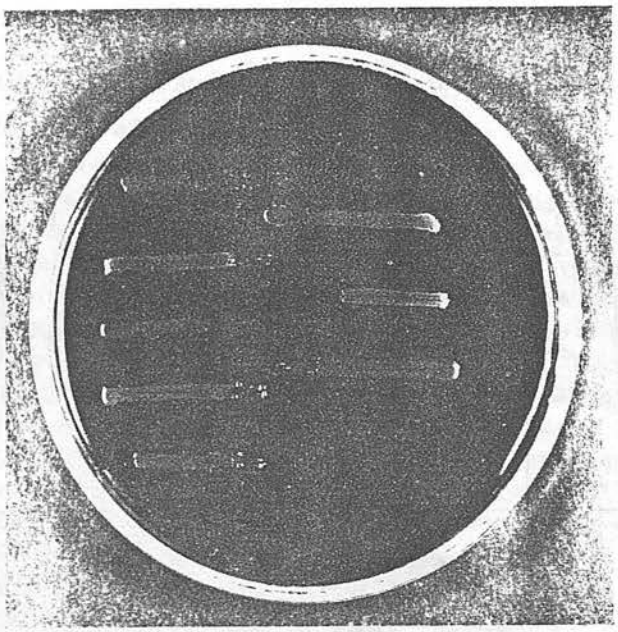

FIG. 3. Pyocin typing plate of a strain of *Pseudomonas aeruginosa* belonging to type 1, i.e. only indicator strain 6 (top right) remains uninhibited. Note extended inhibition zone against indicator strains 3 and 7.

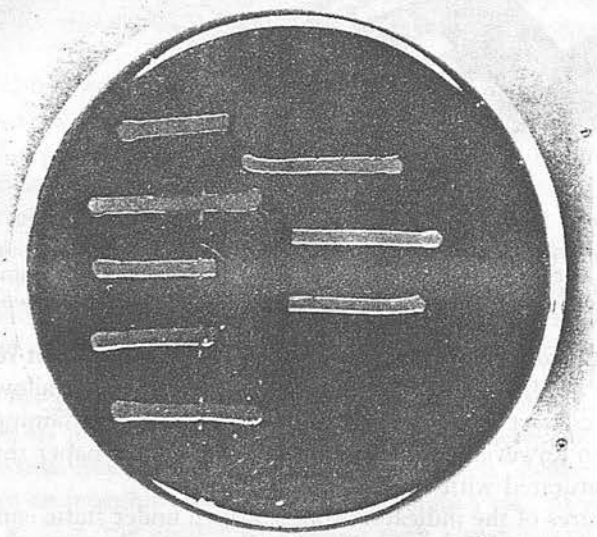

FIG. 4. Pyocin typing plate of a strain of *Pseudomonas aeruginosa* belonging to type 16. The zones of inhibition against indicator strains 1, 3, 4, 7 and 8 are uniform in size.

are streaked across a second plate from left to right. The plate is then reincubated at 37°C for 18 h.

Any pyocins produced by the original inoculum diffuse into the medium during the first period of incubation and then exert their inhibitory action on the indicator strains during the subsequent incubation (Figs. 3–4). The pyocin types of the strains under examination are recognized from the patterns of inhibition which they produce on the eight indicator strains, no. 1–8 (Table I) and the five additional indicator strains, A–E (Table II).

TABLE I

Inhibition patterns of pyocin types of *Pseudomonas aeruginosa* using the Gillies and Govan method

Pyocin type	Inhibition of indicator strain no.							
	1	2	3	4	5	6	7	8
1	+	+	+	+	+	−	+	+
2	−	+	−	−	−	−	−	−
3	+	+	+	−	+	−	+	−
4	+	+	+	+	+	−	−	+
5	−	−	−	−	+	−	−	−
6	+	+	+	+	+	−	+	−
7	+	+	+	−	−	−	+	+
8	−	+	+	+	−	−	+	−
9	−	−	−	−	+	−	+	−
10	+	+	+	+	+	+	+	+
11	+	+	+	−	−	−	+	−
12	+	+	−	+	+	−	−	+
13	−	−	−	+	−	−	−	+
14	−	−	+	−	+	−	+	−
15	−	+	−	−	+	−	+	−
16	+	−	+	+	−	−	+	+
17	−	−	+	−	−	−	+	−
18	+	−	+	+	+	−	+	+
19	−	−	+	+	−	−	+	−
20	−	−	−	−	+	+	−	−
21	−	+	−	+	+	−	−	−
22	+	+	+	−	+	+	+	−
23	+	−	−	−	+	−	+	−
24	−	−	+	+	+	−	+	+
25	+	−	+	−	−	−	+	−
26	+	−	−	−	−	−	+	−
27	+	−	+	−	+	−	+	−
28	−	−	−	+	−	−	+	−
29	−	+	−	−	+	−	−	−
30	−	+	+	−	−	−	−	−
31	−	−	−	−	−	−	+	−

TABLE I (*continued*)

Pyocin type	Inhibition of indicator strain no.							
	1	2	3	4	5	6	7	8
32	−	−	−	+	+	−	−	+
33	+	+	+	+	+	+	+	−
34	−	−	−	−	−	−	−	+
35	+	+	−	−	+	−	+	−
36	−	+	−	+	−	−	−	+
37	−	+	+	+	+	−	+	−
38	−	+	+	−	−	−	+	−
39	−	+	+	+	−	−	+	+
40	+	+	−	−	+	−	−	−
41	−	+	+	−	+	−	+	−
42	−	−	+	−	−	−	+	+
43	−	+	+	+	+	−	+	+
44	+	+	+	−	+	−	−	−
45	+	+	+	−	+	−	+	+
46	+	+	+	+	−	−	+	−
47	−	−	+	−	−	+	+	−
48	+	+	−	−	+	+	+	−
49	−	−	+	−	+	−	−	−
50	−	−	+	−	−	−	−	−
51	+	+	+	+	−	−	−	+
52	+	+	−	−	+	+	−	−
53	−	+	−	+	+	−	−	+
54	−	+	+	+	−	−	−	−
55	+	−	−	−	−	−	−	−
56	+	−	−	−	+	−	−	−
57	−	+	+	+	−	−	−	+
58	+	+	+	−	−	−	−	−
59	+	−	+	−	−	−	−	−
60	+	+	−	−	−	−	+	−
61	+	−	−	+	−	−	−	+
62	+	+	+	−	−	+	+	−
63	+	−	+	+	+	−	+	−
64	−	−	+	−	−	−	−	+
65	−	−	+	+	−	−	−	+
66	−	+	+	−	+	−	−	−
67	−	−	−	−	−	+	+	−
68	−	−	−	+	−	−	−	−
69	−	+	+	−	−	+	+	−
70	−	+	−	+	−	−	−	−
71	−	+	−	+	−	−	+	−
72	−	+	−	−	+	+	−	−
73	−	+	−	−	−	+	−	−
74	−	+	+	−	−	−	+	+

TABLE I (*continued*)

Pyocin type	Inhibition of indicator strain no.							
	1	2	3	4	5	6	7	8
75	+	−	+	+	−	+	+	−
76	+	+	−	+	+	+	+	−
77	−	+	+	−	−	−	−	+
78	−	+	−	+	−	+	+	+
79	−	−	−	+	+	+	+	+
80	−	+	−	+	−	−	+	+
81	−	+	−	−	+	−	−	+
82	−	+	−	−	+	−	+	+
83	−	−	−	+	+	−	+	+
84	−	+	−	−	−	−	+	−
85	−	+	−	−	−	−	+	+
86	−	+	+	−	−	+	−	−
87	−	+	+	+	−	+	−	+
88	+	−	−	−	+	−	−	+
89	−	+	+	+	−	+	−	−
90	−	−	+	+	−	−	−	−
91	−	−	+	+	−	+	−	−
92	−	−	+	+	+	−	+	−
93	+	−	−	+	−	−	−	−
94	+	−	−	+	+	−	−	+
95	+	−	+	−	−	+	+	−
96	+	−	+	+	−	−	−	−
97	+	−	+	+	+	−	−	+
98	+	−	+	+	−	−	+	−
99	+	−	+	+	+	+	+	+
100	+	+	−	−	−	+	+	−
101	+	+	−	−	+	−	−	+
102	−	−	−	+	−	+	+	−
103	−	−	−	+	+	+	−	+
104	−	−	−	+	−	−	+	+
105	+	+	+	+	−	−	+	+

+ = Inhibition; − = no inhibition.

D. Additional notes on the use of the pyocin typing technique

1. *Test strain*

The technique described does not stipulate the nature of the test strain inoculum. Experience, however, has shown that the best results are obtained from single colonies taken from non-inhibitory medium, e.g. nutrient agar. If desired, the test strain inoculum may be applied to the

agar surface by means of a sterile swab rather than with a bacteriological loop.

Edmonds *et al.* (1972) used a modified pyocin typing technique whereby the test strains were grown in Oxoid Tryptone Soya Broth at 32°C for 2–4 h before inoculation on to the typing medium. The reason for this additional step is not stated.

2. *Growth conditions during pyocin production*

In the course of developing the standardized pyocin typing technique,

TABLE II

Inhibition patterns of subtypes of common pyocin types using the Govan and Gillies method

Pyocin subtype†	Inhibition of indicator strain				
	A	B	C	D	E
a	+	+	+	+	+
b	−	+	+	+	+
c	−	−	+	+	+
d	+	−	+	+	+
e	−	+	+	−	+
f	−	−	−	−	−
g	−	−	+	−	+
h	−	+	−	+	+
j	+	−	−	−	+
k	−	−	−	−	+
l	−	+	+	−	−
m	+	+	+	−	−
n	+	+	+	−	+
o	−	+	−	−	−
p	+	−	+	+	−
q	+	−	+	−	+
r	+	−	−	+	−
s	−	−	+	+	−
t	+	−	+	−	−
u	−	+	−	+	−
v	−	−	−	+	−
w	+	+	+	+	−
x	−	−	−	+	+
y	−	−	+	−	−
z	+	−	−	−	−

+ = inhibition; − = no inhibition.

† Strains in these subtypes are designated as type 1/a, 5/f, UT/k, etc.

the clarity of inhibition was found to be greatly influenced by the duration and temperature of incubation of the test strain and the culture medium used (Gillies and Govan, 1966; Govan, 1968). There was considerable improvement in the clarity of inhibition when primary incubation was reduced from 48 h to 24 h and finally to 14 h (Fig. 5). In practice, incubation for 14–18 h is satisfactory. Even more important than the duration of incubation was the temperature employed during this phase; regardless of the duration of incubation employed, many strains, particularly those belonging to pyocin types 5 and 16, produced clear-cut inhibition at 32°C but gave inferior results at 35·5°C and failed to show any pyocin activity whatsoever after primary incubation at 37°C. Some strains, however, produced identical inhibition patterns at all three temperatures, though once again, inhibition is more clear-cut at the lower temperatures. Oxoid Tryptone Soya Agar supplemented with 5% defibrinated horse blood was found to be the most satisfactory culture medium for pyocin typing; equally good results have been obtained by the author with BBL Trypticase Soy Agar. Addition of blood is not essential, but provides a good contrast background for the reading of inhibition. No appreciable difference has been found using horse, sheep or human blood. Incorporation of iodoacetic acid, sodium citrate and dipotassium hydrogen phosphate (Wahba, 1963; Darrell and Wahba, 1964) does not improve the clarity of inhibition in the author's experience.

These optimum conditions (32°C for 14–18 h) for spontaneous pyocin production have been confirmed by Tagg and Mushin (1971) and Bergan (1973a). Tripathy and Chadwick (1971) reduced the number of untypable strains encountered by inducing pyocin production with Mitomycin C (0·5 μg/ml) in Difco Tryptose Agar and employing a 6 h incubation period at 32°C. Kohn (1966) reported that removal of the primary growth from the culture medium could be facilitated by inoculating the test strain directly on to Oxoid cellulose acetate strips. Not all pyocins are capable of passing through cellulose acetate spontaneously (Wahba, 1963; Govan, 1968, 1974b; Macpherson and Gillies, 1969) and the use of such membranes is not recommended for routine typing purposes.

3. *Indicator strains*

(a) *Maintenance.* Stock indicator strains can be maintained as freeze-dried cultures or as cultures grown on nutrient agar slopes contained in screw-capped $\frac{1}{4}$ oz bottles and held at 4 or 37°C. Routinely, cultures are maintained on nutrient agar, held at 4°C, and subcultures prepared after 14 days.

The stability of the indicator strains and the maintenance of suitable typing standards should be monitored by including several standard pyocin producer strains of known pyocin type in each group of tests.

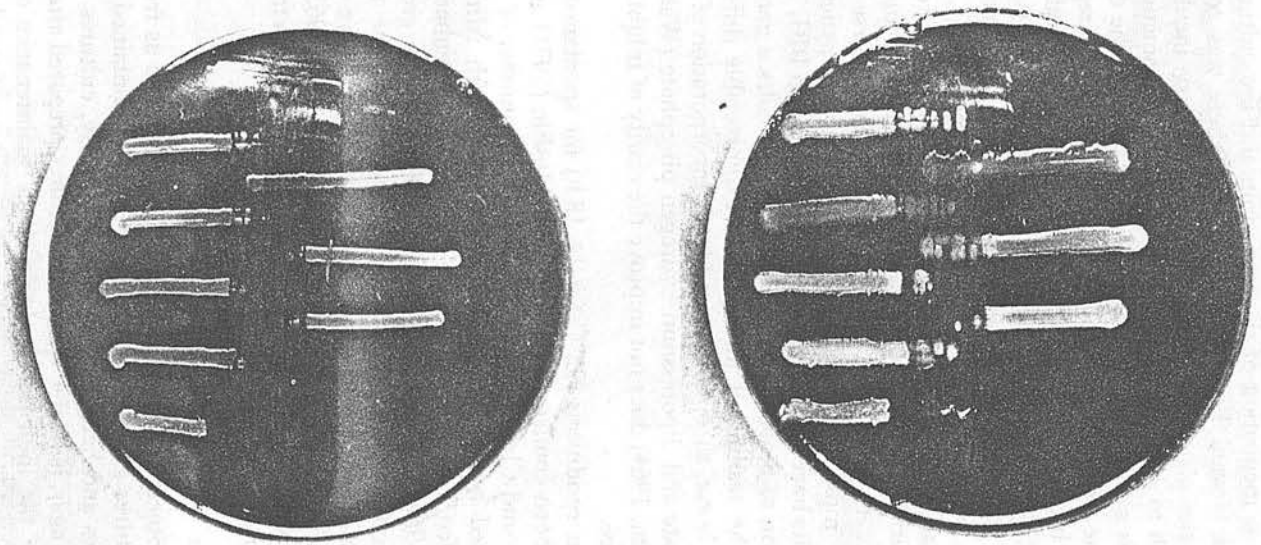

FIG. 5. Pyocin typing plates of a strain of *Pseudomonas aeruginosa* belonging to type 1. Following inoculation with the pyocinogenic strain both plates were incubated at 32°C for either 14 h (left) or 24 h (right). The eight indicator strains were then applied and both plates reincubated for 18 h at 37°C. Note the increased resistant growth against indicator strains 1, 2, 3, 4, 7 and 8 when primary incubation was for 24 h; a wide zone of inhibition is evident in both instances against indicator strain 5 and remains unaffected by the duration of incubation of the pyocinogenic strain.

(b) *Preparation.* A single colony is used to inoculate 2 ml of prewarmed Oxoid Nutrient Broth No. 2 contained in test-tubes measuring $3 \times \frac{3}{8}$ in. and incubated without agitation for 3–4 h at 37°C to yield a log phase culture containing approximately 1×10^8 cells/ml. Pellicle formation is usually minimal in such cultures, nevertheless before use each tube is agitated to ensure uniform turbidity. No significant improvement in the clarity of typing results was observed by the author when 18 h broth cultures were employed as indicators at various dilutions. Indeed, the best results using such diluted cultures were found using a dilution which matched turbidimetrically a standard undiluted 3–4 h broth culture. Bergan (1973a) observed that a 1/100 or 1/150 dilution of an indicator culture gave better results than undiluted cultures. It is difficult to assess the value of this modification since the parent 3 h broth culture had been prepared under vigorous agitation and therefore initially would contain a larger bacterial population than those employed in the standardized typing technique, i.e. after growth in test-tubes without agitation.

4. *Application of the indicator strains*

When pyocin typing is carried out on a large number of strains, the application of indicator strains individually by hand is tedious and time-consuming. Several types of applicator apparatus have been described (Wahba and Lidwell, 1963; Merrikin and Terry, 1969; Tagg and Mushin, 1971). Since 1967 we have routinely used a simple, yet effective, instrument (Brown, 1973a) which allows the processing of approximately 200 typing plates/h. The apparatus is inexpensive to manufacture, easy to sterilize and possesses a simplicity in keeping with the typing technique itself.

The apparatus (Fig. 2) consists of an aluminium stand to hold $3 \times \frac{3}{8}$ in. tubes of indicator strains and a rubber block containing bacteriological wire loops to spread these indicators. The tube holder is made from 32 S.W.G. sheet aluminium measuring $6\frac{7}{8} \times 3\frac{3}{4}$ in.; five holes each of diameter $\frac{7}{16}$ and $\frac{5}{8}$ in. between centres, are drilled out along the lines A and F and the end of the sheet cut off along line F to leave semi-circular notches. The sheet is then shaped to form the holder, with the area between lines D and E acting as the base.

The loop holder is a rubber eraser measuring $3\frac{1}{2} \times \frac{5}{8} \times \frac{3}{8}$ in. and the loops are made from 24 S.W.G. Ni-chrome wire. Each wire is fashioned from a $2\frac{3}{4}$ in. length. The end to be inserted in the rubber is turned back on itself for $\frac{1}{8}$ in. for better grip. A loop of $\frac{1}{12}$ in. diameter is made at the other end and the five wires are inserted into the rubber block at $\frac{5}{8}$ in. intervals to a depth of $\frac{1}{2}$ in. The free distal $\frac{1}{2}$ in. of the loops is curved to an angle of approximately 30° to allow easier inoculation of the culture plate and yet still permit the loops' insertion into the tubes of indicator broth culture.

The loops are sterilized to red heat in a bunsen flame and the charged loops are applied to the culture plate and drawn across it from the centre of the producer strain growth area to the edge of the plate. The loops are recharged after each inoculation without being resterilized. This model was designed for the application of five indicators to one side and another loopholder consisting of three loops is used to apply indicator strains to the other side. A composite model consisting of eight loops has also been employed.

5. *Incubation conditions following application of the indicator strains*

It was recommended (Gillies and Govan, 1966) that incubation of the test strain should be at 32°C for 14–18 h to ensure maximum spontaneous pyocin production, but incubation for a similar period at 37°C should be employed following addition of the indicator strains. Edmonds *et al.* (1972) and Shriniwas (1974) employed 32°C and a 14–18 h incubation period for pyocin production and during the subsequent incubation period. No reason is given for the use of 32°C during this second incubation period. The use of various periods and temperatures of incubation following the indicator strain application have been studied (Govan, 1968) and 14–18 h at 37°C found to give optimum results.

6. *Reading of results*

The style adopted for assignment of a strain of *Ps. aeruginosa* to a particular pyocin type is to decide qualitatively whether inhibition of an indicator strain has taken place (+) or whether no inhibition is present (−) and then allot a pyocin type by consulting the pattern charts.

The inhibition reactions encountered in pyocin typing are, (a) complete inhibition corresponding to the growth area of the test strain and with little or no resistant growth, (b) inhibition with many resistant colonies, (c) inhibition zones containing areas of confluent resistant growth.

The high molecular weight R and F pyocins do not diffuse readily through agar and give rise to zones of inhibition corresponding closely to the original growth area of the producer strain. The low molecular weight pyocins, however, give rise to areas of inhibition extending beyond the original growth area. Compare the extent of inhibition of indicator strains 3 and 7 (Fig. 3) and indicator strain 5 (Fig. 5) with that against the other indicator strains.

Typing results can be more fully recorded by noting both (a) the presence of inhibition over the producer growth area only (+) or inhibition extending beyond this area (+ + +), and (b) the presence of confluent resistant growth (RG) or significant numbers of resistant colonies (RC).

Although details of zone size and resistant growth do not alter the particular pyocin type allotted to a strain of *Ps. aeruginosa*, such information is often of considerable value in confirming the relationship of strains in epidemic situations. It should be noted that the amount of resistant growth can vary when a strain is typed on separate occasions. It is advisable, therefore, to compare the nature of inhibition obtained with two or more isolates, only when these are tested on the same occasion.

III. VALIDITY OF THE TECHNIQUE

Ideally, a typing technique should give reproducible results which are epidemiologically valid. It should also be applicable on an international level and allow the characterization of the maximum number of strains tested into a reasonable number of distinct types.

A. Epidemiological validity

The indices of reliability used in determining the validity of this method of characterizing strains of *Ps. aeruginosa* for epidemiological purposes were:

In vitro. Constancy of pyocin production after prolonged storage and/or subculture.

In vivo. (a) Constancy of pyocin type in replicate isolates from the same site in a given patient. (b) Uniformity of pyocin type in strains from an epidemic outbreak.

Considerable evidence that the technique described fulfils these criteria was obtained in studies of many clinical isolates over a three year period (Govan, 1968). Eight strains of *Ps. aeruginosa*, one each from pyocin types 1, 3, 5, 9, 10, 11, 16 and 31 were subcultured on nutrient agar once every 14 days and typed once a week for over two years without any alteration in the type pattern or quality of inhibition produced. In a much larger series of 260 strains, however, representing 22 pyocin types and stored for periods ranging from three months to three years, at room temperature, variations were noted. On retyping, 15 strains (5·7%) produced patterns of inhibition which differed from the original. All but two of these strains, however, had been stored for more than six months and in the two exceptions loss of activity towards only one indicator strain was observed.

Replicate isolates from the same site in 530 patients (2523 strains) treated in hospital were of the same pyocin type as the original isolate but in 68 instances (268 strains) different pyocin type patterns were encountered on different days of testing. In some instances this variation in type involved only one isolate differing from the majority. The interval between isolations varied from one day to six months and the number of replicates

1 to 55. In the case of patients treated at home the stability of pyocin production is even more impressive. Twenty-seven patients (59 strains) showed no variation in the pyocin type of replicate isolates from the same site and in only one instance was variation observed.

An examination of replicate isolates made on different days from different sites in the same patient also suggested considerable stability of pyocin production *in vivo*. Such replicate isolates from 168 hospitalized patients (519 strains) were of the same type in 133 patients and of different types in 35 patients (129 strains). One patient over a six month period produced 137 isolates of *Ps. aeruginosa* from nine types of specimen and a further 89 isolates from culture plates exposed at his bedside and all belonged to pyocin type 35.

The reason that not all isolates from the same site in a patient are of the same pyocin type could be due to simultaneous infection with more than one strain, instability of pyocin production or instability of sensitivity or resistance to pyocins in the indicator strains. When an average of six colonies were typed from each diagnostic plate, it was found that more than one pyocin type was found more frequently in hospital patients (10·2%) than in patients treated at home (3·8%). Deighton *et al.* (1971) found more than one pyocin type of *Ps. aeruginosa* from the same site in 15% of hospital patients and Heckman *et al.* (1972) in 13% of similar patients; no home-treated patients were present in these series. In view of the considerable stability of pyocin production both *in vitro* and *in vivo* and the difference in results obtained from hospital patients and those treated at home it is suggested (Govan, 1968; Govan and Gillies, 1969) that, in the majority of cases, the presence of more than one type in a specimen is due to multiple infection and reflects the high incidence of different strains of *Ps. aeruginosa* in the hospital environment.

It would be unreasonable to assume that loss of pyocin producing ability never occurs *in vivo*. Instability of pyocin production after storage has been reported by Hamon *et al.* (1961). A change in pyocin type after storage, noted in a minority of strains, usually entails loss of inhibition of one or more indicator strains rather than a complete loss of pyocin production (Govan, 1968; Zabransky and Day, 1969; Merrikin and Terry, 1972). Since approximately 50% of strains produce more than one form of pyocin simultaneously (Govan, unpublished), loss of inhibition against certain indicator strains could be due to the loss of the genetic determinant for one pyocin only.

B. Validation of the typing technique with respect to percentage of strains typed, type distribution and international application

The data presented in Table III summarizes the application of this

TABLE III

Pyocin typing of 10,708 strains of *Pseudomonas aeruginosa* in 11 countries using the method of Gillies and Govan

Country	No. of strains examined	Percentage distribution of most frequent pyocin types				No. of pyocin types encountered	Percentage of untypable strains	Percentage of strains allocated to one or other of the 37 types	Reference
		1	3	5	10				
Australia									
Victoria	1114†	30	21	4	11		9	82	Tagg and
New South Wales	219†	37	16	2	23	25	6	89	Mushin (1971)
Singapore	99†	47	10	0	17		10	85	
Canada									
Toronto	1820†	46	8	3	10	NS	10	NS	Duncan and Booth (1975)
Kingston	336†	43	10	4	9	21	11	87	Tripathy and Chadwick (1971)
Scotland	2396†	34	25	8	3	35	8	87	Govan and Gillies (1969)
Hungary	1043†	29	20	6	15	32	9	83	Csiszar and Lanyi (1970)
England	156†	42	11	5	11	15	4	84	Al-Dujaili and Harris (1974)
Holland									
Amsterdam	593	33	11	3	4	18	7	87	Siem (1972)
Arnhem	299	33	23	7	8	16	7	85	
Germany	210	18	16	9	7	NS	NS	NS	Neussel (1971)
Israel	199	40	8	5	11	18	5	87	Mushin and Ziv (1973)
Norway	486	32	20	14	6	NS	3	NS	Bergan (1973a)
U.S.A.									
Albany	238	31	10	14	8	20	10	76	Baltch and Griffin (1972
Milwaukee	1500	52	7	3	11	NS	12	85	Heckman *et. al.* (1972)

† Only one strain from each patient included.
NS = not stated.

pyocin typing technique in the examination of 10,708 strains of *Ps. aeruginosa* in 15 centres located in 11 countries. In assessing the validity of the technique in epidemiological studies, two items are worthy of comment and further consideration; (a) The set of indicator strains isolated in Scotland, labelled no. 1–8, have proved suitable for use on a world-wide basis and allow a high proportion of strains to be allocated to one or other of the 37 types. (b) Strains belonging to pyocin types 1, 3, 5, 10 and those designated untypable (UT) are those most commonly encountered, regardless of the geographical location.

1. *Pyocin type inhibition patterns 38–105.*

Originally, Gillies and Govan (1966) described 36 inhibition patterns encountered with the eight indicator strains and later introduced a 37th (Govan and Gillies, 1969). Each type had been confirmed on the basis of maintenance of the typing pattern (a) after strains had been subcultured and retested (b) in replicate isolates made from the same site in a patient.

Since 1964 over 15,000 strains of *Ps. aeruginosa* have been examined in this laboratory and many inhibition patterns have been encountered other than the 37 previously described. Because, with few exceptions, they are only seldom encountered, these potential new pyocin type patterns have not been published. However, because several of these patterns have also been encountered by other workers an extended list of 105 inhibition patterns is included on this occasion. This also contains patterns of inhibition encountered only by other workers.

It should be emphasized that the validity of these new pyocin types is based only on the stability of the inhibition pattern on repeated testing. In the absence of epidemiological data, the number of isolations of these new types have been included to allow estimation of their relative importance.

Bergan (1975) introduced the new pyocin type patterns 38–51 on the basis of at least four isolations. We have also encountered most of these new types on more than four occasions the only exception being type 49 which we have never encountered. In Australia, Tagg and Mushin (1971) isolated more than four strains belonging to types 46, 47 and 48, and type 47 has also been encountered on four occasions in Holland (Siem, 1972).

In addition, the following new pyocin types are suggested. Types 52–61 isolated by us on more than four occasions with type 52 also encountered by Siem (1972) and type 60 by Tagg and Mushin (1971). Types 61–69 were isolated on three occasions and include types 62, 68 and 69 isolated by Tagg and Mushin (1971); type 69 has also been observed by Siem (1972). Types 70–78 were isolated on two occasions and types 79–104 on one occasion only. Siem (1972) reported seven isolates of type 80 and more

than three isolations of type 88. Strains belonging to type 105 have been observed by Tagg and Mushin (1971) on more than four occasions.

2. *Subdivision of common pyocin types*

Universally, the most common pyocin inhibition pattern encountered is that of pyocin type I (Table III) and such strains together with those belonging to types 3, 5, 10 and UT account for 58% (Siem, 1972) to 85% (Heckman *et al.*, 1972) of all isolates. Clustering of so many isolates into relatively few types reduces the value of this pyocin typing method for epidemiological purposes and further subdivisions of these types would be beneficial.

In studies of pyocinogeny and the nature of the various pyocins produced by strains belonging to the common pyocin types, it became apparent that these common types were not homogeneous groups, but could be subdivided, e.g. compare the size of inhibition zones obtained with two strains of pyocin type 1 in Figs. 3 and 5.

The use of five additional indicator strains, labelled A–E, allowed subdivision of 795 strains belonging to type 1 into eight subtypes (Govan and Gillies, 1969). The technique was validated using the same criteria as were employed for the primary typing method and has proved a valuable additional aid for further characterization (Phillips *et al.*, 1968; Smith and Tuffnell, 1970; Rose *et al.*, 1971; Mushin and Ziv, 1973; Duncan and Booth, 1975).

To investigate the possibility of subdividing the other common pyocin types 2400 consecutive isolates of *Ps. aeruginosa* were examined for pyocin production against the eight primary indicator strains, the five indicators A–E and four additional strains, two of which had been used in a successful subdivision of strains belonging to pyocin type 5 (Brown, 1973b).

The subtyping indicators A–E, used in conjunction with the primary set, were sufficient to subdivide strains belonging to types 3, 5 and 10. In addition it allowed detection of pyocin activity, and therefore subdivision, in certain strains previously designated UT (untypable). The patterns of inhibition observed with indicators A–E have therefore been extended (Table II) and currently consist of 25 patterns labelled a–z. These include all the inhibition patterns obtained with the types 1, 3, 5, 10 and UT against this subtyping set. It should be noted that an inhibition pattern obtained with this set may be shared by two strains which have already been distinguished by their inhibition pattern against indicator strains no. 1–8. No confusion arises since type designation follows the convention type 1/a, type 3/e, type UT/k, etc.

Five hundred and sixteen strains belonging to pyocin type 1 could be allocated to one or other of the eight subtypes (1/a–1/h) already described

(Govan and Gillies, 1969) but a further 47 strains were distributed between a further 13 subtypes. Eleven of these 47 strains produced the inhibition pattern representing type 1/v and similar strains have been described by Rose *et al.* (1971) and Mushin and Ziv (1973). Strains belonging to types now designated 1/u and 1/y have also been isolated (Mushin and Ziv, 1973).

One hundred and forty-two of 289 strains belonging to pyocin type 3 could be allocated to type 3/e but types 3/a, 3/b, 3/l and 3/n are also represented.

Although they inhibit only one indicator strain of the primary set and might be considered to form a homogeneous group, strains of type 5 could be allocated to three subtypes, namely, 5/f, 5/j and 5/k.

One hundred and fifty-five strains belonging to pyocin type 10 could be allocated to eight subtypes predominantly 10/a, 10/b and 10/c.

Of particular value was the observation that of 170 strains previously designated UT, i.e. which had shown no pyocin activity against the eight primary indicators, 46 produced inhibition against one or more of the indicators A–E and could be allocated to at least four groups, namely UT/k, UT/l, UT/y and UT/z. The possibility of finding further indicators to detect activity in the remaining untypable strains was diminished by the evidence that these strains did not produce pyocin activity against any of the additional four indicator strains used in the investigation.

Ziv *et al.* (1971) have already used two of their own isolates as indicator strains and subdivided types 1, 3 and 10 each into two subgroups. The author, however, recommends the general use of indicator strains A–E for subtyping purposes. These strains have been shown capable of subdividing each of the common types into a reasonable number of subgroups and have already been distributed widely because of their present value in the subdivision of strains of pyocin type 1.

IV. TYPING OF MUCOID STRAINS OF *PSEUDOMONAS AERUGINOSA* BY PYOCIN PRODUCTION

A modified version of the standardized pyocin typing technique is used for one purpose only, namely to characterize the very mucoid strains of *Ps. aeruginosa* often isolated from patients suffering from cystic fibrosis (Fig. 6). In view of the failure, on many occasions, to detect any inhibition pattern against the indicator strains Schwarzman and Boring (1971) assumed that such strains were apyocinogenic. The present author considers it more likely, however, that such strains did produce pyocins but that these were prevented from diffusing into the medium by the mucus surrounding the bacterial surface.

The following modified technique (Williams and Govan, 1973) was found

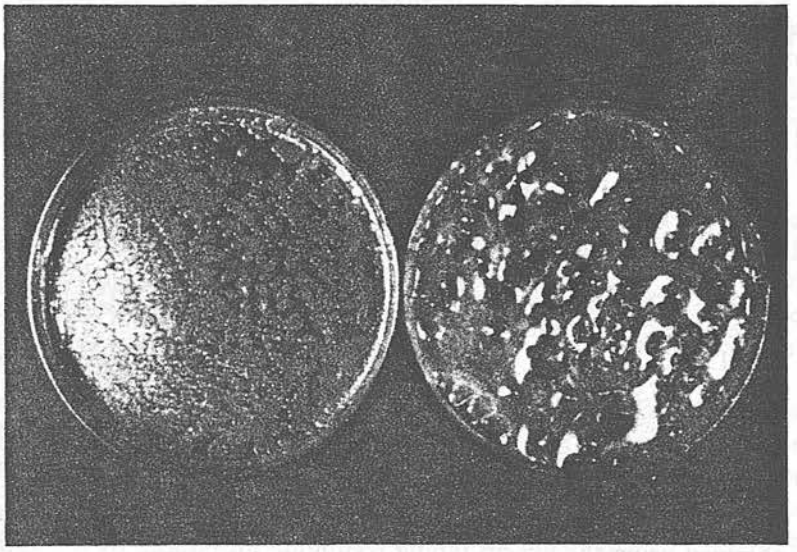

FIG. 6. Non-mucoid (left) and mucoid (right) strains of *Pseudomonas aeruginosa* grown on Oxoid Tryptone Soya Agar for 18 h at 32°C.

useful in typing mucoid strains. It consists of growing the mucoid strain in liquid culture and then assaying the cell-free supernate for pyocin activity against the standard indicator strains. The method is as follows.

A. Production of pyocins in liquid culture

A 100 ml volume of Oxoid Tryptone Soya Broth contained in a 2 litre flask is inoculated with 2 ml of an overnight broth culture of the mucoid strain and incubated at 32°C with vigorous agitation for 4 h. Pyocin production can be increased by addition of the inducing agent Mitomycin C (1·5 μg/ml final concentration) and further incubation for 2 h. The culture is centrifuged to remove cells and the supernate, containing pyocins, sterilized with 5% (v/v) chloroform for 15 min and decanted.

B. Detection of pyocin activity

Four-hour broth cultures of the indicator strains are used to prepare lawns on 9 ml nutrient agar contained in plastic Petri dishes (diameter 9 cm). Drops (0·02 ml) of serial two-fold dilutions of supernatants in sterile physiological saline are spotted on to well-dried indicator lawns. The drops are allowed to dry and the plates incubated for 18 h at 37°C. Pyocin activity is indicated by zones of inhibition on the indicator lawns.

The application of a range of dilutions to the indicator lawns allows

simultaneous recognition of phage activity which can be a useful additional epidemiological marker.

This technique is recommended for use only with mucoid *Ps. aeruginosa*; it is more time-consuming and requires more basic materials than the standardized pyocin typing technique. Provided that suitable agitation is used, the volume of primary culture could probably be reduced. Identical inhibition patterns, corresponding to recognized pyocin types, were obtained using induced and uninduced cultures.

V. CORRELATION BETWEEN TYPING METHODS

Regardless of the phage or pyocin typing scheme employed there appears to be little correlation between results of phage or pyocin typing (Sjoberg and Lindberg, 1967, 1968; Govan, 1968; Farmer and Herman, 1969; Bergan, 1973b). Considerable correlation does exist between certain pyocin types and serotypes.

Wahba (1965) noted that strains belonging to his pyocin types G, K, L, O and P belonged in general to the Wahba serotype 14 and to Habs serotypes 1, 3, 9 and 6 respectively. Further confirmation of correlation has been obtained using the pyocin typing technique introduced by the author and the serotyping schemes of Habs (1957) and Lanyi (1966). To avoid confusion in the following discussion, the serotype nomenclature of Lanyi has been converted to the corresponding Habs serotype designations. A significant correlation has been found between strains belonging to pyocin type 3 and serotype 6 (Govan, 1968; Csiszar and Lanyi, 1970; Siem, 1972; Bergan, 1973a). The ability of pyocin typing to subdivide particular serotypes and the relationship between specific pyocin types and a serotype is also evident from the following. Siem (1972) noted that of 149 strains of the unusual pyocin type 2, 131 belonged to serotype 6 and Govan (1968) found that all nine pyocin type 17 strains examined and all three pyocin type 19 strains also belonged to serotype 6. Csiszar and Lanyi (1970) observed that 12 of 13 pyocin type 29 strains and 14 of 25 pyocin type 5 strains belonged to serotype 3. Bergan (1973a) confirmed the latter correlation observing that 48 of 66 pyocin type 5 strains belonged to serotype 3. Pyocin typing carried out by Siem (1972) further confirmed this correlation and simultaneously showed a considerable subdivision of serotype 3 into five pyocin types; 17 of 36 pyocin type 5 strains, 10 of 11 pyocin type 9 strains, all five pyocin type 15 strains, 8 of 12 pyocin type 29 strains and 12 of 14 pyocin type 35 strains all belonged to serotype 3. Siem also confirmed that 10 of 11 strains of pyocin type 11 belonged to serotype 1. Initially there appeared to be little correlation between strains of pyocin type 1 and any particular serotype. However, when such strains are allocated to subtypes on the basis

of their activity against indicator strains A–E, certain correlations emerge. Csiszar and Lanyi (1970) observed that pyocin types 1/c and 1/d accounted for 80% of all strains of serotype 2; 15 of 17 strains belonging to pyocin type 1/f were of serotype 9 and 15 of 34 strains belonging to pyocin type 1/b were of serotype 11. Al-Dujaili and Harris (1974) noted that 11 of 15 strains belonging to pyocin type 1/h were of serotype 11.

Since it has already been shown that strains of pyocin types 3 and 5 may be subdivided using indicator strains A–E, it is not unexpected that the correlation between these primary types and serotypes 6 and 3 respectively is less than absolute. It would be interesting to observe the correlation results when subdivision of these pyocin types is investigated in conjunction with serotyping.

Pyocin typing as described here examines the ability of strains to produce a substance whereas serotyping is determined by the presence of antigens on the cell surface. The fact that the two techniques are based on different biological systems and yet demonstrate a degree of correlation does not present a conflict in regard to their value for epidemiological studies. If all the causal strains in an epidemic have a single ancestral origin and the two methods of typing used are considered reliable, then correlation should be absolute. The lack of complete correlation between a serotype and a pyocin type suggests the ability of one technique to subdivide the other, but could also result from occasional instability in the biological determinants of one or other technique.

VI. ALTERNATIVE METHODS OF PYOCIN TYPING

A different approach to pyocin typing has been to characterize strains of *Ps. aeruginosa* on the basis of their sensitivity to pyocins.

Lewis (1967) grew seven pyocin producer strains in agar for 18 h at 30°C, then examined the sensitivity of the test strain to the pyocins produced, by incorporating it in an agar overlay. Growth inhibition in the top layer appeared over primary growths and for 169 strains examined 17 types were distinguished.

The use of stock preparations of pyocins, resulting in a technique resembling phage typing, has received more attention. Osman (1965) used pyocin preparations obtained from four producer strains and differentiated 101 isolates into ten pyocin sensitivity patterns. A more elaborate two-stage technique was described by Farmer and Herman (1969). Firstly, pyocin and phage production was induced in a log phase culture of the test strain using Mitomycin C and the lysate tested for inhibitory activity against a set of 27 indicator strains. Secondly, 24 standard pyocin-phage lysates were employed to test the sensitivity of the test strain which had

been prepared under carefully standardized conditions. In this way an "epidemiological fingerprint" consisting of 51 operational characteristics could be established for each isolate. Each of the 157 strains examined was typable by this method and the "fingerprinting" technique was considered a sensitive tool for epidemiological studies.

Later in conjunction with other workers, Farmer considered that pyocin sensitivity was too unstable a property for use in epidemiological typing (Bobo *et al.*, 1973) and a simplified version of the "fingerprinting" technique based on pyocin production only was described (Jones *et al.*, 1974). In this method, pyocins are produced from test strains grown for 18 h at 32°C in Trypticase Soy Broth (without glucose) incorporating 1% potassium nitrate. These authors considered that the ability of most strains of *Ps. aeruginosa* to use nitrate as a terminal electron acceptor and thus grow uniformly throughout the broth eliminated the need for mechanical shaking during pyocin production and also induction with Mitomycin C. A set of 18 indicator strains was chosen from 250 strains, including 60 strains already used in other pyocin typing systems; the final set included 11 of the latter. The simplified technique gave the identical epidemiological results when used to type 23 isolates of *Ps. aeruginosa* from a nursery outbreak, previously investigated using the more elaborate "fingerprinting" method.

Rampling *et al.* (1975) described a technique based on pyocin sensitivity in which pyocin-phage lysates were rendered "phage-free" by ultraviolet irradiation; 27 such lysates were used to divide 105 isolates into 40 sensitivity patterns. When the reproducibility of the pyocin sensitivity patterns was examined by testing six control strains eight times in one experiment, only two strains gave consistent results.

VII. APPLICATIONS OF THE GILLIES AND GOVAN PYOCIN TYPING TECHNIQUE

Pyocin typing (Gillies and Govan, 1966; Govan and Gillies, 1969) has proved to be a valuable aid in epidemiological studies of *Ps. aeruginosa*. The technique has been used to determine the incidence of exogenously and endogenously acquired infections and to elucidate the sources, reservoirs and mode of spread of the organism in the hospital environment.

In an early application of the technique (Govan, 1968) two long-term episodes of cross-infection in two adjacent wards were detected retrospectively. During a 16-month period, strains of the relatively uncommon pyocin type 35 accounted for 26 (72%) of the 36 cases of infection with *Ps. aeruginosa* in unit A. Concurrently in unit B, strains of the equally rare

pyocin type 29 were isolated from 23 (70%) of the 33 similar cases of infection.

Outbreaks involving many patients in a short period of time are easily recognized. Insidious outbreaks of cross-infection, such as those just described, in which only a few cases are present simultaneously, are more difficult to detect and demonstrate the value of constant monitoring of infections with pyocin typing.

The use of pyocin typing has implicated faulty sterilization techniques and many inanimate vehicles in the spread of infections due to *Ps. aeruginosa*. Improper sanitization of urine bottles and milk feed stoppers was the cause of two large outbreaks (Govan, 1968). Duncan and Booth (1965) encountered an outbreak of infection due to an uncommon subtype of type 1. The causal organisms were isolated from rubber urine collection bags and the surrounds of the physiotherapy treatment pool. All three outbreaks ceased following improved sanitization procedures.

With hospital equipment, e.g. respirators, sterilization may be more apparent than real. Tinne *et al.* (1967) described an outbreak of respiratory tract infection in a cardiac surgery unit which affected seven cases with three deaths. All cases were due to pyocin type 10 strains and post-operative mechanical ventilation implicated. It was significant that the last three cases of infection occurred after "sterilization" of the respirator by disinfection and exposure to ethylene oxide. The unit was closed, sanitized and intricate equipment dismantled before exposure to ethylene oxide. On reopening the unit another case of respiratory tract infection with a type 10 strain was encountered raising doubts whether these measures had been effective. It was found, however, that this patient had been present in the unit during the outbreak. The organism was eradicated from this patient's sputum by chemotherapy but not before cross-infection had occurred to the patient in the adjoining bed.

The role of the infected patient and human carrier following successful sterilization of implicated apparatus was further emphasized by Fierer *et al.* (1967) in the first reported use of pyocin typing in the U.S.A. The original cause of an epidemic which involved 22 new-born infants and resulted in two deaths, was found to be delivery room resuscitation equipment that had been contaminated with *Ps. aeruginosa* via a wash-sink aerator. After disinfection the causal strain was never again isolated in the nursery except from infected babies. Cross-infection through the contaminated hands of personnel was considered the most likely means of transmission to infants not resuscitated at birth and for the 12 new cases encountered after eradication of the causal organism from the equipment. Deighton *et al.* (1971) used pyocin typing to show that infected hands of personnel contributed to the spread of infection during bathing of infants. Repeated

outbreaks of cross-infection were encountered and it was shown that objects and persons in contact with infected patients became contaminated with the causal strain. In contrast, Baltch and Griffin (1972) found no evidence of cross-infection with *Ps. aeruginosa* in a comprehensive study lasting 12 months; they were unable to detect the same pyocin type in several patients on one ward or room at a given time.

Although there was clear evidence for cross-infection in extended-care and urology wards, Duncan and Booth (1975) were tempted to conclude from pyocin typing results, obtained in a three year investigation, that endogenous rather than exogenous spread was the most common method of spread in *Ps. aeruginosa* infections. Patients acquire an increased faecal carriage of *Ps. aeruginosa* following admission to hospital (Shooter *et al.*, 1966) and pyocin typing has demonstrated that faecal carriage may lead to endogenous infection (Govan, 1968; Deighton *et al.*, 1971). The manner in which colonization of the gut occurs is not clearly understood but may originate in contaminated foods.

More unusual evidence for endogenously acquired infection derives from a microbiological study of submarine crews during long patrols. Morris and Fallon (1973) encountered an outbreak of otitis externa which was shown by pyocin typing to be due to the same strain of *Ps. aeruginosa* as that isolated a few days previously from apparently healthy throats.

Heckman *et al.* (1972) made valuable use of pyocin typing to determine the sites of colonization of patients with the same pyocin type. In addition to a study of sites and patterns of colonization, results from 17 patients demonstrated correlation between clinical diagnosis and the pyocin types of organisms isolated from one or more ante-mortem sites and post-mortem material.

Williams and Govan (1973) used both the standard technique and the modified method, described in this Chapter, to investigate the relationship between mucoid and non-mucoid strains of *Ps. aeruginosa* isolated from the sputa of children with cystic fibrosis. Mucoid strains are frequently observed in the sputa from such children (Doggett, 1969). Mucoid and non-mucoid strains of *Ps. aeruginosa* are often isolated from the same specimen, and as the infection progresses the mucoid strain predominates with a concomitant deterioration in the condition of the patient (Doggett *et al.*, 1966). Mucoid and non-mucoid strains isolated from the same specimen belonged to the same pyocin type suggesting that the mucoid variant was derived from the non-mucoid strain.

Pyocin typing has been used in studies of chemotherapeutic agents and in the therapeutic use of pyocins themselves, to determine relapse or reinfection (Brumfitt *et al.*, 1967; Phillips *et al.*, 1968; Govan, 1968; Williams, 1974). Shulman *et al.* (1971) used pyocin typing to demonstrate

the emergence and spread, in a burns unit, of a Gentamycin-resistant strain of *Ps. aeruginosa* belonging to pyocin type 5. The Gentamycin-resistant strain did not spread to areas of the hospital which did not use Gentamycin and the outbreak was dramatically reduced following discontinuation of the routine use of the antibiotic.

Pyocin typing has been shown to be applicable in the study of *Ps. aeruginosa* from non-human sources such as turkeys and horses (Govan, unpublished) and in cattle and many other animals (Mushin and Ziv, 1973).

The use of pyocin typing in many studies of the epidemiology of *Ps. aeruginosa* infections reveals numerous routes, sources and vectors. Several common factors emerge, however, which are important if such infections are to be prevented or controlled.

1. The hospital environment usually harbours many different pyocin types of *Ps. aeruginosa* and, without a suitable method of characterizing the organism, it is impossible to monitor infections or investigate and control outbreaks.

2. Except in episodes of cross-infection, the distribution of pyocin types encountered in infections in a unit, over a period of time, follows no regular pattern. The epidemiology of nosocomial infections due to *Ps. aeruginosa* is therefore different from similar staphylococcal infections where a large proportion of infections are caused by a few resident "hospital" staphylococci.

VIII. BIOLOGICAL PROPERTIES AND PYOCIN TYPES

No statistically valid evidence has been reported to correlate particular pyocin types of *Ps. aeruginosa* with pigment production and colonial morphology (Csiszar and Lanyi, 1970), preference for a particular site or type of infection (Heckman *et al.*, 1972; Duncan and Booth, 1975) or virulence (Baltch and Griffin, 1972; Al Dujaili and Harris, 1975).

IX. THE FUTURE

A. International standardization of pyocin typing

Formal pleas have been made for international standardization of a single technique of pyocin typing and for a mnemonic system of type nomenclature (Herman and Farmer, 1970; Farmer, 1970).

Table III illustrates the widespread use of the Gillies and Govan technique and suggests that *de facto* standardization already exists. To the author's knowledge the indicator strains have already been distributed from this laboratory to more than 200 centres.

B. Choice of a particular typing method

No single typing technique for *Ps. aeruginosa*, phage typing, pyocin typing or serotyping has proved completely satisfactory and in many epidemiological studies strains have been examined using more than one typing method.

Phage typing requires preparation, maintenance and standardization of phage stocks and of the three methods, is probably the most laborious to undertake. The method is less reproducible than pyocin typing and serotyping and sensitivity to phage is altered by changes in colonial morphology due to dissociation (Shionoya and Homma, 1968). An advantage of phage typing is that it divides strains into a large number of phage types. Bobo *et al.* (1973) investigated an outbreak of *Ps. aeruginosa* infection in a nursery using five typing methods including phage typing. Pyocin production and serotyping proved to be the most useful and stable markers.

Serotyping shows good reproducibility, but the preparation or purchase of antisera is relatively expensive. The main disadvantages of this technique are that it does not provide as large a number of distinct types as the other methods and, in addition, the majority of strains can be allocated to only a few common types.

Similarly, one of the main criticisms of the Gillies and Govan pyocin typing scheme was that the majority of isolates examined belonged to a relatively few common pyocin types. Subdivision of the most common type, pyocin type 1 (Govan and Gillies, 1969), has greatly improved the sensitivity of the technique. Even better differentiation is now possible following the introduction of a scheme for subdividing the other common pyocin types using the existing indicator strains A–E. A disadvantage of this method of typing is that the technique requires three days to complete.

Following the improvements outlined in this Chapter, pyocin typing now has the necessary properties to recommend it as the sole typing system for *Ps. aeruginosa* in all laboratories. It is suggested, however, that reference centres use both pyocin typing and one other technique. Phage typing has much to offer but, if serotyping was chosen, this would allow further analysis of the obvious correlation between certain serotypes and pyocin types.

C. Future developments

Pyocin typing benefits considerably from basic knowledge of pyocins and pyocinogeny and will continue to do so in future. Already the nature of the individual pyocins produced alone or in combination by strains belonging to several pyocin types has been determined (Govan, 1973b and unpublished; Brown, 1973b). Electron microscopy, pyocin-resistant

mutants, diffusibility and trypsin sensitivity can be used to determine heterogeneity of pyocin production.

Subdivision of the more common pyocin types, using indicator strains A–E, increases the sensitivity of the technique and further subdivision of these and other pyocin types is probable. The number of untypable strains may be further reduced by selection of further indicator strains but a lack of detectable pyocin activity *per se* is a useful epidemiological marker. The introduction of certain R factors into a pyocinogenic strain of *Ps. aeruginosa* can lead to the apparent loss of pyocin production (Jacoby, 1974; Govan, unpublished), and further investigation of this phenomenon could be relevant to pyocin typing in epidemiological investigations.

Although the optimum condition may have been found for pyocin production it seems reasonable that future use of pyocin typing techniques will encounter typing patterns not yet published.

D. Pyocin(e) or aeruginocin(e) typing

The name "pyocine" was first introduced by Jacob (1954) in a paper, written in French, to describe a bacteriocin of the species *Pseudomonas pyocyanea* (the alternative species epithet for *Pseudomonas aeruginosa*). At present, the term "pyocin" or the French form "pyocine" is used by many workers. In 1970 the Judicial Commission of the International Committee on the Nomenclature of Bacteria accepted the name *Pseudomonas aeruginosa* in place of *Pseudomonas pyocyanea*. To avoid confusion the term "pyocin typing" has been retained throughout this Chapter but it seems logical that in future the more correct term would be "aeruginocin typing".

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The Instability of Mucoid *Pseudomonas aeruginosa*: Fluctuation Test and Improved Stability of the Mucoid Form in Shaken Culture

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INTRODUCTION

The first description of mucoid *Pseudomonas aeruginosa* by Sonnenschein (1927) also reported the segregation of non-mucoid revertants. Subsequently, the instability of mucoid strains isolated either from patients or *in vitro*, and cultured on agar or in broth, has been well documented (Henriksen, 1948; Zierdt & Schmidt, 1964; Martin, 1973; Govan, 1975); little is known, however, of the mechanism underlying instability.

The ability to maintain *P. aeruginosa* in the mucoid state *in vitro* is essential in studies of this unusual form. Govan (1975) described improved stability when mucoid *P. aeruginosa* was grown in the presence of surfactants, such as lecithin or sodium deoxycholate. In this report we describe improved stability in nutrient broth cultures, suitably agitated, and suggest an explanation for the instability of the mucoid form. We report the use of a fluctuation test and growth rate experiments to indicate that, in unshaken broth cultures, instability can be explained on the basis of spontaneous mutation to the non-mucoid form, followed by a growth rate advantage for non-mucoid revertants. Under conditions where stability is improved, as in the case of shaken cultures, this growth rate advantage is not so apparent.

METHODS

Bacteria. Strain PAO568 is a mucoid variant of the non-mucoid *Pseudomonas aeruginosa* strain PAO381 (*leu-38 str-6* FP2; Stanisich & Holloway, 1969) and produces an acetylated alginate-like exopolysaccharide composed of mannuronic and guluronic acids (Govan & Fyfe, 1978). A non-mucoid revertant, PAO547, was obtained by subculture from the spreading, non-mucoid growth which appeared at the edge of a mucoid colony after several days growth on nutrient agar at 37 °C. Strain PAO548 is a prototrophic derivative of PAO547 obtained by transduction using phage F116L (Krishnapillai, 1971) propagated on the strain PAO1 (Holloway, 1955).

Media. Nutrient broth (NB) was Oxoid no. 2 (code CM67) with 0·5 % (w/v) yeast extract (Oxoid) and nutrient agar (NA) was Columbia Agar Base (Oxoid, CM331). Desoxycholate citrate agar (DCA) was Oxoid CM227. Minimal agar (MA) was that described by Vogel & Bonner (1956).

Transduction. The method used was that described by Holloway *et al.* (1962).

Fluctuation test. Fluctuation analysis was based on the method described by Luria & Delbruck (1943). A mucoid colony from an 18 h culture of PAO568, grown on DCA, was suspended in 2 ml physiological saline and diluted in saline such that addition of 0·1 ml of the suspension to 10 ml NB (contained in a 150 ml bottle) produced an initial density of less than 10 cells ml^{-1}. Cultures were incubated for 30 h at 37 °C without shaking and then vortexed briefly to obtain a uniform suspension. The numbers of mucoid and non-mucoid colony-forming units were measured as follows. Samples (0·2 ml) were diluted in physiological saline and 0·1 ml was spread on NA and incubated for 18 h at 37 °C. The percentage of non-mucoid revertants was calculated from the average count obtained from three plates, at a dilution yielding 100 to 200 colonies per plate.

To investigate the stability of the mucoid form in shaken broth cultures, the inoculum was prepared as above but cultures were incubated in an orbital incubator (Gallenkamp) at 140 rev. min^{-1}.

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Growth rate experiment. The culture vessels and medium used were as described for the fluctuation test. Incubation was at 37 °C and the culture vessels were shaken at 140 rev. min^{-1} or unshaken. Strains PAO568 and PAO548 were grown from single colonies, inoculated into NB and shaken for 18 h at 37 °C, diluted in physiological saline and inoculated together into 10 ml NB to give initial densities of 10^6 and 10^3 cells ml^{-1}, respectively. Viable counts of mucoid and non-mucoid colony-forming units were determined at 1·5 h intervals by removing 0·1 ml from the culture, diluting appropriately in saline and plating 0·1 ml in triplicate on NA and MA.

RESULTS AND DISCUSSION

Results of a fluctuation test showed that the mean percentage of non-mucoid revertants in 12 independent cultures of PAO568 was 37·8 with a variance of 390·1, whereas for 12 samples taken from a single culture, the mean percentage was 43·9 with a variance of 14·7. This experiment clearly demonstrated the instability of PAO568 in unshaken NB cultures incubated for 30 h at 37 °C. In addition, the difference in variance between the 12 independent cultures and 12 samples from the same culture indicated a clonal distribution of non-mucoid revertants among the mucoid populations. In contrast, the percentage of non-mucoid revertants observed in each of 12 cultures of PAO568 inoculated in a similar manner but incubated with shaking for 30 h was less than 0·2%.

An explanation for the instability of PAO568 could be that spontaneous mutations result in non-mucoid revertants and that revertants have a growth rate advantage over the mucoid parent strain which is more pronounced in unshaken than in shaken cultures. To test this hypothesis the growth rates of PAO568 and of a non-mucoid revertant were studied in a competitive situation (see Methods). The non-mucoid revertant used was the prototrophic transductant PAO548. This strain was derived for the experiment from the auxotrophic non-mucoid revertant PAO547 so that viable counts of the non-mucoid strain could be determined using unsupplemented MA plates and thus be distinguished from the auxotrophic non-mucoid revertants arising from PAO568 during the course of the experiment. A preliminary experiment was performed to compare the growth rates of PAO547 and PAO548 in shaken and unshaken NB cultures incubated at 37 °C. Results obtained for shaken cultures of both strains indicated a generation time during the exponential growth phase of 25·0 ($\pm$ 2·0) min. In unshaken culture, PAO547 and PAO548 showed similar growth curves with a mean generation time for both strains of 30·0 ($\pm$ 4·0) min during the first 6 h growth. From these results it was concluded that PAO548 exhibited similar growth characteristics to PAO547 in NB culture.

Growth curves were then obtained for PAO568 and PAO548 grown competitively in unshaken and shaken cultures (Fig. 1). The viable counts of PAO568 were determined by counting the mucoid colonies on NA plates, whereas colonies of PAO548 were counted on MA.

In unshaken culture, during the period of maximum growth from 1·5 to 6 h, the mean generation times of PAO568 and PAO548 were 37·5 and 30·5 min, respectively. Within the limits of probable error this allows for only a slight growth rate advantage for the non-mucoid strain during this period. After 6 h, however, as the growth rate of the mucoid strain approached zero, there was clear evidence of a growth rate advantage for PAO548 as it continued to grow until 24 h. During the period between 6 and 12 h the mean generation time of PAO568 was 97·5 min compared with 60·0 min for the non-mucoid strain. The percentage of colony-forming units of PAO548 in the unshaken culture increased from 0·2% at the beginning of the experiment to 6·0% after 12 h incubation and reached 52% after 24 h.

In contrast, the growth rate advantage of PAO548 over the mucoid strain was less evident in shaken culture. The generation time of PAO568 between 1·5 and 6 h was 26·7 min compared with 23·7 min for PAO548. After 6 h the growth rate of PAO568 approached zero only slightly ahead of the non-mucoid strain. By 12 h the percentage of colony-forming

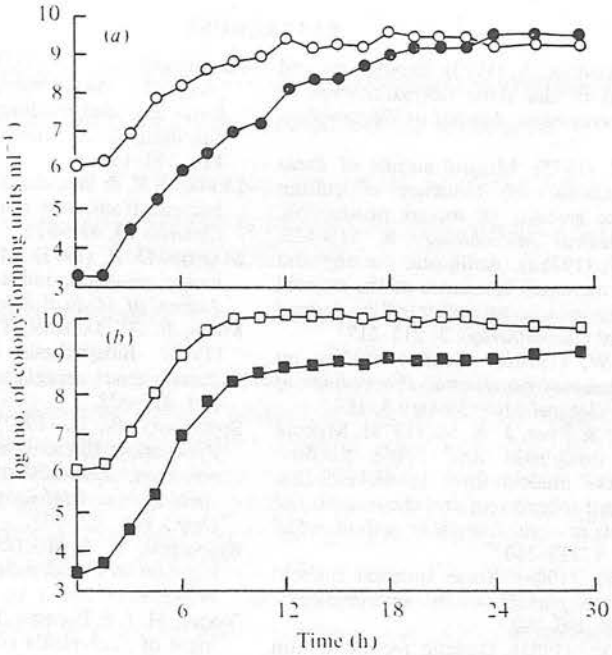

Fig. 1. Growth curves of *Pseudomonas aeruginosa* strains PAO568 (○, □) and PAO548 (●, ■) grown together in unshaken (*a*) and shaken (*b*) NB cultures.

units of PAO548 in the shaken culture was 3·1% and by 24 h had only risen to 13·4%.

We have previously provided evidence that mucoid variants can arise from non-mucoid *P. aeruginosa* via spontaneous mutation and that mucoid variants can be isolated *in vitro* by selecting for increased resistance to carbenicillin (Govan, 1976*a*, *b*; Govan & Fyfe, 1978). Conversely, we now suggest that the instability of mucoid *P. aeruginosa* also results from spontaneous mutation, back to the non-mucoid form which then has a selective growth advantage in unshaken cultures.

Mian *et al.* (1978) observed that during continuous culture of mucoid *P. aeruginosa*, non-mucoid variants arose in the mucoid population and increased as a proportion of the total number of organisms. The rate of this increase was thought to be consistent with mutation followed by selective advantage of the non-mucoid strain.

The effect of exopolysaccharide synthesis *per se* on the growth and instability of mucoid *P. aeruginosa* grown without shaking was suggested by further observations. Exopolysaccharide could be detected, by alcohol precipitation (Evans & Linker, 1973), in culture supernates from shaken and unshaken NB cultures from the early stationary phase. In contrast, no exopolysaccharide could be detected when PAO568 was grown in minimal broth (Govan, 1976*b*) or anaerobically in NB supplemented with 0·4% (w/v) KNO_3, and under these conditions PAO568 remained stable.

Several explanations can be offered to explain the differences in the stability of mucoid organisms in shaken or unshaken culture. It is possible that physical dispersion of cell-bound polysaccharide in shaken culture results in improved oxygen and nutrient uptake. Another possible explanation is that the polysaccharide surrounding the cell acts as a diffusion barrier to oxygen transfer and results in a higher growth rate for mucoid organisms in the increased dissolved oxygen concentration maintained in shaken culture.

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Alginate Synthesis in Mucoid *Pseudomonas aeruginosa*: a Chromosomal Locus Involved in Control

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Mucoid variants of *Pseudomonas aeruginosa* isolated *in vitro* or *in vivo* could be classified into two phenotypic groups based on whether alginate was produced on a chemically defined medium. Mucoid strains yielded lower recombination frequencies than the non-mucoid parent when used as donors in FP2-mediated plate matings. The mucoid characteristic (*muc*) was co-inherited by a proportion of recombinants selected for the inheritance of chromosomal markers *his-5075*$^+$ or *cys-5605*$^+$. The results of further experiments using either a mucoid recipient or a mucoid donor carrying plasmid R68.45 suggested that the control of alginate production in *P. aeruginosa* involves at least one chromosomal locus.

INTRODUCTION

Mucoid strains of *Pseudomonas aeruginosa* are frequently associated with chronic pulmonary infections in patients with cystic fibrosis (CF), but are rarely encountered in nature or in other clinical situations (Doggett, 1969). Doggett *et al.* (1966) reported that the non-mucoid form of *P. aeruginosa* precedes the mucoid form in the CF lung, but the latter eventually predominates and is associated with clinical deterioration. Mucoid and non-mucoid strains isolated from the same patient have been shown to belong to the same pyocin type (Williams & Govan, 1973) and serotype (Diaz *et al.*, 1970) suggesting that they are variants of the same strain.

Several explanations have been proposed for the emergence of mucoid strains *in vivo*. These include induction by phage (Martin, 1973) and selection of a mutant by prolonged antibiotic therapy or some other factor (Doggett & Harrison, 1969; Govan & Fyfe, 1978). Markowitz *et al.* (1978) suggested that the genetic basis of alginate production by mucoid *P. aeruginosa* may be plasmid-determined, but concluded that there was no evidence for this.

In *Escherichia coli* K12, capsular polysaccharide production is controlled by three regulator genes, *CapR*, *CapS* and *CapT*. Mutations in any of these loci result in a mucoid phenotype, and mapping studies have shown them to be situated in three distinct regions of the chromosome (Hua & Markovitz, 1972).

We have previously reported that mucoid variants of *P. aeruginosa* strain PAO and other non-mucoid strains can be isolated *in vitro* by selecting for carbenicillin resistance (Govan & Fyfe, 1978) or resistance to a virulent phage (Govan, 1975), and that the exopolysaccharide produced by such variants is an alginate-like polymer, similar to that produced by clinical isolates. The ability to produce alginate is transferable between strains of *P. aeruginosa* by conjugation (Govan, 1976; Fyfe & Govan, 1978).

This paper reports further studies on the genetic basis of alginate production in *P. aeruginosa* using mucoid derivatives of genetically marked PAO strains.

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Table 1. *Strains of Pseudomonas aeruginosa used in this study*

Strain	Genotype*	Description or reference
PAO8	*met-28 ilv-202 str-1*, FP$^-$	Isaac & Holloway (1968)
PAO381	*leu-38 str-2*, FP2$^+$	Stanisich & Holloway (1969)
PAO568	*leu-38 str-2 muc-2*, FP2$^+$	Mucoid derivative of PAO381 isolated following incubation with phage M6 (Govan, 1975)
PAO578	*leu-38 str-2 muc-22*, FP2$^+$	Mucoid derivative of PAO381 isolated by the method of Govan & Fyfe (1978)
PAO579	*leu-38 str-2 muc-23*, FP2$^+$	Mucoid derivative of PAO381 isolated by the method of Govan & Fyfe (1978)
PAO581	*leu-38 str-2 muc-25*, FP2$^+$	Mucoid derivative of PAO381 isolated following incubation with phage E79 (Govan, 1975)
PAO2018	*met-28 ilv-202 str-1 muc-35*, FP$^-$	Mucoid derivative of PAO8 isolated by the method of Govan & Fyfe (1978)
PAO2020	*cys-5605 argA171 pro-67 his-52 nal-25*, FP$^-$	His$^-$ derivative of a *his-5075*$^+$ transductant of PAO2022
PAO2021	*cys-5605 his-5075 argA171 pro-67 nal-25 muc-36*, FP$^-$	Mucoid derivative of PAO2022 isolated by the method of Govan & Fyfe (1978)
PAO2022	*cys-5605 his-5075 argA171 pro-67 nal-25*, FP$^-$	Pro$^-$ NAL^r derivative of PAO540 (Haas *et al.*, 1977)
PAO2023	*cys-5605 his-5075 argA171 pro-67 nal-25 car-10*, FP$^-$	Car$^-$ derivative of PAO2022
PAO2050	*his-5075 argA171 pro-67 nal-25 muc-35*, FP$^-$, R$^-$	Mucoid Cys$^+$ recombinant from PAO2018(R68.45)× PAO2022

* The genotype symbols designate the following: *arg*, arginine; *car*, carbamoylphosphate synthase; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine/valine; *leu*, leucine; *met*, methionine; *muc*, mucoid (alginate-producing); *nal*, nalidixic acid resistance; *pro*, proline; *str*, streptomycin resistance. FP$^-$, R$^-$, recipient strain; FP2$^+$, donor strain carrying the sex factor FP2.

METHODS

Bacteria and bacteriophage. The PAO strains used in this study are shown in Table 1. Donor strains carrying the plasmid R68.45 were constructed according to Haas & Holloway (1976). Nalidixic acid-resistant mutants were isolated according to Haas & Holloway (1976). Phage F116L (Krishnapillai, 1971) was used for all transductions. Phage PR4 (Stanisich, 1974) was used in the isolation of strains which had lost R68.45. Phages E79 and M6, obtained from Professor B. W. Holloway, were used in the isolation of mucoid strains using the method of Govan (1975). Clinical strains of mucoid *P. aeruginosa* were obtained from patients attending the CF clinic at the Royal Hospital for Sick Children, Edinburgh and were supplied by Dr Kenneth Watson.

Media and cultural conditions. Nutrient Broth (NB) was Oxoid no. 2 supplemented with 0·5 % (w/v) yeast extract (Oxoid), Pseudomonas Isolation Agar (PIA) was from Difco and Minimal Agar (MA) was as described by Vogel & Bonner (1956). Amino acid supplements were used at a concentration of 1 mM, 50 mM stock solutions being kept over chloroform. Antibiotics used were carbenicillin (Pyopen; Beecham), kanamycin (Bristol Laboratories), tetracycline (Glaxo) and nalidixic acid (NAL) (Winthrop).

Broth cultures were grown in McCartney bottles on an orbital incubator (Gallenkamp) at 140 rev. min^{-1}, and all cultures were incubated at 37 °C.

Transductions were performed according to the method of Krishnapillai (1971).

Plate matings were performed as described by Stanisich & Holloway (1972).

Interrupted matings were performed as described by Haas *et al.* (1977).

Recombinant analysis. This was performed to determine the percentage co-inheritance of unselected markers. For each selected marker, 100 recombinants were purified by streaking on to fresh selective MA. Inheritance of unselected auxotrophic markers was scored by transferring recombinants to appropriately supplemented MA. Inheritance of *muc* or *muc*$^+$ was scored by transferring the recombinants to PIA, except for matings using the donors PAO579 and PAO581. In these cases, mucoid recombinants could be recognized on the mating plates, and transfer to PIA before scoring made no difference to the result obtained.

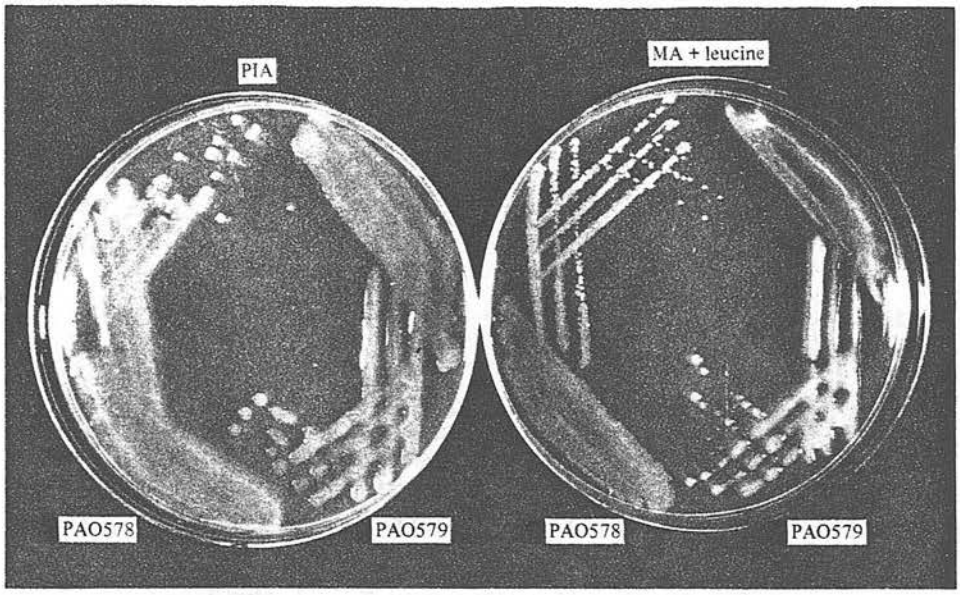

Fig. 1. Phenotypic differences between two mucoid variants of PAO381. Strains PAO578 and PAO579 after 24 h incubation on PIA and MA + leucine: PAO579 produces copious alginate under both conditions, whereas PAO578 appears rough and non-mucoid on MA + leucine.

RESULTS

Phenotypic differences between mucoid strains

The mucoid strains investigated produced copious exopolysaccharide on PIA after 24 h incubation and resembled colonial type 5 described by Phillips (1969); they could be readily distinguished from the slimy growth often observed following extended incubation of *P. aeruginosa* in media containing high concentrations of carbohydrate. However, when 100 independent, spontaneous mucoid derivatives of PAO381 were cultured on MA (+leucine), only 40 % were mucoid after 24 h, while the remaining 60 % appeared rough and non-mucoid; when transferred back to PIA, the two groups were both mucoid. Strains PAO579 and PAO581 are examples of the first category (group 1) and PAO568 and PAO578 are examples of the second group (group 2). Figure 1 shows PAO578 and PAO579 after 24 h incubation on PIA and MA +leucine.

Mucoid strains isolated from the sputa of patients with cystic fibrosis could also be classified into groups 1 and 2, and in several cases appeared concurrently in the same specimen.

Mucoid strains as donors and recipients in FP2*-mediated plate matings*

Figure 2 is a chromosome map of *P. aeruginosa* PAO showing the locations of markers relevant to this study. To examine the effect of alginate production on chromosome transfer, a series of plate matings was performed using mucoid strains as donors or recipients.

Table 2 shows the recombination frequencies obtained using the non-mucoid recipient strain PAO2022 and the mucoid donors PAO568, PAO578, PAO579 and PAO581. Strain PAO381 was included as a control donor. The mucoid donors yielded lower recombination frequencies than PAO381 for all selected markers. Non-mucoid revertants of these strains had recovered donor ability, giving recombination frequencies equivalent to those obtained using PAO381.

When mucoid derivatives of PAO2022 were used as recipients in plate matings with PAO381, recombination frequencies were similar to those obtained in the PAO381 × PAO2022 mating. Hence alginate production does not impair recipient ability.

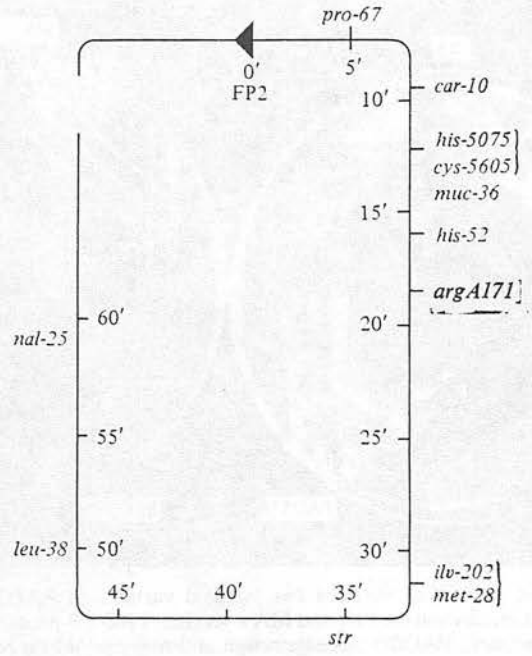

Fig. 2. Chromosome map of *P. aeruginosa* strain PAO showing the markers relevant to this study (based on Holloway *et al.*, 1979). See legend to Table 1 for genotype symbols. Very close linkage exists between the following pairs of markers: *pro-67* and *proB*, *car-10* and *car-9*, *his-5075* and *hisI*, *his-52* and *hisII*.

Table 2. *Frequency of recombinants obtained in plate matings between* PAO2022 *and mucoid derivatives of* PAO381

Selected marker	No. of recombinants per 10^8 donors				
	× PAO381	× PAO568	× PAO578	× PAO579	× PAO581
pro-67$^+$	1186	25	81	13	47
his-5075$^+$	98	9	14	3	17
cys-5605$^+$	81	7	8	2	9
argA171$^+$	958	100	108	36	119

Co-inheritance of muc with his-5075 and cys-5605

Examination of the recombinants from the PAO579 × PAO2022 and PAO581 × PAO2022 matings revealed mixtures of mucoid and non-mucoid clones. All the recombinants from the matings using PAO568 and PAO578 appeared non-mucoid on the selection plates. However, when purified and replica-plated to PIA, a proportion was mucoid.

Table 3 shows the percentage co-inheritance of alginate production with each of the selected markers in these plate matings. All mucoid clones from the mating plates were checked for NAL^r to ensure that they were recombinants and not *leu*$^+$ revertants of the donors.

In a reciprocal plate mating between a non-mucoid donor strain (PAO381) and a group 2 mucoid recipient (PAO2021), co-inheritance of *muc-36*$^+$ (non-mucoid) with *his-5075*$^+$ and *cys-5605*$^+$ was observed. Analysis of recombinants (outlined in Table 4) indicated a location for *muc-36* distal to *cys-5605* rather than between *his-5075* and *pro-67*, since the co-inheritance of *muc*$^+$ by *cys*$^+$*pro*$^+$ and *his*$^+$*pro*$^+$ double recombinants was low. However, low co-inheritance of *muc*, *his* and *cys* was obtained when the selected marker was *argA171*.

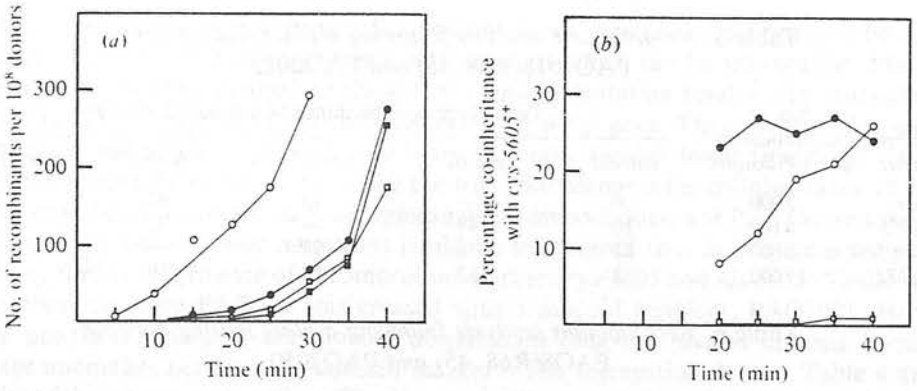

Fig. 3. Interrupted mating between PAO381 and the mucoid recipient PAO2021. (*a*) Time of entry kinetics of *pro-67* (○), *cys-5605* (●), *his-5075* (□) and *argA171* (■). (*b*) Percentage co-inheritance with *cys-5605*$^+$ of the unselected markers *muc-36*$^+$ (○), *pro-67*$^+$ (●) and *argA171*$^+$ (□).

Table 3. *Co-inheritance of muc mutations with selected auxotrophic markers following plate matings between mucoid donors and the non-mucoid recipient* PAO2022

	Percentage of recombinants co-inheriting *muc*			
Donor	*pro-67*$^+$	*his-5075*$^+$	*cys-5605*$^+$	*argA171*$^+$
PAO568 (*muc-2*)	1	21	15	<1
PAO578 (*muc-22*)	1	11	11	<1
PAO579 (*muc-23*)	2	21	32	<1
PAO581 (*muc-25*)	<1	7	14	<1

Table 4. *Recombinant analysis following a plate mating between* PAO381 *and the mucoid recipient* PAO2021

Selected marker	No. of recombinants per 10^8 donors	Percentage co-inheritance of unselected marker				
		muc-36$^+$	*pro-67*$^+$	*his-5075*$^+$	*cys-5605*$^+$	*argA171*$^+$
pro-67$^+$	2182	1	—	2	2	1
his-5075$^+$	164	15	34	—	90	2
cys-5605$^+$	155	18	30	93	—	1
argA171$^+$	1273	<1	<1	<1	<1	—

So confirmation of the proposed location of *muc-36* could not be obtained by the analysis of *arg*$^+$*cys*$^+$ and *arg*$^+$*his*$^+$ double recombinants.

Co-inheritance of unselected auxotrophic markers was not affected by alginate production by either the donor or recipient in these plate matings.

Transfer of muc-36$^+$ *during an interrupted mating*

To obtain further evidence for the location of *muc-36* distal to *cys-5605* an interrupted mating was performed between PAO381 and PAO2021 (Fig. 3). Time of entry kinetics displayed by the selected markers *pro-67*$^+$, *his-5075*$^+$, *cys-5605*$^+$ and *argA171*$^+$ (Fig. 3*a*) were consistent with the published order and times of entry for these loci (Holloway *et al.*, 1979). The transfer of *muc-36*$^+$ could not be measured directly as a selection procedure is not available. However, the *cys*$^+$ recombinants obtained at each interruption time were scored for co-inheritance of *muc*$^+$ as well as the proximal marker *pro*$^+$ and the distal marker *arg*$^+$ (Fig. 3*b*). The percentage co-inheritance of *pro*$^+$ by *cys*$^+$ recombinants was approximately 25 %, irrespective of the interruption time. Conversely, the percentage co-inheritance of *muc*$^+$ increased significantly with mating time, indicating a time of entry later than for *cys*.

Table 5. *Recombinant analysis following a plate mating between* PAO2018(R68.45) *and* PAO2022

Selected marker	No. of recombinants per 10^8 donors	Percentage co-inheritance of unselected marker				
		muc-35	*pro-67*$^+$	*his-5075*$^+$	*cys-5605*$^+$	*argA171*$^+$
pro-67$^+$	3000	6	—	43	42	3
his-5075$^+$	4100	16	49	—	92	2
cys-5605$^+$	5600	22	46	93	—	4
argA171$^+$	19000	2	13	10	8	—

Table 6. *Recombinant analysis following a plate mating between* PAO8(R68.45) *and* PAO2050

Selected marker	No. of recombinants per 10^8 donors	Percentage co-inheritance of unselected marker			
		muc-35$^+$	*pro-67*$^+$	*his-5075*$^+$	*argA171*$^+$
pro-67$^+$	1500	5	—	31	2
his-5075$^+$	3100	26	49	—	7
argA171$^+$	17000	3	7	4	—

However, low co-inheritance of *arg*$^+$ was again obtained, and when the *arg*$^+$ recombinants were scored for *cys*$^+$ and *muc*$^+$, <1 % co-inheritance was observed.

Mapping muc-35 using R68.45

In a further attempt to map a *muc* locus unambiguously, a series of plate matings was performed using donor strains carrying R68.45. The group 2 mucoid strain PAO2018 (R68.45) was crossed with PAO2022, and the recombination frequencies and co-inheritance of unselected markers are shown in Table 5. One of the *cys*$^+$ recombinants from this mating had inherited *muc-35* and R68.45, but was still *pro his arg*. Strain PAO2050 was derived from this recombinant by isolating a clone resistant to the phage PR4. This clone had acquired sensitivity to carbenicillin, tetracycline and kanamycin and could act as a recipient for R68.45. Hence, it was concluded that PAO2050 was an R$^-$ derivative of the original *cys*$^+$ recombinant. A plate mating was performed between PAO8(R68.45) and PAO2050 in which *muc-35* was a recipient marker rather than a donor marker, as in the previous mating. The results are shown in Table 6.

Linkage of muc-35 to other markers

Since *muc-35* showed linkage to *his-5075* and *cys-5605* in R68.45-mediated plate matings, it was expected that co-inheritance of *muc-35* would also be observed with other markers in the 8 to 15 min region of the PAO chromosome. Hence, plate matings were performed using PAO2018(R68.45) as donor and the non-mucoid recipient strains PAO2020 (*pro-67 cys-5605 his-52 argA171*) and PAO2023 (*pro-67 car-10 his-5075 cys-5605 argA171*). Recombinant analysis following these matings indicated that co-inheritance of *muc-35* with *his-52* and *car-10* was low (3 % in each case).

Attempts to isolate new auxotrophic markers in the 8 to 15 min region were unsuccessful.

DISCUSSION

The mucoid PAO strains and mucoid strains of *P. aeruginosa* isolated from CF patients could be divided into two major groups based on alginate production or non-production on MA after 24 h at 37 °C. Further characterization of these strains should reveal the extent of phenotypic variation and lead to an understanding of the nature of the control system.

Production or non-production of alginate cannot be selected for in plate or interrupted

mating experiments. As in the case of *E. coli*, the mucoid determinants can only be mapped indirectly by measuring co-inheritance with markers which can be selected for. Markovitz *et al.* (1967) used this method to show that *CapS*, a mutation resulting in mucoidness of *E. coli* K12, was located on the chromosome near a *trp* gene. They performed a series of interrupted and uninterrupted matings between a non-mucoid donor (*trp*$^+$ *CapS*$^+$ Hfr) and a mucoid recipient (*trp CapS*) screening the *trp*$^+$ recombinants for co-inheritance of *CapS*$^+$.

Our initial experiments with *P. aeruginosa* using mucoid donors (FP2$^+$) showed that four, independently isolated *muc* mutations could be transferred to a non-mucoid recipient by selecting for the inheritance of chromosomal markers *cys-5605* and *his-5075*. Similar results were obtained when PAO381 was crossed with a mucoid recipient, PAO2021. However, more precise mapping of the mucoid determinant (*muc-36*) proved difficult because of linkage anomalies between the selected markers. The segregation data in Table 4 and the results of the interrupted mating (Fig. 3) serve to illustrate this problem. Although *argA171* enters approximately 7 min after *his-5075* and *cys-5605*, *arg*$^+$ recombinants show no co-inheritance of the earlier markers.

Low linkage between markers with similar times of entry has been previously described in PAO, and several hypotheses have been put forward to explain this observation. These included the 'two linkage group' theory (Stanisich & Holloway, 1969; Loutit, 1969) and the 'two origin' hypothesis (Holloway *et al.*, 1975); however, insufficient evidence has been obtained to support either of these alternatives. The development of an improved technique for FP2-mediated interrupted matings (Haas *et al.*, 1977) and the introduction of R68.45 into PAO genetics have provided alternative approaches to chromosome mapping in *P. aeruginosa*.

We attempted to map *muc-35* by means of R68.45-mediated plate matings; however, the results were essentially the same as those obtained using FP2.

A possible explanation for the low linkage between *argA171* and the proximal markers in our experiments could be that the recipient strain PAO2022 was derived from PAO540, which had been mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on two occasions. However, this is unlikely because we have obtained similar results using PAT strains with comparable markers.

Despite these anomalies, which are not in themselves associated with alginate production in *P. aeruginosa* strain PAO, this investigation has shown that all the *muc* mutations examined are linked to the chromosomal markers *pro-67*, *his-5075* and *cys-5605*, and that at least one, *muc-36*, is distal to *cys-5605* and probably at about 14 min. A catabolic marker, *puuB*, is also reported to be in the 14 min region (Matsumoto *et al.*, 1978) but we have not yet determined whether *muc-36* is closely linked to this locus.

Before the number of genes involved in the control of alginate production can be determined, a closely linked marker will need to be found which can be selected for in matings and transductions.

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SHORT COMMUNICATION

Isolation of Alginate-producing Mutants of *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas mendocina*

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Spontaneous alginate-producing (*muc*) variants were isolated from strains of *Pseudomonas fluorescens*, *P. putida* and *P. mendocina* at a frequency of 1 in 10^8 by selecting for carbenicillin resistance. The infrared spectrum of the bacterial exopolysaccharide was typical of an acetylated alginate similar to that previously described in *Azotobacter vinelandii* and in mucoid variants of *P. aeruginosa*. Mucoid variants were not isolated from *P. stutzeri*, *P. pseudoalcaligenes*, *P. testosteroni*, *P. diminuta*, *P. acidovorans*, *P. cepacia* or *P. maltophilia*.

INTRODUCTION

Only two species of bacteria, *Azotobacter vinelandii* (Gorin & Spencer, 1966) and *Pseudomonas aeruginosa* (Evans & Linker, 1973), are known to produce alginic acid, a (1→4)-linked linear copolymer of β-D-mannuronic and α-L-guluronic acids. Alginate production in *P. aeruginosa* is restricted to mucoid variants which are rarely encountered except in association with chronic pulmonary infection in patients with cystic fibrosis (Doggett, 1969).

We have previously reported that mucoid mutants of *P. aeruginosa* can be isolated in vitro from wild-type, non-mucoid strains by selecting for carbenicillin resistance (Govan & Fyfe, 1978) or resistance to a virulent phage (Martin, 1973; Govan, 1975). Mutations responsible for this change in phenotype can be mapped on the chromosome (Fyfe & Govan, 1980). This evidence suggested that wild-type, non-mucoid strains of *P. aeruginosa* carry the genetic information necessary for alginate production but it is normally repressed.

The aim of the present study was to determine whether alginate-producing mutants occur in other species of *Pseudomonas*. We report for the first time the isolation of mutant strains of *P. putida*, *P. fluorescens* and *P. mendocina* producing an alginate-like exopolysaccharide similar to that obtained from *A. vinelandii* and mucoid *P. aeruginosa*.

METHODS

The strains used (Table 1) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. The species selected represent the five rRNA homology groups of pseudomonads described by Stanier *et al.* (1977).

Media and methods used to determine minimum inhibitory concentrations of carbenicillin and to isolate mucoid mutants were those described previously (Govan & Fyfe, 1978; Fyfe & Govan, 1980) with the following modifications. The temperature employed for growth was 30 °C and Pseudomonas Isolation Agar (Difco) was

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Table 1. *Strains of Pseudomonas used in this study*

Species	Strain number and reference	
P. testosteroni	NCIB 8893 Strain 79	Stanier *et al.* (1966)
P. cepacia	NCIB 9135 Strain 425	Stanier *et al.* (1966)
P. acidovorans	NCIB 9681 Type strain	Stanier *et al.* (1966)
P. pseudoalcaligenes	NCIB 9946 Type strain	Stanier *et al.* (1966)
P. putida	NCIB 10007 Strain C1-B	Stanier *et al.* (1966)
P. fluorescens	NCIB 10525 Strain 12	Stanier *et al.* (1966)
P. stutzeri	NCIB 9040	Wilkinson (1970)
P. maltophilia	NCIB 9203 Type strain	Hugh & Ryschenkow (1961)
P. diminuta	NCIB 9393 Type strain	Leifson & Hugh (1954)
P. mendocina	NCIB 10541 Strain CH-50	Palleroni *et al.* (1970)

replaced by medium A for growth of strains NCIB 10541, NCIB 9040, NCIB 9946, NCIB 8893 and NCIB 9393. Medium A comprised 20 g Difco Bacto-peptone, 1·4 g $MgCl_2$, 10 g K_2SO_4, 20 ml glycerol, 9 g Oxoid agar no. 1 and 980 ml water. Peptone gluconate broth was 1 % (w/v) Difco Bacto-peptone supplemented with 2 % (w/v) sodium gluconate.

Infrared spectroscopy of the sodium salt of the exopolysaccharides was carried out by the KBr disc method (Fillipov & Kohn, 1974).

RESULTS

Spontaneous mucoid mutants resembling the colonial type 5 of Phillips (1969) were isolated, by selecting for enhanced carbenicillin resistance, at a frequency of approximately 1 in 10^8 cells from *P. putida* NCIB 10007, *P. fluorescens* NCIB 10525 and *P. mendocina* NCIB 10541. In further studies with strain NCIB 10541 this frequency was increased 40-fold following mutagenesis with ethyl methanesulphonate. Despite repeated attempts, including mutagenesis, no mucoid variants were obtained from *P. stutzeri* NCIB 9040, *P. pseudoalcaligenes* NCIB 9946, *P. maltophilia* NCIB 9203, *P. acidovorans* NCIB 9681, *P. cepacia* NCIB 9135, *P. diminuta* NCIB 9393 and *P. testosteroni* NCIB 8893.

The mucoid variants of strains NCIB 10007, NCIB 10525 and NCIB 10541 did not require the continued presence of carbenicillin for mucoid colonial growth. All of the 90 mucoid variants isolated in this study produced mucoid colonial growth on minimal agar (see Methods) within 24 h. When the variants were grown for 18 h in peptone gluconate broth, cell-free supernates contained an exopolysaccharide which precipitated on addition of 3 vol. 95 % (v/v) ethanol and redissolved in water to form a viscid solution which rapidly gelled on addition of 0·1% (w/v) $CaCl_2$. No such exopolysaccharide was obtained from similar cultures of the wild-type parent strains. Infrared spectroscopy of the exopolysaccharide from mucoid mutants of each of the three species gave spectra typical of acetylated alginate similar to that previously reported for mucoid *P. aeruginosa* and *A. vinelandii* (Evans & Linker, 1973).

DISCUSSION

As a result of this study the number of bacterial species known to possess alginate-producing potential has been increased. The isolation of alginate-producing or *muc* mutants (Fyfe & Govan, 1980) in *P. fluorescens, P. putida* and *P. mendocina* provides a valuable aid to studies of the biosynthesis and regulation of *Pseudomonas* alginates in species less virulent than *P. aeruginosa* and in which alginate synthesis has more favourable carbon conversion efficiencies than in *A. vinelandii* (Jarman *et al.*, 1978).

Taxonomically, the three pseudomonad species from which *muc* mutants were isolated belong to the same RNA homology group, designated group I by Palleroni (1978). The isolation of *muc* mutants in *P. mendocina* is particularly interesting. On the basis of DNA

homology this species is the nearest genetic neighbour to *P. aeruginosa* (Stanier *et al.*, 1977). *Pseudomonas mendocina* was not isolated during an extensive investigation of pseudomonads causing infection in humans (Gilardi, 1972) and all strains of the species investigated by Palleroni *et al.* (1970) were obtained from non-clinical sources.

Our failure to isolate *muc* mutants from the other pseudomonads examined is not definitive evidence that such mutants do not exist. The occurrence of *muc* mutants in some species could be strain dependent. This was not found to occur, however, with *muc* mutants of *P. aeruginosa* (Govan & Fyfe, 1978). In addition, no *muc* mutants were found when a further eight strains of *P. cepacia* were investigated whilst *muc* variants were isolated from a single additional strain of *P. putida* (strain NCIB 9494) (J. R. W. Govan & J. A. M. Fyfe, unpublished results).

We have previously reported (Fyfe & Govan, 1980; Govan *et al.*, 1981) that the mucoid strains of *P. aeruginosa* isolated in vitro or from cystic fibrosis patients are heterogeneous with respect to the nutritional factors necessary for alginate production. The *muc* mutants isolated in this study from *P. fluorescens*, *P. putida* or *P. mendocina* produced alginate within 24 h on minimal medium and thus resembled group I variants of *P. aeruginosa* (Fyfe & Govan, 1980).

Similarities in the instability of *muc* variants to form non-mucoid revertants and in the biosynthesis and rheological properties of the bacterial alginate have been noted in *muc* variants of *P. mendocina* (A. Hacking, personal communication) compared to previous reports for *P. aeruginosa* (Govan, 1975; Mian *et al.*, 1978). Further studies are necessary in this area and to determine if the mutations responsible for alginate synthesis in *P. fluorescens*, *P. putida* and *P. mendocina* involve at least one chromosomal locus as in *P. aeruginosa* (Fyfe & Govan, 1980).

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Heterogeneity of Antibiotic Resistance in Mucoid Isolates of *Pseudomonas aeruginosa* Obtained from Cystic Fibrosis Patients: Role of Outer Membrane Proteins

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Mucoid *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients are very heterogeneous and include a class which is hypersusceptible to carbenicillin (minimum inhibitory concentration, ≤1 μg/ml). Hypersusceptible mucoid *P. aeruginosa* isolates were found in 12 of 22 cystic fibrosis patients examined. In cystic fibrosis patients having both resistant and hypersusceptible mucoid strains, 24 of 54 mucoid colonies obtained from a sputum sample were found to belong to the hypersusceptible class. In most instances, hypersusceptible and resistant strains isolated from the same sputum sample were indistinguishable, aside from their antibiotic susceptibilities, by classical methods. A particular pair of mucoid isolates (one hypersusceptible and one resistant) was chosen for further study. The hypersusceptibility was not limited to carbenicillin but was found to extend to other penicillins, tetracycline, and trimethoprim but not to the aminoglycosides gentamicin and tobramycin. The hypersusceptibility of the mucoid strain was found to be unrelated to amount or ability to synthesize alginate. The hypersusceptible strain was found to have two additional outer membrane proteins (32,000 and 25,000 daltons) as compared with the resistant strain. The 32,000-dalton protein, termed protein N1, was found to be correlated to the hypersusceptibility phenotype, as all spontaneous mutants of the hypersusceptible mucoid strain which were capable of growing in the presence of 50 μg of carbenicillin per ml had lost the 32,000-dalton outer membrane protein. The possible origins of the hypersusceptibility phenotype and the implications of the heterogeneity of mucoid *P. aeruginosa* in the pathogenesis of *P. aeruginosa* are discussed.

Pseudomonas aeruginosa is noted for its innate resistance to numerous antibiotics, with mucoid isolates of *P. aeruginosa* generally being somewhat more resistant to antibiotics and surfactants than nonmucoid isolates (7). Typically, the minimum inhibitory concentration of carbenicillin (carbenicillin is widely used in antipseudomonas therapy [12]) against *P. aeruginosa* isolated from various sources falls within the range of 25 to 50 μg/ml (4, 12). However, May and Ingold (18) reported the existence of a hypersusceptible class of *P. aeruginosa* isolates from the respiratory tract which were inhibited by 6 μg of carbenicillin per ml. Hypersusceptible strains of *P. aeruginosa* accounted for 33% of the 111 strains examined, and the incidence of hypersusceptibility differed little in mucoid strains as compared with nonmucoid strains. In a subsequent study, Berche et al. (4) examined the susceptibility of 47 mucoid and 71 nonmucoid *P. aeruginosa* isolates to 18 antibiotics. Both mucoid and nonmucoid strains could be divided into two separate classes, one class consisting of strains very susceptible to most antibiotics and a second class containing more resistant strains. The mucoid strains were divided almost equally between the two classes, 45 and 55%, respectively (4). The nonmucoid strains were much more homogeneous, as 89% of the strains belonged to the resistant class (4). Although the strains were isolated from a variety of clinical sources, the majority of the susceptible strains were obtained from sputum. We and other workers studying the association of *P. aeruginosa* with chronic debilitating respiratory infection in patients with cystic fibrosis (CF) have also reported the isolation of hypersusceptible *P. aeruginosa* and, in addition, the simultaneous isolation of hypersusceptible and more resistant strains from individual sputa; in most instances, hypersusceptible and resistant strains isolated from the same sputum sample belonged

to the same pyocin type or serotype (29, 31; J. R. W. Govan, J. A. M. Fyfe, K. Lam, and J. W. Costerton, J. Infect. Dis., in press; J. R. W. Govan and J. A. M. Fyfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D45, p. 47; J. R. W. Govan, J. A. M. Fyfe, R. Irvin, and J. W. Costerton, Proc. Cong. Cystic Fibrosis 8th, Toronto, p. 18a, 1980). The basis of the hypersusceptibility of these strains has not been established.

Previously, antibiotic hypersusceptibility in gram-negative bacteria has been correlated with alterations in the outer membrane. In particular, abbreviation of the lipopolysaccharide has been correlated with both the loss of outer membrane proteins (1, 13, 30) and increased susceptibility to numerous antibiotics (21, 26). Hypersusceptibility to antibiotics has also been reported to occur when the lipopolysaccharide has been altered but when the outer membrane proteins have remained constant (5). Outer membrane permeability has also been found to be altered by changes in the outer membrane protein composition (2, 22, 27, 33). Thus, the susceptibility of the hypersusceptible mucoid *P. aeruginosa* strains to a wide range of antibiotics was examined to confirm that the hypersusceptibility was likely due to increased outer membrane permeability, and subsequently, the outer membrane composition was examined, and the hypersusceptibility was correlated to the presence of additional outer membrane proteins.

MATERIALS AND METHODS

Organisms. The strains of *P. aeruginosa* used in this study were isolated from the sputa of patients with CF who were attending the CF clinic at the Royal Hospital for Sick Children, Edinburgh, Scotland. All strains investigated were isolated and maintained on Pseudomonas Isolation Agar (PIA; Difco Laboratories) and identified as *P. aeruginosa* by the production of pyocyanin and oxidase and the ability to grow at 42°C. Approximately 100 colonies of *P. aeruginosa* from each sputum sample were investigated. The mucoid strains 492a and 492c were chosen as typical examples of isolates occurring simultaneously in individual CF sputa and exhibiting normal resistance to carbenicillin (492a) and hypersusceptibility to carbenicillin (492c). In this study, the term mucoid indicates variants which produced copious mucoid growth on agar medium within 24 h of incubation at 37°C and resembled *P. aeruginosa* colonial type 5 (24). Spontaneous nonmucoid revertants were obtained by subculture from cultures grown in nutrient broth (Oxoid no. 2 with 0.5% Oxoid yeast extract; Oxoid Ltd.) at 37°C without agitation. Only one revertant was chosen from each broth culture to avoid siblings. Spontaneous variants which could grow in the presence of 50 μg of carbenicillin per ml were obtained from hypersusceptible strain 492c by plating 10^7 cells on Diagnostic Sensitivity Test Agar (Oxoid) containing 50 μg of carbenicillin per ml and incubating at 37°C for 48 h.

Antimicrobial susceptibility. The antibiotics were incorporated into Diagnostic Sensitivity Test Agar, held at 45°C, dispensed in 20-ml volumes in plastic petri dishes, and used within 24 h. Inoculum was prepared by suspending in 1 ml of saline a single colony from a PIA plate incubated for 24 h at 37°C. This suspension was diluted 1/100 to give approximately 10^4 organisms per ml. The inoculum was applied with a Mast multipoint inoculator. After the plates had been incubated at 37°C for 18 h, the minimum inhibitory concentration was calculated as the minimum concentration of antibiotic which inhibited growth of the organism. Susceptibility to carbenicillin was also investigated with a broth dilution technique. The inoculum used contained 10^4 cells cultured as described below for the extraction of bacterial exopolysaccharide.

Exopolysaccharide extraction. Mucoid strains were grown in 1% peptone (Difco)–2% sodium gluconate overnight at 37°C in an orbital incubator (Gallemkampf) at 140 rpm. A 1% inoculum of the culture was introduced into fresh medium, and incubation was continued for 24 h. Extraction and characterization of the exopolysaccharide as bacterial alginate was as previously described (9).

Pyocin typing. The pyocin typing technique used was that described by Williams and Govan (35) and incorporated the revised scheme of Govan (8).

Outer membrane preparations. Outer membranes were prepared by a modification of the method of Mühlradt and Golecki (19). Harvested cells were initially treated with toluene (3% [vol/vol] in 0.1 M cacodylate buffer [pH 6.80]) for 3 h at room temperature to ensure accessibility of Triton X-100 to the cytoplasmic membrane. Toluene-treated cells were then exposed to 1% Triton X-100 [vol/vol in 10 mM tris(hydroxymethyl)aminomethane buffer (pH 7.00) containing 8 mM $MgSO_4$ and 20% sucrose] for 18 h at room temperature (11). The outer membrane and peptidoglycan were then washed three times with 0.1 M cacodylate buffer (pH 6.80), treated with 1 mg of deoxyribonuclease I (Sigma Chemical Co.)–0.5 mg of ribonuclease (Sigma)–1 mg of lysozyme per ml for 3 h at room temperature, washed again three times, and suspended in distilled H_2O at a final protein concentration of 5 mg/ml.

SDS-polyacrylamide gel electrophoresis. The sodium dodecyl sulfate (SDS)-discontinuous polyacrylamide gel electrophoresis system J 4179 of Neville and Glossman (20) was used for analysis of protein components. Samples contained 5 mg of protein per ml as determined by the method of Lowry et al. (15) with bovine serum albumin as the standard. Samples were normally solubilized by adding 10 mg of Na_2CO_3–40 mg of SDS–100 μl of 2-mercaptoethanol to 1 ml of sample and boiling for 20 min. Samples were occasionally solubilized in the above mixture at 60°C for 20 min, in the above mixture minus 2-mercaptoethanol for 20 min with boiling, or alternately by precipitating the protein with an equal volume of 15% (wt/vol) trichloroacetic acid, neutralizing with 0.1 M NaOH, and solubilizing by boiling with SDS-2 mercaptoethanol for 20 min (10). Solubilized samples (10 μl each) were applied to a 20-cm slab gel consisting of 11 or 14% acrylamide and 0.1% bis-acrylamide and electro-

phoresized with a constant current of 50 mA. Gels were subsequently stained with Coomassie brilliant blue overnight and destained electrophoretically. Molecular weight assignments were made as described by Weber and Osborn (32) with the Daltons Mark VI SDS molecular weight marker kit (Sigma).

Electron microscopy. Cultures for electron microscopy were grown on PIA overnight at 37°C. Single colonies were scraped off the surface and suspended in either 0.1 M cacodylate buffer (pH 6.80) containing 0.05% ruthenium red and 0.1% glutaraldehyde or cacodylate buffer containing only 0.1% glutaraldehyde. Samples were then processed as described previously except that they were not enrobed in agar (23). All specimens were examined with an AE1 801 electron microscope operating with an accelerating potential of 60 kV.

RESULTS

Bacteriological investigation of 33 sputum samples from 22 CF patients chronically infected with *P. aeruginosa* showed that 12 patients harbored hypersusceptible strains that were inhibited by 1 μg of carbenicillin per ml or less. In eight of these patients, strains of *P. aeruginosa* exhibiting normal resistance (i.e., inhibition by 20 to 80 μg of carbenicillin per ml) were isolated simultaneously from the same sputum sample. Hypersusceptible strains were found among mucoid strains (24 of 54 examined) and nonmucoid strains (11 of 24 examined). In contrast, no hypersusceptible *P. aeruginosa* strains were found in 216 strains obtained from a wide variety of clinical, but non-respiratory, sources in non-CF patients. Four CF patients were infected with mucoid *P. aeruginosa* belonging to both hypersusceptible and normal classes. In each patient, the normal and hypersusceptible strains belonged to the same pyocin type.

A typical pair of mucoid strains, 492a and 492c, isolated from the same sputum sample was chosen for further study. Both strains were shown to belong to pyocin type 1/b and were indistinguishable on the basis of colonial appearance. Strain 492a exhibited normal resistance to carbenicillin, whereas strain 492c represented the hypersusceptible class.

The difference in susceptibility of strains 492a and 492c towards carbenicillin was also observed with a broth dilution technique. Strain 492c was inhibited by 1 μg of carbenicillin per ml, whereas strain 492a was inhibited by 50 μg/ml, when the strains were grown in 1% peptone–2% sodium gluconate. The exopolysaccharide produced by both 492a and 492c grown on Diagnostic Sensitivity Test Agar or in 1% peptone–2% sodium gluconate was identified by infrared spectroscopy as an alginate-like polymer of mannuronic and guluronic acids. Strain 492c produced five times as much exopolysaccharide as strain 492a in 1% peptone–2% sodium gluconate (without carbenicillin) (Table 1). The hypersusceptibility of strain 492c was not directly associated with alginate production because six nonmucoid revertants from which no alginate could be detected were as susceptible to carbenicillin as strain 492c.

A further investigation was then made to determine if the hypersusceptibility of strain 492c extended to other antibiotics. Table 1 summarizes the minimum inhibitory concentrations of various antibiotics against strains 492a and 492c and illustrates that hypersusceptibility extended to other penicillins, tetracycline, and trimethoprim but not to the aminoglycosides gentamicin and tobramycin.

Isolates 492a and 492c produced copious amounts of ruthenium red-staining exopolysaccharide when grown on PIA or Diagnostic Sensitivity Test Agar (data not shown). Spontaneous mucoid mutants of strain 492c that grew in the presence of 50 μg of carbenicillin per ml also produced large amounts of ruthenium red-staining exopolysaccaride when grown on PIA (data not shown). Nonmucoid revertants obtained from isolate 492c produced no ruthenium red-staining exopolysaccharide when grown on PIA (data not shown) and yet were still susceptible to less than 1 μg of carbenicillin per ml. The nonmucoid revertant obtained from isolate 492a produced no ruthenium red-staining material when grown on PIA (data not shown) and was not hypersusceptible to carbenicillin. These morphological observations confirmed the mucoid phenotype which was originally assigned by the colony morphology of the strain on PIA.

TABLE 1. *In vitro susceptibility of two mucoid strains (492a and 492c) of P. aeruginosa isolated from the same CF sputum sample*[a]

Antibiotic	MIC (μg/ml) against strain:	
	492a	492c
Carbenicillin	40	0.4
Azlocillin	8	0.2
Piperacillin	6	0.2
Ticarcillin	8	0.3
Methicillin	200	4
Gentamicin	4	2.5
Tobramycin	0.7	0.7
Tetracycline	12	2
Trimethoprim	50	4

[a] Both strains belong to pyocin type 1/b. The amount of alginate obtained from each strain grown in 1% peptone (Difco)–2% sodium gluconate for 24 h at 37°C was 2.33 mg per mg of dry cells for 492a and 11.43 mg per mg of dry cells for 492c. Medium, Diagnostic Sensitivity Test Agar. Inoculum, 10^4 cells.

An examination of the outer membrane protein composition of strains 492a and 492c revealed the presence of two additional outer membrane proteins of 32,000 and 25,000 daltons in the outer membrane of strain 492c (Fig. 1). These additional outer membrane proteins did not appear to be heat modifiable or to contain disulfide bonds and did not appear to constitute a solubilization artifact (Fig. 2). Resistant mutants obtained from isolate 492c by selecion of mucoid colonies on Diagnostic Sensitivity Test Agar containing 50 μg of carbenicillin per ml lost the 32,000-dalton outer membrane protein but not the 25,000-dalton outer membrane protein (Fig. 3). The presence or absence of an extensive capsule had no effect on the outer membrane protein composition (data not shown).

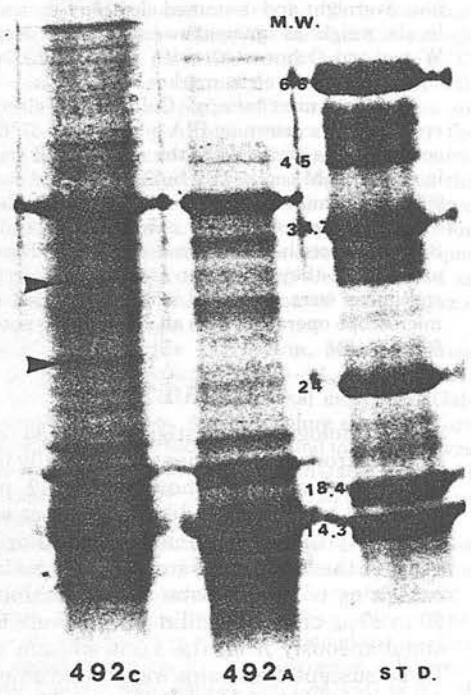

FIG. 1. *SDS-polyacrylamide gel electrophoresis (14% acrylamide gel) of outer membranes of strain 492a (mucoid resistant) and strain 492c, which has two additional proteins with apparent molecular weights of 32,000 and 25,000 (arrows). Molecular weight (M.W.) markers are 66,000 (66), 45,000 (45), 34,700 (34.7), 24,000 (24), 18,400 (18.4), and 14,300 (14.3). STD, Standard.*

DISCUSSION

This investigation of hypersusceptibility in mucoid *P. aeruginosa* might appear to contradict our previous research (9), in which we reported that mucoid strains exhibit enhanced resistance to antibiotics such as carbenicillin. The explanation is that mucoid *P. aeruginosa* strains are extremely heterogeneous. Although the association of mucoid *P. aeruginosa* with respiratory infection in CF patients has been known since the early report of Doggett (6), only recently has the heterogeneity of mucoid strains, in general and also within individual CF patients, been appreciated (29, 31; Govan and Fyfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D45, p. 47; Govan et al., Proc. Cong. Cystic Fibrosis 8th, Toronto, p. 18a, 1980). Some mucoid strains of *P. aeruginosa* are demonstrably more resistant to penicillins and aminoglycosides than related nonmucoid strains. As a consequence, alginate-producing mucoid variants can be isolated readily in vitro from nonmucoid strains by selecting for enhanced resistance to carbenicillin or aminoglycosides (9). Individual isogenic variants, however, although sharing a common property of alginate production, may differ in other properties, such as the nutritional requirements necessary for alginate production. In our experience, it has not been uncommon to obtain from CF patients isolates that appear nonmucoid on blood agar but in reality are alginate-producing mutants if grown on PIA.

It should be emphasized also that nonmucoid revertants are heterogeneous. Some revertants retain the antibiotic resistance of the mucoid parent, whereas others are more susceptible (Govan et al., in press).

Our survey on the incidence of *P. aeruginosa* strains hypersusceptible to carbenicillin in CF patients confirmed the original report of the significant incidence of such strains in isolates from sputa (18). The incidence of hypersusceptible variants in association with more resistant strains in 55% of the 22 CF patients examined was slightly greater than the 38% incidence reported by Seale et al. (29). This could be due, however, to our intensive search involving 100 colonies from each sputum sample.

Thomassen et al. (31) reported that individual CF sputa often produced one or more colonial types of *P. aeruginosa*: classic, rough, mucoid, gelatinous, dwarf, and enterobacter. Different colonial forms of *P. aeruginosa* isolated from the same sputum sample differed in antibiotic susceptibility, and it was suggested that susceptibility tests on isolates from CF patients should be performed on each colonial type. The results which we have reported in this investigation, exemplified by the similar colonial appearance of strains 492a and 492c, indicate that the heterogeneity of *P. aeruginosa* in CF patients de-

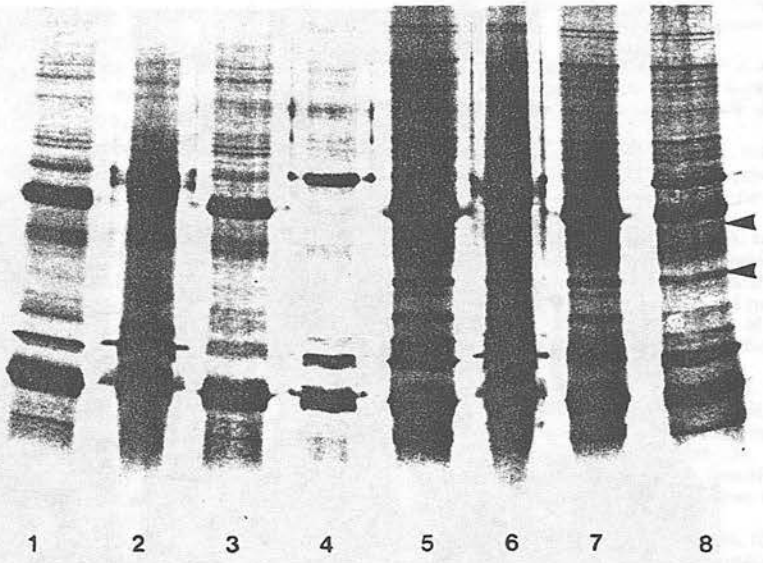

FIG. 2. *SDS-polyacrylamide gel electrophoresis (14% acrylamide gel) of outer membrane proteins of strain 492a (lanes 1 to 4) and strain 492c (lanes 5 to 8) solubilized by the following procedures: lanes 1 and 5, boiling for 20 min in the presence of SDS and 2-mercaptoethanol; lanes 2 and 6, boiling for 20 min in the presence of SDS; lanes 3 and 7, solubilizing at 60°C for 20 min in the presence of SDS and 2-mercaptoethanol; and lanes 4 and 8, precipitating with trichloroacetic acid then solubilizing as for lanes 1 and 5. Note that the apparent molecular weights of the two additional proteins (arrows) of strain 492c were not altered by the different solubilizations.*

mands even further consideration and examination of multiple examples of mucoid and nonmucoid colonial types.

The ability to act as donors in bacterial conjugation, to produce protease, and to agglutinate in the presence of homologous antisera is considerably diminished in mucoid variants as compared with their isogenic nonmucoid revertants. In contrast, the hypersusceptibility of strain 492c and other such strains was retained by nonmucoid revertants and not directly associated with alginate production. A close relationship, however, between the original mutation responsible for alginate production and hypersusceptibility cannot be discounted.

Although this investigation, together with those of May and Ingold (18) and Berche et al. (4), has shown hypersusceptible variants to be equally divided between mucoid and nonmucoid strains, it is not possible to distinguish between nonmucoid revertants which have arisen in vivo and the classic nonmucoid *P. aeruginosa* responsible for the original infection (unpublished observations). Thus, nonmucoid hypersusceptible strains isolated from CF patients could be revertants of a mucoid hypersusceptible variant and not true wild-type *P. aeruginosa*, as the hypersusceptibility phenotype is uncorrelated to mucoidy. Hypersusceptible strains are seldom encountered among nonmucoid strains of *P. aeruginosa* isolated from non-respiratory sources, but further investigation is necessary to establish the incidence of hypersusceptible strains among nonmucoid strains from respiratory sources in which no mucoid variants have been present.

The presence of hypersusceptible and normal variants within an individual sputum sample cannot be explained by simultaneous infection with more than one strain of *P. aeruginosa* or by cross-infection. No single pyocin type was responsible for the majority of normal or hypersusceptible strains investigated. Strains 492a and 492c, which were chosen for further study, not only belonged to the same pyocin type but were indistinguishable in colonial appearance from the uncommon pigment pyorubrin after incubation for 3 days at 37°C on PIA. Similarly, other pairs of resistant and hypersusceptible strains isolated from individual sputa appeared to be isogenic (unpublished data).

Increased susceptibility or hypersusceptibility to a wide range of antibiotics in gram-negative bacteria has generally been correlated to a cell envelope alteration, such as an abbreviation of the lipopolysaccharide (21, 26), a septation mutation (33), or an alteration in the lipopolysaccharide (5). As changes in outer membrane

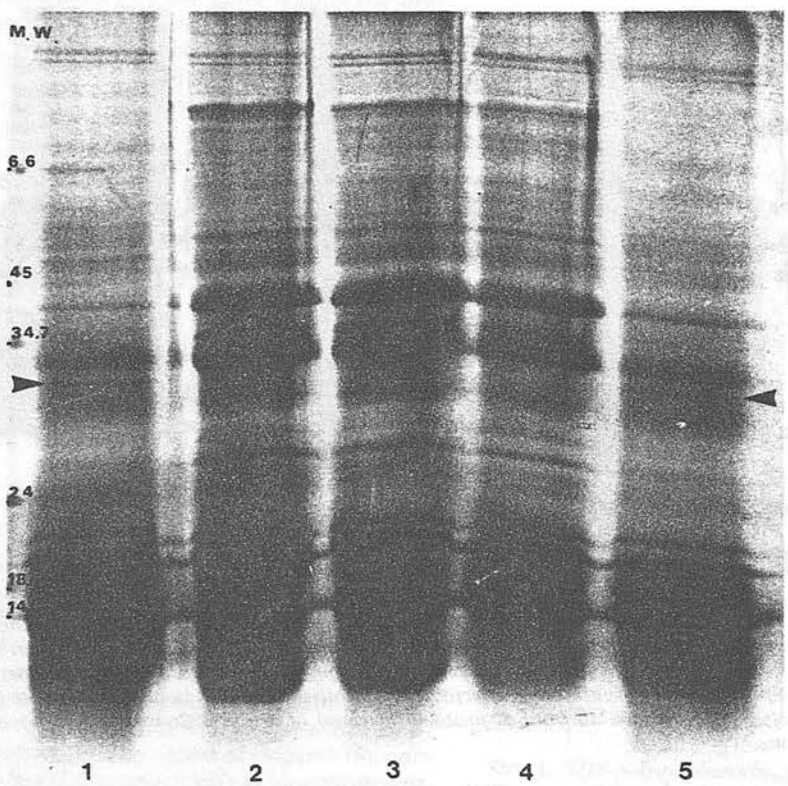

FIG. 3. *SDS-polyacrylamide gel electrophoresis (14% acrylamide gel) of outer membrane proteins of strain 492c (mucoid hypersusceptible) (lanes 1 and 5) and three mucoid resistant mutants (lanes 2, 3, and 4) obtained from strain 492c. Note that the mucoid resistant strains have lost the 32,000-dalton protein (arrows). See legend to Fig. 1 for explanation of numbers at left.*

permeability characteristics could be due to an alteration in the outer membrane protein composition, the outer membrane proteins of isolates 492a and 492c were compared.

The outer membrane protein compositions of isolates 492a and 492c are remarkably similar and differ only in the two additional outer membrane proteins with apparent molecular weights of 32,000 and 25,000 in isolate 492c (we propose to call the 32,000-dalton protein N1 and the 25,000-dalton protein N2 to conform with the nomenclature established by Hancock and Carey [10]) as compared with isolate 492a and in the relative abundance of a few outer membrane proteins (Fig. 1). Proteins N1 and N2 are not heat modifiable or 2-mercaptoethanol modifiable (Fig. 2), and thus protein N2 does not appear to be protein G (10). Protein N1 appears to be involved in the hypersusceptibility phenotype of isolate 492c, as all resistant mutants obtained from isolate 492c either completely lacked protein N1 or at least had a considerably reduced level of protein N1, whereas those outer membrane proteins of isolate 492c normally found in greater abundance as compared with isolate 492a were not reduced in abundance in the resistant mutants (Fig. 1 and 3). Thus, the outer membrane of isolate 492c is altered by the addition of two proteins as compared with a very similar isolate obtained from the same sputum sample.

The existence of a hypersusceptible strain of *P. aeruginosa* in a patient undergoing antibiotic therapy is perplexing and suggests that, if the hypersusceptibility is expressed in vivo, the isolate was not exposed to any significant level of antibiotic (25). Further, the extreme relatedness of isolate 492c to isolate 492a suggests that isolate 492c was originally isolate 492a but was modified due to the incorporation of a plasmid or temperate phage, with a resultant transition in outer membrane protein composition (3, 17, 27, 34). The origin of the hypersusceptibility of isolate 492c is presently under investigation.

If hypersusceptibility is expressed in vivo this would be further support for our previous sug-

gestion (9) that no single reason may explain the emergence of mucoid *P. aeruginosa* in CF patients, but that the reasons may be complex and interrelated, involving the consequences of long-term antibiotic therapy and the selective advantage of alginate in blocking *Pseudomonas* receptors on alveolar macrophages (J. W. R. Govan and J. A. M. Fyfe, manuscript in preparation) and phagocytic digestion (28).

An appreciation of heterogeneity and the existence and incidence of hypersusceptible *P. aeruginosa* in CF patients is important not only in understanding the reasons for the emergence of the mucoid form after primary infection with a nonmucoid isolate but in the pathogenesis and optimum treatment of existing infection.

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Alginate Biosynthesis by *Pseudomonas mendocina*

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Exopolysaccharide-synthesizing variants of *Pseudomonas mendocina* NCIB 10541 were isolated on media containing carbenicillin. The exopolysaccharide was identified as alginic acid with a mannuronic acid : guluronic acid ratio of 1 : 2. These strains lost the ability to produce alginate at a high frequency, but more stable mutants which produced increased amounts of polysaccharide could be isolated by subsequent mutagenesis. High concentrations of polysaccharide (approximately 20 g l^{-1}) were obtained in nitrogen-limited continuous culture with a minimal glucose medium. In common with other bacterial alginates, the polymer is acetylated and has similar rheological properties to alginate from brown algae. An alginate lyase activity was present in cultures at sufficient specific activities to result in a low molecular weight, low viscosity polymer with rheology similar to printing grade alginate. This degradation was overcome by incorporation of a proteolytic enzyme into the growth medium without adverse effects on bacterial or polysaccharide yields. As an organism for the study of alginate biosynthesis, *P. mendocina* possesses advantages over *Azotobacter vinelandii* or *Pseudomonas aeruginosa* in terms of yield, strain stability, and absence of known pathogenicity.

INTRODUCTION

Alginic acid is a (1→4)-linked linear copolymer of β-D-mannuronic and β-L-guluronic acid synthesized by several species of marine algae (Booth, 1975) and produced as an exopolysaccharide by *Azotobacter vinelandii* (Gorin & Spencer, 1966) and *Pseudomonas aeruginosa* (Evans & Linker, 1973). *Azotobacter vinelandii* strains are usually mucoid when isolated and retain the ability to synthesize polysaccharide through repeated subculture (Jarman, 1979), although polysaccharide-negative mutants have been isolated and these remain viable under laboratory conditions (Sutherland, 1972). Alginate is also found in the cell walls of *Azotobacter* cysts (Sadoff, 1975).

Strains of *P. aeruginosa* do not in general produce alginate, but mucoid, alginate-producing variants are frequently isolated from the sputum of patients with cystic fibrosis (Doggett & Harrison, 1969). The environmental factors proposed to account for their gradual emergence in such patients include prolonged antibiotic therapy (Doggett & Harrison, 1969; Govan & Fyfe, 1978; Kulczcki *et al.*, 1978) and immunological selection through the protective effect of alginate on phagocytosis by alveolar macrophages (Schwarzmann & Boring, 1971) and reduced pulmonary clearance (Govan *et al.*, 1983). Alginate-producing mutants can be isolated *in vitro* by selection for resistance to phages (Martin, 1973; Govan, 1975) or antibiotics (Govan & Fyfe, 1978). Mucoid variants isolated *in vivo* or *in vitro* are often unstable; non-mucoid revertants are frequently found at high frequencies because of a growth rate advantage over the parent strain (Govan, 1975). Stability can be improved by growth in the presence of surfactants or with

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agitation (Govan, 1975). Large-scale microbial alginate synthesis is not feasible with *P. aeruginosa* because it is an opportunistic pathogen. Only poor carbon conversion efficiencies have been obtained with *A. vinelandii* (Deavin *et al.*, 1977; Jarman *et al.*, 1978).

More recently, alginate-synthesizing strains of *P. fluorescens*, *P. mendocina* and *P. putida* have been isolated by selection on growth media containing carbenicillin (Govan *et al.*, 1981). The present paper describes an investigation of alginate production by the saprophytic species *P. mendocina* (Palleroni *et al.*, 1970) for which no pathogenic role has been reported (Gilardi, 1972) and which has been very rarely isolated from clinical material (Farmer, 1976).

METHODS

Growth and maintenance of organisms. *Pseudomonas mendocina* (NCIB 10541) was routinely subcultured on nutrient agar slopes. *Pseudomonas aeruginosa* PsB (Mian *et al.*, 1978) was maintained on *Pseudomonas* isolation agar. Mucoid strains of *P. mendocina*, Muc 18, C7, U1 (NCIB 11687) and U3 (NCIB 11688) were maintained on agar (1·5%, w/v) containing a mineral salts medium (Jayasuria, 1955) plus glucose (2%, w/v) for maximum polysaccharide synthesis. Viability on this medium is poor because of acid production and subculturing must be carried out at monthly intervals. For growth of shake flask cultures using the same minimal medium, sodium gluconate (2%, w/v) as carbon source did not result in acid production. The medium used in continuous culture contained ($g\,l^{-1}$): glucose, 90; $(NH_4)_2HPO_4$, 1·25; K_2HPO_4, 1·25; $Ca(OH)_2$, 0·05; $Mg(OH)_2$, 0·07; $MgSO_4.7H_2O$, 0·14; citric acid, 0·99; yeast extract, 0·2; $MnSO_4.4H_2O$, $1{\cdot}1 \times 10^{-3}$; $FeSO_4.7H_2O$, $3{\cdot}6 \times 10^{-3}$; $ZnSO_4.7H_2O$, $0{\cdot}7 \times 10^{-3}$; $CaSO_4.5H_2O$, $0{\cdot}25 \times 10^{-3}$; $CoSO_4.7H_2O$, $0{\cdot}28 \times 10^{-3}$; H_3BO_4, $0{\cdot}06 \times 10^{-3}$, pH 7·0. Glucose was autoclaved separately to avoid charring.

Carbenicillin resistance. An exponential phase culture of *P. mendocina* in nutrient broth was serially diluted and spread on to nutrient agar plates containing carbenicillin, initially in the range 0 to 1000 $\mu g\,ml^{-1}$ in multiples of 100 $\mu g\,ml^{-1}$. This gave an approximate indication of the MIC which was defined further by a second set of determinations over a narrower range in multiples of 20 $\mu g\,ml^{-1}$.

Serial dilutions of an exponential phase culture of *P. mendocina* in nutrient broth were spread over nutrient agar plates containing 1·5 times the MIC of carbenicillin. The plates were examined after 36 h incubation at 30 °C. Resistant mucoid clones selected on the basis of their raised, glistening appearance were purified on minimal agar containing glucose (2%, w/v). The colony appearance of these isolates was recorded on deoxycholate/citrate agar, *Pseudomonas* isolation agar, minimal glucose agar (0·2, 1·0 and 2·0%, w/v), minimal gluconate agar (2·0%, w/v), nutrient agar and nutrient agar incorporating Tween 80 (1·0%, w/v) or Brij 35 (1·0%, w/v).

Mutagenesis. An exponential phase culture of *P. mendocina* in glucose (10 mM) mineral salts medium was harvested by centrifugation, washed in 0·5 M-Tris/maleate buffer, pH 6·0 and resuspended in 9 ml of this buffer to $0{\cdot}5 \times 10^9$ cells ml^{-1}. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) dissolved in the same buffer was added to a final concentration of 20 $\mu g\,ml^{-1}$. After incubation for 30 min at 30 °C, the cells were harvested by centrifugation, washed in mineral salts medium and resuspended in 100 ml salts medium plus glucose (2%, w/v). Measurements of viable cell concentration were made at all stages in this procedure. Cultures were grown for 24 h at 30 °C, the cells were collected by centrifugation, washed, resuspended in mineral salts medium, diluted and spread on to glucose (2%, w/v)/mineral salts agar plates to give 20 to 50 colonies per plate. Colony morphologies were examined after 4 to 6 d incubation at 30 °C.

Continuous culture. A laboratory fermentation vessel (L. H. Engineering Ltd, Stoke Poges, Buckinghamshire; type 1/1000) modified for continuous culture (working volume = 2·5 l) was used. The pH was maintained at 7·0 by the automatic addition of 2 M-NaOH. The temperature was 30 °C. Foaming was controlled by the addition of a silicone antifoaming agent. Air was supplied at 1 l min^{-1} and the culture was mixed at impeller speeds of 300 to 750 r.p.m. so as to maintain the dissolved oxygen at about 20% of saturation.

For continuous culture, medium addition was started when the batch culture reached the stationary phase. The dilution rate was adjusted by alteration of the rate of flow of medium. Following a change in conditions, a steady state was considered to exist when cell dry weight and oxygen uptake had remained constant for three residence times. Glucose concentrations in culture broths were determined by the glucose oxidase method using a Model 23A glucose analyser (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.)

Cell dry weight. Bacterial cells and exopolysaccharide could only be separated in the presence of a chelating agent. Culture broth (40 ml) was mixed with 5 M-NaCl (0·8 ml) and 0·5 M-EDTA pH 7·0 (0·8 ml) and after standing for 10 min, was centrifuged at 24000 *g* for 40 min. The supernatant was removed, the precipitate was resuspended in distilled water and centrifuged as before. The final precipitate was dried at 105 °C to constant weight.

Polysaccharide determination. The first supernatant from the cell dry weight determination (25 ml) was added to propan-2-ol (75 ml). After mixing and standing for 10 min, the precipitate obtained was filtered on a pre-weighed Whatman GF/A filter disc which was dried *in vacuo* for 24 h and re-weighed.

Analysis of exopolysaccharide. Infra-red spectral analysis of the sodium salt of the polysaccharide was carried out by the KBr disc method (Fillipov & Kohn, 1974) using a Perkin-Elmer 457 infrared spectrometer.

The acetate content of the polymer was determined by the hydroxamate method of McComb & McCready (1957).

Molecular weight profiles of polysaccharide were obtained by gel electrophoresis using 6% (w/v) polyacrylamide (Bucke, 1974).

Rheology. For a non-Newtonian fluid the apparent viscosity, η, is dependent upon the rate of shear and can be defined by: $\eta = K(d\gamma/dt)^n$ (Van Wazer *et al.*, 1963) where K is the consistency index (apparent viscosity at a shear rate of 1 s^{-1}), $d\gamma/dt$ is the rate of shear and n is the flow behaviour index. The higher the value of K, the more viscous the fluid. The flow behaviour index varies from 1 to 0 for pseudoplastic fluids; the lower its value the more pronounced are the non-Newtonian properties of the fluid.

Culture viscosity measurements were made using a model HAT cone-and-plate microviscometer (Wells-Brookfield Engineering Laboratories Inc., Stoughton, Mass., U.S.A.) at 25 °C. For more viscous solutions a model HBT was used. The apparent viscosities were determined over a range of shear rates from 3·75 to 750 s^{-1} and K was obtained by extrapolation of a log–log plot of apparent viscosity against shear rate.

A more detailed rheological examination was made using a Rheomat-30 rotational viscometer with a cone-and-plate system (Contraves A. G., Zürich, Switzerland). A 1% (w/v) polysaccharide solution in (*a*) distilled water and (*b*) 100 mM-EDTA, pH 7·0, was prepared by continuous agitation for 1 h using a top-stirrer. Solutions were examined at 30 shear rates and values of K and n obtained from shear rate versus viscosity plots.

Preparation of cell-free extracts. Cells were harvested in the late-exponential phase of growth, washed in 0·1 M-phosphate buffer (pH 7·0) and suspended in four times their wet weight of the same buffer. In viscous cultures, 0·01 M-EDTA (sodium salt, pH 7·0) was added to the wash buffer. The cells were sonically disrupted by 4 × 20 s pulses (20 kHz) in a tube chilled to 0 °C using an NaCl freezing mixture. The supernatant fluids were used for enzyme assays after centrifugation at 40000 *g* for 45 min at 0 °C.

Assay of alginate lyase activity. This assay measures unsaturated uronic acids released from the polysaccharide using the periodate/thiobarbituric acid method (Weissbach & Hurwitz, 1959). Reaction mixtures (2·0 ml) contained 0·1% (w/v) Manugel in 0·1 M-potassium phosphate buffer (pH 7·0). Assays were started by the addition of bacterial extract (1–2 mg protein). Control mixtures lacking enzyme or alginate were included. Samples (0·2 ml) were withdrawn from reaction mixtures after 10, 20 and 30 min incubation at 30 °C. The reaction was stopped by the addition of 62·5 mM-H_2SO_4 (0·2 ml) and the sample was mixed with 0·02 M-periodic acid in 62·5 mM-H_2SO_4 (0·2 ml). After 20 min incubation at room temperature, 2% (w/v) sodium arsenite in 0·5 M-HCl (0·5 ml) was added. The mixture was shaken and, after standing for 2 min, 0·3% (w/v) thiobarbituric acid (2 ml) was added followed by heating for 10 min at 100 °C. After cooling, the absorbance at 549 nm was measured against a reagent blank. Enzyme activities are defined as nmol β-formyl pyruvic acid liberated (mg protein)$^{-1}$ min^{-1}; 10 nmol β-formyl pyruvic acid produces an A_{549} of 0·29 under these conditions (Preiss & Ashwell, 1962).

Chemicals. Commercial algal alginates (Manugel and Manutex) were obtained from Alginate Industries, Girvan, Ayrshire, U.K. Deoxycholate/citrate agar was from Oxoid, *Pseudomonas* isolation agar from BBL, carbenicillin from Beecham Pharmaceuticals and Neutrase from Novo Industri, Bagsvaerd, Denmark.

RESULTS

Isolation of mucoid strains

The MIC of carbenicillin for the wild-type strain of *P. mendocina* in the single cell dilution technique on nutrient agar was 80 μg ml^{-1}. When suspensions of this strain were plated on to nutrient agar containing carbenicillin (120 μg ml^{-1}), resistant colonies grew after 3 to 4 d incubation at 30 °C as described by Govan *et al.* (1981). Mucoid colonies (e.g. Muc 18; C7; Table 1) were identified by their raised glistening appearance on glucose (2%, w/v) minimal agar. Resistant colonies could not be obtained by plating directly on to glucose minimal agar containing carbenicillin even at concentrations as low as 50 μg ml^{-1}. The capsular polysaccharide was provisionally identified as alginate by the formation of a white flocculent precipitate in 0·05 M-H_2SO_4 and by gelation in 0·01 M-$CaCl_2$. Confirmation was later obtained by IR analysis and rheology.

Of the media tested, capsule production (as judged by colony morphology) in the mucoid strains was only apparent in mineral salts containing more than 1% (w/v) glucose, or on deoxycholate/citrate agar. The active components of deoxycholate/citrate medium appeared to be sodium deoxycholate and sodium thiosulphate, although neither alone was as effective in eliciting polysaccharide synthesis as the complete formulation. Incorporation of other

Table 1. *Alginate production by various strains of P. mendocina in shake flask cultures*

All strains were grown in gluconate (2%, w/v)/minimal salts medium. Propan-2-ol precipitation was carried out after 4 d incubation at 30 °C.

Strain	Alginate produced (g propan-2-ol precipitate l^{-1})
Wild-type (NCIB 10541)	0·45
Muc18 (First stage carbenicillin-resistant isolate)	2·0
C7 (First stage carbenicillin-resistant isolate)	1·6
U1 (NCIB 11687) (Nitrosoguanidine mutagenesis of Muc18)	2·8
U3 (NCIB 11688) (Nitrosoguanidine mutagenesis of Muc18)	3·2

Table 2. *Alginate synthesis by mutant U1 growing in continuous culture under nitrogen limitation at various dilution rates*

Culture conditions and medium were as described in Methods. Glucose was supplied at 90 g l^{-1} and the dissolved oxygen tension was maintained at approximately 20% of saturation. The yield of polysaccharide is defined as the ratio of the propan-2-ol precipitate to the glucose consumed by the culture.

Dilution rate (h^{-1})	Cell dry weight (g l^{-1})	Propan-2-ol precipitate (g l^{-1})	Cell: polysaccharide ratio	Residual glucose (g l^{-1})	Glucose used by culture (g l^{-1})	Yield of polysaccharide
0·03	2·7	17·5	6·5	33·5	56·5	0·31
0·04	2·5	18·0	7·5	32·0	58	0·31
0·06	2·5	23·5	9·2	25·5	64·5	0·36

surfactants, for example Tween 80 (polyoxyethylene sorbitan mono-oleate) or Brij 35 (polyoxyethylene-23-lauryl ether), into growth media was not effective. Colonies did not, however, appear mucoid on gluconate (2%, w/v) minimal agar, but alginate could be recovered from liquid cultures using this medium. An alginate-synthesizing strain of *P. aeruginosa* PsB appeared mucoid on all media tested.

When *P. mendocina* cultures grown on a non-inducing medium were transferred back to 2% glucose or deoxycholate/citrate agar the mucoid appearance was regained, but all strains tested were unstable. Constant segregation to small non-mucoid colonies occurred but instability was particularly apparent in liquid culture. In some cases less than 20% of the population retained polysaccharide synthesis after 60 mass doublings.

In an attempt to improve stability and polysaccharide yield, nitrosoguanidine-induced mutagenesis was carried out on one carbenicillin-resistant isolate (Muc 18). Distinct large spreading mucoid colonies could be observed amongst survivors of the mutation. A total of 35 such isolates was obtained by screening 10^5 clones on glucose (2%, w/v)/mineral salts agar without selective enrichment. Consistently greater propan-2-ol precipitates were obtained in shake flask cultures of these strains than in strain Muc18 or any of the first stage carbenicillin-resistant isolates (Table 1). Shake flask culture using glucose as sole carbon source was difficult to reproduce because of high glucose dehydrogenase activity and release into the medium of gluconic acid which inhibited growth by lowering the pH. Increasing the buffering capacity of the medium inhibited polysaccharide synthesis, so replacement of glucose by sodium gluconate was adopted as a standard procedure, but polysaccharide concentrations were never as high as using glucose in a pH-controlled fermenter. Approximately 50% of the large mucoid clones were unstable in shake flask culture as judged by the rapid appearance of small colonies, but the remainder (e.g. U1, U3; Table 1) were successfully subcultured for 70 mass doublings.

Polysaccharide production in continuous culture

High concentrations of polysaccharide were achieved by growth of one of the large colony isolates (strain U1) in continuous culture (Table 2). Optimal rates of polysaccharide synthesis

Table 3. *Stability of exopolysaccharide synthesis by mutant U1 in continuous culture*

Culture conditions were as described in Methods. The dilution rate was maintained at an average of 0·05 h^{-1} and dissolved oxygen tension at 20% of saturation.

Time after inoculation (h)	Propan-2-ol precipitate (g l^{-1})	Percentage of small colonies
0	0	0
25·6	15·6	0
52	19·0	5
122	15·1	5
197	18·5	17
298	20·3	21
381	18·4	35

Table 4. *Effect of incorporation of various amounts of a proteolytic enzyme on culture viscosity*

Cultures were maintained at a dilution rate of 0·05 h^{-1} and dissolved oxygen tension at approximately 20% of saturation. Other culture conditions and medium were as described in Methods.

Neutrase (Anson units l^{-1})	Propan-2-ol precipitate (g l^{-1})	Cell dry weight (g l^{-1})	Culture consistency index, K (mPa s^n)
0	18·5	3·4	32
0·1	21·0	3·6	4700
0·25	19·6	3·0	5100

were obtained by maintaining the dissolved oxygen tension between 18 and 25% of saturation. Below this range polysaccharide concentration declined while above it concentrations were maintained but carbon conversion efficiencies from glucose fell, presumably because of increased substrate oxidation. Polysaccharide concentration increased with dilution rate to 0·06 h^{-1} but then fell sharply to less than 10 g l^{-1} at 0·08 h^{-1}, although cell dry weight remained constant. Nitrogen was established as the limiting nutrient in the medium used by adding 1 g NH_4Cl l^{-1} to a culture at a steady state. Cell density rose from 2·7 g l^{-1} to 4·1 g l^{-1} after 12 h but then remained constant due to the establishment of a new limit.

When the mutant U1 was maintained at a constant dilution rate of 0·5 h^{-1}, strain instability as estimated by the appearance of small colonies was apparent (Table 3). This was not accompanied, however, by a corresponding drop in polysaccharide concentration, although when polysaccharide synthesis by these small colony types was measured in shake flask culture it was less than 50% of the starter culture of mutant U1.

Degradation of polysaccharide

All strains of *P. mendocina* used in this work were found to possess an alginate lyase activity which was present at specific activities of between 0·2 and 0·35 nmol min^{-1} (mg protein)$^{-1}$ in crude extracts independent of polysaccharide synthesis or growth substrate. The specific activities also remained unaltered throughout the course of continuous fermentations. Despite high concentrations of alginate, culture viscosities (as estimated by the culture consistency index) remained low unless a proteolytic enzyme was incorporated into the medium (Table 4). Addition of 'Neutrase' increased culture viscosities substantially. This enzyme preparation did not have an adverse effect on cell growth or polysaccharide synthesis.

Evidence was also obtained that the alginate lyase activity was normally intracellular and was only released by cell lysis. PAGE of polysaccharide indicated only high molecular weight material in exponential phase batch cultures. Lower molecular weight bands only appeared after 12 to 24 h in the stationary phase. Addition of sonicated cell extracts to high viscosity cultures resulted in a rapid drop in viscosity and faster migrating bands on electrophoresis. Similar results were obtained when polysaccharide breakdown was estimated by the concentration of free uronic acid groups using the thiobarbituric acid assay.

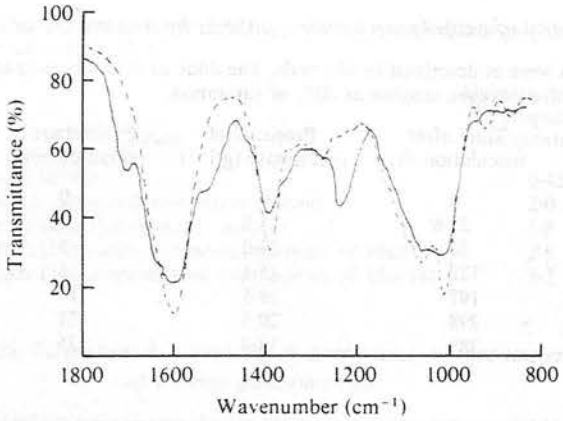

Fig. 1. Infra-red spectra of exopolysaccharide from *P. mendocina* grown in continuous culture under nitrogen limitation (——) and commercial algal alginate (– – – –).

Table 5. *Rheology of alginate samples*

All samples were prepared as 1% (w/v) solutions and rheology measurements made as described in Methods.

Sample	Solvent	Consistency index, K (mPa s^n)	Flow behaviour index (n)
Nitrogen-limited culture without protease	H_2O	9·6	0·86
	EDTA (100 mM)	8·4	0·86
Nitrogen-limited culture plus protease	H_2O	8043	0·27
	EDTA (100 mM)	557	0·43
Low molecular weight algal alginate (Manutex F)	H_2O	31·4	0·91
	EDTA (100 mM)	12·8	0·98

Polysaccharide characterization

The IR spectrum of exopolysaccharide from mutant U1 grown in continuous culture differs from algal alginate by having additional absorption peaks at 1250 cm^{-1} and 1720 cm^{-1} which correspond to *O*-acetyl ester (Fig. 1). It is similar to the spectra of alginate from *P. aeruginosa* and *A. vinelandii* (Evans & Linker, 1973; Mian *et al.*, 1978). The acetate content of the polymer was determined to be 12% (w/v) and an approximate mannuronic : guluronic acid ratio of 1 : 2 was estimated from the absorbances at 1125 cm^{-1} and 1030 cm^{-1}.

The rheological data (Table 5) indicate that without the addition of a proteolytic enzyme the *P. mendocina* polysaccharide has similar properties, including low pseudoplasticity, to the low molecular weight commercial alginate obtained from brown algae. With addition of protease a high viscosity product with similar properties to the high molecular weight algal product is obtained. Addition of EDTA lowers viscosity by chelation of divalent metal ions which form cross-links between chains.

DISCUSSION

Alginic acid can be synthesized by variants of *P. mendocina* selected in the presence of normally inhibitory concentrations of carbenicillin (Govan *et al.*, 1981). A similar response has been observed in some strains of *P. aeruginosa* (Govan & Fyfe, 1978). The mucoid variants of *P.*

mendocina are unstable. Stability and yields of polysaccharide can be improved by selection of large mucoid clones after mutagenesis of carbenicillin-selected isolates. The reasons for this response to carbenicillin are unclear. Continued selection on carbenicillin attempted in this study resulted in the eventual dominance of non-mucoid resistant strains. Similarly, attempts to stabilize polysaccharide synthesis using surfactants were unsuccessful although this technique was used with *P. aeruginosa* (Govan, 1975). Polysaccharide synthesis by *P. mendocina* was also dependent upon growth medium, high concentrations of glucose giving the best results, whereas a mucoid strain of *P. aeruginosa* synthesized exopolysaccharide on all growth media tested.

The polymer from *P. mendocina* is partially acetylated, in common with that from *A. vinelandii* (Larsen & Haug, 1971) and *P. aeruginosa* (Evans & Linker, 1973) but unlike that from the brown algae. This does not, however, seem to result in gross differences in rheology. The similarity in structure of a polysaccharide from such a range of organisms is interesting in view of the synthesis of very different exopolysaccharides by different wild-type strains of *P. aeruginosa* (Brown *et al.*, 1969; Bartell *et al.*, 1970).

The presence of an alginate lyase, albeit at very low intracellular activities, dramatically reduces the viscosity of culture broths. This suggests an endo-enzyme making possibly only a few breaks in a long chain. A similar effect has been observed in *A. vinelandii* cultures (Jarman, 1979). An inducible alginate lyase has been studied in *Beneckea pelagia* which is able to utilize alginate as a carbon source (Pitt & Raisbeck, 1978), but *P. mendocina* in common with *A. vinelandii* (Davidson, 1975) possesses low specific activities of this enzyme and is unable to grow on alginate. In *A. vinelandii*, however, a role for the enzyme in breaking down alginate during encystment or cyst germination can be postulated. Lyase activity has been reported in *P. putida* and *P. maltophila* (Sutherland & Keen, 1981) but not in *P. aeruginosa*. In this work intracellular activities similar to those in *P. mendocina* were detected in the alginate-synthesizing *P. aeruginosa* PsB although this organism does not degrade polymer in fermentations (Mian *et al.*, 1978). The difference may lie in a lower rate of cell lysis or in a poor activity of the enzyme towards an acetylated polymer.

Alginate lyase activity can be overcome in continuous culture of *P. mendocina* by incorporation of a protease in the growth medium and concentrations of alginate of 20 $g\,l^{-1}$ were maintained for 400 h at a dilution rate of 0·05 h^{-1}. The stability of this strain of *P. mendocina* was considerably better than that of *P. aeruginosa* PsB where 45% non-mucoid colonies were apparent after 112 h (Mian *et al.*, 1978). Higher polysaccharide:cell ratios were also obtained in this work. Alginate synthesis by *A. vinelandii* suffers from poor carbon conversion efficiencies because the organism synthesizes poly-β-hydroxybutyrate (Jarman *et al.*, 1978), and at higher oxygen tensions has very high respiration rates which result in low polysaccharide synthesis (Deavin *et al.*, 1977). At present, therefore, *P. mendocina* appears to be the most suitable organism to study bacterial alginate synthesis on a larger scale.

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Heterogeneity and Reduction in Pulmonary Clearance of Mucoid *Pseudomonas aeruginosa*

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Mucoid, alginate-producing *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis were studied to determine the extent of heterogeneity of the isolates within individual sputa. Considerable heterogeneity involving cultural requirements for mucoid colonial growth was observed, and sensitivity to the β-lactam antibiotic carbenicillin was also variable. For determining if the presence of alginate increased pulmonary survival of the bacteria, groups of rats were infected by transtracheal instillations of equivalent numbers of mucoid or nonmucoid *P. aeruginosa*, and survival was measured as the percentage of inoculum colony-forming units cultured from lung homogenates. Increased pulmonary survival of mucoid *P. aeruginosa* was observed in animals killed 3 hr or 6 hr after infection with unwashed bacteria. No difference in survival between mucoid and nonmucoid cells was observed when bacteria were washed prior to instillation. It was concluded that a single mucoid colony isolated from a sputum does not fully represent the population of mucoid *P. aeruginosa* within the patient and that pulmonary killing of mucoid *P. aeruginosa* can be less efficient than that for nonmucoid strains.

In the late 1960s the pioneering research of Doggett and Harrison [1] revealed the association of mucoid *Pseudomonas aeruginosa* and chronic, severe pulmonary infection in patients with cystic fibrosis. Today, *P. aeruginosa* has become the most troublesome respiratory pathogen of patients with cystic fibrosis [2, 3]. Although research in the last decade has greatly increased our basic knowledge of the properties of this organism, the natural history of pseudomonas infection in patients with cystic fibrosis is not fully understood.

Clinical and bacteriologic studies of patients with cystic fibrosis have indicated that initial infection occurs with a classic, nonmucoid strain of *P. aeruginosa*. Subsequently, a mucoid form emerges and becomes predominant with concomitant pulmonary deterioration and a poor prognosis for the patient [1–4]. Nonmucoid and mucoid forms isolated from the same patient usually belong to the same pyocin type [5] and serotype [6].

The exopolysaccharides produced by mucoid *P. aeruginosa* isolated from 10 patients with cystic fibrosis were shown to be similar and to consist of an acetylated alginate [7]. In contrast to pseudomonas slime [8], purified pseudomonas alginate is nontoxic when injected ip into mice (authors' unpublished results). Several in vitro studies have indicated that the alginate may be antiphagocytic [9, 10]. It would seem probable, therefore, that the function of the alginate is protective in vivo rather than invasive.

It has been reported [3] that the continuous use of antibiotics seems to contribute to the emergence of mucoid strains. Some mucoid variants have been reported to be slightly more resistant to β-lactam antibiotics and aminoglycosides than are related nonmucoid strains, and the isolation of mucoid, alginate-producing mutants of *P. aeruginosa* [11] and other *Pseudomonas* species [12] with use of a technique based on enhanced resistance of these mutants to carbenicillin has been described. It must be emphasized that generalizations cannot be made about the resistance of mucoid strains or their nonmucoid revertants. Some revertants retain the enhanced resistance to carbenicillin of the mucoid parent strain [13]; more striking, perhaps, is that very sensitive strains of both mucoid and nonmucoid forms of

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P. aeruginosa frequently are isolated from sputa [14].

This paper reports on the heterogeneity of mucoid *P. aeruginosa* isolated in vitro and from patients with cystic fibrosis and on the use of transtracheal inoculation in rats to compare the pulmonary clearance of isogenic mucoid and nonmucoid strains. The significance of the results with respect to present knowledge of pseudomonas infection in patients with cystic fibrosis is then discussed.

Materials and Methods

Pseudomonas aeruginosa strains. Mucoid strains of *P. aeruginosa* were isolated from sputa obtained from patients with cystic fibrosis attending the cystic fibrosis clinic at the Royal Hospital for Sick Children, Edinburgh. The mucoid mutant strain PAO 579 [15] was isolated from a culture of strain PAO 381 with use of a carbenicillin selection technique [11].

Heterogeneity of mucoid P. aeruginosa in sputa of patients with cystic fibrosis. Sputa of patients were treated with sputolysin (Calbiochem-Behring, Bishop's Stortford, England), diluted in sterile physiologic saline, and plated on Pseudomonas isolation agar (PIA; Difco, Detroit, Mich.). After incubation for 24 hr at 37 C, 100 mucoid cfu resembling Phillips colonial type 5 [16] were subcultured onto nutrient agar (NA; Columbia agar base, Oxoid, London), blood agar (BA; Columbia agar supplemented with 5% sterile human blood), minimal agar (MA, [17]), deoxycholate citrate agar (DCA; Oxoid CM227), and diagnostic sensitivity test agar (Oxoid) containing 5 μg of carbenicillin/ml (Beecham Research Laboratories, Brentford, England) and examined for mucoid growth after 24 hr at 37 C.

Pseudomonas alginate. The exopolysaccharide produced by mucoid strains grown on PIA was extracted, purified to an asbestos-like powder, and identified as alginate as previously described [11]. Gelatinization of the exopolysaccharide was examined by preparing a 1% aqueous solution from dried alginate and adding 3 m*M* $CaCl_2$.

Animal studies. Pathogen-free male Sprague-Dawley rats (200 g) were obtained from OLAC 1976 (Bicester, England). The technique used for transtracheal inoculation of bacteria into a distal bronchus was that previously described [18]. Cultures of strains PAO 381 and PAO 579 were grown in a chemically defined medium [19] with 1% sodium gluconate as the carbon source and were incubated in an orbital incubator (Gallenkamp, London, England) at 140 rpm at 37 C for 18 hr to stationary phase. Strain PAO 579, but not strain PAO 381, readily produces extracellular alginate under these in vitro conditions, but no clearly defined bacterial capsule is observed in preparations stained with india-ink or ruthenium red. The bacterial cultures were diluted 1:100 in PBS (pH 7.0); the 0.1-m inoculum contained $\sim 10^6$ cfu. Washed bacteria were prepared by two cycles of centrifugation at 3,000 *g* and resuspension of bacteria in PBS to yield 10^7 cfu/ml.

Bacterial quantitation. Animals were killed with ip pentobarbital, and both lungs and the heart were removed aseptically and washed with sterile PBS. The lungs were then excised and placed in 10 ml of sterile PBS. The tissues were homogenized in a Waring blender. Serial dilutions of the homogenate in PBS were plated on DSTA, and cfu were counted after overnight incubation at 37 C.

Pyocin typing. The pyocin-typing technique used was that described by Williams and Govan [5], with the incorporation of the revised scheme of Govan [20].

Results

Heterogeneity of mucoid P. aeruginosa. The heterogeneity of mucoid *P. aeruginosa* was discovered by the isolation of variants that produced alginate only when grown on certain media and, in addition, by the identification of mucoid variants that were sensitive to 5 μg of carbenicillin/ml.

Table 1 summarizes the characteristics of four groups of "medium-dependent variants" of *P. aeruginosa* isolated from patients with cystic fibrosis. These variants were recognized by their ability to produce mucoid growth resembling the Phillips colonial type 5 [16] after incubation at 37 C for 24 hr. Mucoid mutants belonging to groups 1–4 were successfully isolated in vitro from the nonmucoid strain PAO 381 as single-step mutations with use of the carbenicillin selection technique. The exopolysaccharide from each mutant gelled rapidly in the presence of Ca^{++} (figure 1) and was identified as an acetylated alginate-like polymer of mannuronic and guluronic acid.

Table 2 summarizes the incidence of each mucoid group and of the strains of *P. aeruginosa*

Table 1. Characterization of medium-dependent mucoid variants of *Pseudomonas aeruginosa* isolated in vitro and from patients with cystic fibrosis.

Variant group	Type of growth in indicated medium*			
	PIA	NA/BA	DCA	MA
1	+	+	+	+
2	+	+	+	−
3	+	+	−	−
4	+	−	−	−

* + = mucoid growth resembling Phillips colonial type 5 after incubation at 37 C for 24 hr; − = nonmucoid growth; PIA = pseudomonas isolation agar; NA = nutrient agar; BA = blood agar; DCA = desoxycholate citrate agar; and MA = minimal agar [17]. See Materials and Methods for further information about the media.

Table 2. Heterogeneity of mucoid *Pseudomonas aeruginosa* isolates from 100 sputum samples obtained from 17 patients with cystic fibrosis.

Group of mucoid isolate*	Percentage of sputum samples harboring group	Percentage of patients
1	89	100
2	24	65
3	10	35
4	11	29
Carbenicillin sensitive†	36	65

NOTE. Data are based on the analysis of 100 mucoid colonies from each sputum sample.

* See table 1 for characterization of mucoid groups.

† MICs of carbenicillin for isolates were <5 μg/ml.

sensitive to 5 μg of carbenicillin/ml among all strains isolated from 17 patients with cystic fibrosis who were chronically infected with this organism. Thirty-five sputum samples obtained from three patients presented a homogenous population of group 1 mucoid strains of *P. aeruginosa*. Indeed, variants belonging to group 1 were by far the most common; they were isolated from all 17 patients and from 89% of the individual sputa. Variants belonging to group 2 were isolated from 65% of patients; group 3 variants, from 35%; and group 4, from 29%. Strains of *P. aeruginosa* sensitive to 5 μg of carbenicillin/ml were isolated from 65% of the patients. This characteristic was independent of the mucoid group. Although most patients harbored mucoid strains from more than one group simultaneously, no clear transition was observed from one group to another. Group 1 variants usually appeared first in the course of infection and accounted for the majority of *P. aeruginosa* isolated from an individual specimen or patient. However, mucoid variants sensitive to <5 μg of carbenicillin/ml also were found frequently in large numbers and in one patient accounted for all isolates of *P. aeruginosa*. Heterogeneity could not be explained on the basis of mixed infections with more than one strain of *P. aeruginosa*. In most patients, mucoid variants of different classes belonged to the same pyocin type.

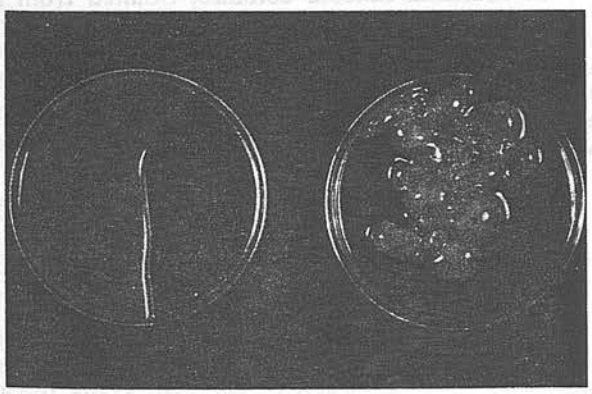

Figure 1. Viscid 1% aqueous solution of alginate extracted from *Pseudomonas aeruginosa* strain PAO 579 (*left*) and the gel that forms (*right*) in the presence of 3-m*M* Ca^{++}.

Pulmonary clearance of P. aeruginosa. We attempted to identify a possible advantage for mucoid *P. aeruginosa* in the respiratory tract by investigating the clearance of isogenic mucoid and nonmucoid bacteria from rat lungs. Table 3 compares the survival of the group 1 mucoid mutant strain PAO 579 or the nonmucoid parent strain PAO 381 of *P. aeruginosa* in homogenized lungs after transtracheal inoculation. No viable *P. aeruginosa* were recovered 24 hr after inoculation with 10^6 cfu of washed or unwashed bacteria, and no animals died. Further experiments showed that few bacteria were recovered after 6 hr. A typical result for lungs sampled at 3 hr and 6 hr indicated that the mucoid strain PAO 579 was cleared less efficiently than the nonmucoid strain PAO 381 ($P < .01$, Student's t test). No significant difference in the clearance rate of strains PAO 579 and PAO 381 was observed when washed cell suspensions were used. Washing significantly increased the clearance of strain PAO 579 ($P < .001$) but not of strain PAO 381.

Table 3. Survival of isogenic mucoid and nonmucoid *Pseudomonas aeruginosa* in rat lungs after transtracheal instillation.

Time after instillation (hr)	Percentage of indicated inoculum recovered*			
	PAO 579 (mucoid)		PAO 381	
	Washed	Unwashed	Washed	Unwashed
3	0.32 ± 0.04	19.3 ± 2.15	0.30 ± 0.03	1.6 ± 0.62
6	. . .	4.1 ± 0.54	. . .	0.27 ± 0.02

* Expressed as percentage of inoculum cfu recovered from lung homogenates. Values are expressed as mean ± SE; each group comprised five animals.

Discussion

It is arguable that in no other infection does *P. aeruginosa* exhibit the complexity and range of biological properties that are observed in chronic pulmonary infection in patients with cystic fibrosis; however, an appreciation of this complexity of such infection is essential not only to an understanding of the natural history of the infection but also to an explanation of the apparent contradictions in the literature that result from generalizations on this subject, in particular those dealing with the nature of the mucoid form.

Mucoid *P. aeruginosa* frequently are assumed to be a homogeneous class of the species possessing basic common properties. Increasingly, however, genetic and physiologic studies have revealed the heterogeneity of this unusual form of the species.

Genetic studies [15, 21] of isogenic mucoid mutants of PAO strains have shown that at least two chromosomal loci are involved in the control of alginate synthesis. Individual mucoid strains isolated in vitro or from patients with cystic fibrosis differ considerably in the amount of alginate produced under defined conditions; in the degree of acetylation of the alginate, which is proportional to viscosity of the polymer; and in the optimal conditions for alginate synthesis [22].

Extreme sensitivity to carbenicillin in *P. aeruginosa* has previously been observed and its association with respiratory isolates noted [23]. The phenomenon frequently is encountered in mucoid strains but is not dependent on alginate production [14].

The significance of heterogeneity in *P. aeruginosa* isolated from patients with cystic fibrosis is further emphasized by the heterogeneous population of mucoid variants found within individual sputum samples from patients with cystic fibrosis. Heterogeneity cannot be explained by simultaneous infection with more than one strain of *P. aeruginosa*.

The nature of heterogeneity reported in this paper involves forms of mucoid *P. aeruginosa* that possess identical colonial appearance when grown on PIA. Other reports [24, 25] have described heterogeneity among strains isolated from individual sputa of patients with cystic fibrosis with regard to colonial appearance (e.g., rough, mucoid, or dwarf) and have noted differences in antibiotic susceptibility associated with these different colonial types. It was suggested [24] that studies of antibiotic susceptibility should be performed on several colonies of each colonial type of *P. aeruginosa* isolated from an individual sputum sample. We would support this suggestion and further emphasize that up to 100-fold differences in susceptibility to β-lactam antibiotics can be found in mucoid colonies, isolated from the same sputum, that are indistinguishable in colonial appearance [14].

Two recent reports have provided valuable insight into the natural history of *P. aeruginosa* infection in patients with cystic fibrosis, in particular the initial selective adhesion and colonization of the upper respiratory tract by nonmucoid strains of *P. aeruginosa* and the subsequent emergence of the mucoid form. Woods et al. [26] reported that in vitro adherence of *P. aeruginosa* to buccal epithelial cells was significantly higher in cells obtained from patients with cystic fibrosis than in those from control subjects. Mucoid *P. aeruginosa* adhered in significantly lower numbers than did nonmucoid forms, and this pili-mediated adherence varied directly with the loss of

protease-sensitive fibronectin from the cell surface and the increased levels of salivary proteases found in patients with cystic fibrosis. In contrast, Marcus and Baker [27] observed that in ciliated hamster tracheal epithelium mucoid *P. aeruginosa* adhered to the cilia and formed mucoid microcolonies, whereas nonmucoid forms showed little or no adherence.

The results reported in this paper on the clearance of *P. aeruginosa* from the lower airways of rats suggest a further selective advantage for the mucoid form. We deliberately used the nonmucoid parent strain PAO 381 as the control for these experiments because of the considerable physiologic and genetic heterogeneity found in nonmucoid revertants [13, 28]. As was previously reported by Blackwood and Pennington [29], we found no significant difference between mucoid and nonmucoid organisms when animals were infected with washed cell suspensions; we did observe a difference, however, with unwashed suspensions. An explanation for these results may lie in the nature of alginate production in *P. aeruginosa*. In contrast to the classic cell-bound capsule of *Klebsiella*, the alginate produced by *P. aeruginosa* in vitro is in the form of a loosely associated slime or peripheral capsule [30]. After centrifugation most of the alginate remains in the culture supernate. In the course of the short experimental period, there would be little prospect for alginate synthesis in vivo.

The results reported here do not establish that alginate per se is responsible for enhanced pulmonary survival of mucoid strains. The ability of pseudomonas alginate to gel in normal pulmonary levels of Ca^{++} is striking. Further studies are required to assess the influence of the characteristically increased levels of Ca^{++} found in patients with cystic fibrosis and the role of cell-free and cell-associated alginate in the clearance of *P. aeruginosa*.

It is difficult to assess the relevance of in vitro experiments and animal studies to human disease. Nevertheless, since a reduced rate of pulmonary clearance for mucoid *P. aeruginosa* has been observed in healthy rats, the selective advantage for the mucoid form might be even greater in the compromised lungs of patients with cystic fibrosis.

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Focus on Research: Number 48

Pseudomonas Aeruginosa and Respiratory Infection in Cystic Fibrosis

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The susceptibility of patients with cystic fibrosis (CF) to chronic respiratory infections due to particular bacterial pathogens has been recognised since the first descriptions of CF more than four decades ago. Today, the vicious cycle of recurrent exacerbations and progressive pulmonary deterioration has assumed even greater prominance as the major influence on mortality and quality of life in CF patients. Does this apparent lack of progress indicate the ultimate futility of the efforts of those involved in the management and prevention of respiratory infection? Is it simply the price of progress and greater life expectancy or does it emphasise the need for continuing reappraisal of the complex and subtle association between opportunist infection and a uniquely compromised human host? More constructively, is there evidence that we need to revise our attitudes regarding the significance and control of infection and is there an argument for considering new initiatives?

In other areas of CF research, e.g. in the search for the basic defect, respected and well intentioned researchers have reported 'breakthroughs' which unfortunately could not be reproduced in other centres. On the subject of respiratory infection in CF patients, reproducibility of data concerning incidence and treatment is not often the subject of dispute. Instead the problem is one of interpretation. What does the clinical and bacteriological data actually signify? As in politics all shades of opinion are possible and inevitable. Similarly, on the difficult topic of respiratory infection in CF, differences in interpretation are inevitable. It must be emphasised that the views expressed in this article may not be shared by all those involved in CF management and research. Nevertheless the interpretations and conclusions expressed are not those of the author alone, but have evolved through collaboration and discussion with respected colleagues exprienced in the problems of CF.

Why are there so many 'grey areas' in the subject of respiratory infection? Why do we hear, all too frequently, in discussions of anti-biotic and vaccine therapy or the mechanisms of bacterial pathogenicity, the statement "this does not apply to CF"? To understand the reasons for this situation it is necessary to review briefly the development of our understanding of the role of bacteria in disease.

In the early days of clinical microbiology strict conditions were laid down to prove that a particular microorganism was the cause of a particular disease. These conditions known as Koch's postulates required that the microbe be found in the body in all cases of the disease, that it be isolated from a case and grown in a series of pure cultures *in vitro* and that it reproduce the disease on the inoculation of a pure culture into a suitable healthy host. If the microorganism could not be isolated and cultured *in vitro,* then alternatively, serological observations namely the detection and an increase in titre of antibodies specific for the microorganism could also be taken as evidence.

It is arguable that these early dogmas of clinical microbiology should not be so strictly applied to today's major problems of infection which frequently involve the role of opportunist microorganisms as the cause of infection in patients already compromised by underlying diseases or iatrogenically by therapeutic procedures such as surgery or chemotherapy which are necessary in the management of the disease. In CF, the problem of interpretation of bacteriological findings is particularly difficult on the controversial subject of the significance of *Pseudomonas aeruginosa* in causing respiratory deterioration in CF patients. In this case we have the undisputed association of a well recognised, adaptable opportunist pathogen and a host whose compromised status involves a subtlety and complexity which probably extends beyond criteria normally recognised as responsible for increasing susceptibility to bacterial infection.

Host Factors

In CF patients the host factors responsible for initiation of early infection and for later recurrences cannot be identified with certainty. It is postulated that infection occurs because the sticky bronchial secretions characteristic of the underlying disease and exacerbated by previous infection lead to failure in normal pulmonary clearance of bacteria. This itself may be only partially correct since there is some evidence that the secretions are normal in the absence of infection. With respect to other immunological defence mechanisms no specific primary defect has been demonstrated in CF patients which can explain their increased susceptibility to pulmonary infections. Alveolar macrophages have been reported to malfunction in CF patients although results are conflicting and may well represent another secondary manifestation of the disease.

It seems reasonable to argue that the susceptibility of a CF patient to respiratory infection results not from a single factor but from a combination of the abnormalities associated with the disease and the multiple forms of therapy involved in its management. Progress in life expectancy, changes in management procedures concerning the use of antibiotics and dietary supplements, the influence of viral infections and the electrolyte environment in the CF respiratory tract may all combine to account for the initiation of infection and the changing pattern of infection observed.

The changing pattern of bacterial infection.

To many people involved in CF the pattern of bacterial infection in CF patients was fixed in the early days of CF research, when, in 1946 di Sant'Agnese and Andersen reported the bacteriological results of 14 post mortem examinations. In the majority of cases *Staphylococcus aureus* was the predominant pathogen. In only a single case was *Haemophilus influenzae* or *P. aeruginosa* observed. Significantly, however, all but two cases involved patients less than a year old and the child from whom *P. aeruginosa* was isolated was one of the oldest cases, aged 32 months. These results led to the belief that *Staph. aureus* was the principal pathogen in CF patients and consequently the practice of long term prophylactic antibiotic therapy directed against this microorganism. This traditional assumption is still held by many involved in the management of CF despite later studies which indicated that CF patients are equally susceptible to other respiratory pathogens. Undoubtedly in later studies a changing pattern of

infection was obvious. In 1963, Iacocca *et al.* reported that *Staph. aureus* with an incidence of 85% remained predominant but that *P. aeruginosa* (65%) and *H. influenzae* (21%) were an increasing cause for concern. In 1978, after a 15 year survey Kulczycki *et al.* concluded "In the beginning of the study, there was a prevalence of *Staph. aureus* in all groups studied. At the conclusion of the study, however, *Staph. aureus* disappeared from the patients with *P. aeruginosa* and from those with mucoid *P. aeruginosa* and was not recovered at post mortem examination. The continuous use of antibiotics seemed to contribute to the persistence of *P. aeruginosa* and the appearance of mucoid strains of *P. aeruginosa*."

Why has the pattern of bacterial infection in CF changed and what lessons can be learned for the future? The nature of management of the CF patient is an evolving process rather than a static one. For example, it can be argued that improved life expectancy alone would influence the significance to be placed on infection with *Staph. aureus*. This microorganism has always been a significant pathogen in the early years of life and remains so today. Thus it would be reasonably expected to play a significant role in the early days of CF. Does it follow, however, that it should remain the most significant pathogen for the CF patient as life expectancy improved and the population of CF patients moved out of infancy? Similarly, the assumption that the increase in *P. aeruginosa* is a direct result of changes in the microorganism rather than in the CF patient needs careful scrutiny; it is arguable, for example, that the incidence of *P. aeruginosa* in very young CF patients differs little today from that observed in 1946. Obviously, infections due to *Staph. aureus* must never be underestimated and require appropriate antibiotic therapy but is continuous prophylactic therapy necessary and at worst a contributor of later and more intractable infection due to *P. aeruginosa*. It is clearly evident from the studies of Iacocca *et al.* (1963) and Kulczycki *et al.* (1978) that the incidence of persistent infection due to *P. aeruginosa* is highest in those patients who have continuous antibiotic therapy. This is no different from experience in many other opportunist infections in other compromised patients. Nor, it would appear, does the lack of such therapy prejudice the CF patient to staphylococcal infection. In the Edinburgh clinic, at least, the present practice of treating only confirmed clinical infection with *Staph. aureus* has not resulted in an increased incidence of staphylococcal infection but in addition the incidence of *P. aeruginosa,* even on an age-related basis, remains significantly low.

The role of P. aeruginosa in CF

Another controversial and difficult question to answer in simple terms is the role of *P. aeruginosa* in CF patients.

In the late 60's and through the 70's as the incidence of *P. aeruginosa* increased so also was the emergence and establishment of mucoid *P. aeruginosa* in CF patients increasingly recognised. Doggett (1969), Hoiby (1975) and other researchers noted that following primary colonisation with classic non-mucoid *P. aeruginosa,* mucoid forms of the same microorganism emerged and came to predominate with a corresponding increase in pulmonary deterioration. This association of mucoid *P. aeruginosa* and CF became so evident that it was suggested as a diagnosic sign of CF itself (Reynolds *et al.,* 1976). In 1982, the correlation of mucoid *P. aeruginosa* with a poor prognosis was further confirmed in a statistical study by Henry *et al.* which concluded "Whilst identification of mucoid forms of *P. aeruginosa* in the respiratory tract of children and adolescents with cystic fibrosis is an unfavourable factor, non-mucoid forms appear to be of no major significance". This conclusion, however, only confirms the association of mucoid *P. aeruginosa* and significant infection. It could be explained by the suggestion that the microorganism is present as a result of pulmonary deterioration rather than a direct cause. This chicken and egg dilemma is not easy to answer but deserves our attention.

To proceed further we must return to some basic aspects of clinical microbiology, e.g. to the difference in interpretation between the presence of a classic pathogenic microorganism such as the tubercle bacillus and an opportunist such as *P. aeruginosa.* In the former case, the presence of a single identifiable cell is considered sufficient for diagnosis and treatment. This does not apply, however, to opportunist infections whose very nature involves a more subtle upset in equilibrium between host defences and microbial hyenas. In this case the load of challenge directed against the host and the particular susceptibility of the latter combine to dictate the eventual outcome. As a consequence to judge the significance of mucoid *P. aeruginosa* in a CF patient or to assess the efficacy of antibiotic treatment merely by the presence or absence of the microorganism may give rise to misinterpretation of the organisms real significance. For this reason the presence of *P. aeruginosa* should be examined on a quantitative basis and with close collaboration between microbiologist and clinician regarding the clinical status of the patient.

Several of these considerations are illustrated in Fig. 1 which concerns a single exacerbation of respiratory infection in a CF patient whose previous history had been closely monitored. This patient was admitted to hospital with an exacerbation of infection and in poor clinical condition. Bacteriological examination revealed a sputum count of 10^8 organisms/ml of a mucoid *P. aeruginosa* which the patient had harboured for some time. In addition, on this occasion and for the first time, a classic non-mucoid *P. aeruginosa* was also present in significant numbers. Typing (fingerprinting) of the two organisms revealed that they were of two distinct pyocin types and thus unrelated. On antibiotic treatment the patient showed marked clinical improvement paralleled by a significant reduction in the bacterial count. Indeed, the non-mucoid strain was eradicated within a week and did not return subsequently. Although both mucoid and non-mucoid *P. aeruginosa* exhibited similar sensitivity towards the antibiotic, as judged by standard *in vitro* examination, the number of mucoid bacteria was reduced more slowly. Unusually, on this occasion, resistant forms of the mucoid strain appeared as a small proportion of the population and then came to predominate. This was paralleled by an increase in the bacterial count and clinical deterioration.

This particular episode emphasises several aspects of CF bacteriology. Firstly, the clinical improvement in the patient cannot be simply explained by hospitalisation, secondly as is so often observed in many antibiotic regimes, clinical improvement was evident but the mucoid *P. aeruginosa* were not eliminated. Quantitatively, however, the population had been reduced to less than .001% of the original. This episode also illustrates the individuality of the CF patient. One might conclude from the results that this particular mucoid strain of *P. aeruginosa* possessed a degree of virulence which could be reproduced if transmitted to another patient. This type of generalisation cannot be made in CF however, since the patient's younger sibling harboured the same organism in high numbers with no apparent clinical consequences at the time. It is possible that a second pathogen was present which was not identified and accounted for these findings. In our view this was not likely but remains as an example of the difficulty of making definitive conclusions in CF.

If the presence of mucoid *P. aeruginosa* is an unfavourable factor for the CF patient because it is a cause of tissue damage then we must find the best means to prevent or control the infection. At present, it is a common and outwardly reasonable practice to restrict antibiotic therapy against *P. aeruginosa* to obvious clinical infection due to mucoid *P. aeruginosa*. The progress of such infections however is insidious and in view of the widely held agreement that such treatment, even with the most active antibiotics, can at best be palliative and seldom eradicates the microorganism it could be argued that treatment is applied too late. On this subject it is interesting to note a recent study (Szaff *et al.,* 1983) which demonstrated significantly improved survival and quality of life when CF patients were examined at three monthly intervals and if found to harbour

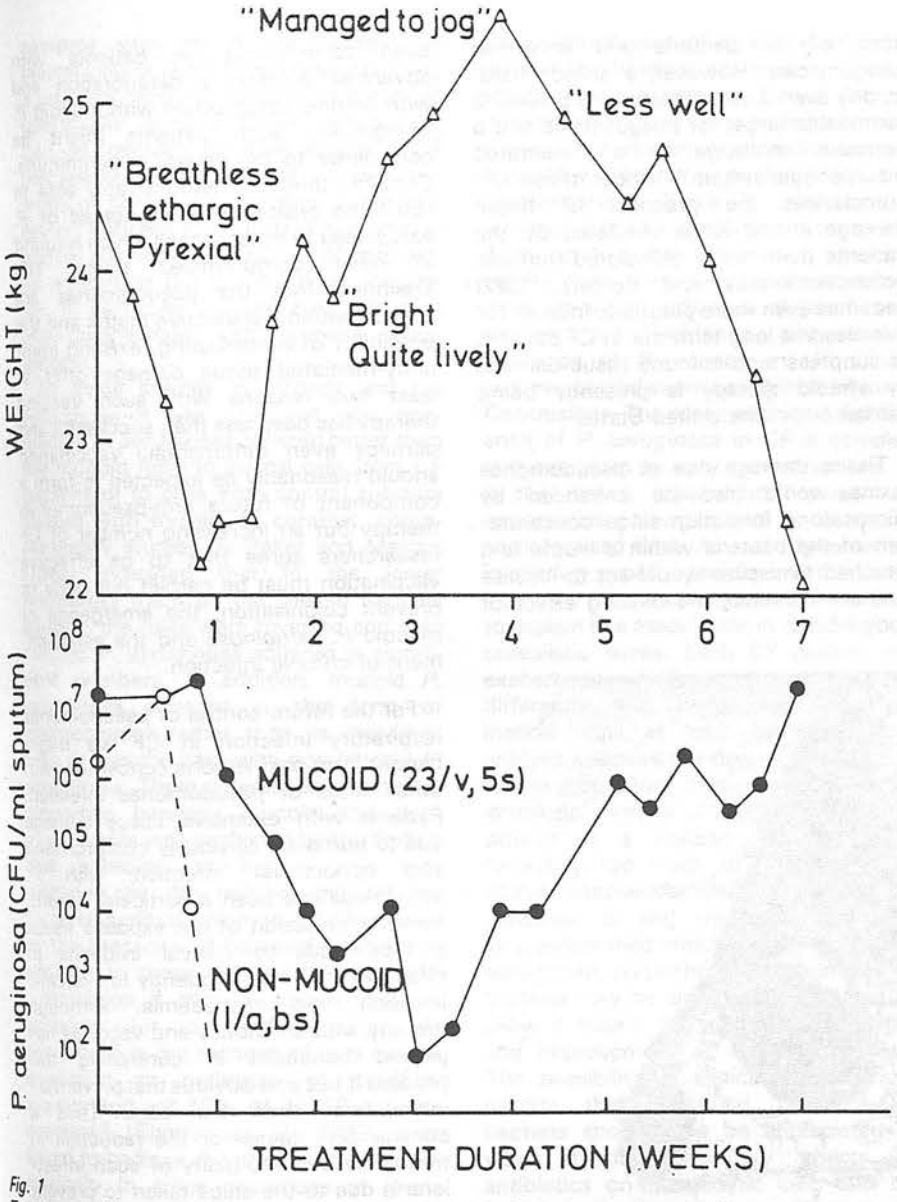

Fig. 1
Treatment of an exacerbation of respiratory infection due to P. aeruginosa. *Note the correlation between weight, clinical condition and the numbers of* P. aeruginosa *found in the patient's sputum. Note also the eradication of a newly acquired non-mucoid* P. aeruginosa *but the eventual increase of the mucoid strain.*

P. aeruginosa were treated with appropriate antibiotics even in the absence of obvious clinical infections. If only mucoid *P. aeruginosa* are of clinical significance does this provide a clue to the mechanism of pathogenicity and explain why infection in CF patients with *P. aeruginosa* is so different from the many other opportunistic infections caused by the classical, non-mucoid form of this microorganism?

Mucoid *P. aeruginosa* are rarely isolated except from the lungs of CF patients. By the time of post mortem examination Kulczycki *et al.* (1978) observed that almost 100% of patients who died of pulmonary tract complications had cultures positive for *P. aeruginosa*, 90% of which were mucoid *P. aeruginosa*. Other unusual properties observed in *P. aeruginosa* at the transition from colonisation to apparent infection include heterogeneity in the population of *P. aeruginosa* within an individual CF patient and the expression of unusual properties including hypersensitivity towards certain antibiotics, loss of serotype reaction, expression of a new somatic antigen and sensitivity to normal human serum (Irvin *et al.*, 1981; Penketh *et al.*, 1983).

In contrast to their role in CF, typical non-mucoid strains of *P. aeruginosa* are notoriously successful opportunist pathogens causing a wide range of infections which may be localised or spread systemically resulting in septicaemia and high mortality. Tissue damage in such infections is due to the release of various extracellular toxins including elastase and the more potent exotoxin A which resembles diphtheria toxin in its inhibitory effect on eucaryotic protein synthesis. Two striking differences become immediately apparent in the case of respiratory infections in the CF patient. Firstly, that infection is restricted to the respiratory tract and secondly the more virulent role for mucoid *P. aeruginosa* which *in vitro,* at least, appear less toxigenic. In addition, although the use of antibiotics can bring about clinical improvement, mucoid *P. aeruginosa* are seldom eradicated. The problem is not one of enhanced resistance; on the contrary, mutants hypersensitive to antibiotics are frequently found in CF patients (Irvin *et al.*, 1981). A possible explanation for these observations is that *in vivo,* mucoid *P. aeruginosa* are not present as individual cells but, as shown in Fig. 2, exist as microcolonies protected by the peculiar physical properties of the bacterial mucoid material.

The bacterial mucoid material is a polysaccharde composed of alginate, a polymer of mannuronic and guluronic acids. In some reports pseudomonas alginate has been confused with other exopolysaccharides produced spontaneously by all *P. aeruginosa.* It must be emphasised that the mucoid nature of *P. aeruginosa* isolated from CF patients

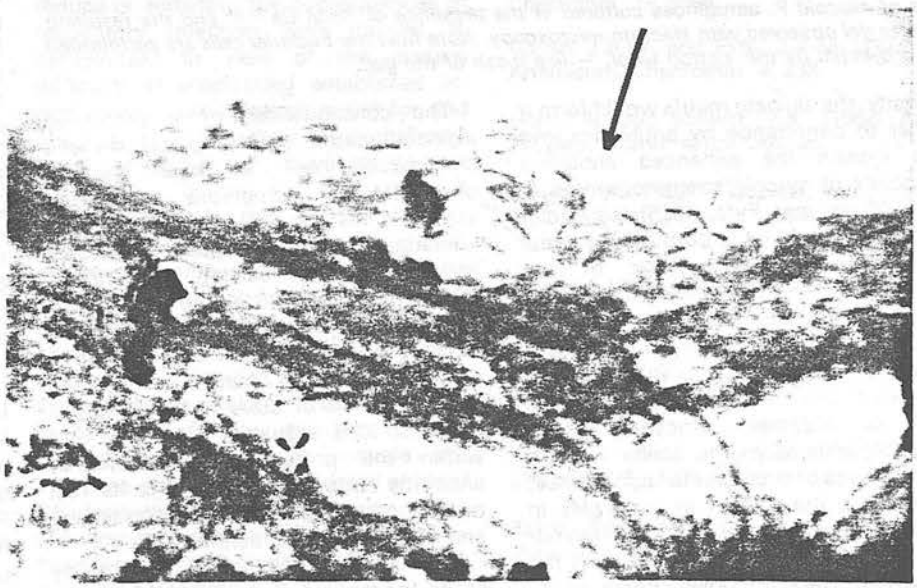

Fig. 2
Gram-stained smear of sputum from a CF patient harbouring mucoid P. aeruginosa. *Non-mucoid* P. aeruginosa *would normally appear distributed across the field as free bacilli. Note, in contrast, how the mucoid* P. aeruginosa *(arrowed) are enclosed within a microcolony which is apparently attached to bronchial material.*

results from alginate biosynthesis caused by bacterial mutation (Fyfe and Govan, 1980) and that the alginate is distinct from other pseudomonas polysaccharides and also from the capsular polysaccharides of *Klebsiella* and mucoid *Escherichia coli.* Alginates from viscid aqueous solutions but even more significantly they are remarkable gelling agents rapidly forming stable gels in the presence of electrolytes such as Ca^{++}. In the absence of suitable electrolytes pseudomonas alginate is produced as an extracellular slime and is easily removed by washing. In the presence of Ca^{++}, however, at concentrations as low as 1mM the cells become embedded in a protective matrix or microcolony surrounded by their own alginate (Fig. 3). If this is the explanation for the microcolony mode observed in Fig. 2 we can explain a number of other observations associated with the presence of *P. aeruginosa* in CF patients.

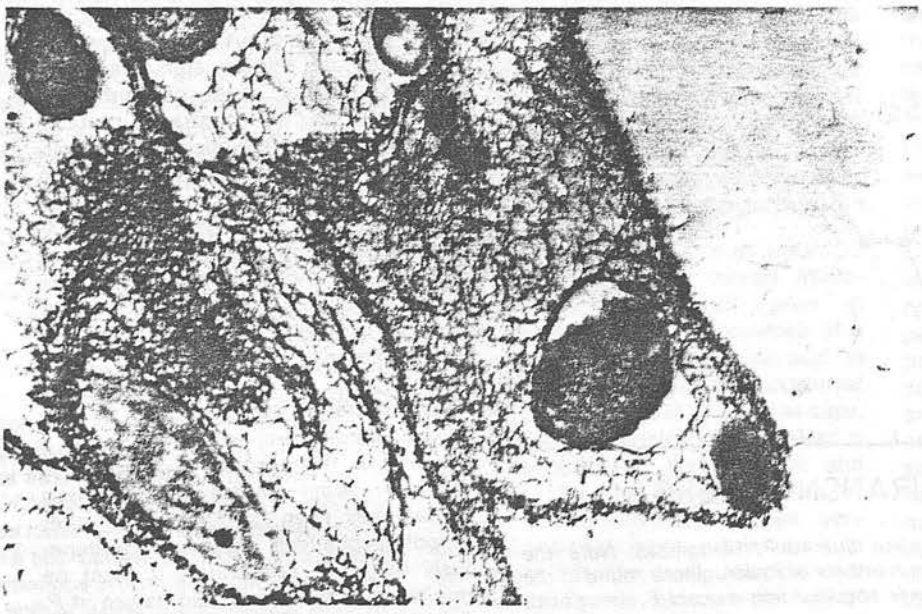

Fig. 3
Cells of mucoid P. aeruginosa *cultured in the presence of 1mM* Ca^{++} *and the resulting alginate gel observed with electron-microscopy. Note how the bacterial cells are surrounded and protected by the 'cotton wool' — like mesh of the gel.*

Firstly, the alginate matrix would form a barrier to penetration by antibiotics and thus explain the enhanced antibiotic resistance of mucoid strains observed *in vitro* (Govan and Fyfe, 1978); secondly, the matrix would constitute a substantial barrier against phagocytosis of the bacteria by the lung defences. In the microcolony mode we can also appreciate how much mucoid *P. aeruginosa* would present a more difficult problem for the phagocytic cell than capsulate organisms such as *Klebsiella* whose capsular polysaccharide lacks the ability to form gels. Perhaps of even greater significance, however, is the role of alginate gels in explaining the potential for an inflammatory mechanism of tissue damage in the CF patient. Recent studies (Bryan *et al.,* 1983) have shown that the pseudomonas alginate is antigenic and that the sera of CF patients contains specific anti-alginate antibodies. Under normal circumstances this would be beneficial allowing opsonisation of the bacteria and enhanced phagocytosis. However, a gelled microcolony even if opsonised would present a formidable target for phagocytosis and a constant challenge to a "frustrated macrophage system." Under these circumstances the prospect of tissue damage in the lungs mediated by the patients own highly stimulated immune defences (Hoiby and Schiotz, 1982) becomes even more plausible. Indeed, for this reason a long term trial in CF patients to suppress auto-immune tissue damage by steroid therapy is presently being carried out in the United States.

Tissue damage due to pseudomonas toxins would also be enhanced by microcolony formation since concentration of the bacteria within a matrix and attached to tissues would act to localise toxins and reduce the diluting effect of body fluids.

The consequences of a protective alginate matrix may also explain the considerable cell envelope changes observed in *P. aeruginosa,* e.g. serum sensitivity and the expression of polyagglutinating antigen (Penketh *et al.,* 1983) and which are associated with chronic infection and clinical severity. The bacterial alginate binds iron which is essential to bacterial metabolism and a protective alginate matrix would also impede access of other nutrients to the bacteria. It is arguable, therefore, that within the protection of a matrix, antibiotic hypersensitivity exerts its real role by providing enhanced permeability and uptake for essential nutrients.

The consequences of a microcolony mode for mucoid *P. aeruginosa* within a CF lung would also have significant bearing on the potential of vaccine therapy. To date, vaccination has not proved successful in CF but perhaps with reason. The vaccine trials have generally been carried out on patients with advanced pulmonary deterioration and with chronic colonisation with mucoid *P. aeruginosa.* Such patients might be considered to be already hyperimmune. Certainly they are demonstrably able to resist the typical systemic spread of *P. aeruginosa* to other organs which is found in other compromised hosts. The likelihood that the pseudomonas are already within a protective matrix and the possibility of exacerbating, existing immunity-mediated tissue damage offer at least two reasons why such vaccine therapy has been less than successful and perhaps even detrimental. Vaccination should reasonably be expected to form a component of future anti-pseudomonas therapy but an increasing number of CF researchers agree that to be effective vaccination must be carried out early to prevent colonisation, the emergence of mucoid *P. aeruginosa* and the establishment of chronic infection.

For the future control of pseudomonas respiratory infection in CF we might benefit from past lessons concerned with other kinds of pseudomonas infection. Patients with extensive tissue damage due to burns are obviously compromised and opportunist infection with *P. aeruginosa* has been a particular hazard. Initial colonisation of the exposed tissue surface leads to clinical evidence of infection and later frequently to systemic invasion and septicaemia. Although therapy with antibiotics and vaccines has proved beneficial in controlling this process it became obvious that prevention was easier than the cure. Thus a considerable cause for the reduction in frequency and morbidity of such infections is due to the steps taken to prevent and control colonisation. In CF, therefore, should we accept the implied message of Henry *et al.* (1982) and ignore the asymptomatic colonisation of the CF patient with non-mucoid *P. aeruginosa?* I think this would be a mistake. From a genetic viewpoint, at least two of the phenotypic characteristics of *P. aeruginosa* associated with advanced chronic infection, alginate production and hypersensitivity to antibiotics are due to bacterial mutations. In order for such mutations to occur and be selected, a resident pseudomonas population would be essential but not necessarily situated in the lung itself. Where and why might colonisation occur?

In 1980, Woods *et al.* reported the important observation that in CF patients levels of salivary proteases were raised and that buccal cells from CF patients allowed significantly greater adhesion of *P. aeruginosa in vitro* than similar cells from control subjects. Previous studies (Johnanson *et al.,* 1979) had shown that control cells behaved similarly to CF cells if their normal coating of fibronectin was reduced by treatment with the proteolytic enzyme, trypsin. Do the raised protease levels observed in the saliva of CF patients allow exposure of suitable bacterial

receptor sites for *P. aeruginosa?* Are buccal cells or other sites in the oral cavity a possible reservoir of early colonisation specifically suitable for *P. aeruginosa?* Can we speculate on other aspects of CF management which would increase the levels of proteolytic enzymes in the oral cavity? It is relevant to bear in mind that in normal subjects the presence of fibronectin modulates the oral bacterial population, in particular by mediating adhesion and colonisation with harmless streptococci.

In these studies by Woods and his colleagues it was observed that non-mucoid *P. aeruginosa* adhered better than the mucoid form to buccal cells from CF patients or to cells from control subjects treated with trypsin. In contrast, complementary studies by Baker and Marcus (1982) indicated that in the lower respiratory tract, on the ciliated tracheal surface, the roles were reversed and only mucoid *P. aeruginosa* adhered in significant numbers. In addition, mucoid *P. aeruginosa* adhered in the form of microcolonies rather than as individual cells as was the case with non-mucoid *P. aeruginosa*. There differences in adhesive properties between mucoid and non-mucoid cells was explained by the finding that adhesion to buccal cells was mediated by the hair-like pili on the bacterial surface which would be masked by the presence of bacterial alginate. In addition to these two sites of potential colonisation if the CF patient there is evidence for a third location. *P. aeruginosa* is not recognised as a normal inhabitant or pathogen of maxillary sinuses and yet 68% of 20 CF patients examined (Shapiro *et al.,* 1982) were found to harbour *P. aeruginosa* asymptomatically in sinus secretions at levels greater than 10^4 organisms/ml. In addition, even at this level of the respiratory tract 30% of strains were found to be mucoid.

Is there life after P. aeruginosa?

Would the control and prevention of infection due to *P. aeruginosa* merely lead to the emergence of other pathogens. This is often asked but not so obvious a sequelae in CF as in other compromised hosts. If other opportunist pathogens, e.g. yeasts, *Klebsiella, Legionella* were to be a future problem they should have made their presence known already. This is not the case to any great extent. It could be argued, instead, that the present day CF patient presents a uniquely compromised host which combined with the considerable adaptability of *P. aeruginosa* ensures the predominance of colonisation and infection with this microorganism. The subject of the role of *P. cepacia* does require consideration but is arguably not a cause for immediate alarm. In a few centres, *P. cepacia* has been isolated in increasing numbers in the last decade, particularly from older CF patients and associated with pulmonary deterioration. The prospect of *P. cepacia* as yet another CF pathogen is a daunting one, particularly in view of its innate antibiotic resistance. However, the present concern stems from experience in a limited number of clinics. Typing or fingerprinting schemes for epidemiological analysis are only now becoming available for this microorganism. Since *P. cepacia* is a common hospital contaminant the possibility remains that the present situation results from long term endemic cross-infection within individual clinics. Nevertheless, the incidence and clinical relevance of *P. cepacia* in UK clinics requires careful examination in the future.

Conclusions. The association and significance of *P. aeruginosa* in CF is complex and not given to easy generalisations. It is the classic association between an opportunist pathogen and an unusually compromised host. As such, 'grey areas' in relation to pathogenicity and treatment may be a natural consequence if we seek to explain this association in too simple or unrealistic terms. Each CF patient and exacerbation of infection may well behave differently and bacteriological examination must at least by quantitative analysis measure the degree of challenge to the respiratory tract. The efficacy of antibiotic therapy should also be considered in a realistic manner. It is expecting too much of even the most active antipseudomonas antibiotics to penetrate to and eradicate completely long established infection with mucoid *P. aeruginosa*. Nevertheless the numbers of bacteria may be significantly reduced to allow a respite for pulmonary defences and improvement in clinical condition. The possibility of clinical improvement despite the continued presence of bacteria should also be appreciated in view of the secondary effects of antibiotics on phagocytic cells and on lowering the toxigenicity of bacterial pathogens.

What else can we do constructively to reduce or eliminate the consequences of respiratory infection with mucoid *P. aeruginosa?* In view of the present difficulty of eradicating established infection are new initiatives called for and are they feasible? Herein lies a challenge to all involved in CF. If we accept that infection, or colonisation is detrimental irrespective of whether tissue damage results from bacterial toxins or an over exuberant immune response then we need to determine the steps involved from colonisation to infection. With such knowledge we could then direct our efforts to the weakest point in the chain. These efforts might necessiate the broadening of our attitudes towards respiratory infection to include the influence of apparently unrelated areas of CF, from the role of electrolytes on the pseudomonas alginate to possible influences of dietary supplements on early bacterial colonisation.

This article has contained disputed and undisputed interpretations regarding the role of *P. aeruginosa* in CF but also arguments based on experience and interpretation. In the understanding and management of opportunist infection, progress seldom comes from dramatic 'break-throughs' but rather from patience, realism and a degree of boldness to consider new approaches. These virtues are obviously exercised already by those involved in the many diverse aspects of CF research and management but have no less a role to play on the subject of respiratory infection. Ideally, if the basic defect of cystic fibrosis was understood and removed the question of the role of *P. aeruginosa* would not even exist.

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SYNTHESIS, REGULATION AND BIOLOGICAL FUNCTION OF BACTERIAL ALGINATE

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INTRODUCTION

Historically, interest in bacterial exopolysaccharides has been chiefly medically orientated. The association of capsule production with virulence has been noted in several important bacterial pathogens, the earliest example being Streptococcus pneumoniae (pneumococcus). The virulence of pneumococci for man and animals was found to depend on production of a polysaccharide capsule which surrounds the organism and protects it from phagocytosis. Non-capsulate mutants of pneumococci occur spontaneously and are easily recognised on agar medium as they lack the glossy, mucoid appearance characteristic of wild-type, capsulate strains. These non-capsulate mutants are easily phagocytosed in vivo and are avirulent. Griffiths (ref. 1) showed in 1928 that when a mixture of live, non-capsulate and dead, capsulate pneumococci was injected into mice, some of the mice died of septicaemia and from the blood of these mice, live capsulate pneumococci were isolated. This was the first demonstration of genetic transformation and was thought by Griffith to be mediated by the polysaccharide itself. However, it was later shown by Avery (ref. 2) that DNA was responsible.

Further evidence for the role of capsules as virulence factors was obtained by comparative observations on capsulate and non-capsulate variants derived from the same strain of various other bacterial species.

Examples of bacterial species where evidence has been obtained for the significant virulence-enhancing role of a capsule are Bacillus arthracis, Yersinia pestis, Klebsiella pneumoniae, Haemophilus influenzae and various species of Streptococci. It must be emphasised that capsule production per se is not sufficient to ensure virulence in a bacterial species but it undoubtedly contributes to the overall pathogenic mechanism.

From the earliest studies it became evident that many harmless, saprophytic bacteria, including those found in soil or aquatic environments were heavily capsulate. The role of exopolysaccharides in nature has not been clearly established and is probably diverse and complex. It has been suggested that

they may protect against dessication, phagocytosis and phage attack (refs. 3, 4), or high oxygen tension (ref. 5), participate in uptake of metal ions (refs. 3, 4), as adhesive agents (ref. 6) or ATP sinks (ref. 7) or be involved in interactions between plants and bacteria (ref. 8) or have a possible role in developmental systems such as those found in Myxobacteria (ref. 9).

Industrial microbiologists have become increasingly aware of the commercial potential of procaryotic exopolysaccharides as gelling and emulsifying agents but at the same time are faced with the troublesome consequences of exopolysaccharide production through the formation of bacterial aggregates which reduce fluid flow in a variety of industrial pipe systems. This phenomenon of bacterial adhesion through the agency of exopolysaccharides poses a particular problem in pipe systems which are not readily accessible such as those found in deep water and/or associated with oil recovery. It is fascinating to have observed in the last decade the role of exopolysaccharides in the almost ecumenical development of the various fields of microbiology. Studies by industrial and agricultural microbiologists on the role of exopolysaccharides in bacterial adhesion as an industrial hazard, or as a mechanism of specific adhesion to specific plant hosts, would appear to have re-awakened medical microbiologists to the significant role of exopolysaccharide-mediated adhesion in explaining the localisation of many pathogenic bacteria to specific anatomical sites and surfaces.

The consequences of the virtual explosion of interest in adhesion as a virulence determinant for specific bacterial pathogens have been observed in several well-known bacterial infections. The adherence and localisation of Streptococcus mutans to the surface of teeth following conversion of dietary sucrose to a glucan "cement matrix", and the consequent dental caries which ensues, is only one of many striking examples in medical microbiology of the importance of bacterial exopolysaccharides in localisation and adherence of bacteria to specific human or animal tissues. Knowledge of the determinants of microbial pathogenicity in human, plant and animal hosts is often thwarted by the limitation of in vitro experiments and the necessity to assess results in the context of their relevance in vivo.

The role of exopolysaccharide adhesion in nature and the limitations of in vitro observations were noted by Costerton et al. (ref. 10). The authors drew attention to the fact that certain bacteria cultured for long periods in laboratory conditions lost certain properties normally possessed in their natural environment. Thus microbes that apparently produce no exopolysaccharide in vitro appear to have lost the ability. In nature, the same microbes adhere to a variety of surfaces ranging from the bovine intestine to rocks in fast moving streams by a tangle of fibres of exopolysaccharide that extends from

the bacterial surface to form a felt-like glycocalyx surrounding an individual cell or colony of cells.

On first consideration a precise distinction between the terms capsule and slime as they are presently used and the term glycocalyx might appear semantic and unnecessary. However, it is arguable that the concept of a distinct bacterial glycocalyx and its role in adhesion *in vivo* has merit. The term glycocalyx may be useful if it defines those cell-associated exopolysaccharides or protein complexes produced *in vivo* which resemble bacterial capsules or slime but which are lost or difficult to observe *in vitro*.

Within the wide diversity of microbial exopolysaccharides both in relation to composition and function, bacterial alginate occupies an unusual and fascinating position.

Alginate is a 1, 4 linked copolymer of β-D-mannuronic acid and its C-5 epimer α-L-guluronic acid (Fig. 1), and is a commercially important polysaccharide with gelling and colloidal properties. It has a variety of uses, e.g. as an ingredient of photographic emulsions and dental impression material and as an additive in various foodstuffs, e.g. ice-cream, jellies and beer (for maintaining a good head).

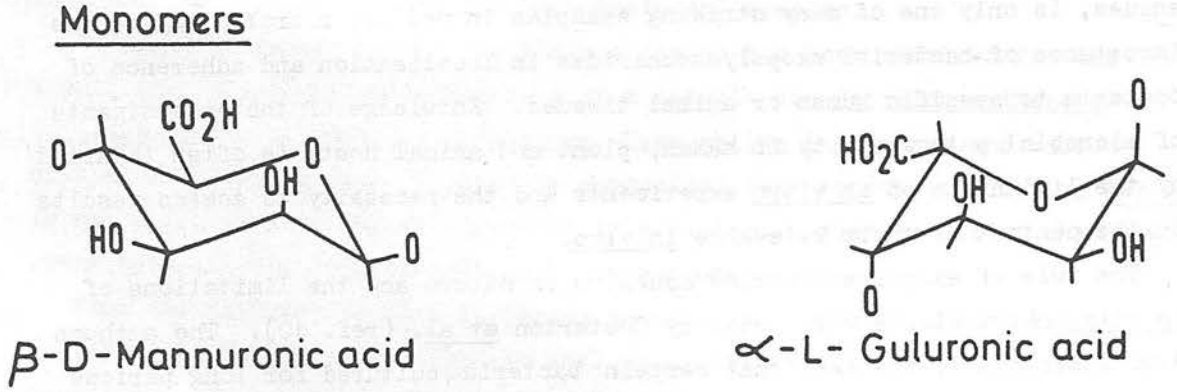

Fig. 1. The structure of alginic acid.

Together with cellulose, hyaluronic acid and sialic acid, alginate is one of the few polymers synthesised by eucaryotic and procaryotic systems. The present source of commercial alginate is eucaryotic namely from marine algae. However, amongst the procaryotes, two bacterial genera are known to contain species capable of alginate production, Azotobacter and Pseudomonas. The present chapter attempts to collate our current scanty knowledge of bacterial alginate in particular its synthesis, regulation and biological significance.

BIOSYNTHESIS AND FUNCTION OF BACTERIAL ALGINATE

The genera Pseudomonas and Azotobacter

Members of the genus Pseudomonas (refs. 11, 12) are common inhabitants of soil, fresh water and marine environments. Some species cause diseases of plants, while others are occasional human and animal pathogens. The cells are gram negative straight or curved rods 0.5-1 μm by 1.5-4 μm and are motile by means of polar flagella. They are strict aerobes, except for those species which can use denitrification as a means of anaerobic respiration. They are among the most metabolically versatile organisms known and are able to utilise a wide variety of organic compounds as sole sources of carbon and energy. The most commonly encountered species belong to the fluorescent group, e.g. P. aeruginosa, P. fluorescens and P. putida. These produce characteristic, water soluble, fluorescent yellow-green pigments. The G + C content of the DNA ranges from 58-70 moles %.

Members of the genus Azotobacter (refs. 11, 12) are also soil and water inhabitants and are able to fix N_2 under aerobic conditions. The cells are gram negative plump rods or cocci, characteristically paired and often containing granules of poly-β-hydroxybutyrate. In young cultures the cells are motile by means of polar or peritrichous flagella. Three species, i.e. A. chroococcum, A. beijerinckii and A. vinelandii produce distinctive resting cells called microcysts which are resistant to dessication. Most strains of this genus characteristically produce large amounts of exopolysaccharide and give smooth glistening colonies on agar medium (Fig. 2). Some strains produce a green fluorescent pigment and the G + C content of the DNA ranges from 63-66 moles %.

The discovery of bacterial alginates

The synthesis of alginate-like polymers by bacteria was first reported in 1964, when Linker and Jones (ref. 13) isolated and partially characterised the exopolysaccharide from a mucoid strain of Pseudomonas aeruginosa (Fig. 3) isolated from the sputum of a patient with cystic fibrosis (CF). They showed using paper chromatography that the substance was a polyuronide with properties

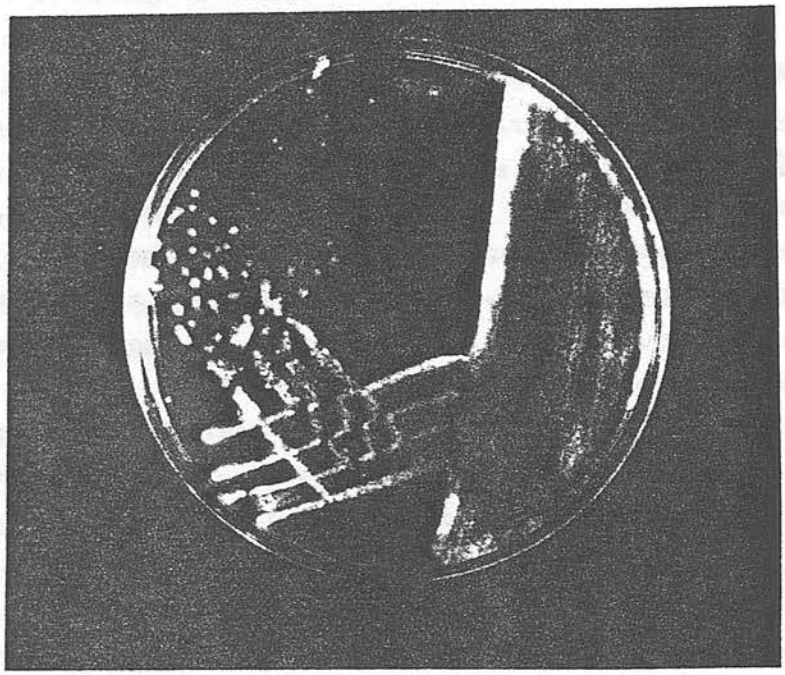

Fig. 2. A strain of A. vinelandii on nitrogen-free medium with glucose.

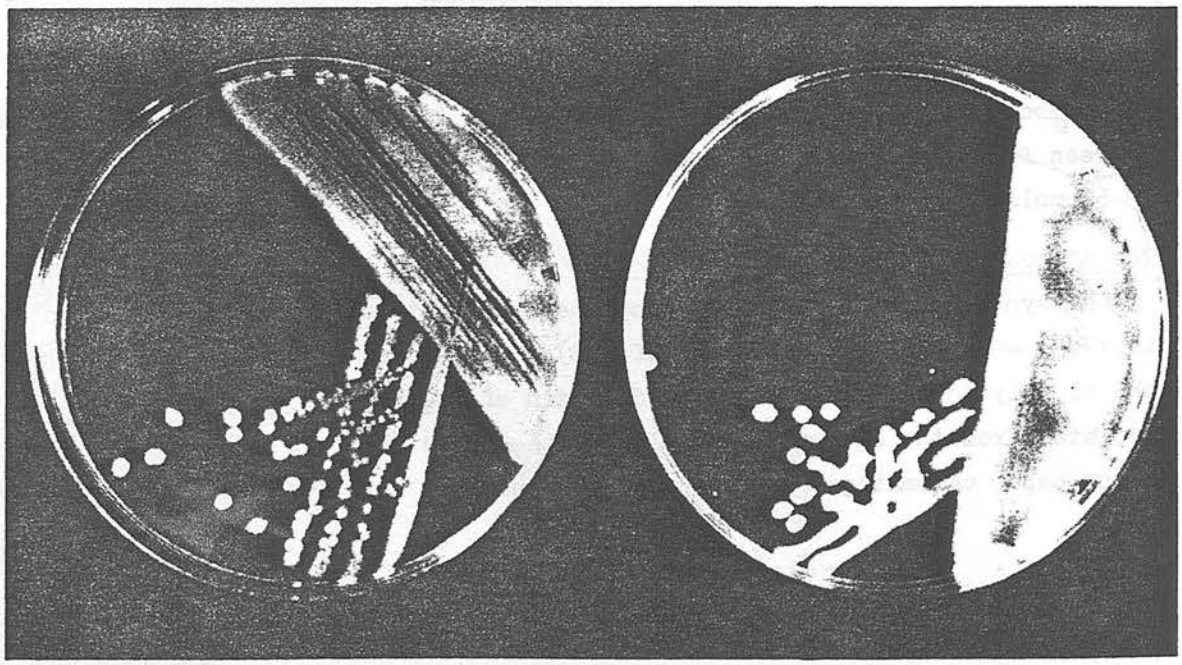

Fig. 3. Clinical isolates of P. aeruginosa grown for 24 h on Difco Pseudomonas Isolation Agar (PIA). On the left, a classical non-mucoid strain and on the right, an alginate-producing mucoid strain.

similar to alginic acid. This finding was confirmed and extended by Carlson and Matthews (ref. 14) in 1966 in a study of 13 mucoid strains of P. aeruginosa from CF and non-CF sources. All were found to produce mannuronic and guluronic acids but the authors were unable to say at this stage whether these were present as homopolymers or as a heteropolymer. Linker and Jones (ref. 15) further analysed the polymer from their strain and demonstrated the presence of acetyl groups as well as confirming the strong resemblance to algal alginate on the basis of composition, structure and physical properties.

In the same year (1966), Gorin and Spencer (ref. 16) demonstrated that the polysaccharide produced by a strain of A. vinelandii was similar in most respects to algal alginate, but was partly acetylated.

Biosynthesis of alginate by A. vinelandii

The production of copious amounts of capsular polysaccharide is characteristic of the majority of strains of A. vinelandii isolated from the environment and the identification of this capsular material as an alginate-like polymer aroused the interest of industrial biochemists interested in developing an alternative source of this useful compound. Such workers sought to elucidate the biosynthetic pathway and the conditions leading to maximal production of alginate (refs. 17-23).

Haug and Larsen (refs. 17, 18) showed that polymannuronic acid is the first polymeric product of alginate biosynthesis produced by vegetative cells of A. vinelandii and that the final polymer contains regions of homo and heteropolymeric blocks, much like the alginate from brown algae.

As part of a project to investigate the commercial feasibility of producing alginate by fermentation, Pindar and Bucke (ref. 19) determined the sequence of reactions involved in alginate biosynthesis from sucrose in A. vinelandii NCIB 9068 by feeding radioactive intermediates to cell-free extracts of the bacteria and by studying the individual enzymes. The pathway previously determined by Lin and Hassid (refs. 24, 25) for the biosynthesis of alginate in the brown algae Fucus gardneri was used as a starting point (Fig. 4).

It should be noted that in this study it was concluded that the epimerisation of mannuronic acid to guluronic acid took place at the monomer level, but in a later study Madgwick et al. (ref. 26) were able to extract a polymannuronic 5-epimerase from brown algae, the presence of which had been hard to prove because the algal enzymes are difficult to extract due to the presence of large amounts of sulphated polysaccharides and phenolic compounds.

The results obtained by Pindar and Bucke for their strain of A. vinelandii suggested the pathway set out in Fig. 5.

No evidence for the formation of free GDP-guluronic acid was found at any stage in the reaction - the intermediate between GDP-mannose and the polymeric

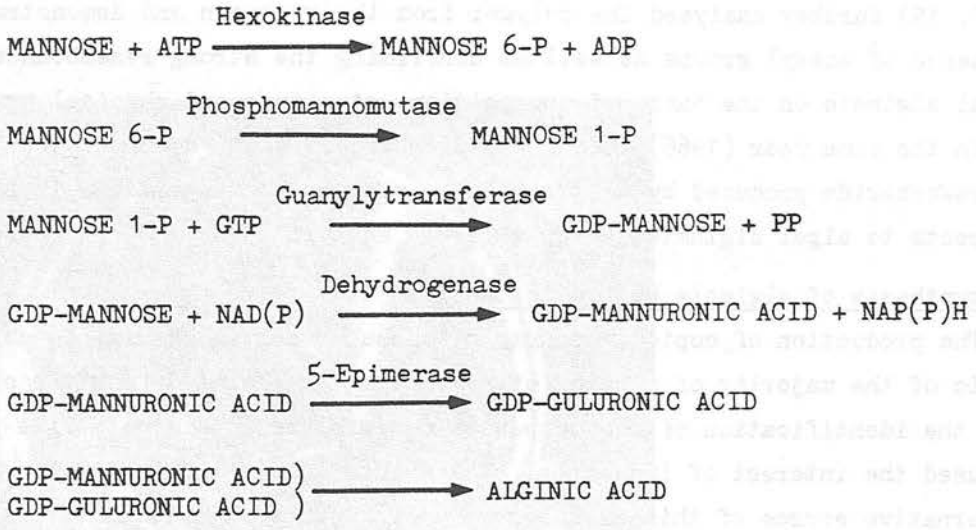

Fig. 4. Biosynthetic pathway for the production of alginic acid from mannose in Fucus gardneri (ref. 25).

material was GDP-mannuronic acid. The composition of the final polymeric product was analysed using partial acid hydrolysis to isolate homopolymeric blocks followed by fractionation at pH 2.85 to separate polymannuronic acid blocks from polyguluronic acid blocks. This procedure indicated that 86% of the polymeric product was in the form of an alternating sequence of mannuronic and guluronic acid units, 10.4% was polymannuronic acid blocks and 3.6% polyguluronic acid blocks, although this composition varied with growth conditions.

The authors did not determine at what stage the acetyl groups were introduced or their association with mannuronic acid or guluronic acid units. The physical location of the polymannuronate epimerase was also to be determined.

Davidson et al. (ref. 27) later showed that the acetyl groups were associated with mannuronic acid residues and suggested that they may play a role in protecting certain of these residues from epimerisation.

An investigation of the physiology of alginate production by A. vinelandii NCIB 9068 (ref. 20) revealed that in batch culture, under phosphate-deficient conditions, alginate synthesis occurred during growth and ceased when the cells entered stationary phase. In a continuous culture system under phosphate limitation the rate of alginate synthesis was independent of specific growth rate. However, by manipulating the conditions of fermentation, e.g. by altering the calcium ion concentration, the molecular weight and viscosity of the product could be varied. Likewise, when growth was limited by a variety of nutrients (ref. 21) including carbon source (sucrose), the rate of alginate synthesis varied only slightly. The only exception was oxygen limitation which was detrimental to alginate synthesis. In addition, when oxygen

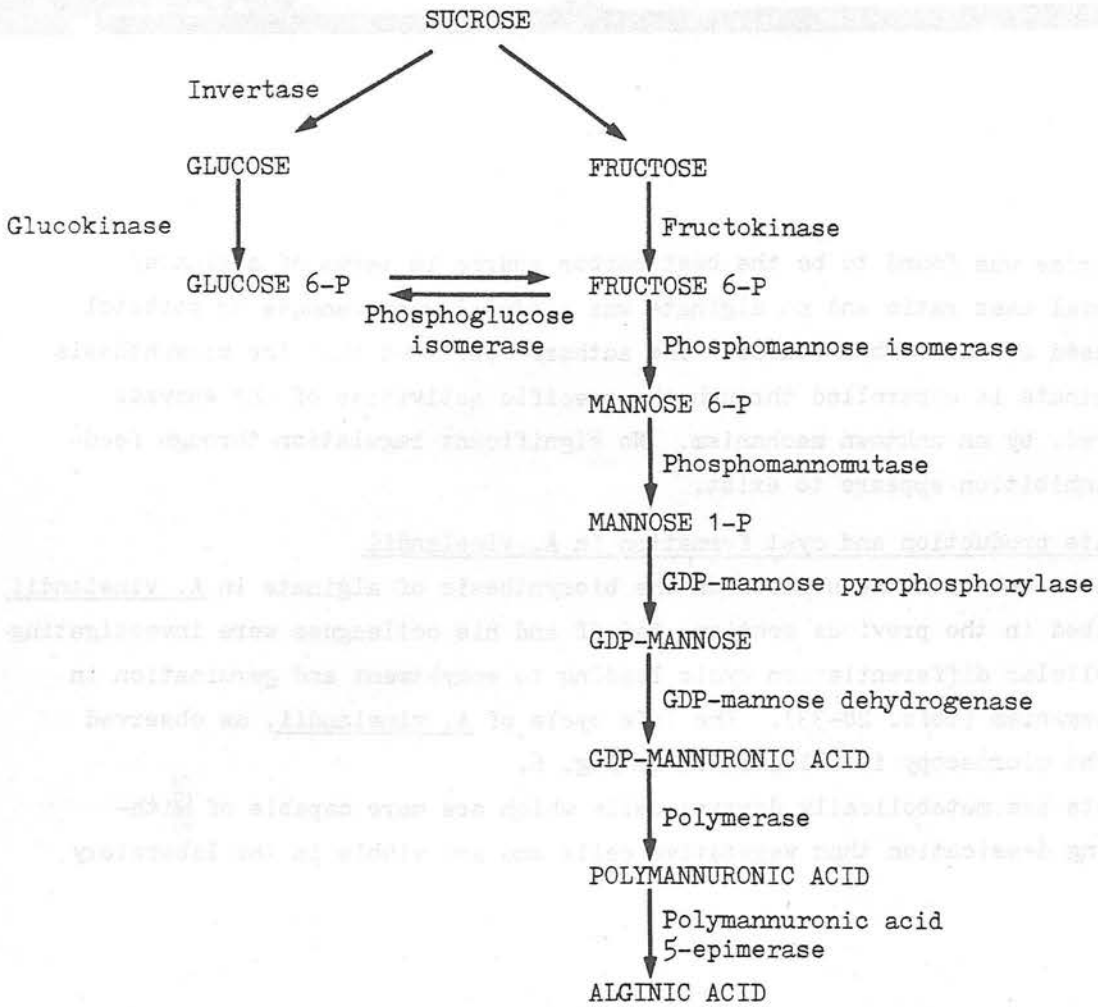

Fig. 5. Pathway of biosynthesis of alginic acid in A. vinelandii (ref. 19).

availability was increased by altering the fermenter agitator speed, the specific respiration rate went up and the conversion efficiency for sucrose into alginate fell from an optimum of 40% to 8% (ref. 22).

In some bacteria, exopolysaccharide synthesis appears to share common precursors and co-factors with cell wall synthesis (ref. 9), hence competition for these intermediates would take place and rate of polysaccharide synthesis would be influenced by growth rate. However, the apparent independence of the rate of alginate synthesis from specific growth rate in A. vinelandii 9068 suggests that this system may not operate in this case (ref. 22).

A later study (ref. 23) using a mutant of strain 9068 with enhanced ability to synthesise alginate, showed that in this strain, alginate production continued after the cessation of growth. Activities of the enzymes phosphomannose isomerase, GDP mannose pyrophosphorylase and GDP mannose dehydrogenase were found to correlate with the amount of alginate produced.

Sucrose was found to be the best carbon source in terms of alginate/bacterial mass ratio and no alginate was produced when mannose or sorbitol were used as the carbon source. The authors concluded that the biosynthesis of alginate is controlled through the specific activities of the enzymes involved, by an unknown mechanism. No significant regulation through feedback inhibition appears to exist.

Alginate production and cyst formation in A. vinelandii

Concurrent with the studies on the biosynthesis of alginate in A. vinelandii described in the previous section, Sadoff and his colleagues were investigating the cellular differentiation cycle leading to encystment and germination in this organism (refs. 28-33). The life cycle of A. vinelandii, as observed by light microscopy is illustrated in Fig. 6.

Cysts are metabolically dormant cells which are more capable of withstanding dessication than vegetative cells and are viable in the laboratory

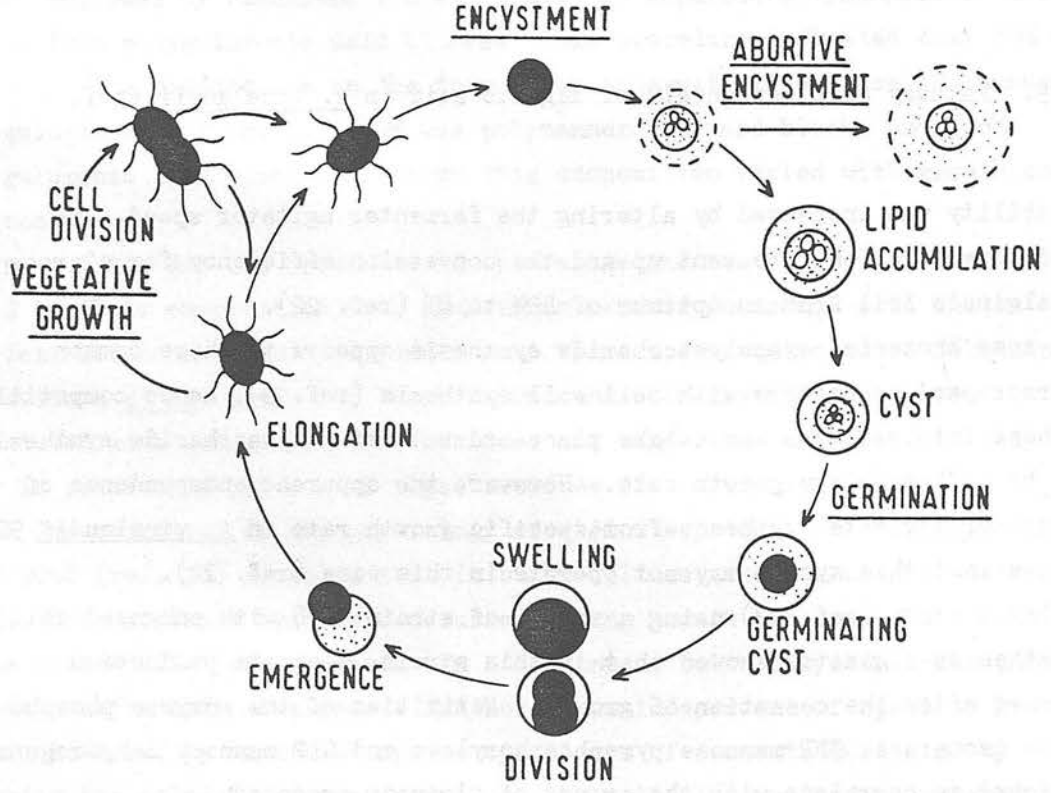

Fig. 6. Schematic diagram of the life cycle of A. vinelandii taken from Sadoff (ref. 33).

for at least 10 years kept in dry soil. They are also more resistant to radiation and sonication than vegetative cells. However, unlike the endospores of gram positive organisms, cysts are not heat resistant.

In 1968, Lin and Sadoff (ref. 28) reported the association of a viscous polymer with the encystment process of A. vinelandii ATCC 12837. Two other groups (refs. 34, 35) later published electronmicrographs of cysts stained with ruthenium red revealing polysaccharide capsular material external to the exine (outer coat) region (Fig. 7). Densely stained material along with unstained regions were seen in the vicinity of the exine, while the intine (inner coat) was also heavily stained.

The relationship of capsule production and cyst formation in A. vinelandii ATCC 12837 was demonstrated by Eklund et al. (ref. 36) using three independent methods.

1. When a phage-induced polysaccharide depolymerase (capable of depolymerising the capsular material) was added to encystment medium, the cells were unable to form typical cysts, exine coats were partially destroyed and intine was greatly reduced.

2. Under conditions unfavourable to polysaccharide production, i.e. when ammonium nitrate was present in the culture medium, encystment did not take place.

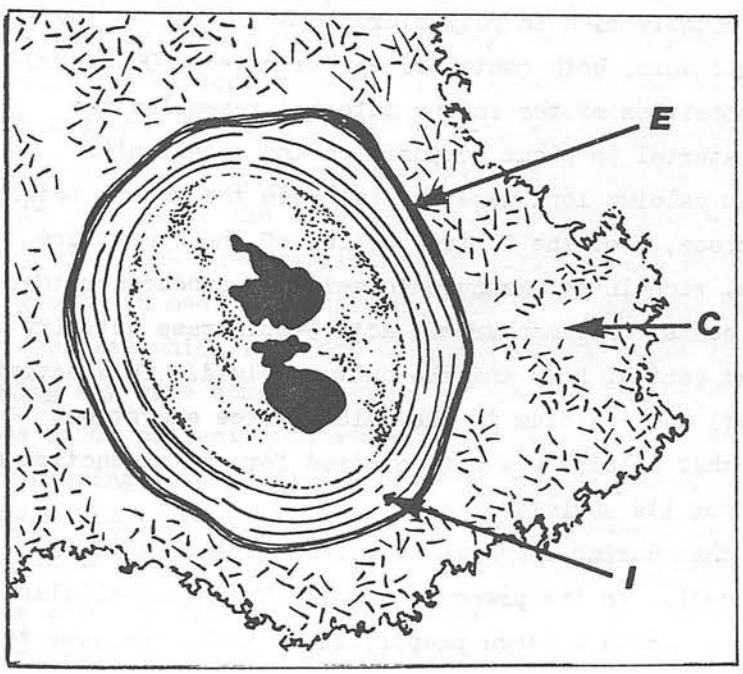

Fig. 7. Diagram of a ruthenium red stained cyst of A. vinelandii based on an electronmicrograph (ref. 34). C, capsular material, E, exine coat, I, intine coat.

3. Non-capsulate mutants of A. vinelandii 12837 were unable to form cysts. Hence, although capsule production per se is not necessarily followed by encystment, it is a mandatory requirement for encystment to occur.

Chemical analysis of cyst fractions revealed that the intine and exine are rich in protein, carbohydrate and lipid and contain high levels of calcium and lesser amounts of magnesium (ref. 30). It had also been noted that the suspension of mature cysts in EDTA led to the disruption of the exine layer (refs. 29, 37).

Page and Sadoff (ref. 32) examined the role of cations in the structure and integrity of mature cysts and the role of calcium in the encystment process. As part of this study, cyst fractions were examined for the presence of uronic acids in the form of alginate and for polymannuronic acid 5-epimerase activity. Mannuronic and guluronic acids in sequences characteristic of alginate were demonstrated in both the intine and exine cyst fractions. Proportions of the two uronic acids were found to differ between the two fractions - the exine was notably rich in polyguluronic acid whereas the intine was richer in polymannuronic acid, both contained approximately 50% hetero-polymeric blocks. The composition of the intine material resembled the vegetative cell capsular material in block composition and mannuronic/guluronic acid ratio. When calcium ions were omitted from the growth medium, abortive encystment took place, i.e. the central bodies of the cysts were normal but a viscous slime, rich in polymannuronic acid was produced rather than the intine and exine coats. Polymannuronic acid 5-epimerase activity was detected in the mature cyst central body and the culture fluid. This activity was stimulated by the addition of calcium to the calcium-free encystment culture fluid, suggesting that calcium was not required for the production of the enzyme per se, rather for its activity.

The authors postulated that during encystment, polymannuronic acid is initially secreted by the cell. In the presence of calcium, extracellular polymannuronic 5-epimerase converts a large proportion of these residues to guluronic acid. Guluronic acid residues have a high calcium binding capacity (ref. 38) and alginate containing a significant amount of guluronic acid forms a gel in the presence of calcium ions due to the formation of inter-chain linkages. Alginate in this gel form has an even higher selectivity for calcium ions. Hence the coalescence of the exine coat would reduce the availability of calcium for epimerisation of the intine coat, so the proportion of guluronic acid in this fraction would be lower.

The role of the exine coat would appear to be the maintenance of cyst rigidity and resistance to dessication. In addition, the properties of radiation and sonication resistance are dependent on an intact exine (ref. 33). The function of the intine layer is less obvious, however, during germination

in young cultures of mucoid P. aeruginosa, and further compounded by the poor pigmentation associated with growth on these common diagnostic media. An alternative explanation is that the rarity of mucoid P. aeruginosa was genuine. Today, mucoid strains are almost solely isolated from the sputum produced in chronic respiratory infections in CF patients and indeed the presence of mucoid P. aeruginosa has been suggested as a diagnostic feature of the disease in older, previously undiagnosed patients (ref. 45). In 1948, when Henriksen (ref. 44) described the isolation of a single mucoid strain, the life expectancy of a CF patient was measured in months, today many patients survive into adulthood. The increased isolation of mucoid P. aeruginosa reported by Elston and Hoffman in 1967 (ref. 46) most probably resulted from the significant improvement in management and antibiotic therapy leading to a longer survival and a greater reservoir for potential isolation of these strains.

Elston and Hoffman (ref. 46) noted that capsulation of mucoid P. aeruginosa was not a constant finding, that when seen by the India ink technique (ref. 47) capsules were irregular, indistinct and shown by only some of the individual cells in a culture. We have observed that the presence or absence of a discrete capsule for some strains is dependent on the level of Ca^{++} ions in the growth medium. As is the case with A. vinelandii (ref. 48), growth of the alginate-producing P. aeruginosa 492a (ref. 49) in low levels of calcium resulted in release of the exopolysaccharide from the cells. On centrifugation, this remained in the supernatant fraction and the cells appeared non-capsulate. However, growth in medium containing 1 mM Ca^{++} resulted in the formation of discrete capsules which remained attached to the cells even after washing. This finding may have some relevance to the clinical situation as the bronchial secretions of CF patients contain elevated levels of calcium.

The pioneering work of Doggett and colleagues (refs. 40, 50, 51) in the 1960's which first revealed the association of mucoid P. aeruginosa and CF was accompanied by studies on the composition and properties of the exopolysaccharide which by the end of the decade had been identified as an acetylated heteropolymer of mannuronic and guluronic acids (refs. 13-15, 52) resembling algal alginate and similar to the polymer found in A. vinelandii (ref. 16).

From the earliest studies on P. aeruginosa, classic non-mucoid strains belonging to Phillips colonial types 1 to 4 (ref. 41) have been known to produce viscid, slimy broth cultures particularly when incubation is prolonged and in medium with a high carbon content. Indeed, slime production is so characteristic of the species that its synthesis in medium containing 4% potassium gluconate has been long used as a diagnostic feature (refs. 41, 53). Unfortunately, in recent years, the terms mucoid material and slime have been used indiscriminately and synonymously and considerable ambiguity can be found

there is a gradual loss of its ability to react with electron dense fixatives and stains (ref. 39) suggesting that the contents of the intine may be used as growth substrates.

Alginate-producing strains of P. aeruginosa

The increasing interest during the last decade in alginate-producing strains of P. aeruginosa has arisen for reasons which are in striking contrast to alginate synthesis in A. vinelandii. As already indicated, alginate production is characteristic of the majority of strains of A. vinelandii. In contrast, alginate production in P. aeruginosa is extremely uncommon in strains isolated from a wide variety of human, animal and environmental sources (ref. 40). The important exception to this rarity is the frequent emergence of alginate-producing P. aeruginosa in the lungs of CF patients. The significance of this now classic association in CF is that the increasing predominance of alginate-producing pseudomonas in the lungs is paralleled with clinical deterioration, increasing refractoriness to treatment with antibiotics and a poor prognosis. Unfortunately, concurrent with the increased importance attributed to alginate-producing P. aeruginosa in the last decade, there has arisen considerable confusion in the literature regarding the nature of pseudomonas exopolysaccharide, in particular the distinction between alginate and the more general term slime which historically has been used to describe the viscid liquid phase associated with broth cultures of P. aeruginosa grown under particular conditions. For the purpose of clarity, an attempt will be made to rationalise the use of the terms mucoid, slime and alginate in the light of present knowledge.

The initial recognition of a bacterial characteristic is often made at the colonial level. In 1969, Phillips (ref. 41), described six colonial types of P. aeruginosa, viz., typical, coliform-like, rough, rugose, mucoid and dwarf. The mucoid form (Fig. 3) which produced a large gelatinous colony within 24 h at 37°C on blood agar and which was shown to be associated with alginate production (ref. 13) was observed in only two of the 128 isolates of P. aeruginosa. The single representative of the dwarf type arose as a variant from one of the mucoid isolates.

Mucoid strains of P. aeruginosa have been known to microbiologists since their first reported isolation in 1927 by Sonnenschein (ref. 42). Until the 1960's however, only a few further isolations were reported (refs. 43, 44). The relative rarity of mucoid P. aeruginosa prior to the 1960's could be explained by their colonial resemblance to Klebsiella aerogenes and their misdiagnosis as this species. In the case of blood agar and MacConkey agar cultures this misdiagnosis is undoubtedly still being made, partly because of the poor production of the characteristic pigments pyocyanin and fluorescein

in the literature (ref. 54). This confusion is further compounded by a lack of uniformity in the results of analyses carried out on slime extracted from non-mucoid P. aeruginosa (Table 1).

TABLE 1

Analysis of extracellular slime from non-mucoid strains of P. aeruginosa

Strain	Components	Reference
OSU 64	mannose, DNA, RNA, protein	55
B1	mannose, glucose, rhamnose, galactose, glucosamine, galactosamine, glucuronic acid, DNA, RNA	56, 57
OSU 64 NCTC 6750, 7244, 8203, 1999	mannose, glucose, rhamnose, glucosamine, glucuronic acid, hyaluronic acid, DNA, RNA, protein	58
36 clinical isolates	mannose, glucose, fucose, galactose, ribose, rhamnose, glucosamine, galactosamine	59

A review of the literature indicates that whilst pseudomonas slime undoubtedly exists and plays a part as a virulence determinant (refs. 57, 60), the exact composition of this loosely-defined material is extremely variable and dependent upon the strain, the cultural conditions and the method of analysis. For the purpose of this chapter the term slime is restricted to the viscid material consisting of DNA, protein and various polysaccharides which is produced characteristically by all strains of P. aeruginosa. The term mucoid is restricted to those strains producing the characteristic colonial type 5 of Phillips within 24 h on agar media and producing an acetylated alginate-like heteropolymer of mannuronic and guluronic acids.

The isolation of mucoid P. aeruginosa in vivo and in vitro

The association of mucoid strains of P. aeruginosa with chronic pulmonary infection in CF patients is well recognised (refs. 40, 50, 61-63). Evidence for their emergence in vivo was first provided by Doggett et al. (ref. 50) who observed that in individual CF patients, initial colonisation is by non-mucoid P. aeruginosa but, during the course of infection, mucoid isolates gradually emerge and eventually come to predominate. In addition, when the two forms are found in the same specimen, they belong to the same serotype (ref. 63) and pyocin type (ref. 64).

In contrast to their emergence in vivo, when cultured in vitro, mucoid strains of P. aeruginosa tend to revert to the non-mucoid form. Zierdt

and Schmidt (ref. 65) reported that serial transfer on solid medium gave rise to the rapid loss of the mucoid character.

The first report of *in vitro* isolation of mucoid variants was that of Martin (ref. 66) who, during the course of phage typing, noted rings of slimy, mucoid growth around areas of phage lysis, while the background lawn remained non-mucoid. Subculture from these slimy areas resulted in pure cultures of mucoid colonies which looked and behaved like clinical isolates.

These mucoid variants had the same phage type and serotype as the non-mucoid parent and thus were sensitive to the phage responsible for their emergence. From this, Martin concluded that the mucoid variants had not been selected from the non-mucoid population, but rather the presence of phage in the lytic cycle was necessary for the continued expression of the mucoid character. The term "pseudolysogeny" was used to describe this phenomenon. Martin postulated that phage could also be responsible for the emergence of mucoid strains *in vivo*, the source of which would be other strains of *P. aeruginosa*, but no evidence for such mixed infections was presented.

An investigation of the influence of various substances on the stability of mucoid *P. aeruginosa* *in vitro* (ref. 67) revealed that clinical and phage-derived strains maintain their mucoid colonial form when serially subcultured on desoxycholate citrate agar, or in sodium deoxycholate broth. However, the genetic basis for the emergence of the mucoid characteristic remained obscure.

In 1978 we published an alternative method for isolating mucoid variants *in vitro* without the use of phage (ref. 68). This method was based on the observation that mucoid strains are slightly more resistant to some antibiotics than the related non-mucoid forms (ref. 69). The selective agent of choice is carbenicillin and the isolation procedure involves the following steps: (Fig. 8).

1. Approximately 10^7 cells from an 18 h nutrient broth culture of *P. aeruginosa* are spread on to the surface of nutrient agar plates containing carbenicillin at a concentration of 1.5 times the MIC of the strain.

2. After 18 h incubation at 37°C, the resistant colonies are replica plated from the antibiotic containing medium to PIA plates containing no antibiotic.

3. After 18 h incubation at 37°C, the mucoid variants can be recognised as distinctive, watery colonies against a background of non-mucoid growth (Fig. 9).

The frequency of isolation of mucoid variants from *P. aeruginosa* strain PAO using this procedure is approximately 1 in 10^7 cells and this can be increased 40-fold following mutagenesis with ethyl methanesulphonate (EMS). These mucoid variants do not require the continued presence of the antibiotic for mucoid colonial growth and infra-red spectroscopy of the exopolysaccharide has indicated an acetylated polymer of mannuronic and guluronic acids.

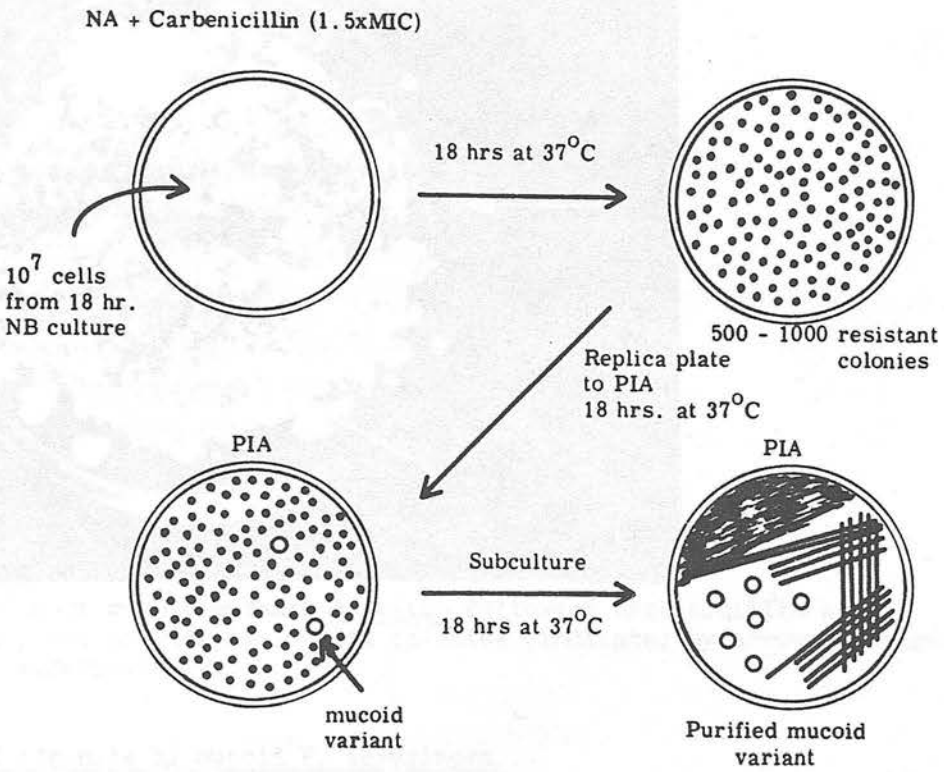

Fig. 8. Technique for the isolation of mucoid variants of P. aeruginosa in vitro by selection for resistance to carbenicillin (ref. 68).

Carbenicillin is not the only antibiotic which can be used to select such mucoid variants - other penicillins and aminoglycosides were also used successfully at a concentration of 1.5 times the MIC for the non-mucoid strain.

The ability to isolate mucoid derivatives of well characterised strains and maintain them in vitro has led to progress in the physiological and genetic studies of alginate synthesis in mucoid P. aeruginosa. In addition, a modified version of the selection procedure has been used to isolate alginate-producing variants of P. putida, P. fluorescens and P. mendocina, in which species alginate biosynthesis had not previously been reported (ref. 70).

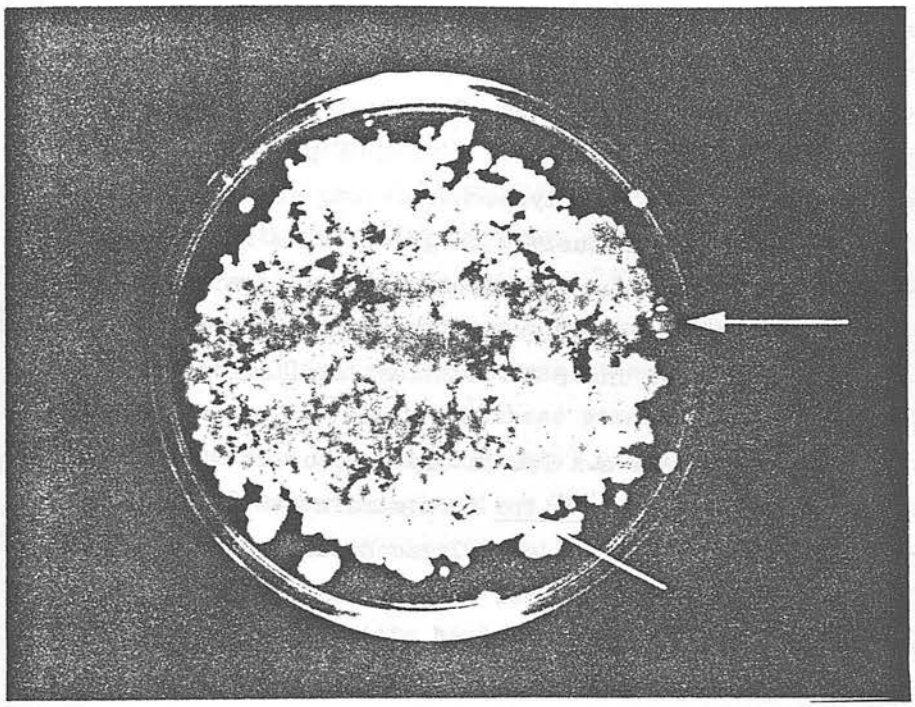

Fig. 9. Isolation of mucoid mutants in vitro following carbenicillin semi-selection. PIA plate showing two mucoid colonies (indicated by arrows) amongst a background of non-mucoid growth.

Biosynthesis of alginate by mucoid P. aeruginosa

Early studies (refs. 13, 15) which identified alginate from mucoid P. aeruginosa employed clinical isolates cultured on Oxoid sensitivity test agar for 24 h at 37°C. The first investigation in which the effect of growth conditions on pseudomonas alginate biosynthesis was examined was that of Evans and Linker (ref. 52). These authors grew three mucoid clinical isolates of P. aeruginosa on various agar media and incubated at 12°C, 25°C or 37°C. Determination of maximal exopolysaccharide production was subjective, i.e. by visual estimation of the ratio of transparent material to the amount of opaque cellular material of a colony and the amount of bacterial exopolysaccharide observed by the India ink technique (ref. 47).

For each strain, a lower incubation temperature resulted in more exopolysaccharide per cell, regardless of growth medium. Exopolysaccharide production was enhanced by growth on MacConkey plates containing sodium chloride or glycerol. Large batches of exopolysaccharide for analysis were obtained by seeding the organisms on to MacConkey agar supplemented with 3% glycerol and incubating at 25°C for four days.

Analysis of the material obtained in this way revealed that different strains varied widely according to the mannuronic/guluronic acid ratio of the exopolysaccharide. However, organisms isolated from the same patient at different

times appeared to produce similar polymers. All the polymers were acetylated and the acetyl content was proportional to the mannuronic acid content. Molecular weights of all the pseudomonas alginates were higher than those of the algal alginates tested.

A later investigation of the biosynthesis of alginate in batch culture (ref. 71) by a mucoid P. aeruginosa, isolated from a CF patient, showed that in a glucose-yeast extract medium, exopolysaccharide was produced throughout the growth phase and ceased simultaneously with growth as a result of glucose exhaustion. In continuous culture, under nitrogen limited conditions, both cell and exopolysaccharide concentrations were largely independent of dilution rate. Polysaccharide was produced under all nutrient limitations tested including carbon limitation.

An early observation made in our own laboratory in studies of mucoid, alginate-producing mutants isolated in vitro from strain PA0381 (ref. 72) was that certain mutants appeared to produce exopolysaccharide on both minimal and complex media, e.g. PA0579, whilst other mutants, e.g. PA0568 failed to produce exopolysaccharide on minimal medium (ref. 73). On this basis, mucoid strains can be classified into groups 1 and 2 where PA0579 represents group 1 and PA0568, group 2. Fig. 10 shows the colonial appearance of PA0568 and PA0579 after 24 h incubation at 37°C on PIA and minimal agar.

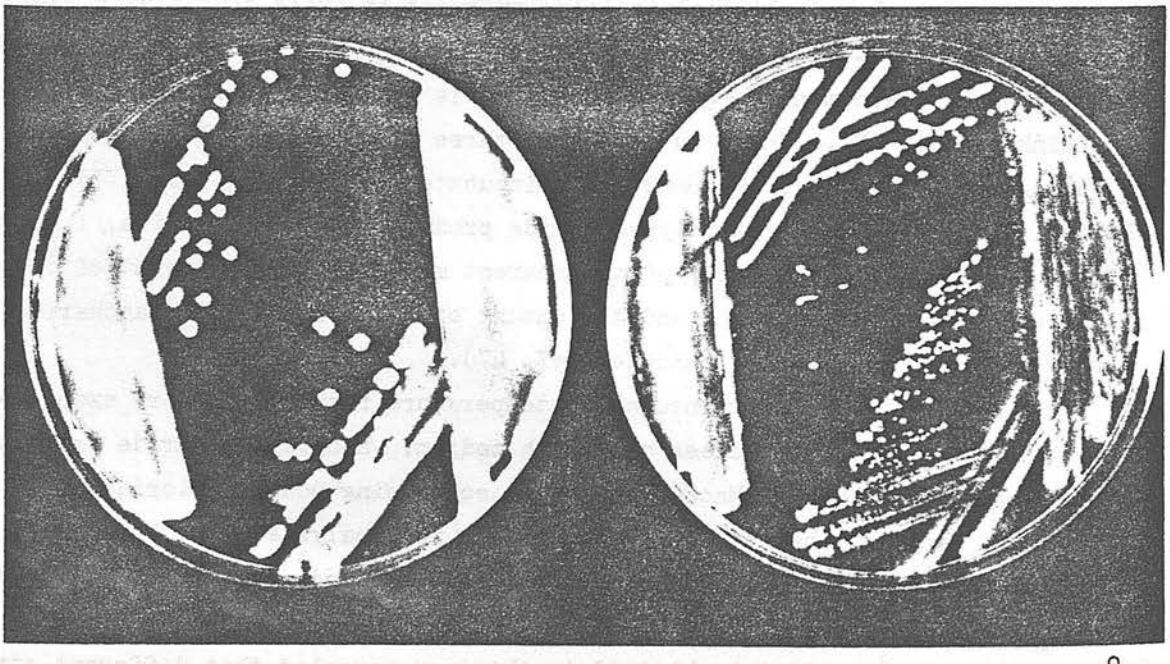

Fig. 10. Two mucoid mutants PA0568 and PA0579 after 24 h incubation at 37°C on PIA (left plate) and minimal agar (right plate). The group 1 mutant PA0579 on the left of each plate produces alginate on both media, whereas the group 2 mutant PA0568 on the right of each plate appears non-mucoid on the minimal agar.

We have also observed that mucoid P. aeruginosa isolated from the sputa of patients with CF can also be classified into these two groups, and in many patients, both forms are isolated simultaneously from the same specimen (ref. 74).

Piggott (ref. 75) studied alginate biosynthesis in a number of mucoid mutants derived from PA0381, including PA0568 and PA0579. In batch culture, in yeast-extract medium with gluconate, polymer production in the majority of strains was not growth associated. In addition, the conditions for optimal alginate production varied markedly between strains. Some strains produced alginate equally well at 30°C and 37°C, whereas others produced best at 37°C. When the composition of the polymers from these strains (collected after 48 h growth in yeast extract medium with 2% gluconate) was analysed, all samples had a high mannuronic acid content ranging from 75% to 95%. The degree of acetylation and viscosity also varied significantly and a linear relationship was observed between % acetylation and viscosity over the range 2.3-8.6% acetate content. In every aspect of alginate biosynthesis studied, the mutants varied considerably although all were derived from the same parent.

Piggott et al. reported enzyme analyses on PA0381 and four of the mucoid mutants (ref. 76). The results obtained for three enzymes, phosphomannose isomerase, GDP-mannose pyrophosphorylase and GDP-mannose dehydrogenase involved in the alginate biosynthetic pathway are shown in Table 2.

TABLE 2

Enzyme levels in P. aeruginosa PA0381 and four mucoid derivatives. Results are expressed in μmoles product formed mg protein^{-1} min^{-1} (ref. 76).

	PA0381	PA0579	PA0578	PA0568	PA0585
Phosphomannose isomerase	22	380	360	280	41
GDP-mannose pyrophosphorylase	6	21	23	9	33
GDP-mannose dehydrogenase	1	17	6	2	6

The low levels of activity of the GDP-mannose metabolising enzymes in PA0568 were increased by the addition of 0.5 μM fluoride indicating the probable presence of a nucleotide hydrolysing enzyme. Attempts to isolate an epimerase from these strains were unsuccessful.

Similar enzyme analyses of four non-mucoid revertants of PA0579 revealed no detectable GDP-mannose pyrophosphorylase or GDP-mannose dehydrogenase while phosphomannose isomerase levels remained elevated.

GENETICS OF BACTERIAL ALGINATE BIOSYNTHESIS

Genetics of P. aeruginosa and A. vinelandii

Genetic analysis of any organism is dependent on (a) the ability to obtain and recognise stable genetic variation both natural and induced, and (b) a system of gene transfer and recombination.

For many years, bacterial genetics was synonymous with Escherichia coli genetics following the pioneering work of Lederberg and Tatum (refs. 77-79). Interest in the genetics of organisms other than E. coli is more recent. The genus Pseudomonas attracted the attention of geneticists because of its biochemical diversity and significance as an opportunist pathogen. Gene transfer by means of conjugation was first described in P. aeruginosa in 1956 (ref. 80), transducing phages were also isolated and characterised (ref. 81) and genetic mapping studies ensued. Most of these studies have used two strains PAO and PAT originally described by Holloway (ref. 82). Strain PAO was isolated from a patient in Australia in 1954. Strain PAT was originally isolated in South Africa in 1950 and carried the sex factor now known as FP2. Several extensive reviews reporting the progress in genetic analysis of P. aeruginosa have been published (refs. 83-87).

For over 20 years, mapping of the PAO chromosome was hampered by the inability to demonstrate genetic circularity. Unlike F, the original E. coli sex factor, which can mobilise the chromosome from many different sites, FP2 apparently had a single origin of transfer, so that accurate mapping of markers by plate and interrupted mating techniques was only possible for the 0' to 40' region (ref. 87).

In strain PAT, however, several R plasmids were found that exhibited chromosome mobilising ability (Cma) (ref. 88). Mapping data obtained using the plasmids FP2, R91-5 (Inc. P-10) and R68 (Inc. P-1) together with transductional analysis, allowed a genetically circular linkage map to be constructed for this strain (refs. 89, 90). The isolation of plasmid R68.45, a variant of R68 with efficient Cma for strain PAO (refs. 91, 92, for review see ref. 93) proved a major step forward in PAO genetics, as it was shown to mobilise the chromosome from multiple origins. Subsequently, a genetically circular map of the PAO chromosome was constructed (ref. 94, Fig. 11). Indeed, because of its broad host range, R68.45 has been a useful tool in the genetic analysis of various other bacterial species including Rhizobium leguminosarum (ref. 95), R. meliloti (ref. 96), R. trifolii (ref. 97) and Rhodopseudomonas spharoides (ref. 98).

Genetic studies of A. vinelandii have had an even more chequered development. Several workers have reported the isolation of auxotrophs, (refs. 99-101), antibiotic resistant mutants (ref. 100) and mutants unable to fix nitrogen (refs. 101-103), but certain other kinds of mutants have proved extremely

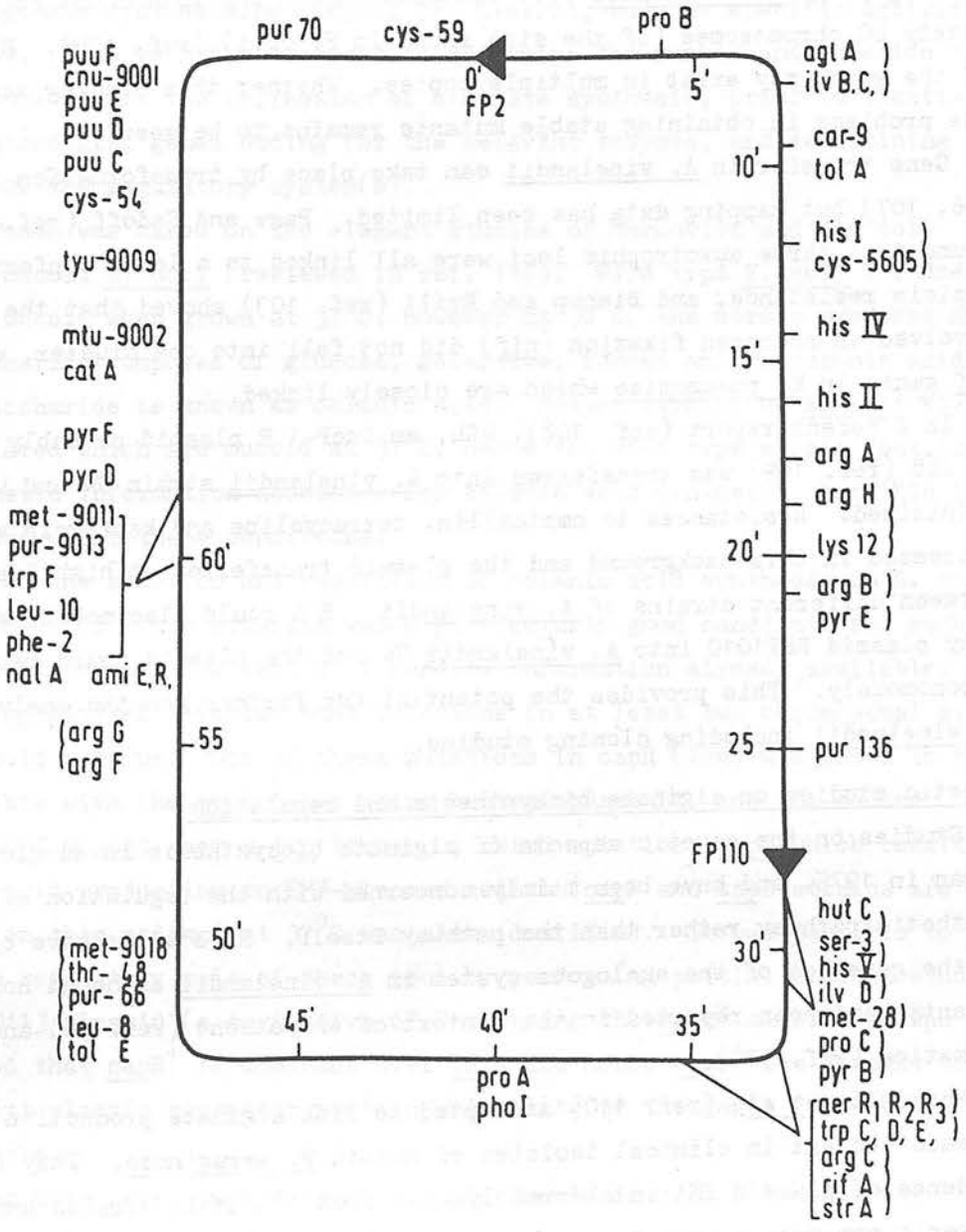

Fig. 11. Chromosome map of P. aeruginosa PAO (ref. 94). Markers whose location is indicated by a bar joined to the map were located by interrupted matings using FP2 donors. Round brackets indicate that markers are cotransducible with one or more of the phages F116, F116L, G101 and E79. Marker abbreviations are as follows:- Anabolic markers: arg, arginine; car, carbamoylphosphate synthase; cys, cysteine; his, histidine; ilv, isoleucine valine; leu, leucine; lys, lysine; met, methionine; phe, phenylalanine; pro, proline; pur, purine; pyr, pyrimidine; ser, serine; thr, threonine; trp, tryptophan. Catabolic markers: ami, amidase; cat, catechol; cnu, carnosine; hut, histidine; mtu, mannitol; puu, purine; tyu, tyrosine. Resistance markers: agl, aminoglycoside; nal, naladixic acid; rif, rifampicin; str, streptomycin. Other markers: aer, aeruginocin production; pho, alkaline phosphatase; tol, aeruginocin tolerant.

difficult to isolate, e.g. those blocked in β-hydroxybutyrate metabolism (ref. 104). The reasons for this are not completely understood, but genetic instability of the mutant DNA is thought to be responsible rather than a problem with mutagenesis per se (ref. 105). It has been estimated that a mid log phase A. vinelandii cell contains enough DNA to account for approximately 40 chromosomes (of the size found in E. coli) (ref. 104). Hence, many of the genes may exist in multiple copies. Whether this finding accounts for the problems in obtaining stable mutants remains to be seen.

Gene transfer in A. vinelandii can take place by transformation (refs. 101, 106, 107) but mapping data has been limited. Page and Sadoff (ref. 101) found that three auxotrophic loci were all linked to a locus conferring rifampicin resistance, and Bishop and Brill (ref. 103) showed that the genes involved in nitrogen fixation (nif) did not fall into one cluster, unlike the nif genes in K. pneumoniae which are closely linked.

In a recent report (ref. 108), RP4, an IncP-1 R plasmid probably identical to R68 (ref. 109) was transferred into A. vinelandii strain UW and was stably maintained. Resistances to ampicillin, tetracycline and kanamycin were all expressed in this background and the plasmid transferred at high frequency between different strains of A. vinelandii. RP4 could also mobilise the multicopy plasmid RSF1010 into A. vinelandii UW and the plasmid could replicate autonomously. This provides the potential for further genetic analysis of A. vinelandii including cloning studies.

Genetic studies on alginate biosynthesis and regulation

Studies on the genetic aspects of alginate biosynthesis in mucoid P. aeruginosa began in 1975, and have been mainly concerned with the regulation of the biosynthetic pathway rather than the pathway itself. No studies have been reported on the genetics of the analogous system in A. vinelandii although non-capsulated mutants have been reported in the context of encystment (ref. 36) and transformation (ref. 101).

Markowitz et al. (ref. 110) attempted to link alginate production with plasmid content in clinical isolates of mucoid P. aeruginosa. They sought evidence of plasmid DNA in cleared lysates from 18 mucoid strains and their isogenic non-mucoid revertants, but were unsuccessful. In addition, the spontaneous loss of the mucoid character was not significantly increased by plasmid curing regimens, e.g. treatment with ethidium bromide, further indicating the lack of plasmid involvement.

Work in our laboratory (refs. 67, 68, 73, 74, 111-114), aimed to test the hypothesis that all wild type P. aeruginosa have the necessary genetic information for alginate synthesis and that this is normally repressed. Hence, mucoid P. aeruginosa would arise following a spontaneous mutation in one of an unknown number of regulator genes. An alternative hypothesis is that wild type

P. aeruginosa have a defective enzyme in the alginate biosynthetic pathway and mucoid strains have a normal one. However, enzyme analysis of a non-mucoid P. aeruginosa (PAO381) described in a previous section (Table 2), suggested that the alginate synthesising enzymes are present, but the specific activities are very low. Thus we have attempted to determine the number and location of genes responsible for the repression of alginate synthesis, prior to identifying the structural genes coding for the relevant enzymes, and determining the nature of the regulatory system(s).

Our approach was based on the elegant studies of Markovitz and his colleagues on mucoid E. coli (reviewed in ref. 115). Wild type E. coli K12 does not appear mucoid when grown at 37°C, however at 30°C, the strain produces an exopolysaccharide composed of glucose, galactose, fucose and glucuronic acid. This polysaccharide is known as colanic acid. Mutant strains of E. coli K12 can be isolated which are mucoid at 37°C, hence the wild type strain contains all the genetic information necessary for colanic acid synthesis, but this is not expressed under these conditions.

Studies on the genetics and regulation of colanic acid synthesis in E. coli K12 began in 1962. This organism was a particularly good candidate for such an analysis because of the wealth of genetic information already available. Early mapping studies revealed that mutations in at least two chromosomal sites yielded mucoid strains. One of these mutations in capR (lon) was shown to be cotransducible with the proC locus using bacteriophage P1, and the second mutation, capS mapped near a trp locus. A third mutation, capT which resulted in colanic acid production on EMB-glucose medium (capR and capS strains are non-mucoid on this medium) at 37°C was also identified. It was possible to confirm the regulatory role of these loci by performing partial diploid studies. Using the F'13 plasmid (a derivative of F carrying the $capR^+$ allele) it was demonstrated that $capR^+$ is dominant over capR and hence $capR^+$ was thought to specify a cytoplasmic repressor protein which acts on the genes for colanic acid synthesis.

A working model for the system, controlling colanic acid synthesis was proposed, i.e. there are several targets on the E. coli chromosome, capR, capS, and capT whose function is to directly or indirectly switch on or off the enzymes involved in colanic acid synthesis. The aim was then to determine whether the control is at the level of transcription or translation and to determine the nature of the regulatory products.

As well as switching on colanic acid synthesis, the capR mutation has a number of pleiotropic effects. CapR strains are UV sensitive, resistant to low levels of tetracycline, chloramphenicol and puromycin and do not allow phages λ and P1 to replicate in the plasmid mode. These properties are not the direct result of colanic acid synthesis, but reflect the diverse role of the

capR gene product. CapS and capT mutants do not share these properties. Recently, (ref. 116) the capR$^+$ gene has been cloned and its product identified as a 94K protein, thought to be under autoregulatory control.

Obviously, our brief outline of the genetic studies on mucoid E. coli has been a greatly simplified version and is not meant to give a detailed account of the regulation of colanic acid synthesis. However, the rationale for these studies in E. coli provided the basis for our approach to the genetics of alginate synthesis in P. aeruginosa, although, at the time of our initial studies, the background genetic information available for P. aeruginosa was far less comprehensive than for E. coli.

The development of the semi-selection technique for isolation of alginate-producing (muc) mutants of P. aeruginosa, already described (Fig. 8), allowed us to put muc mutations into various genetic backgrounds. Table 3 describes the strains relevant to our genetic studies.

TABLE 3

PAO strains used in genetic studies on alginate production

Strain	*Genotype	Origin and/or Reference
PAO381	leu-38, str-2, FP2	ref. 72
PAO568	leu-38, str-2, muc-2, FP2	Group 2 mucoid mutant of PAO381 (ref. 73)
PAO578	leu-38, str-2, muc-22, FP2	Group 2 mucoid mutant of PAO381 (ref. 73)
PAO579	leu-38, str-2, muc-23, FP2	Group 1 mucoid mutant of PAO381 (ref. 73)
PAO585	leu-38, str-2, muc-37, FP2	Group 1 mucoid mutant of PAO381 (our laboratory)
PAO954	met-9011, ami E200, oru-292	Obtained from D. Haas
PAO964	ami-151, hut-C107, pru-354	Obtained from D. Haas
PAO1042	pur-67, thr-9001, cys-59, pro-65	ref. 94
PAO2021	cys-5605, his-5075, argA171, pro-67, nal-25, muc-36, FP$^-$	Group 2 mucoid mutant of PAO2022 (ref. 73)
PAO2022	cys-5605, his-5075, argA171, pro-67, nal-25, FP$^-$	ref. 73

*The genotype symbols used are the same as for Fig. 11. The following pairs of markers are closely linked:- ilv-202 and ilvD, leu-38 and leu-8, str-2 and strA, thr-9001 and thr-48, pro-67, pro-65 and proB, nal-25 and nalA. Markers not appearing on the chromosome map are pur, proline utilisation, oru, ornithine utilisation, muc, alginate synthesis.

PAO381 was chosen as the initial genetic background for the mapping of muc loci, as it is a single auxotroph (leu-38), carrying the sex factor FP2 and thus could act as donor in mating experiments.

It should be emphasised at this point that unlike auxotrophic or catabolic markers, muc cannot be selected for in matings or transductions. The small increase in carbenicillin resistance associated with muc is not sufficient to provide a specific selection of recombinants from the large number of recipient bacteria. Hence, the mapping of muc loci is dependent on the demonstration of linkage to known selectable markers.

We postulated that the muc mutation in PA0579 (muc-23) would be located somewhere on the PAO chromosome, so the strain was used as a donor in plate matings (ref. 117) with various multiply marked recipient strains (non-mucoid, muc^+), selecting for a range of auxotrophic markers in different regions of the chromosome (non-circular at this stage). A significant early result from these matings was that the recombination frequencies for all markers were 10 to 50 fold lower than those obtained using the isogenic non-mucoid donor. However, when a mucoid strain, e.g. PA02021, was used as recipient in a plate mating with PA0381, the numbers of recombinants were not similarly reduced. Later studies involving mucoid donors in plate matings mediated by R68.45 suggested that donorability is not impaired to the same extent using this sex factor. Markowitz et al. (ref. 110) have reported that, in broth matings, alginate synthesis by either the donor or recipient had little or no effect on the transfer of certain R plasmids. The mechanism involved in the inhibition of FP2-mediated chromosome transfer by alginate synthesis has yet to be determined.

When PA0579 was crossed with the muc^+ recipient PA02022, approximately 30% of the recombinants obtained on selection for $cys\text{-}5605^+$ and $his\text{-}5075^+$ were mucoid. These mucoid recombinants were easily distinguished from the non-mucoid variety on the actual mating plates (minimal agar supplemented with the appropriate amino acids), thus showing the same, group 1, characteristics as PA0579. When a similar cross was performed using the group 2 mucoid donor, PA0578, similar linkage of muc-22 to cys-5605 and his-5075 was observed. However, the recombinants had to be transferred to PIA for scoring of muc. This confirmed that the difference between group 1 and group 2 mucoid strains is a result of the mutation leading to alginate production rather than a secondary change modifying the phenotype.

The cross between PA0381 and the mucoid recipient PA02021 (muc-36) likewise indicated linkage of the muc^+ allele to cys-5605 and his-5075. Table 4 shows the results of recombinant analysis.

These results indicated a chromosomal location for muc-36 at a point distal to cys-5605 and his-5075 with respect to the origin of transfer. This was confirmed by interrupted matings (ref. 118) in which cys^+ recombinants obtained at each interruption time were scored for co-inheritance of muc^+, as well as the proximal marker pro^+ and the distal marker arg^+ (Figs. 12a and b).

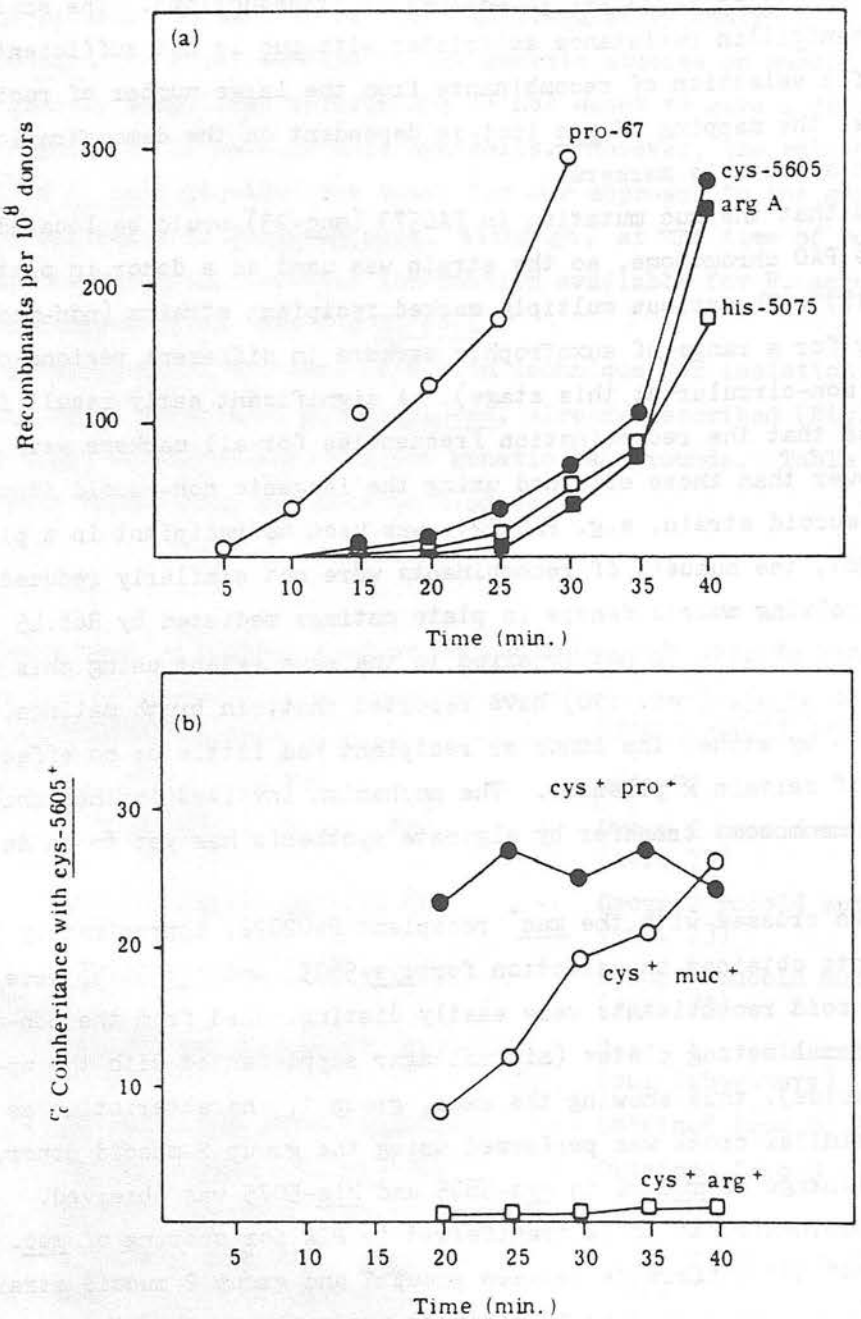

Fig. 12. Interrupted mating between PA0381 and the mucoid recipient PA02021 (muc-36) (a) Time of entry kinetics of pro-67 (○), cys-5605 (●), his-5075 (□), and argA171 (■) (b) Percentage coinheritance with cys-5605$^+$ of the unselected markers muc-36$^+$ (○), pro-67$^+$ (●) and argA171$^+$ (□) (ref. 73).

TABLE 4

Recombinant analysis following a plate mating between PA0381 and the mucoid recipient PA02021 (ref. 73)

Selected marker	No. of recombinants per 10^8 donors	Percentage co-inheritance of unselected marker				
		muc-36$^+$	pro-67$^+$	his-5075$^+$	cys-5605$^+$	argA171$^+$
pro-67$^+$	2182	1	-	2	2	1
his-5075$^+$	164	15	34	-	90	2
cys-5605$^+$	155	18	30	93	-	1
argA171$^+$	1273	<1	<1	<1	<1	-

The percentage co-inheritance of pro$^+$ by cys$^+$ recombinants was approximately 25% irrespective of interruption time, but the percentage co-inheritance of muc$^+$ increased with mating time, indicating a later time of entry.

However, an anomaly in the linkage data prevented a precise chromosomal location being assigned for muc-36. On the basis of the published map (ref. 94, Fig. 11) muc-36 should have been located at approximately 14 mins, however, the expected linkage to argA171 could not be demonstrated. In retrospect, a clue to the problem was provided by the recombination frequencies obtained in the plate mating (Table 4). The numbers of recombinants obtained on selection for cys$^+$ and his$^+$ were almost 10-fold lower than for arg$^+$, a marker with a later time of entry!

In 1981, demonstration of genetic circularity of the PA0 chromosome (ref. 95) and the suggestion that FP2 may have additional origins of transfer allowed further progress to be made in the mapping of muc loci. Although cys-5605 and his-5075 (hisI) remained at 12' on the revised PA0 map (ref. 94, Fig. 11), this location was based on time of entry in FP2-mediated interrupted matings, assuming a single origin and direction of chromosome transfer. In view of the anomalous recombination frequencies obtained for these two markers in plate matings, it seemed possible that FP2 might have a second minor origin from which these markers were being transferred. The revised chromosome map also showed two newly isolated cys mutations:- cys-54 and cys-59. Linkage between proB and cys-59 could be demonstrated in FP2-mediated plate matings, and it was subsequently shown that cys-59 and cys-5605 are mutations in the same, or closely linked loci (L. Soldati and D. Haas, personal communication).

When crosses were performed between the mucoid donors PA0578 and PA0579 with the recipient strain PA01042 (cys-59, muc$^+$), recombinant analysis, shown in Table 5, provided evidence for two separate muc loci (Fig. 13).

No selectable markers have been mapped between cys-59 and proB as far as we know, hence no further mapping of muc-23 has been possible and the location with respect to the major FP2 origin is provisional.

TABLE 5

Recombinant analysis following plate matings between the donors PA0578 and PA0579 and recipient strain PA01042

Donor	Selected Marker	% Co-inheritance of unselected marker			
		muc	pro$^+$	cys$^+$	pur$^+$
PA0578 (muc-22)	pro-65$^+$	1	-	4	<1
	cys-59$^+$	34	33	-	6
	pur-70$^+$	10	2	5	-
PA0579 (muc-23)	pro-65$^+$	8	-	4	<1
	cys-59$^+$	38	35	-	10
	pur-70$^+$	2	2	4	-

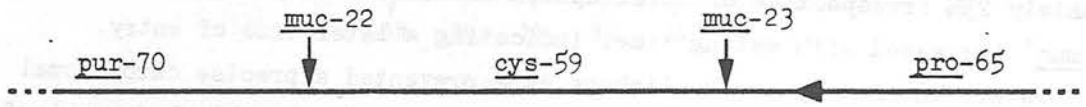

Fig. 13. Approximate locations of muc-22 and muc-23 based on plate mating data.

It has been possible, however, to proceed further with the mapping of muc-22 using transductional analysis, due to the work of Soldati and Haas (manuscript in preparation) who had isolated and mapped several catabolic loci in the region between pur-70 and cys-59 (Fig. 14). Oru-292 and pru-354 are involved in the utilisation of ornithine and proline respectively and show 60% linkage in R68.45-mediated crosses.

Fig. 14. Selectable markers in the region pur-70 to cys-59.

It is extremely difficult to obtain high titres of transducing phage grown in the usual way on mucoid strains, presumably because the phage receptors on the bacterial surface are blocked or the phage immobilised in the viscid environment surrounding the cells. Thus, before muc-22 could be mapped by transduction, it was necessary to isolate a non-mucoid derivative of PA0578 which had arisen due to a second, unlinked mutation switching off alginate production. Such "suppressed" revertants are still able to transfer the

muc allele in matings, giving rise to mucoid recombinants on selection for the appropriate markers. These kinds of strains are relatively common amongst non-mucoid revertants and their nature will be discussed later.

Phage F116L was grown on a revertant of PA0578 and used to transduce PA0954 and PA0964 selecting for oru$^+$ or pru$^+$; transductants were then scored for the co-inheritance of muc-22. 20% cotransduction was obtained between pru-354 and muc-22, while no mucoid transductants were observed on selection for oru$^+$. Similarly, 43% cotransduction was obtained between pru-354 and muc-2 (PA0568).

Four mucoid mutants (all group 2) of PA0964 were isolated and these were transduced with F116L grown on PA01, selecting for pru$^+$. In each case, 30-50% of the transductants were muc$^+$ (i.e. non-mucoid) indicating a similar location for these muc mutations. Two of these strains were used as recipients in three factor crosses to determine the marker order for oru, pru and muc. R68.45 was transferred into PA0954 and this strain was crossed with the two mucoid derivatives of PA0964, selecting for pru$^+$. Recombinants were then scored for co-inheritance of oru and muc$^+$. The results indicated that both muc mutations in these strains were to the right of pru-354 (Fig. 15).

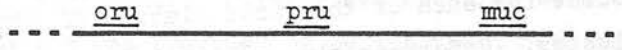

Fig. 15. Marker order determined from 3-factor crosses.

On the basis of results obtained so far, we can conclude that at least two regions on the PAO chromosome are involved in the repression of alginate synthesis. Mutations in either of these loci (or clusters of loci) result in derepression of the enzymes in the alginate biosynthetic pathway.

The nature of the repression of alginate synthesis in wild type (non-mucoid) strains has still to be determined. Partial diploid studies using R68.45 in a recA background (ref. 119) or involving P. putida (ref. 120) have been reported, and now that at least one muc locus is known to be closely linked to a selectable marker (pru-354), it should be possible to determine whether a cytoplasmic repressor, analogous to the capR gene product, is coded for by this muc locus. Cloning studies in P. aeruginosa have also been reported recently (ref. 121), thus further adding to the techniques available for the genetic analysis of this organism.

Indirect evidence for the protein nature of the capR$^+$ cytoplasmic repressor in E. coli was obtained by growing wild type strains on amino acid analogues, e.g. para-fluorophenylalanine (FPA) (ref. 122). When grown on certain

concentrations of FPA at 37°C, wild type E. coli K12 produced colanic acid, presumably because the analogue was incorporated into the capR$^+$ (or capT$^+$) gene product, resulting in a biologically inactive repressor.

Attempts to find levels of FPA derepressing alginate production in wild type P. aeruginosa have so far proved unsuccessful (ref. 75, Govan and Fyfe unpublished data).

A different approach to the genetic analysis of alginate production in P. aeruginosa has been described by Ohman and Chakrabarty (ref. 123). They attempted to develop a genetic system in a clinical mucoid isolate and map the loci (alg) responsible for the instability of alginate production in this strain.

Using a fluctuation test we have shown that instability in PA0568 can be explained on the basis of spontaneous mutations back to the non-mucoid form (ref. 113). A variety of mutations capable of "switching off" alginate production would be expected, including mutations in the structural genes coding for the biosynthetic enzymes leading to polymannuronic acid, so the approach taken by Ohman and Chakrabarty combined with enzyme analyses would prove valuable in the identification of genes involved in this system.

Ohman and Chakrabarty identified three classes of alg mutants based on colonial appearance and the results of genetic crosses between alg donors and recipients, some of which produced alg$^+$ recombinants. They concluded that the alg loci responsible for each of the three classes of non-mucoid mutants were located in a cluster. Unfortunately, no comparison was made between the markers employed in this system and those appearing on the PAO chromosome map (Fig. 11). Also, no attempt was made to determine the nature of the genes being studied. Hence, it is difficult to relate this work to our studies using PAO.

The possibility that PAO mucoid strains might be genetically distinct from mucoid P. aeruginosa isolated in vivo has been dispelled to a large extent by our finding that a muc locus in the clinical strain 492c (ref. 49) has been mapped in a R68.45-mediated inter-strain cross (with PAO1042) and is located between cys-59 and pur-70 (Fyfe and Govan, unpublished data).

THE SIGNIFICANCE OF BACTERIAL ALGINATE

A taxonomic relationship between Azotobacter and Pseudomonas?

In any consideration of the biosynthesis, regulation and function of bacterial alginate it is difficult to avoid the intriguing and obvious question as to why this unusual polymer, normally associated with marine algae, is uniquely produced in the case of procaryotes by members of two such diverse genera as Azotobacter and Pseudomonas. It is interesting to speculate on the origin, evolution and maintenance of the genes responsible for alginate biosynthesis

and regulation, the biological function of the polymer and even a possible taxonomic relationship between the soil-inhabiting, nitrogen fixing A. vinelandii and the opportunist pathogen P. aeruginosa.

Although comparisons of the genera Pseudomonas and Azotobacter are not commonly found in research publications several studies have recently revealed close relationships between species of these two genera, on the basis of various characteristics. Durham et al. (ref. 124) compared the three enzymes that initiate metabolism of protocatechuate in Azotobacter and Pseudomonas and revealed a close immunological relatedness of isofunctional proteins. Furthermore, they found that in both Azotobacter and Pseudomonas species of the "fluorescent" and "cepacia" groups, β-ketoadipate induces all the enzymes of the protocatechuate pathway (except protocatechuate oxygenase), a regulatory property which sets these organisms apart from other bacteria. The authors concluded that a closely related set of genes may code for the protocatechuate pathway in Azotobacter and Pseudomonas.

While studying the antigenicity of various outer membrane molecules in P. aeruginosa, Hancock et al. (ref. 125) using a monoclonal antibody, demonstrated that a single antigenic site on the major outer membrane lipoprotein, H2, is shared by various strains of P. aeruginosa, P. fluorescens, P. putida and a strain of A. vinelandii. Further evidence for evolutionary conservation of genetic information was provided by De Vos (ref. 126) who has demonstrated significant rRNA homology between A. vinelandii and a variety of fluorescent pseudomonads.

The biological function of bacterial alginate

We have already discussed evidence for a structural function for bacterial alginate in the metabolically dormant microcysts of A. vinelandii. This does not explain, however, the abundant production of extracellular alginate in metabolically active vegetative cells of this species. It is even more difficult to explain the evolutionary retention of alginate biosynthesis and regulation in P. aeruginosa, P. putida, P. fluorescens and P. mendocina. It would seem likely that in vegetative cells alginate has no single function but rather that the polymer, as with other exopolysaccharides (ref. 127), may contribute a range of properties whose specific roles might depend upon the environment. Evidence suggests that, in A. vinelandii, alginate does not serve as an overflow metabolite (ref. 22) but rather that it may act as a protective barrier against heavy metal toxicity (ref. 128), as a diffusion barrier to oxygen (ref. 5), as an ion-exchange system (ref. 129) with enhanced selectivity for Ca^{++}, particularly when alginate is in the gelled state (refs. 38, 130) or provide the bacterium with a hydrophilic, negatively charged coating which provides protection against attack and adverse environmental conditions (refs. 22, 127). Reference has already been made to the possible

utilisation of alginate as a nutrient source in the germination of Azotobacter cysts (ref. 39). Although an alginate lyase can be detected in cultures of A. vinelandii, the biological significance of this enzyme is uncertain. Couperwhite and McCallum (ref. 131) suggested that alginate could serve as a storage polymer although further confirmation for this role has not been found (ref. 22).

In the case of P. aeruginosa, Piggott found no evidence for the production of alginate lyases by mucoid or non-mucoid strains (ref. 75) although alginases have been detected in P. putida and P. maltophilia (ref. 132). No natural, ecological niche for alginate-producing pseudomonads, with the exception of the CF lung, is known. This suggests that in the majority of habitats alginate biosynthesis provides no advantage to the organism. However, in the unusually compromised environment of the CF lung, alginate must confer some advantage. Evidence is now available which suggests a multifactorial role for pseudomonas alginate in CF and provides a classic example of the ability and versatility of P. aeruginosa to act as an opportunist pathogen.

It is interesting to note that although P. cepacia can also be isolated from some CF patients, alginate-producing strains of this species have not been reported. This is in agreement with our observation that alginate-producing P. cepacia could not be isolated in vitro (ref. 70) and suggests that alginate biosynthesis is not a characteristic of this species or, if repressed, is controlled by a mechanism fundamentally different from that found in P. aeruginosa.

The adaptibility of P. aeruginosa and the significance of alginate biosynthesis in this species can be more fully appreciated by a knowledge of the pathological features found in CF patients. CF is an inherited, autosomal recessive disease characterised by symptoms which include high sweat electrolyte levels, pancreatic insufficiency and in the respiratory tract, raised levels of Ca^{++} and Mg^{++} in bronchial secretions which in turn are abnormally viscid due to the presence of highly sulphated glycoproteins (refs. 133, 134). These abnormally viscid bronchial secretions depress the normal non-specific immune defences of the lung in particular the rapid removal of bacteria and foreign matter via the ciliated epithelium of the upper respiratory tract. As we have already discussed in an earlier section of this chapter, during the course of chronic respiratory infection in CF patients, alginate-producing mutants of P. aeruginosa gradually emerge in the majority of patients. The emergence and eventual predominance of alginate-producing pseudomonas correlates with a poor prognosis (ref. 135, 136) suggesting that in CF patients the alginate acts as a virulence determinant. Doggett and Harrison (ref. 51) detected pseudomonas alginate in the sputum of CF patients confirming in vivo synthesis of the polymer. Various studies have suggested mechanisms by which

alginate contributes to the virulence of P. aeruginosa in the respiratory tract, e.g. in bacterial adhesion to the surface of ciliated tracheal epithelium (ref. 137), resistance to attack by host phagocytes (refs. 138-140), resistance to pulmonary surfactant (ref. 67) and to antibiotics (refs. 68, 69) and in reduced pulmonary clearance of P. aeruginosa (ref. 74). To these roles as an adhesin and protective agent it is arguable to add the potential for pathological damage residing in the physical properties of alginate. Aqueous solutions of pseudomonas alginate are extremely viscid and gel rapidly in the presence of physiological levels of Ca^{++} (ref. 74). It is not difficult to imagine the detrimental effect of the polymer's rheological properties in a lung already characterised by sticky bronchial secretions and stasis, and to appreciate, albeit reluctantly, this particular form of bacterial adaptation in a species whose versatility is already well recognised.

FUTURE STUDIES

Many questions remain to be answered concerning the biosynthesis and regulation of alginate in A. vinelandii and P. aeruginosa. Physiological studies have been few and the results obtained dependent to some extent on the strains used. The relationship between cell wall biosynthesis and alginate production has not been fully investigated. In A. vinelandii strain 9068, the rate of alginate synthesis is relatively independent of specific growth rate, suggesting that in these bacteria, competition for precursors between alginate synthesis and cell wall synthesis does not have a great influence on the rate of alginate synthesis (ref. 22). In P. aeruginosa, however, strains which do not produce detectable alginate during log phase, have a higher specific growth rate than those strains exhibiting growth association of alginate synthesis.

No studies have been reported on the genetics of alginate biosynthesis and regulation in A. vinelandii and progress in this area is dependent on further development of the genetic system in this organism.

In P. aeruginosa, the techniques are now available for a detailed analysis of the alginate biosynthetic pathway, along with the added component of its repression in wild type strains. We know that at least two genes involved in the repression of the alginate biosynthetic enzymes are located on the P. aeruginosa chromosome in the region near the major FP2 origin, however, the nature of the gene products has yet to be determined. Partial diploid analysis should reveal whether the muc loci, so far identified, code for repressor proteins or some other regulatory product. Cloning studies will be required if these gene products are to be fully identified.

Yet another question to be answered involves the genetic significance of the different mucoid phenotypes in P. aeruginosa, i.e. groups 1 and 2. In

E. coli, capT mucoid mutants synthesise colanic acid when grown on EMB-glucose agar at 37°C, whereas capR and capS mutants do not. In addition, capR and capS mutants are distinguishable on the basis of other characteristics, e.g. UV sensitivity. Our initial mapping results using PA0578 and PA0579 suggested that strains with mutations at one of the two different sites, (represented by muc-22 and muc-23, Fig. 13) could be distinguished on the basis of alginate synthesis on minimal agar. However, further work has shown that several group 1 mutations are closely linked to pru-354 (unpublished data). The medium component responsible for differential alginate synthesis on minimal agar has not been fully determined. However, we have some evidence that Mg^{++} concentration may play a role, as increased Mg^{++} concentration leads to increased alginate synthesis in some group 2 mutants. No enzyme analyses have been performed on group 2 strains grown in minimal medium, so it is not known at which stage alginate synthesis is blocked.

Apart from increased resistance to various antibiotics and surfactants, indicating cell membrane changes, none of the other pleiotrophic effects detected in capR mutants of E. coli, e.g. UV sensitivity, have been observed in mucoid P. aeruginosa.

Determination of the role of acetylation in the alginate biosynthetic pathway will require the isolation of mutants whose alginate lacks acetyl groups. It will be interesting to see if such alginates have a high guluronic acid content, thus providing evidence for the role of acetylation, postulated by Davidson et al. (ref. 27), to protect certain mannuronic acid residues from epimerisation.

Finally, what of the potential of bacterial alginate as an alternative to the present marine algal product. Clearly bacterial alginates exhibit all the physical properties necessary for the commercial applications of the polymer. The problems may lie in the production of the bacterial polymer on a large scale.

A. vinelandii has been examined in this context and the results reviewed elsewhere (ref. 20). Production of alginate on a large scale by this organism could turn out to be fairly costly as the efficiency of conversion of the carbon source to alginate is low, especially under conditions where respiration rate is high. Genetic manipulation of the organism may overcome this problem.

Amongst the alginate-producing pseudomonads, mucoid P. aeruginosa are strain dependent in respect of the conditions leading to maximal alginate yield. Some strains synthesise alginate during the growth phase, and at first sight would appear good candidates for alginate production in a continuous culture system. However, these strains are extremely unstable and revert back to the non-mucoid state due to the growth advantage of non-mucoid revertants (ref. 113).

Strains which synthesise alginate only in late log phase and stationary phase tend to be more stable, but perhaps more suitable for batch production.

Although advantages in the use of mucoid P. aeruginosa for production of alginate include the relative efficiency of alginate synthesis in terms of carbon conversion, and the possibilities of genetic manipulation, the problems of this organism's potential as an opportunist pathogen must be a major hazard. Alginate producing mutants of P. putida, P. fluorescens and in particular P. mendocina would perhaps be more acceptable, because of these species' relatively infrequent association with infections.

However, physiological studies are required to determine the optimal conditions for alginate synthesis by these species, and any similarity to the genetics and regulation in P. aeruginosa is open to speculation.

It is probable that future studies of bacterial alginate will continue to emphasise the diverse and occasionally conflicting research aims of modern microbiologists. Biochemists and industrial microbiologists will undoubtedly seek to improve the yield, viscosity and gelling potential of alginate as a commercially valuable polymer, especially if supply of the raw source of marine alginate becomes scarce or commercially uneconomic. In contrast, recognising mucoid P. aeruginosa as an important pathogen in the lungs of CF patients, medical microbiologists are already actively seeking means to interfere with alginate production in vivo in order to aid antibiotic therapy and reduce the organism's virulence.

Irrespective of the reasons for studying bacterial alginate, each field of microbiology will certainly benefit from a greater knowledge and understanding of the synthesis, regulation and function of bacterial alginate, both in vitro and in vivo.

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Ceftazidime in cystic fibrosis: clinical, microbiological and immunological studies

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We have investigated the use of the new cephalosporin ceftazidime for the treatment of pseudomonas infection in cystic fibrosis, using 100 to 240 mg/kg intravenously daily. The clinical and microbiological results of 18 courses of therapy, lasting from 1 to 4 weeks have been satisfactory, particularly since the patients had previously proved refractory to treatment with most other appropriate antibiotics. However, in common with other anti-pseudomonal antibiotics, a first course of ceftazidime proved the most successful, subsequent courses being less effective. Three patients died because of chronic respiratory infection.

In cystic fibrosis pseudomonal infections rapidly relapse after therapy and therefore we have been examining the interactions of antibiotics with relevant host defence mechanisms. In one patient who received a 4 g infusion of ceftazidime there was enhancement of neutrophil phagocytosis-triggered chemiluminescence.

We conclude that, used in high doses, ceftazidime is a useful addition to the available antibiotics for therapy of pseudomonas infection in cystic fibrosis.

Introduction

Recurrent respiratory infection in patients with cystic fibrosis is a major factor which leads to both morbidity and mortality. *Pseudomonas aeruginosa*, particularly mucoid forms, are the organisms most frequently cultured from the sputum of cystic fibrosis patients in whom there is pre-existent lung damage. Experience has shown that therapy with antibiotics active against *Ps. aeruginosa* can produce significant clinical improvement in these subjects, as measured in terms of exercise tolerance, body weight or the degree of productive cough (Høiby *et al.*, 1982). However, therapy rarely, if ever, eradicates the organism and some subjects may have sputum colonization with it for years without major respiratory disability. Furthermore major lung damage can occur in cystic fibrosis in the absence of chronic pseudomonas infection. These findings raise doubt as to the role of the organism in the aetiology of respiratory damage in this disease. What we believe is that pseudomonas infection is a most important factor in the development of lung damage in cystic fibrosis but that other mechanisms operate too, such as an inappropriate host response, contributing to lung damage (Hunninghake *et al.*, 1979).

Ceftazidime offers several advantages for the management of cystic fibrosis, particularly its good activity against *Ps. aeruginosa* and evidence of low toxicity (Capel-Edwards *et al.*, 1981; Foord, 1983). This latter aspect is an important consideration because the patients are likely to need repeated therapy against *Ps. aeruginosa* over many years,

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leading to a cumulatively high dosage. The cephalosporins in general have advantages over the aminohexoses in this respect. In this paper we report preliminary clinical and microbiological experience with ceftazidime during and after 18 courses of intravenous therapy in high dosage. Since relapse is the rule, not an exception, following therapy of cystic fibrosis respiratory infection, there may be a local defect of host defence mechanisms within the lung. Consequently we have started systematic studies of the interaction of ceftazidime with phagocytic cells both *in vivo* and *in vitro*.

Methods

Patients

Eight cystic fibrosis subjects aged from 5 to 36 years took part in this study. Five of these individuals received further courses during later exacerbations, two patients receiving a total of 5 and 4 courses of therapy, respectively, each lasting for at least 20 days. All patients received ceftazidime intravenously in doses ranging from 100 mg/kg/day to 240 mg/kg/day, usually in 3 divided doses. Tables I and II summarize the clinical details.

Table I. Patients treated with ceftazidime

Subject	Age	Sex	Weight (kg)	Daily dose (g)	Duration of therapy (days)	Predominant organism
P.F.	16	M	41	6	7	Mucoid *Ps. aeruginosa*
R.F.	14	M	34	6	30	Mucoid *Ps. aeruginosa*
T.H.	11	M	22·6	4·5	21	Mucoid *Ps. aeruginosa*
S.R.	5	F	12·7	3	23	Mucoid *Ps. aeruginosa*
J.S.	15	F	35·4	6	31	Mucoid *Ps. aeruginosa*
M.T.	12	F	25	4·5	13	Mucoid *Ps. aeruginosa*
K.D.	6	F	15	3	10	Non-mucoid *Ps. aeruginosa*
J.C.	35	F	41	4 or 6	10	Non-mucoid *Pseudomonas* sp.

Microbiology

Sputum samples obtained after vigorous physiotherapy were taken to the laboratory within 2 h and processed by a standard method, to give absolute viable counts of individual bacterial species in sputum. Minimum inhibitory concentrations (MICs) of ceftazidime were measured by a plate diffusion technique. Assays of ceftazidime in serum and sputum were performed by plate diffusion (Bennett *et al.*, 1966), with *Proteus morganii* as test organism and comparison with standard concentrations of ceftazidime (333 mg/l to 0·46 mg/l) in physiological buffered saline at pH 7·4.

Immunology

These have been detailed elsewhere (Grant *et al.*, 1983; Raeburn *et al.*, 1980; van Furth, van Zwet & Raeburn, 1979) but in principle all tests are *in-vitro* reflections of the

Table II. Results of treatment of patients with cystic fibrosis, with ceftazidime

Subject	Clinical results		Viable counts of *Ps. aeruginosa*		Further course of therapy	Course of illness
	(a) Symptomatic	(b) Weight gain during treatment (kg)	Before treatment	After treatment		
P.F.	Improved in 4 days	3	2×10^6	10^3	None	Died 8/12 later
R.F.	Sputum volume halved in 6 days	4	3×10^7	2×10^2	4	Moderate deterioration in 11/12
T.H.	Improved in 3 days	2·2	10^7	10^3	3	Deterioration over 11/12
J.S.	Improved in 2 days	2·7	10^7	5×10^3	1	Died 4/12 later
S.R.	Slight improvement at 6 days	2·3	10^8	−ve	1	Died 6/52 later
M.T.	Well and very active in 5 days	2	10^6	−ve	1	Slight deterioration in 8/12
K.D.	Improved in 3 days	0·5	10^6	−ve	None	No further admission in 10/12
J.C.	Improved exercise tolerance in 3 days	None	10^7	10^7	Intermittent	Previous deterioration halted

interactions thought to occur *in vivo* between micro-organisms and phagocytic cells. The effect of ceftazidime was studied *in vivo* on blood taken before and after intravenous infusions or *in vitro* in experiments which assayed the effect of pre-incubation of phagocytes for 1 h with prepared ceftazidime concentrations. The chemiluminescence response of phagocytes to zymosan or *Staph. aureus*, preopsonized with human AB serum, was measured (Kowolik & Grant, 1983) as a further measure of the antimicrobial and metabolic potential (Allen, Stjernholm & Steele 1972).

Results

The clinical response to ceftazidime in these subjects compared favourably with our previous experience of therapy with either gentamicin or tobramycin (Raeburn & McCrae, 1974; McCrae, Raeburn & Hanson, 1976). It should also be noted that all but two patients (PF and KD) had received previous treatment with tobramycin. Clinical improvement did not usually take place until at least 3 days after the start of ceftazidime therapy; to an extent this was related to the severity of lung disease. Table II summarizes the clinical and microbiological findings.

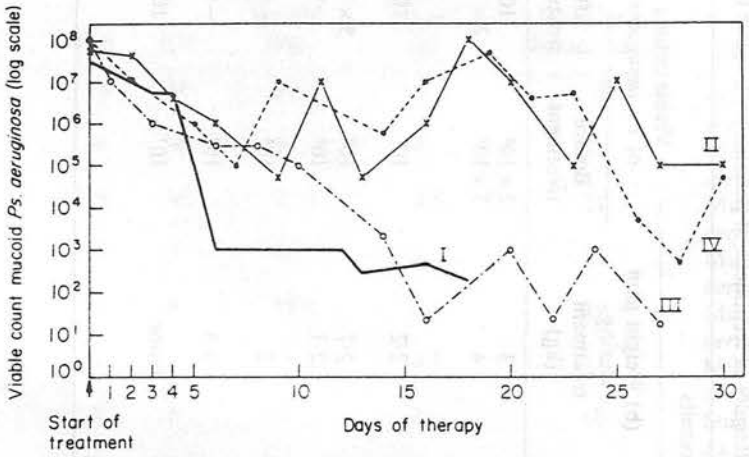

Figure 1. Reduction in viable *Ps. aeruginosa* from the sputum in one cystic fibrosis patient during four separate courses of ceftazidime. Resistance to the antibiotic does not explain the slower response in later courses of therapy but an increased dosage (course III) had some benefit. I, 1st course 6 g/day (——); II, 2nd course 6 g/day (x—x); III, 3rd course 9 g/day (○·—·○); IV, 4th course 6 g/day (●--●).

Serial viable counts of pseudomonas showed a decrease in all but one subject (Table II). This patient could not be admitted to hospital and she received a daily infusion over 1 h of 4 g ceftazidime, prepared with water for injection in the standard way and then diluted with 100 ml normal saline. This treatment was continued for 10 days. Figure 1 shows the serial viable counts during 4 consecutive courses of ceftazidime (I–IV) in a 14-year old male receiving either 2 or 3 g 8-h. It is clear that the rate of decrease in viable *Ps. aeruginosa* slows after the first course and that a higher dose partially corrects the slower response. This change from the response of the first course could not be attributed to resistance to ceftazidime.

The leucocyte function studies are at a very early stage but so far there is no evidence that ceftazidime (*in vivo* or *in vitro*) alters phagocytosis or intracellular killing of *Staph. aureus* by blood phagocytes. Neutrophil luminol-dependent chemiluminescence, has been

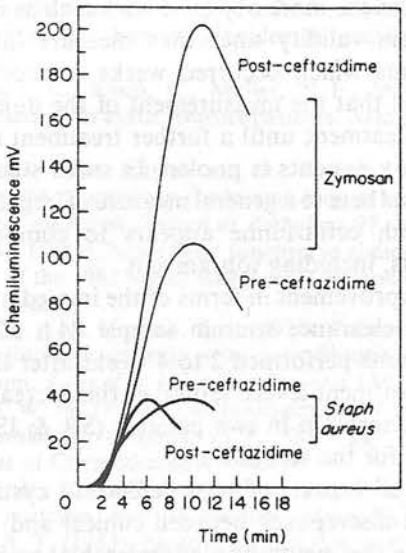

Figure 2. The chemiluminescent output after zymosan or *Staph. aureus* stimulation is proportional to the metabolic capacity of neutrophils to produce microbicidal reactive oxygen species and to perform later microbicidal reactions such as chlorination via myeloperoxidase activity. The serum level at the time of the post-ceftazidime sample was 200 mg/l.

Table III. *In-vitro* activity of ceftazidime on chemiluminescence

Cells	Ceftazidime concn (mg/l)	Peak chemiluminescence (mv)	% change from baseline
Granulocytes	None	171·4	—
	50	173·3	+1·11
	100	186·9	+9·04
	200	162·9	−4·96
Monocytes	None	30·54	—
	50	27·97	−8·42
	100	24·21	−20·73
	200	19·68	−35·56

measured on three occasions in one subject (JC) before and immediately after 4 g infusions of ceftazidime. On each occasion there was an increase in light output following ceftazidime with opsonized zymosan. By contrast there was no difference in the response to opsonised *Staph. aureus* (Figure 2). *In-vitro* studies with neutrophils from healthy donors or from the patient JC show that, in general, moderate concentrations of ceftazidime (about 150 to 200 mg/l) can enhance the chemiluminescence response to zymosan. Corresponding experiments on monocytes consistently show a concentration-dependent depression by ceftazidime of chemiluminescence output (Table III).

Discussion

The different antibiotic regimes for the management of *Ps. aeruginosa* infection in cystic fibrosis are difficult to compare mainly because many relevant criteria of improvement

are highly subjective. Even the more objective tests such as measurements of respiratory function are of uncertain validity since they measure lung changes that may have originated with infections which occurred weeks or months previously. Despite its subjective aspect, we feel that the measurement of the duration of improvement from the end of a course of treatment until a further treatment is required is a useful guide provided data from many patients is pooled. In small studies this is not possible and we restrict our assessments here to a general measure of improvement as well as the weight gain. In the doses used, ceftazidime appears to compare favourably with other anti-pseudomonas agents, including tobramycin.

The bacteriological improvement in terms of the immediate fall in viable count *during treatment*, and up to a clearance sputum sample 24 h after the last dose, was also impressive. However counts performed 2 to 4 weeks after therapy had all increased, in some cases to the pre-treatment levels. However this increase was only associated with comparable clinical deterioration in two patients (SR & JS), both of whom had been treated with ceftazidime for the second time.

We are studying several aspects of host defence in cystic fibrosis in an attempt to understand the frequent discrepancy between clinical and bacteriological results. The preliminary data indicate that neutrophil antimicrobial activity is unaffected by ceftazidime. The significance of the ceftazidime-associated enhancement of neutrophil responses to opsonised zymosan in the chemiluminescence assay and depression of the monocyte responses requires clarification and further experiments are in progress. It may be relevant that ceftazidime, in concentrations up to 200 mg/l, does not inhibit phytohaemagglutinin-induced lymphocyte transformation (Forsgren, 1981). Clearly a positive interaction between an effective anti-pseudomonas antibiotic and the host defence system of the cystic fibrosis patient without long term immunological effects would be of great importance. We have recently shown that treatment with the 'second-generation' cephalosporin, cefaclor, can lead to enhanced activity of the neutrophil enzyme myeloperoxidase and that in certain subjects this may influence antimicrobial function (Grant *et al.*, 1983). The effect of ceftazidime on myeloperoxidase activity in cystic fibrosis is still being studied.

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Chromosomal Loci Associated with Antibiotic Hypersensitivity in Pulmonary Isolates of *Pseudomonas aeruginosa*

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492a and 492c were two strains of *Pseudomonas aeruginosa* isolated from the sputum of a patient with cystic fibrosis. The strains were closely related but expressed different antibiograms. 492c was hypersensitive (10–100 times more sensitive than 492a) to the β-lactam antibiotics carbenicillin, methicillin, flucloxacillin, mecillinam and cefuroxime and the non-β-lactam, nalidixic acid. 492c also showed enhanced sensitivity (4–8 times more sensitive than 492a) to chloramphenicol, trimethoprim and novobiocin. 492a and PAO8 expressed similar levels of antibiotic resistance, except for trimethoprim, to which 492a was five times more sensitive than PAO8. Two genes associated with antibiotic hypersensitivity were mapped in the 30 min region of the chromosome, by means of R68.45-mediated plate matings between a Leu$^-$ mutant of 492c and PAO8, followed by transductional analysis using phage F116L. The first of these genes, *blsA1*, was closely linked to *nalB*, and in a PAO background, was associated with hypersensitivity to the β-lactams and a moderate increase in sensitivity to chloramphenicol, trimethoprim, nalidixic acid and novobiocin. A further increase in sensitivity to the latter three antibiotics was associated with the second gene, *tpsA1*, which mapped between *ser-3* and *hisV*. This gene could also be transferred to PAO from 492a, thus 492c could have arisen from 492a *in vivo* following a single chromosomal mutation at the *blsA* locus. Isolation of a *blsA* mutant of PAO969 provided further evidence for this theory.

INTRODUCTION

One of the characteristics most commonly associated with *Pseudomonas aeruginosa* is an intrinsic resistance to antimicrobial agents, in particular, the β-lactam antibiotics. Indeed, this property is generally held responsible for the emergence of *P. aeruginosa* in the last three decades as a clinically important opportunist pathogen.

The development of carbenicillin, the first semi-synthetic penicillin with significant anti-pseudomonas activity, was a significant advance in chemotherapy. However, the minimal inhibitory concentration (MIC) of carbenicillin for *P. aeruginosa* is generally 25–50 μg ml^{-1} (Knudsen *et al.*, 1967), which is beyond the levels attainable in soft tissues, e.g. the lung (Marks *et al.*, 1971).

May & Ingold (1972) reported that strains of *P. aeruginosa* isolated from sputum were often considerably more sensitive to carbenicillin than isolates from other sources. In a study involving 111 sputum isolates from patients with cystic fibrosis (CF), chronic bronchitis and bronchiectasis, they found that 35% of strains were sensitive to 6 μg carbenicillin ml^{-1} and some had MICs as low as 0·7 μg ml^{-1}.

Abbreviations: CEF, cefuroxime; CF, cystic fibrosis; DSTA, Diagnostic Sensitivity Test Agar; NA, nutrient agar; NB, nutrient broth; TP, trimethoprim.

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Berche *et al.* (1979) reported a study on antibiotic susceptibilities of mucoid and non-mucoid strains of *P. aeruginosa*. They compared 47 mucoid with 71 non-mucoid isolates on the basis of sensitivities to 18 antibiotics and concluded that both groups could be divided into two distinct classes on this basis. Class A contained strains significantly more resistant to antibiotics such as the aminoglycosides and tetracycline, than those strains allocated to Class B.

While studying the association of *P. aeruginosa* with chronic respiratory infection in CF patients, we have also reported the isolation of strains unusually sensitive (hypersensitive) to a range of antibiotics including the β-lactams carbenicillin, azlocillin, methicillin and also to trimethoprim (Irvin *et al.*, 1981). The carbenicillin MICs of these strains were < 1 μg ml^{-1} and thus comparable with those reported by May & Ingold (1972). In addition, we reported that hypersensitive and 'normal' isolates could be obtained from the same sputum specimen and that these strains were invariably of the same pyocin type, indicating a close relationship.

Recently, there has been an increasing interest in the genetic basis of virulence in *P. aeruginosa* and various potential virulence factors have been mapped using the genetically characterized strain, PAO. For example, several chromosomal genes involved in exotoxin A production have been mapped following isolation of toxin-deficient PAO mutants, and the use of classical genetic techniques (Gray & Vasil, 1981*a*; Hanne *et al.*, 1983). Similarly, a chromosomal gene controlling phospholipase C production has been identified (Gray & Vasil, 1981*b*). Both of these potential virulence factors are normally expressed in PAO, but others may be expressed only following a mutation, for example, alginate production leading to a mucoid phenotype which is so common in *P. aeruginosa* isolated from the sputa of CF patients. Mucoid mutants of PAO have been isolated *in vitro* and two chromosomal loci involved in the control of alginate synthesis have been mapped (Govan & Fyfe, 1978; Fyfe & Govan, 1980; J. A. M. Fyfe & J. R. W. Govan, unpublished).

A third approach to the genetic analysis of virulence involves the transfer of the relevant gene(s) from a clinical isolate into a PAO background. This approach is applicable to both chromosomally-encoded and plasmid-encoded characteristics, and has been used in the genetic analysis of extracellular protease production (Wretlind *et al.*, 1983) and transposable antibiotic resistance (Sinclair & Holloway, 1982).

In the course of studying the intrinsic antibiotic resistance of *P. aeruginosa* strain PAO, various loci associated with this characteristic have been mapped on the chromosome (Mills & Holloway, 1976; Okii *et al.*, 1983; Matsumoto & Terawaki, 1982). Functional aspects of the *P. aeruginosa* outer membrane have been examined using an antibiotic 'supersusceptible' mutant of strain 799 (Zimmerman, 1980; Angus *et al.*, 1982; Darveau & Hancock, 1983). However, genetic analysis of this strain proved unsuccessful (Angus *et al.*, 1982).

The genetic basis of antibiotic hypersensitivity as expressed in clinical isolates of *P. aeruginosa* has not, to our knowledge, been reported. May & Ingold (1972) postulated that the normal resistance to carbenicillin might be plasmid-encoded, and that this plasmid may sometimes be lost during growth in the respiratory tract. However, no evidence was presented to support this hypothesis. The present paper describes the genetic mapping of antibiotic hypersensitivity in the clinical isolate 492c (Irvin *et al.*, 1981) by means of inter-strain crosses with suitably marked PAO recipients, followed by transductional analysis using phage F116L (Krishnapillai, 1971). A preliminary report of this work has been presented (Fyfe & Govan, 1983).

METHODS

Bacteria and bacteriophages. The bacterial strains used in this study are shown in Table 1. Donor strains carrying the plasmid R68.45 were constructed according to Haas & Holloway (1976). Phage F116L (Krishnapillai, 1971) was used for transduction and F116c for strain construction.

Media and cultural conditions. Nutrient broth (NB), nutrient agar (NA), and minimal agar have been described previously (Fyfe & Govan, 1980). Amino acid supplements were added at a concentration of 1 mM. The antibiotics used were carbenicillin (Pyopen; Beecham), methicillin (Celbenin; Beecham), benzyl penicillin (Crystapen; Glaxo), ampicillin (Penbritin; Beecham), flucloxacillin (Floxapen; Beecham), mecillinam (Leo Pharmaceuticals), cefuroxime (Zinacef; Glaxo), cefoxitin (Mefoxin; MSD), tetracycline (Glaxo), trimethoprim lactate (Wellcome), nalidixic acid (Winthrop), novobiocin (Sigma), rifampicin (Roche), streptomycin (Glaxo), gentamicin (Roussel),

Table 1. *Strains of P. aeruginosa used in this study*

Strain	Genotype/Description*	Reference
PAO1	Prototroph, *chl-2*	Holloway (1969)
PAO2	*ser-3*	Isaac & Holloway (1968)
PAO4	*arg-47 pyrB52*	B. W. Holloway collection
PAO8	*met-28 ilv-202 str-1*	Isaac & Holloway (1968)
PAO222	*met-28 trp-6 lys-12 his-4 ilv-226 pro-82*	Haas & Holloway (1976)
PAO969	*proC130*	Rella & Haas (1982)
PAO6002	*met-9011 amiE200 nalB4*	Rella & Haas (1982)
PAO6006	*proC130 nalB9*	Rella & Haas (1982)
PAO6511	*proC130 blsA2*	This paper
PAO6524	*proC130 nalB4* derivative constructed using F116c	This paper
PAO6526	Pro$^+$ transductant of PAO6511 constructed using F116c	This paper
GMA037	*hisV5037*	Mee & Lee (1967)
492a	Clinical isolate, mucoid, prototrophic, *tpsA*	Irvin *et al.* (1981)
492a Leu$^-$	Leucine auxotroph isolated from 492a following EMS mutagenesis	This paper
492c	Clinical isolate, mucoid, prototrophic, *blsA1 tpsA1*	Irvin *et al.* (1981)
492c Leu$^-$	Leucine auxotroph isolated from 492c following EMS mutagenesis	This paper
PAJ1	Prototrophic, *tpsA1 blsA1* recombinant from cross 492c Leu$^-$R68.45 × PAO8	This paper
PAJ2	Prototrophic, *blsA1* recombinant from cross 492c Leu$^-$R68.45 × PAO8	This paper
PAJ3	Prototrophic, *tpsA1* recombinant from cross 492c Leu$^-$R68.45 × PAO8	This paper
PAJ5	*met-28 tpsA1*, recombinant from cross 492c Leu$^-$R68.45 × PAO8	This paper
PAJ6	*ilv-202 tpsA1*, transductant of PAJ5 using F116c grown on PAO8 *met*$^+$	This paper

* Genotype symbols are the same as those used for *E. coli*; *blsA* designates sensitivity to β-lactam antibiotics, *tpsA* sensitivity to trimethoprim, *ami* acetamide utilization and *str* resistance to streptomycin.

tobramycin (Lilley), and kanamycin (Bristol Laboratories). All antibiotics were incorporated in Diagnostic Sensitivity Test Agar (DSTA; Oxoid) at the appropriate concentration.

Broth cultures were grown in McCartney bottles on an orbital incubator (Gallenkamp) at 140 r.p.m., and all cultures were incubated at 37 °C, unless stated otherwise.

Isolation of mutants. Auxotrophic mutants were isolated after EMS mutagenesis (Watson & Holloway, 1976) and carbenicillin enrichment (Watson & Holloway, 1978).

Carbenicillin-hypersensitive mutants were isolated following EMS mutagenesis and screening of colonies by replica plating onto DSTA plates containing 5 μg carbenicillin ml^{-1}.

Antibiotic sensitivity testing. Exponential phase NB cultures were diluted in physiological saline to a concentration of 10^5 organisms ml^{-1}. A multiple inoculator (Mast) was used to dispense samples (containing 10^2–10^3 cells) onto DSTA plates appropriately supplemented with the antibiotics. The MIC was read as the lowest concentration of antibiotic that caused complete growth inhibition after 18 h incubation at 37 °C.

Plate matings. The procedure used was that described by Stanisich & Holloway (1972). Prior to inter-strain matings, NB cultures of the recipient strains were incubated for 18 h at 43 °C (Rolfe & Holloway, 1966).

Transductions. These were performed according to the method of Krishnapillai (1971).

RESULTS

Antibiotic sensitivities

It has previously been reported that 492a and 492c differ in their sensitivities to carbenicillin, azlocillin, piperacillin, ticarcillin, methicillin, tetracycline and trimethoprim (Irvin *et al.*, 1981).

We determined the MICs of an extended range of antibiotics for 492a and 492c, and the results are shown in Table 2. Strain PAO8 was also included to confirm that the MICs obtained for this strain were comparable with 492a and not the hypersensitive 492c. However, 492a was five times more sensitive to trimethoprim than PAO8. Table 2 shows that 492c is generally hypersensitive to the β-lactam antibiotics with the following exceptions: benzyl penicillin,

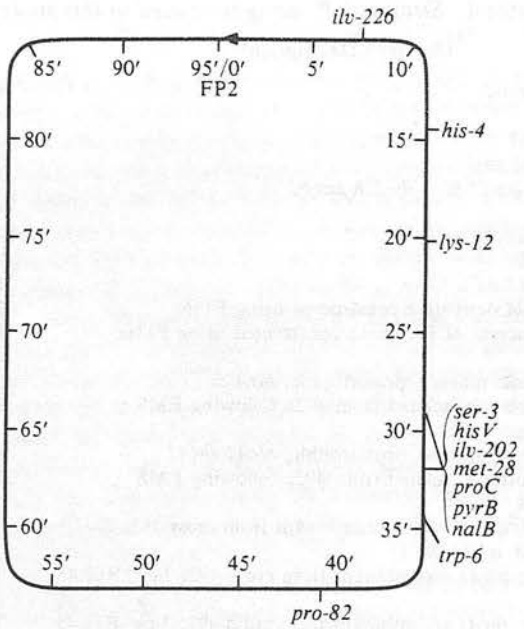

Fig. 1. Genetic map of *P. aeruginosa* showing markers relevant to this study. Based on Holloway & Crockett (1982).

Table 2. *Sensitivities of P. aeruginosa strains 492a, 492c and PAO8 to antibiotics*

Antibiotic Strain ...	MIC (μg ml^{-1}) 492a	492c	PAO8
Carbenicillin	80	0·6	40
Methicillin	>500	10	500
Benzyl penicillin	500	500	>1000
Ampicillin	100	100	200
Flucloxacillin	>500	50	>500
Mecillinam	500	10	500
Cefuroxime	>500	10	400
Cefoxitin	400	400	500
Cephaloridine	500	500	>1000
Chloramphenicol	40	10	60
Tetracycline	10	6	10
Trimethoprim	40	5	200
Nalidixic acid	60	2	80
Novobiocin	>500	100	500
Rifampicin	40	40	20
Streptomycin	40	40	>500*
Gentamicin	0·6	0·6	0·6
Tobramycin	0·4	0·4	0·2
Kanamycin	60	60	60

* PAO8 is resistant to streptomycin due to the *str-1* locus.

ampicillin, cefoxitin and cephaloridine. Increased sensitivity to trimethoprim was confirmed for 492c, and also noted for nalidixic acid and novobiocin. No differences between 492a and 492c were observed for rifampicin, streptomycin, gentamicin, tobramycin or kanamycin.

Transfer of antibiotic hypersensitivity from 492c to PAO

Figure 1 is a chromosome map of *P. aeruginosa* PAO showing the locations of markers relevant to this study. A donor derivative of 492c was prepared by isolating a Leu$^-$ mutant, and

Table 3. *Results of recombinant analysis following a plate mating between 492c Leu⁻ (R68.45) and PAO8*

Selected marker	Percentage co-inheritance of unselected character			
	CEF^{s}*	TP^{s}†	Met^{+}	Ilv^{+}
met-28^{+}	80	82	–	77
ilv-202	75	88	87	–

* CEF^{s} designates sensitivity to 100 μg cefuroxime ml^{-1}.
† TP^{s} designates sensitivity to 100 μg trimethoprim ml^{-1}.

transferring R68.45 into this strain. Plate matings were performed between 492c Leu⁻ (R68.45) and the multiple-marker recipient PAO222. Selection was made for each of the auxotrophic markers and 100 recombinants for each marker were scored for co-inheritance of cefuroxime sensitivity (on DSTA plus 100 μg cefuroxime ml^{-1}). Increased sensitivity to cefuroxime rather than carbenicillin was chosen initially to avoid any problems associated with the inheritance of R68.45 by some of the recombinants (R68.45 codes for a type IIIa β-lactamase which is active against carbenicillin but not cefuroxime). Recombinant analysis revealed that 56% of the *met-28*$^{+}$ colonies were sensitive to 100 μg cefuroxime ml^{-1} and likewise 20% of the *trp-6*$^{+}$ colonies. Less than 1% of the recombinants for the other markers, i.e. *ilv-226*, *his-4*, *lys-12* and *pro-82* had co-inherited this characteristic.

In order to map the locus associated with increased cefuroxime sensitivity more precisely, a plate mating was performed between 492c Leu⁻ (R68.45) and PAO8 (*met-28 ilv-202*). Selection was made for both auxotrophic markers and recombinants scored for both cefuroxime and trimethoprim sensitivity (on DSTA plus 100 μg trimethoprim ml^{-1}). The results of this recombinant analysis are shown in Table 3, and indicate that cefuroxime sensitivity (CEF^{s}) and trimethoprim sensitivity (TP^{s}) do not always cosegregate. All CEF^{s} recombinants were TP^{s}, but a percentage of the TP^{s} colonies were CEF^{r}.

The MICs for representatives of each recombinant class, i.e. CEF^{s} TP^{s}, CEF^{r} TP^{s} and CEF^{r} TP^{r}, were determined using those antibiotics to which 492c is hypersensitive, i.e. carbenicillin, methicillin, flucloxacillin, mecillinam, cefuroxime, chloramphenicol, trimethoprim, nalidixic acid and novobiocin.

All CEF^{r} TP^{r} recombinants (three tested) were indistinguishable from PAO8 on the basis of MICs to these antibiotics. The CEF^{s} TP^{s} group (eight tested) could be subdivided on the basis of their trimethoprim, nalidixic acid and novobiocin MICs. Three recombinants were very sensitive to these antibiotics with trimethoprim and novobiocin MICs of 10 μg ml^{-1}, and a nalidixic acid MIC of 5 μg ml^{-1}. The other CEF^{s} TP^{s} recombinants were moderately sensitive with the following MICs: trimethoprim 60 μg ml^{-1}, novobiocin 50 μg ml^{-1}, nalidixic acid 20 μg ml^{-1}. All eight recombinants were equally sensitive to the β-lactam antibiotics and chloramphenicol, and with the exception of cefuroxime were as sensitive as 492c. The third class of recombinant, CEF^{r} TP^{s} (five tested), displayed moderate sensitivity to trimethoprim, nalidixic acid and novobiocin, with MICs of 60 μg ml^{-1}, 20 μg ml^{-1} and 100 μg ml^{-1}, respectively.

From these results, we postulated that the antibiotic hypersensitive phenotype of 492c is determined by two separate genes, both linked to *met-28* and *ilv-202*, when transferred into PAO8. We propose to call these genes *blsA1*, coding for hypersensitivity to certain β-lactams and a moderate sensitivity to trimethoprim, nalidixic acid and novobiocin; and *tpsA1* coding for increased sensitivity to the latter three antibiotics but not the β-lactams. Table 4 shows the relevant MIC values for PAO8 and three typical recombinant strains PAJ1 (*blsA1 tpsA1*), PAJ2 (*blsA1*) and PAJ3 (*tpsA1*).

Transfer of tpsA1 from 492a to PAO8

As shown in Table 2, the trimethoprim MIC for 492a is fivefold less than for PAO8. In addition, the increased sensitivity of 492c to trimethoprim, compared with 492a (eightfold), is of the same order as the MIC difference between PAJ1 and PAJ3 (sixfold) (see Table 4).

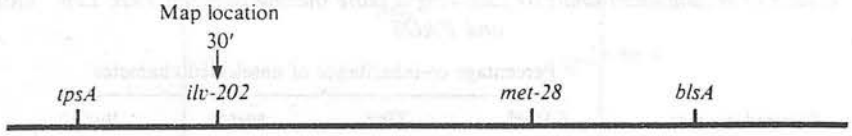

Fig. 2. Approximate locations of *blsA* and *tpsA* on the basis of results obtained from plate matings between 492c Leu⁻ (R68.45) and PAO8.

Table 4. *Antibiotic sensitivities of PAO8 and typical recombinant strains PAJ1, PAJ2 and PAJ3*

Antibiotic	Strain ...	MIC (μg ml⁻¹) PAO8	PAJ1 (*blsA1 tpsA1*)	PAJ2 (*blsA1*)	PAJ3 (*tpsA*)
Carbenicillin		40	0·4	0·4	40
Methicillin		500	10	10	500
Flucloxacillin		>500	50	50	>500
Mecillinam		500	10	10	500
Cefuroxime		400	100	100	400
Chloramphenicol		40	20	20	40
Trimethoprim		200	10	60	60
Nalidixic acid		80	5	20	20
Novobiocin		500	10	50	10

On this basis, we proposed that *tpsA1* is present in both 492a and 492c, and that the antibiotic hypersensitivity of the latter strain can be explained by a mutation at the *blsA* locus, i.e. there is only a single gene difference between 492a and 492c accounting for antibiotic hypersensitivity.

Using the mapping procedure already described for 492c, a donor derivative of 492a [492a Leu⁻ (R68.45)] was plate mated with PAO8, and the *met*⁺ and *ilv*⁺ recombinants scored on DSTA containing either 100 μg trimethoprim ml⁻¹ or 100 μg cefuroxime ml⁻¹. As expected, a proportion of recombinants failed to grow well on trimethoprim, though all grew well on cefuroxime.

Mapping of blsA and tpsA by transduction using phage F116L

From the plate mating results, it was possible to assign approximate chromosomal locations to *blsA1* and *tpsA1* relative to *ilv-202* and *met-28* (see Fig. 2). However, to map these loci more precisely with respect to other markers in the 30 min region, transductional analysis was performed.

In the case of *blsA1*, a preparation of F116L was grown on PAJ2 and used to transduce recipient strains carrying the auxotrophic markers *met-28* (PAO8), *ilv-202* (PAO8), *proC130* (PAO969) and *pyrB52* (PAO4). Selection was made for these markers and cotransduction of *blsA1* scored on DSTA plus 5 μg carbenicillin ml⁻¹.

In the case of *tpsA1*, transducing preparations of F116L could not be propagated on any recombinants containing this marker, as they did not appear to support vegetative growth. However, *tpsA1* strains retained the receptors for this phage as they were good recipients in F116L-mediated transductions, so in order to map this locus, an *ilv-202 tpsA1* derivative, PAJ6, was constructed (via the intermediate strain PAJ5) and used as recipient in transductions mediated by F116L propagated on either PAO1, PAO2 (*ser-3*) or GMA 037 (*hisV5037*). In each case selection was made for *ilv*⁺ transductants and these were scored for co-inheritance of *tpsA*⁺ and where appropriate, the unselected auxotrophic markers. Figure 3 shows the locations obtained for *blsA* and *tpsA*.

Isolation of a blsA mutant of PAO969

Working on the hypothesis that 492c could have arisen from 492a by a single step mutation at the *blsA* locus, we postulated that a similar *blsA* mutant could be isolated from a PAO strain.

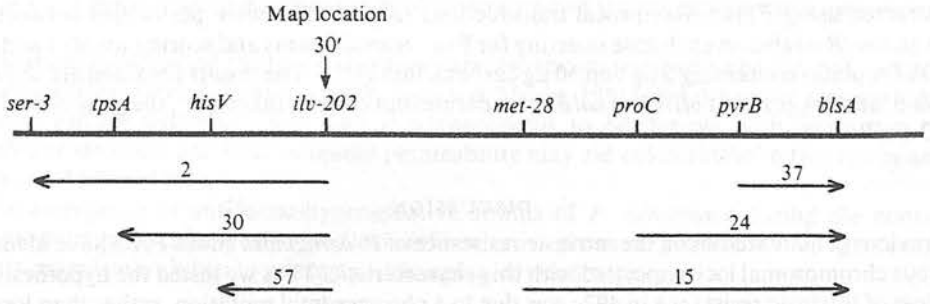

Fig. 3. Mapping of *tpsA1* and *blsA1* by transduction. Values indicate percentage cotransduction using phage F116L (the mean of several experiments scoring at least 200 transductants). Arrowheads point to the unselected marker.

Table 5. *Results of F116L-mediated transductions to determine the relationship between nalB and blsA*

F116L grown on:	Recipient	Percentage cotransduction with *proC*+*		
		blsA2	*nalB4*	Wild-type
PAO1 (*nalB4*+ *blsA2*+)	PAO6511 (*blsA2*)	–	–	33
	PAO6524 (*nalB4*)	–	–	43
PAO6002 (*nalB4*)	PAO6511 (*blsA2*)	–	27	<0·5
PAO6526 (*blsA2*)	PAO6524 (*nalB4*)	19	–	3

* In each case, selection was made for *proC*+ and 200 transductants were scored for the co-inheritance of *nalB4*+/− and *blsA2*+/− on DSTA plus 50 μg and 5 μg carbenicillin ml^{-1}, respectively.

PAO969 was mutagenized with EMS and the potential mutant colonies screened for hypersensitivity to carbenicillin by replica plating to DSTA plates containing 5 μg carbenicillin ml^{-1}. From approximately 5000 colonies screened, one failed to grow on medium containing carbenicillin. Fortuitously, this mutant (PAO6511) exhibited a similar antibiogram to the *blsA1* recombinants, i.e. PAO6511 was highly sensitive to carbenicillin, moderately sensitive to cefuroxime, trimethoprim, nalidixic acid, novobiocin and chloramphenicol, but remained resistant to cefoxitin, cephaloridine and benzyl penicillin. However, PAO6511 was more resistant to methicillin, mecillinam and flucloxacillin than a *blsA1* transductant of PAO969.

F116L transductions revealed 33% cotransduction between *proC* and the mutant locus. Furthermore, when F116L propagated on PAJ2 was used to transduce PAO6511, selecting for *proC*+, <0·5% of the transductants were able to grow on 5 μg carbenicillin ml^{-1}, indicating a very close linkage between *blsA1* and the PAO mutation, which, on this basis, we have designated *blsA2*.

Relationship between blsA and nalB

Rella & Haas (1982) have described a class of mutants (*nalB*) in strain PAO which are resistant to nalidixic acid, novobiocin, carbenicillin and various other β-lactams. We examined two *nalB* mutants PAO6002 (*nalB4*) and PAO6006 (*nalB9*) to determine whether they showed increased resistance to cefuroxime, trimethoprim, methicillin and chloramphenicol. Both strains showed increased resistance to all four antibiotics with the following relative MICs with respect to PAO969: cefuroxime, ×3; trimethoprim, ×6; methicillin, ×5; chloramphenicol, ×10.

nalB has been mapped at 32 min on the PAO chromosome, distal to *pyrB* using G101 transduction (Rella & Haas, 1982), so we determined the location of *blsA2* with respect to this locus, using strains PAO6511 and PAO6002. In order to perform reciprocal transductions, a *proC* derivative of PAO6002 (PAO6524) and a *proC*+ derivative of PAO6511 (PAO6526) were

constructed using F116c. Reciprocal transductions using F116L were performed between the *blsA* and *nalB* strains, in each case selecting for Pro$^+$ transductants and scoring for *blsA* and *nalB* on DSTA plates containing 5 μg and 50 μg carbenicillin ml^{-1}. The results obtained are shown in Table 5, and suggest that *blsA* and *nalB* are separate, but closely linked loci, the gene order being *proC–nalB–blsA*.

DISCUSSION

Previous genetic studies on the intrinsic resistance of *P. aeruginosa* strain PAO have identified various chromosomal loci associated with this characteristic. Thus we tested the hypothesis that the loss of intrinsic resistance in 492c was due to a chromosomal mutation, rather than loss of a plasmid as suggested by May & Ingold (1972). Inter-strain crosses between 492c Leu$^-$ (R68.45) and PAO8 identified two distinct loci, in the 30 min region of the chromosome, associated with antibiotic sensitivity. The first of these, *blsA*, is associated with sensitivity to the β-lactams carbenicillin, methicillin, flucloxacillin, mecillinam and cefuroxime, and also the non-β-lactams chloramphenicol, trimethoprim, nalidixic acid and novobiocin and cannot be transferred to PAO from 492a.

The isolation of a *blsA* derivative of PAO following EMS mutagenesis provides further evidence that 492c could have arisen from 492a *in vivo* following a single-step mutation which was then selected out in the lung environment. This mutation–selection mechanism is also responsible for the emergence of mucoid strains of *P. aeruginosa* during the course of chronic pulmonary infections in CF patients. All strains of *P. aeruginosa* have the necessary structural genes to synthesize alginate, but these are normally subject to repression. Mutation in a regulator gene close to the major FP2 origin results in derepression of alginate synthesis giving rise to a mucoid strain (Govan & Fyfe, 1978; Fyfe & Govan, 1980; J. A. M. Fyfe & J. R. W. Govan, unpublished).

Three major questions remain to be answered regarding antibiotic hypersensitivity associated with respiratory isolates. Firstly, whether all such isolates have the same mutation as 492c or whether they form a genetically heterogeneous group. We have examined several pairs of strains (i.e. hypersensitive and 'normal' from the same sputum specimen) isolated from different patients on the basis of antibiotic sensitivities and in general, the antibiograms have been similar to 492a and 492c (results not shown). Genetic analysis of one other hypersensitive strain gave identical results to those obtained with 492c, even though this strain was isolated from a different patient and was of a different pyocin type. However, further work is required to determine whether the *blsA* locus is the only one involved in clinically associated antibiotic hypersensitivity.

Secondly, we would like to know the structural basis of this characteristic. We have previously reported that 492c has two extra outer membrane proteins when compared with 492a (Irvin *et al.*, 1981), but further studies have failed to confirm that these are associated with antibiotic sensitivity.

Several studies on the structural basis of antibiotic hypersensitivity in *P. aeruginosa* have been reported (Noguchi *et al.*, 1980; Curtis *et al.*, 1981; Angus *et al.*, 1982; Zimmerman, 1980; Kropinski *et al.*, 1982; Darveau *et al.*, 1983) and these have suggested that increased permeability of the cell envelope is responsible for this characteristic. In the case of 799/61, a 'supersusceptible' mutant of strain 799 (Zimmerman, 1979), alterations in the LPS composition have been detected (Kropinski *et al.*, 1982; Darveau *et al.*, 1983). Similar mutants (*envA*) have been reported in *Escherichia coli* (Grundstrom *et al.*, 1980; Boman *et al.*, 1974).

The close linkage of *blsA* and *nalB* on the *P. aeruginosa* chromosome is interesting in view of the fact that *nalB* mutants are more resistant to those antibiotics to which *blsA* strains are hypersensitive, and it is tempting to speculate that these two loci form part of a gene cluster controlling outer membrane permeability in this organism.

Finally, the clinical and biological significance of antibiotic hypersensitivity remains to be elucidated. Superficially it appears paradoxical that hypersensitive strains arise in patients undergoing aggressive antibiotic therapy, often with β-lactams. The lung, however, is a

notoriously difficult site in which to achieve therapeutic levels of anti-pseudomonas antibiotics, and in this environment the vulnerability of hypersensitive strains may be more apparent than real.

Mutations to increased antibiotic sensitivity are also encountered amongst naturally occurring gonococci (Eisenstein & Sparling, 1978; Lysko & Morse, 1981) and it has been suggested that mucosal surface pathogens may find it advantageous to be flexible with respect to outer membrane structure and that increased permeability may aid colonization in this environment (Lysko & Morse, 1981).

The emergence of antibiotic-hypersensitive strains of *P. aeruginosa* during the course of chronic pulmonary infections, regardless of the selective pressure, provides yet another example of this organism's ability to adapt to a changing environment.

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Revised Pyocin Typing Method for *Pseudomonas aeruginosa*

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In the Gillies and Govan method of pyocin typing for *Pseudomonas aeruginosa* a cross-streaking technique was used, and 105 main types and 25 subtypes were identified by the patterns of inhibition observed on 13 indicator strains. Disadvantages of the technique included the need to remove test strain growth before application of the indicator strains, the 48-h period needed to obtain a result, and the inability to reliably type mucoid *P. aeruginosa*. Recent studies have enabled us to overcome these disadvantages and significantly improve the speed and application of pyocin typing. Our revised technique utilizes the same 13 indicator strains which are already used internationally. Test strains were rapidly applied to the surface of agar plates with a multiple inoculator. After incubation for 6 h and exposure to chloroform, the indicator strains were applied in agar overlays without prior removal of the test strain growth. After 18 h of incubation, the pyocin type was recognized by inhibition of particular indicator strains. Additionally, the activity of particulate (R and F) and nonparticulate (S) pyocins could be distinguished on the basis of inhibition zone size, which thus allowed further discrimination. The revised technique allows typing within 24 h, increases the number of identifiable types, and can be used to type mucoid strains.

In the last three decades, *Pseudomonas aeruginosa* has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients (12). At particular risk are patients who have suffered major trauma or burns and are exposed to intensive care units (16). Also at risk are normal individuals exposed to a compromising occupational or recreational environment, e.g., a deep-sea diving bell, in which outbreaks of acute and painful otitis externa due to *P. aeruginosa* have been reported (1), or the jacuzzi, in which *P. aeruginosa* has been implicated in the irritating skin rash known as "hot tub" or jacuzzi syndrome (17).

The increased importance of *P. aeruginosa* as an opportunistic pathogen, together with its well-recognized and characteristic ubiquitous nature, gives rise to many instances in which reliable and discriminating typing or "fingerprinting" of strains is required to investigate outbreaks of nosocomial infection and to aid effective infection control.

Several biological criteria have been assessed for typing *P. aeruginosa*, including pigmentation, antibiograms, and phage sensitivity. However, the two most reliable and generally accepted methods are serotyping and pyocin (aeruginocin) typing (2, 14). A number of pyocin typing methods have been described, but in independent comparative experience and reviews (2, 11) it has been suggested that the most suitable method is that developed in our laboratory (4, 7, 8).

In our pyocin typing technique (7), a cross-streaking method is used which can identify 105 main types on the basis of pyocin production by test strains and the recognition of different inhibition patterns observed against eight indicators, labeled 1 through 8, and further subdivision into 25 possible subtypes with five additional indicator strains, labeled A through E. Thus, the discriminatory potential of the method is good and superior to serotyping. However, in epidemiological studies, as with serotyping, the majority of strains fall into a limited number of types or subtypes. Thus, further discrimination would be an advantage. Other disadvantages of this method are (i) the 48 h required to obtain a result; (ii) the need to remove the producer strain growth before application of the indicator strains, which is a messy and time-consuming procedure; (iii) the inability to reliably type mucoid strains of *P. aeruginosa*, which have become a serious problem in respiratory infections in patients with cystic fibrosis; we have previously described a modified pyocin typing method for mucoid *P. aeruginosa* (18), but the method involves preparation of pyocin-containing extracts from aerated broth cultures, and although simple to perform, it is time consuming and labor intensive; and (iv) the fact that the technique, as first described in 1966 (4), did not distinguish between the different classes of pyocin produced by *P. aeruginosa*, i.e., the particulate R- and F-pyocins (5, 6) and the diffusible S-pyocins (9). It has been suggested (7) that the ability to distinguish between particulate and nonparticulate pyocins on the basis of inhibition zone size would provide additional valuable strain discrimination in pyocin typing.

Our continued studies on the production and detection of individual pyocins, including their production by mucoid *P. aeruginosa*, have led to the reduction or elimination of these disadvantages and, thus, to significant improvements in the speed, sensitivity, and application of pyocin typing for epidemiological purposes.

This paper follows a preliminary report (J. A. M. Fyfe and J. R. W. Govan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C35, p. 317) and describes the development and evaluation of a revised technique for pyocin typing. In the revised technique, the use of the original 13 indicator strains and the inhibition patterns previously described (7) are retained. The main practical modifications to the previously described cross-streaking technique (7) are that the test strains are applied by a spotting method and the indicator strains are incorporated in agar overlays without prior removal of the test strain growth.

MATERIALS AND METHODS

Bacterial strains. Fifty pyocinogenic strains of *P. aeruginosa* were employed initially to compare the pyocin typing results obtained by the standard cross-streaking method and a modified spotting method. Thereafter, an additional 500 clinical isolates of *P. aeruginosa*, including mucoid strains, were used to evaluate the use of the spotting method.

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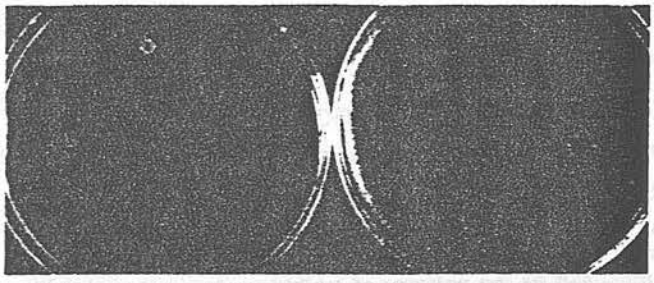

FIG. 1. Examination for pyocin activity against indicator strain 8 in four strains of *P. aeruginosa* by the spotting method. The left plate shows the test strains A, B, C, and D after 6 h of incubation at 30°C and before addition of the indicator strain. The right plate shows the inhibition zones produced by those strains after addition of the indicator strain in an agar overlay and subsequent incubation for 18 h at 37°C. Strain A shows no pyocin activity (−); strain B shows S-pyocin activity (+) characterized by an inhibition zone extending beyond the area of original growth; strain C shows a restricted inhibition zone characteristic of R- and F-pyocins (+); strain D shows a mottled inhibition zone typical of phage activity (-Ø).

Pyocin typing by the cross-streaking method. Pyocin typing by the cross-streaking method was carried out as previously described (7). As suggested in this review, the recognition of S-pyocin activity was incorporated into the typing scheme. Thus, a test strain was allotted to a pyocin type not only on the basis of the pattern of inhibition observed against the 13 standard indicator strains, but it was further characterized by noting the presence of classic S-pyocin activity which causes a zone of inhibition that extends beyond the original growth area of the producer strain. Hence, for example, a strain of pyocin type 1/a producing S-pyocin against indicator strains 7 and B is designated type 1/a ($S_{7,B}$).

Pyocin typing by the spotting method. Strains of *P. aeruginosa* to be typed were streaked for single colonies onto nutrient agar (Columbia agar base; Oxoid Ltd., Basingstoke, London, England) and incubated at 37°C overnight. The single colonies that arose from each test strain were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1 ml of sterile physiological saline (absorbance at 550 nm, ≈0.5).

A multipoint inoculator (model A400; Denley Instruments Ltd., Sussex, England), incorporating 21 stainless steel pins (one being a marker pin; diameter of each pin, 2 mm; pins were set 16 mm apart), was used to dispense 1-μl volumes of the bacterial suspensions onto a set of 13 plates (diameter, 90 mm) each containing 10 ml of tryptone soy agar (Oxoid). In this way, 20 test strains could be typed simultaneously against each indicator strain. After the spots dried, usually within a few minutes, the plates were incubated at 30°C for 6 h. Filter paper disks (5 cm; Whatman, Inc., England) were impregnated with chloroform, and the plates were placed over the disks for 15 min to allow the chloroform vapor to kill the bacteria. The plates were then exposed to air for an additional 15 min to eliminate residual chloroform vapor. Cultures of the indicator strains, grown without agitation in nutrient broth (Oxoid no. 2) for 4 h at 37°C to a population size of approximately 10^7 organisms per ml, were applied to the plates by adding 0.1 ml of each bacterial indicator culture to 2.5 ml of molten, semisolid agar (1% peptone; Difco Laboratories, Detroit, Mich., in 0.5% agar; Oxoid L 11) held at 45°C and poured as overlays (NB, a separate indicator strain, was applied to each plate). When the overlays had set, the plates were incubated for 18 h at 37°C, and the pyocin types were determined, as with the cross-streaking method, on the basis of the indicator strains inhibited. T size of the inhibition zones was also taken into account the purpose of more detailed strain comparison (Fig. 1) a determination of S-pyocin activity incorporated into typing results as in the cross-streaking method.

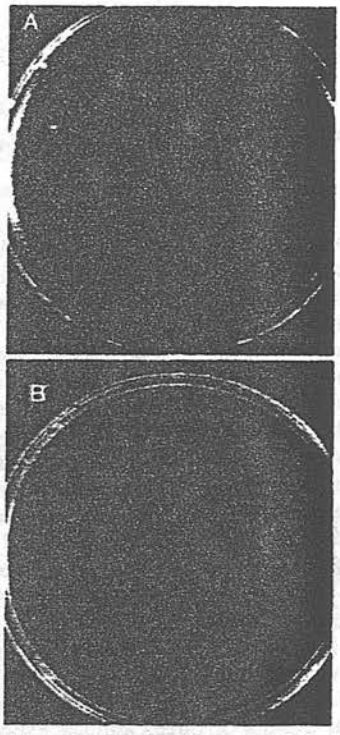

FIG. 2. Typical routine typing plates obtained by the spot method. (A) Indicator strain 5; (B) indicator strain B.

RESULTS

Detection of pyocin activity by the spotting method. Incu tion of the test strains at 30°C on tryptone soy agar for resulted in detectable pyocin production on the basis of cl zones of inhibition of an indicator strain (Fig. 1). S-ty pyocin production could be distinguished from R- and types on the basis of zone size. The area of growth af inoculation with a multipoint inoculator had a diameter c mm. Inhibition zones due to R- or F-type pyocins rang from 5 to 7 mm in diameter and had a sharp edge. S-ty pyocins produced zones of 9 to 12 mm in diameter, and the often had a diffuse edge. Phage activity could generally distinguished from pyocin activity, as the former gave rise a mottled inhibition zone. Two typing plates on which t same 20 test strains were overlaid with indicator strain (Fig. 2a) and indicator strain B (Fig. 2b) are shown. Comp ison of test strains grown on the same set of plates is eas and more meaningful than comparison of those grown individual plates, as in the cross-streaking method.

Comparison between spotting and cross-streaking metho For comparative purposes, 50 strains of *P. aeruginosa* we typed by both methods. Twenty-seven strains gave identi results, including the detection of S-type pyocin activi Nine strains gave the same pyocin type, although the cro streaking method failed to detect S-pyocin activity agai one or more indicators. Fourteen strains gave a differe pyocin type by the two methods. In 12 strains, pyoc activity was detected by the spotting method, which failed

be detected by the cross-streaking method, and in the remaining 2 strains pyocin activity was detected by the cross-streaking but not the spotting method.

Value of S-pyocin production as a further epidemiological discriminator. An additional 500 clinical isolates of *P. aeruginosa* were pyocin typed by the spotting method. The majority of these strains (99%) were typable, and 74% produced S-pyocin activity against 1 or more of the 13 indicator strains. All indicators showed some sensitivity to S-pyocins, and 63 different "S-type patterns" were distinguished. The most common of these were S_5 (11.3% of those strains with S-pyocin activity), $S_{5,8}$ (9.7%), S_7 (8.9%), and $S_{7,8}$ (8.6%).

As an example of the improved discrimination provided by recognition of S-pyocin activity, 34 isolates of *P. aeruginosa*, belonging to the common pyocin type, 1/b, could be divided into 10 distinct groups on the basis of their S-pattern.

Typing of mucoid strains by the spotting method. Thirty mucoid strains of *P. aeruginosa* isolated from the sputa of six patients with cystic fibrosis were pyocin typed by the spotting method. These included multiple isolates from individual sputa. All 30 strains gave clear typing patterns, with 13 strains producing S-pyocins. Multiple isolates from five of the patients were shown to be of the same type (different types for each patient), whereas the sixth patient harbored strains of two distinct types, 29/f(S_5) and 13/k.

DISCUSSION

The revised spotting method of pyocin typing described in this report has advantages over the cross-streaking method previously described (7). (i) The time required to obtain a typing result is reduced from 48 to 24 h. (ii) Up to 20 isolates can be directly compared on the same set of typing plates. This is ideal for comparative typing of multiple colonies from a single specimen to investigate mixed-type infection or to compare isolates from a single epidemic outbreak. (iii) Inhibitory activity due to S-pyocins can be more readily distinguished from R- and F-pyocin activity than when the cross-streaking method is used, thus providing greater discrimination in epidemiological studies. In our study, the epidemiological value of including detection of S-pyocin activity has been emphasized not only by the increased discrimination which it provides but also by the high frequency of S-pyocin production (74%) observed in the 500 test strains examined. This incidence is higher than that reported in a study carried out in India (38%); in the latter survey, however, only 29 strains were examined, and the methods for pyocin production and detection included different cultural conditions and indicator strains (15). (iv) There is no requirement to remove producer strain growth before application of the indicator strains; hence, the method is less tedious and time consuming. Similarly, the application of the indicator strains in agar overlays rather than as cross-streaks is more efficient. (v) Finally, the spotting method is more suitable for typing mucoid *P. aeruginosa*.

An earlier spotting method for pyocin studies described by Kageyama, in which different cultural conditions were used (10), included induction of pyocin production by exposure of the producer strain to UV light. We made a comparative study of six standard reference strains with a range of UV doses and found that under the conditions of the revised typing technique described in this report, no significant advantage was gained for typing purposes by inclusion of an induction stage (unpublished data). In addition, for typing purposes, an induction stage to enhance pyocin production in apparently apyocinogenic strains and thus to reduce the number of untypable strains was unnecessary due to the very low incidence of such strains (1%) found in our survey.

In a wider context, the question remains as to which is the most suitable typing system for epidemiological studies of *P. aeruginosa*, and realistically, is any one system adequate? Despite the improvements in pyocin typing described in this paper, the method still does not match the rapidity of the other most-suitable typing method, serotyping.

P. aeruginosa is serologically heterogeneous, and identification of group-specific heat-stable lipopolysaccharide antigens by agglutination forms the basis of O-serotyping procedures. Several systems have been described and their use reviewed (2, 11, 14). O-serogroup sera are available commercially, but they are expensive and the most widely used system (Difco) requires a set of antigen suspensions for characterizing the sera. In addition, the sera can only be purchased as a complete set of 17 sera.

A major disadvantage of O-serotyping is that the discriminatory power is only fair (3); further discrimination can be provided by detection of H-antigens, but the procedures for H-typing are beyond the scope of many laboratories (14). The typability of *P. aeruginosa* by O-serological typing is usually over 90%, but serotyping is often unsatisfactory for mucoid *P. aeruginosa* in which O-antigens may be masked, typing of colonial dissociants in which serological changes occur within a single culture, and typing polyagglutinable *P. aeruginosa*. The latter, together with mucoid *P. aeruginosa*, forms less than 5% of clinical isolates but is frequently observed in patients with cystic fibrosis (13).

By our use of the revised pyocin typing technique described in this report in combination with O-serotyping it is concluded that neither system provides all the requirements of the ideal typing system for *P. aeruginosa* (unpublished data). We suggest that both systems offer significant contributions to epidemiological studies. O-serotyping provides a rapid indication of antigenic differences when these occur. In an epidemic situation, however, the value of serotyping is limited unless the strains isolated belong to unusual serotypes. Pyocin typing, as described in this paper, requires a period of 24 h to achieve a result but provides adequate discrimination on which to base more confident epidemiological judgment. We suggest that this revised technique provides an improved method for both epidemiological studies of *P. aeruginosa* and for basic studies on the wide range of pyocin activity which can be found in this species.

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respiratory tract infections. In fact 95% of positive results both clinically and bacteriologically were obtained. No difference was observed between the 2 administration groups. Though only a small number of patients were treated with a single daily dose, the results reached encourage one to consider the single 2 g injection of cefotetan as an effective antibiotic dosage schedule for respiratory tract infections. Moreover, cefotetan was well-tolerated and no clinical or biological side effects related to the treatment were observed.

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In Vitro Activity of Ciprofloxacin Against Pseudomonas Strains Isolated from Patients with Cystic Fibrosis and Preliminary Treatment Results

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INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease primarily of exocrine glands and is one of the most lethal inherited childhood disorders. The susceptibility of CF patients to chronic respiratory infections due to particular bacterial pathogens has been recognised since the first descriptions in 1946. Today, the vicious cycle of recurrent exacerbations and progressive pulmonary deterioration has assumed even greater prominence as the major influence on mortality and quality of life in CF patients.

Pseudomonas aeruginosa is the most troublesome respiratory pathogen in CF patients. Clinical and bacteriological studies indicate that initial colonization occurs with a classic non-mucoid strain. Subsequently, mucoid alginate-producing mutants emerge and become predominant with concomittant pulmonary deterioration and poor prognosis[1]. *In vitro*, mucoid *P. aeruginosa* are usually slightly more resistant to aminoglycoside and β-lactam antibiotics than related non-mucoid strains[3,5]. Infections due to mucoid *P. aeruginosa* are notoriously intractable despite aggressive therapy with high dosages of antibiotics which appear appropriate on the basis of *in vitro* sensitivity tests. Once established, the organisms may be reduced in numbers but are seldom eradicated. Available evidence suggests that the failure to eliminate bacteria is not due to bacterial resistance but to poor penetration of antibiotics into pulmonary sites and the ability of mucoid *P. aeruginosa in vivo* to form bacterial microcolonies embedded in a gelled alginate matrix. In CF patients, acquired antibiotic resistance is seldom a problem. In contrast, most CF patients with advanced chronic infection, harbour mucoid *aeruginosa* which contain mutations (*bls, tps*) conferring enhanced sensitivity to β-lactams and trimethoprim[2,4], (Goven,

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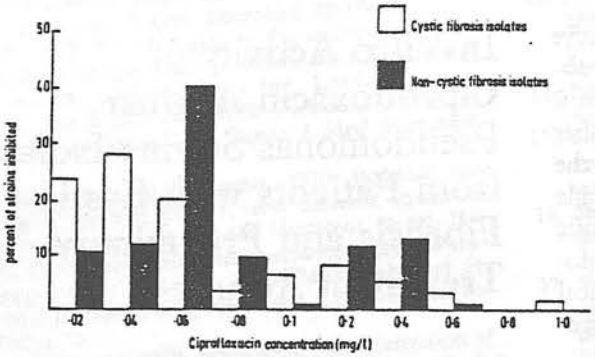

Figure 1 - Comparative *in vitro* activity of ciprofloxacin against 60 cystic fibrosis and 188 non-cystic fibrosis isolates of *Pseudomonas aeruginosa*.
Medium. DSTA (Oxoid). Inoculum 10^4.

shed data). Our *in vitro* and *in vivo* studies of the new oral quinolone derivative ciprofloxacin (Bay 09867, Bayer) indicate it to have high antipseudomonal activity and to be a potential new therapeutic agent in the management of respiratory infections in patients with CF.

RESULTS

Comparative in vitro activitity of ciprofloxacin against cystic fibrosis and non-cystic fibrosis isolates of P. aeruginosa

The very high degree of *in vitro* activity of ciprofloxacin against 60 CF and 188 non-CF isolates of *P. aeruginosa* is shown in *Figure 1*. The MICs for the non-CF isolates in particular also presented a bimodal distribution (mode 0.06 and 0.3 mg/l). No correlation was found between a particular non-CF population and resistance to other antibiotics; all 188 non-CF isolates had MICs for tobramycin and ceftazidime of <1.0 and <5.0 mg/l respectively. The cumulative MICs for the CF isolates were slightly less than the corresponding values for non-CF isolates at most concentrations of ciprofloxacin investigated between 0.02 and 1.0 mg/l (e.g. MIC^{50} 0.04 and 0.06 mg/l respectively). Although the differences in MICs were small they are interesting, and perhaps unexpected, in view of the fact that most CF isolates were of mucoid strains and all patients from whom the strains were isolated had received frequent antibiotic therapy with a range of antipseudomonal agents.

Influence of mutations involving hypersensitivity (bls, tps) and alginate biosynthesis (muc) on the sensitivity of P. aeruginosa to carbenicillin (Beecham), nalidixic acid (Sterling) and ciprofloxacin (Bayer)

It was considered that a possible explanation for the enhanced sensitivity of CF isolates in general might be that many CF isolates of *P. aeruginosa* contain *bls* and *tps* mutations which result in enhanced sensitivity to a number of antibiotics and also that sensitivity to ciprofloxacin might be unaffected by the *muc* mutation which is responsible for alginate biosynthesis. *Table 1* shows how the presence of *bls* in the CF isolate 492c increases sensitivity to carbenicillin by a factor of 130 but also increases sensitivity to the quinolones nalidixic acid (30*) and ciprofloxacin (2.5*). Transfer of *bls* from 492c into PAO8 increases sensitivity of the recombinant strain PAJ2 to all three antibiotics; transfer of *tps* increases

TABLE 1 - *Inlfluence of mutations involving hypersensitivity (bls, tps) and alginate biosynthesis (muc) on the sensitivity of P. aeruginosa to carbenicillin, nalidixic acid and ciprofloxacin*

Strain	Description*	MIC (mg/l) Carbenicillin	Nalidixic acid	Ciprofloxacin
492a	CF isolate, *muc, tps*	80	60	0.1
492c	CF isolate, *muc, bls, tps*	0.6	2	0.04
PAO8	nonmucoid	40	80	0.08
PAJ1	*bls, tps* recombinant from cross 492c *leu*⁻R68.45 × PAO8	0.4	5	0.04
PAJ2	*bls* recombinant from cross 492c *leu*⁻R.68.45 × PAO8	0.4	20	0.04
PAJ3	*tps* recombinant from cross 492c *leu*⁻R.68.45 × PAO8	40	20	0.04
PAO381	nonmucoid	40	80	0.08
PAO579	mucoid (*muc*) derivative of PAO381	60	80	0.08

Medium DSTA (Oxoid); Inoculum 10^4; *tps* sensitivity to trimethoprim.
* References: Fyfe, J.A.M. & Govan, J.R.W. Journal of General Microbiology *119*, 443-450, 1980 and *130*, 825-834, 1984.

sensitivity to the quinolones but not to carbenicillin whilst the transfer of both *bls* and *tps* further enhances sensitivity to nalidixic acid but not to cpirofloxacin or carbenicillin.

Clinical studies of 13 courses of oral ciprofloxacin in five patients with cystic fibrosis

The five CF patients treated with oral ciprofloxacin had long-established and intractable respiratory colonization (viable counts 10^8 bacteria/ml sputum) due to *P. aeruginosa* (4 patients) or *Pseudomonas cepacia* (1 patient, MIC 0.6 mg/l). All five patients had previously received multiple courses of tobramycin, azlocillin or ceftazidime either singly or in combination and by i.v. or aerosol ad ministration. A series of 13 courses of oral ciprofloxacin was given with dosages ranging from 100 to 500 mg t.d.s. and duration from seven to 46 days. The results of these treatments can be summarised as follows:

1) Each patient showed obvious clinical improvement in activity and respiratory function and average weight gains of 2.5 kg over 14 days. Quantitative counts of *Pseudomonas* showed reduction to between 10^6 and 10^3 organisms/ml and in one patient *P. aeruginosa* was eradicated.

2) The social and therapeutic benefits of oral ciprofloxacin were considerable in comparison with i.v. or aerosolised antibiotics. The drug was acceptable to all of the patients and no serious side-effects including joint pain (one patient had minor heartburn) was observed even on prolonged administration at a dosage of 500 mg t.d.s.

3) In two patients the strain of *P. aeruginosa* isolated prior to treatment showed a 10* and 16* increase in resistance to ciprofloxacin at the completion of treatment (MIC values increasing from 0.06 to 0.6 and from 0.06 to 1.0). This resistance disappeared, however, after cessation of treatment and the original sensitivity was observed after 30 days.

CONCLUSION

Ciprofloxacin shows a very high degree of activity against both CF and non-CF strains of *P. aeruginosa*. Our preliminary clinical studies in five CF patients with long-established pseudomonas colonization demonstrated a reduction in bacterial numbers, clinical improvement and no serious side-effects. We conclude that oral ciprofloxacin will prove beneficial in the treatment of exacerbations of respiratory infection due to mucoid *P. aeruginosa* in CF patients. We also speculate, however, that a valuable potential future role for oral ciprofloxacin might be in the control of early colonization with classic non-mucoid *P. aeruginosa*.

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Influence of Antibiotics, Alginate Biosynthesis and Hypersensitivity Mutations on *Pseudomonas* Virulence Factors Associated with Respiratory Infections in Patients with Cystic Fibrosis

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Factors influencing production of pseudomonas proteases were studied to improve understanding of the pathogenesis and management of pseudomonas respiratory infections in patients with cystic fibrosis. Alginate appeared to slow the release of protease from mucoid P. aeruginosa. Protease production was suppressed by the hypersensitivity mutation tps and by growth in subinhibitory, concentrations of antibiotics particularly the 4-quinolone ciprofloxacin. Protease suppression also occurred in subinhibitory concentrations of acriflavine, acridine orange and in ciprofloxacin resistant mutants of PAO 1 suggesting that one mechanism of antibiotic-induced protease suppression involves interference with DNA-gyrase.

Pseudomonas aeruginosa is now the most troublesome respiratory pathogen in patients with cystic fibrosis (CF). Infections due to mucoid, alginate-producing mutants are notoriously intractable despite aggressive therapy with high dosages of antibiotics which appear appropriate on the basis of in vitro tests but which in vivo seldom reach lethal concentrations for the infecting bacteria. Nevertheless, even when bacterial numbers isolated from sputa are not reduced, many patients show clinical improvement. We sought evidence to explain this phenomenon by investigating the influence of subinhibitory concentrations of antibiotics on pseudomonas proteases which are held to be virulence factors in chronic lung infections. Evidence suggests that pathogenesis of respiratory infection in CF patients involves growth of mucoid pseudomonas within protective alginate microcolonies and that tissue damage is due, in part at least, to the action of bacterial exoenzymes and an overexuberant immune response (1). We also took into account the possible influence on protease activity of mutations responsible for alginate biosynthesis (muc) and antibiotic hypersensitivity (bls, tps) which are found in many CF isolates of P. aeruginosa. The CF isolates 492a (muc, tps) and 492c (muc, bls, tps) together with isogenic derivatives of the PAO series were used (2). The influence of antibiotics was studied using PAO 1 and fifty non-mucoid clinical isolates of P. aeruginosa. Protease activity was investigated using agar diffusion assays in tryptone soy agar with skimmed milk or elastin as substrate and spectrophotometrically based on dye release from orcein-elastin.

RESULTS

In agar diffusion assays with skimmed milk or elastin substrates, little protease activity was observed with mucoid P. aeruginosa 492a, 492c and PAO 579 after 24 h incubation; activity gradually appeared on further incubation. In contrast, non-mucoid revertants of these isolates showed large zones of proteolysis similar to controls within 24 h. When the protease activity of the non-mucoid strain PAO1 was investigated there was a linear reduction in the zone of proteolysis when 0, 0.5, 1 and 2% sodium alginate was incorporated into the medium.

Studies of the proteolytic zone diameters in revertants of 492a and 492c showed a 50% increase in 492c suggesting that the bls mutation might enhance protease release. However, further studies using recombinants of PAO 8 obtained from crosses 492c leu.R68.45 x PAO 8 indicated that tps reduced zone diameters by one third when transferred to PAO 8 alone or in combination with bls. Of fifty clinical isolates of P. aeruginosa, 92% showed reduced or no proteolytic activity in the presence of ciprofloxacin at concentrations less than 50% of the MIC value; fewer strains showed this antibiotic-induced suppression with subinhibitory concentrations of tobramycin (60%), azlocillin (42%) or ceftazidime (24%). With individual antibiotics the degree of protease suppression was strain specific and independent of the MIC value. This phenomenon could be strikingly illustrated for ceftazidime by growth of two strains with similar MIC values in agar medium containing a gradient of ceftazidime at subinhibitory concentrations. Whilst one strain showed linear reduction in the protease zone until no zone was observed, the second strain showed no reduction in protease activity. Suppression was most obvious with ciprofloxacin; in some strains reduction in proteolytic zones commenced at concentrations as low as 5% of the MIC value. In strain PAO 1 there was also a linear decrease in zone diameter in the presence of subinhibitory concentrations of acridine orange and acriflavine. In addition, a ciprofloxacin resistant mutant of PAO 1 (MIC value raised from 0.06 to 0.6 mg/l) produced a proteolytic zone only 25% the diameter of the parent strain. The effect of antibiotic-induced protease suppression could not be simply explained by growth inhibition. In quantitative assays using dye release from orcein-elastin and relating elastase activity to the same $\log_{10}$ value of CFU, subinhibitory concentrations of ceftazidime had little or no effect on viable counts yet elastase production was less than 50% of the control value. These experiments however, indicated that the effect of subinhibitory concentrations on protease production could be complex. In some strains protease production could be both suppressed at one subinhibitory concentration and enhanced by a lower subinhibitory concentration.

CONCLUSIONS

In vitro, protease activity in P. aeruginosa is influenced by bacterial characteristics associated with P. aeruginosa isolates from CF patients, and by subinhibitory concentrations of antibiotics. Alginate appears to interfere with the release of protease from mucoid strains but in vivo could concentrate the protease and localise its slow release at the bronchial surface. Protease suppression by the tps mutation is particularly interesting since this mutation has other functions (including suppression of phage replication) and is itself inactivated by subinhibitory concentrations of ciprofloxacin (unpublished results). Our results of antibiotic-induced protease suppression confirm the few other reports on this topic (3,4) and suggest that this is an important topic for future study at both a microbiological and clinical level. It seems likely that the superior protease suppression by ciprofloxacin results from its interference with DNA-gyrase and thus conceivably interference with gene expression. In clinical terms, these results suggest that future assessments of antibiotic therapy in CF patients and the rationale for the choice of antibiotic might beneficially not be restricted solely to antibacterial activity.

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Typing of *Pseudomonas cepacia* by Bacteriocin Susceptibility and Production

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The significance of *Pseudomonas cepacia* as an opportunistic pathogen in immunocompromised patients has become increasingly recognized. Particularly disturbing is its increased incidence, reported by several North American centers, in respiratory tract cultures from patients with cystic fibrosis. Epidemiological studies of *P. cepacia* have been hampered by a lack of typing methods. In this paper we report the development of a typing scheme based on bacteriocin production and susceptibility. For bacteriocin production, test isolates of *P. cepacia* were rapidly applied to the surfaces of agar plates with a multiple inoculator. After incubation of these test isolates for 5.5 h and their exposure to chloroform, indicator strains were applied in agar overlays without prior removal of the test strain growth. After 18 h of incubation, inhibition zones caused by bacteriocin activity were recognized. A similar procedure was used to examine the bacteriocin susceptibility of the test strain. The bacteriocin type of the test strain was defined based on its bacteriocin production as judged by zones of inhibition against a set of eight indicator strains and by susceptibility or resistance of the test strain to bacteriocin produced by six producer strains. Of 373 strains of *P. cepacia*, 95.2% were typed into a total of 44 type combinations. Bacteriocin typing provided a suitable procedure for epidemiological studies of colonization or infection by *P. cepacia*. The technique described in this paper was simple to perform, gave a result within 24 h, provided good strain discrimination, and was suitable for clinical, environmental, and phytopathogenic strains.

Pseudomonas cepacia is arguably the most nutritionally versatile species of the genus *Pseudomonas* and the species most resistant to antibiotics, antiseptics, and disinfectants (3, 12, 14). The organism is even able to utilize penicillin G as a sole carbon source (2). Isolation of *P. cepacia* from clinical specimens and hospital environments, particularly from wet surfaces, equipment, pharmaceutical solutions, and antiseptics, has became increasingly common, and the presence of *P. cepacia* in these areas has been shown to lead to colonization and infection (13, 15, 16). The virulence of *P. cepacia* is low, and most clinical isolations are not associated with symptoms, but in 1980, Randall (15) emphasized that the species "... could become a potentially lethal pathogen in a compromised patient." Subsequently, this observation was strikingly confirmed when several centers in North America noted an increasing percentage of *P. cepacia* in respiratory tract cultures from patients with cystic fibrosis; the incidence was highest either at postmortem examination or immediately before death (L. L. Mackenzie and P. H. Gilligan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C34, p. 317), and infection with *P. cepacia* had become a major clinical problem (9). Epidemiological investigations and judgments have been hampered by a lack of suitable typing or "fingerprinting" methods for *P. cepacia*. Gonzalez and Vidaver (5) divided 30 strains into two distinct groups based on differences in bacteriocin production and in maceration of onion slices; all six clinical isolates were negative for these characteristics. Recently, a serotyping system has been described (8) which allows differentiation of 282 isolates into seven somatic (O) and five flagellar (H) groups, with 98.5% of strains being typable by this method. To achieve greater discrimination, we developed an additional or alternative typing system based on susceptibility to and production of bacteriocins. The technique described in this paper was simple to perform and allowed differentiation of 373 isolates of *P. cepacia* into 44 cepaciacin types, with 95.2% of strains being typable by this method.

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MATERIALS AND METHODS

Bacterial strains. A total of 243 pseudomonads, consisting of 130 *P. cepacia* (including ATCC 25416, 25609, and 17759), 98 *P. fluorescens*/*P. putida* (including NCIB 1007 and 10525), 10 *P. maltophilia* (*Xanthomonas maltophilia*; including ATCC 13637), and 1 each of *P. mendocina* (NCIB 10541), *P. diminuta* (ATCC 11568), *P. pseudoalcaligenes* (NCIB 9946), *P. testosteroni* (NCIB 9393), and *P. stutzeri* (NCIB 9040), were used to select suitable producer and indicator strains which either produced bacteriocins active against *P. cepacia* or were susceptible to the bacteriocins produced by strains of *P. cepacia* (Table 1). The 373 strains of *P. cepacia* which were typed represented one strain from each patient, plant, or environmental specimen. These strains had also been isolated from different geographical locations and included strains generously provided by H. Monteil, Institut de Bacteriologie de la Faculte de Medicin de Strasborg, Strassburg, France; J. D. Klinger, Rainbow Babies Childrens Hospital, Cleveland, Ohio; A. King, St. Thomas Hospital, London; and T. Pitt, Public Health Laboratory, Colindale, London. The identity of the strains was confirmed with the arginine glucose medium of Stewart (17), the API 20NE system (Analytab Products, Plainview, N.Y.), and the Uni-N/F-Tek multiple medium circular plate (Flow Laboratories, Inc., McLean, Va.). *P. aeruginosa* 430 and *P. aeruginosa* indicator strains 5 and 6 were from the laboratory collection (6), and strains PAO3047 and PML15161 (11) were kindly supplied by M. Kageyama, Mitsubishi-Kasei Institute of Life Sciences, Tokyo.

TABLE 1. Producer and indicator strains for bacteriocin typing of *P. cepacia*

Strain type and no.	Strain name	Source (reference)
Producer (CP)		
1	*P. cepacia* JC2346	Govan and Harris
2	*P. cepacia* B12417	H. Monteil
3	*P. cepacia* B68061	H. Monteil
4	*P. fluorescens* NPF26	Govan and Harris
5	*P. cepacia* 18979	H. Monteil
6	*P. cepacia* 77-16	J. Klinger
Indicator (CS)		
1	*P. cepacia* B12290	H. Monteil
2	*P. cepacia* PC8604	H. Monteil
3	*P. fluorescens* NPF26	Govan and Harris
4	*P. cepacia* B68303	H. Monteil
5	*P. cepacia* B68061	H. Monteil
6	*P. cepacia* G2	Govan and Harris
7	*P. putida* PAW85	Bayley et al. (1)
8	*P. cepacia* D36	H. Monteil

Typing of *P. cepacia* by production of bacteriocin. The method used for typing *P. cepacia* by bacteriocin production was based on our technique for pyocin typing of *P. aeruginosa* (4). The strains of *P. cepacia* to be typed were streaked for single colonies onto nutrient agar (Columbia agar base; Oxoid Ltd., Basingstoke, London) and incubated at 30°C overnight. The single colonies that arose from each test strain were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1 ml of sterile physiological saline (absorbance at 550 nm, 0.5).

A multipoint inoculator (model A400; Denley Instruments Ltd., Sussex, England), incorporating 21 stainless-steel pins (one being a marker pin; diameter of each pin, 2 mm; pin distance apart, 16 mm), was used to dispense 1-μl volumes of the bacterial suspensions onto a set of eight plates (diameter, 90 mm) each containing 10 ml of tryptone soy agar (Oxoid). In this way 20 test strains could be typed simultaneously against each indicator strain. After the spots dried, usually within a few minutes, the plates were incubated at 30°C for 5.5 h. Filter paper disks (5 cm; Whatman, Inc., England) were impregnated with chloroform, and the plates were placed over the disks for 15 min to allow the chloroform vapor to kill the bacteria. The plates were then exposed to air for an additional 15 min to eliminate residual chloroform vapor. Cultures of the indicator strains, grown without agitation in nutrient broth (Oxoid no. 2) for 4 h at 30°C to a population size of approximately 10^7 organisms per ml, were applied to the plates by adding 0.1 ml of each bacterial indicator culture to 2.5 ml of molten, semisolid agar (1% Proteose Peptone [Difco Laboratories, Detroit, Mich.] in 0.5% agar [Oxoid L11]) held at 45°C and poured as overlays (a separate indicator strain was applied to each plate). When the overlays had set, the plates were incubated for 18 h at 30°C, and the cepaciacin producer type (P1, 2, etc.) was determined by the patterns of inhibition produced on the eight indicator strains (Table 2, Fig. 1).

Typing by susceptibility of the test strain to bacteriocin. The technique used to type strains by their susceptibility to bacteriocin was similar to that described above for typing by bacteriocin production except that the test strain was prepared as described for an indicator strain and that its susceptibility to the bacteriocins produced by six standard producer strains (CP1 through CP6) was determined. The cepaciacin susceptibility types (S1, 2, etc.) were determined by the susceptibility pattern of the test strain against the standard producer strains (Table 3).

Cepaciacin susceptibility to trypsin. Trypsin (Difco) was incorporated into molten tryptone soy agar held at 45°C at a concentration of 50 mg/ml. The influence of trypsin on the bacteriocin activity of the six producer strains (CP1 through CP6) was investigated. Controls included non-trypsin-containing medium and strains of *P. aeruginosa* producing both trypsin-resistant and trypsin-sensitive bacteriocins. *P. aeruginosa* 430 produces the phage-tail-like, trypsin-resistant pyocins F1 and R2, which are active against *P. aeruginosa* indicator strains 5 and 6, respectively (6, 11); *P. aeruginosa* PAO3047 produces the trypsin-sensitive pyocin S2, which is active against strain PML15161.

Production and induction of cepaciacin in liquid medium. The medium used was sodium glutamate broth (10). Of an overnight culture of the bacteriocinogenic strain, 1 ml was added to 20 ml of sterile broth and incubated in a 100-ml flask in an orbital incubator (model 1H460; Gallenkamp, London) at 140 rpm for 2 h at 30°C. Mitomycin C was added to a final concentration of 5 μg/ml, and incubation was continued for an additional 2 h. The culture was centrifuged at 1,430 × *g*

TABLE 2. Inhibition patterns of cepaciacin producer types of *P. cepacia*

Cepaciacin producer type (P)	Inhibition of indicator strain (CS) no.[a]:							
	1	2	3	4	5	6	7	8
1	+	−	−	+	−	+	+	−
2	−	−	+	−	+	+	−	−
3	−	−	−	+	+	+	+	−
4	+	−	+	+	+	+	−	+
5	−	+	−	−	+	+	−	+
6	+	+	−	−	+	+	−	+
7	+	−	−	+	−	−	−	−
8	−	−	−	+	−	−	−	−
9	−	−	−	−	−	+	−	−
10	−	−	−	−	−	+	−	+
11	+	−	−	−	−	−	−	−
12	+	−	−	−	−	+	−	−
13	+	−	−	+	−	+	−	−
14	+	−	+	+	−	+	−	−

[a] +, Inhibition; −, no inhibition.

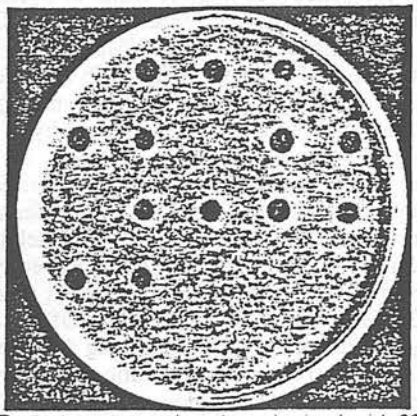

FIG. 1. Typical routine typing plate obtained with 20 test strains of *P. cepacia* tested for bacteriocin activity against indicator strain CS1; 13 strains show inhibition of CS1.

for 30 min to remove bacterial cells, and the supernatant was membrane filtered (0.2 μm; Millipore Corp., London).

Assay of cepaciacin activity. The procedure used for the assay of cepaciacin activity was that described by Govan (7) for an assay of pyocin activity with *P. cepacia* CS1 used as the bacteriocin-susceptible strain.

Electron microscopy. Mitomycin C-induced cepaciacin preparations were centrifuged for 90 min at 95,000 × *g*, and the pellets were gently suspended in 1 M ammonium acetate (pH 7.0). Equal volumes of each suspension and 2% potassium phosphotungstate (pH 7.0) were mixed on the surface of a clean glass slide, and a drop of the mixture was transferred to the surface of a collodion-coated grid. After 30 s, excess fluid was removed, and the preparation was allowed to dry. Preparations were viewed with a Hitachi electron microscope at 50 kV.

RESULTS

Development of a bacteriocin typing technique. Initial studies carried out on 243 pseudomonads, including 130 isolates of *P. cepacia*, indicated that the strains of *P. cepacia* could be differentiated based on their production of and susceptibility to bacteriocins. Examples were also observed of the susceptibility of *P. cepacia* to bacteriocins produced by other pseudomonad species and, in turn, of the production of bacteriocins by *P. cepacia* strains active against other species. Based on these observations, a preliminary set of producer and indicator strains was assembled. Using these strains, we investigated the use of different media, temperatures, and periods of incubation to obtain optimal conditions for the production and detection of bacteriocins suitable for typing *P. cepacia*; the selected technique is described above. We next sought to improve strain discrimination and reduce the number of untypable strains by testing an additional 100 previously untested strains of *P. cepacia* for their ability to act as producer or indicator strains. By a process of elimination and selection, we assembled a final set of eight indicator strains (CS1 to CS8) and six producer strains (CP1 to CP6). The origin and description of these strains is given in Table 1.

TABLE 3. Inhibition patterns of cepaciacin susceptibility types of *P. cepacia*

Cepaciacin susceptibility type (S)	Inhibition pattern produced on test strain by producer strain (CP) no.[a]:					
	1	2	3	4	5	6
1	+	+	+	−	−	+
2	+	+	−	+	−	−
3	+	−	+	−	+	+
4	+	+	−	−	+	+
5	−	−	−	+	−	−
6	+	+	+	−	+	−
7	+	−	+	+	+	−
8	+	+	−	−	−	−
9	−	+	−	+	−	−
10	−	+	−	−	+	−
11	+	+	−	+	+	−
12	+	+	−	−	−	+
13	+	−	−	−	+	+
14	+	−	+	+	+	+
15	−	−	+	+	+	−
16	+	+	+	−	+	+
17	+	+	−	−	+	−
18	+	−	+	−	−	−
19	−	−	−	+	+	+
20	+	−	+	+	−	−
21	−	−	−	−	+	−
22	+	−	−	−	−	+
23	+	+	−	+	+	+

[a] +, Inhibition; −, no inhibition.

TABLE 4. Distribution of cepaciacin types of 186 nonepidemic *P. cepacia* isolates from human, environmental, and phytopathogenic specimens[a]

Cepaciacin type[b]	No. of strains[c] from:			Total no. (%)
	France	U.S.	U.K.	
S3/P0	27	8	1	36 (19.5)
S2/P0	10	4	9	23 (12.4)
S16/P0		15	1	16 (8.5)
S7/P6	12			12 (6.5)
S1/P0	7	3		10 (5.4)
S13/P0	5	2		7 (3.8)
S14/P6	7			7 (3.8)
S4/P13	4			4 (2.2)
S5/P4	1	1	2	4 (2.2)
S0/P4	2		2	4 (2.2)
S8/P0	3			3 (1.6)
S9/P11	3			3 (1.6)
S11/P13	3			3 (1.6)
S17/P13	3			3 (1.6)
S2/P11			2	2 (1.1)
S7/P5	1			1 (1.1)
S18/P0		1	1	2 (1.1)
S20/P0			2	2 (1.1)
S22/P0		1	1	2 (1.1)
S0/P0	10	3	3	16 (8.5)
Other types[d]	13	8	4	25 (13.4)

[a] Only one strain from each specimen or from each epidemic outbreak is represented.

[b] For example, a strain designated as type S7/P6 gives a susceptibility pattern 7 (Table 3) and a production pattern 6 (Table 2).

[c] France, from the collection of Dr H. Monteil; U.S., from the collection of J. Klinger; U.K., from T. Pitt and A. King and from strains isolated in our laboratory.

[d] One each of types S2/P13, S2/P3, S3/P6, S3/P8, S4/P1, S4/P12, S5/P0, S6/P13, S6/P0, S6/P9, S10/P13, S11/P12, S12/P13, S13/P9, S13/P10, S13/P14, S16/P7, S17/P7, S19/P0, S19/P4, S21/P0, S23/P12, S23/P6, S0/P2.

Bacteriocin typing of *P. cepacia*. In the collection of 373 isolates of *P. cepacia* investigated, a total of 23 susceptibility types (S1, S2, etc.) and 14 producer types (P1, P2, etc.) were distinguished. By combining the characteristics of bacteriocin susceptibility and production, we divided 95.2% of the isolates into a total of 44 type combinations with 19 types represented by more than a single isolate. The results of typing demonstrated epidemics of cross-infection among clinical isolates obtained from certain hospitals. For example, 128 strains of *P. cepacia* were recovered from 128 patients in an intensive care unit over a period, and all strains were cepaciacin type S3/P0. Similarly, all 30 strains recovered from 30 patients in a different intensive care unit were cepaciacin type S7/P6. The distribution of 186 nonepidemic *P. cepacia* isolates from human, environmental, and phytopathogenic specimens is shown in Table 4. Clinical, environmental, and phytopathogenic strains could be typed based on bacteriocin susceptibility and production.

Reproducibility of typing patterns. All 373 strains of *P. cepacia* were retyped after 6 months of storage at −70°C. In addition, 60 strains (maintained on minimal medium at 12 to 15°C) were typed on six occasions during this 6-month period. No differences were observed with strains maintained at low room temperature (12 to 15°C), and in the single retyping of the entire collection, only three strains showed a

pattern of production or susceptibility which differed from the original; in each instance this difference involved only a single reaction.

Nature of the bacteriocins of *P. cepacia*. When the cultural conditions of the typing technique were used, without exception every zone of inhibition observed in the typing of 373 strains of *P. cepacia* appeared as a narrow band extending only 1 to 2 mm from the outer edge of the producer growth, and each zone had a sharp edge (Fig. 1). Similar zones were observed with the R and F pyocins of *P. aeruginosa* 430, whereas the S pyocin of strain PAO3047 produced zones of 5 to 8 mm and had a diffuse edge. The exposure of the bacteriocins produced by the six *P. cepacia* producer strains, CP1 through CP6, to trypsin had no effect on their inhibitory activity; a similar result was observed in the case of the R and F pyocins of *P. aeruginosa* 430, whereas the inhibitory activity of the S pyocin of *P. aeruginosa* PAO3047 was completely lost in the presence of trypsin. When producer strains CP1 and CP2 were grown in sodium glutamate broth and their inhibitory activity was assayed against the indicator CS1, titers of activity were in the range of 8 to 16; in the presence of mitomycin C at 5 μg/ml, bacteriocin activity was induced to 256 titers. Titers of activity were taken as the highest dilutions of the bacteriocin preparation that gave clear zones of inhibition of the indicator strain. When bacteriocin-containing preparations were centrifuged at 95,000 × *g* for 90 min, bacteriocin activity was found to be sedimentable, with no residual activity remaining in the supernatant. Electron microscopy of the sedimented pellets revealed contractile, phage-tail-like bacteriocins resembling the R pyocins of *P. aeruginosa* (7). No evidence of plaques of inhibition due to phage activity was observed in the course of these assays.

DISCUSSION

In the early stages of this investigation, considerable difficulty was encountered in maintaining the viability of *P. cepacia* cultures during storage at 4°C. Although cultures remained viable as suspensions at −70°C in 10% skimmed milk (Oxoid), in contrast to our experience with *P. aeruginosa*, most *P. cepacia* died rapidly when maintained on nutrient agar slopes at 4°C. Since it is an advantage to maintain cultures for several days for experimental, typing, and transport purposes, it was necessary to find a suitable storage medium. We found that cultures of *P. cepacia* could be adequately maintained for at least 6 months at low room temperature (12 to 15°C) on synthetic minimal media: a suitable medium was the minimal medium of Vogel and Bonner (18).

The technique for bacteriocin typing of *P. cepacia* described in this report was simple to perform and required no special materials for its performance. Strain discrimination was good and an improvement on existing methods of typing. In vitro, both production of and susceptibility to bacteriocins were stable characteristics of *P. cepacia*. Other indices of reliability for a satisfactory typing technique are the constancy of type in replicate isolates obtained on different days from the same site in the same patient and the degree of uniformity of type in strains from the same epidemic focus. In the case of *P. cepacia*, evidence for such in vivo stability was difficult to acquire since the large collection of strains used to develop the technique had of necessity been collected over a period of years for a variety of purposes and since epidemiological data were not always available. However, in several patients colonized with *P. cepacia* over a period of 6 months, in vivo stability could be demonstrated. The second index of reliability was more strikingly demonstrated in the case of the Strassburg isolates, in which the high incidence of cepaciacin type S3/P0 and S7/P6 resulted from two epidemic episodes and, in each epidemic, the uniformity of the strains was confirmed by serotyping (8).

The good reproducibility of the typing results may be due to the typing procedure, which was developed from a similar technique which has been used successfully for the pyocin typing of *P. aeruginosa*. Cepaciacin typing, however, revealed several interesting comparisons regarding the bacteriocinogeny of these two pseudomonads. In the case of *P. aeruginosa*, production of bacteriocins is very common and found in over 95% of isolates (4). In contrast, production of bacteriocins by *P. cepacia* was less common and was observed in only 30% of isolates. Since the detection of bacteriocins requires the use of suitable indicator strains, it could be argued that the low bacteriocinogeny of *P. cepacia* isolates resulted from inadequate detection. This seems unlikely, however, since several unsuccessful attempts were made to detect bacteriocin activity in nonproducers by testing a large number of potential indicator strains and by induction with mitomycin C. Consequently, the typing of isolates based on bacteriocin production, as is the case with *P. aeruginosa*, was not found to be suitable for *P. cepacia*; therefore, to obtain maximum typability and discrimination, cepaciacin typing was based on both bacteriocin production and susceptibility. A second comparison of bacteriocinogeny between *P. aeruginosa* and *P. cepacia* concerns the nature of the bacteriocins per se. Under the conditions of the typing procedure, the type of inhibition zone observed with *P. cepacia* was without exception a narrow, sharp-edged zone, resistant to the presence of trypsin and similar to the zones produced by the trypsin-resistant, phage-tail-like R and F pyocins of *P. aeruginosa* (6). No evidence was found in *P. cepacia* of the wide, diffuse-edged zones resulting from low-molecular-weight S pyocins, which have been observed in over 70% of *P. aeruginosa* isolates (4). Electron microscopy of mitomycin C-induced preparations from each of the six producer strains of *P. cepacia* (CP1 through CP6) confirmed that the cepaciacins did indeed resemble the phage-tail-like, contractile R pyocins. Cepaciacin typing was suitable for clinical, environmental, and phytopathogenic isolates of *P. cepacia*. This observation appears to conflict with the report of Gonzalez and Vidaver (5), who found that only environmental and phytopathogenic isolates are bacteriocinogenic and then only in liquid media. The likely explanation for this discrepancy lies in the small number of clinical isolates investigated by these authors and the fact that the technique described in this report is probably more suited to the production and detection of cepaciacins.

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In Vivo Significance of Bacteriocins and Bacteriocin Receptors

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Bacteriocins are protein or protein-complex antibiotics produced by a wide variety of bacterial species. By conventional definition, bacteriocins differ from most other antibiotics in that the producer strain is immune to the action of its own bacteriocin and the inhibitory activity of individual bacteriocins is directed only to bacteria which are closely related to the strains which produce them. Bacteriocin production is regulated by plasmid or chromosomal elements and bacteriocin activity is initiated by adsorption of bacteriocin to specific outer membrane receptors on susceptible cells. In Darwinian terms, production of bacteriocin by a bacterial strain, within a particular ecological niche, could be considered advantageous by ensuring elimination of other closely related, and thus competitive, bacteria. In contrast, conservation of bacteriocin receptors appears suicidal if their only function is to initiate cell death. The paper will illustrate the ubiquity of bacteriocins and discuss evidence for their *in vivo* function in terms of bacterial survival. Evidence will also be presented to indicate that bacteriocin receptors in *Escherichia coli* and *Pseudomonas aeruginosa* have important alternative physiological functions in outer-membrane mediated nutrient uptake, particularly with respect to bacterial iron metabolism.

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INTRODUCTION

In general, bacteriocins are defined as protein or protein-complex antibiotics whose bactericidal activity is directed towards strains of the same or closely related species as the producer bacterium. Further properties which distinguish bacteriocins from other antibiotics include immunity of bacteriocinogenic strains to the lethal effect of their own bacteriocins and the role of cell surface receptors in the initiation of bacteriocin activity. In many bacterial species, bacteriocin synthesis is plasmid-mediated and regulated such that only a few cells in the culture actively produce bacteriocin, a process that often results in partial lysis. Such regulation can often be overcome by SOS-system inducing agents and thus many bacteriocins can be induced by UV irradiation or mitomycin C. The most intensively studied bacteriocins are undoubtedly the colicins produced by certain strains of *Escherichia coli* and other enterobacteria (1, 2). However, since the definition of a bacteriocin involves essentially functional properties, the term bacteriocin has come to be used to describe many bacterial antagonists observed in a wide variety of Gram-negative and Gram-positive bacteria. In addition to colicin V, originally described by Gratia in 1925, are now included various other colicins, pyocins or aeruginocins (*Pseudomonas aeruginosa*), megacins (*Bacillus megaterium*), pesticins (*Yersinia pestis*), ulcerins (*Corynebacterium ulcerans*), klebicins (*Klebsiella pneumoniae*), cloacins (*Enterobacter cloacae*) and many other agents including various vibriocins, staphylococcins and streptococcins. Thus the term bacteriocin now encompasses a large heterogeneous group of bacterial antagonists which includes classic colicin-like agents and also more complex agents such as the phage-tail-like R and F pyocins of *P. aeruginosa* (3, 4) and proticin 3 of *Proteus mirabilis* (5). In addition, a class of low molecular weight bacteriocins from enterobacteria with properties distinct from colicins has been described and termed microcins (6, 7).

Most studies on bacteriocins have been concerned with essentially *in vitro* aspects and have been reported in several comprehensive reviews (1, 2). Four general areas of research can be identified:

1. The molecular basis of bacteriocin synthesis and the modes of action of individual bacteriocins.
2. Studies on the characteristics of Col plasmids (colicin-producing) and their use as vectors in gene transfer.
3. Descriptions of new bacteriocins in bacterial species previously not known to be bacteriocinogenic or the description of new bacteriocins exhibiting novel properties.
4. The use of bacteriocin production or susceptibility as the basis of typing systems to differentiate or fingerprint strains of the same species.

In contrast, this brief review will attempt to focus on the *in vivo* and ecological relevance of bacteriocins and, in particular, will take account of important multifunctional roles for bacteriocin receptors.

In Darwinian terms, it could be argued that bacteriocin production might provide an *in vitro* and *in vivo* survival advantage for bacteria on the basis that inter-strain competition would be keenest amongst closely-related bacteria sharing similar growth requirements in a particular ecological niche or human tissue. In contrast, on first consideration, it seems puzzling that so many bacteria possess bacteriocin receptors whose genetic conservation and function result in cell death.

THE ASSOCIATION OF COLV PLASMIDS AND VIRULENCE IN *ESCHERICHIA COLI*

Strains of *E. coli* responsible for generalised infections and death in livestock and humans usually harbour plasmids, that enhance virulence and code for colicin V but not other colicins; curing of the plasmids, e.g. pColV-K30, reduces virulence in both animal and human experiments whilst transfer of the plasmids enhances virulence in previously avirulent strains (8). There is no evidence, however, to establish a correlation between production of the bacteriocin *per se* and virulence (9). The enhanced virulence and selective advantage of ColV plasmids is apparently linked to other properties mediated by the plasmids which include serum resistance (10), ability to promote adherence *in vitro* to mouse intestinal epithelium (11) and probably of most importance, the provision of a highly efficient uptake and transport system for Fe^{3+} (12, 13).

Iron is an essential requirement for the establishment and maintenance of bacterial infections (14–16). Since Fe^{3+} is insoluble or complexed by host iron-binding proteins transferrin and lactoferrin in plasma or tissue fluids, commensal and pathogenic bacteria must compete for the sequestered Fe^{3+} by production of efficient chelators capable of competing with the high association constants (about 10^{36}) of transferrin and lactoferrin. Subsequently the bacteria must be capable of rescuing the Fe^{3+} from the siderophore-iron complex and transporting the iron into the cell. In many bacteria, it has been found that siderophore-iron complexes initially bind to specific outer membrane proteins, which are produced in large amounts only under iron-deficient conditions. The function of such receptors is to bring the Fe(III) complex to, or through, the cell envelope where iron undergoes a reductive separation from the chelator (17).

Studies of the plasmid pColV-K30 have shown clearly that the plasmid is associated with synthesis of the hydroxamate siderophore, aerobactin, and a corresponding bacterial receptor system which involves outer membrane proteins of molecular weights 74,000 and 50,000 (13, 18, 19). Both outer membrane proteins appear necessary for iron assimilation in iron-deficient conditions. What is equally fascinating, however, in an ecological sense, is that the

74,000 protein also acts as the cell receptor for another bacteriocin, cloacin DF13 (13, 19, 20).

It has now become apparent that this multifunctional role for bacteriocin receptors involving the contrasting properties of killing and nutrition, is not restricted to colicin V and the aerobactin system, but includes other bacterial nutrients (2) such as vitamin B12 (colicins E1, E2 and E3), nucleosides (colicin K) and the siderophores enterobactin (colicins B and D) and ferrichrome (colicin M).

This multifunctional role for bacteriocin receptors is intriguing and goes some way to explain their evolutionary conservation and to discount an apparently suicidal role in bacteria if their only function was as bacteriocin receptors.

It is arguable that if bacteriocinogeny or susceptibility to the lethal effects of bacteriocins within a bacterial species was to be of *in vitro* or *in vivo* significance then strains exhibiting these properties should be found frequently, at least in some environmental niches or infected tissue. With this rationale, studies in my own laboratory have focused on *P. aeruginosa*. This ubiquitous, opportunistic pathogen has provided further insight into the extent to which bacterial iron uptake systems are associated with susceptibility to bacteriocins and also emphasizes the need to consider and appreciate the influence of environmental factors in experiments designed to assess the significance of bacteriocins and bacteriocin receptors *in vivo*.

PYOCINS OF *PSEUDOMONAS AERUGINOSA*

Bacteriocin production and susceptibility are arguably more commonly observed in strains of *P. aeruginosa* than in most other bacterial species. In addition, the classes of bacteriocin-like agents produced represent the combined range found in other species. Phage-tail-like R and F pyocins (3, 4, 21) are produced by more than 90 per cent of clinical isolates and colicin-like S pyocins (22–25) are produced by over 70 per cent of strains (25); susceptibility to these bacteriocins is found in almost 100 per cent of strains. Some strains of *P. aeruginosa* (22) also produce a class of low molecular weight, trypsin resistant bacteriocins resembling the microcins of the enterobacteria (6, 7). Kageyama and his colleagues have reported (26) that the efficiency of killing by pyocin S2 was very low when the pyocin S2-susceptible strain *P. aeruginosa* PML 1550 was grown in an iron-rich medium; the capacity of these cells to adsorb pyocin S2 was also reduced. Culture under iron deficient conditions (1 μM or less) was necessary to produce a fully-sensitive cell population and was accompanied by the appearance of an outer membrane protein which appeared to act as the receptor for pyocin S2. Competition experiments between pyocin S2 and culture supernatant containing the yellow-green pseudomonas siderophore, pyoverdin, suggested that the pyocin S2 receptor was not associated with pyoverdin-mediated iron assimilation. Our own studies have expanded these observations on the pyocin susceptibility of strain PML 1516 and have shown a similiar increase in susceptibility to another unrelated pyocin, pyocin Sa, under iron deficient conditions. In addition, in competitive growth experiments involving the pyocin Sa producer strain 1003 and 1516, the survival of 1516 decreases in a dose-dependent manner as the Fe^{3+} content of the culture medium is reduced. Further studies in my laboratory suggest that the phenomenon is common in S pyocins but that low iron concentration does not enhance the killing ability of other classes of pyocins including the R and F pyocins and those resembling the microcins. More detailed studies are presently being conducted to determine the nature of the relationship between S pyocins, and pseudomonas siderophores. In addition, the ubiquitous nature of *P. aeruginosa* will allow studies to determine the relative frequency of the different classes of pyocins produced by isolates from various environmental and *in vivo* locations. In ecological terms, it is interesting to note that bacteriocinogeny in strains of *P. aeruginosa* appears more frequently in clinical than in environ-

mental isolates. Since pyocin production appears to be unaffected by iron concentration, at least *in vitro*, it is possible that carefully designed *in vivo* studies might show that susceptibility to pyocins may play a significant role in the establishment of pyocin-producing strains, particularly in those infected tissues where the organism is known to grow under iron limitation, such as the chronically colonised lungs of patients with cystic fibrosis (27).

Whilst there is no evidence at present to suggest that the bactericidal activity of colicins or pyocins plays a significant role *in vivo*, there is some evidence available to suggest an *in vivo* role for some other bacteriocins, in particular the bacteriocins produced by various species of streptococci and the microcins of the enterobacteria.

BACTERIOCINS AND DENTAL CARIES

There is both *in vitro* and *in vivo* evidence that bacteriocins produced by *Streptococcus mutans* and other oral streptococci may contribute to the ecology of dental caries. Bacteriocinogeny is frequently observed in *S. mutans* and up to 80% of strains examined have demonstrated an ability to produce mutacins (28, 29). A bacteriocinogenic strain of *S. mutans* selectively killed sensitive *S. sanguis* in artificial plaque formed on glass rods (30). In plaque formed on a tooth in an artificial mouth, bacteriocinogenic *S. salivarius* overwhelmed a strain of *S. sanguis* which was sensitive to the bacteriocin in conventional tests (31). However, mutants of *S. salivarius* which were deficient in bacteriocin production *in vitro* also antagonised the *S. sanguis* and it was concluded that the effect of bacteriocins *in vivo* might be modified by environmental factors. In addition, the relative infrequency of bacteriocinogeny in *S. salivarius* strains and the inhibition of strains relatively resistant to the bacteriocin suggested that other ecological factors might be involved. With *S. mutans*, however, bacteriocinogenic strains more readily colonized the dental plaque of specific pathogen-free rats and suggested that bacteriocin production might be an important factor in the successful invasion of plaque by this species (32). In studies of the acquisition of *S. mutans* by infant monkeys and the development of dental caries within the animal colony the predominance of specific *S. mutans* strains within the animal colony was considered at least in part to be due to the production of a bacteriocin active against other strains of *S. mutans* and also other species of oral plaque-associated streptococci (33). Recently, another interesting bacteriocin from *S. mutans* has been described (34) which possessed some properties similar to the microcins observed in enterobacteria (6, 7). This bacteriocin, JH1000 inhibited 123 out of 124 strains of *S. mutans*. In animal studies using mutant strains which produced increased or decreased yields of bacteriocin, the ability of the bacteriocinogenic strain to superinfect or preemptively colonize the oral cavities of rats corresponded with the amount of bacteriocin produced *in vitro*.

BACTERIOCINS AND THE HUMAN INTESTINE

Among the diverse ecological niches found *in vivo*, the human intestinal tract harbours a well defined community of microorganisms that undergoes considerable transitions in composition and in the relative abundance of distinct species under normal and pathological conditions. In such transitions newly colonizing bacteria are often able to displace other closely related species by mechanisms which remain poorly understood (35). In such an ecosystem it is tempting to postulate a role for bacteriocins as agents of strain displacement and colonization resistance. Unfortunately, as has been discussed earlier in this article, no clear evidence to support this hypothesis has been presented, at least as far as colicins are concerned. However, studies of a more recently described class of bacteriocin-like agents once again expand the range of bacteriocinogenic agents but also provide some *in vitro* and *in vivo* evidence for an ecological role. The term microcin was introduced in 1976 (6) to

describe a class of low molecular weight antibiotics produced mainly by enterobacteria of faecal origin. Microcins have several properties (6, 7) that distinguish them from the more extensively characterised colicins. They are smaller with molecular weights between 200 and 5,000 dalton, their synthesis unlike most colicins is not lethal for the producing cell and they cannot be induced by mitomycin C. In most cases microcin production has been shown to be plasmid mediated and the activity of purified microcins to be relatively resistant to heat, extremes of pH and to proteases such as trypsin. The spectrum of activity of microcins is usually broader than colicins and purified preparations have shown activity against non-enteric bacteria including Gram-positive organisms. Whilst anaerobiosis and the high proteolytic activity of the intestinal tract would not favour many colicins, the relative stability of microcins would be an advantage for *in vivo* function (36). Significantly, the presence of particular microcins as free products within faecal samples has been shown to correlate with the presence of similar microcin-producing bacteria in the same sample (5, 37). From studies on bacterial colonization of new borne babies there is some evidence that microcins may be involved in colonization resistance and transitions between bacterial strains *in vivo* (37). *In vitro* evidence also suggests a role for microcins in inter-species successions and a degree of self regulation of microcin activity (38). Mixed batch cultures of both microcin producing *K. pneumoniae* and microcin-sensitive *E. coli* strains showed that microcin excretion into the medium allowed the producer strain to predominate. Furthermore, when nutrients became depleted a microcin antagonist was produced by the microcin-producing strain which allowed recovery of viability in the sensitive strains.

Although microcins have been associated mainly with faecal strains of enterobacteria, similar bacteriocin-like agents have been described in *S. mutans* isolated from human saliva (34) and it is arguable that the class of S-like, trypsin resistant and non inducible pyocins of *P. aeruginosa*, e.g. pyocin S40 (22), might also be considered as a class of microcins.

CONCLUDING REMARKS

In any rational consideration of possible *in vivo* roles for bacteriocins or bacteriocin receptors, the reviewer is immediately faced with the problem "What is a bacteriocin? How many bacteriocin-like agents do I include?" Such considerations only augment the already exacting task facing any study of *in vivo* behaviour, namely the influence of multiple and perhaps little understood environmental factors. Given the present ready acceptance of so many bacterial antagonists within the class termed bacteriocins, a comprehensive review of all known bacteriocins and *in vivo* related studies was considered well beyond the scope of this article. I have thus avoided discussion of *in vivo* roles for the phage-tail-like bacteriocins, though I readily acknowledge that some of these agents, such as proticin 3, have been implicated as virulence factors for *P. mirabilis* in urinary tract (5). Whilst setting aside these phage-like bacteriocins on this occasion, it seems pertinent in discussing ecological roles, to query why such agents are often dismissed from bacteriocin reviews and given the derogatory term "defective phages". In the case of the R and F pyocins of *P. aeruginosa*, they appear to far outnumber the known pseudomonas phages. In addition, their high incidence in *P. aeruginosa*, and appearance as headless, contractile or flexuous phage particles has undoubtedly been conserved on a significant scale.

No attempt has been made either to discuss the possible therapeutic role of bacteriocins since this topic has been discussed previously (39, 40) and would require consideration of a much wider range of *in vivo* factors in addition to those considered in this article.

The primary aim of this short review has been to consider not only the bactericidal activity of bacteriocins *in vivo* but to encourage a broader view of these interesting antibiotics, including the *in vivo* relevance of bacteriocin receptors. Further studies are required to provide convincing evidence of an *in vivo* role for the bactericidal action of bacteriocins.

Meanwhile, bacteriocin receptors, far from being suicidal proteins, demonstrate multifactorial properties which have undoubted *in vivo* significance, particularly enhancement of bacterial iron uptake, and also pose interesting questions regarding bacterial evolution.

ACKNOWLEDGEMENTS

The author readily acknowledges the contribution made by the published articles of many bacteriocin researchers in the preparation of this article. Grateful acknowledgement is also made to Gillian Harris for technical assistance and to Dr Makoto Kageyama for the gifts of bacterial strains and helpful correspondence over the years.

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CASE REPORT

Pseudomonas cepacia – fatal pulmonary infection in a patient with cystic fibrosis

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Summary

A 9-year-old girl with cystic fibrosis (CF) was admitted with an exacerbation of respiratory infection and subsequently died. At death *Pseudomonas cepacia* was cultured from her sputum, in large numbers, 10^7 colony forming units/ml.

Introduction

Patients with cystic fibrosis (CF) are characteristically susceptible to debilitating, life-threatening exacerbations of respiratory infection due to *Staphylococcus aureus*, *Haemophilus influenza* and *Pseudomonas aeruginosa*. Recently, a disturbing increase in the incidence of *P. cepacia* as a cause of fatal respiratory infection in CF patients has been reported by several centres in North America.[1–4] We believe that this is the first report of a death in the U.K. due to this organism.

Case report

A young girl was diagnosed as having CF at age 3 years. Initial respiratory tract pathogens isolated were *S. aureus* and *H. influenzae*. Repeated courses of antibiotic therapy, neither continuous nor by aerosol, kept exacerbations under control for many months.

A pseudomonas-like organism was first isolated from sputum in September 1981 and grown intermittently until March 1984, after which time it persisted. The organism was not *P. aeruginosa* but species identification was not done initially. When carriage of the organism became chronic, however, further laboratory tests were done with the API 20NE system (Analytab Products, Plainview, NY), and the Uni-N/F-Tex multiple medium circular plate (Flow Laboratories, Inc. Mclean, VA). The organism was identified as *P. cepacia* belonging to bacteriocin type S20/PO.[5]

From December 1984 the child's condition deteriorated more rapidly requiring admission to hospital every 3–4 weeks. *Staphylococcus aureus* and *H. influenzae*, present in the sputum with *P. cepacia*, were reduced in numbers by antibiotics and occasionally not found at all, whereas *P. cepacia* persisted.

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On the final admission there had been rapid clinical deterioration over a period of 24 h with fever, dyspnoea, cyanosis and profuse production of purulent sputum. A chest X-ray showed new areas of consolidation which increased over the next 48 h. The white blood cell (WBC) count was elevated at $12{\cdot}8 \times 10^9$/l (65% neutrophils), as was the erythrocyte sedimentation rate (ESR) at 60 mm/h. As is characteristic of respiratory exacerbations in CF patients, bacteria were not found in cultures of blood. Cultures of sputum yielded *H. influenzae* and *P. cepacia* at counts of 5×10^7 and 10^7 CFU/ml respectively. Intravenous ceftazadime therapy resulted in the rapid eradication of *H. influenzae* from the sputum but *P. cepacia* persisted at 10^7 CFU/ml.

The patient's condition continued to deteriorate with further increase in WBC to $17{\cdot}6 \times 10^9$/l (84% neutrophils) and the ESR to 65 mm/h. The haemoglobin concentration fell to 9·7 g/dl. Fever, tachypnoea and cyanosis persisted with increasing mental confusion. She died 4 days after admission.

Discussion

The rapid terminal course of events observed in this patient with CF is in significant contrast to the usual chronic terminal illness seen in this disease, including that associated with *H. influenzae*. However, it is characteristic of the clinical course following colonisation with *P. cepacia* as reported from Canada.[1, 4] Three clinical categories have been described; (i) chronic asymptomatic carriage, (ii) progressive deterioration over many months, and (iii) rapid fatal deterioration.

There is increasing concern about *P. cepacia* in North America[1–4], but to date deaths due to this organism in patients with CF have not been reported in the U.K. Our patient fell into group (iii) and confirms the need for vigilance in detecting and accurately identifying *P. cepacia* as well as recognising its possible emerging role as another respiratory pathogen in CF.

(We thank Mrs C. Doherty and Mrs G. Harris for technical assistance.)

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Pseudomonas aeruginosa and cystic fibrosis: unusual bacterial adaptation and pathogenesis

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Pseudomonas aeruginosa *is an adaptable, saprophytic bacterium with the potential to cause a variety of opportunistic infections in compromised hosts. In patients with cystic fibrosis, chronic pulmonary colonization with mucoid alginate-producing mutants of* P. aeruginosa *is a major cause of morbidity and mortality and is an interesting example of microbial adaptation and host-bacterium interaction.*

Introduction

Pseudomonas aeruginosa is an adaptable bacterial saprophyte with the greatest potential, among the pseudomonads, to cause opportunistic infections in humans, animals, insects and plants. The organism exhibits innate resistance to a wide range of antimicrobial agents and considerable ability to survive in, or adapt to, a variety of often hostile ecological niches. In man, the ongoing development and sophistication of medical science has resulted in the emergence of *P. aeruginosa*, in the last few decades, as an opportunistic pathogen *par excellence* responsible for a diverse range of infections in patients compromised by underlying disease, chemotherapy or occupational and recreational factors. Such opportunistic infections include acute pneumonia and bacteraemia in burn and cancer patients and painful and socially disastrous ear infection in deep-sea divers working in the confined environment of saturation chambers. More recently, the warm, aerated aqueous environment of the recreational whirlpool or jacuzzi has provided ideal cultural conditions for this essentially aquatic microbe and an increased incidence of an irritating skin condition known as 'jacuzzi rash'. The pathogenic factors and host interactions of *P. aeruginosa* vary within individual types of infection, but include chemotaxis, endotoxin and a considerable and diverse armoury of extracellular products including exotoxins, proteases, phospholipase and leucocidin. Arguably, one of the most important pathogenic roles of *P. aeruginosa*, and that which illustrates the most subtle and complex form of pathogenesis, is the debilitating and life-threatening exacerbations of pulmonary infection which follow the gradual colonization of the lungs of patients with cystic fibrosis by mucoid, alginate-producing mutants of *P. aeruginosa*.[1-3]

Pulmonary infection in cystic fibrosis

Cystic fibrosis is an inherited, autosomal recessive disease of exocrine gland function and usually presents at birth or early childhood as a gastrointestinal or pulmonary disorder.[4] The condition is predominantly observed in caucasians and has an incidence of about 1 in 2000 live births. Cystic fibrosis is now a major cause of distress and mortality in children and is the most lethal of the inherited diseases. Since the first descriptions of the disease in the 1940s when life expectancy was measured in months, significant advances in management of patients have been achieved and now many cystic fibrosis patients survive to adulthood. Control of the gastrointestinal problems which result from fibrosis of the pancreatic ducts and deficiency in pancreatic enzymes can be achieved readily by adding pancreatic supplements to a high calorific diet. Rigorous daily physiotherapy and exercise is also encouraged to assist the removal of the abnormally viscid bronchial secretions, which are characteristic of the disease, and to reduce colonization of pulmonary airway surfaces by microorganisms. Several bacterial species, namely *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa* have shown themselves to be particularly adept at colonizing cystic fibrosis lungs. In general, the two former species can be treated effectively with oral antibiotics; *P. aeruginosa*, in contrast, usually proves particularly intractable to treatment even when aggressive i.v. therapy is used with the most potent anti-pseudomonal agents. Failure to control colonization with *P. aeruginosa* is now a major cause of pulmonary debilitation in patients and an obstacle to improvement in the quality of life and life expectancy for cystic fibrosis patients.[5] The incidence of pseudomonal colonization in cystic fibrosis patients varies in individual centres but often involves a majority of patients and may reach almost 100%. Accumulated research and clinical experience indicates that future management of pulmonary disease in cystic fibrosis patients would benefit from a better understanding of the nature and pathogenesis of *P. aeruginosa* in the cystic fibrosis lung.

Pseudomonas and cystic fibrosis

Considerable differences are observed in the association of *P. aeruginosa* in cystic fibrosis patients compared to that observed in other pseudomonas infections. Typical, non-mucoid strains which can cause fatal bacteraemia and pneumonia, together with debilitating surface and

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urinary infections in other patients, are asymptomatic in cystic fibrosis lungs. Secondly, in contrast to other immunologically compromised patients, pseudomonas infections in cystic fibrosis patients remain localized in the respiratory tract: infection of other tissues and bacteraemia are rare. Accumulated evidence[6] suggests that initial asymptomatic, and often intermittent, colonization of the upper respiratory tract with non-mucoid strains of *P. aeruginosa* precedes the emergence and establishment of chronic colonization with mucoid variants of the original strain which have a copiously mucoid colonial appearance (Figure 1). Despite raised serum levels of precipitins against *P. aeruginosa*, this transformation is associated with progressive respiratory distress and a poor prognosis;[7] in most cases, post-mortem examination reveals endobronchial suppuration, cor pulmonale and the presence of mucoid *P. aeruginosa* in infected pulmonary foci.[8] Since the mucoid form of *P. aeruginosa* is so strikingly associated with cystic fibrosis as to be almost diagnostic of the disease, considerable research has been directed towards the nature and biosynthesis of the bacterial mucoid material. Various studies have indicated that the mucoid material is an acetylated, alginate-like polymer comprising homogeneous or mixed blocks of mannuronic and guluronic acids.[9,10] Pseudomonas alginate is similar to the alginates produced by several species of marine algae and by *Azotobacter vinelandii*. Research in the author's laboratory and in other centres has indicated that biosynthesis of the pseudomonas alginate is controlled by chromosomal genes.[11,12] In addition, since the alginate confers enhanced resistance to certain antibiotics, it has been possible to develop a technique[13] for isolating alginate-producing mutants of *P. aeruginosa in vitro* from any non-mucoid isolate, and for producing evidence that the ability to produce alginate extends to other pseudomonas species including *P. fluorescens, P. putida, P. mendocina*, but not *P. stutzeri, P. cepacia, P. pseudoalkaligenes, P. testosteroni, P. diminuta, P. maltophilia* or *P. acidivorans*. Like the other alginates, pseudomonas alginate forms viscid, aqueous solutions and rapidly forms stable gels in the presence of Ca^{++} at concentrations as low as 3 mM.[14] As colonization of the cystic fibrosis lung by mucoid *P. aeruginosa* becomes chronic other unusual characteristics are expressed, including sensitivity to killing by serum,[15] loss of O-antigen specificity[15] and hypersusceptibility[14,16] to a range of antimicrobial agents (Figure 2). The heterogeneity of a pseudomonas population within a single cystic fibrosis patient may be enhanced further by the nature of the bacterial mutation responsible for alginate production. This can result in differences in the ratio of mannuronic and guluronic acids within the polymer, and hence its gelling ability. In addition, mucoid *P. aeruginosa* are characteristically unstable *in vitro* as a result of faster growing non-mucoid revertants. Such non-mucoid revertants may exhibit genetic properties (including suppressor mutations) distinct from the parent strain[11] and can also be found in cystic fibrosis patients. Consideration of these unusual pseudomonal characteristics, which are associated with poor clinical status in cystic fibrosis patients, and the special properties of the bacterial alginate help to explain some of the complex and puzzling pathogenic and microbiological features associated with pseudomonas colonization of the cystic fibrosis lung. Firstly, hypersusceptible mutants of *P. aeruginosa* are uniquely associated with chronic respiratory infections and can be isolated from over 50% of cystic fibrosis patients. The proportion of such mutants in an individual patient varies but may (paradoxically) increase during antibiotic therapy and in some patients reach 100%.[14] Secondly, despite evidence for the presence of an antibody response directed towards the alginate and other cellular and extracellular products of *P. aeruginosa*,[17,18] the cystic fibrosis lung continues to deteriorate. Thirdly, intermittent treatment of cystic fibrosis patients with steroids, which in other patients often results in pseudomonas infection, appears to produce clinical benefit by reducing pulmonary inflammation.[19] Finally, in contrast to acute lung infections with non-mucoid *P.*

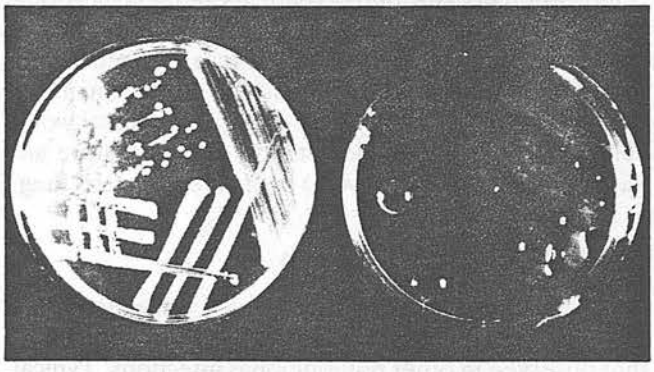

Figure 1. *Typical non-mucoid colonial form of* P. aeruginosa *and mucoid, alginate-producing mutant.*

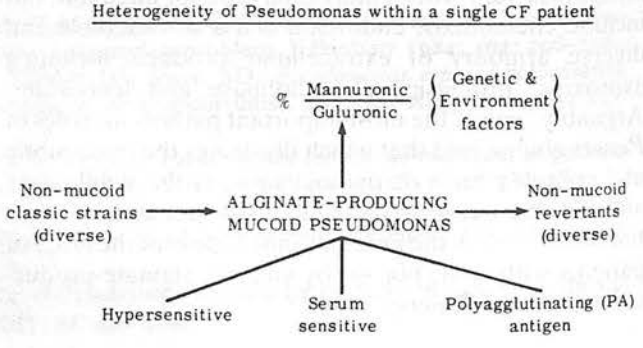

Figure 2. *Characteristics of* P. aeruginosa *isolated from cystic fibrosis patients include alginate biosynthesis, hypersensitivity to antibiotics, serum sensitivity and a polyagglutinating antigen. The rheological properties of the pseudomonas alginates are influenced genetically by mutations which give rise to different percentages of mannuronic and guluronic acids in the alginate polymer and by environmental factors, particularly Ca^{++} ions. Non-mucoid revertants may also exhibit genetic characteristics distinct from the original infecting isolate.*

aeruginosa in which the bacteria are seen microscopically to be widely dispersed throughout the sputum, the mucoid organisms in cystic fibrosis patients often appear as microcolonies (Figure 3) adhering to the bronchial mucosa.[2,20]

Figure 4 illustrates a hypothetical, but plausible, model for mucoid *P. aeruginosa* in a cystic fibrosis patient which takes into account the clinical and microbiological features of the host-bacterium association, the special properties of the bacterial alginate and the other unusual pseudomonas characteristics associated with advanced colonization of the cystic fibrosis lung. Mucoid *P. aeruginosa* have been shown to adhere more readily than non-mucoid forms to tracheal epithelium, particularly when the tracheal surface is damaged.[21,22] In addition, provided the bacterial alginate is not removed by centrifugation and washing, evidence from transtracheal inoculation of pseudomonas into animal lungs indicates that mucoid mutants are removed less rapidly than isogenic non-mucoid forms.[14] Culture of alginate-producing *P. aeruginosa* on agar-based media containing Ca^{++} causes the normally mucoid colonial appearance to assume a more compact gelatinous form (Figure 5a). When mucoid *P. aeruginosa* are cultured *in vitro* and examined by phase-contrast microscopy the bacterial alginate takes the form of an extracellular slime which can be extracted by alcohol precipitation (Figure 5b). The presence of low concentrations of Ca^{++}, however, rapidly results in the formation of bacteria-

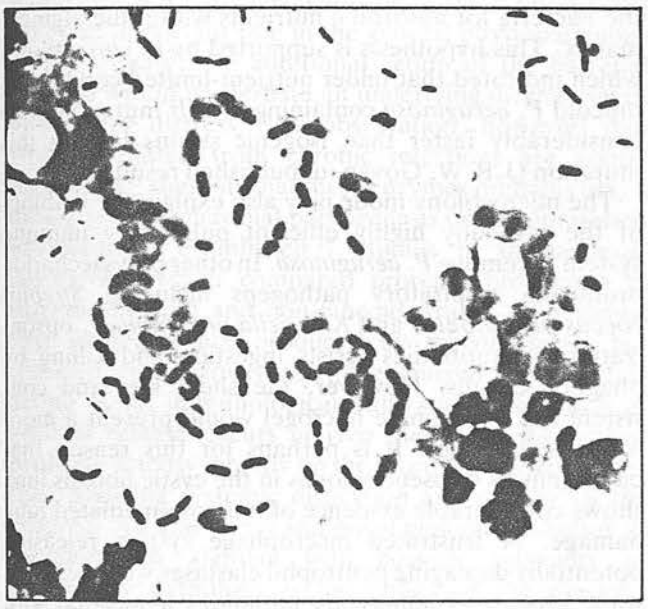

Figure 3. *Gram-stained sputum from a cystic fibrosis patient harbouring mucoid* P. aeruginosa. *Non-mucoid* P. aeruginosa *typically appear randomly distributed across the field. In contrast, mucoid* P. aeruginosa *are usually clustered in gelatinous-like microcolonies attached to respiratory mucosa, ×2250.*

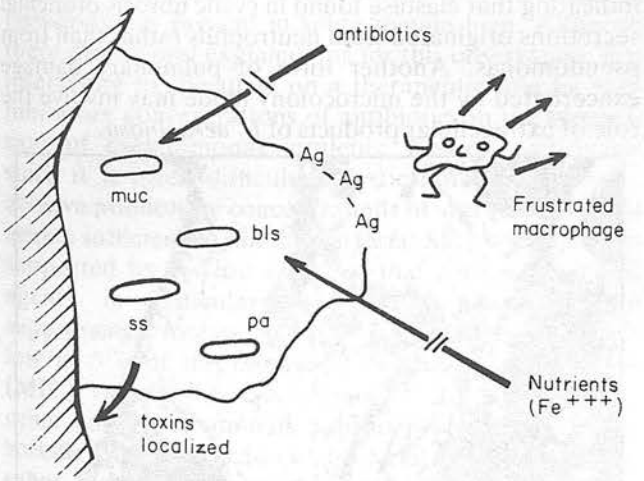

Figure 4. *Microcolony model for mucoid* P. aeruginosa *colonization within a cystic fibrosis lung includes attachment of pseudomonas alginate to respiratory mucosa and containment of bacteria exhibiting alginate production (muc), antibiotic hypersensitivity (bls), serum sensitivity (ss) and polyagglutinating antigen (pa). Other properties of the bacterial microcolony, consistent with* in vitro *and* in vivo *observations, include a diffusion barrier to antibiotics and bacterial nutrients, slow localized release of bacterial toxins and a frustrated and potentially damaging immune response exacerbated by the antigenic mass (Ag) of the bacteria-containing alginate polymer.*

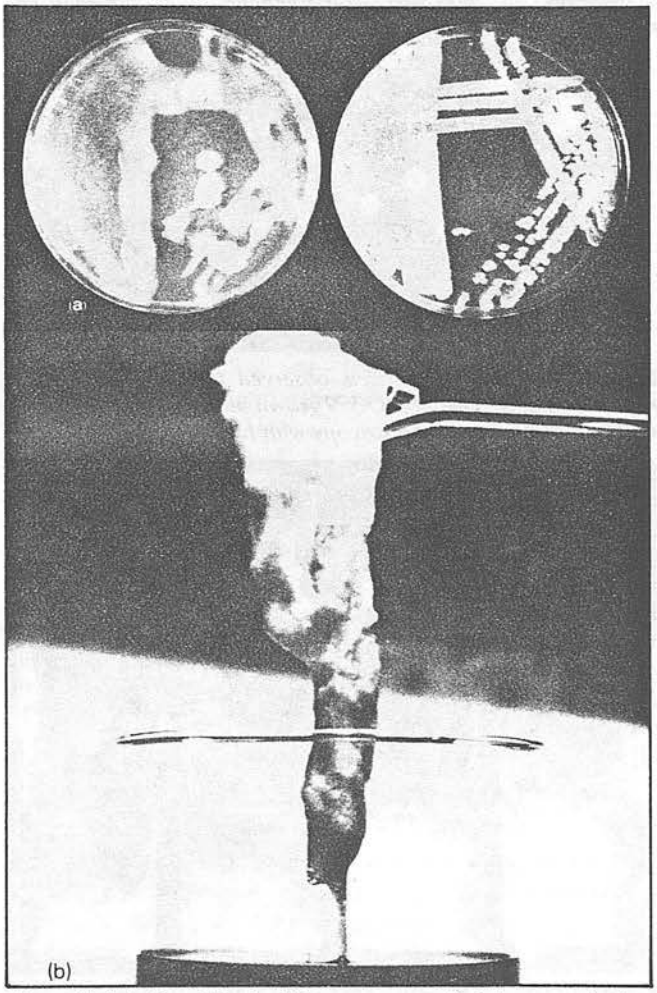

Figure 5. *(a) Mucoid colonial appearance of alginate-producing* P. aeruginosa *PAO 581 grown on a Ca^{++}-free medium (left) and the gelatinous form of the same strain in the presence of 3 mM Ca^{++}. (b) Alginate obtained by alcohol-extraction from mucoid* P. aeruginosa.

containing microgels (Figure 6) which in liquid culture rapidly sediment (Figure 7). By electron microscopy, the microgels are seen as a cotton wool-like mesh enclosing the bacterial cells (Figure 8). On this evidence it seems arguable that the bacterial microcolonies which are observed microscopically in the sputum of cystic fibrosis patients result from the combined gelling ability of the bacterial alginate and the presence of raised Ca^{++} concentrations which are characteristic of the disease. Once established within the microcolony, the enhanced resistance of mucoid *P. aeruginosa* to antimicrobial agents might be explained by a diffusion barrier, but more likely results from antagonism of the antibiotics by the high ionic content of the alginate gel.[23] A diffusion barrier, however, and the iron-chelating ability of alginates might explain the observation of Brown *et al.*[24] that mucoid *P. aeruginosa* in the lungs of cystic fibrosis patients appear to grow under iron-limited conditions. Within the microcolony, the persistence of *P. aeruginosa* sensitive to serum and hypersusceptible to antibiotics becomes more plausible. The almost total restriction of hypersusceptible pseudomonas to chronic colonization of the respiratory tract is intriguing. The phenomenon is frequently observed in mucoid strains of *P. aeruginosa* but is not dependent on alginate production.[14] *In vivo*, it is possible that the selective advantage of the *bls* mutation responsible for this characteristic[16] lies in conferring greater permeability on the bacteria for absorbing nutrients within the alginate matrix. This hypothesis is supported by *in vitro* studies which indicated that under nutrient-limited conditions, mucoid *P. aeruginosa* containing the *bls* mutation grew considerably faster than isogenic strains lacking this mutation (J.R.W. Govan, unpublished results).

The microcolony mode may also explain the inability of the normally highly efficient pulmonary immune system to remove *P. aeruginosa*. In other polysaccharide producing respiratory pathogens including *Streptococcus pneumoniae* and *Klebsiella pneumoniae*, opsonization by antibodies assists ingestion and killing by phagocytic cells. However, the sheer size and consistency of an alginate microgel would present a more formidable target. It is perhaps for this reason that pathogenesis of pseudomonas in the cystic fibrosis lung shows considerable evidence of immune-mediated lung damage. A frustrated macrophage system releasing potentially damaging neutrophil elastases would explain some aspects of pulmonary pathology associated with pseudomonas colonization (and certainly the beneficial effects recently reported from an ongoing trial in which patients receive intermittent steroid therapy).[19] Immune-mediated pulmonary damage is also consistent with the findings of Suter *et al.*[25] who found evidence indicating that elastase found in cystic fibrosis bronchial secretions originated from neutrophils rather than from pseudomonas. Another form of pulmonary damage exacerbated by the microcolony mode may involve the role of extracellular products of *P. aeruginosa*.

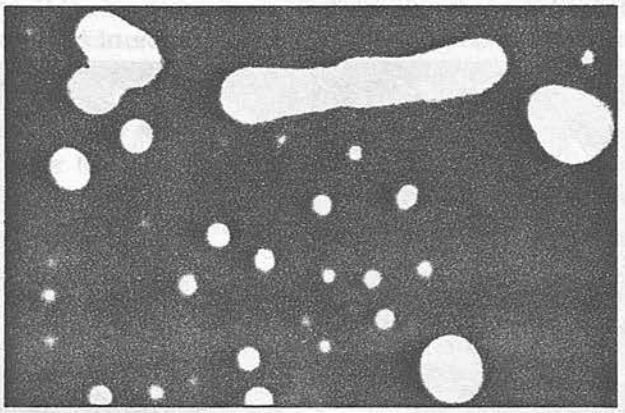

Figure 6. *Alginate microgels observed in liquid cultures of mucoid* P. aeruginosa *PAO 579 grown in the presence of 3 mM Ca^{++}. Phase-contrast microscopy with India ink, ×500.*

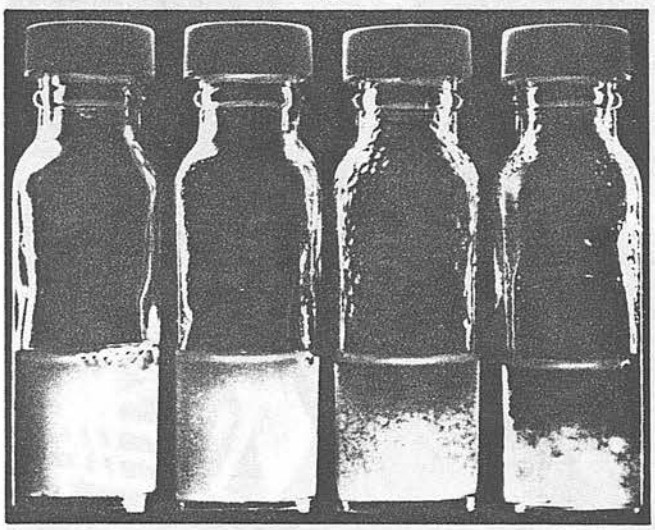

Figure 7. *Mucoid* P. aeruginosa *PAO 579 grown in the presence of 0, 3, 7 and 10 mM Ca^{++}. At 3 mM Ca^{++}, the gelatinous aggregates are visible by microscopy but at the higher concentrations the microgels are visible macroscopically and rapidly sediment.*

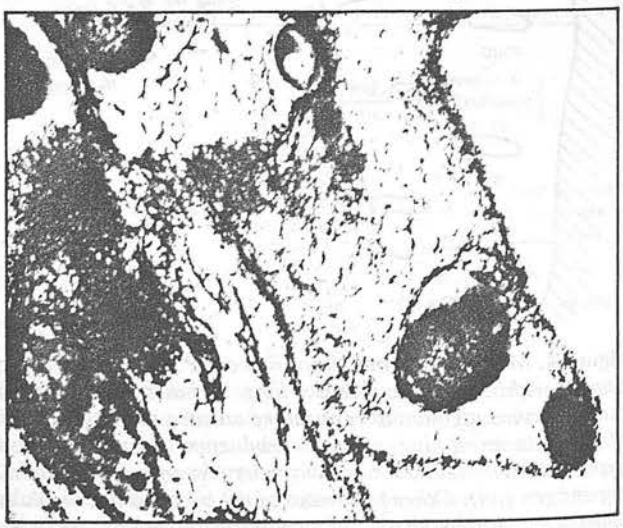

Figure 8. *Electron micrograph of microgels formed by mucoid* P. aeruginosa *PAO 579 in the presence of 3 mM Ca^{++}. Note how the bacteria are enclosed within a cotton wool-like alginate matrix. Ruthenium red stain, ×45 000.*

Accumulated evidence suggests that pseudomonas virulence factors e.g. proteases, may be important in the early stages of lung colonization, causing tissue damage, inactivation of immunoglobulin and facilitating the spread of infection.[26,27] Of particular interest is the observation of Klinger *et al.* that pseudomonas proteases evoke mucin release from tracheal epithelium.[28] Since the production of abnormally viscid bronchial secretions is a basic characteristic of cystic fibrosis and plays an important role in the general physiopathology of the disease, the additional action of the bacterial enzyme would exacerbate an underlying mechanism of pulmonary distress. The observation, however, that strains isolated from chronic infections are poorly proteolytic, suggests that these enzymes probably play a minor role in microbial pathogenesis once colonization has become established.[29] Studies in the authors' laboratory, which compared protease production in isogenic mucoid and non-mucoid strains, have shown that proteases are produced by mucoid forms but released more slowly as if the mucoid material was acting as an immunological adjuvant. If this form of gradual release occurs *in vivo* then it would localize protease activity directly on the bronchial surface rather than allowing systemic dispersion and dilution of the enzyme. An additional and novel biological function of pseudomonas alginate with possible relevance to respiratory pathology is the ability of the exopolysaccharide to enhance extracellular bacterial lipase activity, in a time- and concentration-dependent manner.[30]

Finally, it is a common observation that cystic fibrosis patients obtain clinical benefit from treatment with anti-pseudomonal agents even when the numbers of pseudomonas present in sputa remain high. Although there are several explanations for this observation, it is interesting to speculate on a therapeutic role for sub-inhibitory concentrations of antibiotics in the suppression of pseudomonas virulence factors, particularly since it is often difficult in cystic fibrosis patients to achieve pulmonary concentrations of anti-pseudomonal agents sufficient to kill the bacteria. Such speculation is supported by *in vitro* evidence that anti-pseudomonal agents, in particular ciprofloxacin, are capable of suppressing protease biosynthesis at concentrations as low as 5% of the minimum inhibitory concentration (MIC) value.[31] The superiority of ciprofloxacin over other anti-pseudomonals is probably explained by its specific inhibitory action on bacterial DNA gyrase since other gyrase inhibitors, e.g. acriflavine and acridine orange have similar effects and, in addition, isolates of *P. aeruginosa* which have developed increased resistance to ciprofloxacin have protease activity which is considerably reduced.[31]

Conclusions and future prospects

On first consideration there are many more likely microbial candidates than *P. aeruginosa* which might be expected to play a pathogenic role in the respiratory tracts of cystic fibrosis patients e.g. such typical respiratory opportunists as *S. pneumoniae*, *K. pneumoniae*, mycoplasmas or legionellae. The fact that these organisms do not present a significant problem in cystic fibrosis together with the range of unusual bacterial characteristics which are associated with chronic pseudomonas colonization, suggest that it is the adaptability of *P. aeruginosa* that allows it to be so adept at persisting in the cystic fibrosis lung. If this is the case, then it is encouraging that the ultimate prevention or control of colonization by *P. aeruginosa* would not inevitably lead to replacement by other microbial opportunists. One such opportunistic pathogen, *P. cepacia*, has been isolated in recent years from an increasing proportion of cystic fibrosis patients and has been associated with fatalities. The problem, to date, appears to be limited to a few North American clinics, and the future role of this organism requires careful monitoring. The association of *P. aeruginosa* and cystic fibrosis creates difficult problems for both clinicians and microbiologists in their attempts to understand and provide the best form of management for the patient. In a more general sense, however, the association poses interesting microbiological considerations. The biosynthesis of pseudomonas alginate is arguably the most important factor, allowing the bacteria to attach, persist and directly and indirectly bring about lung damage. This leads to a more general consideration of the regulation, biosynthesis and properties of this unusual polymer and its genetic conservation in only a few pseudomonads. Apart from the lungs of cystic fibrosis patients there is no evidence for any other ecological niche for alginate-producing *P. aeruginosa* and alginate biosynthesis in other pseudomonas species has only been described following genetic manipulation *in vitro*.[11] The biological function of alginate in the only other bacterial source, namely *A. vinelandii*, had been clearly demonstrated to be associated with calcium-dependent encystment.[32] Attempts in our laboratory to induce encystment in mucoid *P. aeruginosa* have been unsuccessful but there is some evidence which demonstrates geneological similarities between *P. aeruginosa* and *A. vinelandii*, particularly with respect to outer membrane proteins.[33] As an alternative approach, we considered that in view of the prevalence of pseudomonads in aquatic environments and the high water content of the alginate gel, that alginate biosynthesis might be genetically conserved as a means of resistance to dehydration. This hypothesis is supported by recent evidence from our own studies which showed that mucoid *P. aeruginosa*, induced to a microgel form by the presence of Ca^{++}, has a greater resistance to dehydration than isogenic non-mucoid forms or the mucoid form in the absence of Ca^{++}.

The present failure in cystic fibrosis patients to cope satisfactorily with established colonization by mucoid *P. aeruginosa*, either by aggressive therapy with the most potent anti-pseudomonal agents or by available

vaccines, should provide the impetus to consider new approaches to the management of pseudomonas colonization. The metabolically wasteful and apparently suicidal nature of alginate biosynthesis, hypersusceptibility, serum sensitivity and polyagglutination are unquestionably associated with the ability of *P. aeruginosa* to maintain colonization in the cystic fibrosis lung despite an immune response which is demonstrably effective in preventing the development of systemic infection. Since these bacterial properties are not usually observed in pseudomonas isolated from other clinical or environmental sources and since the two former properties are known to be the result of chromosomal mutations, it seems arguable that early therapy, whether based on vaccination or antibiotics, might prove a worthwhile strategy with the rationale to reduce the microbial reservoir from which such mutants arise.

As an alternative and complementary approach to the strategy of preventing or controlling early colonization we need to elucidate the host-bacterium interactions which account for the propensity of *P. aeruginosa* to colonize the cystic fibrosis lung. Potentially important clues to such an understanding were provided by the *in vitro* observations of Woods *et al.*[34] which indicated the following:

(1) non-mucoid *P. aeruginosa* adhered significantly better to buccal cells from cystic fibrosis patients than to cells from controls and mucoid pseudomonas adhered less readily;

(2) increased bacterial adherence *in vitro* varied directly with the loss of the protease-sensitive glycoprotein, fibronectin from the cell surface, as well as increased levels of salivary proteases in cystic fibrosis patients;

(3) adhesion of pseudomonas to non-cystic fibrosis buccal cells could be promoted by prior exposure to trypsin.

These observations by Woods *et al.* suggested that buccal cells might form the site and reservoir of early colonization of cystic fibrosis patients by typical non-mucoid forms of *P. aeruginosa* and provide easily accessible material to monitor the initial stages of colonization. Secondly, the promotion of bacterial adhesion by trypsin suggested that cystic fibrosis patients might be compromised not only in an immunological sense, but also by dietary factors. In addition to neutrophil proteases, a source of the raised cystic fibrosis respiratory proteases observed by Woods *et al.* could be the pancreatic supplements which form an essential dietary component for many cystic fibrosis patients. At present, the *in vivo* significance of buccal cell adhesion requires further evaluation. In our own centre, we have been able to reproduce adhesion *in vitro* in both cystic fibrosis buccal cells and in trypsin-treated controls and have successfully cultured the attached organisms from washed cells. In contrast, however, we have been able to culture pseudomonas on only two occasions from buccal cells obtained directly from 88 cystic fibrosis patients who, at the time, were not receiving antibiotic treatment.

Finally, recent reports that culture filtrates of *P. aeruginosa* slow human ciliary beating *in vitro*[35] and that there is an association between pseudomonas alginate and pulmonary lectins[36] illustrates progress in our understanding but also suggests that pseudomonas colonization of cystic fibrosis patients is complex and results from a combination of various host-bacterium factors including adhesion mechanisms selective for both non-mucoid and mucoid *P. aeruginosa*.

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Infection

Reprint

Rational Parameters for Antibiotic Therapy in Patients with Cystic Fibrosis

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Rational Parameters for Antibiotic Therapy in Patients with Cystic Fibrosis

Summary: Data from the literature and the authors' experiences were used to review aspects of antibiotic therapy of patients with cystic fibrosis; attention was paid to *in vitro* antimicrobial susceptibility tests and assessment of therapy directed against mucoid *Pseudomonas aeruginosa*. The heterogeneity of *P. aeruginosa* within single sputa with respect to antibiotic susceptibility is stressed. Quantitative viable counts of bacteria based on an analysis of homogenised sputum is recommended. The mode of *in vivo* growth of mucoid *P. aeruginosa* is discussed to explain the survival of hypersusceptible *P. aeruginosa in vivo*, and the clinical benefit observed in the absence of a significant reduction of the pathogen. The value of ceftazidime in the treatment of exacerbations due to *Haemophilus influenzae* is emphasised. The social benefits from oral administration of ciprofloxacin also emphasises that the patient's quality of life must also be considered.

Zusammenfassung: *Parameter für eine rationelle Antibiotikatherapie bei Patienten mit zystischer Fibrose.* In einer Übersicht werden Daten aus der Literatur und eigene Erfahrungen in der Antibiotikatherapie bei Patienten mit zystischer Fibrose dargestellt. Die *In vitro*-Testung und klinische Beurteilung der Therapie von Infektionen durch mukoide Stämme von *Pseudomonas aeruginosa* finden besondere Beachtung. *P. aeruginosa*-Isolate aus einer einzigen Sputumprobe weisen im Hinblick auf ihre Antibiotikaempfindlichkeit eine bemerkenswerte Heterogenität auf. Keimzahlenbestimmungen sollten an homogenisierten Sputumproben vorgenommen werden. Das *In vivo*-Wachstumsverhalten mukoider *P. aeruginosa*-Stämme gibt Aufschluß über die Beobachtung, daß hochempfindliche Stämme von *P. aeruginosa in vivo* überleben. Auf das Phänomen der klinischen Besserung ohne signifikante Erregerreduktion wird eingegangen. Hervorgehoben wird der Wert von Ceftazidim in der Therapie akuter Exazerbationen durch *Haemophilus influenzae*. Der soziale Gewinn einer oralen Therapie mit Ciprofloxacin weist auf die Bedeutung der Lebensqualität für den Patienten hin.

Introduction

"Perhaps, nowhere in the management of patients with cystic fibrosis is there more controversy than in the therapy of their pulmonary disease" (1).
Chronic debilitating respiratory infection is the main threat to both the quality of life and life expectancy in patients with cystic fibrosis (CF). In CF patients the normally highly effective immune defences of the respiratory tract are not only overwhelmed but, arguably, make a significant contribution to the pathogenesis of the infection. The microbial pathogen most responsible for morbidity and mortality in CF patients is *Pseudomonas aeruginosa* (2). Furthermore, despite very significant increases achieved in the potency of antipseudomonal antibiotics, the organism remains notoriously intractable to antibiotic therapy and, once established in the lungs of a CF patient, is seldom if ever eradicated. A better understanding of the mechanisms of pathogenicity of *P. aeruginosa* in the CF respiratory tract is essential if advances are to be made in the prevention of and effective treatment for this organism and if optimum use is to be made of the bacteriological laboratory to make *in vitro* assessments of the choice and effectiveness of particular antibiotics.
Therapeutic strategies including choice of antipseudomonal agent, dosage and route of administration have recently been authoritatively reviewed (3). The aims of this paper are to focus on other rational parameters for antimicrobial therapy in CF patients, particularly on therapy against *P. aeruginosa* taking into account state of the art knowledge of the complex association of the organism in the respiratory tracts of CF patients.

In Vitro Susceptibility Tests of Pseudomonas aeruginosa from CF Patients

One of the most striking aspects of the natural history of *P. aeruginosa* and its association with CF patients is the adaptation and heterogeneity exhibited by the organism as colonisation of the lung develops to a chronic state. In the early stages of colonisation the pseudomonas population is usually homogeneous with respect to colonial appearance, antigenicity and antimicrobial susceptibility; later, however, considerable heterogeneity is observed in these characteristics and eventually the pseudomonas population is very atypical compared with other pseudomonas infections. In addition to the characteristic emergence and establishment of mucoid strains derepressed for alginate biosynthesis, colonies cultured from single sputa may exhibit a range of colonial appearance and antibiotic susceptibility whilst investigation of colonies sharing a similar appearance may also reveal differences in serum sen-

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Table 1: Susceptibility of individual colonial isolates of mucoid *Pseudomonas aeruginosa* from a patient with cystic fibrosis treated with combined azlocillin and tobramycin.

Isolates	Colony designation	MIC (mg/l)[a] Azlocillin	Tobramycin
	1	6	2
	2	20	2
Pre-therapy	3	4	6
	4	1	4
	5	10	6
	6	2	4
	7	80	8
After 5 days	8	4	2
	9	80	2
	10	6	20

a: Antibiotics incorporated into Difco Mueller Hinton agar supplemented with $CaCl_2$ (2.5 mM) and $MgSO_4$ (0.75 mM). Inoculum 10^4 cfu.

Table 2: Effect of medium on the susceptibility of individual colonial isolates of *Pseudomonas aeruginosa* from two patients with cystic fibrosis.

Patient	Colony designation	MIC (mg/l)[a] Azlocillin STA[b]	Azlocillin MH + Ca,Mg[c]	Tobramycin STA	Tobramycin MH + Ca,Mg
A	1	0.2	1	0.3	2
	2	0.4	20	0.4	1
B	1	6	20	0.6	4
	2	4	8	0.5	2

a: Inoculum 10^4 cfu;
b: Diagnostic sensitivity test agar (Oxoid);
c: Mueller Hinton agar (Difco) supplemented with $CaCl_2$ (2.5 mM) and $MgSO_4$ (0.75 mM).

sitivity, antigenicity and a considerable degree of heterogeneity with respect to antimicrobial susceptibility (4–7). In most instances the colonies exhibit the same bacteriocin type and thus heterogeneity cannot be simply explained by multiple infection with different strains of *P. aeruginosa* (4).

Thus *in vitro* susceptibility tests based on the examination of a single colonial representative may not reflect the true population of pseudomonas within the sputum. Table 1 illustrates the range of MIC values obtained from different colonies sharing similar appearance and isolated from individual sputa before and during therapy with azlocillin and tobramycin. Based on single colony assessments this course of treatment could be interpreted to have caused increased resistance, increased sensitivity or no change in bacterial susceptibility. The phenomenon cannot be explained on the basis of a mixed infection with different strains since all the colonies examined belong to the same pyocin type and serotype.

The influence of culture medium on the results of antibiotic susceptibility tests is well known and may also be significant in the interpretation of such tests applicable to CF patients. *Bryan* and *Rabin* (8) suggested the addition of calcium and magnesium to susceptibility test medium to more realistically resemble the ionic milieu of the CF lung. The addition of calcium was reported by these authors to antagonise the aminoglycosides. In our own experience we have noted that calcium, at the levels suggested by *Bryan* and *Rabin*, also significantly reduces susceptibility to the ureidopenicillin azlocillin but, in contrast to the well recognised antibiotic-associated antagonism found with the aminoglycosides, antagonism with respect to azlocillin is strain dependent and even varies with different colonies obtained from the same sputum (Table 2). Whilst these observations make the interpretation of *in vitro* susceptibility tests considerably more difficult compared to other pseudomonas infections, further confusion has also surrounded the influence of the bacterial mucoid material or alginate on susceptibility to antibiotics. The reasons for the heterogeneity of antimicrobial sensitivity observed in CF isolates of *P. aeruginosa* is little understood but can be shown, *in vitro*, to be influenced, in part at least, by the presence of the *muc* mutation involved in alginate synthesis (9) and the *bls* and *tps* mutations associated with hypersusceptibility to β-lactams and trimethoprim and various other antimicrobial agents, excluding the aminoglycosides (10). If susceptibility tests are performed with a degree of sensitivity greater than that obtained using simple doubling dilutions of the antibiotics then production of alginate can be shown to decrease susceptibility to β-lactams and aminoglycosides by approximately 50% (11); indeed, this property has been used to form the basis of a technique for the isolation of alginate-producing mutants of *P. aeruginosa in vitro* from any non-mucoid *P. aeruginosa* and also from various other pseudomonas species which had not previously been known capable of producing this unusual bacterial polysaccharide (11, 12). However, if a mucoid isolate also contains the *bls* and *tps* mutations then the effect of alginate is rendered insignificant and results in unusual *P. aeruginosa* isolates which are mucoid and at the same time considerably more susceptible to most antibiotics than even typical non CF isolates (10, 13). Although such hypersusceptibility is generally associated with mucoid colonial forms the two characteristics are independent and regulated by distinct chromosomal mutations (9, 10).

Table 3 illustrates the considerable differences in antimicrobial susceptibility *in vitro* between mucoid *P. aeruginosa* strains 492a and 492c (hypersusceptible) which were isolated from the same sputum and belong to the same pyocin type and serotype and also the influence on susceptibility when the hypersusceptibility and mucoid mutations are transferred into a PAO background.

Hypersusceptibility would appear to be associated with adaptation of pseudomonas to the respiratory tract since isolates with this property are almost exclusively obtained from respiratory specimens (4).

For the reasons discussed above we recommend that antibiotic susceptibility tests on *P. aeruginosa* isolates from

Table 3: Influence of mutations involving hypersusceptibility (*bls, tps*) and alginate biosynthesis (*muc*) on the susceptibility of *Pseudomonas aeruginosa* to carbenicillin, trimethoprim, nalidixic acid and ciprofloxacin.

Strain	Description	MIC[a] Carb[b]	Trim[c]	Nal[d]	Cip[e]
492a	CF isolate, *muc, tps*	80	40	60	0.1
492c	CF isolate, *muc, bls, tps*	0.6	0.5	2	0.04
PA08	nonmucoid	40	200	80	0.08
PAJ1	*bls, tps* recombinant from cross 492c *leu*-R68.45 × PA08	0.4	10	5	0.04
PAJ2	*bls* recombinant from cross 492c *leu*-R68.45 × PA08	0.4	60	20	0.04
PAJ3	*tps* recombinant from cross 492c *leu*-R68.45 × PA08	40	60	20	0.04
PA0381	nonmucoid	40	200	80	0.08
PA0579	mucoid (*muc*) derivative of PA0381	60	300	80	0.08

a: Diagnostic sensitivity test agar (Oxoid). Inoculum 10^4 cfu;
b: Carbenicillin;
c: Trimethoprim;
d: Nalidixic acid;
e: Ciprofloxacin.

CF patients should include examination of multiple colonies. Although it has been suggested previously that the basis of the choice of colonies to be tested should be differences in colonial appearance (6, 7), it must be stressed that colonies with similar appearance may demonstrate considerable differences in susceptibility. Undoubtedly, these proposals involve a greater workload and expense than is customary in most laboratories. In our own clinic we have compromised by routinely testing 20 colonial representatives at the beginning and during the course of antibiotic treatment and for this purpose find the use of a multipoint inoculator very suitable.

Finally, it is generally accepted that the reasons for *in vitro* susceptibility testing is to determine the degree of susceptibility of a bacterium and, in association with the known achievable, nontoxic tissue concentrations, to make judgements on the most suitable agent for therapy. It is almost axiomatic then that increased sensitivity of an organism *in vitro* should be reflected by more successful elimination from the infected tissue. Paradoxically, however, in many CF patients the proportion of pseudomonas exhibiting the hypersusceptibility mutations *bls* and *tps* increases with the chronicity of colonisation and, equally surprising, the proportion of such hypersensitive pseudomonas within an individual patient may often increase during the course of therapy (*Govan,* unpublished data).

Assessment of Antimicrobial Therapy

In most instances the success or failure of antibiotic therapy is judged on a qualitative basis i.e. the eradication of the bacterial pathogen. However it is arguable that this approach requires modifying in the case of infections due to opportunistic pathogens and particularly in the case of respiratory colonisation due to *P. aeruginosa* in CF patients. In these patients the eventual outcome of infection may depend upon the bacterial load placed upon the lung and the pulmonary condition of the CF patient prior to the episode of exacerbation. As a consequence, to judge the significance of *P. aeruginosa* isolated from CF sputum for diagnostic purpose or in assessment of treatment solely on the basis of the presence or absence of the microorganism is insufficient. For this reason the use of quantitative bacteriological assessment of CF sputum flora is to be encouraged not only to judge the significance of *P. aeruginosa* or other pathogens, including *Staphylococcus aureus* and *Haemophilus influenzae,* as a cause of a particular episode of exacerbation but also to assess the therapeutic efficacy of a particular course of antibiotic treatment. Unlike the rapid and successful elimination of *H. influenzae* from CF sputa with ceftazidime, antipseudomonal therapy normally shows a more gradual reduction in the numbers of bacteria present in the sputum. Close collaboration between the clinician and the microbiologist during such treatments is essential to allow ongoing assessment of the therapeutic value of the particular treatment. The significance of the bacterial load on a CF patient during a particular exacerbation and the slow but significant reduction in the numbers of mucoid *P. aeruginosa* are illustrated in Figure 1 which concerns an exacerbation in a CF patient whose previous bacteriological status had been closely monitored. On admittance, the patient was in poor clinical condition. Quantitative examination of his homogenised sputum for a range of respiratory pathogens revealed only mucoid and nonmucoid forms of *P. aeruginosa* at greater than 10^7 cfu/ml. The patient had harboured the mucoid organism for some time but on this occasion, and for the first time, a classic nonmucoid strain of *P. aeruginosa* was also present in significant numbers. Pyocin typing (14) of the two colonial forms showed that they were of different pyocin types and thus unrelated. On treatment with ceftazidime the patient showed marked clinical improvement paralleled by a significant reduction in the bacterial count. The nonmucoid strain was rapidly eradicated and did not return subsequently, whereas reduction of the mucoid organism, to barely detectable levels, occurred more slowly. Although in this case the two organisms were equally sensitive to the antibiotic on the basis of *in vitro* susceptibility tests, the number of mucoid organisms was reduced more slowly and never eradicated. Monitoring of the pseudomonas showed initially a mucoid population which was homogeneous with respect to *in vitro* sensitivity and with an MIC value of 1 mg/l ceftazidime. Gradually more resistant forms appeared with MIC values up to 20 mg/l and this was paralleled with an increase in the pseudomonas population and a deterioration in the patient's condition. In our experience over the last ten years the above episode is characteristic of the treatment of *P. aeruginosa* in CF. Treatment of exacerbations with a variety of antipseudomonal agents usually results in clinical improve-

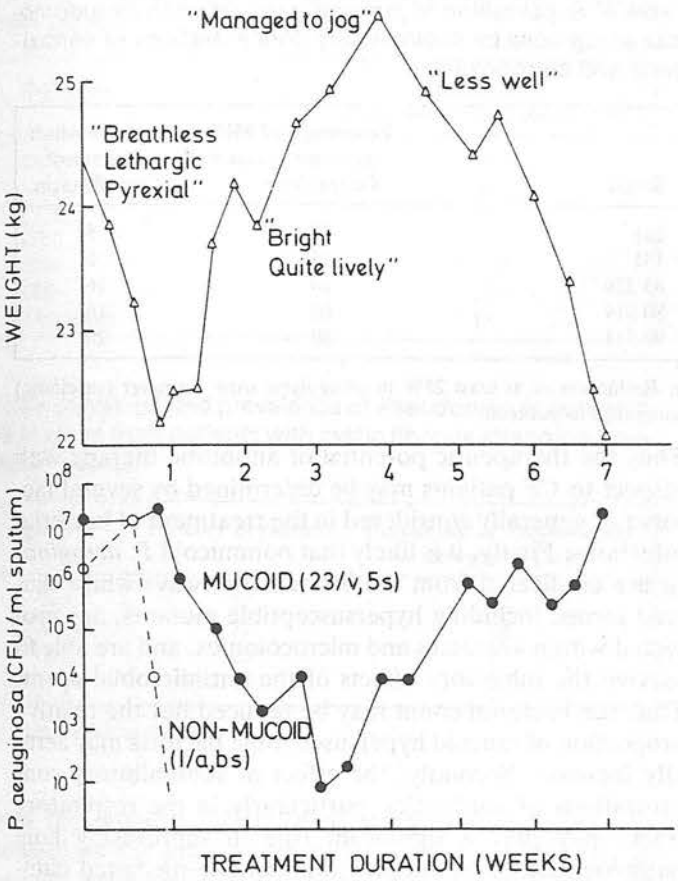

Figure 1: Treatment of an exacerbation of respiratory infection due to *P. aeruginosa*. Note the correlation between weight, clinical status and the numbers of mucoid *P. aeruginosa* isolated from the patient's sputum. Note also the eradication of the newly acquired nonmucoid *P. aeruginosa* (pyocin type 1/a, bs) but the eventual increase of the mucoid strain (pyocin type 23/v, 5s).

ment, including reduced sputum viscosity and volume, weight gain, reduced fever and C-reactive protein levels, and improved pulmonary function. This clinical recovery is associated with a reduction but seldom long term eradication of mucoid *P. aeruginosa*. The emergence of enhanced resistance is also common but it has to be emphasised that this is almost never high level resistance. Typically it involves increases of MIC values of ten to twenty fold. On initial treatment the resistance is often temporary with the bacterial population showing the original level of susceptibility on the patient's next admittance to hospital; however, as repeated courses of the antibiotic are given and although considerable heterogeneity in MIC values can be observed within a single sputum, there is usually a gradual decrease in the base level of susceptibility in the pseudomonas population.

It could be argued that the emergence of resistance to an antibiotic in pseudomonas isolates from a CF patient, might be considered to have repercussions distinct from other patients. Experience shows that as *P. aeruginosa* adapts and establishes chronic colonisation in the CF lung or in the course of intensive antimicrobial therapy, the colonial appearance of the organism may become very atypical and almost nonviable. Similarly it has been observed that strains which have acquired resistance to ciprofloxacin exhibit severely reduced protease biosynthesis (15). Thus if we accept that *P. aeruginosa,* once established in the CF lung, is seldom eradicated, therapeutic benefit, albeit not ideal, may follow from interference with the viability of the organism or, in the case of some resistant forms, a reduction in bacterial virulence. Finally, the incidence of resistance in particular clinics may reflect resistance acquired as a result of therapy or cross infection of individual patients with an already resistant strain. To distinguish these events and to monitor the influence of cross infection in general a policy of epidemiological typing or fingerprinting is useful. For this purpose pyocin typing (14) is recommended in preference to serotyping in view of the incidence of polyagglutinating variants of *P. aeruginosa* in CF patients and the difficulty of serotyping mucoid isolates (5). To date, in the Edinburgh clinics we have had little evidence of multiresistant strains of *P. aeruginosa.* In addition, monitoring of isolates by pyocin typing has shown that, with the exception of siblings, no two patients are presently colonised with the same strain of *P. aeruginosa.*

Figure 1 illustrates the typical bacterial killing following initial use of a new antipseudomonal agent. An alternative course of events however almost invariably occurs when the same antibiotic is used in subsequent exacerbations. On these occasions clinical improvement is associated with a slight reduction in the pseudomonas count but, whilst the patient continues to improve, no further significant decrease in the bacteria occurs unless the dosage of the antibiotic is increased (16). This type of result is puzzling for two reasons. Firstly, the failure to reduce the bacterial count generally cannot be simply explained on the basis of the emergence of resistance since there may be no change in the MIC values obtained. Secondly, we need to explain the apparent clinical improvement in the absence of a significant reduction in bacterial numbers. It seems likely that the key to an understanding of these apparent anomalies and to the paradox that not only may the percentage of hypersusceptible *P. aeruginosa* in a sputum increase during therapy but that hypersusceptible forms increase as colonisation progresses (17), lies in a greater appreciation of the behaviour and pathogenic mechanisms of *P. aeruginosa* in the CF patient.

Growth of Mucoid Pseudomonas aeruginosa In Vivo

Microscopy of sputa from acute respiratory infections with nonmucoid *P. aeruginosa* in nonCF patients and in the early stages of colonisation of CF patients usually show the bacteria as individual cells. In contrast, mucoid *P. aeruginosa* are frequently observed *in vivo* as microcolonies (2, 18, 19) protected by the peculiar physical properties of the bacterial mucoid material. Pseudomonas alginate is composed of a polymer of mannuronic and glu-

ronic acids (20) and is quite distinct from the capsular polysaccharides of *Klebsiella pneumoniae* and mucoid forms of *Escherichia coli.* Alginate forms viscous aqueous solutions but, even more significant, rapidly gels in the presence of electrolytes such as calcium (17, 19). In the absence of calcium, or in the presence of calcium chelators, e.g. EDTA, pseudomonas alginate is produced as a noncapsular extracellular "slime" and is easily removed from the bacteria by washing (17). However, in the presence of calcium as low as lmM the cells become embedded in a protective matrix or microcolony surrounded by their own alginate (19). If this is the basis for the microcolony formation observed in Gram films of pseudomonas-containing CF sputa then a number of observations associated with the presence of mucoid *P. aeruginosa* in the CF patient may be explained. Firstly, the alginate is known to enhance binding to tracheal epithelium (21, 22) and in the form of a gel might form a physical and ionic barrier to penetration by antibiotics in an environment in which high concentrations of antibiotics are already difficult to achieve. Secondly, even though pseudomonas alginate is antigenic (23), the matrix could still provide a substantial barrier to phagocytosis. Perhaps, of even greater significance, however, is the potential role of the alginate gel to promote an inflammatory and immune-mediated mechanism of tissue damage in the CF patient. Studies by *Bryan* et al. (23) showed that the sera of CF patients contained anti-alginate antibodies. Under normal circumstances the presence of such antibodies would be beneficial allowing opsonisation of the bacteria and enhanced phagocytosis. However, a gelled microcolony would present a formidable target for phagocytosis and a constant challenge to a "frustrated macrophage". Under these circumstances the suggestion of tissue damage in the lungs brought about by a patient's own highly stimulated immune response (24, 25) becomes even more plausible and might explain the poor results previously obtained with vaccination and the beneficial results of steroid therapy reported in the United States (26). It could also be speculated that tissue damage due to known pseudomonas virulence factors in the respiratory tract, eg exotoxin A and proteases (27) might also be enhanced by microcolony formation since concentration of the bacteria within a matrix and attachment to bronchial epithelium would act to localise the toxins and reduce the diluting effect of body fluids. Such a mechanism is supported by our own *in vitro* studies of protease production in isogenic mucoid and nonmucoid strains of *P. aeruginosa.* Although the proteases are readily excreted by nonmucoid strains, excretion from mucoid forms is a more gradual process as if the alginate was acting like an adjuvant to release the proteases gradually. In yet another context histopathological studies of postmortem material from CF patients and from animal studies (28, 29), involving chronic respiratory infection with *P. aeruginosa,* demonstrate considerable lung consolidation and abscess formation presenting yet another barrier to antibiotic penetration.

Table 4: Suppression of protease production in *Pseudomonas aeruginosa* by subinhibitory concentrations of ceftazidime and ciprofloxacin.

Strain	Percentage of MIC value above which protease production is suppressed[a]	
	Ceftazidime	Ciprofloxacin
281	25	5
131	25	5
83 229	40	16
80 919	60	16
90 711	50	20

a: Reduction of at least 25% in proteolytic zone diameter (caseinase) compared to control.

Thus the therapeutic potential of antibiotic therapy with respect to CF patients may be determined by several factors not generally considered in the treatment of bacterial infections. Firstly, it is likely that nonmucoid *P. aeruginosa* are eradicated from the bronchial airways whilst mucoid forms, including hypersusceptible mutants, are protected within abscesses and microcolonies, and are able to survive the inhibitory effects of the antimicrobial agent. Thus the bacterial count may be reduced but the relative proportion of mucoid hypersusceptible bacteria may actually increase. Secondly, the effect of subinhibitory concentrations of antibiotics, particularly in the respiratory tract, may play a significant role in suppressing lung pathology either by suppressing immune-mediated damage or, in addition, by suppressing the biosynthesis of the bacterial toxins. Table 4 illustrates the considerable suppression of pseudomonas protease by subinhibitory concentrations of ceftazidime and ciprofloxacin. It would seem that the superiority of the quinolone is explained by its effect on the bacterial gyrase since other gyrase inhibitors, e.g. acriflavine and acridine orange have similar effects (15) and isolates of *P. aeruginosa* which have developed increased resistance to ciprofloxacin during therapy have a considerably reduced protease activity (*Govan,* unpublished data).

In conclusion, the therapeutic benefits of antipseudomonas antibiotics may be assessed by the ability of the antibiotic to reduce the load of organisms in sputa at least temporarily but this requires quantitative measurement of the load of pseudomonas within the homogenised sputum rather than merely noting the presence or absence of the organism. However, it should be also borne in mind that antibiotics may exert biological and therapeutic effects in other ways and that subinhibitory concentrations may well act *in vivo* to suppress toxin biosynthesis and, like steroids, lessen immune mediated damage.

The Influence of Antibiotic Therapy on the Incidence of Pseudomonas aeruginosa in CF Patients

In a discussion of the parameters and problems associated with the use of antibiotics in the treatment of pseudomonas infection in CF patients it is perhaps relevant to dis-

Table 5: Prevalence of *Pseudomonas aeruginosa* in sputa from patients with cystic fibrosis.

Reference	Incidence of *Pseudomonas aeruginosa* (%)
(31)	70
(2)	64
(32)	32
(33)	77
(34)	70–90
(35)	17
This study	17

Table 6: Age-related prevalence of *Pseudomonas aeruginosa* in sputa from patients with cystic fibrosis attending Edinburgh clinics.

Age[a] (yr)	Number of patients	Prevalence of *Pseudomonas aeruginosa* (%)
0–5	27	0
6–10	27	14
11–15	25	24
n ≥ 16	23	30

a: Range 4/12 to 26 years.

cuss the possible influence of antimicrobial therapy in general on the gradual emergence of this organism to its present dominance in CF respiratory infections. Whilst there has been considerable debate in the last few decades regarding the pathological significance of *P. aeruginosa* there is little doubt that the incidence of the organism in CF patients has risen inexorably to approximately 70% in many centres and almost 100% in some clinics (2, 28, 30). Nevertheless, considerable differences can be observed in the incidence of chronic colonisation with *P. aeruginosa* from different centres (Table 5). Several explanations could be considered to account for such differences in incidence. Since the incidence of *P. aeruginosa* has been reported to increase with age (30), then it follows that clinics with high adolescent populations would yield a higher incidence than in paediatric clinics. Alternatively, ecological pressure due to antistaphylococcal therapy has also been suggested as a significant cause of the increased incidence of *P. aeruginosa* in CF patients in the last two decades and for the selection of mucoid variants (28, 36, 37). In reality, the reasons underlying the incidence of *P. aeruginosa* in any one centre might well be multifactorial. Nevertheless, it is arguable that the low incidence of the organism in the Edinburgh clinics (Tables 5 and 6) regardless of age group reflects an antibiotic policy which deliberately does not include prophylactic antistaphylococcal therapy but restricts treatment to clinical evidence of exacerbation and the isolation of *S. aureus* from sputum at greater than 10^6 cfu/ml (36). Two other aspects of this controversial topic need to be considered. Is a policy of continuous antistaphylococcal therapy successful in reducing the incidence of *S. aureus* and, alternatively, does a policy of nonprophylaxis result in an increased incidence of *S. aureus?* The answer to both these questions would appear to be no, since at 38% the incidence of *S. aureus* in the Edinburgh clinics is no higher than that reported in other studies (2, 30).

Therapy Against Haemophilus influenzae

Respiratory exacerbations in CF patients due to *H. influenzae* may be shown to occur with some frequency, particularly if bacteriological examination includes culture of suitable dilutions of homogenised sputum on appropriate culture medium, e.g. chocolate blood agar incorporating bacitracin and incubation in 10% CO_2. Antimicrobial therapy of such exacerbations, however, also reveals a degree of complexity involving factors distinct from those involved in the treatment of *P. aeruginosa*. Whilst a particular episode of exacerbation may be considered due to *H. influenzae* and sputum culture produce a growth of greater than 10^6 cfu (36) it is not uncommon to detect simultaneous colonisation with smaller numbers of *S. aureus* and/or *P. aeruginosa*. In such episodes apparently appropriate β-lactam therapy of a non β-lactamase producing *H. influenzae* may be antagonised by the presence of *S. aureus* and *P. aeruginosa*. In our experience with 20 patients, one of the most successful and reliable forms of antihaemophilus therapy, whether the organism exists alone or in combination with *S. aureus* or *P. aeruginosa,* is the use of ceftazidime. This third generation cephalosporin is not promoted nor usually considered for treatment of respiratory infection due to *H. influenzae*. Nevertheless, in practice, in exacerbations where prior treatments with amoxycillin or augmentin have failed, the use of ceftazidime invariably results in eradication of large numbers of *H. influenzae* from sputa and clinical recovery within two to three days.

Conclusion

Many questions and controversial aspects of antimicrobial therapy in CF respiratory infections remain to be answered. This present review is not intended to be an exhaustive discussion of all aspects of antimicrobial therapy in CF. The review has not included a discussion of the dosages and route of administration of antibiotics nor has the problem of the development and incidence of multiresistant strains of *P. aeruginosa* been covered since these important topics introduce factors beyond the scope of this review. The aim of this review has been to encourage a wider and more openminded approach to various parameters, in particular to take into account the realities of present therapy and the subtle nature and pathogenesis of chronic lung infection caused by *P. aeruginosa* in CF lungs. Given the considerable heterogeneity of *P. aeruginosa* in CF patients with respect to antimicrobial susceptibility and the high incidence of hypersusceptibility, it could be argued that, in a conventional sense, susceptibil-

ity testing is of little value. For this reason, one of the most important factors in the therapy of CF patients is close collaboration between the microbiologist and the clinician regarding the bacteriological and clinical background of each individual patient.

The characteristic intractability of mucoid *P. aeruginosa* once it is established in the CF lung and despite significant advances in the potency of antipseudomonal agents is now well recognised. It would seem important, therefore, that future aims should be to determine the factors which contribute to the establishment of colonisation and which account for the differences in incidence of *P. aeruginosa* in different centres. The relatively low pseudomonas incidence in Melbourne and in the Edinburgh clinics (Tables 5 and 6) indicates that colonisation need not inevitably involve the majority of CF patients. Whilst several factors could account for this result it seems reasonable that the role of continuous antistaphylococcal therapy and other potential factors including cross infection needs to be clearly determined, ideally by multicentered studies.

Since at least two pseudomonal factors associated with chronic colonisation, namely, derepressed alginate production and antibiotic hypersusceptibility, result from bacterial mutations it is arguable that early therapy directed against asymptomatic colonisation with nonmucoid pseudomonas might be an important strategy to reduce the microbial reservoir from which such mutants emerge; early antipseudomonal therapy is currently practiced in several clinics (38, 39). A preventive strategy would ideally involve vaccination but, until the development of a suitable vaccine, presently available antibiotics will remain the main therapeutic agents. The parameters involved in antipseudomonal therapy at early stages of colonisation would probably be different from those discussed for chronic infection. Heterogeneity of the pseudomonas isolates would not need to be considered in respect of antimicrobial susceptibility tests. With such a therapeutic approach aerosolised antibiotics may well prove effective but even more exciting, at least for adolescent patients, we might find a valuable role for new oral agents such as ciprofloxacin.

In assessments of antimicrobial therapy in CF the review has attempted to show that both clinicians and microbiologists need to take account of the relatively complex bacteriology of the CF lung and appreciate that the beneficial action of antipseudomonal agents may result from *in vivo* activity other than the expected eradication of the bacterial pathogen.

The therapeutic value of ceftazidime in the treatment of exacerbations due to *P. aeruginosa* is well recognised and appreciated as is the high financial cost of this agent; the potential problems of emerging resistance to the drug require careful monitoring. One of the most consistent and encouraging observations which has been discussed in the review is the value of ceftazidime in the treatment of exacerbations due to *H. influenzae* which have not responded to treatment with conventional antihaemophilus agents. Finally, initial clinical experience with the new 4-quinolone antibiotic ciprofloxacin is also encouraging and emphasises important social parameters in the treatment of CF patients. For the adolescent patient, the development of ciprofloxacin, the first antipseudomonal agent available in an oral form, has allowed the option for careful administration on an outpatient basis and introduces an important social benefit in antipseudomonal therapy.

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Leading article

Ciprofloxacin therapy in cystic fibrosis

Chronic respiratory colonisation with mucoid *Pseudomonas aeruginosa* is a common and important complication of cystic fibrosis (CF) and a major cause of debilitating exacerbations and progressive lung deterioration. Studies of recent years have confirmed that treatment that reduces the load of *P. aeruginosa* is beneficial, not only subjectively but objectively, with improvements in spirometry, arterial blood gases, chest x-rays, weight and C-reactive protein levels. Since anti-pseudomonal therapy has in the past been dependent upon parenteral antibiotics it has always had the disadvantage of necessitating recurrent admissions to hospital, possibly for several weeks. In the past five years this difficulty has been approached by the development of home intravenous therapy, often administered by the patients themselves or the family.

The recent development of agents with very high in-vitro activity against *P. aeruginosa* and which are available in oral form, holds out the promise of successful home therapy in patients with CF (Scully *et al.*, 1986). A theoretical limitation of such treatment is the risk of developing bacterial resistance to the antibiotic.

All these points were considered and discussed at a recent Workshop in Edinburgh at which the experience of treatment of over 150 CF patients in the United Kingdom with ciprofloxacin was pooled and evaluated. In in-vitro tests ciprofloxacin is one of the most active anti-pseudomonal agents and its MIC for many strains of *P. aeruginosa* is 100 to 1000 times lower than that of carbenicillin. One decision of the workshop was that standardised techniques for testing the in-vitro sensitivity of *P. aeruginosa* to ciprofloxacin were essential. Agar-based serial dilution tests are preferred to disc methods. In addition, if disc methods are to be used for screening, a disc with a lower concentration than the 5 μg disc in precent use (Barry *et al.*, 1985) may distinguish more clearly between sensitive and resistant strains.

Although several studies revealed the isolation of strains with reduced susceptibility to ciprofloxacin (MIC increased 20 fold) during therapy, the clinical significance of these 'resistant' isolates remains to be determined. The resistance was often observed to be unstable, being lost on cessation of treatment. In some patients there was a gradual decrease in the susceptibility of the pseudomonas population following multiple courses of ciprofloxacin.

A further observation of the workshop was that more information is needed about the total clearance, apparent volume of distribution and bioavailability of ciprofloxacin in patients with cystic fibrosis. To obtain such data, studies involving intravenous as well as oral administration are necessary. After oral doses of 500 or 1000 mg, mean peak plasma concentrations of 2·5 and 5·6 mg/l are achieved at 2·5 and 1·9 h, respectively. Sputum levels peak at around 4 h, and are approximately 2·1 mg/l after 1000 mg by mouth (Goldfarb *et al.*, 1986). The extent to which ciprofloxacin is excreted unchanged following oral administration varies considerably; for example, in seven patients, aged 10–23 years, the percentage recovered from the urine at 8 hours was 24–51% (Alfaham *et al.*, 1986). The high values for renal clearance of ciprofloxacin reported by Bender *et al.* (1986) suggest that tubular secretion plays an important role in the excretion of unchanged drug.

Dr Margaret Hodson, from the Brompton Hospital, summarised her controlled trial of 40 adult patients who received ten days therapy, either with oral ciprofloxacin as a single agent or with the combination of azlocillin and gentamicin administered intravenously (Hodson *et al.*, 1987). It was shown that both treatments produced significant improvement in lung function tests and sputum volumes. However, after ciprofloxacin therapy, improvement was maintained for longer periods than after the two parenteral drugs. Other clinical experience quoted at the Workshop showed that treatment courses of as long as 28 weeks (including one in a ten-year-old boy) did not have any disturbing toxic effects.

The most generally accepted parameter to assess the efficacy of antibiotic treatment is the demonstration of good in-vitro activity against the bacterial pathogen. *In vivo* there should then be reduction or elimination of bacteria following antibiotic administration at a dosage calculated to provide a lethal concentration in the infected tissues. The workshop raised wider issues in respect of ciprofloxacin usage in CF and suggested potential benefits in a broader approach to antibiotic therapy.

There is a clear need to determine the effects of different dosage and treatment duration on the emergence of, and persistence of, resistance to ciprofloxacin. Intriguingly, however, there is also in-vitro evidence that resistant isolates show reduced ability to produce extracellular virulence factors; such isolates might be less pathogenic than the original sensitive strain (Govan & Doherty, 1985). The common observation that CF patients obtain clinical benefit from treatment with antipseudomonal agents, even when the number of 'sensitive' pseudomonas remains high during and after treatment, and the beneficial use of ciprofloxacin reported by most participants at the workshop, might be partly explained by in-vitro evidence that antibiotics, in particular ciprofloxacin, are capable of suppressing pseudomonas protease activity at concentrations as low as 5% of the MIC (Govan & Doherty, 1985; Govan & Harris, 1986). Because of evidence that ciprofloxacin may cause arthropathy in young experimental animals, there has been a hesitation to use the antibiotic in children. However, the workers from the University Hospital of Wales (Drs Goodchild, Alfaham and Gajeweska) reported its use in five patients aged less than 18 years. In one female of 35 kg weight and aged 16 there was a transient arthropathy of both knees after three weeks of ciprofloxacin in a dose of 1500 mg/day. All symptoms subsided within two weeks of stopping the drug. Reports at the Workshop, by Dr Goodchild (U.H.W.), Dr Redmond (Belfast) and Dr McCrae (Edinburgh) showed that the drug had been given to over 30 paediatric patients and the above case was the only arthropathy that was probably linked with ciprofloxacin; it should be noted that arthropathy occurs in up to 10% of older CF patients. There was a good therapeutic response to ciprofloxacin in over half these children.

The Workshop ended with a plea that more detailed assessments of individual CF patients be made before and after therapy, using all possible objective measurements which include spirometry, sputum volume, body weight and, possibly, exercise tests, arterial blood gas analysis or ear oximetry. It was suggested that a protocol should be prepared which summarised the indications for and the dosage of ciprofloxacin in the CF patient. If further experience could then be collated nationally the most effective regimens for ciprofloxacin usage would soon evolve. Such further studies are undoubtedly required, but ciprofloxacin is at this time an agent to be considered positively in the treatment of *P. aeruginosa* infection in teenage or adult CF patients.

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A Fatal Transfusion Reaction Associated with Blood Contaminated with *Pseudomonas fluorescens*

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Abstract. A fatal transfusion reaction due to contamination of platelet-depleted whole blood with *Pseudomonas fluorescens* is reported. Routine sterility testing on blood products and environmental microbiological monitoring suggested no source for the contaminating organism, as has been the case for the majority of reported incidents of this type. The value of routine sterility testing in the prevention and investigation of such incidents is discussed.

The introduction of presterilised plastic packs [1] has greatly reduced the incidence of sepsis associated with blood transfusion, but there continue to be occasional reports of such events, most commonly associated with *Pseudomonas fluorescens* in cold-stored red cells [2–14] but with gram-positive organisms in platelet concentrates stored at 22°C [15–27, 31] (tables I, II). Due to the very low frequency of such events, some authorities have dropped any requirement for routine sterility testing of blood donations [28], whereas others still recommend this [29, 30]. In view of recent concern over the sterility of platelet concentrates [31], this Centre continues to perform routine sterility testing on outdated donations and environmental monitoring of the microbial load in blood processing areas. Since the introduction of the current technique, based on that in the *European Pharmacopoeia* [30], we have tested over 1,500 units and found only 1 confirmed case of contamination due to *Staphylococcus epidermidis* in a platelet concentrate. Pseudomonas contamination has never been observed, even on initial testing, although the method used allowed detection of the organism described below.

The implicated donation was taken on the 26th October 1986 from a regular donor, whose previous donations had not been associated with any adverse reactions. It was processed to platelet-depeleted whole blood (PDWB) and platelet concentrate, the latter being transfused uneventfully 2 days later. The PDWB was eventually transfused 32 days after donation, after being issued and returned to the blood bank 3 times and transported to London as part of a bulk issue from Edinburgh. Prior to transfusion, it may have spent up to 7 h out of the blood bank, although transport conditions and external storage should have ensured that it remained cooled for the majority of this time.

The propositus, a 84-year-old woman with fractures of both femora, received transfusion for anaemia during conservative treatment prior to planned surgery. Transfusion with the implicated PDWB was started at a fresh site, following uneventful transfusion of 2 units of blood. The patient rapidly became unwell, felt cold and vomited with a pulse of 110 but no pyrexia. Transfusion was immediately stopped and infusion of dextrose-saline with potassium started, but she became hypotensive, anuric and developed disseminated intravascular coagulation. Although treated with cefuroxime and gentamicin with hydrocortisone (and later with piperacillin), following demonstration of gram-negative rods in her blood, her condition worsened and she died 5 days later. *P. fluorescens* was identified both in the patient's blood and the residual PDWB.

Investigations at the Regional Centre, instigated by three senior staff not involved in blood processing, confirmed the presence of *P. fluorescens* in both the PDWB and the patient's blood, but not in a separate sample taken at donation. This organism grew at 4, 37 and even 42°C and flourished at room temperature and 30°C; it was resistant to cefuroxime and trimethoprim, but sensitive to gentamicin and tetracycline. Extensive investigation failed to isolate any such organism from the blood-processing areas, nor did environmental surveillance at the time of preparation of the implicated unit reveal anything unusual. No defect was found in the implicated

Table I. Transfusion reactions associated with bacterial contamination of red cell units collected in plastic packs

Reference	Year	Organism	Number of cases			Source
			cultured from patient	cultured from donation	deaths	
Thuillier and Gandrille[1] [2]	1969	*Flavobacterium* sp.	0	2	NS	? pack
Marski[1] [3]	1977	*P. mirabilis*	1	NS	0	ND
Honig and Bove [4]	1980	pseudomonad	0	1	1	ND
Taylor et al. [5,6]	1980/1984	*P. putida*	0	1	0	? local anaesthetic
Stenhouse and Milner [7]	1982	*Y. enterocolitica*	1	2	1	ND
Khabbaz et al. [8]	1984	*P. fluorescens*	2	3	2	ND
Phillips et al. [9]	1984	*P. fluorescens*	0	1	1	ND
Tabor and Gerety [10]	1984	*P. fluorescens*	2	4	1	ND
		P. putida	0	1	1	ND
Gibaud et al. [11]	1984	*P. fluorescens*	NS	1	0	ND
Puckett [12, 13]	1985/1986	*P. fluorescens*	1	1	1	pack defect
		P. fluorescens	>2	>2	2	anaesthetic gun
Murray et al. [14]	1987	*P. fluorescens*	2	2	1	ND
		P. fluorescens	2	NS	NS	ND
Current study		*P. fluorescens*	1	1	1	ND
		Total	>14	>22	12	

ND = Not determined; NS = not stated.
[1] Unclear whether donation in plastic pack or glass.

pack, nor have any adverse reports reached the manufacturer for the batch of packs to which this one belonged. Following this incident, the procedures for skin decontamination at donation have been reviewed, steps have been taken to further reduce any time donations spent out of refrigeration and a policy of placing packs in a plastic outer bag, just after donation, has been instituted.

In common with many previous reports (table I), no source of the organism responsible for this incident has been identified. The rarity of such incidents makes it unlikely that routine monitoring of donations for sterility will reduce their occurrence. Environmental monitoring may be a more useful approach to identifying potential contaminants but was not revealing for the present case.

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Table II. Transfusion reaction associated with bacterial contamination of other blood components prepared from blood collected in plastic packs

Reference	Year	Organism	Number of cases			Source
			cultured from patient	cultured from component	deaths	
Platelet concentrate						
Buchholz et al. [15, 16]	1971/1973	*E. cloacae*	2	2	0	ND
		Serratia sp.	1	1	1	ND
		Flavobacterium sp.	1	1	0	ND
		S. epidermidis	2	2	1	? skin
Rhame et al. [17]	1973	*S. cholerae-suis*	7	3	2	Donor
Blajchman et al. [18]	1979	*S. marcescens*	3	2	1	Pilot tube
Honig and Bove [4]	1980	gram-positive cocci	0	1	1	ND
Heal et al. [19–21]	1986	*S. aureus*	1	1	0	ND
	1984/1987	Salmonella heidelberg	1	1	1	Donor
Van Lierde et al. [22]	1985	*S. marcescens*	1	1	0	ND
FDA [31]	1986	NS	NS	NS	19	NS
Arnow et al. [23]	1986	*E. coli*	1	1	0	At donation
		coagulase-negative staphylococci	0	1	0	ND
Anderson et al. [24]	1986	Staphylococci/ enterococci	4	3	0	Skin dimple
Braine et al. [25]	1986	*S. epidermidis*	1	2	0	ND
		S. epidermis and Flavobacterium	NS	1	0	ND
		S. viridans	NS	1	0	ND
		Total	25	24	26	
Cryoprecipitate						
Rhame et al. [26]	1979	*P. cepacia*	3	(2)	0	Water bath
Fresh-frozen plasma						
Casewell et al. [27]	1981	*P. aeruginosa*	1	(1)	(1)	Water bath

NS=Not stated; ND=not determined; ( )=indirect association.
[1] FDA data may include data presented elsewhere in the table.

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CHAPTER 7

Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis

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Introduction

Our present knowledge of the control of alginate biosynthesis by *Pseudomonas aeruginosa* comes primarily from its involvement in cystic fibrosis (CF) disease resulting in one of the most bizarre and intractable bacterial infections known to modern science (Deretic *et al.*, 1987a).

Arguably one of the most unusual and intractable pathogenic associations between a microbe and a host is the gradual emergence and establishment of mucoid, alginate-producing variants of *P.aeruginosa* (MPA) in the lungs of CF patients and the debilitating and tissue damaging sequelae. This review will describe alginate biosynthesis and other unusual characteristics exhibited by *P.aeruginosa* in CF, present a model for pathogenesis and discuss therapeutic strategies.

Cystic fibrosis

This is an inherited autosomal defect affecting exocrine gland function and is one of the most common lethal genetic disorders in Caucasian populations (Davis and Di Sant'Agnese, 1980). Approximately one in every 20 individuals is a carrier (frequency one in 2000 live births); there are some 4550 patients in the UK. The disease is manifested when a child inherits the mutant gene from both carrier parents. In addition to pancreatic insufficiency, an important sympton is the overproduction of abnormally viscid tracheobronchial mucus, which results in poor mucociliary clearance of the bronchial airways, inevitable bacterial colonization, pulmonary inflammation, production of copious viscid sputum, chronic obstructive lung disease and progressive lung deterioration. When CF was first recognized and described in the 1940s, life expectancy in patients with the disease was measured in months. In the last decade, however, because of improvements in management, in particular in dietary supplements, effective physiotherapy and the use of increasingly potent antimicrobials, the life expectancy of the CF child has extended into adulthood.

Much effort has been directed towards the elucidation of the molecular genetics and pathophysiology of CF. Recently, the use of specific DNA probes has successfully indicated a localization of the CF gene close to the proto-oncogene *met* on chromosome 7 (White *et al.*, 1985). Concurrently, the use of classical techniques for cloning mammalian genes has resulted in the successful isolation of the CF antigen gene from chromosome 1 (van Heyningen *et al.*, 1985). It should be stressed that the mutation in the CF antigen gene is linked to, but not the cause of CF. Evidence indicates a defective Cl^- transport in CF epithelial cells (Welsh and Liedtke, 1986; Case, 1986) with an overproduction of a normal serum protein, the CF antigen (Bullock *et al.*, 1982). Following the purification of the CF antigen protein and sequencing of its amino acid terminus, Dorin *et al.* (1987) used a highly redundant oligomeric DNA probe to isolate complementary DNA clones encoding the CF antigen. The deduced total amino acid sequence of CF antigen reveals extensive similarities to several Ca^{2+}-binding proteins and it has been tentatively suggested that the CF antigen is part of a macromolecular signal-transducing complex coupled to the Cl^- channel with the CF gene product occurring as another component of the complex (Goodfellow, 1987).

These physiological studies seem far removed from the major clinical problem faced by the CF patient. However, as a consequence of the basic defects in the CF patient, the CF respiratory mucosae presents a rather different environment to a potential pathogen than that in normal individuals. Tracheobronchial mucin purified from CF sputa has been shown to possess different chromatographic and rheological properties from non-CF sputa (Rose *et al.*, 1987). The volume and rheological properties of bronchial mucus, which play an important part in preventing bacterial colonization, are also affected by several interrelated factors including repeated episodes of exacerbation and concomitant inflammation, the presence of DNA from resident bacteria and from inflammatory immune cells, raised levels of electrolytes in particular Na^+ and Ca^{2+} and a physiological status akin to dehydration (Knowles *et al.*, 1983).

General microbiology of CF

Most of the exacerbations causing morbidity and respiratory symptoms in CF patients are due to bacterial infections, but infections with viruses, chlamydia and mycoplasma may also contribute to the progressive deterioration of the lungs; allergic aspergillosis occurs in only a few patients (Friend, 1986). *Staphylococcus aureus*, *Haemophilus influenzae* and *P.aeruginosa* have a predilection for the CF lung. In general, but not exclusively, *Staph.aureus* is considered to be the initial pathogen, but in older patients *H.influenzae* and *P.aeruginosa* begin to predominate; MPA (*Figure 1a* and *b*), in particular, are associated with progressive chronic respiratory infection (Henry *et al.*, 1982; Hoiby, 1984; Pier *et al.*, 1987). The presence of large numbers of *H.influenzae* in sputum (1×10^7 c.f.u. ml^{-1}) is usually associated with considerable morbidity; disturbance of respiratory cilia by *Haemophilus* (Wilson *et al.*, 1985) may also assist subsequent colonization by *P.aeruginosa*. Without doubt, in the last 20 years, *P.aeruginosa* has emerged as the major cause of anxiety and morbidity in CF. The pathogenic status and *modus operandi* of this classic microbial opportunist is by no means obvious as illustrated by the wry inquiry '*Pseudomonas aeruginosa* in cystic fibrosis: sylph or sycophant' (Fick, 1981).

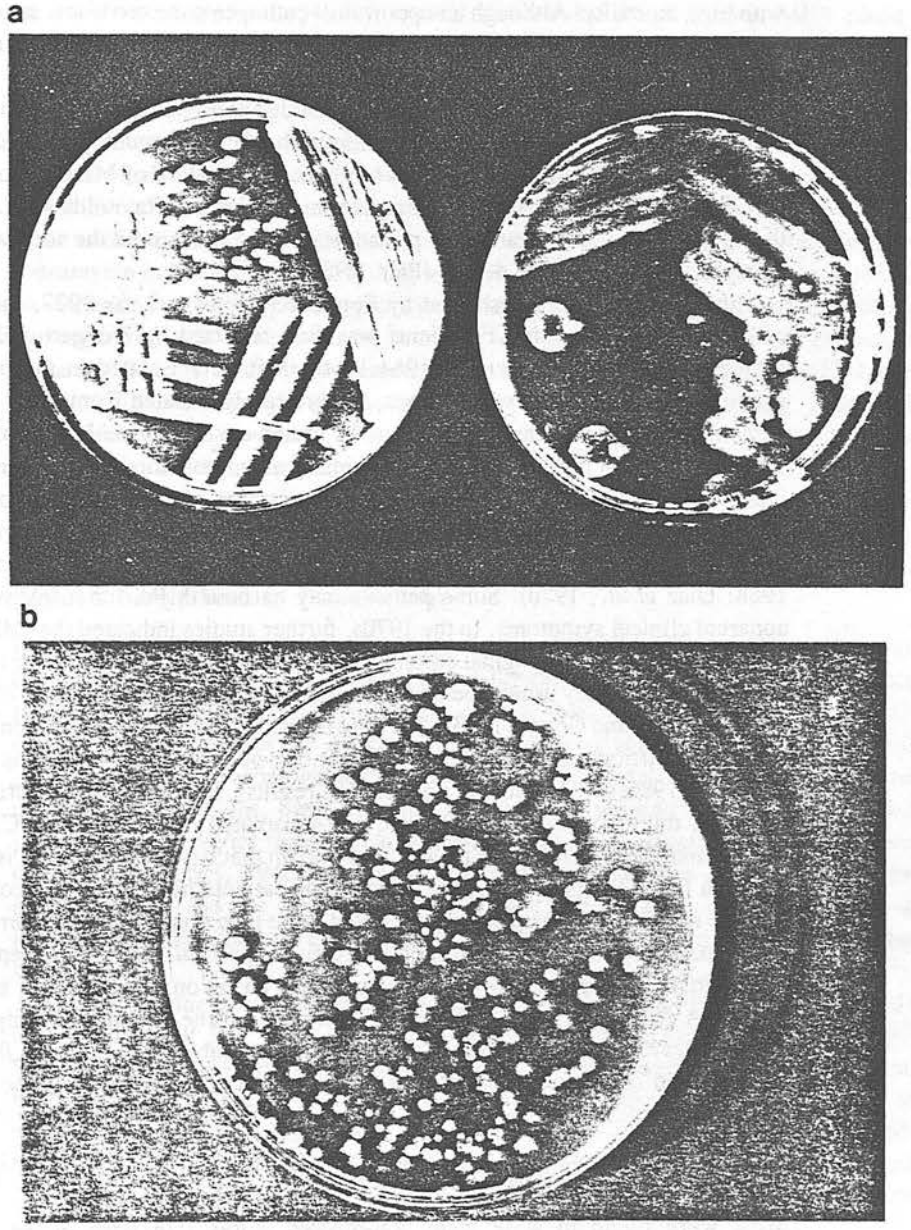

Figure 1. (**a**) Typical non-mucoid colonial form of *P.aeruginosa* and mucoid, alginate-producing variant (MPA) grown on Pseudomonas isolation agar (PIA. Difco) for 48 h at 37°C. Photograph taken from Govan and Harris (1986) *Microbiological Sciences*. **3**. 302–308. By permission. (**b**) Sputum culture on PIA from a CF patient showing the emergence of the mucoid colonial form: previous cultures had produced only non-mucoid *P.aeruginosa*.

P.aeruginosa and CF

P.aeruginosa (PA) is an adaptable saprophyte and a significant opportunist pathogen causing localized or systemic infections in compromised patients. Pseudomonal infections

are often troublesome, intractable to treatment and, in the case of septicaemia, associated with high mortality. Although an opportunist pathogen par excellence, statistical studies have indicated that typical strains of PA are of little clinical significance in CF patients (Henry *et al.*, 1982).

Septicaemia and non-respiratory infections due to PA are rarely encountered in CF patients; in contrast, chronic colonization with MPA is arguably the most important cause of morbidity and mortality. Not only is the isolation of MPA almost diagnostic of CF in young adults with chronic pulmonary disease (Reynolds *et al.*, 1976), but the appearance of the organism in pre-adolescence has acquired the somewhat ominous status as a 'harbinger of death' (Pier, 1986).

Although MPA were described by Sonnenschein as early as 1927, the association of mucoid isolates with CF patients was first reported by Doggett and co-workers (Doggett, 1969; Doggett *et al.*, 1964,1966). MPA are isolated in up to 90% of CF patients colonized with *Pseudomonas*, but are rarely isolated from other infections in human beings, plants, animals, or from the varied environmental habitat of the species.

Doggett *et al.* (1966) observed that primary asymptomatic colonization occurred in individual patients with typical non-mucoid strains but later MPA appeared. The establishment of MPA was also associated with the appearance of antipseudomonal serum antibodies and increased severity of the patient's pulmonary status (Burns and May, 1968; Diaz *et al.*, 1970). Some patients may harbour MPA for many years with no apparent clinical symptoms. In the 1970s, further studies indicated that MPA appeared to be derived from the original colonizing strain; when the two forms are isolated from the same sputum they usually belong to the same serotype (Diaz *et al.*, 1970) and pyocin type (Williams and Govan, 1973). The time taken for the mucoid conversion in individual patients is difficult to assess accurately but can be as short as 3 months (Friend and Newsom, 1986; J.R.W.Govan, unpublished results). In the author's laboratory, pyocin typing of multiple colonies of PA and MPA, isolated from individual CF sputa on a longitudinal basis from 32 CF patients, has shown that: (i) cross-infection is rare, except between CF siblings; and (ii) the majority of patients have remained colonized with a single strain: only nine patients harboured more than one strain either simultaneously, or in separate episodes of colonization. Recently, Ogle *et al.* (1987) have reported similar results in 15 CF patients using a typing system based on Southern blot hybridization and DNA fragments derived from the exotoxin A gene and surrounding sequences. These observations indicate that mucoid variants probably arise from PA *in vivo*, either by induction of alginate biosynthesis in the whole population or by the selection of spontaneous mutants.

Isolation of mucoid *P.aeruginosa in vitro*

MPA were found to arise from non-mucoid cultures *in vitro* in the presence of bacteriophage (Martin, 1973; Govan, 1975). At the time, there was considerable controversy as to whether the mechanism of phage-associated mucoid conversion was due to selection of pre-existing mutants, or, as Martin suggested, that the continuous presence of phage was required and mucoid conversion due to a form of pseudolysogeny.

In contrast to the gradual establishment of MPA in the CF patient, mucoid strains isolated from CF patients and those obtained *in vitro* by the action of phage are usually unstable and give rise to non-mucoid revertants after incubation for more than 24 h

or when subcultured serially. Our studies showed that phage-derived MPA could be maintained in the mucoid form without the presence of phage and that stability of MPA, isolated from CF sputa or phage-derived, was improved if the strains were cultured on surfactant-containing media (Govan, 1975) or incubated in broth cultures with aeration. Fluctuation tests indicated that instability resulted from spontaneous non-mucoid mutants which had a growth rate advantage in non-aerated cultures (Govan *et al.*, 1979). In addition to the *in vitro* conversion to mucoid phenotype through the agency of phage, other contemporary studies indicated a 1.5-fold increase in MIC of carbenicillin and tobramycin in MPA compared to isogenic non-mucoid strains. These observations led to evidence that MPA, phenotypically similar to clinical and phage-derived isolates, can arise from non-mucoid parent strains at a frequency of one in 10^7 by exposure to these antibiotics and that the frequency could be increased by mutagenesis (Govan and Fyfe, 1978). Similarly, alginate-producing mutants were isolated from other pseudomonads not previously known to produce alginate (Govan *et al.*, 1981) and low levels of alginate have also been detected in some plant-pathogenic pseudomonads (Fett *et al.*, 1986). These studies indicate that the various genes for alginate biosynthesis are present in probably all PA and certain other species of *Pseudomonas*.

Alginate biosynthesis in *Pseudomonas*

Despite the isolation of PA from a wide range of environmental niches and various tissue sites in infected hosts, the species is not typically associated with a mucoid colonial morphology and alginate biosynthesis at readily detectable levels. In contrast, MPA emerge in up to 90% of CF patients, in up to 40% of sputum isolates from non-CF patients with chronic obstructive lung disease and occasionally (up to 10%), in chronic urinary tract infections. Since evidence suggests that alginate is the most convincing pseudomonal virulence factor associated with pulmonary exacerbations in CF patients (Govan and Harris, 1986), an appreciation of the properties of this unusual exopolysaccharide and the factors responsible for its regulation and biosynthesis are important to understand the pathogenesis of MPA in CF lungs and to devise therapeutic strategies.

Alginate produced by MPA is a linear copolymer of *O*-acetylated $\beta(1-4)$-linked D-mannuronic acid and its C-5 epimer L-guluronic acid (Evans and Linker, 1973; Sutherland, 1986). When alginate biosynthesis is de-repressed or 'switched on', some MPA produce very high levels of alginate, for example 200 μg mg^{-1} of wet cell weight (Deretic *et al.*, 1986), with carbon conversion efficiencies approaching 65% (Mian *et al.*, 1978). A striking characteristic of microbial alginates is their high viscosity in solution and the ability to form rigid stable gels in the presence of divalent cations, particularly Ca^{2+} (Rees, 1972).

Alginate extracted from MPA strains from CF patients is highly acetylated (Sherbrock-Cox *et al.*, 1984; Dunne and Buckmire, 1985) and analysis by proton NMR indicates that the pseudomonal alginate lacks repetitive sequences of L-guluronate giving the polymer gels an elastic rather than a brittle physical nature (Gacesa and Russell, personal communication).

Culture of MPA on agar-based media containing Ca^{2+} causes the normal mucoid colonial appearance to assume a more compact gelatinous appearance (Govan and Harris, 1986). When MPA are cultured *in vitro* and examined by phase-contrast microscopy,

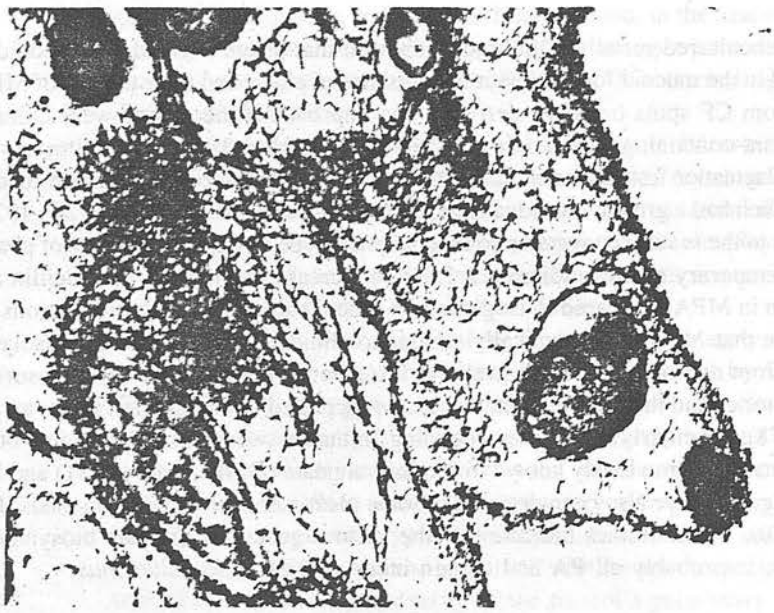

Figure 2. Electron micrograph of microgels formed by the mucoid *P.aeruginosa* PA0579 in the presence of 3 mM Ca^{2+}. Note how the bacteria are enclosed within a cotton wool-like alginate matrix. Ruthenium red stain. ×342 000. Photograph taken from Govan and Harris (1986) *Microbiological Sciences*. **3**. 302–308. By permission.

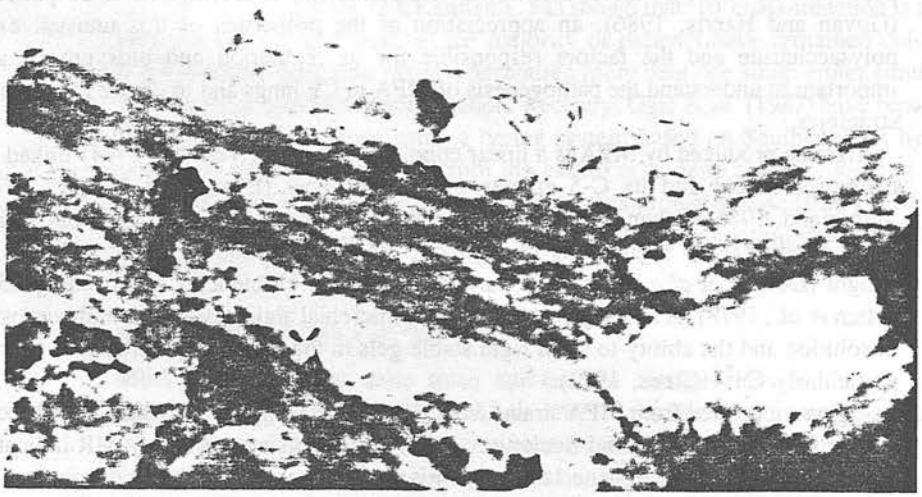

Figure 3. Gram-stained sputum from a CF patient harbouring mucoid *P.aeruginosa*. Non-mucoid *P.aeruginosa* typically appear distributed randomly across the field. In contrast, mucoid *P.aeruginosa* can be seen in gelatinous microcolonies attached to the bronchial mucosa. ×1620.

the bacterial alginate appears as an extracellular slime. The presence of Ca^{2+}, at concentrations as low as 3 mM, rapidly results in the formation of bacteria-containing microgels which rapidly sediment in liquid culture. By electron microscopy, the microgels are seen as cotton wool-like mesh enclosing the bacterial cells (*Figure 2*). It could be speculated that the bacterial microcolonies (Figure 3) observed microscopically in the sputum of CF patients (Hoiby, 1982; Govan, 1983; Govan and Harris, 1986) and in post-mortem lungs (Lam *et al.*, 1980) result from the gelling potential of the bacterial alginate and the presence of raised Ca^{2+} and DNA concentrations which are a basic characteristic of CF disease (Chernick and Barbero, 1959; Matthews *et al.*, 1963). A recent interesting observation on the relationship between Ca^{2+} and human DNA on the rheological properties of tracheobronchial mucus is that addition of pseudomonal alginate increased the viscoelasticity of CF mucus but not the mucus from patients with chronic bronchitis; in addition, the phenomenon was only apparent in the presence of at least 4 mM Ca^{2+} (James *et al.*, personal communication).

Alginate biosynthetic pathway

The biosynthesis of alginate has been studied in the alga *Fucus garderi* (Lin and Hassid, 1966) and in *Azotobacter vinelandii* (Pindar and Bucke, 1975). Accumulated evidence suggests that a similar biosynthetic pathway operates in mucoid *P.aeruginosa* (Piggott *et al.*, 1981; Pugashetti *et al.*, 1983; Sa'-Correa *et al.*, 1978; Wang *et al.*, 1987) and in alginate-producing mutants of *P.mendocina* (Anderson *et al.*, 1987).

Biochemical evidence indicates that the Entner–Doudoroff pathway is a major pathway for alginate biosynthesis in *Pseudomonas* (Banerjee *et al.*, 1983; Lynn and Sokatch, 1984). Enzyme analyses suggest that the pathway commences by conversion of the glycolytic intermediate fructose 6-phosphate to mannose 6-phosphate then proceeds as outlined in *Figure 4a*. Four of the alginate biosynthetic enzymes (PMI, PMM, GMP and GMD , see figure legend for full names) have been shown to be normally present at low levels in PA cell-free extracts and raised in mucoid strains (Pigott *et al.*, 1981; Sutherland, 1982; Sa'-Correa *et al.*, 1987).

Genetics of alginate biosynthesis in P.aeruginosa

There are a number of genes whose function is essential for production and regulation of alginate biosynthesis in *P.aeruginosa* (*Figure 4b*). Various approaches have indicated that these genes are located on the *P.aeruginosa* PAO chromosome in at least three main clusters. In this review, the time of entry of the known alginate genes and associated linked markers are recalibrated to accommodate the recently revised 75 min map drawn up by O'Hoy and Krishnapillai (1987).

In our laboratory, MPA were derived *in vitro* from *P.aeruginosa* PAO and the mucoid colony phenotype *muc* transferred to the multiply marked recipient *P.aeruginosa* PAO222 by FP2 and R68.45-mediated conjugation and by transduction with phage F116L (Krishnapillai, 1971). From these studies (Fyfe and Govan, 1980,1981,1983) two distinct *muc* loci were located in the 70 region of the PAO chromosome on either side of the markers *his*1 and *cys*-59 (*Figure 4b*). MPA can be distinguished phenotypically on the basis of medium-dependent mucoid colonial growth (Govan *et al.*, 1983). Group 1 mutations, represented by *muc*-23 which maps to the right of *cys*-59, yields

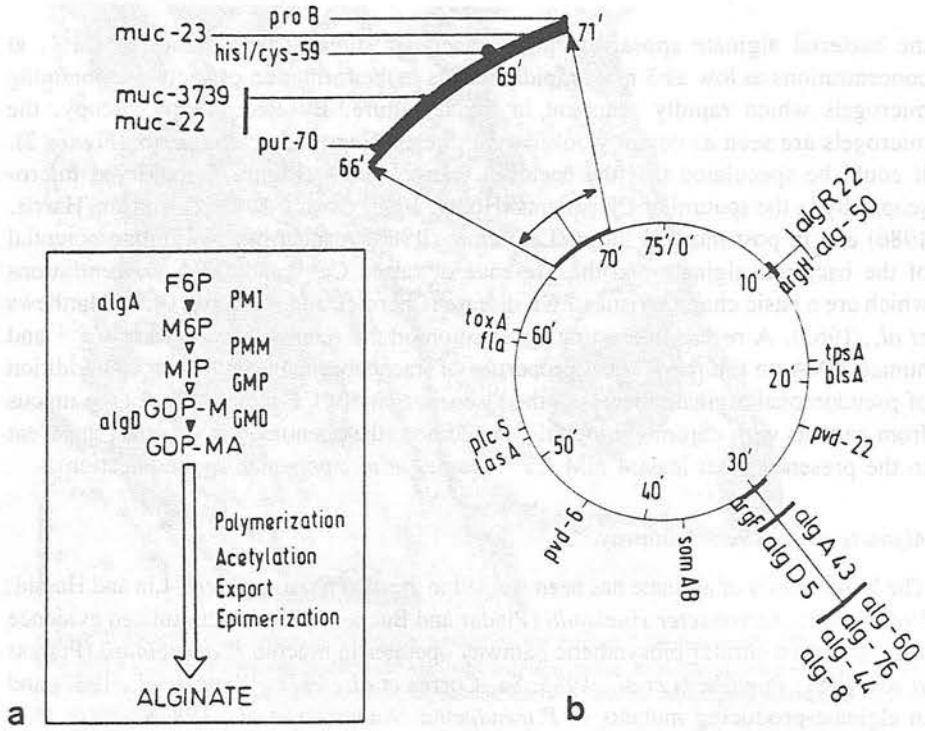

Figure 4. (a) Alginate biosynthetic pathway. The final steps include polymerization, export, epimerization and acetylation. *alg*A, gene encoding PMI; *alg*D, gene encoding GMD. F6P, fructose 6-phosphate; PMI, phosphomannose isomerase; M6P, mannose 6-phosphate; PMM, phosphomannose mutase; M1P, mannose 1-phosphate; GMP, GDP-mannose pyrophosphorylase; GDP-M, GDP-mannose; GMD, GDP-mannose dehydrogenase; GDP-MA, GDP-mannuronic acid. (b) Chromosome map of *P.aeruginosa* PAO illustrating location of the three known gene clusters (at 10, 30 and 70 min) involved in alginate biosynthesis (*alg*) and transfer of the mucoid colony phenotype (*muc*) after Fyfe and Govan, 1983; Fyfe, 1985; MacGeorge *et al.*, 1986; Deretic *et al.*, 1987a,b,c). Other structural genes involved in the virulence of *P.aeruginosa* are also included (Fyfe and Govan, 1984; Fyfe, 1985; Haas *et al.*, 1987); *tps*A, and *bls*A, genes encoding hypersusceptibility to trimethoprim and β-lactam antibiotics respectively; *pvd*, pyoverdin production; *som*, somatic antigens; *las*A, elastase; *plc*S phospholipase C, *fla*, flagella; *tox*A, exotoxin A; *arg*H, *arg*F, arginine catabolic genes; *pur*, purine; *his*1, histidine; *cys*, cysteine; *pro*B, proline. The loci, based on time of entry, have been relocated from the previous standard map (Holloway and Morgan, 1986) to accommodate the revised map recalibrated to 75 min without changes of marker order (O'Hoy and Krishnapillai, 1987).

mucoid colonies on minimal agar whereas group 2 mutations (*muc*-22) which maps to the left of *cys*-59, yields non-mucoid colonies on minimal agar; however, since group 1 and group 2 mutations have been mapped to the left of *cys*-59, clearly more than one locus is involved in the 3 min region between *pur*-70 and *cys*-59. Although mucoid phenotypes isolated *in vitro* exhibited the same range of medium-dependent alginate biosynthetic characteristics as isolates from CF patients, there was no assurance that the PAO *muc* mutations bore any genetic relationship to the mutations responsible for alginate biosynthesis in CF strains. This doubt was dispelled by the observation that a *muc* locus in the CF isolate 492c could be transferred by R68.45-mediated interstrain matings with PAO 1042 and mapped between *cys*-59 and *pur*-70 (Fyfe and Govan, 1983). Having isolated derivatives of the plasmid R68.45 containing the *pru*-54 marker

(at 67) from non-mucoid PAO derivatives, Fyfe (1985) observed that these plasmids were able to 'switch off' alginate production in mucoid PAO variants with *muc*$^+$ mutations linked to *pru*. This suggested that the plasmids could be used as probes to determine whether an unknown *muc* mutation in CF isolates mapped at this region or elsewhere. Subsequently, the probe pJF4 was found to 'switch off' alginate biosynthesis in four out of five mucoid clinical strains isolated from CF patients. The exception, strain 492a may well contain a *muc*$^+$ mutation similar to that in PAO 579 (*muc*-23) which as previously indicated maps between 69 and 71 min.

Recently, in a second approach, employing plasmid pM060-mediated conjugation to PAO recipients, MacGeorge *et al.* (1986) showed that a locus responsible for alginate biosynthesis in five independent mucoid CF isolates could be transferred to PAO, and in each case was located on the PAO chromosome in the 66–71 region (e.g. *muc*-3739, *Figure 4b*). It was not possible in this study to determine if the same locus was involved in each case.

A third approach, initiated by Chakrabarty, Ohman and colleagues was to take a mucoid CF isolate designated FRD and introduce into it auxotrophic and non-mucoid mutations, termed *alg*, which resulted in a non-mucoid phenotype, then with the use of chromosome-mobilizing plasmids attempt to map the loci involved in repression of alginate biosynthesis. These studies, incorporating cloning, complementation and enzyme analyses have provided a fascinating insight into the molecular biology of alginate biosynthesis and only a brief appraisal will be attempted in this review. Ohman and Chakrabarty (1981) reported three closely linked *alg* loci in strain FRD that could mutate to suppress the mucoid phenotype. In their study, the *alg* loci were determined by linkage to a range of auxotrophic markers generated in FRD: thus it was not possible to relate the location of the *alg* cluster to any particular region of the PAO chromosome which has been extensively mapped and is widely accepted as a reference for genetic studies. With another CF isolate, Darzins *et al.* (1985a) were able to employ a clone from a cosmid gene bank, identified by its ability to complement the *alg*-suppressor mutation (namely, restore the mucoid phenotype) and which carried the PAO *arg*H$^+$ wild-type gene, located at 10 min. Three other *alg* loci were identified using complementation by different clones but no chromosomal location was determined. Goldberg and Ohman (1987) described an *alg*B mutation, at 10 min on the PAO chromosome which is involved in high level alginate production but not directly involved in the biosynthetic pathway. Flynn and Ohman (1988) described the cloning of an *alg*S gene mapping near *his*1 which turns on alginate production in strain FRD.

Complementation studies indicated that the *his*1$^+$ gene of strain FRD was the same as that in strain PAO, indicating that *alg*S maps close to the previously described *muc* mutations in the same 70 region in PAO and FRD. Flynn and Ohman speculated that *alg*S might act as a genetic switch to control expression of a closely linked *alg*T mutation in a *cis*-active fashion and that the *alg*T gene product in turn promotes the activation of distal genes in the alginate pathway.

Historically, it is interesting that the existence of mutations resulting in loss of mucoid phenotype, distinct from *muc* mutations at 70 and from alginate biosynthetic genes, had been previously suspected and utilized (Fyfe and Govan, 1980,1983). 'Suppressed' non-mucoid PAO mutants were able to transfer the *muc*$^+$ alleles in matings, and give rise to mucoid recombinants on selection for appropriate markers in the 70 region; these

mutants also provided suitable strains for phage preparations capable of transducing the *muc* alleles into PAO (Fyfe and Govan, 1983). Suppressed variants are relatively common amongst non-mucoid revertants of *P.aeruginosa* obtained from *in vitro* cultures; such variants also arise during *in vivo* growth (J.R.W.Govan, unpublished results) when MPA are used to establish chronic pulmonary infection in rat lungs using the agar bead model described by Cash *et al.* (1979).

Further insight into alginate regulation in *P.aeruginosa* followed the cloning, sequencing and characterization (Darzins *et al.*, 1985b; Wang *et al.*, 1987; Deretic *et al.*, 1987a,b,c) of the two genes for the enzymes PMI, (*alg*A) and GMD (*alg*D) located in a third alginate cluster near *arg*F at 30 min. The over-expression of the *alg*A gene also results in derepression of PMM and GMP activity but not of the essential enzyme GMD (Sa'-Correa *et al.*, 1987). A segment of the clustered alginate region at this locus and distal to the *pmi* gene contains *alg*D which was shown to be transcriptionally active in MPA but inactive in spontaneous non-mucoid revertants (Deretic *et al.*, 1987a,b,c). Using transcriptional fusion, Deretic *et al.* (1987c) found the *alg*D promoter, an important regulator of alginate biosynthesis, to be under positive control by an *alg*R gene which had previously been located (Darzins and Chakrabarty, 1984) at 10 min. Measurements of the specific activity of the two other enzymes GMP and PMM in *alg* mutants located at this locus suggest that the remaining *alg* genes *alg*-8, *alg*-44, *alg*-76 and *alg*-60 do not encode either PMM or GMP but are involved in post GDP−mannuronic acid (MA) processing events including MA transport, polymerization, secretion, epimerization and acetylation (Wang *et al.*, 1987).

Antigenic variation of *P.aeruginosa* in CF

Typical PA strains, isolated from clinical and environmental sources, possess smooth lipopolysaccharide (LPS) as the dominant epitope on the bacterial surface and can be differentiated into 17 O-serovars on the basis of O-antigen specificity (Pitt, 1986). The LPS of such strains comprises repeating sugar units in the LPS side chain, linked to a conserved core polysaccharide, which in turn, is bound to a lipid A moiety embedded deeper in the outer membrane. Loss of the O-side chain by a strain results in loss of specific O-serovar antigen and exposes lipid A which in turn is capable of activating complement and causes the cell to become sensitive to the bactericidal action of normal human serum. Serum-sensitive PA strains are rarely isolated but, in contrast, account for up to 80% of CF pseudomonal isolates. Serum-sensitive PA show no O-antigen specificity and are usually termed polyagglutinating since they may agglutinate with several of the distinct type-specific antisera (Penketh *et al.*, 1983; Hancock *et al.*, 1983). Thus PA isolates which are usually serum resistant and exhibit a specific O-serovar lose these properties as colonization becomes established. Penketh *et al.* (1983) showed that severely debilitated patients harboured PA at the transitional stage between smooth and rough LPS chemotype. As with alginate biosynthesis, antigenic surface variation and serum sensitivity are associated with the respiratory tract and found not only in CF but also in chronic pseudomonal colonization in non-CF patients with bronchiectasis. Antigenic variation of PA in the respiratory tract may well suppress the development of an effective antibody response whilst it has been argued that the high incidence of serum-sensitive strains could account for the low incidence of septicaemia in CF patients. Against such an explanation, however, Thomassen and Demko (1981) demonstrated

that some CF sera contained blocking antibodies which neutralized the bactericidal activity of the patient's serum for the homologous PA strain. Whilst antigenic variation might confer a selective advantage on *Pseudomonas* within the CF lung, studies by Cryz *et al.* (1984), using a burned mouse model and i.p. inoculation, indicated that an LPS-defective variant was 1000-fold less virulent than the parent strain.

Antibiotic hypersusceptibility of *P.aeruginosa* in CF

PA is associated with an intrinsic resistance to many antimicrobial agents, indeed this property is generally held responsible for the emergence of the species as a significant opportunist pathogen. In the CF lung, we need to consider another paradoxical pseudomonal characteristic, namely the frequency and survival of variants exhibiting hypersusceptibility to a range of antibiotics.

The development of carbenicillin, the first semisynthetic penicillin with good anti-pseudomonal activity was a significant advance in therapy. However, MIC values of carbenicillin for *P.aeruginosa* which are generally 25–50 μg ml^{-1} (Knudsen *et al.*, 1967), were still higher than the levels attainable in bronchial secretions (Marks *et al.*, 1971). May and Ingold (1972) reported that *P.aeruginosa* isolates from sputum were often considerably more sensitive to carbenicillin than isolates from other sources. In their study of 111 sputum isolates of *P.aeruginosa* from patients with CF, chronic bronchitis or bronchiectasis, they found 35% of strains sensitive to 6 μg ml^{-1} carbenicillin and some isolates to less than 1 μg ml^{-1}. In our studies of the association of *P.aeruginosa* and CF, we also reported strains unusually sensitive (hypersusceptible) to a range of antibiotics including the β-lactams carbenicillin, methicillin, azlocillin and also cefuroxime, trimethoprim, nalidixic acid and tetracycline (Irvin *et al.*, 1981; Govan *et al.*, 1983).

Hypersusceptible isolates were observed in 24 out of 54 respiratory isolates of MPA and in 11 of 24 non-mucoid respiratory isolates. Hypersusceptible strains were usually isolated in association with organisms of normal susceptibility belonging to the same pyocin type indicating a close relationship. Non-mucoid revertants retained their hypersusceptibility. In contrast, no hypersusceptible PA were found in 216 non-respiratory isolates obtained from various clinical sites in non-CF patients or from the environment. Thus hypersusceptibility is not directly linked with alginate production and is apparently confined to the pulmonary environment. Further studies also indicated that hypersusceptibility is also independent of serum sensitivity and antigenic variation (Fyfe and Govan, 1984).

As with alginate biosynthesis, antigenic variation and serum sensitivity, hypersusceptibility is observed with increased frequency in patients with established pseudomonal colonization. Paradoxically, hypersusceptible PA are not eliminated during antibiotic therapy and may even increase during antipseudomonal therapy. The percentage of hypersusceptible isolates within an individual sputum varies but can account for almost 100% of the pseudomonas population. Hypersusceptibility is most obvious with carbenicillin and becomes less significant as antipseudomonal activity of individual antibiotics increases; in the case of ciprofloxacin the most active of the available antipseudomonal agents, hypersusceptible isolates are only 4-fold more sensitive than normal isolates. Using enhanced sensitivity to cefuroxime as a marker, mutations encoding hypersusceptibility were transferred from the CF strain 492c into *P.aeruginosa*

PAO by plasmid R68.45-mediated conjugation (Fyfe and Govan, 1984; Fyfe, 1985). Transductional analysis using phage F116L (Krishnapillai, 1971) indicated the location of two loci (*bls* and *tps*) on the PAO chromosome linked to a *met* marker at 18 min (*Figure 4*). The *tps* mutation which enhances susceptibility to trimethoprim but not the β-lactams was also found to be present in the non-hypersusceptible strain 492a, isolated from the same CF sputum. On the hypothesis that 492c could have arisen from 492a by a single-step mutation at the *bls*A locus, we postulated that a similar *bls*A mutant could be isolated from PAO; this was subsequently demonstrated following mutagenesis with ethylmethane sulphonate and the mutant strain PAO 6511 shown to exhibit the same antibiogram as the PAO recombinants from the inter-strain crosses between 492c and PAO. In general, hypersusceptible PA isolated from CF patients exhibit the same range of hypersusceptible antibiogram. It remains to be determined however if all hypersusceptible strains contain the same *bls* and *tps* mutations.

The structural basis of hypersusceptibility is not known though the range of different antibiotics involved suggests some form of relatively non-specific increase in permeability. It has previously been reported that the prototype hypersusceptible CF isolate 492c has two extra outer membrane proteins when compared with the 'normal' strain 492a isolated from the same sputum (Irvin *et al.*, 1981); further studies, however, failed to confirm that these proteins are associated with hypersusceptibility (Fyfe and Govan, 1983). Zimmerman (1980) reported the development of hypersusceptibility *in vitro* in strain Z/61 following mutagenesis. Mapping of the genetic determinants for hypersusceptibility in Z/61 (Fyfe, 1985; Angus *et al.*, 1987) followed transfer of mutations from Z/61 to PAO strains. One mutation (*abs*A) was 40% co-transducible with the *pro*-82 marker at 26 min and gave rise to hypersusceptibility to a wide range of antibiotics including β-lactams, gentamicin and hydrophobic agents. Another mutation (*abs*B), associated with hypersusceptibility to β-lactams, and gentamicin but not to hydrophobic agents, was co-transducible with *met*-28 and *pro*C at 20 min close to the *bls*A mutation encoding hypersusceptibility in the CF isolate 492c (Fyfe and Govan, 1983). Both *bls*A and *abs*B are close to *nal*B which codes for resistance to nalidixic acid, novobiocin and various β-lactams due to decreased permeability to these agents (Rella and Haas, 1982). The molecular basis of hypersusceptibility in Z/61 and in CF isolates remains obscure. SDS–PAGE studies suggest that hypersusceptibility may be associated with altered patterns of O-antigen distribution whilst the *abs*B mutation caused a structurally undefined alteration in the physical interaction of EDTA and gentamicin with the outer membrane (Darveau and Hancock, 1983; Angus *et al.*, 1987). To date, there has been no genetic analysis of the other LPS-associated properties, namely serum sensitivity and antigenic variation; it seems likely however that these are genetically unrelated to hypersusceptibility.

The clinical relevance of hypersusceptibility in patients with CF can be considered in two ways. It is well recognized in CF microbiology that considerable heterogeneity of colonial morphology and antibiotic susceptibility is observed when multiple colonies of PA are examined from a single CF sputum (Thomassen *et al.*, 1979; Seale *et al.*, 1979; Govan *et al.*, 1987) and it has been suggested that susceptibility tests on isolates from CF patients should be performed on each colonial type. Hypersusceptibility should be considered as an extreme form of this heterogeneity but its significance in anti-microbial susceptibility testing lies in the fact that hypersusceptible and non-

hypersusceptible PA are usually of similar colonial appearance. Thus heterogeneity PA strains in CF patients demands even further consideration including examination of multiple examples of mucoid and non-mucoid colonial types. The paradoxical emergence and survival of hypersusceptible strains in the face of often aggressive anti-pseudomonal therapy with potent antipseudomonal agents will be discussed later in considering the microcolony mode of growth of *P.aeruginosa* in the CF lung. Interestingly, hypersusceptibility confers a considerable growth rate advantage under certain cultural conditions. The MPA strains 492a and 492c represent a typical pair of normal (492a) and hypersusceptible (492c) isolates from a CF patient; they are both mucoid, serum-sensitive, polyagglutinating and belong to the same pyocin type. When cultured in rich complex media, for example nutrient broth with yeast extract, little difference can be detected in the growth rate of the two organisms. In contrast, when grown in minimal media, or under diauxic growth conditions, 492c grows significantly faster and has a shorter lag phase than 492a. When the *bls* and *tps* mutations present in 492c were transferred into PAO (Fyfe and Govan, 1983) and the growth rate of the hypersusceptible recombinant PAJ1 examined, no growth rate advantage was observed. However, when a mucoid derivative of PAJ1, termed PAJ1M, was isolated by exposure to carbenicillin (Govan and Fyfe, 1978), and compared with mucoid, non-hypersusceptible recombinants, PAJ1M was found to have the same growth rate advantage as the parent strain 492c (J.R.W.Govan, unpublished results). It seems likely that the growth rate advantage, associated with the *bls* and *tps* mutations, occurs only in association with the presence of alginate. Perhaps of more clinical relevance is the observation that 492c grows considerably better than 492a *in vitro* when tracheobronchial mucin is used as the sole nutrient.

Growth of *P.aeruginosa* in the CF lung

From the evidence presented in this review it seems reasonable to speculate that a combination of adaptive properties account for the survival and pathogenicity of PA in the CF lung environment; it is also arguable that alginate biosynthesis and the rheological properties of this bacterial polymer are the most important factors in understanding the mechanisms of pathogenesis and in developing therapeutic stratagems.

In contrast to acute pulmonary infections in non-CF patients in which the pseudomonas are observed, by microscopy, to be widely dispersed throughout the sputum, the MPA present in CF *post-mortem* lung (Lam *et al.*, 1980) and in CF sputum (Hoiby, 1982; Govan, 1983; Govan and Harris, 1986) can often be seen as large microcolonies adhering to bronchial mucosa (*Figure 3*) and surrounded by phagocytes. From evidence in the CF literature it is possible to use this concept of a microcolony form of growth *in vivo* (originally proposed in a wider environmental context by Costerton *et al.*, 1978) to construct a hypothetical but plausible model which takes account of the unusual properties associated with isolates of *Pseudomonas* and the survival and pathophysiology of pseudomonal infection in the CF lung. Additional bizarre clinical observations which have been made concerning *Pseudomonas* and CF and which require explanation include the following: first, despite a considerable reputation as a microbial hyena, capable of causing a wide range of opportunistic infections, non-mucoid PA are of no clinical significance in CF patients; secondly, despite evidence for the presence of antibodies

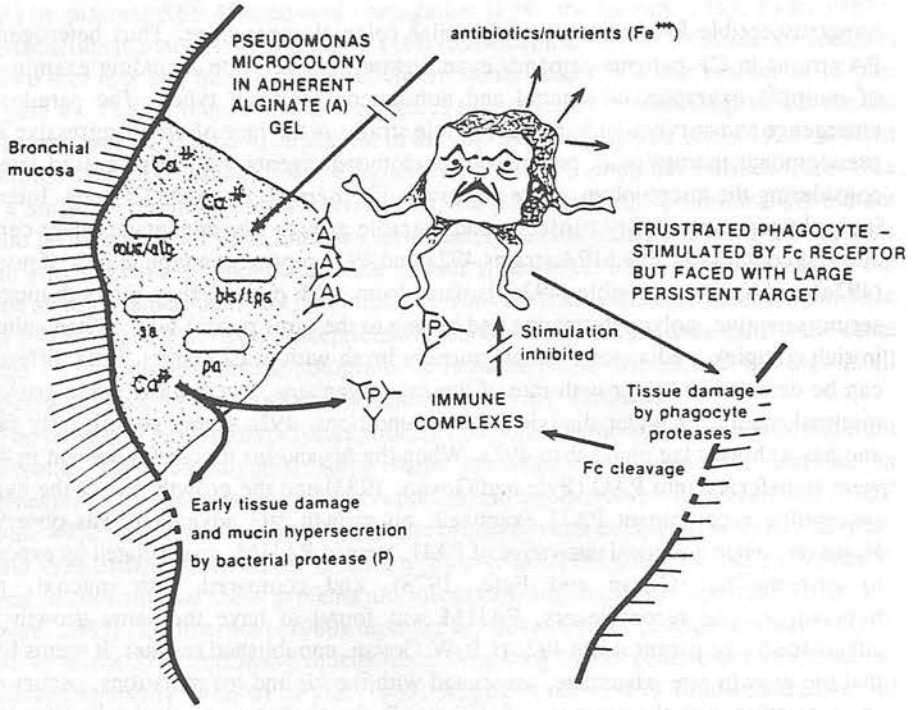

Figure 5. Microcolony model for survival and pathogenesis of mucoid *P.aeruginosa* within a CF lung includes attachment of bacterial alginate to bronchial mucosa and containment of bacteria exhibiting alginate production (*muc* and *alg*), antibiotic hypersusceptibility (*tps* and *bls*), serum sensitivity (ss) and polyagglutinating antigen (pa) within a Ca^{2+}-dependent microgel composed of alginate, DNA and tracheobronchial mucin. Other properties of the bacterial microcolony consistent with *in vitro* and *in vivo* observations, include a physical and electrolyte barrier to antibiotics and a reduced nutrient availability, slow localized release of bacterial proteases (P) and rhamnolipids and a damaging immune-mediated response exacerbated by the size, antigenicity and persistence of the alginate matrix (A) and the action of immune complexes associated with pseudomonas antigens. The model also incorporates tissue damage by phagocyte proteases and the proposals by Hoiby *et al.* (1986) to explain intermittent exacerbations based on subsequent Fc cleavage of immune complexes by the proteases and inhibition of phagocyte stimulation.

against alginate and other cellular and extracellular pseudomonas antigens (Bryan *et al.*, 1983; Schiotz, 1982; Pier *et al.*, 1987), the CF lung continues to deteriorate whilst the patient is very effectively protected from other localized or systemic pseudomonal infections; thirdly, the use of antipseudomonal antibiotics may result in clinical benefit, including a rapid reduction in C-reactive protein levels, in the absence of any significant reduction in the pseudomonal population (Govan *et al.*, 1987). Finally, vaccination of patients already harbouring *Pseudomonas* may exacerbate pulmonary deterioration (Langford and Hiller, 1984) whilst steroid therapy, a regimen which in other compromised patients is a classic precursor of opportunistic infection, paradoxically produces clinical benefit in CF patients (Auerbach *et al.*, 1985).

The microcolony model (*Figure 5*) accommodates the observation that MPA adhere more readily than non-mucoid variants to tracheal epithelium particularly when the tracheal surface is damaged (Marcus and Baker, 1985; Ramphal and Pier, 1985). In

healthy individuals, the presence of a pseudomonal receptor on tracheobronchial mucin would have a protective effect ensuring removal of bacteria by mucociliary clearance. In CF however, the abnormally viscid mucus layer would hinder such clearance resulting in colonization. Pseudomonal alginate has been shown to bind to tracheal epithelial cells in a specific manner (Doig *et al.*, 1987) and MPA have been shown to bind directly to the tips of human cilia with only minimal binding to unciliated epithelial cells (Franklin *et al.*, 1987). In addition, provided the bacterial alginate is not removed by centrifugation and washing, evidence from intratracheal inoculation into rat lungs indicates that MPA are removed less rapidly than isogenic non-mucoid strains (Govan *et al.*, 1983). In such experiments, when 1×10^6 c.f.u. of MPA are inoculated, the bacteria are usually cleared from the lungs within 12 h. In contrast, when MPA are cultured in the presence of Ca^{2+}, and the microgels instilled into the rat lungs, bacterial survival is increased to several days; this reduced clearance resembles, but does not match, the establishment of chronic lung infection which occurs when non-mucoid *Pseudomonas*, enclosed in agar beads, are inoculated into rat lungs (Cash *et al.*, 1979). It is possible that the bacterial microcolonies which are observed in CF patients result from the gelling potential of pseudomonal alginate and the raised Ca^{2+} and DNA in the CF lung mucosa which is characteristic of the disease. Within the microcolony, the decreased susceptibility of MPA (in the absence of *bls* and *tps* mutations) is probably not due to a diffusion barrier or binding by alginate but to antagonism of antibiotics by the high electrolyte content of the alginate gel (Slack and Nichols, 1981; Govan *et al.*, 1987) or other biofilm-associated mechanisms (Nichols *et al.*, 1988). The diffusion barrier and the iron-chelating properties of alginate might explain the observation, based on a study of outer membrane proteins, that MPA in the lungs of CF patients grow under iron-limited conditions (Brown *et al.*, 1984). Within the protection of the microcolony, the persistence of antibiotic-susceptible and serum-sensitive *Pseudomonas* becomes more plausible. It is possible that in this pulmonary environment normal wild-type *Pseudomonas*, growing freely within the bronchial lumen, are more exposed to bactericidal agents than more susceptible cells growing within the microcolony. Such protection, however could result in poor nutrient availability and suggests that the selective advantage for the *bls* and *tps* mutations *in vivo* lies in conferring greater permeability for limited nutrients within the alginate matrix. This possibility is supported by *in vitro* experiments which showed that, under nutrient-limited conditions, five independent hypersusceptible MPA isolates isolated from CF sputa grew significantly faster than related non-hypersusceptible mucoid strains (J.R.W.Govan, unpublished results).

The microcolony mode also explains the inability of the normally highly efficient pulmonary immune system to remove *Pseudomonas* and accounts for the observed pathophysiology of the CF lung. With other exopolysaccharide-producing respiratory pathogens, including *Strep.pneumoniae and Klebsiella pneumoniae*, opsonization by specific antibodies assists ingestion and killing by phagocytic cells. However, in the CF lung, the size and gelatinous nature of the alginate microcolony, would present a formidable and persistent target. In addition, the ability of pseudomonal alginate to reduce phagocytosis is well documented (Schwarzmann and Boring, 1971; Oliver and Weir, 1983). More recently, convincing quantitative evidence has been presented indicating that evasion of host defences is probably due to the physical rather than the

biochemical properties of the alginate (Simpson *et al.*, 1987). In another study, Learn *et al.* (1987) showed that purified pseudomonal alginate was more effective than seaweed alginate in inhibiting luminol-dependent chemi-luminescence of neutrophils. Further evidence suggested that the effect was due to the ability of the pseudomonal alginate to scavenge hypochlorite and indicates that alginate could enhance virulence by scavenging the phagocyte-generated oxidant HOCl. Frustrated phagocytic cells, particularly pulmonary neutrophils, stimulated by immune complexes (IC) or altered immunoglobulins and releasing elastase and toxic oxygen radicals may explain the considerable evidence for immune-mediated damage in CF lungs (Kharazmi *et al.*, 1986a,b,1987; Doring *et al.*, 1986; Dasgupta *et al.*, 1987a,b) and the potential beneficial effects of anti-inflammatory agents (Auerbach *et al.*, 1985; Kharazmi *et al.*, 1988; Jensen *et al.*, 1988). The presence of IC formed from pseudomonal antigens in CF lungs and a type III hypersensitivity reaction was originally suggested by the high number of corresponding specific antibodies in CF patients and which were associated with poor lung status (Schiotz, 1982); later studies provided evidence of IC composed in part of soluble pseudomonal antigens (Doring *et al.*, 1984; Hoiby *et al.*, 1986) including proteases. Polymorphonuclear leukocytes play an important role in IC-mediated tissue damage. The role of IC in CF lung damage is supported by the presence in CF bronchial lavage fluids, in advanced infection, of more than 99% polymorphs compared to less than 1% in normal individuals (Moss and Lewiston, 1985; Thomassen, 1985). Hoiby *et al.* (1986) have proposed that polymorph activation in chronic inflammatory states in CF is regulated by a feedback mechanism which could explain the cyclic remissions and relapses in CF patients. Hoiby *et al.* (1986) suggested that activation of polymorphs by immune complexes leads to release of lysosomal elastase which cleaves free immunoglobulins and ICs which then do not participate in further polymorph activation; the observation that levels of ICs and polymorph elastase activities were reciprocal in CF patients supports this mechanism (Doring *et al.*, 1986).

Other experimental approaches suggest that pseudomonal proteases may be important virulence factors in CF particularly in the early stages of lung colonization, causing tissue damage, inactivation of immunoglobulin and α_1-antitrypsin, suppression of human leukocyte-mediated killing of *P.aeruginosa* and thus facilitating the spread of the infection (Doring *et al.*, 1981; Baker, 1982; Kharazmi *et al.*, 1986a). Of particular interest is the observation that pseudomonal protease evokes mucin release from tracheal epithelium (Klinger *et al.*, 1984). Since the production of abnormal mucin and viscid bronchial secretions are basic characteristics of CF and play an important role in its pathophysiology, the additional action of the bacterial enzyme would exacerbate an existing mechanism of pulmonary damage. Studies in the author's laboratory, which compared protease production in isogenic strains indicated that proteases are released more slowly from cultures of MPA, indicating that alginate might act as an immunological adjuvant (Govan and Doherty, 1985). If this form of gradual release occurs *in vivo*, then adherent pseudomonal microcolonies would localize and concentrate protease activity near the bronchial surface rather than allowing systemic dispersion and dilution. An additional and interesting biological function reported for pseudomonal alginate with possible relevance to tissue damage is the ability of the polymer to enhance lipase activity in a time- and concentration-dependent manner (Wingender and Winkler, 1984).

In view of the high phospholipid content in the respiratory tract, it would seem rational to consider a role for phospholipase as a potential pseudomonal colonizing factor. In studies of CF patients, Granstrom *et al.* (1984) reported a correlation between chronic colonization with PA and elevated serum antibody titres to phospholipase C and a possible relationship between clinical signs of exacerbation and increased serum antibody titres against pseudomonal elastase and exotoxin A. Since biosynthesis of proteases and exotoxin A, but not phospholipase C, is increased during iron-limited growth (Vasil, 1986), it is possible that phospholipase C acts as an initial colonizing factor for PA giving rise to the early phospholipase antibodies detected by Hollsing *et al.* (1987). Later, in conditions of iron-limited growth associated with MPA in the CF lung (Brown *et al.*, 1984), biosynthesis of proteases and exotoxin A would be enhanced. In recent studies, Kownatski *et al.* (1987) found rhamnolipids (heat-stable haemolysin) in sputum from CF patients chronically colonized with PA. The rhamnolipids were absent in non-colonized patients; high concentrations correlated with poor clinical studies and levels were reduced following the use of antipseudomonal therapy and clinical improvement. Rhamnolipids are known to inactivate tracheal ciliary axonemes (Hingley *et al.*, 1986), derange airway epithelial ion transport (Stutts *et al.*, 1986) and enhance mucin release *in vitro* (Adler *et al.*, 1983). In addition, since these small detergent-like substances are not antigenic *per se*, the authors speculated that they might evade the patient's immune defences and act as an additional pathogenic factor in the CF lung.

It is a common observation that antibiotic treatment of pulmonary exacerbations due to *P.aeruginosa* in CF patients results in improved lung function and reduction in the levels of C-reactive protein to normal, even when the numbers of *Pseduomonas* present in sputum remain high (Glass *et al.*, 1988). A possible explanation could be that although the desired effect of antimicrobials is to cause bacterial killing, in a wider biological sense, antimicrobials may act therapeutically on account of their ability to modify the inflammatory response (Berger, 1985). Another possibility is a therapeutic role for subinhibitory concentrations of antibiotics in suppression of pseudomonal virulence and tissue damaging inflammatory factors, including rhamnolipids and protease, particularly since it is difficult to achieve bactericidal concentrations of antipseudomonal agents in the CF lung (Bergogne-Berezin, 1987). This speculation is supported by evidence that sublethal concentrations of antipseudomonal agents inhibit adherence of MPA to tracheobronchial mucin and intact hamster tracheal epithelium (Vishwanath *et al.*, 1987; Geers and Baker, 1987a,b). Studies in the author's laboratory suggest that the mechanisms responsible for suppression of pseudomonal proteases are subtle and the phenomenon is not simply explained by reduced growth or interference with release of proteases. Antipseudomonal agents, in particular ciprofloxacin, are capable of suppressing pseudomonal protease biosynthesis *in vitro* at concentrations as low as 5% of the MIC value (Govan *et al.*, 1987) and suppression has also been demonstrated *in vivo* (Dalhoff and Doring, 1985). Suppression is strain-dependent (*Figure 6*) and can be independent of the MIC values. In addition, some pseudomonal isolates which have developed resistance to ciprofloxacin or ceftazidime, as a result of antipseudomonal therapy, have a much reduced protease activity (*Figure 7*; Govan and Doherty, 1985). To date, there is little evidence that such resistant isolates are less virulent than the original sensitive strains. Nevertheless, these observations raise interesting questions regarding the clinical relevance of antimicrobial resistance in CF isolates. It is tempting

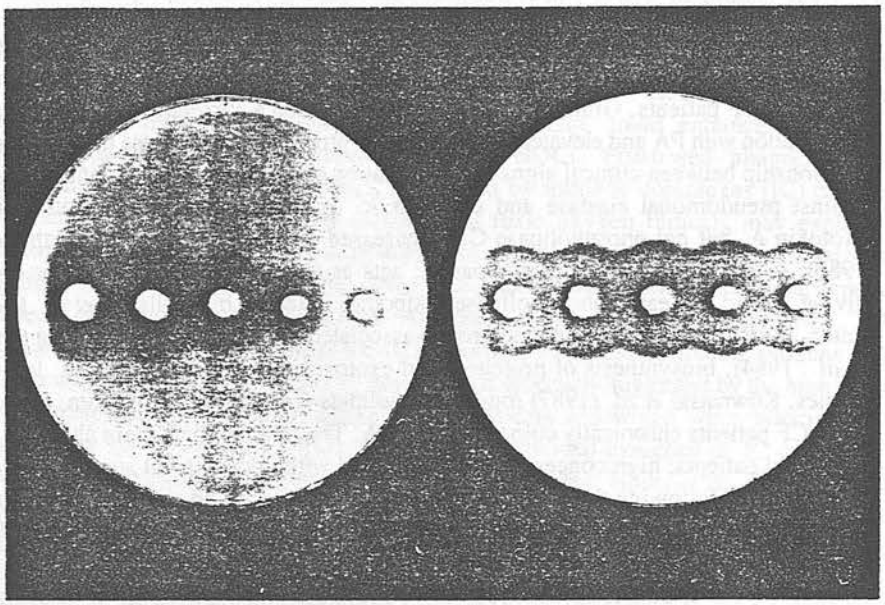

Figure 6. Protease activity of two *P.aeruginosa* isolates from CF patients grown on casein-containing agar in a sublethal concentration gradient of ciprofloxacin. The antibiotic concentrations are similar in each plate and increase from left to right: the MIC of the antibiotic is similar for both strains. Note how the protease activity in the left plate is suppressed by increasing sublethal concentrations of ciprofloxacin whilst the other remains unaffected. Suppression of protease can also be demonstrated with other antibiotics and with elastin as the substrate.

to speculate that since sensitive strains of *P.aeruginosa* in CF patients are characteristically intractable to antipseudomonal therapy, the presence of resistant, but less virulent organisms, could be less of a problem, at least for the CF patient.

Future stratagems and prospects for vaccination

Pseudomonas isolated from CF patients with advanced and pulmonary colonization are highly atypical when compared to isolates from other pseudomonal infections (Luzar *et al.*, 1985; Pitt, 1986; Friend, 1986; Govan and Harris, 1986). CF isolates are typically mucoid, hypersusceptible to antibiotics and normal serum, possess defective LPS and are often non-motile. In a physiological sense the bacteria appear to be as compromised as the CF host! The fact that early colonization with typical strains is not associated with virulence suggests that the well recognized adaptability of the species allows it to persist in the CF patient despite an active immune response and aggressive therapy with potent antipseudomonal agents. The apparent requirement of these unusual characteristics for survival and pathogenesis of *Pseudomonas* in the CF lung and the relative absence of other typical respiratory pathogens, is a hopeful indication that prevention or control of *Pseudomonas* would not inevitably lead to replacement by other microbial opportunists. A potential threat has been the increased isolation and morbidity associated wtih *P.cepacia* in North America (Isles *et al.*, 1984; Thomassen *et al.*, 1985) and in the UK (Glass and Govan, 1986). *P.cepacia* isolated from CF patients do not

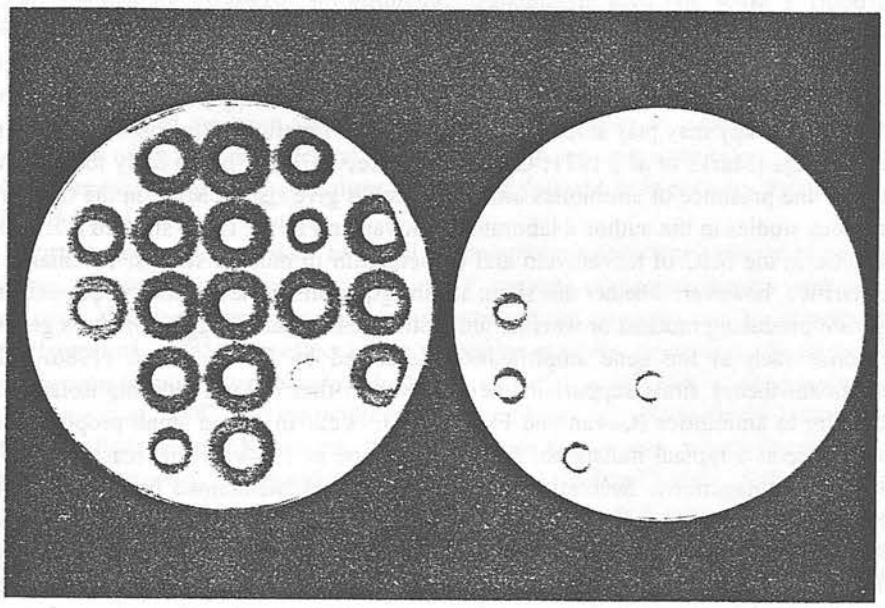

Figure 7. Protease activity on casein agar of 20 respiratory isolates of *P.aeruginosa* sensitive to ciprofloxacin and ceftazidime (left) compared to activity in 20 multiresistant isolates from CF patients following antipseudomonal therapy (right). Note the poor proteolytic activity of the multiresistant strains.

exhibit the phenotypic properties associated with PA isolates. Reassuringly, no alginate-producing *P.cepacia* have been reported and our attempts to isolate such mutants *in vitro* have been unsuccessful (J.R.W.Govan, unpublished results).

From the available evidence, there seems little doubt that the biosynthesis and rheological properties of pseudomonal alginate are the most important virulence factors in CF infections allowing the bacteria to attach, persist and, directly and indirectly, cause pulmonary deterioration. With the exception of the essential role of the *alg*R gene in the activation of the *alg*D promoter, the interplay between the three alginate gene clusters and the CF lung environment in the regulation and biosynthesis of alginate is little understood. Evidence suggests that a mechanism other than (or in addition to) random mutation is responsible for alginate regulation. A chromosomal region of at least 16.8 kb, spanning *alg*R, was found to be capable of inducing alginate biosynthesis when amplified in a non-mucoid *rec*2 *P.aeruginosa* by exposure to kanamycin (Deretic *et al.*, 1986). It has been suggested that the *alg*R regulatory gene could be controlled by the *muc* loci at 70 min. The *alg*R product shows 30% homology with only two proteins, *ntr*C and *omp*R, both of which regulate gene expression using external sensors. It is possible that a unique nitrogen compound in CF can somehow bind with the *alg*R gene product and turn it on in the presence of a flipped switch located at the 70 min region (Chakrabarty, personal communication). A specific CF lung factor capable of de-repressing pseudomonal alginate biosynthesis has always been an attractive hypothesis. In earlier studies in the author's laboratory unsuccessful attempts were made to induce alginate biosynthesis in non-mucoid strains by growth in CF sputa already

harbouring MPA and thus presumably containing the necessary alginate-enhancing factor.

As an alternative mechanism for mucoid conversion, antibiotic-induced gene amplification of alginate biosynthesis (Deretic *et al.*, 1986) supports the suggestion that antibiotic therapy may play at least some role in the promotion of the mucoid phenotype in CF lungs (Marks *et al.*, 1971; Govan and Fyfe, 1978). It is too early to speculate on how the presence of antibiotics and phage could give rise to MPA in the CF lung. Previous studies in the author's laboratory (Govan and Fyfe, 1978) showed a 1.5-fold increase in the MIC of tobramycin and carbenicillin in mucoid strains. It remains to be clarified, however, whether this slight advantage results in the selection of pre-existing alginate-producing mutants or whether antibiotic stress actually triggers *in vivo* a genetic response such as the gene amplification described by Deretic *et al.* (1986). The mutational theory finds support in the observation that mucoid variants isolated by exposure to antibiotics (Govan and Fyfe, 1978), occur in only a small proportion of the culture at a typical mutational frequency of one in 10^7 which increases 100-fold following mutagenesis. Secondly, CF mucoid isolates maintained under laboratory conditions do not demonstrate amplification of the *alg*-22 region (Deretic *et al.*, 1986). Thirdly, there is no doubt that mucoid variants, phenotypically resembling CF isolates and containing *muc* mutations at 70 min in the same region as CF mucoid strains, can be isolated by exposure to phage and do not require the continued presence of phage for alginate biosynthesis (Fyfe and Govan, 1983). The association of phage with conversion to the mucoid form *in vivo* remains obscure. Phages have been demonstrated in CF sputa (Tejedor *et al.*, 1982) and phages isolated from MPA were better able to convert non-mucoid strains to the mucoid form than phages from non-mucoid CF isolates (Miller and Renata Rubero, 1984).

Are non-mucoid Pseudomonas of no pathogenic significance?

On clinical evidence, most clinicians and microbiologists would agree with the statement of Henry *et al.* (1982), 'Whilst identification of mucoid forms of *P.aeruginosa* in the respiratory tract of children and adolescents with cystic fibrosis is an unfavourable factor, non-mucoid forms appear to be of no clinical significance'. The relevance of this conclusion to future CF research concerning either microbial pathogenesis or therapeutic strategies is worth discussion. The present failure in CF patients to eradicate MPA by aggressive and prolonged therapy using the most potent antipseudomonal agents (the most recent antibiotic, ciprofloxacin being 2000 times more active than carbenicillin) indicates that alternative stratagems to prevent the early asymptomatic colonization in CF patients might be fruitful. The known pseudomonal characteristics associated with pulmonary exacerbations in CF are quite distinct from those expressed in the early stages of CF colonization and in other non-CF infections with the exception of patients with chronic bronchitis. Since at least two of these virulence or survival factors are known to result from chromosomal mutations, it seems arguable that future therapeutic stratagems would benefit from elucidation of the factors involved in initial colonization by non-mucoid *Pseudomonas* with the rationale to reduce the microbial reservoir from which the virulent CF phenotypes arise. Because of the pathogenic significance of mucoid strains, however, the host–bacterium interactions responsible for early colonization

by non-mucoid strains have been comparatively neglected. Potentially important clues to our understanding were provided by the *in vitro* observations of Woods *et al.* (1982) suggesting that buccal cells might form the reservoir of early colonization. In our own studies, although we were able to reproduce the Woods *et al.* observations *in vitro*, the results of an *in vivo* investigation in CF patients was not convincing. We cultured *Pseudomonas* on only two occasions from 88 CF patients; both patients also harboured *Pseudomonas* in their sputum.

Further studies are necessary to reveal the bacterial–host factors involved in the initial stages of pseudomonal colonization in CF patients. Potential factors include depression of ciliary beat by pseudomonal extracellular products and by *H.influenzae* (Wilson *et al.*, 1985); the effect of the phenazine pigment, pyocyanin, on superoxide production by polymorphonuclear leukocytes (Miller *et al.*, 1987); phospholipase C (Hollsing *et al.*, 1987) and the inhibition of natural killer cell activity by the pseudomonal elastase (Pedersen and Kharazmi, 1987). Adhesion factors may include the association between pulmonary lectins and the bacterial alginate (Gilboa-Garber, 1986; Ceri *et al.*, 1986; Ko *et al.*, 1987), sialic acid-associated aggregation of *P.aeruginosa* (Komiyama *et al.*, 1987) and probably of most significance, an association between *Pseudomonas* and CF tracheobronchial mucin (Ramphal and Vishwanath, 1987; Ramphal *et al.*, 1987).

Prospects for vaccination

Undoubtedly, the development of a successful vaccine would be the ideal stratagem to prevent pseudomonal infection in CF. A successful vaccine would confer long-lasting local protection at the mucosal surfaces of the respiratory tract and be administered prior to the establishment of *Pseudomonas* in the CF lung. Such a vaccine would probably not be similar to other vaccines such as that against meningococcal meningitis, which allows colonization but not invasion (Baltimore, 1985).

CF patients clearly are capable of mounting a serum antibody response against cellular and extracellular pseudomonal antigens (Hoiby, 1977; Hancock *et al.*, 1984; Brown *et al.*, 1984; Pedersen *et al.*, 1987) and the relevance of this response to immune-mediated lung damage has been discussed. Although antipseudomonal immunoglobulin can be detected in lung tissue (Speert *et al.*, 1988) they apparently fail to result in clearance of *P.aeruginosa* from CF lungs. Although basically the humoral and cellular CF host defences appear to be intact, immunity may be reduced by the ability of pseudomonal elastase to act as an IgG protease (Doring *et al.*, 1981) and the observation that serum and sputum antipseudomonal IgG may be deficient in the Fc portion resulting in reduced opsonization (Fick *et al.* 1984).

Recent studies using different approaches have provided promising clues to the future development of a vaccine against *Pseudomonas* through the use of an animal model arguably relevant to CF lung infection and the demonstration of protective antibodies in CF patients directed against pseudomonal alginate. In the first approach, Sordelli *et al.* (1987) and Hooke *et al.* (1987) developed a live vaccine strain by constructing temperature-sensitive PA mutants with reduced replication at mammalian body temperature. Animals immunized with such mutants by aerosol or intranasal inoculations were subsequently protected against aerosol challenge by a PA strain of the same O-immunotype. The mouse model used in these studies involved lung clearance rather

than systemic infection and seems relevant to the prevention of early colonization of CF patients. The main drawback to this vaccine model is the O-immunotype specificity of protection; arguably, CF patients are exposed to all 17 O-immunotypes of PA.

A second approach to vaccination has involved the detection of opsonophagocytic antibodies against pseudomonal alginate in both animals and CF patients. In animals, Ames *et al.* (1985) showed that alginate from a single MPA strain induced an opsonic killing response in serum against a variety of MPA strains. More recently, Pier *et al.* (1987) reported an association between opsonophagocytic killing antibodies in the sera of relatively healthy CF adolescents (> 12 years), directed against alginate from a single MPA and a lack of detectable pseudomonal colonization. This demonstration of apparent *in vivo* protection against colonization by various pseudomonal immunotypes, together with the potential use of non-cellular immunogen which is readily expressed *in vitro* is encouraging. The results suggest that protective alginate antibodies are produced in CF patients despite considerable diversity in the uronic acid composition and acetylation of alginate obtained from CF isolates (Sherbrock-Cox *et al.*, 1984). This is an interesting and unexpected observation since Irvin and Ceri (1985) found that a monoclonal antibody, raised against a formalin-fixed MPA had an apparent specificity for L-guluronic residues in an ELISA, but did not react uniformly with all pseudomonal alginates, suggesting that acetylation and uronic acid diversity may interfere with antibody binding and define specific epitopes. The monoclonal antibody also reacted with purified outer membrane indicating that some alginate or L-guluronic acid is intimately associated with outer membrane. The ratio of mannuronic to guluronic acid in the MPA strain used by Pier *et al.* was approximately 1:1.

Until further studies are carried out the potentially important observations of Pier *et al.* (1987) should be viewed with cautious optimism. First, Pier *et al.* acknowledge that their non-colonized patients were drawn non-randomly from North American clinics with large CF populations and might not be representative; alternatively, these non-colonized patients might represent a subset with a less severe form of disease and with alginate antibodies present as a marker of this condition. Secondly, it is puzzling how CF patients harbour antibodies to alginate in the absence of previous colonization by MPA and when early and transient colonization would involve typical non-mucoid strains. In previous studies, Bryan *et al.* (1983) used purified alginate from a MPA to sensitize enzyme-linked immunoabsorbant plates and found high titres of alginate antibodies in the sera of CF patients colonized with MPA; there was also a high frequency of low antibody titres in the normal non-CF population. Speert *et al.* (1984) using a similar assay system reported that normal controls had virtually no alginate antibodies but CF patients colonized with MPA had levels of antibody which correlated with the length of colonization. Recently, Speert *et al.* (1988) found that *Pseudomonas* in *post-mortem* CF lung tissue reacted strongly with polyclonal and monoclonal antibodies against pseudomonal alginate. In the study by Pier *et al.* (1987) the titres of opsonophagocytic killing antibody specific for alginate in non-colonized CF adolescents were generally low but not detectable in non-CF controls or young non-colonized CF patients. In addition, although adolescent patients with chronic pseudomonal colonization had higher titres of opsonophagocytic killing antibody than non-colonized patients, these antibodies were not specific for the alginate antigen; all sera exhibited antibodies to other pseudomonal antigens.

On the basis of the genetic evidence presented in this review and the presence of low levels of alginate biosynthetic enzymes in non-mucoid strains it is reasonable to conclude that alginate biosynthesis does not occur *de novo* in MPA but results from de-repression with typical non-mucoid colony types producing trace amounts of the polymer. A recent report provides further evidence that alginate biosynthesis probably occurs in all PA strains. Anastassiou *et al.* (1987) reported that slime material from a non-mucoid revertant derived from the MPA strain MS contained 16% uronic acid compared to 65% in the mucoid parent strain. In addition, the extracellular slime from six other non-mucoid PA isolated from non-CF patients also contained 8–14% uronic acids which yielded similar IR spectra to the purified alginate from strain MS. If alginate biosynthesis is a property shared by all PA strains it would be interesting to determine if opsonophagocytic killing antibodies to alginate are present in the sera of non-CF patients with chronic pseudomonal infections.

From the evidence presented by Pier *et al.* (1987) it appears that protective alginate-specific antibodies are produced in some CF patients as a result of transient pseudomonal colonization and when the alginate is presented in association with growth of the bacteria *in vivo*. As an immunogen, however, purified alginate is usually very viscous and some researchers have been unable to detect alginate-specific antibodies when the polymer is inoculated into animals. Pseudomonal alginate is readily produced by MPA *in vitro* and would appear to be an ideal immunogen if it is shown to provide a broad range of protection against the many PA immunotypes. The composition of an alginate-based vaccine immunogen may require some form of conjugate and the most suitable route of administration for CF patients also requires investigation.

Concluding on an optimistic note, it is hoped that the results of Pier *et al.* (1987) can be speedily confirmed and extended to form the basis of a screening assay and a pseudomonal vaccination trial for unprotected CF patients not already colonized.

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Serum C-reactive protein in assessment of pulmonary exacerbations and antimicrobial therapy in cystic fibrosis

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It is well recognized that antibiotic therapy relieves the symptoms of acute pulmonary exacerbations of cystic fibrosis but is seldom accompanied by eradication of mucoid *Pseudomonas aeruginosa* or other bacteria from the sputum[1,2]; *Haemophilus influenzae* is the exception, when treated with ceftazidime.[3] Quantitative sputum culture may allow measurement of the decrease in bacterial load, but clinical improvement can occur without reduction in the numbers of *P. aeruginosa*.[3,4] Thus, on the basis of sputum culture, there is often difficulty in distinguishing *Pseudomonas* colonization from active infection and, accordingly, in assessing the need for, and efficacy of, antimicrobial therapy.[4,5] Clinical assessments in CF patients based on other measurements may also be unreliable.[1,6] Chest radiographs and body weight may not change substantially; the erythrocyte sedimentation rate and leukocyte count are useful but variable, as are pulmonary function test results.

CF	Cystic fibrosis
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate

Elevated C-reactive protein levels (above 10 mg/L) have been shown to be a rapid and reliable measure of inflammatory response in various disease states,[7] but the value of quantitative CRP assays as indices of exacerbation in CF patients chronically colonized by potential bacterial pathogens has received little attention. The aim of our study was to determine whether CF is associated

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Table. Parameters of inflammatory response in CF patients during routine clinic visit (control group, n = 12) and in patients requiring antimicrobial treatment (exacerbation group, n = 12)

CRP (mg/L)			Leukocytes (10^9/L)			ESR* (mm/hr)	
Control group	Exacerbation group		Control group	Exacerbation group		Exacerbation group	
	Pretreatment	Posttreatment		Pretreatment	Posttreatment	Pretreatment	Posttreatment
0.8	38	0.8	16.7	9.2	11.6	40	12
4.7	91	3.3	19.1	13.5	12.4	65	24
2.7	39	2.5	13.2	12.4	6.8	21	21
4.4	14	1.0	ND	14.8	14.1	ND	ND
3.0	31	2.9	5.3	16.8	11.1	40	35
7.4	72	10.0	18.1	16.8	10.5	72	41
4.0	34	10.5	14.8	12.4	8.9	26	18
6.0	96	7.2	12.1	21.4	10.0	26	9
2.7	125	3.0	12.6	20.2	13.2	65	30
1.2	124	2.2	9.6	18.2	12.9	110	1
0.7	53	8.3	8.7	7.0	8.1	25	15
7.5	68	3.6	8.1	17.1	10.0	20	20
Mean 3.8	65	4.6	12.6	15.0	10.8	46	20
SD 2.4	35	3.3	4.2	14.1	2.1	27	12

ND, Not done.
*ESR values for the control group were all within normal range (<10 mm/hr).

with raised CRP levels, or whether respiratory exacerbations increase CRP levels and, if so, whether CRP levels return to normal after antibiotic treatment. In addition, CRP levels in individual patients were measured during periods of pulmonary exacerbation and remission.

METHODS

Patients. In the preliminary study, blood was obtained by venipuncture from 24 CF patients attending the CF clinic at The Royal Hospital for Sick Children, Edinburgh. Serum CRP levels of a control group of 12 CF patients who were not acutely ill were compared with those of 12 debilitated patients, designated the exacerbation group. Patients in the control group were chosen from those attending the monthly CF clinic. The criteria used for inclusion in the control group were reasonable pulmonary status commensurate with the patient's baseline values, no antibiotic therapy in the previous 48 hours, and no symptoms suggesting an imminent exacerbation. Neither cough nor production of sputum was a contraindication for inclusion in the control group if the symptom was typical of the patient's normal status and was not associated with pulmonary distress. Patients with evidence of other intercurrent illness were excluded from the study. Age range in the control group was 8 months to 15 years (mean 8 years).

The exacerbation group comprised 12 consecutive patients with symptoms sufficiently severe to require hospital admission and treatment. All patients in this group had increased cough and sputum production, dyspnea, and weight loss ranging from 2% to 5%. Determinations of forced expiratory volume in 1 second, forced vital capacity, and peak expiratory flow rate indicated a significant deterioration from the patient's normal baseline values; in several patients such tests were not carried out because of acute breathlessness. Only two patients had fever (≥38° C).

The choice of antibiotic treatment was based on bacteriologic analysis of homogenized sputum and confirmation of in vitro susceptibility of pulmonary pathogens to the antimicrobial agent. Treatment was continued until return of pulmonary function to baseline, reduced cough and sputum volume, and increased appetite with a weight gain to preexacerbation level; in the two febrile patients, fever resolved within 72 hours. The CRP levels were measured within 24 hours of the cessation of treatment; in some patients, CRP levels were measured at intervals during the hospital stay. The average duration of therapy was 14 days, with a range of 10 to 21 days. The age range of this group was 12 to 18 years (mean 15 years). No patient received anti-inflammatory drugs.

Informed consent was obtained from each participant's parent or guardian and, when appropriate, from the subjects themselves.

CRP assay. Quantitative CRP assays were ELISA-based immunoassays as described by Salonen.[8] In addition to CRP assays, standard haematologic tests, including determinations of the ESR and leukocyte count, were performed.

Bacterial cultures. Expectorated sputum was obtained after chest physiotherapy, and after liquefaction with Sputolysin (Behring Diagnostics, La Jolla, Calif.), quantitative culture for bacterial respiratory pathogens was grown by standard procedures.

RESULTS

The CRP, leukocyte, and ESR measurements are shown in the Table. Without exception, each of the CRP values obtained in the control sera was below 10 mg/L, which is generally recognized as the upper limit for healthy individuals. The mean CRP level for the control group, 3.8 mg/L (SD 2.4), was in striking contrast to the higher values, ranging from 14 to 125 mg/L (mean 65, SD 35) observed in the exacerbation group before antibiotic treatment. After treatment and resolution of pulmonary symptoms, the CRP levels in the exacerbation group were reduced to normal levels (range 0.8 to 10.5 mg/L, mean 4.6, SD 3.3). The differences in CRP values between the control group and the exacerbation group before therapy, and the differences in CRP values among patients in the exacerbation group before and after therapy, were statistically significant ($p < 0.001$, Student t test). In contrast, the leukocyte count showed a wide scatter between the control and exacerbation groups. In addition, there was no significant difference in leukocyte counts and ESR values in the exacerbation group before and after therapy. In the control group of patients, ESR values were within the normal range (<10 mm/hr) but showed a wide scatter in the exacerbation group before and after therapy.

After these encouraging preliminary results, quantitative CRP levels were studied before and after treatment in a further 28 exacerbations involving a total of 20 CF patients. The CRP levels in patients with an exacerbation were always significantly above the normal value of 10 mg/L. In individual episodes of exacerbation, the CRP levels before treatment varied considerably (range 14 to 500 mg/L) but in all cases returned to within normal limits after therapy. When measured at intervals during hospital stay, the CRP concentration fell rapidly, within 48 hours of the start of successful treatment, but the time taken to reach normal levels in individual patients was variable.

During these studies we looked for evidence of an association between levels of CRP before and after antibiotic treatment and quantitative sputum bacteriologic data. A study of 12 courses of treatment of exacerbation in 12 patients involving three antibiotic regimens indicated that clinical recovery and return of CRP levels to normal limits did not always coincide with a significantly reduced sputum count of bacterial pathogens. In seven of these patients, the sputum counts of mucoid *P. aeruginosa* and *Staphylococcus aureus* remained in excess of 10^6 colony-forming units before and after therapy.

In five patients chronically colonized with mucoid *P. aeruginosa*, it was possible to measure CRP levels during periods of exacerbation, recovery, and relapse. Investigation of CRP levels in these patients over periods ranging from 6 to 10 months, and at monthly intervals in the absence of exacerbation, indicated that increases in serum CRP concentration to levels >10 mg/L coincided with periods of exacerbation, and that levels returned to within normal limits after antibiotic treatment. A rising CRP value in patients with vague malaise was also a good predictor of imminent exacerbation. In the absence of exacerbation, CRP levels in these patients were observed to be within the normal range.

DISCUSSION

An early and significant observation of this investigation was that in the absence of pulmonary exacerbation, CF is not associated with raised CRP values. When it is considered that insidious and gradual pulmonary deterioration is a characteristic feature of CF, this was a surprising result and emphasizes the significant contribution of inflammation in CF pulmonary abnormality. Since most of the exacerbations in our study were not associated with fever, elevated CRP values cannot reflect fever rather than pulmonary exacerbation.

A major problem in the management of CF patients involves judgments of when to initiate and how to assess antimicrobial treatment. Our findings, which demonstrate that CRP concentration is significantly elevated in CF patients at the onset of exacerbation and that a return to normal values is associated with clinical resolution, suggest that CRP values could be a useful and easily measured indicator of the need for initiation and efficacy of antimicrobial therapy. The pretreatment CRP values in individual patients varied widely, and further studies are required to determine whether the differences are associated with the severity of underlying disease or exacerbation, the age of the patient, the type of bacterial pathogen, or other factors. In view of the complexity of *Pseudomonas* pathogenesis in the CF lung,[2] including the importance of indirect immune-mediated tissue damage[9] and the ability of sublethal concentrations of antibiotics to suppress *Pseudomonas* virulence factors,[3] it is not unreasonable that clinical recovery and reduction in CRP values are not always associated with a significant reduction in the numbers of bacterial pathogens. It is also reasonable to

speculate that the quantitative CRP assay might assist with the difficult problem of identifying the relative importance of other forms of CF management, including chest physiotherapy, administration of steroids, nutritional support, and bronchodilators.[10]

Our results confirm and expand an earlier study that suggested the value of CRP and another acute-phase reactant, amyloid-related serum protein, as indicators of lung infection in CF patients. In the study by Marhaug et al.,[11] CRP was measured by a relatively insensitive radial immunodiffusion assay, no quantitative sputum culture for bacterial pathogens was reported, nor were data presented on the levels of amyloid-related protein and CRP in asymptomatic patients before pulmonary exacerbation.

We conclude that quantitative CRP assay is useful in the management of CF patients as an index of acute or impending pulmonary exacerbation and of the response of the patient (rather than the pathogen) to antibiotic therapy.

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C-reactive protein in cystic fibrosis

To the Editor:

We read with interest the report of Glass et al. (J PEDIATR 1988;113:76-9) on C-reactive protein (CRP) concentrations in cystic fibrosis (CF). Although the authors noted the need to relate pretreatment CRP values to severity of underlying disease or exacerbation, they did not report any measure of the severity of the disease in their patients.

We have reported the results of a cross-sectional study of 36 patients with CF.[1] Serum CRP concentrations correlated closely with FEV_1 ($r = -0.78$) as a percentage of predicted value and with Shwachman score ($r = -0.77$). Elevated CRP concentrations were found in 20 of 36 patients, including all 12 patients who had a Shwachman score of less than 66. Only 5 of the 20 with elevated CRP had an exacerbation.

The earlier study by Marhaug et al.[2] also reported both FEV_1 as a percentage of predicted and CRP levels in CF patients. Analysis of their tabulated data reveals that patients with elevated levels of CRP had lower FEV_1 (n = 14; median 23.5%; quartiles 8.5%, 40.5%) than those whose CRP levels were below the limit of sensitivity of their assay (6 mg/L) (n = 14; median FEV_1 63%; quartiles 56%, 83%). This lower range of FEV_1 might have reflected more severe chronic pulmonary damage, an acute exacerbation, or both, at the time of CRP measurement. However, inasmuch as antibiotics were not given to several of these patients, it seems unlikely that exacerbation was considered.

The findings described above might suggest that, in the patients of Glass et al. whose CRP values were normal or became normal with treatment, CF was relatively mild. If so, elevations of CRP in severely affected patients may reflect tissue damage not amenable to treatment. If not, the findings of Glass et al. could be reconciled with our findings and those of Marhaug et al. if treatment substantially reduces tissue damage in patients without exacerbation. In either case, quantitative assay of CRP seems a useful objective index of the response of CF patients to treatment, which, for the patients of Glass et al., included not only antibiotics but also hospitalization.

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Reply

To the Editor:

We were pleased to read the contemporary and independent study by Moreton and Kennedy confirming the value of CRP measurements as a simple, objective indicator of pulmonary exacerbation in CF patients. The two studies are complementary rather than contentious. The Moreton-Kennedy study contains interesting information suggesting a relatively low CRP range in normal, healthy children; we did not investigate such individuals but assumed a normal level below 10 mg/L, quoted in previous studies in healthy individuals. In the Moreton-Kennedy study, however, the fact that CRP was measured from serum taken with the patient under anesthesia requires consideration. Our own study confirmed the value of CRP as a supplementary indicator, not only of exacerbation, but also for assessment of antimicrobial therapy in association with quantitative sputum culture for microbial pathogens; this is particularly useful in the management of patients with long-standing colonization with mucoid *Pseudomonas aeruginosa*, in whom antibiotics may reduce the production of virulence factors and exacerbations resolve without any significant reduction of bacterial flora.

The discrepancies in the two studies, which we intrepret as the basis for Moreton and Kennedy's letter, are (1) the presence of elevated CRP in CF patients with no evidence of exacerbation, based on existing measurements, (2) the suggestion that our patients might have milder disease and that therapy might not reduce CRP in patients with severe tissue damage, and (3) the inference in the last sentence that hospitalization per se, rather than antimicrobial therapy, might account for remission of exacerbation (and reduction in CRP).

We stressed in our original article and in a subsequent report[1] that serial CRP measurements and longitudinal studies were of greater value than single assays. In longitudinal studies of six patients, elevated CRP levels correlated with pulmonary exacerbations; rising CRP levels in patients with vague malaise was also a good predictor of imminent exacerbation. In their original article, Moreton and Kennedy did not take into account the earlier CRP study of Marhaug et al. (1983); we believe that the report by Marhaug et al. does not contain sufficient clinical or bacteriologic data to judge whether exacerbation was present. Furthermore, several patients in our studies have severe CF disease as shown by weight and height measurements, chest radiography, and pulmonary function tests with significantly below-normal results, and they require frequent and intensive antimicrobial therapy. Longitudinal studies in six such patients showed a response to antimicrobial therapy and a return of elevated CRP levels to normal. Finally, we noted a similar reduction in CRP levels and remission of exacerbation in patients who were not hospitalized but who received intravenous antipseudomonal therapy at home. We agree that further longitudinal studies of CRP are necessary to determine possible age-related differences and CRP profiles in individual patients.

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Skin crease formation and lack of formation

To the Editor:

Stevens et al. (J PEDIATR 1988;113:128-32) concluded that the cause of absent digital flexion creases at birth cannot be the result of carbon monoxide exposure at 13 menstrual weeks gestation, because flexion creases are well defined by 12 menstrual weeks gestation. Hence they assert that one may reasonably conclude that the flexion creases present in the 13-week finger will be maintained throughout the remaining weeks of pregnancy despite paralysis of finger flexion at 13 weeks. This conclusion is not justified if that crease development is caused by movement resulting in folding of the skin; at 13 weeks, histologic examination of digital creases does not reveal evidence of thinning of dermal and subdermal tissues (which may be explained by deformational forces caused by flexion), evident at histologic examination at later ages, noted by Popich and Smith.[1] Hence no permanent subcutaneous dermal thinning has occurred. More important, however, is the relative size of an interdigital crease at 13 weeks gestation compared with the size of an interdigital crease at term. At 13 weeks the crease is less than 1 mm in length (approximately 0.7 to 0.8 mm); what process of growth (other than deformational forces) may one postulate to explain the expansion, greater than 10 times in length, of a flexion crease over the entire volar surface of the interdigital space at term?

I believe that it is not reasonable to assume that a deformation (shallow skin crease) present at 13 weeks on a small surface *area* of skin would be discernible at birth, when the proportional area occupied by the crease has been stretched approximately 100 times the original area, unless the same deformational force had been in effect during this time. I conclude that the lack of skin creases in this infant *is more likely* to be the result of a known, serious environmental insult at 13 weeks gestation, because tiny creases present at that time would be expected to be obliterated as the skin area expanded 100 times. Appropriate counseling for the parents should state both possibilities. The case reported by Stevens et al. merits full description, because only three case reports of first-trimester carbon monoxide exposure not causing maternal coma exist, and one was associated with malformations (arthrogryposis, reduced muscle mass, joint laxity).[2,3]

Available data strongly support the theory that skin creases are formed by skin deformation because of fetal movement, as outlined by Popich and Smith.[1] I have been examining human embryos and fetuses with real-time ultrasound for the last three years, and the superb descriptions of embryonic and fetal movement provided from live products of conception as described by Humphrey[4] are readily discernible. I have visualized digital flexion and extension at 11 (menstrual) weeks. The data suggesting a direct genetic component to skin crease formation are indirect and weak. The authors incorrectly state that two crease variations have been observed that cannot be explained by this theory. First, the unusual positioning of an extra interphalangeal transverse crease between the metacarpophalangeal and proximal interphalangeal creases of the fifth finger, seen in 4 of 551 people,[5] is most likely due to skin buckling with resultant crease formation at that unusual spot. I predict that these individuals had longer-than-usual phalanges. Abnormal physical shortening of a phalanx (from a genetic or nongenetic cause) may result in the complete absence of a flexion crease because it is physically impossible for the volar skin to buckle in three places if the surface area is sufficient only for two; most common is the absent fifth proximal interphalangeal crease seen in individuals with Down syndrome and severe hypoplasia of the distal fifth interphalanx. Second, the unusual extra crease on the terminal phalanx, seen more commonly in individuals with sickle cell disease, does not constitute evidence that the crease is caused by the action of homozygous genes, even though it apparently is more commonly seen in homozygotes than in heterozygotes and normal black persons. Black persons have, on average, longer digits than white persons and therefore may be expected to have more flexion creases form on the volar surface of the fingers than individuals with shorter phalanges, because the skin will have more room to buckle with flexion. If hypothesized genes regulating digital growth are in linkage disequilibrium with the sickle cell gene, one may postulate that individuals homozygous for the gene would have longer digits, and hence significantly more flexion creases, than those not homozygous. Hence there is no *a priori* evidence to support the authors' assertion that formation of the extra crease is due to purely genetic factors, rather than the physical deformational forces of finger flexion.

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31

J. R. W. Govan

Pseudomonas

Members of this large diffuse genus of aerobic, Gram-negative, non-fermentative bacilli are widely distributed in nature as saprophytes or as commensals and pathogens for man, plants, animals and insects. In medical microbiology, the genus can be divided into three groups. (i) Species which are opportunist pathogens in patients immunologically compromised by disease or treatment; in this group *P. aeruginosa* (*P. pyocyanea*) is pre-eminent, followed by *P. maltophilia, P. fluorescens, P. putida, P. cepacia* and *P. stutzeri*. (ii) Two species, *P. pseudomallei* and *P. mallei* (both previously known under the generic title *Loefflerella*) cause severe and often fatal infections in healthy persons or animals and must be processed with extreme caution in the laboratory. (iii) The remaining members of the genus, over 100 species, have little or no role as commensals or pathogens for man or animals. Some are plant pathogens or are associated with particular plant hosts, e.g. *P. tomato*; others are of interest because of particular physiological properties. Some species are of doubtful taxonomic status since the genus, in the past, has often served as a convenient dumping ground for taxonomically difficult organisms.

All pseudomonads are aerobic Gram-negative bacilli; motile (except *P. mallei*), with polar flagella, mono- or multi-trichous; oxidase positive (except *P. maltophilia*) and catalase positive. Typically the breakdown of carbohydrates is oxidative. Many species produce characteristic water-soluble pigments. The fluorescent pseudomonads produce fluorescein, a yellow fluorescent pigment, whilst *P. aeruginosa* strains, in addition to fluorescein, can also produce pyocyanin (blue), pyorubrin (red) and pyomelanin (brown). Characteristics that distinguish the species of greatest importance in medicine are listed in Table 31.1.

PSEUDOMONAS AERUGINOSA

P. aeruginosa is a classic opportunist pathogen with innate resistance to many antibiotics and disinfectants. It flourishes as a saprophyte in warm moist situations in the human environment, including sinks, drains, respirators, humidifiers and disinfectant solutions. Isolation of *P. aeruginosa* from healthy carriers or environmental sites is significant only when there is a risk of transfer to compromised patients, e.g. by nurses' hands or respirators. Normally, human faecal carriage of *P. aeruginosa* is low, around 3%; however, carriage increases with the length of stay in hospital, reaching 30% after 3 weeks, and thus can present a distinct risk of endogenous infection.

In patients with no clinical evidence of infection isolation of *P. aeruginosa*, particularly in association with other resistant organisms such as *Candida*, can be a consequence of selection by antibiotic therapy and of little clinical relevance. Infections due to *P. aeruginosa* are seldom encountered in healthy adults but in the last two decades the organism has become increasingly recognized as the aetiological agent in a variety of serious infections in hospitalized patients with impaired immune defences (Neu 1983). Susceptibility to infection with *P. aeruginosa* may also be occupational, e.g. ear infections in divers, or recreational, e.g. whirlpool-associated (jacuzzi) rash (Vogt et al 1982).

Table 31.1 Distinguishing characters of clinically relevant *Pseudomonas* species.

Methods. Arginine dihydrolase after Thornley (1960) or in Stewart's AG medium (1972); maltose and lactose utilization in ammonium salt medium (Cowan 1974, p 109); accumulation of poly β-hydroxybutyrate after Stanier et al (1966); other tests included in API strips or Flow N/F system.

Note. All species motile except *P. mallei*; all species oxidase positive except most strains of *P. maltophilia* and *P. mallei*.

Key: . . ., no result given

	Pyocyanin	Fluorescein	Growth at 42°C	Arginine dihydrolase	Lysine decarboxylase	Gelatinase	Aesculin hydrolysis	Lactose (oxidative)	Maltose (oxidative)	Poly-β-hydroxybutyrate	NO_3 reduction to N_2
P. aeruginosa	+	+	+	+	−	+	−	−	−	−	+
P. fluorescens	−	+	−	+	−	+	−	−	−	−	−
P. putida	−	+	−	+	−	−	−	−	−	−	−
P. maltophilia	−	−	+	−	+	+	+	−	+	−	−
P. stutzeri	−	−	+	−	−	−	−	−	+	−	+
P. cepacia	−	−	+	−	+	+	+	+	+	+	−
P. pseudomallei	−	−	+	+	−	+	+	+	+	+	+
P. mallei	−	−	+	+	−	+	. . .	. . .	+/−	+	+

Despite the emphasis on caution in assessing the clinical significance of *P. aeruginosa*, there are undoubtedly occasions when *P. aeruginosa* causes serious infection. Panophthalmitis can result in partial blindness or loss of an eye. Acute otitis media in the saturation conditions necessary for deep water diving is painful and socially debilitating. Endocarditis and septicaemia are relatively rare but carry a high mortality rate, exceeding 70% in patients compromised by severe burns, cancer or drug addiction. Perhaps the most significant and troublesome pathogenic role for *P. aeruginosa* at the present time is in the chronic debilitating respiratory infections due to mucoid strains that are now a major cause of death in patients with cystic fibrosis. Quantitative estimates of the number of *P. aeruginosa* present in these respiratory infections can be used to indicate the progression from early colonization to infection (10^7 orgs/ml), and to follow the results of antibiotic treatment.

Assessment of the significance of a particular episode or outbreak of infection and the most appropriate management procedures require knowledge and appreciation of both the organism and the host. This can best be achieved by consultations between microbiologist and clinician which take account of the patient's clinical status, the factors responsible for susceptibility to the infection and any virulence factor exhibited by the particular strain of *P. aeruginosa*. Putative virulence factors, known to be produced in vitro and in vivo, include the most active factor, toxin A, but also elastase and other proteases that are of importance in corneal infection and the alginate-like polysaccharide produced by mucoid strains from patients with cystic fibrosis. Typing methods are important in epidemiological studies to determine sources of infection and methods of spread.

Morphology and staining

Gram-negative, non-sporing rods; motile, usually with a single polar flagellum. Fimbriae may be present and are usually polar and non-haemagglutinating. Some *P. aeruginosa*, notably from respiratory tract infections in patients with cystic fibrosis, produce large amounts of alginate, an exopolysaccharide consisting of mannuronic and guluronic acids which gives rise to strikingly mucoid colonies. When such strains are examined in vitro with the India ink technique the exopolysaccharide may appear as a discrete capsule but usually as a loosely bound extracellular matrix. It should be emphasized that the alginate produced by these mucoid colonial forms of *P. aeruginosa* is distinct from pseudomonas slime, a heterogeneous mixture of hexoses which is produced by all strains of the species on prolonged incubation in media with high carbon, low nitrogen content.

Cultural characters

Grows readily on simple media. *P. aeruginosa*, like other members of the genus, is extremely adaptable in nutritional terms and can utilize a very wide range of organic substrates as sources of C and N. Cultures produce a characteristic sweet, musty smell of aminoacetophenone. Strict aerobe, although NO_3 can be used as an electron acceptor to permit anaerobic growth. Colonies are usually very small or extremely flat if growth occurs at all on anaerobic plates. Temperature range 5–42°C, optimum 37°C; *P. aeruginosa*, unlike most other *Pseudomonas* species, will grow in serial subculture at 42°C. Optimum pH 7.4–7.6.

After aerobic incubation on nutrient agar for 24 h at 37°C six distinct colonial types of *P. aeruginosa* are encountered (Phillips 1969). Type 1 is the most common and easily recognized; the colonies are large, low convex, rough in appearance and often oval with the long axis in the line of the inoculum streak; sometimes they are surrounded by a thin serrated 'skirt' of growth. Type 2 colonies are small, domed and smooth in appearance and described as coliform-like. Colony types 3 and 4 are also small and appear rough and rugose respectively. The mucoid alginate-producing type 5 colony is very striking; the large amounts of exopolysaccharide produced during overnight incubation often result in merging colonial growth, and on further incubation the copious exopolysaccharide may drip on to the lid of the inverted Petri dish. The dwarf colony type 6 is the smallest colony form of *P. aeruginosa*; dwarfs are usually variants of

the mucoid colonial form and may appear slightly mucoid.

Colonial dissociation from one colony type to another is frequently observed both on subculture and in primary diagnostic plates and does not necessarily indicate the presence of more than one strain of the species. Dissociation is most frequently observed between colony types 1 and 2 and these forms, together with the dwarf type 6, are often seen as non-mucoid revertants of the mucoid type 5 form. Attempts have been made to associate particular colonial types with environmental or infectious loci. The only generally accepted associations, however, are the frequent isolation of coliform type 2 forms from environmental sources and the very characteristic isolation of mucoid type 5 strains in the course of chronic respiratory infections in patients with cystic fibrosis.

Many strains exhibit the phenomenon known as iridescence, which is observed as a moth-eaten type of colonial lysis with a metallic sheen. Although the phenomenon resembles lysis due to bacteriophage it actually has no association with phage activity. Colonies on blood agar may be surrounded by a zone of haemolysis. On MacConkey agar, *P. aeruginosa* colonies are pale, i.e. non-lactose-fermenters, and, as on blood agar, the characteristic pigments are often poorly observed.

Production of pigments

Demonstration of the presence of the blue pigment pyocyanin is absolute confirmation of a strain as *P. aeruginosa* and thus the major diagnostic test. Pigment-enhancing media should be used (see below). The yellow pigment fluorescein is also produced by many strains, giving the characteristic blue-green appearance of infected pus or cultures. Pyocyanin, fluorescein and the more rarely observed pyomelanin and pyorubrin are easily identified on nutrient or sensitivity test agars, particularly after prolonged incubation.

Biochemical reactions

In general, *P. aeruginosa* appears inert in the usual tests (including API 20E reactions) used for the fermentative Gram-negative bacilli, e.g. indole and H_2S are not produced, Voges-Proskauer and methyl red reactions are negative. Tests that may be used to characterize *P. aeruginosa*, particularly when pyocyanin production is absent or doubtful, are shown in Table 31.1.

Sensitivity to chemical and physical agents

P. aeruginosa survives well in wet environments but is not very resistant to drying and is easily killed by heat. *P. aeruginosa* has exceptional resistance to chemical antibacterials. Consequently the species is a very significant contaminant of pharmaceuticals and cosmetics and its presence in such products must be avoided to prevent inactivation of the medicaments as well as direct damage to the user. In the preservation of ophthalmic solutions, phenylethanol is recommended for use in combination with a suitable broad spectrum antibacterial such as benzalkonium chloride, or chlorocresol, or to a lesser extent chlorhexidine. Other effective combinations include EDTA-benzalkonium and EDTA-chlorocresol.

To reduce surface contamination due to *P. aeruginosa* on instruments and in hospital sinks and drains, care is required in the choice and use of disinfectants. *P. aeruginosa* is resistant to many disinfectants including quaternary ammonium compounds; indeed Dettol and cetrimide can be incorporated in selective media for isolation of pseudomonads. Cidex (a 2% aqueous alkaline solution of glutaraldehyde) is suitably active against *P. aeruginosa* but great care must be taken to avoid accidental over-dilution and to follow the manufacturer's instructions for the preparation and replacement of in-use dilutions.

Antibiotic sensitivity and resistance

P. aeruginosa has high intrinsic resistance to many antibiotics at levels attainable in body tissues. Those antibiotics likely to be most effective are gentamicin, tobramycin and amikacin, carbenicillin and ticarcillin, and the newer ureido-penicillins, azlocillin and piperacillin. Most first and second generation cephalosporins

are poorly active against *P. aeruginosa* but some of the more recent derivatives, e.g. cefsulodin and ceftazidime, may prove useful non-toxic alternatives to the aminoglycosides. Current clinical trials with ciprofloxacin, a new 4-quinolone derivative, suggest that it may provide a major advance as the first highly active anti-pseudomonal effective by oral administration.

Despite the significant improvements in the anti-pseudomonal activity of these antibiotics, larger than normal doses are necessary in severe infections. A further problem with *P. aeruginosa* is that many strains do not respond clinically although apparently sensitive to the antibiotic in vitro. With aminoglycosides this is partly explained by tissue antagonism, the effect of ionized divalent cations and poor entry into tissues. The exopolysaccharide produced by mucoid *P. aeruginosa* gels rapidly in the presence of physiological levels of divalent cations; thus with mucoid *P. aeruginosa* the phenomenon may be partly explained by aggregation of bacteria in vivo as microcolonies embedded within a gelled ionized matrix.

Acquired additional resistance superimposed on natural resistance is also a problem. Plasmid mediated resistance involving modifying enzymes is particularly associated with topical antibiotic use and with sites, e.g. bladder, where high levels of antibiotic are achieved. An additional form of acquired resistance does not involve modifying enzymes but is apparently the result of reduced permeability associated with a change in outer membrane proteins.

Although *P. aeruginosa* is often initially recognized as an organism that appears resistant to commonly used antibiotic disks on primary culture plates, it should be appreciated that this is not always a reliable screening test. A considerable percentage of respiratory tract isolates express a 'hypersensitive' mutation which results in a zone of inhibition with such unlikely anti-pseudomonal antibiotics as ampicillin, trimethoprim, nalidixic acid and cefuroxime, and a larger than normal zone of inhibition with carbenicillin. The MIC values of carbenicillin for hypersensitive *P. aeruginosa* are less than 1 μg/ml, compared with a normal range of 20–60 μg/ml. Hypersensitivity does not include the aminoglycosides and the phenomenon is almost exclusively observed in *P. aeruginosa* strains isolated from chronic respiratory infections (Irvin et al 1981; Fyfe & Govan 1984).

Antigenic characters

17 distinct group-specific heat-stable O antigens and at least 2 heat-labile H antigens are recognized on the basis of standard slide agglutination procedures (Liu et al 1983). Serological characterization is primarily used as an epidemiological typing technique rather than for diagnostic confirmation of species identity.

Bacteriocin production

The bacteriocins of *P. aeruginosa* are called aeruginocins, or more usually *pyocins* after the former species nomenclature, *P. pyocyanea*. Pyocinogeny, the ability to produce pyocins, is found in over 90% of *P. aeruginosa* strains. Four distinct types of pyocin are recognized. R pyocins resemble the tails of contractile phages (Ishii et al 1965; Govan 1974a,b), whilst morphologically distinct rod-shaped, flexuous F pyocins resemble the tails of non-contractile phages (Takeya et al 1969; Govan 1974b). Low molecular weight S pyocins (*c.* 10^5 daltons) also occur; two categories have been described, trypsin-sensitive (Ito et al 1970) and trypsin-resistant (Govan 1978). Within each category of pyocin a variety of individual pyocins can be recognized on the basis of their spectrum of activity against different strains of *P. aeruginosa*. Strains are immune to their own pyocin, but sensitive cells are killed following attachment of pyocin to specific receptors on the cell surface. Individual strains of *P. aeruginosa* may produce more than one category of pyocin and also possess receptors for several different pyocins (Govan 1974b, 1978). Characterization of bacteriocin production is used in epidemiological typing of *P. aeruginosa* strains.

Laboratory diagnosis of P. aeruginosa infection

P. aeruginosa grows well on all normal laboratory media but specific isolation of the organism

from environmental sites or from human, animal or plant sources is best carried out on a medium which contains a selective agent and also constituents to enhance pigment production. Most selective media depend upon the intrinsic resistance of the species to various antibacterial agents, e.g. nalidixic acid, and the organism's ability to withstand and even flourish in the use-dilution of common hospital disinfectants, e.g. Dettol, cetrimide. In our experience one of the most satisfactory media is Pseudomonas Isolation Agar (PIA; Difco) which contains pigment-enhancing components and the selective agent irgasan. An alternative commercially produced selective medium is the cetrimide-containing Pseudosel Agar (BBL); in our experience this has proved too inhibitory and gives a significantly lower yield than PIA. For isolation of *P. aeruginosa* from clinical sources a selective medium, e.g. PIA, can be used alone as the primary medium but superior isolation rates, particularly from nose, throat and faeces, have been reported with the use of an acetamide enrichment broth (Kelly et al 1983).*

Most strains of *P. aeruginosa* are easily recognized on conventional laboratory media by their typical colonial appearance, the characteristic sweet musty odour and the distinctive blue-green appearance produced in the medium by the pigments fluorescein and pyocyanin. In cases when colonies that might be pseudomonas are seen in primary culture, an oxidase test should be done; a positive result is then suggestive of their identity.

When primary culture has been on diagnostic media not conducive to pigment production, e.g. MacConkey medium, colonies may subsequently be recognized as *P. aeruginosa* when antibiotic sensitivity testing is carried out; pigment production is enhanced on sensitivity test agars. The possible presence of *P. aeruginosa* may also be indicated by characteristic resistance to most of the commonly used antibiotics. However, caution should be exercised to recognize hypersensitive variants of *P. aeruginosa* that may be encountered in the respiratory tract (see above).

Demonstration that the organism produces the pigment pyocyanin confirms the identity of a pseudomonad as *P. aeruginosa**. However, a small proportion of strains, usually less than 10%, do not produce any pigment on first isolation, or lose the property on repeated subculture, and thus require further confirmation of identity, e.g. by the tests shown in Table 31.1. The oxidase test (Kovacs 1956), the oxidation-fermentation test (Hugh & Leifson 1953) and the arginine dihydrolase test are of great value in distinguishing non-pigment-producing *P. aeruginosa* and other pseudomonads from *Aeromonas*, *Achromobacter* and Enterobacteriaceae. In such cases a positive diagnosis is aided by noting such reactions as motility, growth in serial subculture at 42°C but not 4°C and ability to reduce nitrate to nitrogen gas. Failure to exhibit any one of these characteristics should cast doubt that the strain is *P. aeruginosa*.

In addition, because of the high percentage of strains that are typable with the serotyping and pyocin typing systems (see below), organisms that do not produce pyocyanin and that neither agglutinate with any of the 17 somatic sera nor produce detectable pyocin activity should be examined carefully and identified as *P. aeruginosa* only if the species characteristics outlined in Table 31.1 are satisfied.

Discriminatory tests have been incorporated in several schemes for the identification of *P. aeruginosa*. A useful single tube composite medium for the characterization of *P. aeruginosa* and other clinically relevant pseudomonads was described by Stewart (1972)*; another suitable scheme is that described by King & Phillips (1978). The rapid (4 h) identification test specific for *P. aeruginosa* described by Davis et al (1983) is based on ability to produce turbid growth in the presence of 50 μg/ml 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan (C-390; Norwich-Eaton). Alternatively, commercially produced multisystems are now available for the accurate identification of pseudomonads, e.g. the N/F two tube screen (Flow) or the more multipurpose API 20E test strip; recommendations regarding the use of multisystems are given below in the section dealing with the identification of pseudomonads other than *P. aeruginosa*.

* Refer to *Methods* at end of this chapter.

Epidemiological typing methods for Pseudomonas aeruginosa

Several biological characters have been assessed for typing purposes, including colonial morphology, pigment production, antibiograms and phage sensitivity; the two most reliable and generally accepted methods are serotyping and bacteriocin typing.

Serotyping. Identification of group-specific heat-stable lipopolysaccharide antigens by agglutination forms the basis of O serotyping procedures. Several systems have been described and their use has been reviewed (Lanyi & Bergan 1978; Brokopp & Farmer 1979; Pitt 1980; Pitt 1988). The major disadvantage of serotyping is its limited discriminating power; only nine serotypes account for over 90% of isolates (Brokopp et al 1977).

Serotyping based on H antigens is also possible. Used alone, H typing is not satisfactory for routine use as nearly 50% of strains share the same H antigen; however, in combination with O grouping it has proved useful for subdividing common O groups (Pitt 1981). Unfortunately the procedures for H antigen typing, namely immunofluorescence, immobilization or agglutination with sera prepared against purified flagella, are outwith the scope of many laboratories.

Although more than 90% of clinical strains can be typed by O serotyping, this technique is unsatisfactory for typing mucoid strains of *P. aeruginosa* since O antigens are masked. It is also unsuitable for comparisons of colonial dissociants, because antigenic changes are observed within a single culture, and for typing polyagglutinable *P. aeruginosa*, i.e. strains that are agglutinated by more than one serum in patterns that are outside the established cross-reactions of the serotype strains. Polyagglutinable and mucoid strains of *P. aeruginosa* form less than 5% of general isolates but are frequently observed in patients with cystic fibrosis.

Bacteriocin typing. Typing of *P. aeruginosa* based on pyocin production is the most popular method for typing *P. aeruginosa* in the hospital laboratory (Pitt 1980). Pyocin typing offers greater discriminating power than serotyping whilst retaining simplicity and reliability. A number of methods have been described but comparative experience (Lanyi & Bergan 1978; Brokopp & Farmer 1979) has indicated that the best method is the standard technique described by Govan & Gillies (1969) as amended by Govan (1978). Subsequently, Govan and colleagues have made further improvements to this method and the revised technique* (Fyfe et al 1984) is now recommended; it is less time-consuming and offers other advantages, particularly in strain discrimination and in typing mucoid strains.

Both serotyping and pyocin typing can contribute significantly to epidemiological studies and in the author's laboratory each system is used when appropriate. Serotyping provides a rapid screening procedure to detect whether antigenic differences are present in strains from an epidemic situation, whilst within 24 h the greater discriminating power of pyocin typing provides a more confident basis for epidemiological interpretations. Pyocin typing is the more suitable method for typing mucoid *P. aeruginosa* or polyagglutinable strains, and for comparisons of colonial disssociants.

In the UK the reference centre for serotyping and phage typing is maintained by Dr T. L. Pitt, at the Central Public Health Laboratory, Colindale, London NW9 5HT; for pyocin typing, indicator strains and information can be obtained from Dr J. R. W. Govan, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.

PSEUDOMONAS MALLEI AND P. PSEUDOMALLEI

Human laboratory-acquired infection with *P. mallei* and *P. pseudomallei* is a hazard and few other organisms are so dangerous to work with (Schlech et al 1981). Both species were categorized by the Howie Report of 1978 as category A pathogens; they are now categorized in containment category 3 and require to be handled with the greatest care and under strict and designated isolation conditions (see Ch. 15).

Pseudomonas mallei

This organism, which has also been known as *Malleomyces mallei* and *Loefflerella mallei*, causes equine glanders, a disease characterized by the formation of nasal abscesses and cutaneous and lymphatic nodules ('farcy buds') and abscesses. Human disease may take the form of an acute fulminant febrile illness or a chronic indolent infection producing abscesses in the respiratory tract or skin. It is not common and almost totally restricted to persons handling horses in those countries from which the disease has not been eradicated, principally in Asia and South America. In its morphology and biochemical reactions *P. mallei* closely resembles *P. pseudomallei* (see Table 31.1) but shows a number of negative differences; it is non-motile, has a narrower range of carbon and energy sources, does not grow on MacConkey medium and is usually oxidase negative. It has been suggested by Redfearn et al (1966) that the two species share a common ancestor, and that the negative differences of *P. mallei* are the result of functional and structural loss-adaptation to a strictly parasitic mode of existence.

Guinea-pigs are susceptible. Intraperitoneal injection of small amounts of a pure culture causes testicular swelling in 2–3 days due to bacillary invasion of the tunica vaginalis, which increases up to the tenth day. The 'Strauss reaction' may be followed by death of the animal.

Pseudomonas pseudomallei

P. pseudomallei (*Malleomyces pseudomallei, Loefflerella pseudomallei*) is the causal agent of melioidosis, a disease of rodents. Man is seldom infected and then usually by eating food contaminated with infected rodent excreta or by direct contact with infected animals. Originally the disease was restricted to SE Asia, but it became of increased importance in the USA as a result of military personnel returning from the Vietnam war. Exacerbations of melioidosis can occur a considerable time after exposure (the disease has been referred to as the Vietnam time bomb) and thus laboratory differentiation from other pseudomonads is important in patients returning from endemic areas. Clinically, the human disease, which is difficult to treat and often fatal, may be a chronic indolent infection resembling glanders (see *P. mallei*) with the formation of caseous nodules, round embolic foci and multiple abscesses in the skin, bone and internal organs, or it may present as a fulminating septicaemia.

On nutrient agar *P. pseudomallei* produces unusual and distinctive rough 'corrugated' colonies (Lapage et al 1968). Biochemically *P. pseudomallei* broadly resembles other pseudomonads but does not produce a definite pigment on nutrient agar and cultures do not fluoresce on media designed to enhance fluorescein production. Like *P. aeruginosa* it grows well at 42°C. Tests for differentiation are shown in Table 31.1.

It should be noted that *P. stutzeri* has a colonial appearance similar to that of *P. pseudomallei* and the two species can be confused as they give similar results in biochemical tests. Both species are motile but *P. stutzeri* has a single polar flagellum whilst *P. pseudomallei* has several polar flagella. Useful differential tests, positive for *P. pseudomallei* but negative for *P. stutzeri*, include gelatin liquefaction, arginine dihydrolase and intracellular accumulation of poly-β-hydroxybutyrate.

Strains of *P. pseudomallei* contain common agglutinating components that are also present in strains of *P. mallei*. Suspect cultures can be identified serologically by agglutination tests, employing rapid slide or tube agglutination techniques. Antisera for these tests and for fluorescent antibody studies, which should be used to confirm the identification of all *P. pseudomallei* isolates, are available from Difco. Recently, Ismail et al (1987) have described a very sensitive method for detecting *P. pseudomallei* exotoxin by an ELISA test based on a monoclonal antitoxin; this test is potentially useful in research and diagnosis of melioidosis.

OTHER PSEUDOMONAS SPECIES

A number of other species, principally *P. maltophilia (Xanthomonas maltophilia), P. putida, P. stutzeri, P. fluorescens* and *P. cepacia*, are

capable of causing opportunist infection in immunologically compromised patients (Gilardi 1972). It should be appreciated that the genus *Pseudomonas* contains an uncomfortably large and heterogeneous group of bacteria. The taxonomic status of many of the 171 species named in Bergey's Manual (1984) is debatable. A dogmatic approach to species identification is usually unnecessary in clinical microbiology but there are circumstances when accurate species identification within the pseudomonads is desirable. Thus *P. cepacia* was originally found as a plant pathogen causing soft rot in onions but is now isolated with increasing frequency from human infections. It causes a distinctive form of trench foot in troops training in swamps (Taplin et al 1971), and in the last 10 years has been implicated in acute and fatal exacerbations of respiratory infection in patients with cystic fibrosis. At present, the problem seems restricted to a few North American clinics treating such patients and it remains to be determined whether *P. cepacia* will emerge as a major pathogen in cystic fibrosis generally or whether the present situation is the result of cross-infection within particular clinics. Previously, epidemiological investigations and judgments have been hampered by a lack of suitable typing methods for *P. cepacia*. The recent development and validation of a bacteriocin typing system for *P. cepacia* (Govan & Harris 1985), based on the technique used successfully for pyocin typing of *P. aeruginosa*, provides a useful aid for future epidemiological studies.

A further reason for careful identification of a pseudomonad arises because of the risk of laboratory-acquired melioidosis. A particularly instructive episode was described by Schlech et al (1981). In this case a technician acquired melioidosis while involved in the isolation of aminoglycoside-degrading enzymes from an organism identified as *P. cepacia* in a local hospital. Further enquiries revealed that the patient from whom the organism had been isolated had been treated for a febrile illness in Vietnam 10 years previously. The patient had died from his undiagnosed illness, and the organism was subsequently identified as *P. pseudomallei*.

Laboratory identification of other Pseudomonas species

Gram-negative non-glucose-fermenting bacilli often grow slowly with optimum growth temperatures less than 37°C and are relatively inert biochemically. Thus, traditional identification schemes are time consuming, requiring prolonged incubation with special media in order to perform the tests listed in Table 31.1.

Within the last decade, however, several commercial manual and automated microsystems have become available, including the API 20E and 20NE test strips, the N/F system (Flow), and the Oxi/Ferm Tube (Roche). No two systems use the same range of tests and none offers ideal identification of all clinically relevant pseudomonads.

All four systems provide excellent identification of *P. aeruginosa*, including non-pigment-producing strains, but accuracy is reduced to 70–95% with other pseudomonads. Comparison of reports in the literature is made difficult by the different collections of strains tested, with occasional reliance on a single species representative. In general, the widely used API 20E system, designed predominantly for the Enterobacteriaceae offers good identification of *P. aeruginosa* and *P. maltophilia* but is less accurate for the other species included in the manufacturer's index, *P. fluorescens, P. putida, P. cepacia* and *P. putrefaciens*. Koestenblatt et al (1982) found the N/F system, developed specifically for non-fermenters, to be more accurate than the Oxi/Ferm system.

It is the author's experience that the N/F system and the more recently introduced API 20NE are the most appropriate systems presently available for identification of the pseudomonads. The N/F system consists of a two tube N/F screen and a plate (Uni-N/F-Tek) containing a number of sealed wells. The two tube screen allows determination of five reactions; 42°C growth tolerance, pyocyanin production, fluorescent pigments, glucose oxidation and N_2 production. The plate, constructed in the shape of a wheel containing independently sealed wells, allows determination of 13 different biochemical parameters. The N/F system shows good ability to

identify individual species, including *P. aeruginosa*, *P. pseudomallei*, *P. fluorescens* (grouped with *P. putida*), *P. maltophilia*, *P. cepacia* and 12 other less common species. The API 20NE system is similar in format to the well known API 20E strips but with tests designed for identification of non-fermenting Gram-negative bacilli; these are not identical to those in the N/F system. In the author's laboratory a comparative investigation of both systems for identification of over 200 pseudomonads suggested that neither system has a clear advantage, although in practice the N/F system was found to be easier to set up and interpret.

Ultimately, the choice of approach, be it manual or automatic, composite multitest system or conventional individual tests, depends upon the facilities and finances available and the degree of accuracy required by clinical circumstances. A compromise, but realistic, approach suitable for the average diagnostic laboratory would be to identify *P. aeruginosa* conventionally by production of pyocyanin and to use the multipurpose API 20E system for confident identification of *P. maltophilia* and non-pigment-producing *P. aeruginosa*. Strains presumptively identified as belonging to other pseudomonas species are then best examined by a system specifically designed for non-fermenters, e.g. the N/F wheel or the API 20NE system, or alternatively sent to a reference laboratory. These commercial systems are relatively expensive. If large collections of strains are to be screened we have found that the single tube composite arginine glucose (AG) medium* (Stewart 1972) gives excellent discrimination between the *P. aeruginosa*/*P. fluorescens*/*P. putida* group, *P. maltophilia*, *P. stutzeri* and *P. cepacia*.

METHODS

Pseudomonas enrichment broth (Kelly et al 1983)

NaCl	5 g
$MgSO_4$	0.2 g
$NH_4H_2PO_4$	1 g
K_2HPO_4	1 g
Acetamide, CH_3CONH_2	20 g
Distilled water	1 litre

Dissolve the acetamide and other ingredients in the distilled water and autoclave at 121°C for 10 min. Incubate swabs or specimens in this medium for 18 h at 37°C and then subculture to Pseudomonas Isolation Agar (PIA; Difco).

Stewart's arginine glucose (AG) medium (Stewart 1972)

Indicator mixture. This contains 0.03 g cresol red (BDH) and 0.02 g bromothymol blue (BDH), dissolved together with heating in 100 ml of 0.01 mol/litre NaOH. Filter the solution after cooling.

Medium

Nutrient broth granules, (Oxoid No. 2)	2 g
L-arginine HCl	1 g
Glucose	10 g
Indicator mixture	60 ml
Agar No. 3 (Oxoid)	8 g
Distilled water	1 litre

Dissolve the ingredients in the water; the pH should be 7.4 before autoclaving. Distribute the melted medium in 9 ml amounts in test tubes (152 × 16 mm) closed with loose metal or plastic caps. After autoclaving at 121°C for 10 min allow the medium to gel with a slope 4 cm long and a butt *c.* 4 cm deep.

Inoculation of AG medium. With a straight wire remove a fleck of culture from nutrient agar

Table 31.2 Reactions produced by *Pseudomonas* species and Enterobacteria in Stewart's Arginine Glucose medium after 24–72 h at 30°C.
Key: Y, yellow (acid production); G, green (pH unaltered); B, blue (slight alkali production); V, violet (strong alkali production).

Organism	Colour of Slope	Colour of Butt
P. aeruginosa, *P. putida*, *P. fluorescens*	Y	V
P. maltophilia, *P. stutzeri*	B	G
P. cepacia	Y	G
Enterobacteria[a]	Y	Y + gas

[a] *Escherichia coli*, *Klebsiella aerogenes*, *Salmonella* spp., *Proteus vulgaris*, *Serratia marcescens*.

plates incubated for 24–48 h at 30°C. Stab this inoculum 4 or 5 times to the foot of the tube of AG medium and then streak the slope well with the tip of the wire. Incubate the cultures at 30°C for 24–72 h. Use the reaction patterns shown in Table 31.2 to identify the organisms.

Production and identification of pseudomonas pigments

Pyocyanin and fluorescein

Most pigment-enhancing media are based on Media A and B described by King et al (1954), for production of the major pigments pyocyanin and fluorescein respectively. Most strains of *P. aeruginosa* produce both pigments and the media were developed to enhance production of one pigment whilst suppressing the other.

Various commercial forms of these media are available, e.g. Medium P (Difco) which enhances production of pyocyanin. Pseudomonas Isolation Agar (PIA; Difco) combines the pyocyanin-enhancing properties of Medium P with the selective agent irgasan (2,4,4 trichloro-2-hydroxyphenylether) and is recommended for demonstration of pyocyanin production in normal practice. Production of fluorescein is best achieved by the use of Medium F (Difco) or of the original King's Medium B. The recipes for the Difco media P, F and PIA, are given below.

Pyocyanin is blue in colour and non-fluorescent; it is formed best in peptone media and is soluble in both chloroform and water. In liquid cultures of *P. aeruginosa* grown without agitation a pyocyanin-containing layer can often be observed in the upper part of the broth at the air/liquid interface. Fluorescein is yellow and fluorescent; it is formed only in the presence of phosphate and is soluble in water but not in chloroform. When pyocyanin is produced in small amounts, or its presence is obscured by other pigments, it can be more readily observed by shaking a few millilitres of chloroform in a broth culture or on an agar slope culture; on standing, pyocyanin (but not fluorescein) will appear in the $CHCl_3$ once the phases have separated out. Fluorescein is best observed by use of a suitable dark chamber, with the cultures illuminated with UV light.

Pyorubrin and pyomelanin

A small percentage of strains of *P. aeruginosa* also produce the red pigment pyorubrin or the brown pyomelanin. Caution should be exercised in the identification of these pigments since acidification of pyocyanin produces a red colour and prolonged exposure of pyocyanin to air produces a brownish oxidation product. Identification of pyorubrin and pyomelanin is aided by the use of specific culture media. Growth in 1% DL-glutamate allows pyorubrin but not pyomelanin production. Davis & Mingioli's minimal salts medium supplemented with 1% tyrosine enhances pyomelanin production but not pyorubrin. Alternatively, Furunculosis Agar (tryptone, 10 g; yeast extract, 5 g; L-tyrosine, 1 g; NaCl, 2.5 g; agar, 15 g/ litre) can also be used to detect pyomelanin production (Ogunnariwo & Hamilton-Miller 1975).

Difco P and F media

Difco medium P

Bacto peptone	20 g
Magnesium chloride	1.4 g
Potassium sulphate	10 g
Bacto agar	15 g
Distilled water	980 ml

Difco medium F

Bacto tryptone	10 g
Bacto proteose peptone No. 3	10 g
Dipotassium phosphate	1.5 g
Magnesium sulphate	1.5 g
Bacto agar	15 g
Distilled water	980 ml

Preparation of media

Dissolve either medium (46.4 g Medium P or 38 g Medium F) in the water and add 20 ml Bacto glycerol. Heat to boiling to dissolve the medium completely. Autoclave for 15 min at 121°C.

Pseudomonas isolation agar (PIA)

Bacto peptone	20 g
Magnesium chloride	1.4 g

Potassium sulphate	10 g
Irgasan, 2,4,4 trichloro-2-hydroxy phenylether	0.025 g
Bacto agar	13.6 g
Distilled water	980 ml

Method. Dissolve 45 g medium in the water and add 20 ml Bacto glycerol. Heat to boiling to dissolve the medium completely. Autoclave for 15 min at 121°C. Final pH 7.0 ± 0.2 at 25°C.

Pyocin typing

The standard procedure (Govan 1978) used a cross-streaking technique in which pyocin production by the test strain was detected by inhibitory activity against a standard set of 13 indicator strains of *P. aeruginosa*. The main disadvantages of the technique are the 72 h period required for a result, the need to remove producer strain growth, the poor recognition of S pyocin activity and the difficulty of typing mucoid strains of *P. aeruginosa*. These problems have been overcome by the development of a revised spotting procedure (Fyfe et al 1984) which retains the use of the same 13 indicator strains and the same typing pattern list as previously. The revised procedure is described below.

1. Grow the strains of *P. aeruginosa* to be typed on nutrient agar (Columbia Agar Base, Oxoid) at 37°C overnight. Use individual colonies of each test strain to prepare bacterial suspensions of 10^8–10^9 organisms in 1 ml of sterile physiological saline (absorbance at 550 nm = 0.5).

2. Use a multipoint inoculator (e.g. Model A400, Denley; this incorporates 21 stainless steel pins of 2 mm diameter set 16 mm apart) to dispense 1 μl volumes of the bacterial suspensions on to a set of 13 plates (diameter 90 mm) each containing 10 ml Tryptone Soya Agar (Oxoid). In this way 20 test strains (1 pin being a marker) can be typed simultaneously against each indicator strain. After the spots have dried, usually within a few minutes, incubate the plates at 30°C for 6 h.

3. Impregnate filter-paper disks (5 cm; Whatman) with chloroform and place the plates over the disks for 15 min to allow chloroform vapour to kill the bacteria. Expose the plates to air for an additional 15 min to eliminate residual chloroform vapour.

4. Prepare cultures of the indicator strains in Nutrient Broth (Oxoid, No. 2) without agitation for 4 h at 37°C to a population size of *c.* 10^7 orgs/ml.

5. Apply a separate indicator strain to each plate by adding 0.1 ml of each bacterial indicator culture to 2.5 ml of molten semi-solid agar (1% Peptone, Difco, in 0.5% agar, Oxoid LII, held at 45°C); then pour it as an overlay. When the overlays have set, incubate the plates for 18 h at 37°C.

6. Determine the pyocin types of the test strains, as with the cross-streaking method, on the basis of the patterns of inhibition observed against the 13 indicator strains (Govan 1978). Note that with the revised technique the sizes of

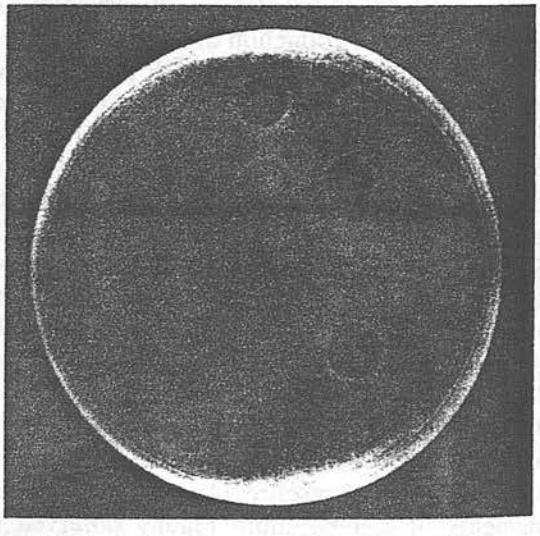

Fig. 31.1 Typical routine typing plate obtained by the revised spotting method for pyocin typing (Fyfe et al 1984). The agar overlay incorporates indicator strain 5. The plate shows the reactions produced by 20 test strains of *P. aeruginosa*. Four strains are surrounded by extensive S pyocin activity; 2 strains show restricted zones of inhibition characteristic of R and F pyocins; the remaining strains have produced no activity against this particular indicator strain.

the inhibition zones are also taken into account for the purpose of more detailed strain comparison, and determination of S pyocin activity is incorporated into the typing results as in the original cross-streaking method (see Fig. 31.1).

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Fecal Isolation of *Pseudomonas aeruginosa* from Patients with Cystic Fibrosis

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Fecal isolation of *Pseudomonas aeruginosa* was observed in 8 of 10 patients with cystic fibrosis who at the time of sampling also exhibited colonization of the respiratory tract. In contrast, *P. aeruginosa* cells were isolated at low frequency (9.1%) from the stools of 44 patients with cystic fibrosis with no previous history of chronic colonization. The results of this study suggest that the gastrointestinal tract is not a significant chronic reservoir of *P. aeruginosa* prior to pulmonary colonization.

A major factor responsible for morbidity and mortality in patients with cystic fibrosis (CF) is the effect of the disease on the lungs, in particular the sequelae of chronic colonization with *Pseudomonas aeruginosa* (9, 14). Initial asymptomatic colonization of the respiratory tract with typical nonmucoid *P. aeruginosa* is followed by phenotypic changes in the organism and subsequent intermittent episodes of exacerbation and progressive pulmonary deterioration (7, 13, 15). Early detection of *P. aeruginosa* is of considerable importance in determining the prognosis for patients with CF, planning therapeutic strategies, and understanding the etiology of pulmonary colonization. In addition, since the major virulence determinant associated with *P. aeruginosa* pathogenesis (derepressed alginate biosynthesis resulting in a mucoid colonial appearance) has been shown to be regulated by the chromosomal mutations *muc* and *alg* (2–4, 7, 12), it seems reasonable to suggest that primary colonization by typical nonmucoid *P. aeruginosa* provides an essential microbial reservoir from which the virulence-associated phenotype arises.

At present, the nature of the site(s) of early asymptomatic colonization remains obscure. Reports of upper respiratory tract colonization sites include maxillary sinuses (18), tongue, buccal mucosa, and saliva (11). Lindemann et al. (11) stressed the difficulty of identifying oral colonization and distinguishing it from sputum contamination because of the high concentration of bacteria in sputum. A number of studies in non-CF patients who are compromised by underlying disease or immune-suppressive therapy have indicated that fecal carriage of *P. aeruginosa* increases the risk of subsequent opportunistic infection (16, 20). There have been few reported studies (10, 17) of the incidence of fecal carriage of *P. aeruginosa* in patients with CF, perhaps reflecting the relative difficulty in obtaining compliance, and these reports provide few details of whether the isolates were mucoid or nonmucoid and no indication of the presence or absence of pulmonary colonization. We considered that a study of fecal carriage of *P. aeruginosa* might provide valuable information, particularly if the study included patients who at the time of investigation did not harbor *P. aeruginosa* in their respiratory tracts.

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MATERIALS AND METHODS

Study group. The study group comprised 54 patients with CF who regularly attend the Edinburgh CF clinics. The investigation was carried out during the period from February to September 1987. All patients had received antibiotic therapy on previous occasions, but no patient was receiving antibiotic treatment at the time of sampling. The age range of the patients was 2 months to 33 years. At the time of sampling, 10 patients were known to be chronically colonized in the upper respiratory tract by mucoid *P. aeruginosa*.

Informed consent was obtained from the parent or guardian of each participant and, when appropriate, from the subjects themselves.

Bacteriology. Stools were mixed with an equal volume of sterile physiological saline and emulsified by vortexing. Fecal emulsion (0.1 ml) was spread onto the surface of *Pseudomonas* isolation agar (PIA; Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 48 h; a further 0.1 ml was inoculated into acetamide enrichment broth (10), and after 24 h of incubation at 37°C, a loopful was removed from the enrichment broth, plated onto PIA, and incubated as described before. Expectorated sputum was obtained after chest physiotherapy, and after liquefaction with sputolysin (Behring Diagnostics, La Jolla, Calif.), quantitative culture for *P. aeruginosa* was carried out by plating 0.1-ml volumes of diluted homogenized sputum onto PIA. *P. aeruginosa* was identified by characteristic features, including production of pyocyanin; when necessary, identification of nonpigmented isolates growing on PIA was by a positive oxidase reaction and identity as *P. aeruginosa* was confirmed by the API 20NE system (API-bioMérieux, Vercieu, France). Ten representative colonies from each *P. aeruginosa*-positive specimen were further characterized by pyocin typing by using a technique suitable for mucoid variants (5).

RESULTS

Ten patients were known to exhibit chronic pulmonary colonization by mucoid and nonmucoid *P. aeruginosa* prior to this investigation. In the study, colonization in these patients was confirmed and ranged from 10^6 to 10^8 CFU/ml of homogenized sputum. Concurrently, *P. aeruginosa*-positive fecal cultures were obtained from eight of these patients and of these eight *P. aeruginosa*-positive stools, six samples harbored mucoid variants. In each case, the *P. aeruginosa*

isolated from the stool was found to belong to the same pyocin type as that present in the sputum. Of four patients who were known to exhibit intermittent pulmonary colonization by nonmucoid *P. aeruginosa*, but whose sputum did not contain *P. aeruginosa* at the time of stool collection, three harbored nonmucoid *P. aeruginosa* in their stools. Forty patients had shown no previous history of respiratory colonization by *P. aeruginosa*, and sputum from these patients was confirmed as *P. aeruginosa*-free at the time of stool culture. From this collection of 40 stool samples, *P. aeruginosa* (nonmucoid) was isolated from only one specimen. In stools and sputum found to be *P. aeruginosa* positive, pyocin typing of 10 colonies from each primary culture plate did not produce evidence of multiple colonization with more than one strain of *P. aeruginosa*.

DISCUSSION

Rates of fecal carriage of *P. aeruginosa* differ widely in published reports and illustrate different carriage rates in different populations (16). In most studies, however, there is general agreement that carriage rates are less than 10% in healthy subjects but may rise to greater than 40% in hospitalized patients (1, 16). In a typical study, Yoshioka et al. (19) found the carriage rate of *P. aeruginosa* in infants delivered at home to be 6% compared with 57% in hospital-born infants. Fecal carriage rates also showed a seasonal variation, ranging in healthy adults from 4.7% in April to 43% in July.

To our knowledge, there has been no attempt to focus on the possibility of gastrointestinal carriage of nonmucoid *P. aeruginosa* as a reservoir for subsequent pulmonary colonization in patients with CF. Kelly et al. (10) reported 9 isolations of *P. aeruginosa* from 34 fecal samples but did not indicate whether isolates were mucoid or whether the patients had concurrent pulmonary colonization. Roy et al. (17) investigated the fecal flora of 23 CF patients and found that 7 patients had *P. aeruginosa* in their stools, 6 of whom were on antibiotics at the time; with one exception, the *P. aeruginosa* isolates were mucoid. In our study, the demonstration of simultaneous fecal carriage of mucoid *P. aeruginosa* belonging to the same pyocin type in 8 of the 10 patients with concurrent pulmonary colonization enhances the evidence of Roy et al. (17) and suggests a considerable degree of intestinal contamination with mucoid *P. aeruginosa* from the respiratory tract. This hypothesis is supported by the fact that mucoid forms of *P. aeruginosa* are rarely found in *P. aeruginosa*-positive stools in non-CF patients nor, in our experience, from the stools of patients with CF in the absence of pulmonary colonization. If the gastrointestinal tract is an important reservoir preceding pulmonary colonization, it would be reasonable to argue that fecal carriage in CF patients would be relatively high compared with that found in healthy individuals. This was not observed in the present study, which showed a fecal isolation rate (9.1%) within the normal range in those patients not exhibiting chronic pulmonary colonization. The relatively low isolation rate could not be attributed to culture techniques in view of the high incidence achieved concurrently in those patients with pulmonary colonization. Similarly, since stool cultures were investigated over a 6-month period from February to September, it seems unlikely that a seasonal variation, as reported by Yoshioka et al. (19), would exert a significant effect. Studies of fecal isolation rates in individual CF centers may be influenced by antibiotic usage as well as by the incidence of pulmonary *P. aeruginosa* colonization which is relatively low in the Edinburgh clinics (8). We deliberately excluded patients on current antibiotic therapy from this study to avoid any suppression of isolation rate by residual antibiotic present in the stools; all patients, however, had received antibiotic therapy on previous occasions. In CF and non-CF patients, colonization of the individual sputum and stools by more than one strain of *P. aeruginosa* has been reported (6, 7) and ranges from 3 to 15%; multiple colonization is more common in hospitalized patients than in patients treated at home. We took this factor into account in the present study but found no evidence of multiple colonization in the *P. aeruginosa*-positive specimens investigated in this study.

The four patients from whom non-mucoid *P. aeruginosa* had been isolated intermittently in the previous 3 years provided an interesting and unexpected result. At the time of sampling, three of these patients harbored nonmucoid *P. aeruginosa* in their stools, although *P. aeruginosa* was not cultured from their sputum. It is arguable that these patients reflect natural carriage or perhaps represent a small subpopulation of CF patients in whom intermittent stool carriage of nonmucoid *P. aeruginosa* precedes pulmonary carriage or vice versa. The future isolation of *P. aeruginosa* from such patients will be followed with interest. From the present study, we conclude that fecal contamination by *P. aeruginosa* resulting from pulmonary colonization occurs in the majority of patients with CF and that in the CF population investigated in this study the gastrointestinal tract does not form an important chronic reservoir of nonmucoid *P. aeruginosa* prior to colonization of the respiratory tract.

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Pseudomonas cepacia Typing Systems: Collaborative Study to Assess Their Potential in Epidemiologic Investigations

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To determine the utility of available *Pseudomonas cepacia* typing systems for confirming the relatedness of isolates, we applied these methods to isolates associated with previously investigated nosocomial outbreaks. We compared chromosome analysis, serologic reactions, biochemical tests, bacteriocin production and susceptibility, and antimicrobial susceptibility in their ability to determine outbreak relatedness. Chromosome analysis, serologic reactions, and biochemical tests were each demonstrated to be epidemiologically useful methods for typing isolates. Determination of the sensitivity and specificity of these typing techniques will facilitate their application in the epidemiologic study of this increasingly important nosocomial pathogen.

Pseudomonas cepacia is an aerobic, glucose-nonfermenting, gram-negative bacillus that has been increasingly recognized as a nosocomial pathogen [1–6]. The organism can proliferate under conditions of minimal nutrition (e.g., distilled water) and can survive in the presence of certain disinfectants [7, 8]. Hospital outbreaks of *P. cepacia* infection have been ascribed to contamination of disinfectant preparations, various aqueous solutions, water reservoirs, and other liquid or moist sources [1, 9–11].

In the burned mouse model, *P. cepacia* appears to be less virulent than *Pseudomonas aeruginosa* [12], and infection in humans is unusual in the presence of intact host defenses. However, recently, *P. cepacia* has emerged as an important pathogen among cystic fibrosis patients in some North American centers; cystic fibrosis patients with respiratory colonization or infection with *P. cepacia* have higher morbidity and mortality than do noncolonized cystic fibrosis patients who are matched for age, sex, and severity of illness [13, 14].

Epidemiologic investigation of epidemic and endemic *P. cepacia* infections has been limited by the lack of a sensitive and specific typing system that allows determination of isolate relatedness [15]. Typing systems based on serologic reactions, bacteriocin susceptibility and production, plasmid profiles, and biochemical reactions have recently been proposed [16–26]. However, there have been no compar-

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The use of trade names is for identification purposes only and does not indicate endorsement by the U.S. Public Health Service or the U.S. Department of Health and Human Services.

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ative studies assessing the validity of these systems as epidemiologic tools in outbreak investigations. Many of these systems have been developed and assessed with sporadic, unrelated isolates rather than those associated with outbreaks [15]. Each typing system has been applied to a different collection of strains, thus precluding comparison of efficacy and validation of proposed classification schemes of the various typing systems.

To determine the utility of available *P. cepacia* typing systems in confirming isolate relatedness, we applied the previously reported typing methods to isolates obtained during six outbreaks investigated by the Hospital Infections Program, Center for Infectious Diseases, Centers for Disease Control (CDC, Atlanta). For one additional outbreak (no. VII), isolates were available from the CDC's reference collection. We report here the results of a comparison of *P. cepacia* typing by chromosome analysis, serologic reaction, biochemical tests, bacteriocin production and susceptibility, and antimicrobial susceptibility.

Materials and Methods

Study design. One hundred one *P. cepacia* isolates were multiply typed in this study. Ninety-two isolates were obtained during the investigation of seven hospital outbreaks, and nine isolates were from diverse sources and were epidemiologically unrelated (table 1).

Ten typing methods were evaluated: chromosome analysis by restriction fragment-length polymorphism (RFLP), four serotyping systems, three different biotyping schemes, bacteriocin susceptibility and production, and antimicrobial susceptibility. Typing results by each method were obtained for the 101 isolates, permitting direct comparison of the various typing systems.

All isolates were confirmed as *P. cepacia* by standard biochemical identification procedures [27]. Isolates were subcultured at CDC, and identical collections of subcultures were distributed simultaneously for typing by each of the collaborators. Isolates were assigned random numbers before distribution so that the investigators would be unaware of the isolates' origins.

Chromosome analysis. Chromosomal DNA was digested with *Eco*RI, and the resultant fragments were separated by electrophoresis in agarose, transferred to nitrocellulose, and hybridized with *Escherichia coli* ribosomal RNA, as previously described [28]. The RFLP among the isolates was analyzed. Isolates within a ribotype differed from each other by three or fewer hybridization bands. Isolates of the same subribotype had identical banding patterns or differed from each other by one hybridization band. Ribotypes were designated by Arabic numbers and subribotypes by capital letters.

Serologic reactions. Four serologic typing systems employing different techniques and antiserum samples were examined. One method included both O (somatic) and H (flagellar) serotyping, two methods involved only O antigens, and one employed

Table 1. Epidemiologic characteristics of *Pseudomonas cepacia* isolates.

Outbreak no.	Year	Location	Reference	Implicated common source	Isolate numbers and source	
					Patient	Environment
I	1975	Georgia	2	Contaminated detergent solution used to clean blood pressure transducers	1	2-10
II	1981	Minnesota	3	Contaminated 2% viscous lidocaine used during bronchoscopy	. . .	11-16
III	1981	Washington	CDC	Inadequately disinfected blood pressure transducers	17-21	22-25
IV	1981	Michigan	1	Inadequately disinfected automatic peritoneal dialysis machines	26-39	40-52
V	1980	New York	4	Contaminated 10% povidone iodine causing pseudobacteremia	53-65	66-70
VI	1974	Maryland	5	Contaminated human serum albumin	71	72-77
VII	1980	Israel	6	Contaminated 0.2% chlorhexidine used for mouth and perineum antisepsis	78-83, 86-89	84-85, 90-93
Unrelated	Various	Various	CDC	None	94-102	

whole live cells as antigen. "Nontypable," "multitypable," and "autoagglutinating" were considered to be distinct serotypes in the typing system analysis.

We used the typing method of Heidt et al. to classify isolates by seven O and five H antigens [18]. The method described by Nakamura et al. was used to type the isolates by a separate set of 10 O antigens [19]. The third serotyping method employed antiserum raised against live cell suspensions of *P. cepacia* [20]. The fourth serotyping system was a modification of the method developed by Jonsson, with an expanded panel of antiserum samples and designation of additional serogroups IV, V, and VI [16, 29].

Biochemical tests. Biologic types were determined according to three separate schemes. In the first system, isolates were classified on the basis of the results of pigment production, nitrate reduction, esculin hydrolysis, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis tests [26]. In the second system, isolates were classified on the basis of growth at 41°C and gelatin liquefaction at 37°C; this system was modified to include an additional biotype (D), defined as growth at 41°C–positive, gelatin hydrolysis–negative [25]. In the third method, isolates were typed by use of the numeric profiles generated by the API Rapid NFT bacterial identification kit (API Analytab Products, Plainview, N.Y.).

Bacteriocin susceptibility and production. Bacteriocin susceptibility and production were determined as previously described [22]. In addition to the bacteriocin susceptibility and production types previously reported, five additional susceptibility types (S24–S28) and one additional production type (P15) were defined as follows: S24, inhibition by producer strains 1, 3, and 6; S25, inhibition by producer strain 1; S26, inhibition by producer strains 1, 2, 3, 4, 5, and 6; S27, inhibition by producer strain 2; S28, inhibition by producer strains 1, 3, and 5; and P15, inhibition of indicator strains 2 and 5.

Antimicrobial susceptibility. Susceptibilities to 40 antimicrobial agents were determined by Kirby-Bauer disk diffusion [30]. Zone diameters were interpreted as susceptible, intermediate, or resistant by the standards of the National Committee for Clinical Laboratory Standards [30]. The following antimicrobial agents were tested: ampicillin, amoxicillin-clavulanic acid, carbenicillin, ticarcillin, ticarcillin-clavulanic acid, piperacillin, mezlocillin, azlocillin, aztreonam, imipenem, cephalothin, cefazolin, cefoxitin, cefamandole, cefotetan, cefuroxime, cefonicid, cefotaxime, cefoperazone, ceftriaxone, ceftazidime, ceftizoxime, moxalactam, gentamicin, tobramycin, amikacin, netilmicin, colistin, trimethoprim-sulfamethoxazole, sulfisoxazole, trimethoprim, ciprofloxacin, norfloxacin, cinoxacin, nalidixic acid, chloramphenicol, rifampin, tetracycline, doxycycline, and minocycline.

Data analysis. From reports and study of isolate sources it was not epidemiologically possible, in the absence of a marker system, to distinguish outbreak types. Therefore, outbreak-related isolates were presumed to be identical for analysis purposes. For each typing system, we defined the most frequent typing result for isolates from each outbreak as the respective outbreak type. We then defined the fraction of isolates with the outbreak type for each outbreak as the respective concordance. Since any isolates not determined to be of the outbreak type represent false-positives (i.e., false determinations of a difference from the other isolates), we compared the outbreak concordance of the typing systems as a relative measure of their specificity (the probability that related strains would be determined to be similar).

We similarly defined the concordance for the unrelated isolates as the fraction of isolates with the most frequent typing result. On the presumption that the unrelated group of isolates were different, we used the unrelated isolate concordance of the typing systems as a relative measure of sensitivity (the probability that unrelated strains would be determined to differ). We considered the number of outbreak types identified by the typing systems as another measure of sensitivity.

Within outbreak, concordances were statistically tested by comparison of proportions adjusted for sampling in groups; unrelated isolate concordances were tested by Fisher's exact test.

Results

Chromosome analysis. There were 23 subribotypes identified among the 101 isolates studied (table 2). Among the seven outbreaks only two had the same most-frequent subribotype (outbreak type), and there were five different subribotypes that were most frequent for the other five outbreaks (table 3). There were seven additional subribotypes among the nine unrelated isolates, of which two (22%) subribotypes were each found in two isolates.

For each of four outbreaks, all outbreak-related isolates were of the same subribotype (100% concordance). For two of the other three outbreaks, no subribotype was found in a majority of isolates from

each outbreak (28% and 43% concordance, respectively).

The 23 subribotypes belonged to 11 ribotypes (table 3). By ribotype, the seven outbreaks could be classified into five outbreak types. There were six ribotypes among the unrelated isolates, of which one was found in three (33%) of the isolates.

For five of the outbreaks, there was 100% concordance of ribotype. The remaining two outbreaks each had one associated isolate that was not of the respective outbreak type. By concordance of outbreak-related isolates, ribotyping was significantly more specific than the other nine typing systems ($P \leq .02$ for each comparison).

Serotyping systems. Seventeen combinations of O and H serotypes were identified among the 101 isolates by the method of Heidt et al. (table 3) [18]. There were six outbreak types among the seven outbreaks by this method, and two (22%) of the unrelated isolates shared the same serotype. Five outbreak serotypes were O,H-typable, and one was O-typable only. In each of two outbreaks, all outbreak-related isolates had the same O,H serotype; the average concordance of the seven outbreaks was 89%.

Five outbreak types were found by serotyping by the method of Nakamura et al. [19]; concordance of the unrelated isolates was 22% (table 3). One hundred percent concordance was found for one outbreak, and concordance for the others ranged from 69% to 96%.

For four of the seven outbreaks, most outbreak-related isolates were nontypable by the serotyping method of McKevitt et al. [20]; two of the remaining three outbreaks shared a single outbreak type (table 2). Four (44%) of the unrelated isolates were also nontypable. The average concordance for the seven outbreaks was 78%.

Five outbreak types, including autoagglutinating and multitypable, were found by the serotyping method of Jonsson [16, 29]; four (44%) of the unrelated isolates were autoagglutinating (table 2). The greatest concordance for the seven outbreaks was 69%, and the least was 29%.

Biotyping schemes. There were five outbreak types among the seven outbreaks by the biotyping method of Richard et al. [26]; concordance of the unrelated isolates was 33% (table 3). All isolates from one outbreak were of the outbreak type, and the average concordance for the seven outbreaks was 80%.

By the method of Esanu and Schubert [25], the outbreak types included all four biotypes; the unrelated isolate concordance was 44% (table 3). For each of two outbreaks, all outbreak-related isolates had the outbreak type, and the concordance for the others ranged from 50% to 94%.

There were 21 API profiles identified among the 101 isolates and five outbreak types by this method among the seven outbreaks (table 3). The unrelated isolate concordance was 33%. For one outbreak, all isolates had the outbreak type; for the other outbreaks, concordance ranged from 29% to 78%.

Bacteriocin typing. Based on bacteriocin susceptibility and production, there were 25 bacteriocin types among the 101 isolates, and each outbreak type was unique. Each of the unrelated isolates had a different bacteriocin type. An averge of 52% of isolates from each outbreak shared the outbreak type. Bacteriocin typing was significantly more sensitive than four of the other methods ($P \leq .041$ for comparisons with serotyping by the methods of McKevitt et al. [20] and Jonsson [16, 29] and biotyping by the methods of Richard et al. [26] and Esanu and Schubert [25]).

Antimicrobial susceptibility. Antimicrobial susceptibility varied substantially between outbreak-related isolates (data not shown). Except in outbreak no. IV, no isolate's antimicrobial susceptibility pattern was repeated in another isolate from the same outbreak (three isolates in outbreak no. IV had the same susceptibility pattern).

The antimicrobial agents to which the isolates were most frequently susceptible were: piperacillin (98%), mezlocillin (95%), azlocillin (90%), ceftazidime (98%), and ceftizoxime (94%), although one (no. 74) was resistant to all of these agents. The frequency of isolate susceptibility to the other antimicrobial agents was less than 90%.

Epidemiologic classification. The typing results generally corroborated similarity of outbreak-related isolates. However, one isolate (no. 77) associated with outbreak no. VI was different from the outbreak type by all typing methods, and one isolate (no. 70) associated with outbreak no. V was different from the outbreak type by all but one method. No epidemiologic features distinguished these isolates from others associated with their respective outbreaks.

Discussion

Endemic and epidemic infections with *P. cepacia* commonly occur [31, 32]. Isolate typing has important applications in epidemiologic investigations to verify outbreaks and to identify the sources or reservoirs. By multiple typing of isolates of established

Table 2. Comparative results of evaluated *Pseudomonas cepacia* typing systems.

		Typing system [reference]										
		Chromosomal*		Serologic					Biochemical			Bacteriocin
Outbreak no.	Isolate no.	SRT [28]	RT	O [18]	H [18]	[19]	[20]	[16]†	[26]‡	[25]	API profile§	[22]
I	1	A	1	2	2	E,G	D	AA	7	C	1477577	S13/P0
	2	A	1	2	2	G	E	III	7	C	1477577	S0/P0
	3	A	1	2	2	G	E	AA	7	C	1477577	S3/P0
	4	A	1	2	2	G	C	III	6	C	1477577	S0/P8
	5	A	1	2	2	G	E	III	7	C	1477577	S0/P0
	6 (nonviable)											
	7	A	1	2	2	G	E	IV	7	C	1477577	S13/P0
	8	A	1	2	2	G	E	IV	7	C	1477577	S0/P0
	9	A	1	2	2	G	E	VI	7	C	1477577	S3/P0
	10	A	1	2	2	G	E	III	7	C	1477577	S25/P0
II	11	I	5	8	7	F	NT	Ia	4GY	C	0067577	S24/P0
	12	I	5	8	7	F	NT	NT	4GY	D	4067577	S3/P0
	13	I	5	8	7	F	NT	AA	4GY	A	0046577	S28/P0
	14	I	5	8	7	F	B	AA	4GY	D	0067777	S28/P0
	15	I	5	8	7	F	NT	AA	4GY	C	4067577	S28/P0
	16	I	5	8	7	F	NT	AA	4GY	C	0067577	S28/P0
III	17	A	1	2	2	NT	E	AA	7	C	1477777	S3/P8
	18	A	1	2	2	G	E	MT	7	C	1467777	S3/P8
	19	A	1	2	1,2	G	E	IV	7	C	1477777	S3/P8
	20	A	1	2	2	G	E	IV	7	C	1477777	S3/P8
	21	A	1	2	2	G	E	AA	7	C	1467777	S3/P8
	22	A	1	2	2	G	NT	IV	7	C	1477777	S3/P0
	23	A	1	2	2	G	E	III	6	C	1477777	S0/P8
	24	A	1	2	2	G	E	IV	7	C	1477777	S3/P8
	25	A	1	2	2	G	E	III	7	C	1477777	S0/P8
IV	26	D	3	3	7	I	D	I	6	D	1067577	S25/P0
	27	D	3	3	7	I	D	I	6	D	1067577	S8/P0
	28	C	3	3	7	I	D	I	4	D	0067577	S8/P0
	29	D	3	3	7	I	NT	I	6	A	1067577	S8/P0
	30	D	3	3	7	I	D	I	4	D	1067577	S8/P0
	31	C	3	3	NT	I	D	MT	4	D	0067577	S8/P0
	32	D	3	3	7	I	D	NT	6	D	1067577	S8/P0
	33	C	3	3	7	I	D	NT	4	D	0067577	S8/P0
	34	C	3	3	7	I	D	I	4	D	0067777	S27/P0
	35	D	3	3	7	I	D	I	6	D	1067577	S8/P0
	36	C	3	AA	NT	MT	D	MT	4	D	1067577	S16/P0
	37	D	3	3	7	I	D	AA	6	B	1067577	S8/P0
	38	D	3	3	7	I	D	I	6	D	1067577	S8/P0
	39	C	3	3	7	I	NT	III	4	D	0067577	S8/P0
	40	C	3	3	7	I	D	I	4	D	0067577	S18/P0
	41	C	3	3	7	I	D	I	4	A	0067577	S8/P0
	42	D	3	3	7	I	D	AA	6	D	0067577	S8/P0
	43	C	3	3	7	I	D	I	4	D	0067577	S25/P0
	44	C	3	3	7	I	D	NT	4	D	0067577	S8/P0
	45	C	3	3	7	I	D	I	4	D	0067777	S25/P0
	46	C	3	3	7	I	D	AA	4	D	0067777	S8/P0
	47	C	3	3	7	I	D	III	4	D	0067777	S8/P0
	48	C	3	3	7	I	D	I	4	D	0067777	S18/P0
	49	C	3	3	7	I	D	I	4	D	0067577	S8/P0
	50	C	3	3	7	I	D	AA	4	D	0067577	S8/P0
	51	D	3	3	7	I	D	I	6	D	1067577	S8/P0
	52	C	3	3	7	I	D	AA	4	B	0067577	S8/P0

(*continued*)

Table 2. (*continued*)

Outbreak no.	Isolate no.	Typing system [reference]										
		Chromosomal*		Serologic					Biochemical			Bacteriocin
		SRT [28]	RT	O [18]	H [18]	[19]	[20]	[16]†	[26]‡	[25]	API profile§	[22]
V	53	F	4	1	NT	D	C	AA	0	A	1046577	S1/P0
	54	E	4	1	NT	D	NT	IV	0	B	0046577	S24/P0
	55	H	4	1	NT	D	NT	NT	0	A	0046577	S1/P0
	56	N	4	1	NT	D	C	AA	0	A	0046577	S1/P0
	57	P	4	1	NT	D	NT	IV	0	A	0057577	S24/P0
	58	G	4	1	NT	D	C	AA	0	B	0056577	S24/P0
	59	O	4	1	NT	D	NT	IV	0	B	0056577	S24/P0
	60	E	4	1	NT	D	NT	IV	0	B	0046577	S24/P0
	61	M	4	1	NT	D	C	Ia	0	A	0046577	S8/P0
	62	E	4	1	NT	D	NT	AA	0	A	0046577	S1/P0
	63	M	4	1	NT	D	NT	AA	0	B	0046577	S1/P0
	64	F	4	1	NT	D	NT	IV	0	A	0046577	S24/P0
	65	E	4	1	NT	D	NT	IV	0	B	0046577	S1/P0
	66	E	4	1	NT	D	NT	Ib	0	B	1046577	S24/P0
	67	G	4	NT	2	MT	D	MT	0	B	0056577	S12/P0
	68	H	4	1	1	D	C	Ia	0	B	0047577	S8/P0
	69	N	4	1	NT	D	NT	V	0	B	0046577	S1/P0
	70	A	1	2	1,2	G	E	IV	7	C	0047577	S3/P8
VI	71	K	5	5	5	E		I	4GY	A	0476577	S28/P5
	72	J	5	5	5	MT	NT	MT	4GY	A	0477577	S18/P6
	73	Q	5	5	5	E	NT	MT	4GY	A	0467577	S21/P6
	74	J	5	5	5	E	C	V	4GY	D	0477577	S28/P6
	75	K	5	5	5	E	NT	AA	5	A	0467577	S28/P15
	76	K	5	5	5	E	NT	IV	1	A	0466577	S28/P15
	77	U	9	AA	NT	D	B	NT	7	D	1467577	S1/P0
VII	78	B	2	8	8	F	C	AA	1	C	0477577	S26 /P0
	79	B	2	8	8	F	E	Ia	1	C	4477577	S26/P0
	80	B	2	9	8	G	NT	III	1	C	0477577	S16/P0
	81	B	2	9	8	G	NT	III	1	C	0477577	S16/P0
	82	B	2	9	8	G	NT	III	1	C	4477777	S16/P0
	83	B	2	9	8	G	NT	III	1	C	4477577	S3/P0
	84	B	2	9	8	G	NT	III	1	C	4477577	S16/P0
	85	B	2	9	8	G	D	III	1	C	0477577	S16/P0
	86	B	2	9	8	G	NT	I	1	C	0477577	S3/P0
	87	B	2	9	8	G	NT	III	1	C	0477577	S16/P0
	88	B	2	9	8	G	NT	III	5	B	0477577	S16/P0
	89	B	2	9	8	G	NT	III	5	C	4477577	S3/P0
	90	B	2	8	8	F	NT	Ia	5	C	4477577	S26/P0
	91	B	2	8	8	F	C	AA	5	C	0477577	S26/P0
	92	B	2	9	8	G	NT	III	5	C	4477577	S16/P0
	93	B	2	9	8	NT	NT	III	5	C	4477577	S16/P0
Unrelated	94	W	11	1,7	2	A	E	IV	1Y	B	4477577	S16/P0
	95	R	6	3	NT	I	D	NT	5	C	1477577	S5/P4
	96	L	5	4	4	NT	NT	V	5	D	1477577	S17/P9
	97	R	6	3	3	I	D	NT	7	C	5477577	S0/P4
	98	J	5	5	5	E	NT	AA	7	B	1477577	S28/P5
	99	L	5	4	NT	C	NT	AA	6	B	5477577	S12/P13
	100	S	7	1,4	2,4	MT	A	AA	7	B	1477777	S27/P3
	101	V	10	1	NT	D	NT	IV	0	A	0056577	S24/P0
	102	T	8	1	NT	D	C	AA	0	D	4456577	S27/P0

NOTE. Typing systems are designated by cited publications; types are defined in respective references, except as noted in text.

* SRT = subribotype; RT = ribotype.

† AA = autoagglutinating; NT = nontypable; MT = multitypable.

‡ GY = greenish-yellow; Y = yellow.

§ Profiles generated by the API Rapid NFT bacterial identification kit (see text).

Table 3. Comparison of number and concordance of types among outbreak-associated and sporadic isolates by evaluated *Pseudomonas cepacia* typing systems.

Method [reference]	Total no. of types	No. of outbreak types*	Within outbreak concordance (%)†		Unrelated isolate concordance (%)‡
			Mean	Range	
Subribotype [28]	23	6	76	28–100	22
Ribotype [28]	11	5	97	86–100	33
Serotypes					
[18]	17	6	89	75–100	22
[19]	10	5	86	69–100	22
[20]	6	3	78	61–93	44
[16]	10	5	50	29–69	44
Biotypes					
[26]	8	5	80	57–100	33
[25]	4	4	79	50–100	44
API profile	21	5	56	29–100	33
Bacteriocin type [22]	25	7	52	29–74	11

* Outbreak type (for each outbreak) = the most frequent type.
† Concordance (for each outbreak) = (frequency of outbreak type/no. of outbreak isolates tested) × 100.
‡ Concordance (for the unrelated isolates) = (frequency of the most frequent type/no. of unrelated isolates tested) × 100.

relatedness with the same typing methods, this study establishes the utility of recently proposed *P. cepacia* typing systems in confirming outbreak associations.

Chromosome analysis—by either the broader categorization of ribotype or the narrower classification of subribotype—was a specific and sensitive typing procedure. By ribotype, only two of the 92 outbreak-related isolates were different from their respective outbreak types, and the epidemiologic determination of outbreak relatedness may be questionable for both isolates because they were also discrepant by the other typing methods. Subribotyping distinguished more outbreak types and had less concordance for the unrelated isolates than ribotyping but was markedly less specific. A significant impediment to epidemiologic application of this powerful technique is the specialized equipment and expertise required.

The serotyping systems also proved to be sensitive and specific. The O,H typing system of Heidt et al. [18] matched subribotype on the measures of sensitivity and was intermediate between subribotype and ribotype on the measure of specificity; the system of Nakamura et al. [19] distinguished one fewer outbreak types and had nearly the same mean concordance within outbreaks by O typing only. While a serotype difference would be less convincing evidence for strain dissimilarity than a ribotype difference, the availability of serotyping facilities may be greater, since sophisticated equipment and training are not required.

Although the two biotyping macromethods of Richard et al. [26] and Esanu and Schubert [25] were somewhat less sensitive and specific than chromosome analysis or serotyping, the component tests are widely available. Without *P. cepacia*-specific tests, biotyping may be achieved by many laboratories. API profiles, on the other hand, were too variable to be useful for epidemiologic typing.

Bacteriocin typing was the most sensitive method for detecting differences between groups of isolates or unrelated isolates. However, bacteriocin susceptibility and production varied substantially among outbreak-related strains.

Antimicrobial susceptibility by disk diffusion was extremely variable among outbreak-related isolates, and it does not appear to be a useful typing method for epidemiologic purposes.

The reservoirs, modes of transmission, and host factors predisposing to infection are still being defined for *P. cepacia*. The sensitive and specific typing techniques demonstrated in this investigation will facilitate epidemiologic study of this increasingly important nosocomial pathogen.

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Alginate and Antibiotics

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The relationship between alginate biosynthesis and antibiotic susceptibility in mucoid *Pseudomonas aeruginosa* (MPA) has been the subject of debate since the pioneering studies of Doggett et al. [1] on the association of MPA and pulmonary colonisation in patients with cystic fibrosis (CF) and early pharmacologic studies in CF patients [2]. From these studies it was concluded that antibiotic therapy might provoke the emergence of MPA; Doggett et al. [1] commented, however, that since an increased incidence of *P. aeruginosa* is usually associated with long-term chemotherapy in chronic illnesses, similar frequencies of MPA would be expected in chronic diseases other than CF if they emerged as a result of antibiotic therapy. The first direct evidence of reduced antibiotic susceptibility in MPA [3] concerned a 1.5 times increase in the minimum inhibitory concentration (MIC) of carbenicillin, flucloxacillin and tobramycin in MPA compared to isogenic nonmucoid strains. In addition to any clinical significance, these observations led to the development of a technique for the isolation of MPA in vitro based on enhanced resistance to carbenicillin and to evidence that MPA, phenotypically similar to clinical isolates, can arise from nonmucoid *P. aeruginosa* (NMPA) at a frequency of one in 10^7 and that the frequency can be increased by mutagenesis. Similarly, alginate-producing mutants were isolated from strains of *Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas mendocina*, species not previously known to produce alginate [4]. These early studies indicated that the various genes for alginate biosynthesis were probably present in all strains of *P. aeruginosa* and certain other species of *Pseudomonas*. Subsequently, the use of the carbenicillin selection technique to isolate mucoid variants from *P. aeruginosa* PAO strains carrying the conjugative plasmids FP2 and R68.45 led to the first demonstration of

transfer of the mucoid phenotype into NMPA and evidence for a cluster of *muc* mutations between 69 and 75 min on the PAO chromosome [5–7]. Based on contemporary evidence it appeared that pre-existing alginate-producing variants could be isolated from most strains of *P. aeruginosa* and, consequently, that mutation rather than induction was responsible for alginate biosynthesis. Later studies indicated that some mucoid variants isolated in vitro or from CF patients were medium dependent with respect to mucoid phenotype, indicating that alginate biosynthesis could be induced by culture conditions [6, 8] and that antibiotics per se could not only be used to select mucoid variants but also be directly responsible for alginate biosynthesis. Deretic et al. [9] reported that gene amplification in the chromosomal *alg*-22 region of the nonmucoid *rec*-2 *P. aeruginosa* PAO2003 upon growth on kanamycin-containing medium led to a stable mucoid phenotype; the influence of antibiotics was further illustrated when it was shown that nonmucoid revertants occurred at low frequency on tetracycline-containing media. The mechanism by which tetracycline contributes to the emergence of revertants is not known; possibilities include a direct effect of the antibiotic on protein synthesis or the enhanced susceptibility of MPA to tetracycline [3]. Although in vitro studies have provided valuable insight into the conservation of alginate biosynthetic genes in the genus *Pseudomonas*, the significance of antibiotics for alginate biosynthesis in vivo is less clear. The question remains whether the reduced susceptibility of MPA allows selection of pre-existing mutants in the CF lung or whether antibiotics at sublethal concentrations in the lung trigger a genetic or physiological response leading to alginate production or suppression. Attempts to generalise on the relationship between alginate and antibiotics are difficult. MPA maintained under laboratory conditions do not demonstrate amplification of the *alg*-22 region [9]. In contrast to alginate induction by kanamycin in *P. aeruginosa* PAO2003, Morris and Brown [10] have recently reported that alginate production in two clinical isolates of MPA was slightly depressed by subinhibitory concentrations of kanamycin and depressed by 60% in the presence of tobramycin; no effect on alginate production was observed with beta-lactams or ciprofloxacin.

It should be emphasised that: (1) Prior to these studies the only in vitro method for isolation of MPA was through the agency of phage [11, 12]; indeed Martin [11] considered that phages were essential for maintenance of the mucoid phenotype through a process resembling pseudolysogeny. (2) The methodology employed to assess antibiotic susceptibility is important.

Firstly, reduced susceptibility is most striking when antipseudomonal activity is relatively low (as with carbenicillin) and the bacterial inoculum and survivors are measured quantitatively as colony-forming units rather than by semiquantitative assays. Secondly, reduced susceptibility of MPA in terms of MIC is only in the order of 50%; this degree of resistance is normally considered to be insignificant in standard susceptibility testing and is not detectable by techniques relying on simple doubling increments of antibiotic concentration. (3) Reduced susceptibility in the presence of alginate biosynthesis is observed in several different classes of antibiotic including beta-lactams and aminoglycosides; in contrast, MPA are more susceptible to tetracyclines than isogenic NMPA [3].

P. aeruginosa is characteristically associated with intrinsic resistance to many antimicrobial agents and this property is generally held responsible for the emergence of the species as a significant opportunistic pathogen. In any discussion of alginate and antibiotics we also need to consider the properties and incidence of isolates exhibiting another unusual phenotype involving hypersusceptibility to a range of antibiotics. Hypersusceptible *P. aeruginosa* were first described by May and Ingold [13] who reported that isolates from sputum were often considerably more susceptible to carbenicillin than isolates from other sources, the MIC of carbenicillin for hypersusceptible strains being < 6 mg/l compared to a general range of 25–50 mg/l. Later studies [14, 15] expanded the range of antibiotics involved in hypersusceptibility to include other beta-lactams, trimethoprim, tetracycline and nalidixic acid; aminoglycosides, however, are not associated with hypersusceptibility. The degree of enhanced susceptibility is most evident with carbenicillin and becomes less significant as the antipseudomonal activity of the antibiotic increases; with ciprofloxacin, hypersusceptible variants are only 2.5-fold more susceptible than normal isolates. Although alginate biosynthesis and hypersusceptibility are frequently present in the same isolate, the two chromosomal mutations (*bls* and *tps*) which encode hypersusceptibility [15] are distinct from those involved in alginate biosynthesis. Hypersusceptibility is almost entirely confined to respiratory isolates of *P. aeruginosa* [14] and, together with alginate biosynthesis, must confer significant advantages for bacterial growth and survival in the respiratory environment. The relevance of hypersusceptibility to the association of alginate and antibiotic susceptibility is significant. Firstly, since the *bls* and *tps* mutations confer significantly more to antibiotic susceptibility than alginate biosynthesis, the presence of the two properties in the same isolate results in an MPA paradoxically more susceptible to many antibiotics than typical NMPA.

Failure to appreciate the independence of these two phenotypes has led to misinterpretation in some studies [16] and contradictory conclusions in the literature. Secondly, strains expressing hypersusceptibility are not uncommon and may be encountered in the majority of respiratory isolates of *P. aeruginosa* [8]; the percentage of variants within an individual sputum varies but can account for 100% of the *P. aeruginosa* population. Finally, and perhaps most puzzling of all, the proportion of hypersusceptible variants within an individual patient does not invariably decrease during antimicrobial therapy but can increase.

Whilst the decreased susceptibility of MPA to certain antibiotics can be demonstrated in vitro, the question of whether the bacterial alginate reduces the penetration of antibiotics in vivo to their target site within an alginate microcolony [7, 17] and the significance of decreased susceptibility in antipseudomonal therapy merits further discussion. The diffusion of aminoglycosides through agar was reported by Slack and Nichols [18] to be retarded by incorporation of algal alginate and by a crude exopolysaccharide preparation from an MPA; the effect was not observed with beta-lactams. It was concluded that the negatively charged polymers might act as ion exchangers in retarding the diffusion of positively charged aminoglycosides to a greater extent than the negatively charged, or neutral, beta-lactams. Subsequently, Tannenbaum et al. [16] observed that only positively charged aminoglycosides exhibit marked binding to *Pseudomonas* alginate and that binding could be reversed in the presence of physiological levels of Na^+ and Cl^+. In a later and more detailed study, Nichols et al. [19] reported the inhibition of (^{3}H)tobramycin diffusion by binding to algal alginate and in a similar manner in terms of tobramycin concentration and maximum saturation to alginate preparations from two MPA isolates. However, the use of physical models to study diffusion into alginate microcolonies or biofilms and to deduce the time taken for a bacterium at the centre of a spherical microcolony or biofilm to be exposed to 90% of the bathing concentration outside the sphere, indicated that binding of the aminoglycoside to the glycocalyx is not a major mechanism of antibiotic resistance within a microcolony or biofilm. Nichols et al. [19] speculated that alternative mechanisms to account for the resistance of MPA could be that bacterial cells in the outer layers of a microcolony adsorb enough antibiotic to increase the penetration time for cells deeper in the matrix; alternatively, cells within the alginate matrix might differ physiologically and have different antimicrobial susceptibility from free-growing bacteria [20]; supporting this hypothesis is the observation that MPA in the CF lung grow under iron-restricted conditions [21]. It is also

relevant that aminoglycosides at subinhibitory concentrations are reported to inhibit the production of iron-chelating siderophores [10] similar to the suppression of *Pseudomonas* proteases by ceftazidime and ciprofloxacin [7, 22, 23] which is strain dependent and can be independent of MIC values [7, 23].

Recently Gordon et al. [24] reported that both algal and *Pseudomonas* alginate behaved similarly with respect to their binding characteristics and showed a strong affinity for aminoglycosides but not for beta-lactams. Binding occurred when the alginate was present either as a solution or as a calcium gel but elimination of binding by physiological levels of salts, as previously reported [16], could not be reproduced. When the diffusion of aminoglycosides or beta-lactams was examined, precipitation of the aminoglycosides occurred after prolonged exposure; no such effect was observed with neutral agar preparations or with beta-lactams. Transport rates of tobramycin through *Pseudomonas* alginate were considerably slower than those for gentamicin and the control without gel. Although no ionic binding effect has been reported, diffusion rates for beta-lactams are at least twice as fast through *Pseudomonas* alginate as through commercial algal alginates emphasising the possible influence of a different monomer block structure.

From in vitro evidence it appears that significant factors controlling the barrier effect of alginate for aminoglycosides include the ratio of antibiotic to alginate which dictates whether the antibiotic can saturate the electrolyte binding sites and ultimately allow the antibiotic through the gel to the target bacterium; this factor was originally suggested by Costerton [25] and supported by evidence [24] in which aminoglycoside transfer through thin alginate gels consists of a lag period prior to a rapid rise in aminoglycoside levels in the receptor compartment. Gordon et al. [24] observed that exposure of alginate to aminoglycoside and subsequent precipitation results in modification of the gel structure and concluded that improved penetration of aminoglycosides as a consequence of gel disruption is likely to be more significant than the problem of impaired diffusion.

Irrespective of the mechanism by which alginate impairs the susceptibility of *P. aeruginosa* to aminoglycosides or beta-lactam antibiotics in vitro, we ultimately have to assess the significance of reduced susceptibility for antipseudomonal therapy in the lungs of a CF patient. Factors relevant to such assessment include the size of the bacterial microcolonies in vivo (which from microscopy and incorporating considerable dehydration vary from <10 to >60 μm diameter), the uronic acid block structure of the alginate, the amount of alginate produced in vivo by the colonising MPA

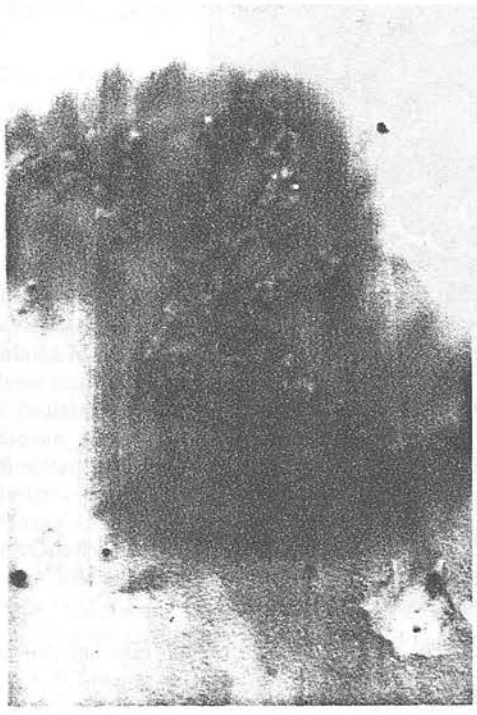

Fig. 1. Electron micrograph of mucoid, alginate-producing *P. aeruginosa* illustrating adherence of purified porcine gastric mucin to the alginate matrix. Mucin spread in monolayer of benzyldimethylalkylammonium chloride, stained with uranyl acetate and shadowed with platinum.

(which from in vitro evidence is dependent on the nature of the alginate mutation involved) and the ionic content of the alginate matrix. Realistically, we also have to take account of the interplay of alginate with host factors including gel formation in the presence of Ca^{2+} ions and enhanced viscosity in the presence of CF bronchial secretions [James, personal commun.], the binding of alginate to mucin (fig. 1), the administration of antibiotic via the airways in the form of aerosols or intravenously by transfusion across the mucosal barrier, and, finally and arguably of greatest significance, the pathological consequences of the host inflammatory response which results in plugging of the major airways (fig. 2), abscess formation and pulmonary consolidation.

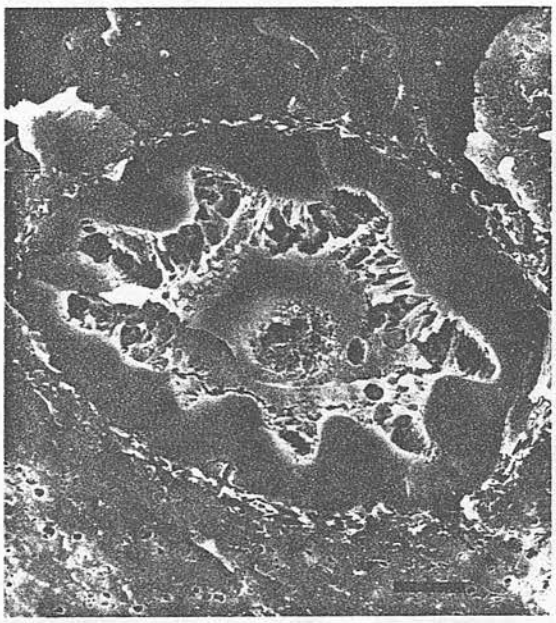

Fig. 2. Plugged bronchial airway with inflammatory infiltrate and surrounding consolidated lung tissue arising from chronic colonisation with mucoid *P. aeruginosa.* Bar = 50μm.

It could be argued that whatever the actual mechanism, a 50% increase in resistance to antipseudomonal agents might be a significant ecological survival factor for MPA in vivo based on poor pulmonary penetration and the difficulty in achieving bactericidal or bacteriostatic concentrations of antibiotics in the bronchial tree [26]. Therapeutic strategies based on acceptance of this problem might include consideration of the enhanced susceptibility of MPA to tetracycline [3] or more appropriate agents with enhanced activity in the mildly acidic environment of the alginate matrix, or the demonstration that EDTA enhances the antipseudomonal activity of antibiotics in vitro [27] and in vivo might disrupt microcolony formation by chelation of Ca^{2+} ions. In conclusion and on the basis of existing evidence, it seems reasonable to conclude that the contribution of pulmonary pharmacokinetics and the patient's inflammatory response far outweigh the contribution of alginate in preventing the penetration of antibiotics to their bacterial target.

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Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: mutations in the *muc* loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli

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Summary

Increased levels of alginate biosynthesis cause mucoidy in *Pseudomonas aeruginosa*, a virulence factor of particular importance in cystic fibrosis. The *algR* gene product, which controls transcription of a key alginate biosynthetic gene, *algD*, is homologous to the activator members of the two-component, environmentally responsive systems (NtrC, OmpR, PhoB, ArcA, etc). In this report, we show that mutations in the *muc* loci, (*muc-2, muc-22*, and *muc-23*, in the standard genetic *P. aeruginosa* strain PAO, as well as a mapped *muc* allele in an isolate from a cystic fibrosis patient) affect transcription of *algD* and *algR*. This influence was strongly dependent on environmental factors. Regulation by nitrogen was observed in all strains examined, but the absolute transcriptional levels, determining the mucoid or non-mucoid status, were strain (*muc* allele)-dependent. Increased concentrations of NaCl in the medium, an osmolyte which is elevated in cystic fibrosis lung secretions, resulted in an increased *algD* transcription and mucoid phenotype in a *muc*-2 strain; the same conditions, however, produced a nonmucoid phenotype in the *muc*-23 background and abolished *algD* transcription. Mutations in the *muc* loci may cause mucoidy by deregulating the normal response of the alginate system to environmental stimuli.

Introduction

A major sequela of cystic fibrosis (CF) disease is an almost inevitable colonization of airways with *Pseudomonas aeruginosa* (Govan, 1988). In the lungs of CF patients, *P. aeruginosa* characteristically assumes a mucoid phenotype caused by hyperproduction of the exopolysaccharide alginate. The protective alginate coating is considered as an important virulence determinant and a major factor contributing to the intractability of *P. aeruginosa* infections in CF (Govan, 1988). Alginate is a negatively charged linear copolymer of D-mannuronic and L-guluronic acids and its synthesis depends on a cluster of biosynthetic genes mapping at 34 min (Deretic *et al.*, 1987a). Transcription of a representative gene from this group of loci, the *algD* gene encoding GDPmannose dehydrogenase (Deretic *et al.*, 1987b), is dependent on the two tightly linked regulatory loci, *algQ* (Deretic and Konyecsni, 1989) and *algR* (Deretic *et al*, 1989a), mapping at the 9-min region of the recalibrated *P. aeruginosa* chromosomal map (Fig. 1) (Darzins and Chakrabarty, 1984). The predicted primary structure of the *algR* gene product (AlgR) shows that AlgR is homologous to the gene products of a number of transcriptional regulators such as *spo0A*, *phoB*, *ompR*, *sfrA*, *virG*, and *ntrC* (Deretic *et al.*, 1989a). These genes control important cellular functions in response to environmental stimuli, and share homologous *N*-terminal domains (Ronson *et al.*, 1987). Thus, it is assumed that the normal levels of alginate biosynthesis are under environmental control and play some as yet undefined physiological or adaptive role.

Alginate-overproducing strains can occasionally be isolated from infection sites other than those of CF, as well as under laboratory conditions (Fyfe and Govan, 1980). These strains show a mucoid colony morphology indistinguishable from that of CF isolates. Several groups have demonstrated that mutations mapping near the *cys-59* marker at 69 min can confer mucoid character in conjugal bacterial crosses (Fig. 1) (Fyfe and Govan, 1980; 1983; MacGeorge *et al.*, 1986). These loci have been designated *muc* by Fyfe and Govan (1980). Recent gene-replacement experiments using a region termed *algST* (Flynn and Ohman, 1988), mapping in the same general area as *muc*, further support the notion that mutations in this region are required for alginate overproduction. Here we have examined how mutations in *muc* affect transcription of the *algD* and *algR* genes.

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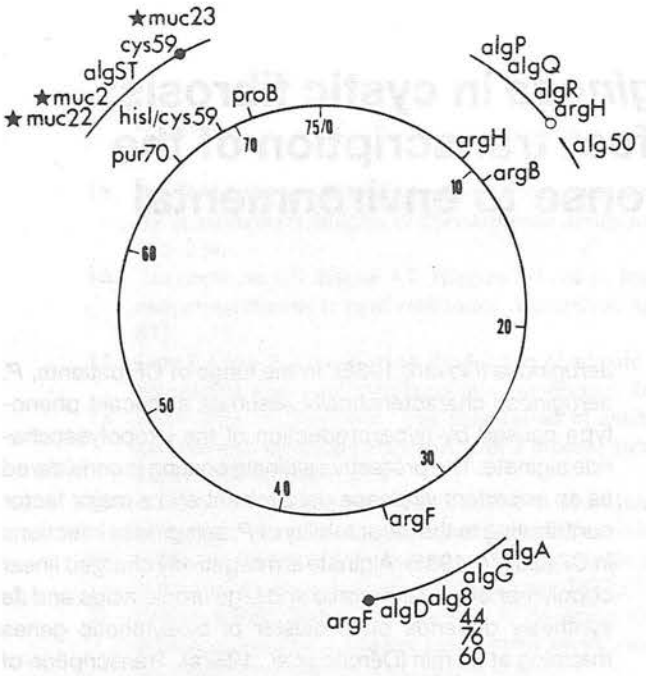

Fig. 1. Genetic map of alginate genes and markers affecting mucoidy in *P. aeruginosa*. The chromosomal map is expressed in minutes and the auxotrophic markers showing linkage to the *alg* and *muc* markers are displayed at positions according to the recalibrated *P. aeruginosa* chromosomal map (O'Hoy and Krishnapillai, 1987). Arcs are expanded regions. Circles indicate positions of auxotrophic markers. Filled symbols indicate that the orientation of the loci has been determined relative to the chromosomal map. The open circle indicates that the orientation of the cluster is not known. The *muc* loci map on both sides of the *cys-59* marker. *muc-25* (PAO581 see Table 1) is not shown; it maps on the same side as *muc-2* and *muc-22* (Fyfe and Govan, 1980). The *algD* gene encodes GDPmannose dehydrogenase and undergoes strong transcriptional activation in mucoid cells (Deretic *et al.*, 1987b). The *algR* gene is a transcriptional activator of *algD*. Two other genes linked to *algR* (*algQ* and *algP*; Deretic and Konyecsni, 1989; Deretic *et al.*, 1989b) are required for *algD* transcription. The *algP, algQ*, and *algR* genes map in a region which is capable of inducing mucoidy when tandemly amplified (Deretic *et al.*, 1986). The *alg-50* marker is reported to map in the proximity of the *algQ-algR* region based on its linkage to *argB* (Goldberg and Ohman, 1987).

Results

Mutations in the muc *loci result in the increased transcription of* algD *and* algR

Mutations in several sites within the chromosomal region surrounding the *cys-59* marker (*muc* and *algST*) have been inferred as the mechanism for the emergence of mucoid *P. aeruginosa* strains in CF lungs (Fyfe and Govan, 1980; 1983; Flynn and Ohman, 1988). A separate line of research has indicated that both *algD* and *algR* (Deretic and Konyecsni, 1989) have to be transcriptionally activated in order for cells to become mucoid. A link between these two events, however, has never been established.

In order to assay *algD* transcription in *P. aeruginosa* derivatives with mutations in the *muc* region, we used triparental bacterial conjugation to introduce an *algD-xylE* transcriptional fusion (plasmid pPAOM3; Konyecsni and Deretic, 1988) into *P. aeruginosa* strains containing different *muc* alleles linked to the *cys-59* marker (Fyfe and Govan, 1980; 1983; Table 2). pPAOM3 was also introduced into a spontaneous nonmucoid revertant (PAO552) of one of the *muc* strains (PAO579). Unlike its mucoid parent, PAO552 is no longer capable of transferring mucoidy in conjugal crosses and is assumed to have lost the *muc* determinant. The levels of the *algD* promoter activity in individual isolates were determined by assaying the reporter gene (*xylE*) activity. As shown in Table 1, all *muc* determinants (Fig. 1) mapping between the *cys-59* and *proB* markers (*muc-23*) and the *cys-59* and *pur-70* markers (*muc-2*, *muc-22*) caused increased expression from the *algD* promoter when cells were grown on *Pseudomonas* isolation agar plates known to enhance alginate synthesis in mucoid *P. aeruginosa*. These results correlated well with our earlier studies using several mucoid *P. aeruginosa* isolates from CF patients and their nonmucoid derivatives. For example, CF201 and CF202, harbouring pPAOM3, showed a typical specific activity of 30–40 U of catechol 2,3-dioxygenase (CDO) (the *xylE* gene product) in mucoid cells and 0.6–1.2 U in nonmucoid cells per mg of protein in whole-cell extracts (Konyecsni and Deretic, 1988).

To further confirm that mutations in the *muc* loci were responsible for the induction of *algD* expression we used S1 nuclease analysis to assess the levels of *algD* transcription in strain PAO381 (nonmucoid parental strain) and its *muc-23* derivative, PAO579 (Fyfe and Govan, 1980; 1983). This also served an additional purpose because we

Table 1. Transcriptional fusion analysis of *algD* expression in *muc* mutants.

Strain	Phenotype/genotype[a]	CDO[b] (U mg^{-1})
PAO579	Alg$^+$ (*muc-23*)	24.6
PAO552	Alg$^-$ PAO579 revertant	0.8
PAO568	Alg$^+$ (*muc-2*)	18.2
PAO578	Alg$^+$ (*muc-22*)	21.0
PAO581	Alg$^+$ (*muc-25*)	36.0
CF201	Alg$^+$ clinical isolate	38.7
CF202	Alg$^-$ CF201 revertant	0.4

a. PAOM3 contains a 1.2 kb *Hin*dIII-*Eco*RI fragment with the *algD* promoter from a PAO2003 derivative, PAOM3 (Konyecsni and Deretic, 1988). This broad host-range plasmid was introduced into *P. aeruginosa* by triparental matings. Strains were grown on PIA plates supplemented with carbenicillin for 20 h at 37°C.

b. The specific activity of catechol 2,3-dioxygenase (CDO) is determined as described in the *Experimental procedures* and expressed in units (U) per mg of total protein in crude extracts. One unit of CDO is defined as the amount of enzyme that oxidizes one micromole of catechol per minute at 24°C.

could not introduce the *algD-xylE* fusion into PAO381. We therefore measured the chromosomal *algD* gene activity in this strain by analysing the abundance of the *algD* mRNA. Using total cellular RNA prepared from PAO381 (*muc*$^+$) and PAO579 (*muc-23*), S1 nuclease protection analysis showed that *algD* was transcriptionally activated in the presence of the *muc-23* allele (Fig. 2A). We have previously observed that another gene, *algR*, undergoes transcriptional activation in mucoid CF isolates (Deretic and Konyecsni, 1989). Using the S1 analysis, we determined the relative activity of the *algR* promoter in *muc*$^+$ (PAO381) and *muc-23* (PAO579) backgrounds. The transcription of the *algR* gene was significantly increased in strain PAO579 (*muc-23*) (Fig. 2B), indicating that this mutation affected not only *algD* but also *algR* expression.

In order to determine whether mutations at the *muc* loci are relevant to the appearance of mucoid *P. aeruginosa* in CF patients, we analysed the effect of a *muc* allele present in a *P. aeruginosa* isolate (492c) obtained from a CF patient. This mutation has been shown to map between *cys-59* and *pur-70*, and is similar to *muc-2* and *muc-22* (Fyfe and Govan, 1983). The 492c isolate displayed patterns of *algD* and *algR* transcription identical to those observed in the *muc* PAO mutants (Fig. 2). Thus, it appears that mutations in the *muc* loci result in increased *algD* and *algR* transcription.

algD *and* algR *promoters are responsive to nitrogen limitation*

The *algR* promoter has been recently mapped using S1 nuclease, reverse transcription and deletion analyses (Deretic *et al.*, 1989b). When the *algD* and *algR* promoters are aligned they show a significant level of similarity (46%) in the first 40 nucleotides upstream of the mRNA start site. In addition, both promoters contain putative σ^{54} (*ntrA*) utilized sequences which are, however, shifted somewhat upstream from the usual −12 and −24 positions in the model examples of *ntr* promoters. To assess the possibility of whether *algD* and *algR* promoters are responsive to nitrogen regulation, we examined their activities on media containing different nitrogen sources. The results of these

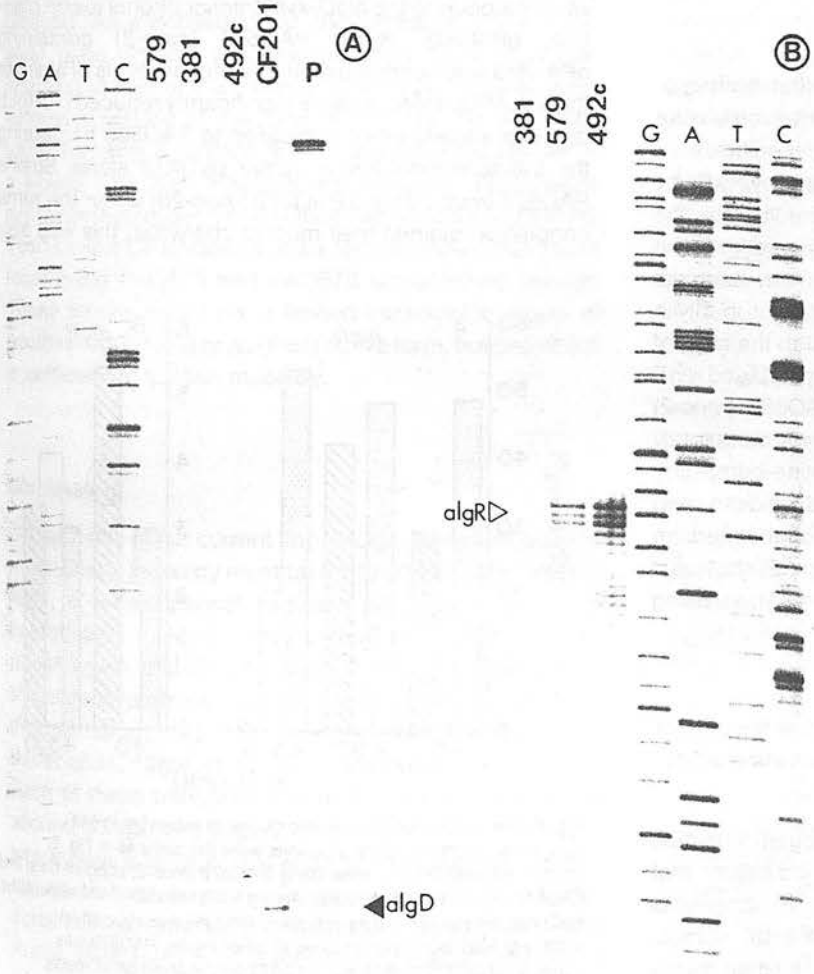

Fig. 2. S1 nuclease analysis of *algD* (A) and *algR* (B) transcription in a *muc* background.
A. The *algD* and *algR* gene transcription levels were determined in strains PAO381 (*muc*$^-$), PAO579 (*muc-23*) and a CF isolate 492c having a *muc* allele mapping on the same side as *muc-2*, *muc-22*, and *muc-25* (see Fig. 1). S1 nuclease analysis was performed using uniformly labelled, single-stranded probes and oligonucleotides specific for the *algR* and *algD* sequences (see the *Experimental procedures*). The products of S1 nuclease reaction were separated on 7% polyacylamide, 8 M urea, next to the sequencing ladder (GATC) produced with the same oligonucleotides and templates used for making hybridization probes. P represents the *algD* probe used for S1 analysis. Arrowheads indicate the positions of the protected portions of *algD* (filled symbols) and *algR* (open symbols) probes (also mRNA start sites) after hybridization with 50 μg of total cellular RNA and digestion with S1 nuclease.

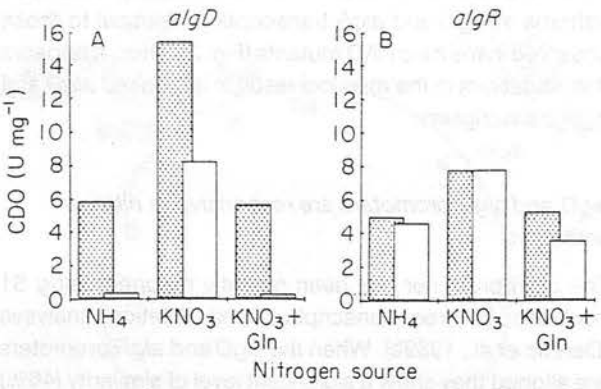

Fig. 3. Responsiveness of the *algD* (A) and *algR* (B) promoters to nitrogen limitation. Strains PAO568 (*muc-2*; open bars) and PAO578 (*muc-22*; stippled bars) harbouring plasmids pPAOM3 (an *algD-xylE* transcriptional fusion) (A), or pP,1 (an *algR-xylE* transcriptional fusion) (B), were grown on minimal medium with the indicated nitrogen sources (ammonia, potassium nitrate, or potassium nitrate plus glutamine). Cells were harvested and the *xylE* gene product, catechol 2,3-deoxygenase (CDO) activity, was determined. Specific activity is defined in Table 1. PAO578 cells were mucoid on nitrate after 24 h and were capable of accumulating alginate only after prolonged incubation on other media. PAO568 was nonmucoid on ammonia and potassium nitrate + glutamine-supplemented media and mucoid on the medium with potassium nitrate only.

experiments, shown in Fig. 3, indicated that both promoters (in particular *algD*) were activated when cells were transferred from an ammonia-containing medium to a nitrate-based medium. This effect could be reversed by transferring cells from the nitrate-only medium to the nitrate plus glutamine medium. This indicated that the nitrogen availability, which is detected in many bacterial species (Magasanik and Neidhardt, 1987) and probably in *Pseudomonas* (Janssen *et al.*, 1981) through the ratio of glutamine to 2-ketoglutarate, was affecting *algD* and *algR* promoter activity. Interestingly, strain PAO568 (*muc*-2) was particularly responsive: this strain was completely nonmucoid on ammonia- or nitrate-glutamine-containing medium but mucoid on the nitrate-based medium, and *algD* transcription was increased 22-fold on the medium containing nitrate only. Strain PAO578 (*muc-22*) showed a less profound response, with *algD* transcription being increased slightly more than two-fold.

Different mutations in the muc *loci determine the type of response of* algD *and* algR *promoters to environmental stimuli*

Variability in responses of *algD* to nitrogen limitation observed with different *muc* strains indicated that mutations in different *muc* loci might render *P. aeruginosa* cells differently responsive to environmental stimuli. Another example of this variability was seen when media of different osmolarity were used to grow PAO568, PAO578, and PAO579 strains. The *muc-2* cells (PAO568) were nonmucoid on LB and could be induced to produce enough alginate to become mucoid with an increase in NaCl concentration. This was accompanied by the induction of *algD* transcription and a very modest response of the *algR* promoter (Figs 4A and 4B). However, strain PAO579 (*muc-23*) showed a completely reverse effect. At the levels of NaCl that induced *algD* in the *muc-2* cells, the *muc-23* cells became nonmucoid and *algD* activity was abolished. A third type of response to osmolarity was seen in strain PAO578 (*muc-22*), which was mucoid under all conditions on LB; the *algD* and *algR* promoters showed very little change in this strain. These results indicated that *algD* and *algR* promoters are responsive to environmental signals in mucoid cells but that the magnitude and direction of response are controlled by the type of *muc* mutations present in the particular mucoid strain.

Promoter multicopy inhibition of alginate production

Another potentially significant difference between *muc-2*, *muc-22*, and *muc-23* strains is their colony morphology when harbouring the *algD-xylE* transcriptional fusion plasmid, pPAOM3. Strain PAO568 (*muc-2*) containing pPAOM3 was nonmucoid on PIA and the levels of alginate that could be detected were significantly reduced (10-fold, data not shown) when compared to PAO568 harbouring the transcriptional fusion vector pVDX18 alone. Strains PAO578 (*muc-22*) and PAO579 (*muc-23*) under the same conditions retained their mucoid character; this was also

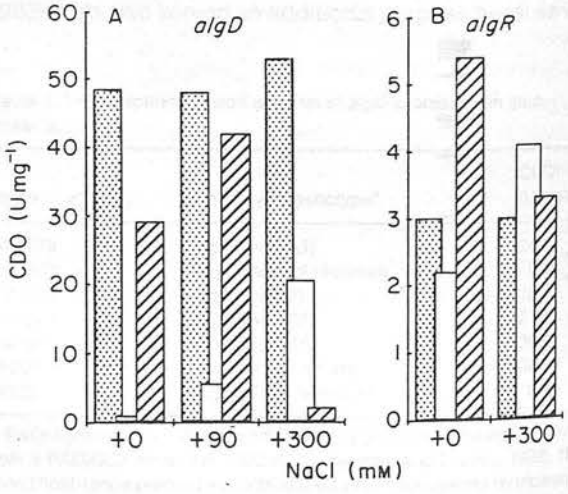

Fig. 4. Differential response of *muc* strains to increases in salt concentration. Transcriptional fusions were the same as in Fig. 3. Strains: PAO568 (*muc-2*; open bars), PAO578 (*muc-2*, stippled bars) and PAO579 (*muc-23*; hatched bars). Added NaCl resulted in the increase of NaCl concentration in LB as indicated. PAO568 was mucoid on +300 mM NaCl but nonmucoid on all other media. PAO579 was nonmucoid on +300 mM NaCl. PAO578 was mucoid on all media.

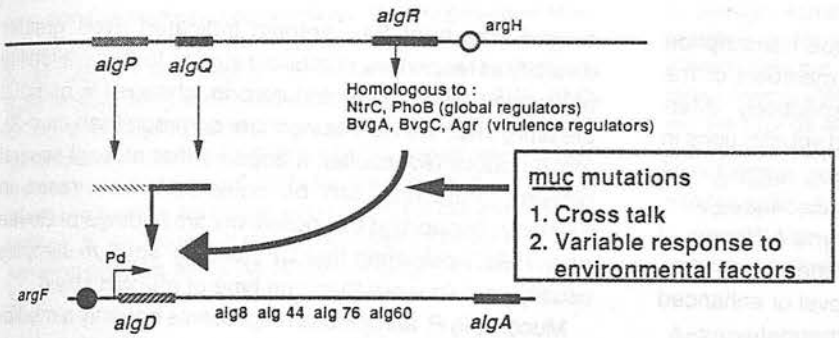

Fig. 5. Model of the regulation of mucoidy. Three closely linked genes, *algP*, *algQ* and *algR*, are required for transcription from P_D, the *algD* promoter. Mutations in the *muc* loci are required for mucoid phenotype. The *algR* gene product is homologous to a number of global transcriptional activators and regulators of virulence in bacteria (Deretic *et al.*, 1989a), which respond to environmental cues. Most of these systems consist of two components: a sensor and a transcriptional regulator (Ronson *et al.*, 1987). It is known (Matsuyama *et al.*, 1986; Ninfa *et al.*, 1988; Iuchi *et al.*, 1989) that the sensory and the regulatory components from different systems can interact in a process called 'cross-talk'. A possibility that mutations in the *muc* loci enhance existing or create novel 'cross-talk' interactions is indicated. This can help explain variable response to environmental conditions (see text) of different *muc* strains. Other possibilities are discussed in the text.

the case with all strains (including PAO568) harbouring pP_r1, the *algR* promoter-*xylE* fusion in pVDX18 (Deretic and Konyecsni, 1989). It is possible that in strain PAO568 the factor(s) *trans*-activating the *algD* promoter are in limited supply and are titrated out by the presence of multiple copies of the *algD* promoter sequences on the plasmid. As a consequence, the functional copy of the *algD* gene on the chromosome may be less active, resulting in loss of the mucoid phenotype. Another characteristic feature of PAO568, which can be explained using the same argument, is that it accumulates alginate at a slower rate than PAO578 and PAO579. Phenotypes similar (in this respect) to PAO568 can be observed among fresh clinical CF isolates, but are less abundant than those resembling PAO578 and PAO579 (unpublished results). These strains may have a limited intracellular supply of positive regulator(s) or its (their) active form, but one which is sufficient to sustain mucoidy.

Discussion

On the basis of our current knowledge, models explaining regulation of mucoidy must take into account the fact that AlgR, a transcriptional regulator of alginate genes, is homologous to NtrC, OmpR, PhoB, VirG, ORF2 of the *phoM* region, etc. (Deretic *et al.*, 1989a). The members of this class of proteins regulate important cellular functions (Ronson *et al.*, 1987), including virulence determinants (Miller *et al.*, 1989), in response to environmental stimuli. Each of these transcriptional regulators interacts with a second component (e.g. NtrB, EnvZ, PhoR, VirA, PhoM), which plays a sensory role and transduces environmental signals probably by acting as a protein kinase of transcriptional activators (Ninfa and Magasanik, 1986; Keener and Kustu, 1988; Hess *et al.*, 1988; Igo and Silhavy, 1988). The sensory component for the alginate system has not yet been identified, although a candidate, *algQ*, has been isolated in the vicinity of the *algR* gene (Deretic and Konyecsni, 1989). However, a similar role for *muc* cannot be ruled out at present. Mutations have been isolated in other two-component regulatory systems, which result in the sensory or regulatory components locked in the 'On' position. This causes constitutive expression of the subordinate genes: the *envZ11* allele with a single amino acid substitution (Thr_{247} to Arg_{247}) causes an OmpC-constitutive phenotype (Matsuyama *et al.*, 1986), and an *ntrB* mutant, *glnL2302*, renders promoters controlling nitrogen assimilation genes constitutively activated (Chen *et al.*, 1982). One plausible model states that similar mutations in *muc* could cause constitutive expression of alginate genes resulting in the mucoid character (Deretic *et al.*, 1989b).

A modification of this model (Fig. 5), which could help explain the diversity in responses of different *muc* strains to environmental conditions, takes into account a process called 'cross-talk'. Based on the homologies among the *N*-terminal domains of the regulators and the *C*-terminal domains of the sensors (Ronson *et al.*, 1987), as well as on the conservation of biochemistry of the phosphorylation process of signal transmission (Hess *et al.*, 1988; Weiss and Magasanik, 1988; Igo and Silhavy, 1988; Ninfa *et al.*, 1988) it has been postulated that regulators can interact with heterologous sensor-kinase proteins (Ninfa *et al.*, 1988). ArcA (alternative symbols Dye and SfrA) can interact with two different sensors, CpxA and ArcB, and control F-pilus synthesis and anaerobic repression of certain aerobic operons in *Escherichia coli* (Iuchi *et al.*, 1989). NtrB can phosphorylate CheY, while CheA will react with NtrC; the former reaction is also the basis for the *glnL2302* ($NtrB^{const}$)-dependent repair of a smooth-swimming phenotype of a *cheA* mutant (Ninfa *et al.*, 1988).

The *envZ11* allele is inducing not only *ompC* transcription but also expression of *lamB* and *phoA*, members of the maltose and phosphate regulons, respectively (Matsuyama *et al.*, 1986). Similarly, amino acid substitutions in the *degU* and *degS* (sensor and regulator, respectively) genes result in the SacU(Hy) phenotype in *Bacillus subtilis*, which overproduces a number of exoenzymes (Henner *et al.*, 1988). It is therefore possible that, like these examples, at least some *muc* mutations result in novel or enhanced 'cross-talk' interactions.

Several alternative, but not mutually exclusive, explanations are also possible. For example, completely opposite responses to osmotic pressure changes of strains PAO568 and PAO579 could be due to the changes in *algD* and *algR* promoter superhelicity, which uncouple transcription initiation from its requirements for ancillary factors. Osmotic pressure changes increase *algD* activity in the *muc-2* cells (see Berry *et al.*, 1989 for similar effects) while the same changes abolish *algD* transcription in the *muc-23* strain (a complete reversal of *muc-2*). The *muc* mutations mapping at the two opposite sides of *cys-59* may be affecting enzymes regulating DNA superhelicity (topoisomerase and gyrase) and altering *algD* and *algR* superhelical density changes in response to increased salt concentration (Higgins *et al.*, 1988). However, the proposed models must await precise information about different *muc* genes and alleles.

The diversity of *muc* mutations underscores a possibility that there are different ways in which *P. aeruginosa* can become mucoid. It has been shown that strains isolated from CF pat ents display a variability of alginate production in response to environmental conditions closely matching different mapped *muc* strains (Fyfe and Govan, 1980; Govan *et al.*, 1983). A recent survey using over 50 clinical isolates from Edinburgh and a smaller sample size from San Antonio indicated even greater diversity of responses of mucoid strains to the availability of nitrogen, phosphate, and carbon, changes in osmotic pressure (*muc-23* type being more common than *muc-2*), etc. (unpublished results). It appears that at least several different mutations can be selected which result in mucoidy. Supporting this possibility are findings of Govan *et al.* (1983) indicating that CF patients' sputum samples usually contain more than one type of mucoid strain.

Mucoidy in *P. aeruginosa* respresents not only a medically important system for studying the control of virulence determinants but may also become a model for studying 'cross-talk' interactions, and other regulatory mechanisms (e.g. phenotypic instability, which is characteristic of mucoid *P. aeruginosa* (Govan, 1988), virulent-to-avirulent phase variation in *Bordetella pertussis* (Miller *et al.*, 1989), and the metastable *phoM*-dependent *phoA* expression in *E. coli* (Wanner *et al.*, 1988)) which involve two-component regulatory systems. Future studies with isolated *muc* genes may help our understanding of these phenomena, and ultimately contribute to better treatments of infections afflicting CF patients.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2. Strains with the known, previously mapped *muc* mutations (Fyfe and Govan, 1980), were verified for their ability to transfer *muc* alleles, by means of FP2 conjugal crosses with PAO540 (FP2$^-$ *cys-5605, his-5075, argA171*) as the recipient and selecting for the *his*$^+$ exconjugants. All strains showed coinheritance of the *muc* alleles with the *his* marker, with frequencies similar to those published previously (Fyfe and Govan, 1980). LB was used to grow *E. coli* and *P. aeruginosa* in liquid culture. For transcriptional

Table 2. Bacterial strains and plasmids.

Strain or plasmid	Relevant markers	Reference
PAO381	*leu-38*, FP2$^-$	Fyfe and Govan (1980)
PAO568	*leu-38, muc-2*, FP2$^-$	Fyfe and Govan (1980)
PAO578	*leu-38, muc-22*, FP2$^-$	Fyfe and Govan (1980)
PAO579	*leu-38, muc-23*, FP2$^-$	Fyfe and Govan (1980)
PAO581	*leu-38, muc-25*, FP2$^-$	Fyfe and Govan (1980)
PAO552	*leu-38*, FP2$^-$, Alg$^-$ PAO579 revertant	Govan (1988)
CF201	Alg$^+$ clinical isolate	Konyecsni and Deretic (1988)
CF202	Alg$^-$ CF201 revertant	Konyecsni and Deretic (1988)
492c	*muc-492c* clinical isolate	Fyfe and Govan (1983)
E. coli		
JM83	Δ*lac-proAB*, Φ80dlacZΔM15	Vieira and Messing (1982)
Plasmids		
pPAOM3	Cb^R, IncQ/P4, *mob*$^+$, *tra*, *algD::XylE*	Konyecsni and Deretic (1988)
pP$_r$1	Cb^R, IncQ/P4, *mob*$^+$, *tra*, *algR::XylE*	Deretic and Konyecsni (1989)
pRK2013	Km^R, ColE1, *mob*, *tra*$^+$ (RK2)	Figurski and Helinski (1979)

fusion studies, cells were grown on solid media containing 1.5% agar. Minimal medium used contained: 100 mM potassium phosphate buffer pH 7.4, 10 mM potassium sulphate, trace elements (2 μM $FeSO_4$, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 8 μM Na_2MoO_4 and 5 μM $MnCl_2$), 1% glucose as the carbon source, and 0.2% KNO_3 or 0.2% $(NH_4)_2SO_4$ as the nitrogen source. When required, glutamine was added to 0.2%. Minimal media were supplemented with 1 mM amino acids for the growth of auxotrophs. When appropriate, antibiotics were used at the following concentrations: ampicillin (40 μg ml^{-1}), carbenicillin (300 μg ml^{-1}), and kanamycin (25 μg ml^{-1}). All cultures were grown aerobically at 37°C.

DNA methods, RNA manipulations and S1 nuclease analysis

Plasmid and phage techniques were performed according to the previously described procedures (Maniatis *et al.*, Konyecsni and Deretic, 1988; Deretic *et al.*, 1989a). Total cellular RNA was prepared by centrifugation in CsCl as previously described (Deretic *et al.*, 1987b). S1 nuclease analysis was performed with single-stranded DNA probes uniformly labelled with ^{32}P. Probes were prepared on templates from the clones of *algD* and *algR* genes in M13 bacteriophage. The *algD*- and *algR*-specific oligonucleotides D1 and R1, respectively, were used as the primers (Deretic and Konyecsni, 1989). Probe-labelling and purification, hybridization with RNA, and S1 nuclease reaction were performed according to the previously published procedures (Deretic *et al.*, 1987c). S1 nuclease products were run on the sequencing gels (7.5% acrylamide, 7 M urea, 100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3) along with the sequencing ladder produced using the same templates and oligonucleotide primers as those used for preparing the S1 nuclease probes.

Conjugal plasmid transfer

Plasmid transfers from *E. coli* to *P. aeruginosa* were performed by triparental filter matings. Overnight cultures of *P. aeruginosa* recipient strains, *E. coli*-carrying transcriptional fusions on pVDX18, a mobilizable broad host-range plasmid, and a separate culture of *E. coli* harbouring the helper plasmid pRK2013 were centrifuged and resuspended in the initial culture volume of LB. 0.3 ml of each culture was used and mixed in 5 ml fresh LB medium, and passed through a 0.45-μm membrane filter. Filters were placed on LB with bacterial filtrates facing air. After overnight incubation at 37°C, bacterial outgrowths were transferred with an inoculating loop onto PIA plates supplemented with 300 μg ml^{-1} of carbenicillin. Plates were incubated for 36 h and colonies sprayed with 100 mM catechol in 50 mM potassium phosphate buffer. Exconjugants harbouring fusions developed a bright yellow colour and were selected for further studies.

Transcriptional fusion and alginate assays

The activity of CDO, the *xylE* gene product, was determined in sonic cell-free extracts from bacterial cultures scraped from the plates after overnight growth. Cells were resuspended in 5 ml of 50 mM potassium phosphate buffer, pH 7.5, 10% acetone, and sonicated for 30 s on ice. Extracts were centrifuged at 11 000 r.p.m. and the supernatants were used to determine protein concentrations following the method of Bradford (1976). Volumes of extracts containing 5 μg of protein were assayed in 3 ml reactions containing 50 mM potassium phosphate buffer, pH 7.5, 0.3 mM catechol, at room temperature. Reaction rates were determined in a Shimadzu UV-160 spectrophotometer by following the change of absorbance at 375 nm. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per min at room temperature. The molar extinction coefficient of 2-hydroxymuconic semialdehyde, the product of the reaction, is 4.4×10^4. Alginate was determined according to the previously published procedure (Knutson and Jeanes, 1968).

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4 Characteristics of mucoid *Pseudomonas aeruginosa in vitro* and *in vivo*

J. R. W. GOVAN

4.1 DISCOVERY AND OCCURRENCE OF MUCOID *P. AERUGINOSA*

Pseudomonas aeruginosa isolates usually appear as non-mucoid colonies when cultured on agar media, but respiratory isolates from patients with cystic fibrosis (CF) characteristically produce mucoid colonies (Fig. 4.1). It is important at this point to make the distinction between the exopolysaccharide produced by mucoid *P. aeruginosa* and slime, which is a loosely defined material with variable composition depending upon the strain and cultural conditions. The distinction is crucial for an understanding of the nature of mucoid *P. aeruginosa* and has not always been appreciated even by those attempting to clarify terminology. Several different morphological types of colony are produced by *P. aeruginosa* and have been described in detail by Phillips (1969). Classic non-mucoid strains, Phillips' colonial types 1–4, produce slimy colonies and viscid broth cultures only when incubation is prolonged and in gluconate media or media with a high carbon content, and such slime production is characteristic of all *P. aeruginosa* (Haynes, 1951). The term 'mucoid' is restricted to those strains producing the large watery colonial type 5 of Phillips (1969) within 24 h on common agar-based media and whose mucoid appearance results from the copious production of the polyuronide, alginate. The debilitating pathological sequelae of colonization by mucoid *P. aeruginosa* are a major cause of morbidity and mortality in CF patients (Høiby, 1984; Pier, 1986; Govan, 1988) and the involvement of *P. aeruginosa* alginate in the complex pulmonary pathogenesis results in 'one of the most bizarre and intractable bacterial infections known to modern science' (Deretic *et al.*, 1987a). This chapter reviews the properties of non-mucoid and mucoid *P. aeruginosa in vitro* and discusses their interrelationships with respect to microbial pathogenesis *in vivo* and antibiotic susceptibility.

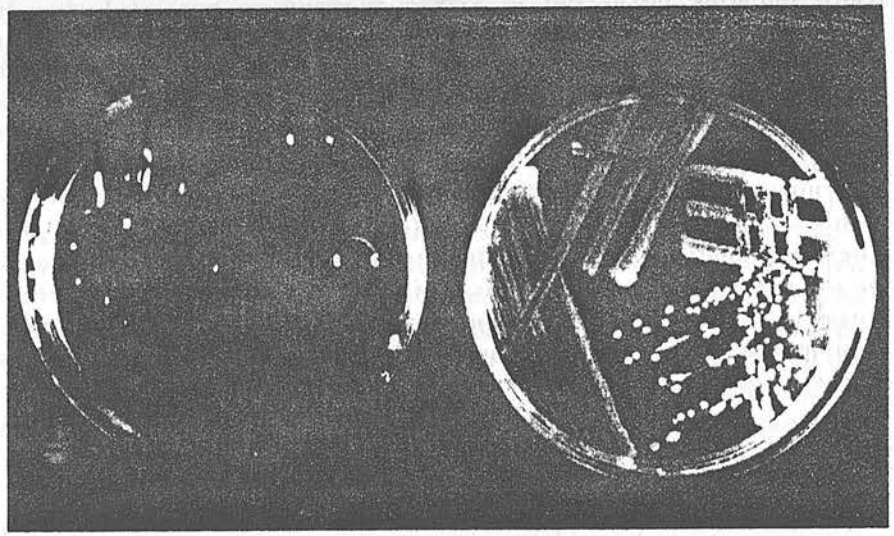

Fig. 4.1 Typical non-mucoid (left) colonial form of *P. aeruginosa* and a mucoid (right), alginate-producing variant grown on *Pseudomonas* isolation agar (Difco) for 48 h at 37°C.

The first description of mucoid *P. aeruginosa* was published by Sonnenschein (1927). Subsequent reports of these pseudomonads were sporadic and superficial (Dahr and Kolb, 1935; Henriksen, 1948; Bullock, 1965). Cetin *et al.* (1965) reported the incidence of mucoid *P. aeruginosa* to be rare (0.8%) in a study of 242 clinical isolates of *P. aeruginosa*. In the first detailed study of mucoid *P. aeruginosa*, Elston and Hoffman (1967) noted a slightly increased incidence of eight mucoid *P. aeruginosa* strains out of 475 clinical isolates (1.7%), and suggested that the increase might be due to a general increase in the isolation of *P. aeruginosa* from compromised patients. Elston and Hoffman submitted the eight mucoid isolates to a wide range of biochemical tests used to characterize *P. aeruginosa* and concluded that mucoid *P. aeruginosa* differed from typical non-mucoid isolates only in morphology. Elston and Hoffman also reported several characteristics of the mucoid isolates which were to become familiar in later studies. These properties included a lack of obvious pigment production by mucoid *P. aeruginosa* on first isolation, as a result of which typical pseudomonad colonial appearance and pigment production cannot be depended upon to identify these bacteria; in addition mucoid *P. aeruginosa* were not even listed in the contemporary edition of Bergey's manual. Elston and Hoffman also suggested that the incidence of mucoid *P. aeruginosa* might be underestimated by at least 50% because of a tendency for isolates to be classified as *Klebsiella*, which mucoid *P. aeruginosa* resembles on blood and MacConkey agar media. Their study also

revealed that mucoid isolates were non-motile, unstable on sub-culture and that motility returned on loss of the mucoid phenotype.

Most of the early isolates of mucoid *P. aeruginosa* reported in the literature had been obtained from sputum or urine. However, the first link between mucoid *P. aeruginosa* and chronic pulmonary colonization in CF patients was reported by Iacocca *et al.* (1963) and by Doggett and colleagues (Doggett, 1969; Doggett *et al.*, 1964, 1966). In a series of studies Doggett and colleagues noted that mucoid *P. aeruginosa* could be isolated in up to 90% of CF patients colonized with *Pseudomonas*, and confirmed that mucoid pseudomonads were rarely isolated from other infections in humans, plants or animals, or from the varied environmental habitat of this ubiquitous organism. In CF patients, primary asymptomatic colonization is typically with non-mucoid *P. aeruginosa*, but later mucoid variants appear. Other studies (Burns and May, 1968; Diaz *et al.*, 1970) revealed that the establishment of mucoid variants was also associated with the appearance of anti-pseudomonal antibodies and increased severity of the patient's pulmonary condition; some patients, however, could harbour mucoid *P. aeruginosa* for many years with no apparent clinical symptoms.

Despite their isolation from a wide range of environmental niches and various tissue sites in infected hosts, strains of *P. aeruginosa* do not typically produce mucoid colonies or readily detectable amounts of alginate. However, in addition to their characteristic isolation from CF patients, it should be noted that mucoid *P. aeruginosa* can be isolated in up to 40% of *Pseudomonas*-positive sputa from non-CF patients with chronic obstructive lung disease and occasionally (up to 10%) from patients with chronic urinary tract infections (Govan, 1988). Moreover, isolates of mucoid *P. aeruginosa* from non-CF patients produce alginate with the same structure as those from CF patients (McAvoy *et al.*, 1989). Despite the large numbers of *P. aeruginosa* isolated from environmental sites, to the author's knowledge no natural niche *in vitro* for mucoid *P. aeruginosa* has been reported. In contrast, accumulated evidence suggests that mucoid variants are so strikingly associated with CF as to be almost diagnostic of the disease (Reynolds *et al.*, 1976); microbiologists involved in studying the bacteriology of the CF respiratory tract could be excused for considering that the mucoid phenotype is the natural state for *P. aeruginosa*. Similarly, alginate is the most convincing virulence factor associated with pulmonary exacerbations due to *P. aeruginosa* in CF patients (Govan and Harris, 1986; Govan, 1988). Thus an understanding of the characteristics of mucoid *P. aeruginosa* and the properties and regulation of alginate are of major importance in understanding the pathogenesis of *P. aeruginosa* in CF patients and in devising therapeutic strategies.

4.2 THE RELATIONSHIP BETWEEN NON-MUCOID AND MUCOID *P. AERUGINOSA IN VIVO*

In the 1970s, individual studies indicated that mucoid *P. aeruginosa* appeared to be derived from the original colonizing strain rather than as *de facto* variants which the patient acquired from an exogenous source. When the two forms were isolated from the same sputum they usually belonged to the same serotype (Diaz *et al.*, 1970) and pyocin type (Williams and Govan, 1973). Zierdt and Williams (1975), however, suggested an alternative hypothesis, namely that 'the mucoid *P. aeruginosa* strain is probably spread from patient to patient, rather than acquiring its mucoid characteristic *de novo* in the CF patient'. Later studies, using more discriminating typing methods, have supported the former hypothesis. In the author's laboratory, a pyocin-typing method revised to accommodate mucoid isolates (Fyfe *et al.*, 1984) has been used to 'fingerprint' multiple colonies of *P. aeruginosa* isolated from individual sputum on a longitudinal basis from 40 CF patients. Our results indicate that cross-infection in the Edinburgh clinics is rare, except between CF siblings; secondly, the majority of CF patients remain colonized with a single strain of *P. aeruginosa* which eventually exhibits the mucoid phenotype and other unusual properties. To date, only nine CF patients have harboured more than one strain, either simultaneously or in separate episodes of colonization. Recently, Ogle *et al.* (1987) reported a similar stability of strain colonization using a new typing system based on Southern blotting and hybridization with a DNA probe derived from the exotoxin A gene and flanking sequences.

In addition to the change to mucoid phenotype, *P. aeruginosa* strains isolated from the individual CF patient are associated with several other unusual phenotypic properties, including sensitivity to killing by serum and loss of O-antigen specificity (Penketh *et al.*, 1983; Hancock *et al.*, 1983), and hypersusceptibility to a range of antimicrobial agents (Govan *et al.*, 1983; Fyfe and Govan, 1984). The heterogeneity of the *P. aeruginosa* population within an individual patient (Fig. 4.2) may also be enhanced by the nature of the mutation responsible for the mucoid phenotype, resulting in alginate with different structures and hence different rheological properties (see Chapter 3). Heterogeneity also extends to non-mucoid revertants which can arise by mutations at several sites in the alginate biosynthetic pathway. Thus, after initial colonization *P. aeruginosa* expresses a considerable degree of phenotypic variation which apparently provides it with a selective advantage in the CF patient.

The time taken for the emergence of mucoid *P. aeruginosa* in individual patients is difficult to measure accurately since conversion is insidious and may occur in the time interval between bacteriological investigations; in

Heterogeneity of Pseudomonas within a single CF patient

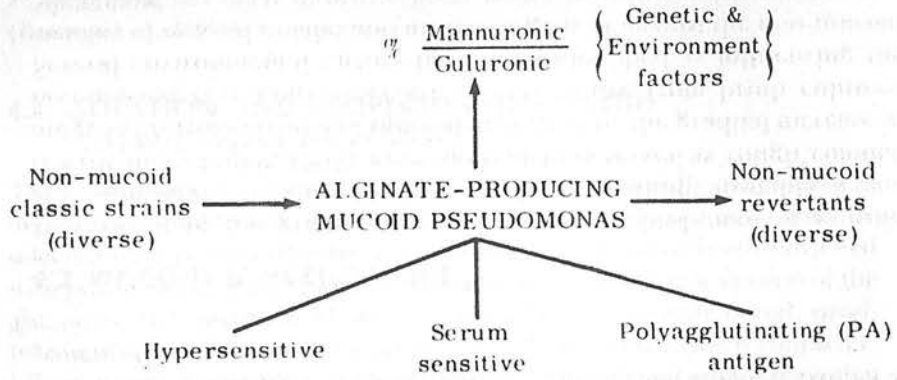

Fig. 4.2 Characteristics of *P. aeruginosa* isolated from cystic fibrosis patients include derepressed alginate biosynthesis, hypersusceptibility to certain antibiotics, serum sensitivity and a polyagglutinating antigen. The rheological properties of the *Pseudomonas* alginates are influenced genetically by chromosomal mutations, which give rise to different percentages and block structures of mannuronic and guluronic acids, and by environmental factors, particularly Ca^{2+} ions. Non-mucoid revertants may also exhibit genetic characteristics distinct from the original colonizing strain. (Reproduced, with permission, from Govan, J. R. W. and Harris, G. (1986) *Microbiol. Sci.* **3** 302–8.)

many patients both non-mucoid and mucoid forms are isolated on initial sputum culture. Recent epidemiological studies have provided direct evidence for transformation of non-mucoid *P. aeruginosa* to the mucoid phenotype *in vivo* and indicate that the time interval between first colonization with a typical non-mucoid strain and the emergence of a mucoid form can be as short as three months. Two CF patients, who had no previous history of *Pseudomonas* colonization, simultaneously became colonized with non-mucoid *P. aeruginosa* whilst using a hydrotherapy pool (Friend and Newsom, 1986). In the author's laboratory pyocin typing suggested that both patients were colonized with the same strain of *P. aeruginosa*, which was also isolated from the pool water together with strains of *P. aeruginosa* belonging to other pyocin types; within three months, a mucoid form of the colonizing strain was isolated from the sputum of one patient (P. A. Friend and J. R. W. Govan, unpublished results). The relationship between these various isolates was subsequently confirmed by DNA probe analysis (M. Vasil, unpublished results). Since initial asymptomatic colonization by non-mucoid *P. aeruginosa* appears to precede subsequent colonization by mucoid variants, the colonizing strain appears to act as a microbial reservoir from which mucoid variants and

the other phenotypes arise (Govan *et al.*, 1984; Govan, 1988). Knowledge of the mechanisms and site(s) of early colonization by non-mucoid *P. aeruginosa* would arguably help to plan strategies to prevent, rather than merely control, pulmonary exacerbations in CF by palliative therapy.

Potentially important clues to the mechanisms of colonization as studied *in vitro* (see Chapter 6 for details) were provided by the observations of Woods *et al.* (1982), who suggested that buccal cells might form a microbial reservoir. First, non-mucoid *P. aeruginosa* adhered significantly better to buccal cells from CF patients than to cells from non-CF patients; secondly, increased bacterial adherence varied directly with loss of the proteinase-sensitive glycoprotein, fibronectin, from the cell surface, as well as increased levels of salivary proteinases in CF patients; thirdly, adhesion of non-mucoid *P. aeruginosa* to non-CF buccal cells could be promoted by prior exposure to trypsin. The promotion of adhesion by trypsin suggested that CF patients might be compromised not only by reduced ability to clear bacteria from the pulmonary airways but also by dietary factors (Govan, 1983; Govan and Harris, 1986). In addition to neutrophil proteinases, a source of the raised respiratory proteinases observed by Woods *et al.* (1982) could be the pancreatic supplements which form an important dietary component for many CF patients. However, from the results of a prospective study *in vivo* in CF patients the significance of buccal colonization as a site of primary colonization remains in doubt (Govan and Harris, 1986). In a related study we also investigated the possibility that colonization of the gastrointestinal tract might act as a reservoir of non-mucoid *P. aeruginosa* in CF, as can occur in *Pseudomonas* infections in non-CF patients. A survey of stool carriage in 54 CF patients suggested that the gastrointestinal tract was not a significant reservoir for non-mucoid *P. aeruginosa* in CF (Agnarsson *et al.*, 1989). Ten patients were known to exhibit chronic pulmonary colonization by mucoid and non-mucoid *P. aeruginosa* prior to the investigation. In eight of these patients non-mucoid and mucoid strains belonging to the same pyocin type were isolated from both stools and sputum. Thus it was not possible to determine whether gut colonization preceded or resulted from colonization of the respiratory tract. Of four patients who were known to exhibit intermittent pulmonary colonization by non-mucoid *P. aeruginosa*, but whose sputum did not contain *P. aeruginosa* at the time of stool collection, three harboured non-mucoid *P. aeruginosa* in their stools. Forty patients had shown no history of respiratory colonization by *P. aeruginosa*; stools from these patients were examined and *P. aeruginosa* (non-mucoid) was isolated from only one specimen. The incidence of stool carriage with non-mucoid *P. aeruginosa* (9.1%) in these 44 patients was near the upper limit of

intestinal *P. aeruginosa* colonization (10%) reported for non-hospitalized individuals (Rhame, 1980) and significantly lower than the 20–54% isolation rate reported for hospitalized patients (Bodey, 1970; Young and Armstrong, 1972). The mechanism of initial colonization by non-mucoid *P. aeruginosa* in CF patients remains obscure (see Chapter 6), but several observations provide potential clues. First, 'the hydrotherapy pool incident' discussed previously provides circumstantial evidence that the colonizing strain might possess colonizing factors absent in the other strains isolated from the water. Similarly, additional epidemiological evidence from the Edinburgh CF clinic indicates that the incidence of unusual pyocin types in *P. aeruginosa* isolates from CF patients (60%) is significantly higher ($p < 0.001$) than the values of approximately 30% observed in non-CF isolates (Tredgett and *et al.*, 1990). Since pyocin typing is based on the production of individual bacteriocins, these results suggest that bacteriocin activity might have a role in colonization *in vivo* (Govan, 1986), or that the chromosomal determinants for particular pyocins are closely linked to those responsible for an unknown colonizing factor, or that a sub-population of *P. aeruginosa* contains appropriate colonizing factors for the CF respiratory tract. The nature of putative colonizing factors in non-mucoid *P. aeruginosa* remains obscure, but potential areas of research for their identification include the ability of non-mucoid strains to attach to tracheobronchial mucin (Ramphal *et al.*, 1987), inactivation of ciliary beat by extracellular products of *P. aeruginosa* and *Haemophilus influenzae* (Wilson *et al.*, 1985) and the role of sialic acid in saliva-mediated aggregation of *P. aeruginosa* isolated from CF patients (Komiyama *et al.*, 1987).

4.3 MUCOID *P. AERUGINOSA*

In contrast to the gradual emergence and establishment of mucoid *P. aeruginosa in vivo*, this phenotype is characteristically unstable *in vitro* (Zierdt and Schmidt, 1964). Reversion can be observed as 'rough' colonial outgrowths from an area of mucoid growth or by the gradual increase in non-mucoid revertants observed on sub-culture from broth cultures. Several environmental factors have been identified as influencing the stability of mucoid isolates and in selecting for or against the non-mucoid phenotype.

On agar media, the incidence of non-mucoid revertants is reduced by the presence of a surfactant, e.g. deoxycholate citrate (Govan, 1975). In broth cultures reversion is reduced by vigorous aeration, and in non-aerated cultures by the addition of surfactants, including phosphatidylcholine, in a dose-dependent manner (Govan, 1975). Fluctuation experiments suggest that instability results from spontaneous non-

mucoid mutants which have a growth-rate advantage in non-aerated cultures (Govan *et al.*, 1979). Subsequent studies by Boyce and Miller (1982b) indicated that oxygen limitation acts as the selective agent for the mucoid phenotype rather than lack of agitation *per se*. In a later study Krieg *et al.* (1986) confirmed that aeration enhances the stability of mucoid *P. aeruginosa* in both batch and continuous culture. In addition, when aeration exceeded 0.5 l/min, the viability of revertants decreased by 50% during a 10-hour period. In contrast, aeration of the mucoid parent strain maintained a totally mucoid population and with no decrease in viability. Aeration of a mixture of non-mucoid and mucoid *P. aeruginosa* resulted in selection of the mucoid form.

Other factors found to enhance reversion to the non-mucoid phenotype include motility (Boyce and Miller, 1982b); this is consistent with the observation of reduced motility and chemotaxis in *P. aeruginosa* isolates from CF patients with advanced colonization (Luzar *et al.*, 1985). Jones *et al.* (1977) reported the enhanced stability of the mucoid phenotype in iron-limited continuous cultures and the tendency of the mucoid form to replace isogenic non-mucoid variants in iron-limited competitive growth experiments. This observation was expanded by Boyce and Miller (1982a) in a study of the effect of cations on the stability of mucoid *P. aeruginosa*, which demonstrated that whilst calcium and magnesium ions had no effect on the growth or stability of mucoid strains the concentration of iron (either Fe^{2+} or Fe^{3+}) had a profound effect on the selection of non-mucoid revertants in unshaken cultures of mucoid *Pseudomonas*. At concentrations of iron greater than 0.01 mM, non-mucoid revertants rapidly accumulated to a greater than 100-fold frequency over the mucoid parent strain. The absolute degree of instability observed varied from experiment to experiment, as would be expected for the selection of a random spontaneously-occurring mutation (Govan, 1975). The exact mechanism for the effect of iron remains obscure. Several possible explanations, including a greater requirement for iron by revertants or greater susceptibility of the mucoid organism to the toxic effects of iron, can be discounted on experimental evidence. The role of iron deprivation as a selective factor for the maintenance of mucoid *P. aeruginosa* is interesting in the light of recent studies on the impact of proteinases on iron uptake by *P. aeruginosa* pyoverdin from transferrin and lactoferrin (Döring *et al.*, 1988). Pyoverdin seems to be the major siderophore of *P. aeruginosa* (Ankenbauer *et al.*, 1985).

Döring *et al.* (1988) observed that pyoverdin was unable to acquire iron from human transferrin or lactoferrin at physiological pH; however, in the presence of *Pseudomonas* elastase, but not polymorphonuclear leucocyte elastase, a rapid release of iron occurred from transferrin but not from lactoferrin. These authors speculated that iron acquisition by

P. aeruginosa might be aggravated by localized infection in the CF lung in which lactoferrin represents the major siderophore and in which *P. aeruginosa* has been shown to grow under iron-restricted conditions (Brown *et al.*, 1984).

4.4 ISOLATION AND PROPERTIES OF MUCOID *P. AERUGINOSA IN VITRO*

The first report of isolation *in vitro* of a mucoid form of *P. aeruginosa* was by Williamson (1956), who observed that mucoid colonies could be selected by repeated transfer of a non-mucoid strain in broth enriched with potassium gluconate and subsequent culture to an agar version of the gluconate-rich medium. However, as has been previously noted, production of a non-alginate 'slime' composed of various hexoses is characteristic of most strains of *P. aeruginosa* (Haynes, 1951) and it seems likely that these 'mucoid' isolates were merely copious slime producers.

Later, during the course of bacteriophage typing, Martin (1973) noted rings of mucoid growth around areas of phage lysis. Sub-culture of these mucoid areas produced pure cultures of mucoid *P. aeruginosa* phenotypically resembling CF isolates. Since phage remained associated with these mucoid cultures, Martin postulated that the variants required the continued presence of phage and that the mucoid phenotype resulted from a form of pseudolysogeny. The association of phage with conversion to the mucoid phenotype *in vivo* remains obscure. Phages have been demonstrated in CF sputa (Tejedor *et al.*, 1982), and phages obtained from mucoid isolates were better able to convert non-mucoid *P. aeruginosa* to the mucoid phenotype than phages from non-mucoid CF isolates (Miller and Rubero, 1984). Govan (1975) confirmed the ability of phage to give rise to mucoid variants, but after suitable washing and sub-culturing to remove contaminating phage the mucoid phenotype was shown to be independent of phage. Thus it appeared that mucoid *P. aeruginosa* arises by selection of pre-existing variants in which the phage receptors are masked by a mucoid surface layer arising from the biosynthesis *de novo* of alginate or alternatively by derepression of alginate biosynthesis to a higher level.

A later study (Govan, 1976) showed a 1.5-fold increase in minimum inhibitory concentration of carbenicillin in mucoid *P. aeruginosa* compared to isogenic non-mucoid strains. This slight difference in susceptibility *in vitro* led to the development of a selection technique for mucoid variants. It also provided evidence that mucoid, alginate-producing mutants, phenotypically resembling both phage-derived and CF isolates, can arise from wild-type parent strains at a frequency of 1 in 10^7 by exposure to carbenicillin or tobramycin at a concentration of 1.5 times the

minimum inhibitory concentration; the frequency can be increased tenfold by mutagenesis with ethylmethanesulphonate (Govan and Fyfe, 1978). In a further series of genetic studies using mucoid variants containing conjugative plasmids, it was shown that the mutations responsible for the mucoid colonial phenotype (*muc*) in a range of phage, antibiotic or clinically derived mucoid isolates were located on the *P. aeruginosa* PAO chromosome in a 'master switch' region located between 66 to 71 min (Fyfe, 1985; Fyfe and Govan, 1980, 1981, 1983; MacGeorge *et al.*, 1986; Govan, 1988). The *muc* mutations are probably localized within the region of the chromosome containing the 'switching' genes *algS* and *algT* (see Fig. 11.2).

The antibiotic-based selection technique was also used to isolate alginate-producing mucoid-colony mutants from *P. fluorescens*, *P. putida* and *P. mendocina*, species not previously known to produce alginate (Govan *et al.*, 1981); low levels of alginate have also been detected in the plant pathogen *P. syringae* (Fett *et al.*, 1986). The reasonable conclusion from these studies is that the genetic capability for alginate biosynthesis is present in probably all strains of *P. aeruginosa* and in certain other closely related *Pseudomonas* species. It is perhaps significant that those species in

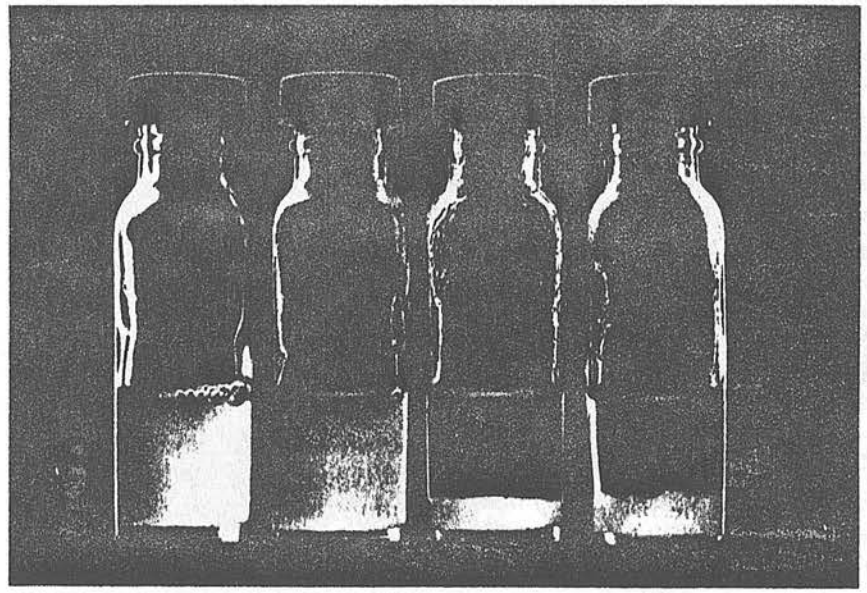

Fig. 4.3 Mucoid *P. aeruginosa* PAO 579 grown in the presence of 0, 3, 7 and 10 mM Ca^{++}. At 3 mM Ca^{++}, the gelatinous aggregates are visible by light microscopy, but at higher concentrations the microgels are visible macroscopically and rapidly sediment.

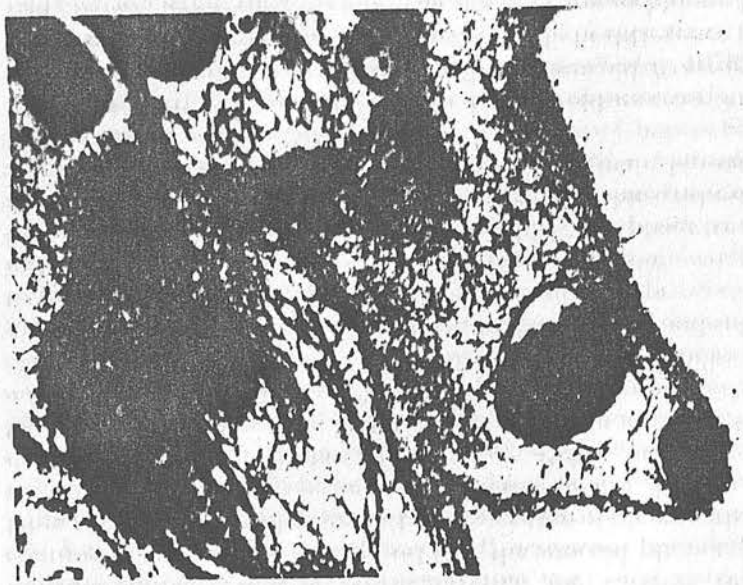

Fig. 4.4 Electron micrograph of microgels formed by mucoid *P. aeruginosa* PAO 579 in the presence of 3 mM Ca^{2+}. Note how the bacteria are enclosed within a cotton-wool-like alginate matrix. Ruthenium red stain, magnification ×45 000. (Reproduced, with permission, from Govan, J. R. W. and Harris, G. (1986) *Microbiol. Sci.* **3**, 302–8.)

which alginate biosynthesis has been demonstrated fall into the same closely linked taxonomic group based on DNA and RNA hybridization (see Chapter 1, and Palleroni *et al.*, 1973).

Culture of mucoid *P. aeruginosa* isolated *in vitro* or from clinical sites has shown that some mucoid variants depend on the culture medium for expression of their mucoid phenotype. Group 1 mucoid *P. aeruginosa* yields mucoid colonies on minimal agar, whereas variants belonging to groups 2 and 3 give rise to non-mucoid colonies unless sub-cultured on more complex media (Fyfe and Govan, 1983; Govan *et al.*, 1983). These observations indicate that the mucoid phenotype can be medium-dependent and inducible; it must be stressed, however, that such induction does not occur in typical wild-type non-mucoid *P. aeruginosa* and requires the presence of *muc* mutations located in a cluster on the *P. aeruginosa* PAO chromosome between 66 and 71 min (Fyfe and Govan, 1983; Fyfe, 1985; MacGeorge *et al.*, 1986).

Culture of mucoid strains on agar-based media containing Ca^{2+} may cause the normally watery and transparent mucoid colony to assume a more compact gelatinous form (Govan and Harris, 1986). The degree of change is strain-dependent and might be explained by differences in the

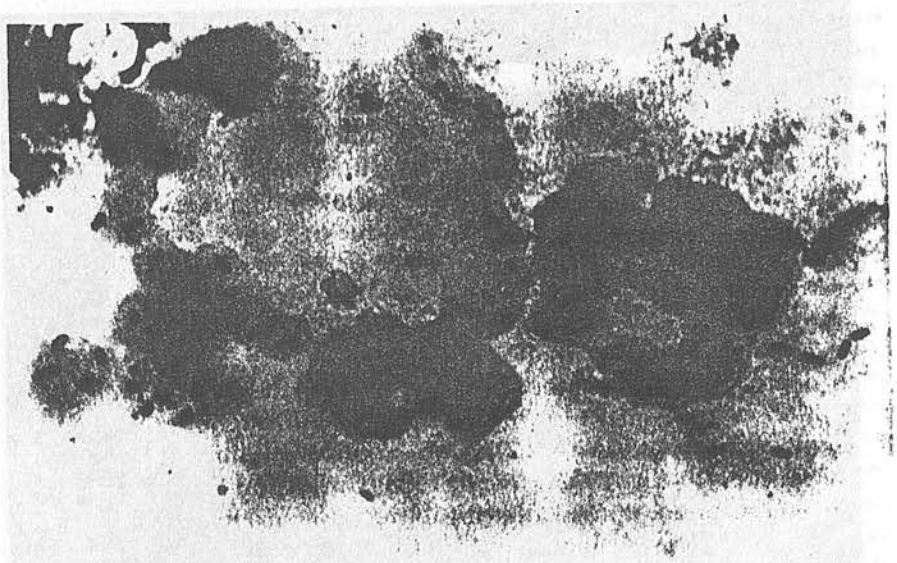

Fig. 4.5 Gram-stained sputum from a cystic fibrosis patient harbouring mucoid *P. aeruginosa*. Non-mucoid *P. aeruginosa* typically appears randomly distributed across the field. In contrast, mucoid *P. aeruginosa* is usually clustered in gelatinous microcolonies and are often attached to respiratory mucosae. Magnification ×2 250.

rheological properties of the alginate which are dependent on the arrangement of the constituent mannuronic and guluronic acids (see Chapter 3). When mucoid strains are cultured *in vitro* and examined by phase-contrast microscopy, the bacterial alginate appears as an extracellular slime. The presence of Ca^{2+} at concentrations as low as 3 mM results in the rapid formation of bacteria-containing microgels which in broth culture rapidly sediment (Govan and Harris, 1986; Fig. 4.3). By electron microscopy, a microgel appears as a cotton-wool-like matrix enclosing bacterial cells (Fig. 4.4). From these observations *in vitro* it could be speculated that the bacterial microcolonies observed in the sputum of CF patients harbouring mucoid *P. aeruginosa* (Fig. 4.5) result from a combination of the adhesive and viscous nature of bacterial polysaccharide and the gelling potential of the polymer in the CF respiratory tract in the presence of Ca^{2+}.

4.5 THE NATURE AND PATHOGENESIS OF MUCOID *P. AERUGINOSA IN VIVO*

One of the most striking observations in the pathogenesis of *P. aeruginosa* is that typical non-mucoid *P. aeruginosa*, which produces a variety of

virulence factors and is associated with a range of localized and systemic infections with significant morbidity and mortality in non-CF patients, is only associated with asymptomatic colonization in the CF lung (Henry *et al.*, 1982). From accumulated evidence (Govan, 1988) it seems reasonable to speculate that the ability of *P. aeruginosa* to colonize and cause significant morbidity in CF patients, and to a lesser degree in patients with chronic obstructive pulmonary disease, is due to a combination of innate and adaptive properties. It is also arguable that alginate biosynthesis and the rheological properties of this unusual bacterial polymer are important factors in understanding the mechanisms of survival and pathogenesis of mucoid *P. aeruginosa* in the CF lung.

In acute pulmonary infections in non-CF patients *P. aeruginosa* is usually observed by microscopy to be dispersed throughout the sputum. In contrast, mucoid strains in CF post-mortem lung (Lam *et al.*, 1980) and in CF sputum (Høiby, 1982; Govan, 1983; Govan and Harris, 1986) are often seen as microcolonies adhering to the bronchial mucosa and which may reach 60 μm in diameter and thus be considerably larger than the surrounding phagocytic cells. The concept of a microcolony or bacterial biofilm mode of growth was originally proposed in a wider environmental context by Costerton *et al.* (1978); in the CF lung the microcolony mode of growth characteristic of mucoid strains provides a plausible model to take account of the various unusual properties associated with *P. aeruginosa* isolates from chronically colonized lungs, the pathogenesis of the pulmonary exacerbations and antibiotic susceptibility *in vivo* (see Chapter 5).

P. aeruginosa proteinases appear to be important virulence factors in CF capable of causing tissue damage directly or indirectly via immune-mediated mechanisms, inactivation of immunoglobulins and α-1 anti-trypsin, suppression of human leucocyte-mediated killing of *P. aeruginosa* and facilitating spread of the infection (Döring *et al.*, 1981; Baker, 1982; Kharazmi *et al.*, 1986). Significantly, *P. aeruginosa* proteinases have also been shown to enhance mucin release from the tracheal epithelium (Klinger *et al.*, 1984). Since production of abnormal mucin is a basic characteristic of CF (Rose *et al.*, 1987) and plays an important role in the pathophysiology of the disease, the action of the bacterial enzyme would exacerbate an existing mechanism of pulmonary damage. Studies *in vitro* indicate that proteinase is released more slowly from mucoid than from isogenic non-mucoid *P. aeruginosa* (Govan and Doherty, 1985). If this slow release also occurs *in vivo*, the alginate microcolony would act as an immunological adjuvant releasing proteinase locally and concentrating tissue damage at the bronchial epithelium (Govan and Harris, 1986). Quantitative assessment of the load of mucoid *P. aeruginosa* in CF sputum can be useful in assessments of anti-pseudomonal therapy in CF patients

(Govan *et al.*, 1987) but in long-established colonization the levels of serum C-reactive protein provide a more reliable index of exacerbation and effective therapy (Glass *et al.*, 1988). From evidence obtained *in vitro* it is possible to speculate that resolution of the pulmonary exacerbation, including a reduction in C-reactive protein to normal levels in the absence of a significant reduction in the numbers of *P. aeruginosa* isolated, is associated with suppression of *Pseudomonas* proteinase by sub-inhibitory concentrations of antibiotics, in particular ceftazidime and ciprofloxacin (Govan and Doherty, 1985; Govan *et al.*, 1987). A recent study has also shown that aminoglycosides, in particular tobramycin, at concentrations that do not affect bacterial growth can inhibit the production of alginate by 60% (Morris and Brown, 1988).

In view of the high phospholipid content of the human respiratory tract, the role of bacterial phospholipases and the influence of pulmonary surfactants on the relationship *in vivo* between non-mucoid and mucoid *P. aeruginosa* seems worth consideration. Granstrom *et al.* (1984) reported that patients colonized with mucoid but not non-mucoid *P. aeruginosa* had high antibody titres against the bacterial proteinases and exotoxin A; in contrast, although 100% of strains of *P. aeruginosa* from patients with CF produced phospholipase C, there was no significant difference in antibody levels against this enzyme between patients colonized with mucoid versus non-mucoid forms, and the titres did not increase with length of colonization. *In vitro*, the biosynthesis of proteinases and exotoxin A, but not phospholipase C, is enhanced during iron-limited growth (Vasil, 1986). It could be speculated that phospholipase C aids initial colonization by non-mucoid *P. aeruginosa*, giving rise to the 'early' phospholipase C antibodies detected by Hollsing *et al.* (1987). Subsequently, during later iron-limited growth associated with chronic colonization by mucoid variants in the CF lung (Brown *et al.*, 1984), biosynthesis of proteinases and exotoxin A would be enhanced. A second consideration, however, concerns the possible influence of lung surfactant as an environmental factor in the emergence and stability of the mucoid phenotype. The characteristic instability of mucoid *P. aeruginosa* has already been emphasized (see sections 4.3 and 4.4). Govan (1975) observed that the stability of mucoid isolates was improved in a dose-dependent manner in the presence of dipalmitoyl-phosphatidylcholine, the major phospholipid in lung surfactant (Brown, 1964). The concentration at which maximum stabilization occurred was approximately that found in human lungs and shown *in vitro* to produce surfactant activity similar to extracts of human lungs (Kuenzig *et al.*, 1965). It is perhaps significant that after pulmonary colonization in CF patients the next highest incidence of mucoid *P. aeruginosa* also occurs in the lungs in patients with chronic obstructive pulmonary disease (Burns and May,

1968) and can comprise 40% of all *P. aeruginosa* isolates from these patients (Govan, 1988). These early studies (Govan, 1975) indicated that stabilization was not simply the result of enhanced inhibition of non-mucoid revertants since non-mucoid forms grew faster than isogenic mucoid variants in competitive growth experiments. The mechanism by which mucoid variants are stabilized in the presence of surfactants might be worthy of renewed investigation as an additional environmental factor to other potential selection mechanisms operating within the CF respiratory tract.

4.6 MUCOID *P. AERUGINOSA* AND ANTIBIOTIC SUSCEPTIBILITY

There have been conflicting reports concerning the effect of the mucoid phenotype on susceptibility of *P. aeruginosa* to antimicrobial agents. In the author's laboratory it was observed that mucoid *P. aeruginosa* was approximately 1.5 times less susceptible than isogenic non-mucoid variants to carbenicillin and tobramycin (Govan, 1976; Govan and Fyfe, 1978). The mechanism for decreased susceptibility remains obscure but is probably not due to a diffusion barrier or binding by alginate, but possibly from antagonism of β-lactams and aminoglycoside antibiotics by the high electrolyte content of the alginate gel (Slack and Nichols, 1981), modification of the alginate gel structure (Gordon *et al.*, 1988), or other biofilm-associated mechanisms (Nichols *et al.*, 1988).

An important phenomenon which explains some of the difficulties in attempting to generalize on the subject of antibiotic susceptibility in mucoid *P. aeruginosa* is the significant incidence of antibiotic hypersusceptibility in respiratory isolates of *P. aeruginosa* and which is frequently associated with the mucoid phenotype (May and Ingold, 1972; Irvin *et al.*, 1981; Govan *et al.*, 1983). The properties and genetics of the *bls* and *tps* mutations responsible for the hypersusceptible phenotype in *P. aeruginosa* have been described (Irvin *et al.*, 1981; Fyfe and Govan, 1984; Govan and Doherty, 1985; Angus *et al.*, 1987). For the purpose of this review the relevance of hypersusceptibility in *P. aeruginosa in vitro* and *in vivo* can be summarized as follows. First, it is well recognized that considerable heterogeneity of antibiotic susceptibility can be found in different isolates from the sputum of one CF patient (Thomassen *et al.*, 1979; Seal *et al.*, 1979; Govan *et al.*, 1987). Hypersusceptibility could be considered as an extreme form of this heterogeneity; hypersusceptibility is independent of the mucoid phenotype and is not associated with colonial morphology. Secondly, contradictory reports on the antibiotic susceptibility of mucoid *P. aeruginosa* can be subject to misinterpretation, because a mucoid isolate with the *bls* mutation is considerably more susceptible to antibiotics than are most normal non-mucoid *P. aeruginosa* despite the mucoid phenotype. Thirdly, the paradoxical emergence and susceptibility of hypersusceptible mucoid *P. aeruginosa* in the face of frequent antibiotic therapy could result from the preferential survival of *P. aeruginosa* growing within an alginate microcolony, whilst the role for *bls in vivo* might be to enhance nutritional uptake within the alginate matrix (Govan, 1988). Evidence obtained *in vitro* also supports the hypothesis that the role for the *bls* mutation *in vivo* is to enhance growth of mucoid variants under nutrient-limited conditions (Govan, 1988). Fourth, although the mutations responsible for hypersusceptibility may appear to confer suicidal properties in the presence of frequent antibiotic therapy, the influence of the microcolony mode and the poor penetration of antibiotics into the infected tissue may have a considerable modifying effect *in vivo*. Finally, the presence of a high proportion of hypersusceptible *P. aeruginosa* in a sputum sample results in a significant reduction of colony-forming units on selective agar media containing antimicrobial agents.

4.7 ALGINATE PRODUCTION *IN VIVO* BY MUCOID *P. AERUGINOSA*

Major advances have been made in our understanding of the association of *P. aeruginosa* and CF, the relationship between mucoid and non-mucoid *P. aeruginosa* and the regulation of alginate biosynthesis (Pitt, 1986; Friend, 1986; Govan, 1988; Deretic *et al.*, 1987a, b, c, 1989; Deretic and Konyecsni, 1989; Flynn and Ohman, 1988a, b; Berry *et al.*, 1988). Recent evidence obtained *in vitro* by the use of NMR spectroscopy on the polysaccharides produced by mucoid and non-mucoid isolates from both CF and non-CF patients indicates that alginate biosynthesis is not restricted to the mucoid colonial phenotype; non-mucoid *P. aeruginosa* and revertants of mucoid isolates were found to produce alginate but at very much lower levels than in mucoid variants (Anastassiou *et al.*, 1987). Similarly, recent studies *in vivo* have demonstrated the presence of apparently protective opsono-phagocytic antibodies specific for alginate in CF patients with no previous history of colonization by mucoid *P. aeruginosa* (Pier *et al.*, 1987). It seems reasonable to conclude from these studies that the mucoid phenotype does not result from acquisition of alginate-biosynthetic genes, but indeed from derepression of a normal regulatory system resulting in a shift from low, barely detectable levels of alginate in non-mucoid strains to high levels in the mucoid phenotype (see Chapters 10 and 11).

The possibility that environmental factors within CF bronchial secretions might induce or switch on alginate biosynthesis has long been an attractive hypothesis, first postulated by Henriksen (1948). In the author's

laboratory, unsuccessful attempts have been made to induce the formation of the mucoid phenotype by repeated sub-culture of labelled non-mucoid *P. aeruginosa* in CF bronchial secretions from which mucoid *P. aeruginosa* had been isolated; similar unsuccessful attempts have been made by many research groups to convert non-mucoid *P. aeruginosa* to the mucoid phenotype by growth in media containing nutrients and electrolytes associated with the CF lung. Ohman and Chakrabarty (1982) found that *P. aeruginosa* growth *in vitro* on sputum-extract medium utilized amino acids and small peptides as major carbon and energy sources. Nevertheless, it could be argued that efforts to induce the mucoid phenotype *in vitro* remain thwarted by basic ignorance of the growth substrates and physiology of *P. aeruginosa* within the CF respiratory tract. Whilst attempts *in vitro* to transform wild-type strains to the mucoid phenotype have been unsuccessful, the induction of mucoid phenotype in strains already containing various *muc* mutations has been demonstrated. Mucoid *P. aeruginosa* belonging to group 2 (Govan *et al.*, 1983; Fyfe and Govan, 1983), represented by *P. aeruginosa* PAO578, does not express the mucoid phenotype on minimal agar but does so on more complex media. The *muc* mutation in PAO 578 (*muc*22) and those responsible for the mucoid phenotype in groups 1 and 3 map between 66 and 71 min on the PAO chromosome, in a region which appears to act as a master switch for alginate biosynthesis. These genes are also referred to as 'switching genes', including *algS* and *algT* (see Fig. 11.2). The *algR* regulatory gene located at 10 min, in association with the closely linked *algQ* gene (Deretic and Konyecsni, 1989), is influenced by *muc* (*algS,T*) mutations at 70 min (Deretic *et al* 1990). It has been proposed that the *algR* gene encodes a positive-acting regulatory protein which mediates alginate production by activation of the *algD* promoter (Deretic *et al.*, 1987c); this in turn leads to production of GDP-mannose dehydrogenase, a key enzyme in alginate biosynthesis (see Chapter 10). Recently, the *algR* gene has been sequenced (Berry *et al.*, 1988; Deretic *et al.*, 1989) and found to have significant sequence homology with several genes known to encode regulatory proteins that respond to environmental stimuli, including nitrogen starvation (*ntrC*), osmolarity (*ompR*), sporulation (*spoOA*) and bacterial chemotaxis (*cheB*). Deretic and Konyecsni (1989) have suggested that *algR* and the recently identified *algQ* may form a two-component regulatory system involved in signal transduction from the environment into transcriptional activation of alginate genes. Berry *et al.* (1988) speculated that environmental factor(s) in the lungs of patients with CF could bind with the *algR* gene product and switch it on in the presence of a flipped switch located at 70 min. From the accumulated evidence it seems reasonable to speculate that alginate biosynthesis *in vivo* and the mucoid phenotype *in vitro* result from the influence of *muc* (*algS,T*) mutations at 70 min superimposed on normal levels of alginate gene regulation (see Chapters 10 and 11 for further discussion).

We have recently investigated cultural conditions responsible for switching on the mucoid phenotype in mucoid PAO mutants and in CF isolates expressing the same medium-dependent mucoid phenotype. With respect to the possible influence of nitrogen compounds on triggering alginate biosynthesis we have observed that group 2 mucoid variants grow as typical rough non-mucoid colonies on minimal agar with ammonium ions as the nitrogen source, but when nitrate is used as the nitrogen source most group 2 variants produce a mucoid phenotype under both aerobic and anaerobic conditions (Fig. 4.6).

Berry *et al.* (1988) investigated osmolarity as a likely contributing factor in triggering alginate biosynthesis in CF patients based on the rationale that one of the prominent features of the CF lung is a high NaCl level (Seymour, 1984). Their hypothesis was supported by the observation *in vitro* that *algD* expression in the mucoid CF isolate 8821 and in the

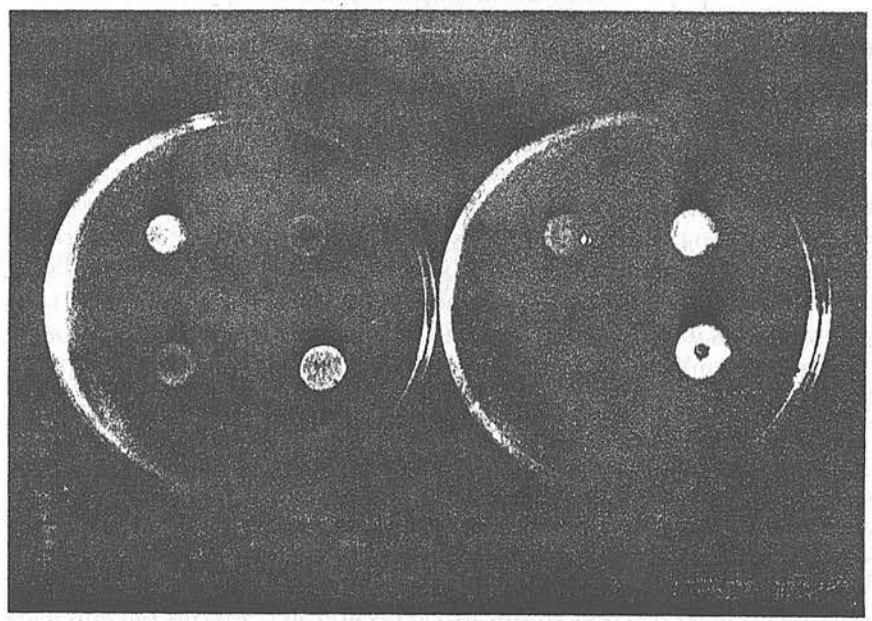

Fig. 4.6 Mucoid *P. aeruginosa* grown on minimal agar for 48 h, incorporating ammonium sulphate (left) or potassium nitrate (right) as the nitrogen source. PAO 579 and the CF isolate 492a (left, top and bottom, respectively) belong to group 1 and produce the mucoid phenotype with both nitrogen sources. In contrast, the group 2 strains PAO 578 and the CF isolate 5188 (right, top and bottom, respectively) produce a non-mucoid phenotype when grown on ammonium sulphate but exhibit the mucoid phenotype when nitrate is used as the nitrogen source.

non-mucoid strain PAO1 increased as the NaCl concentration, and hence osmolarity, of the culture increased. Despite this activation the amount of alginate produced by the phenotypically non-mucoid strain PAO1 increased only slightly under conditions of high osmolarity, suggesting that other factors (possibly *muc* or *algS,T* mutations) are required for full activation of the alginate genes. Recently, we have expanded these observations on the effects of osmolarity using mucoid CF isolates and PAO mutants with different *muc* mutations (Fyfe and Govan, 1983; Govan, 1988) and observed that the effect of NaCl is relatively specific and does not correlate with alginate induction by nitrate. We confirmed the ability of raised NaCl concentrations to increase alginate biosynthesis in PAO 568 (*muc2*) and PAO 578 (*muc22*) and CF492a strains; however, under similar conditions there was reduced alginate biosynthesis in PAO 579 (*muc23*) and PAO 581 (*muc25*) and CF5188 strains (Deretic *et al.*, 1990).

It should be stressed that, whilst the isolation of mucoid *P. aeruginosa* from patients with chronic lung diseases is highly suggestive of CF, mucoid strains are not solely isolated from CF patients nor from the respiratory tract. Thus the influence of NaCl as the sole environmental influence needs to be considered with caution. Nevertheless, the ability of nitrate and NaCl to significantly induce alginate biosynthesis in some mucoid strains of *P. aeruginosa* provides potentially rewarding clues to identify possible environmental factors in the CF lung and, in addition, reveals fascinating parallels with other bacterial systems encoded by environmentally responsive genes (Ronson *et al.*, 1987).

Further studies on the mechanisms by which environmental factors stabilize the mucoid phenotype may also provide useful information. Such studies, however, require an appreciation of the genetic diversity already observed within mucoid *P. aeruginosa* isolated from patients and in mucoid variants of PAO isolated *in vitro* (Govan, 1988; Fyfe and Govan, 1983; MacGeorge *et al.*, 1986; Darzins and Chakrabarty, 1984; Darzins *et al.*, 1985a, b; Goldberg and Ohman, 1987; Deretic *et al.*, 1987a, b, c), as well as the possible presence of other phenotypic properties associated with mucoid *P. aeruginosa* but independent of alginate biosynthesis. For example, non-mucoid revertants resemble typical wild-type non-mucoid *P. aeruginosa* in colonial appearance and might be considered to be similar to the parent strain. Evidence, however, suggests that the revertants can arise from a number of distinct mutations; loss of the mucoid phenotype can arise from repair at the original *muc* locus or from mutations in other regulatory or structural genes. Some revertants, containing conjugative plasmids, are able to transfer the mucoid phenotype to non-mucoid recipients (Fyfe and Govan, 1983); transfer does not occur with wild-type *P. aeruginosa* and suggests the presence of some form of suppression, mediated by a mutation at a site distinct from the *muc* locus. The incidence

of suppressed revertants observed *in vitro* varies in individual mucoid variants of PAO strains from 10 to 70% but remains fairly constant for each mucoid variant. The emergence of suppressed revertants *in vivo* can also be demonstrated in mucoid variants grown *in vivo* (Govan, 1988). The heterogeneity of revertants has significant implications for comparative studies of mucoid and non-mucoid *P. aeruginosa*. Some studies have compared the biological properties of non-mucoid revertants with the mucoid parent strain assuming that revertants are similar to wild-type *P. aeruginosa*. Similarly, when non-mucoid and mucoid isolates from the same sputum are used in comparative studies, it is difficult to determine whether the non-mucoid variant represents the original colonizing *P. aeruginosa* or a non-mucoid revertant. Second, although serum sensitivity, loss of O-antigen specificity and antibiotic hypersusceptibility are independent of the mucoid phenotype, their association with mucoid *P. aeruginosa in vitro* and *in vivo* must be appreciated. For example, in studies of the mechanisms by which environmental factors, including oxygenation, stabilize mucoid variants it was observed that the non-mucoid revertants were more sensitive to the toxic effect of oxygenation than was the mucoid parent strain (Krieg *et al.*, 1986). If, however, the mucoid *P. aeruginosa* strain used in this study also expresses any of the other lipopolysaccharide-associated phenotypes, then the sensitivity of the revertant may reflect the exposure of altered lipopolysaccharide rather than loss of mucoid phenotype *per se*.

In conclusion, the nature of mucoid *P. aeruginosa* presents a fascinating and important microbial challenge: consequently there is a temptation to generalize on the concept of a 'typical mucoid *P. aeruginosa*'. Such generalizations should be resisted, since accumulated evidence suggests that physiological and genetic studies of the nature of mucoid and non-mucoid *P. aeruginosa* using a single representative mucoid isolate or non-mucoid revertant may not entirely reflect the subtleties of alginate biosynthesis *in vitro* or *in vivo*.

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Diagnosis of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis by ELISA for anti-Pseudomonas LPS IgG antibodies

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An enzyme-linked immunosorbant assay (ELISA) with urease enzyme was developed with either a polyvalent pseudomonas smooth lipopolysaccharide (LPS) extract vaccine (PEV-02) or rough LPS (R-LPS) from *P. aeruginosa* rough mutant PAC605. Each ELISA was able to differentiate between sera from cystic fibrosis (CF) patients chronically colonized with *P. aeruginosa* and sera from non-colonized patients. Sera from non-colonized and intermittently colonized CF patients seldom reacted with any of the Pseudomonas LPS, whereas sera from chronically colonized CF patients reacted strongly with most of the sixteen smooth O-serotype vaccine components and with the PAC605 R-LPS, indicating the presence either of a number of different serotype specific IgG antibodies and/or IgG antibodies directed to a common antigenic component of LPS rough core. Absorption studies and immunoblot analysis demonstrated that in sera from CF patients who were chronically colonized with *P. aeruginosa* a significant component of the anti-*P. aeruginosa* antibodies is specific for the core of *P. aeruginosa* LPS and cross reactive with a number of serotypes of *P. aeruginosa* LPS.

Keywords: *Pseudomonas aeruginosa*, ELISA, lipopolysaccharide, anti-LPS antibodies, cystic fibrosis.

Introduction

Pulmonary colonization with *P. aeruginosa* is a major cause of morbidity and mortality in CF patients. Initial asymptomatic and often intermittent colonization of the upper respiratory tract with non-mucoid strains of *P. aeruginosa* precedes chronic colonization with mucoid variants of the original strain. Mucoid *P. aeruginosa* are notoriously intractable to antibiotic therapy and are seldom if ever eradicated despite an apparently competent immune response[1]. Specific antibodies against *P. aeruginosa* antigens, both cellular and extracellular, rise significantly when the infection becomes chronic[2]. Detection of antibodies in some CF patients with early *P. aeruginosa* infection has also been reported[3]. ELISA-based methods to detect an antibody response have proved useful in detecting and monitoring the progress of *P. aeruginosa* infection in patients

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with CF. These ELISA systems used either *P. aeruginosa* whole cells, representing commonly isolated serotypes[4], or a pooled sonicated extract of *P. aeruginosa*[5]. The aim of this study was to produce an ELISA system based on a more defined antigenic preparation and which would be suitable for the detection of an antibody response against all serotypes of *P. aeruginosa*.

We report the development of a rapid and technically simple ELISA with urease-conjugated anti-human IgG antibody for diagnosis of chronic *P. aeruginosa* colonization in CF. The ELISA antigens employed were core R-LPS from rough mutant *P. aeruginosa* PAC605[6] or polyvalent pseudomonas smooth LPS vaccine PEV-02[7] antigens coated to polystyrene microplates.

Materials and methods

Patients

Forty-seven patients with CF attending the paediatric and adult CF clinics in Edinburgh were investigated. Patient's (age range 3–27 years) were classified on the basis of degree of colonization with *P. aeruginosa*: (a) non-colonized patients, i.e. those patients from whom *P. aeruginosa* had never been isolated; (b) intermittently colonized patients, *P. aeruginosa* having been isolated from sputum on one or more occasions but no recognizable pulmonary symptoms attributable to the organism, and (c) chronically colonized patients, *P. aeruginosa* having been isolated continuously from their sputum specimens. Bacteriology was performed on sputum which had been homogenized in sputalysin (Behring). Following appropriate dilutions, *P. aeruginosa* was cultured quantitatively on blood agar and Pseudomonas isolation agar: this procedure allows detection of *P. aeruginosa* at concentrations as low as 10^2 cfu ml^{-1} sputum. Serum obtained from CF patients was stored at − 20°C until analysis. Control serum was also obtained from blood donors at the Blood Transfusion Centre, Edinburgh.

Preparation of LPS

LPS was extracted by the method of Galanos *et al.*[8] as modified by Qureshi *et al.*[9] from washed, freeze-dried bacteria from an overnight culture. The LPS was washed and purified by centrifugation at 100000 **g** for 4 h and freeze-dried.

Diluents and buffers used in ELISA

(1) Coating buffer consisted of 0·05 M carbonate/bicarbonate, pH 9·6.

(2) Post-coat buffer consisted of phosphate-buffered saline pH 7·2 (PBS) containing 5% bovine serum albumin (BSA) (ICN Biomedicals).

(3) Wash buffer consisted of PBS containing 0·05% (v/v) Tween 20.

(4) Dilution buffer consisted of PBS containing 0·05% (v/v) Tween 20, 0·5% BSA and 4% (w/v) polyethylene glycol 6000 (Sigma Chemicals).

All solutions contained 0·02% sodium azide.

ELISA procedure

Polystyrene microplates (Immulon M129A, Dynatech) were coated at 100 µl per well with the following antigens diluted in coating buffer:

(a) polyvalent pseudomonas LPS extract vaccine PEV-02 at 1 μg ml^{-1} (a gift from Dr P. Hambleton, Porton International), which is comprised of cell wall LPS extracts of sixteen international O-serotypes of *P. aeruginosa*, (serotypes Habs 1 to 16) as previously described[7];

(b) the individual vaccine O-serotype components Habs 1–16 used at 1 μg ml^{-1} [10] and

(c) LPS from the rough mutant *P. aeruginosa* PAC605 complexed with polymyxin and dialysed as previously described[11], used at a final concentration of 10 ng ml^{-1}.

Plates were coated overnight at room temperature and washed four times with wash buffer. Plates were then post-coated with post-coat buffer at 100 μl per well overnight at room temperature. After being washed four times with wash buffer, plates were rinsed in distilled water and stored at − 20°C until used.

Sera were diluted 1:200 in dilution buffer and added to coated microplates at 100 μl per well in triplicate. Plates were incubated at 37°C for 90 min before washing four times with wash buffer. Urease-conjugated sheep anti-human-IgG (Commonwealth Serum Laboratories) was diluted 1:500, added at 100 μl per well and plates incubated for a further 90 min at 37°C. Plates were washed four times and rinsed with distilled water before substrate (Sera Lab) at 100 μl per well was added. Plates were incubated for 30 min at room temperature and reactions were stopped by adding 1% (w/v) thimerosal (Sigma) in distilled water (20 μl per well). The optical density (OD) of wells was read at 590 nm on an automated microplate reader (Titertek Multiskan /MC, Flow Laboratories). Final results were expressed after subtraction of the OD of negative controls wells (coated only with BSA post-coat) for each serum.

Absorption of sera with P. aeruginosa *whole cells*

Sera from six CF patients chronically colonized with *P. aeruginosa* were subjected to a series of absorptions with *P. aeruginosa* whole cells. Each serum diluted 1:200 in dilution buffer was added to a pellet of 10^7 harvested and washed bacterial cells. The mixture was resuspended, incubated for 15 min and then recentrifuged. The supernatant was removed and added to another pellet of bacteria and the procedure repeated until antibodies were maximally absorbed (see Fig. 4). Serum IgG anti-pseudomonas LPS antibodies in unabsorbed and absorbed sera were measured by ELISA with either vaccine (PEV-02) or R-LPS from PAC605 as coating antigens.

SDS-PAGE and immunoblotting

The LPS of proteinase K digested whole cell lysates[12] was analysed by electrophoresis using 14% polyacrylamide gels and the buffer system of Laemmli[13]. For immunoblotting LPS was transferred to nitrocellulose membranes (0·2 μm Schleicher and Schuell) by the method of Towbin *et al.*[14]

Results

A range of elevated levels of anti-pseudomonal IgG antibodies directed to both PEV-02 S-LPS and PAC605 R-LPS were demonstrated in sera from twenty patients with CF who were chronically colonized with *P. aeruginosa*. Raised anti-pseudomonal IgG antibody levels were not found in sera from seven intermittently-colonized or twenty non-*P. aeruginosa* colonized CF patients, or in ten healthy adults (non-CF controls)

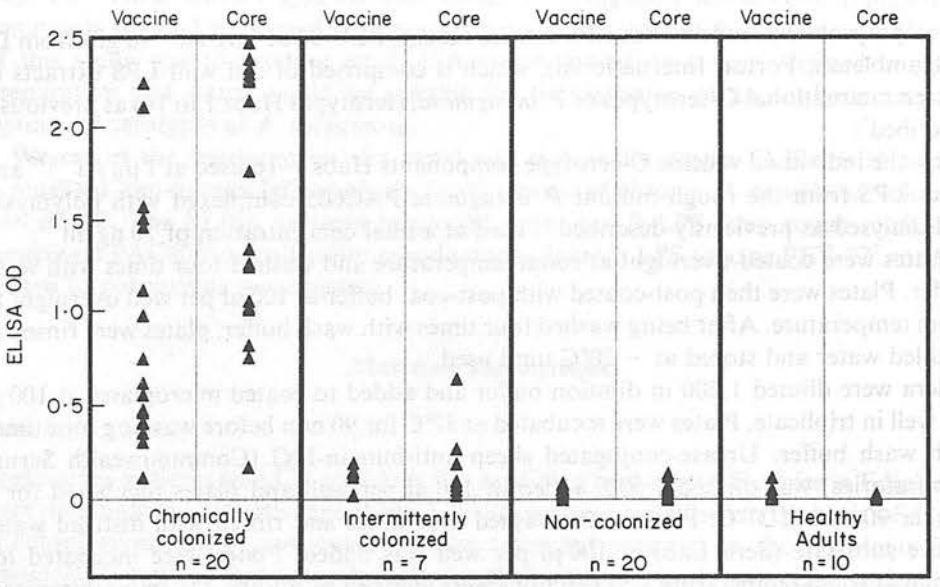

Figure 1. Serum IgG anti-pseudomonas LPS antibodies in 47 CF patients and 10 non-CF individuals measured by ELISA with either heterogeneous polyvalent S-LPS vaccine (PEV-02) or core R-LPS (PAC605) as coating antigens: a total of 20 CF patients chronically colonized with *P. aeruginosa*, 7 intermittently colonized, 20 non-colonized CF patients, and 10 healthy adults (blood donors) are included.

(Fig. 1). The difference in absorbence readings at 590 nm between chronically colonized and intermittently or non-colonized CF patients was highly significant (Chi-squared $P < 0{\cdot}001$). Reproducibility of the assay was checked with 12 serum samples, four from each of the three groups of CF patients, which gave intraplate variation of 4% and day to day variation of 12%.

Serum from patients chronically colonized with *P. aeruginosa* produced a positive reaction with most of the sixteen individual serotype vaccine components. Given the heterogeneity of antibody response, a pronounced antibody response to any one particular serotype was not evident (Fig. 2). In contrast, sera from non-colonized or intermittently-colonized patients produced a barely detectable response.

Western-blot immunoassay of serum from a cystic fibrosis patient with chronic pseudomonas infection against LPS from proteinase-K digests of a number of *P. aeruginosa* strains, including the patients own non-mucoid and mucoid strains, is shown in Fig. 3. A band was obtained against the low molecular weight common core LPS of all the *P. aeruginosa* strains analysed. Bands were also observed with a number of the higher molecular weight O-antigen subunits of the smooth serotype *P. aeruginosa* strains. The reduction in the measurable serum IgG anti-pseudomonas LPS antibody response after previously absorbing sera with PAC605 (incomplete core R-LPS) and PAC608 (complete core R-LPS)[3] (Fig. 4) further indicated the substantial anti-core LPS antibody component in sera from CF patients chronically colonized with *P. aeruginosa*.

Discussion

ELISA based methods for determining antibodies to *P. aeruginosa* have previously proved useful in monitoring both course and treatment of infection in CF[4,5]. The urease

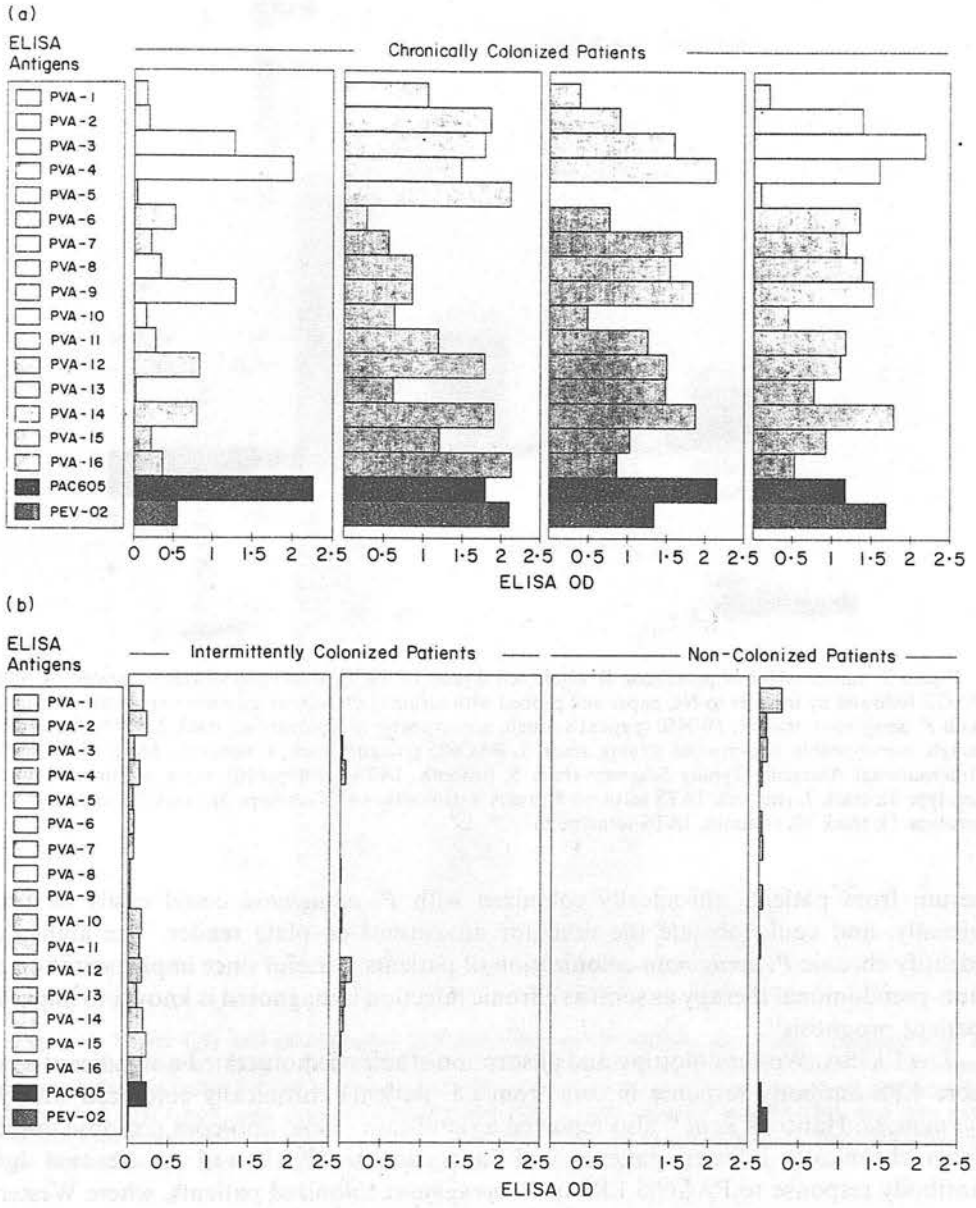

Figure 2. Serum IgG anti-pseudomonas LPS antibodies in eight CF patients which included those (a) chronically and (b) intermittently or non-colonized with *P. aeruginosa* measured by ELISA with individual S-LPS O-serotype vaccine components (PVA-1 to PVA-16), core R-LPS (PAC605) and polyvalent vaccine (PEV-02).

ELISA system which we developed based on either PEV-02 vaccine or the core LPS from *P. aeruginosa* PAC605 was an attempt to refine existing techniques. Our urease ELISA was found to be rapid, reproducible and suitable for routine use, differentiating CF patients chronically colonized with *P. aeruginosa* from intermittently or non-colonized CF patients. The positive colour change of the urease ELISA substrate obtained with

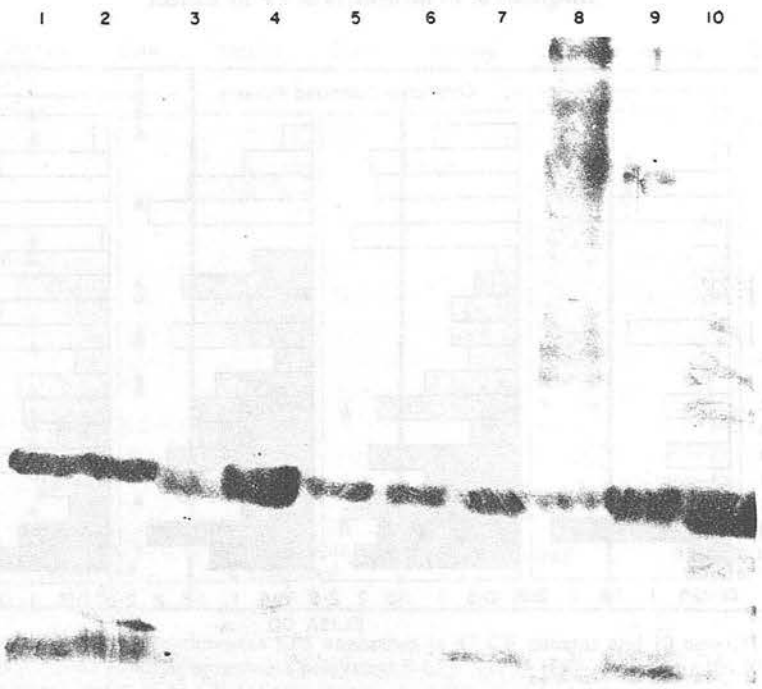

Figure 3. Immunoblot of proteinase K whole cell digests of ten *P. aeruginosa* strains separated by SDS-PAGE followed by transfer to NC paper and probed with serum (1:200) from a patient chronically colonized with *P. aeruginosa*: track 1, JWN50 (patient's rough, non-typeable mucoid strain); track 2, JWN49 (patient's rough, non-typeable non-mucoid strain); track 3, PAC605 (rough); track 4, (smooth, IATS serotype 11) (International Antigenic Typing Scheme); track 5, (smooth, IATS serotype 10); track 6, (smooth, IATS serotype 9); track 7, (smooth, IATS serotype 8); track 8, (smooth, IATS serotype 3); track 9, (smooth, IATS serotype 1); track 10, (smooth, IATS serotype 6).

serum from patients chronically colonized with *P. aeruginosa* could easily be read visually, and could obviate the need for an automated plate reader. The ability to identify chronic *P. aeruginosa* colonization of patients is useful since implementation of anti-pseudomonal therapy as soon as chronic infection is diagnosed is known to improve patient prognosis[15].

The ELISA, Western blotting and absorption studies demonstrated a significant anti-core LPS antibody response in sera from CF patients chronically colonized with *P. aeruginosa*. Hancock *et al.*[16] also reported a significant (74%) anti-core response in sera from chronically infected patients and Jacobson *et al.*[17] showed an elevated IgG antibody response to PAC605 LPS in *P. aeruginosa* colonized patients, where Western blotting demonstrated that sera from most of these patients displayed bands in the rough core LPS region. Strains of *P. aeruginosa* isolated from the sputum of chronically colonized CF patients are frequently rough due to loss of the smooth O-serotype antigen[18]. Exposure of the core antigens coupled with persistent infection may account for the raised anti-core antibodies.

The monovalent cell-wall extract components of the polyvalent *P. aeruginosa* vaccine PEV-02 are predominately LPS[7,19]. Serum anti-pseudomonal IgG antibodies in chronically colonized CF patients, measured using PEV-02 as antigen, may represent (i) antibodies to any of the sixteen O-serotype antigens, (ii) antibodies directed against the common core region of the LPS, or (iii) antibodies to the common A-band LPS of

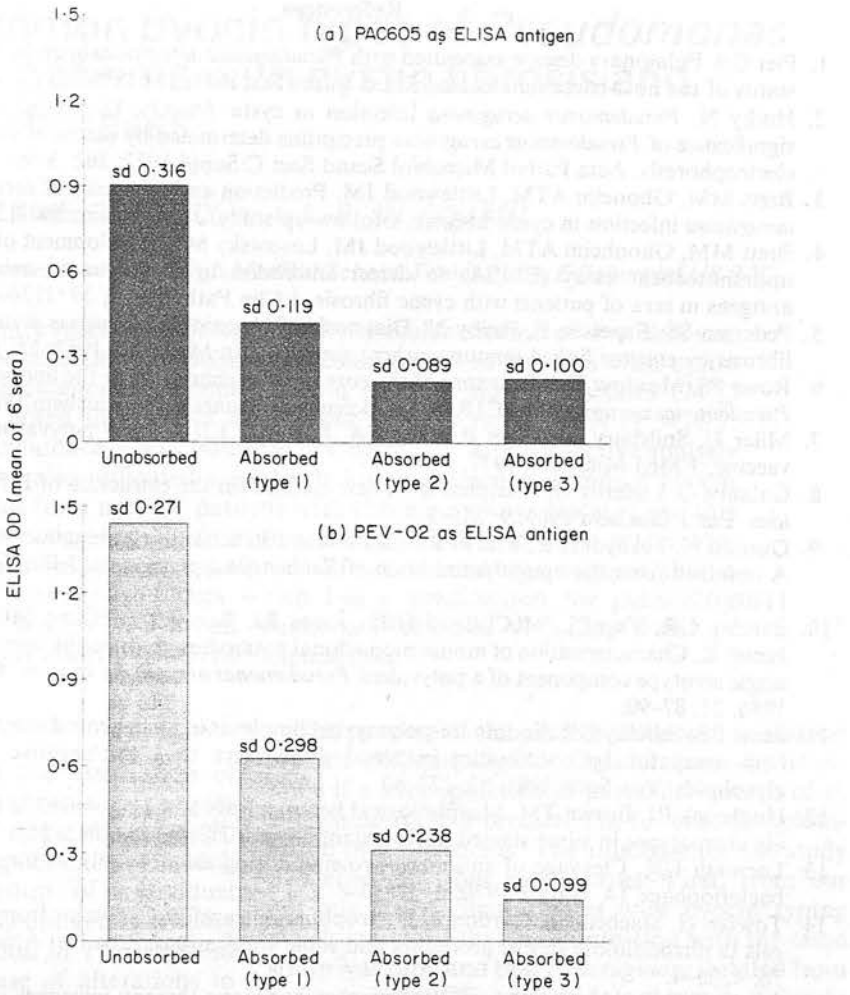

Figure 4. Serum IgG anti-pseudomonas LPS antibodies in CF patients chronically colonized with *P. aeruginosa* measured by ELISA with (a) R-LPS from PAC605 and (b) S-LPS mixture (PEV-02) as coating antigens before and after a series of absorptions with different strains of *P. aeruginosa* whole washed cells. Unabsorbed sera; type-1, sera absorbed with PAC605; type-2, sera absorbed with PAC605, and then with PAC608; type-3, sera absorbed with PAC605, PAC608, and then a pooled mixture of *P. aeruginosa* serotypes 1–16. Optical density readings represent the average from six individual sera.

P. aeruginosa[20] which is particularly expressed by non-typable clinical isolates commonly found in patients with CF[21]. Serum from patients chronically colonized with *P. aeruginosa* produced a positive reaction with most of the sixteen individual serotype vaccine components, again indicating the presence of antibodies directed to a common antigenic component of LPS and/or a number of serotype specific antibodies. The absorption data presented indicates a strong anti core LPS antibody response in chronically colonized patients rather than multiple serotype specific antibodies. The serological evidence presented here and the fact that the majority of CF patients generally remain colonized with a single strain of *P. aeruginosa*[22] suggests that there is a common cross-reactive antibody response in patients chronically colonized with *P. aeruginosa*.

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Incidence of common pyocin types of *Pseudomonas aeruginosa* from patients with cystic fibrosis and chronic airways diseases

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Summary. We sought evidence to determine if particular strains of *Pseudomonas aeruginosa* have a predilection for pulmonary colonisation in patients with cystic fibrosis (CF). The incidence of common pyocin types in non-CF isolates (74%) was similar to that noted in previous reports but differed significantly ($\chi^2 = 16{\cdot}7$, $p < 0{\cdot}001$) from the incidence of 40% observed in CF isolates. A retrospective analysis of respiratory isolates also indicated a relatively low incidence of common pyocin types (44%) in isolates from non-CF patients with chronic airways diseases and this incidence also differed significantly from that observed (73%) in other respiratory isolates from patients in the same hospital. These observations suggest that a subpopulation of *P. aeruginosa* exists which has a predilection for pulmonary colonisation in CF and other chronic pulmonary diseases and may assist in identification of factors affecting bacterial colonisation.

Introduction

Epidemiological studies of the association of *Pseudomonas aeruginosa* and chronic pulmonary colonisation in patients with cystic fibrosis (CF) are greatly helped by typing techniques which indicate the clonal relationship of individual strains. Serotyping, based on O-antigen specificity is not useful for characterisation of *P. aeruginosa* from patients with CF because of alterations in bacterial lipopolysaccharide which are characteristically observed in chronic pulmonary colonisation.[1] Pyocin typing[2,3] which relies on the production and detection of chromosomally determined bacteriocins, DNA probe analysis by Southern blot hybridisation with DNA fragments from the exotoxin A gene,[4] and genome fingerprinting by field inversion gel electrophoresis[5] have been used successfully to determine two important epidemiological factors in patients with CF.[6] Firstly, the majority of patients remain colonised with a single strain of *P. aeruginosa* which gradually evolves to exhibit a range of phenotypic properties including mucoidy due to derepressed alginate biosynthesis, loss of O-antigen specificity, serum sensitivity and hypersusceptibility to a range of antibiotics. Secondly, cross-infection is rare, except between siblings with CF.

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The purpose of the present study was to address a third epidemiological consideration—to determine if a subpopulation of particular strains of *P. aeruginosa* have a predilection for chronic colonisation of CF patients. The rationale for the study had emerged from two sources. First, from our ongoing longitudinal studies with pyocin typing which indicated that cross-infection with the same strain was rare, and that *P. aeruginosa* isolated from patients with CF appeared to have a different pyocin type distribution compared to non-CF isolates. Second, from the evidence from closely documented episodes of primary colonisation by non-mucoid *P. aeruginosa* in patients with CF which suggested that in environments containing multiple strains of *P. aeruginosa* some strains appeared to have enhanced ability to colonise patients.[7]

Materials and methods

Bacteria

A collection of 304 non-CF isolates of *P. aeruginosa* comprised all isolates from sputum and other specimens and anatomical sites in patients treated in three Edinburgh hospitals during 1987. The series of 69 CF isolates of *P. aeruginosa* was from the sputum of patients attending the Edinburgh CF clinics. Subsequently, a retrospective analysis of pyocin type distribution was performed on all respiratory isolates of *P. aeruginosa* which had been isolated from non-CF patients in one hospital during the

period 1986–1988; these isolates comprised 60 strains from patients with chronic airways diseases (CAD) and 52 from patients colonised with *P. aeruginosa* post-operatively. Isolates were identified as *P. aeruginosa* by colonial morphology and production of characteristic pigmentation of Pseudomonas Isolation Agar (Difco, 0927–01); non-pigmented isolates were identified by a positive oxidase reaction[8] and with API 2ONE kits (API Laboratory Products Ltd, Basingstoke).

Pyocin typing

Pyocin typing was performed by our revised technique[3] which incorporates the original type patterns designated earlier in this laboratory. Repeat specimens from the same patient were excluded, as were multiple isolations of a single epidemic strain. A χ^2 statistical analysis was used to determine significant differences in the pyocin type distribution of the different populations; $p<0{\cdot}05$ was considered to represent a statistically significant difference.

Results

Analysis of the distribution of pyocin types revealed a lower incidence of common pyocin types in patients with CF than in isolates from other patients (table I). Amongst 69 isolates from patients with CF, only 28 (40%) produced the common type patterns 1, 3, 5 or 10 based on inhibition of the original set of eight indicator strains. No single pyocin type accounted for a significant proportion of the remaining 41 strains which were distributed amongst 25 pyocin types.

When the incidence of the common type patterns 1, 3, 5 and 10 in CF isolates was compared with that of the non-CF isolates from the three individual hospitals, the results for hospitals 1 and 2 (74% and 70% respectively) were similar to previous reports and differed significantly from the CF isolates ($\chi^2=16{\cdot}69$, $p<0{\cdot}001$ and $\chi^2=13{\cdot}03$, $p<0{\cdot}001$ respectively) whereas isolates from hospital 3 with an incidence of types 1, 3, 5 and 10 at 58% fell midway between the other groups; however, this incidence was still significantly different from that amongst the CF isolates ($\chi^2=5{\cdot}95$; $p<0{\cdot}02$; table I). Although the isolates from patients attending hospital 3 did not contain any strains from patients with CF, further analysis of the sources of *P. aeruginosa* in hospital 3 showed a large proportion of isolates from sputum samples from patients with pulmonary infections. Sputum isolations of *P. aeruginosa* accounted for 39% of all isolates from hospital 3 compared with 7% of isolates from hospitals 1 and 2. Subsequently, in a retrospective study, when all sputum isolates from hospital 3 were excluded from analysis, the incidence of common pyocin types in non-sputum isolates in hospital 3 was 73%; thus, there was no significant difference in the incidence of common pyocin types in all isolates of *P. aeruginosa* in hospital 1 and 2 and non-sputum isolates in hospital 3 ($\chi^2=1{\cdot}3$; $0{\cdot}5>p>0{\cdot}1$ and $\chi^2=0{\cdot}23$, $p>0{\cdot}5$ respectively). This observation suggested that the relatively low incidence of common types 1, 3, 5 and 10 observed in the CF isolates might also be associated with pulmonary colonisation in non-CF patients. This hypothesis was confirmed when a comparison was made of the incidence of common types in patients with CF and sputum isolates obtained from hospital 3 from post-operative patients and patients with chronic airways disease colonised by *P. aeruginosa*. This analysis (table II) indicated no significant difference in the incidence of common pyocin types of *P. aeruginosa* in CF isolates and in isolates from non-CF patients with chronic airways disease including chronic bronchitis, bronchiectasis, asthma and interstitial lung disease; however, the

Table I. Incidence of pyocin types 1, 3, 5 and 10 of *P. aeruginosa* in isolates from sputum from patients with CF, compared with all isolates from non-CF patients in three Edinburgh hospitals

Source	Incidence (%) of pyocin types 1, 3, 5 and 10	p value*
Patients with CF	28 (40)	—
Patients without CF		
in hospital 1	55 (74)	<0·001
in hospital 2	56 (70)	<0·001
in hospital 3	88 (58)	<0·02

* Compared with CF isolates.

Table II. Incidence of pyocin types 1, 3, 5 and 10 of *P. aeruginosa* in isolates from sputum from patients with CF compared with sputum isolates from patients with chronic airways disease (CAD) or post-operative respiratory infection

Source	Incidence (%) of pyocin types 1, 3, 5 and 10	p value*
Patients with CF	28 (40)	—
Patients with CAD	26 (44)	<0·5
Patients with post-operative infection	38 (73)	<0·01

* Compared with CF isolates.

incidence of common pyocin types in acute respiratory infections of post-operative patients was similar to that observed in hospitals 1 and 2.

Detection of the production of low molecular weight S-type pyocins greatly increases the discrimination of pyocin typing.[2,3] Comparison of the production of S-type pyocin activity in the CF isolates and those isolates from non-CF patients treated in the three hospitals indicated that there was no significant difference in the proportion of strains producing S-type activity nor in the S-type inhibition patterns observed with the indicator strains.

Discussion

The evidence from this study indicates that strains of *P. aeruginosa* responsible for pulmonary colonisation in patients with CF and other chronic airways diseases exhibit an incidence of common pyocin types which is significantly lower than noted in previously published reports from world-wide studies in which the same pyocin typing system was used. In these reports[2,9–12] the predominance of pyocin types 1, 3, 5 and 10 is clear and ranges from 58% to 89% not only amongst clinical isolates, but also amongst non-clinical isolates from food and other environmental sources.[10] Indeed, the fact that early studies showed that pyocin types 1, 3, 5 and 10 accounted for the majority of *P. aeruginosa* isolates on a world-wide basis was the rationale behind our introduction of an additional set of indicator strains[13] and the suggestion that detection of S-type pyocin activity allowed further subdivision of these common types and hence improved the discriminative potential of pyocin typing to characterise individual isolates of *P. aeruginosa*.[2,3]

The relatively low incidence of common pyocin types in *P. aeruginosa* isolates from infections in patients with CF and other chronic airways diseases suggests that a subpopulation of *P. aeruginosa*, with as yet unidentified colonising factors, might be responsible for pulmonary colonisation in these patients.

Our results cannot be explained as a peculiarity of the Edinburgh CF clinics since the low incidence of types 1, 3, 5 and 10 was also observed in hospital 3 in isolates from non-CF patients with chronic airways diseases. In addition, a preliminary report from a current independent study on the use of pyocin typing to characterise CF isolates of *P. aeruginosa* has confirmed the relatively low incidence of the common pyocin types in another geographical area (T. L. Pitt, personal communication).

The reasons underlying the predominance of relatively uncommon pyocin types of *P. aeruginosa* in chronic pulmonary colonisation could be associated with factors involved in pyocin production or with the properties of pyocins *per se*. Bacteriocin production and susceptibility are arguably more commonly observed in strains of *P. aeruginosa* than in most other bacterial species; furthermore, the classes of bacteriocins produced represent the combined range found in other species. Phage-tail-like R and F pyocins[14–16] are produced by more than 90% of clinical isolates and colicin-like, trypsin-sensitive S pyocins[2,17] are produced by over 70% of strains;[3] susceptibility to these bacteriocins occurs in 100% of strains. Some strains of *P. aeruginosa* also produce a class of low molecular weight, trypsin-resistant pyocins which resemble the microcins of the enterobacteria.[18] Little is known of the role of pyocins *in vivo* or of their cell surface receptors.[19] From this study, however, we can reasonably conclude that the S pyocins, which share receptors with pseudomonas siderophores,[19,20] and the microcin-like pyocins, are not involved in pulmonary colonisation.

Alternatively, the apparent predilection of uncommon pyocin types of *P. aeruginosa* to establish chronic pulmonary colonisation might merely indicate a subpopulation of strains with properties, which enhance their ability to establish chronic colonisation, but which are independent of pyocin activity. Since all pyocins (R, F, S and microcin-like) studied to date are encoded by chromosomal genes[21,22] (findings further confirmed by our own unpublished observations) we could speculate that genes regulating colonising factors might be closely linked or co-regulated with determinants encoding uncommon pyocin type patterns. However, the range of pyocins produced singly or in combinations by strains of *P. aeruginosa*, is considerable and the role of individual pyocins in relation to the type patterns of inhibition against the indicator strains is little understood. It may be significant that the gene responsible for pyocin R2, which is the major pyocin responsible for production of the common type patterns 1 and 10, is located at 23 min on the PAO chromosome, near loci regulating several *P. aeruginosa* virulence determinants including exotoxin A and the siderophore pyoverdin.[23] At present, however, there is no evidence which links pyocin genes with genetic determinants for pseudomonas colonising factors.

In conclusion, these studies have demonstrated a subpopulation of *P. aeruginosa* with enhanced potential to establish chronic pulmonary colonisation in patients with CF and other chronic

obstructive airways diseases. The basis for this subpopulation is unclear but may include pyocin activity, the genetic determinants for pyocins or other colonising factors. It will be interesting to observe if a similar subpopulation is identified from epidemiological studies with DNA-based typing techniques.

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Mucinophilic and Chemotactic Properties of *Pseudomonas aeruginosa* in Relation to Pulmonary Colonization in Cystic Fibrosis

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Representative isolates of nonmucoid *Pseudomonas aeruginosa* were studied to investigate the hypothesis that mucinophilic and chemotactic properties in this species act as potential factors in the initial stages of pulmonary colonization in patients with cystic fibrosis (CF). Transmission electron microscopy with a surfactant monolayer technique was used in a novel manner to demonstrate the adhesion of all 10 *P. aeruginosa* strains examined to porcine gastric mucin and tracheobronchial mucin from a patient with CF. Control experiments showed that *Escherichia coli* K-12 and single representatives of *Proteus mirabilis* and *Klebsiella aerogenes* did not bind to these mucins. The Adler capillary technique, used to measure bacterial chemotactic response, showed that purified CF mucin acted as a chemoattractant for most *P. aeruginosa* strains, with the exception of the nonmotile mutant M2Fla$^-$ and the nonchemotactic mutant WR-5. The ability of the major sugar and amino acid components of mucin to act as chemoattractants was investigated. The degree of chemotaxis was strain specific; optimum chemotaxis was observed toward serine, alanine, glycine, proline, and threonine. No strain showed chemotaxis to *N*-acetylneuraminic acid, but all strains showed a strain-dependent chemotactic response to the sugars L-fucose, D-galactose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine. These results provide new information on the mucinophilic and chemotactic properties of nonmucoid *P. aeruginosa* and support the hypothesis that these properties could play a role in the initial stages of pulmonary colonization in patients with CF.

Cystic fibrosis (CF) is the most commonly inherited lethal disease of Caucasians and is characterized by disturbances in electrolyte transport from exocrine glands and secretory epithelia, resulting in the accumulation of abnormally viscid tracheobronchial secretions (23, 24). The rheological properties of bronchial mucus are primarily due to its mucin component (21, 33), which comprises glycoprotein macromolecules synthesized by the submucosal glands and goblet cells of the surface epithelium. Mucins consist of a polypeptide core with oligosaccharide side chains attached by *O*-glycosidic linkages (5). Intermolecular associations between mucins result in the formation of viscoelastic gels (36). Mucin hypersecretion is a characteristic feature of the pulmonary airways of patients with CF, and the tenacious viscid secretions (33) are associated with impaired mucociliary clearance and bacterial colonization which, in turn, initiates a vicious cycle of chronic inflammatory reaction. In patients with CF, initial and sometimes intermittent asymptomatic colonization with nonmucoid *Pseudomonas aeruginosa* occurs; eventually, variants expressing a mucoid-colony form (due to copious production of alginate) emerge from the colonizing strain. The establishment of these variants, and that of other phenotypes associated with chronic pulmonary colonization, is a major cause of morbidity and mortality (12, 14, 27). Most research has been directed to the properties and pathogenesis of mucoid *P. aeruginosa*; little attention has been directed to the bacterial and host factors that contribute to the initial stages of asymptomatic colonization which, arguably, forms an important microbial reservoir for the subsequent emergence of mucoid variants (13).

The aim of our study was to seek evidence which supports the hypothesis that chemotactic factors in mucin and mucinophilic properties of *P. aeruginosa* contribute to the initial stages of pulmonary colonization in patients with CF and, thereby, to provide information on which to base rational strategems for vaccination. The study also describes the novel application of an electron microscope technique, developed for the study of mucin structure (36), which demonstrates adhesion of *P. aeruginosa* to mucin monolayers.

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MATERIALS AND METHODS

Bacterial strains. Nonmucoid strains of *P. aeruginosa* J1385 and J1375 were clinical isolates obtained from P. Friend (Public Health Laboratory, Truro, England) and isolated during an investigation of primary respiratory colonization in two patients with CF who had bathed in a hydrotherapy pool contaminated with multiple strains of *P. aeruginosa* (10). J1385 is the colonizing strain which was isolated from both CF patients and from the pool water; J1375 represents one of the four other environmental isolates taken from the water. The clonal relation of these isolates had been previously determined in our laboratory by pyocin typing (11) and confirmed by DNA probe analysis by M. L. Vasil and J. W. Ogle (Health Sciences Center, Denver, Colo.). *P. aeruginosa* JN8, JN47, JN61, and JN62 also express the nonmucoid phenotype. These strains were isolated from the sputa of four CF patients with no previous history of pulmonary colonization by *P. aeruginosa*; each of these four strains belonged to a different pyocin type. Additional strains of *P. aeruginosa* investigated included the well-characterized genetic strain PAO1 (15); WR-5, a nonchemotactic mutant of PAO1; M2Fla$^-$, a nonflagellate mutant of strain M2; and M2Rev, a motile revertant of M2. Strains M2Fla$^-$, M2Rev, and WR-5 were obtained from I. A.

Holder (Shriners Burn Institute, Cincinnati, Ohio). *Escherichia coli* K-12 and individual strains of *Proteus mirabilis* and *Klebsiella aerogenes* were investigated as controls for adherence to mucin monolayers.

Media. Nutrient agar was Columbia agar base (Oxoid Ltd., Basingstoke, England). Nutrient broth was Oxoid no. 2 supplemented with 0.5% (wt/vol) yeast extract. Minimal medium employed for culture of *P. aeruginosa* prior to chemotaxis assays and the chemotaxis medium (CM) used in the assays were as described by Moulton and Montie (25).

Purification of mucins. Sputum from a patient with CF was stored at −20°C, subsequently thawed into ice-cold 0.2 M NaCl–5 mM EDTA–10 mM sodium phosphate buffer (pH 6.5) containing 1 ml of diisopropyl phosphorofluoridate, and then centrifuged at 40,000 × *g* for 30 min at 5°C in a 50 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellet (gel) was then repeatedly extracted with 6 M guanidinium chloride–5 mM EDTA–5 mM *N*-ethylmaleimide–10 mM sodium phosphate buffer (pH 6.5). The mucins were then purified by a two-step isopycnic density gradient procedure, first in CsCl–4 M guanidinium chloride and then in CsCl–0.2 M guanidinium chloride, as described by Carlstedt et al. (3). Purified mucins were dialyzed into 4 M guanidinium chloride–10 mM sodium phosphate buffer (pH 6.5).

Chemotaxis assay. One milliliter of an overnight preculture grown in minimal medium was inoculated into 9 ml of minimal medium and incubated at 37°C for 3 h in an orbital incubator at 120 rpm. The exponentially growing cells were harvested by centrifugation at 4,600 × *g* for 15 min and washed twice with 10 ml of CM before suspension to a density of 2×10^8 CFU (optical density, 0.25 at 590 nm). Chemotaxis was measured by the capillary tube assay described by Adler (1). A small chamber was formed by placing a cover slip on a U-shaped piece of melting-point capillary tubing bonded to a microscope slide. Prior to use in the assays, the chambers, supported in petri dishes, were prewarmed by incubation at 37°C for at least 30 min. The chambers were then filled with 0.4 ml of bacterial suspension in CM. Capillaries (0.25-mm diameter; Phase Separations Ltd., Clwyd, United Kingdom) were filled with potential attractants dissolved in CM. Control capillaries containing CM alone were used in all experiments as a measure of background motility. The open end of a capillary was inserted into the center of a chamber containing the bacterial suspension. After incubation for 30 min (unless otherwise indicated), the capillaries were removed, their sealed ends were carefully broken, and the contents were emptied into saline with the aid of a micropipette bulb (Analtech Inc.) The contents were diluted, and plate counts were made. The accumulation of bacteria in each of two capillaries containing chemoattractant was measured as CFU per capillary, and a mean value was determined. A similar measurement was made from capillaries in which no chemoattractant was present. The ratio of these CFU values (the relative response) was then determined; the ratio normalizes for experimental or day-to-day variations. A meaningful chemotactic response as described by Moulton and Montie (25) was a relative response value of >2.

Potential chemoattractants investigated in this study included mucin purified from tracheobronchial secretions from a CF patient. Before it was used, the mucin preparation, stored as a solution in 4 M guanidinium chloride at 4°C, was diluted in CM (100 μg/ml) and dialyzed against 1 liter of CM at 4°C for 24 h. Other potential chemoattractants investigated were the major amino acids present in mucin, including alanine, aspartic acid, cysteine, glutamic acid, serine, glycine, threonine, and proline, and the major mucin sugars, including L-fucose, D-galactose, *N*-acetyl-D-galactosamine (GalNAc), and *N*-acetyl-D-glucosamine (GlcNAc). All chemicals were supplied by BDH Chemicals, Poole, United Kingdom, and were of the highest purity available. *N*-Acetylneuraminic acid (NANA; Koch-Light, Haverhill, United Kingdom) was also examined. Solutions were made in CM and, with the exception of mucin, were filtered through a 0.2-μm-pore-size membrane filter (Schleicher & Schuell, Dassell, Federal Republic of Germany).

Electron microscopy. Visualization of the mucin-bacterium association was attempted by a modification of the surfactant monolayer technique described by Sheehan et al. (36) for the study of mucin architecture. Purified porcine gastric mucin (BDH) was used in the preliminary experiments because it is reported to resemble human tracheobronchial mucin in its carbohydrate, amino acid, and sulfate ester composition (2, 34). Subsequent experiments were focused on CF respiratory mucin purified as described above. A log-phase culture of *P. aeruginosa* grown in nutrient broth was centrifuged at 4,600 × *g* for 15 min and washed twice in 1% (wt/vol) ammonium acetate in distilled water. The pellet was gently suspended in 15 ml of the porcine gastric or CF mucin solution (0.05 μg/ml in 50 mM magnesium acetate), and the mixture was transferred to a plastic petri dish and incubated for 30 min at 37°C. To prepare mucin monolayers, a small amount of fine graphite powder (BDH) was sprinkled on the surface. A mucin monolayer was obtained by touching the surface of the mucin solution with the tip of a pipette containing 1 μl of 1% (wt/vol) benzyldimethylalkyl-ammonium chloride (BAC) in distilled water. The surfactant nature of the BAC propels the carbon particles to the side of the dish, leaving a fine surface monolayer of mucin in BAC. After 15 min, carbon-coated 400-mesh electron microscope grids were touched to the surface of the monolayer, stained for 1 to 2 s in a solution of uranyl acetate (1 mM in 95% [vol/vol] ethanol), and washed for 1 to 2 s in ethanol. The grids were then allowed to dry in air before being subjected to unidirectional shadowing with platinum at a grazing angle of 7 to 12°. Transmission electron microscopy was performed with a 12A microscope (Hitachi) at 75 K. *E. coli* K-12, *Proteus mirabilis*, and *K. aerogenes* were prepared and examined in a similar manner.

RESULTS

Adherence of *P. aeruginosa* to mucin monolayers. Adherence of *P. aeruginosa* to both porcine and CF mucin monolayers was observed with each of the 10 strains of *P. aeruginosa* examined, including the nonmotile and nonchemotactic mutants. Initial experiments, performed with porcine gastric mucin, showed *P. aeruginosa* cells to be associated and often entangled in a highly aggregated mucin matrix (Fig. 1). When these experiments were repeated with mucin that had been purified from the tracheobronchial secretions of a CF patient, *P. aeruginosa* adhesion was again observed. CF mucin appeared in an aggregated form attached to the bacterial cell surface (Fig. 2A, B, and C). Attachment of aggregated mucin fragments to flagella was also observed (Fig. 2C). Many of the bacterial cells observed in the monolayers possessed flagella, and phase-contrast microscopy confirmed that all the bacteria, except strain M2Fla$^-$, were initially motile when incubated in the presence of mucin. As a control for random association or simple entanglement between mucin and bacterial cells, cultures of *E. coli* K-12, *Proteus mirabilis*, and *K. aerogenes* were

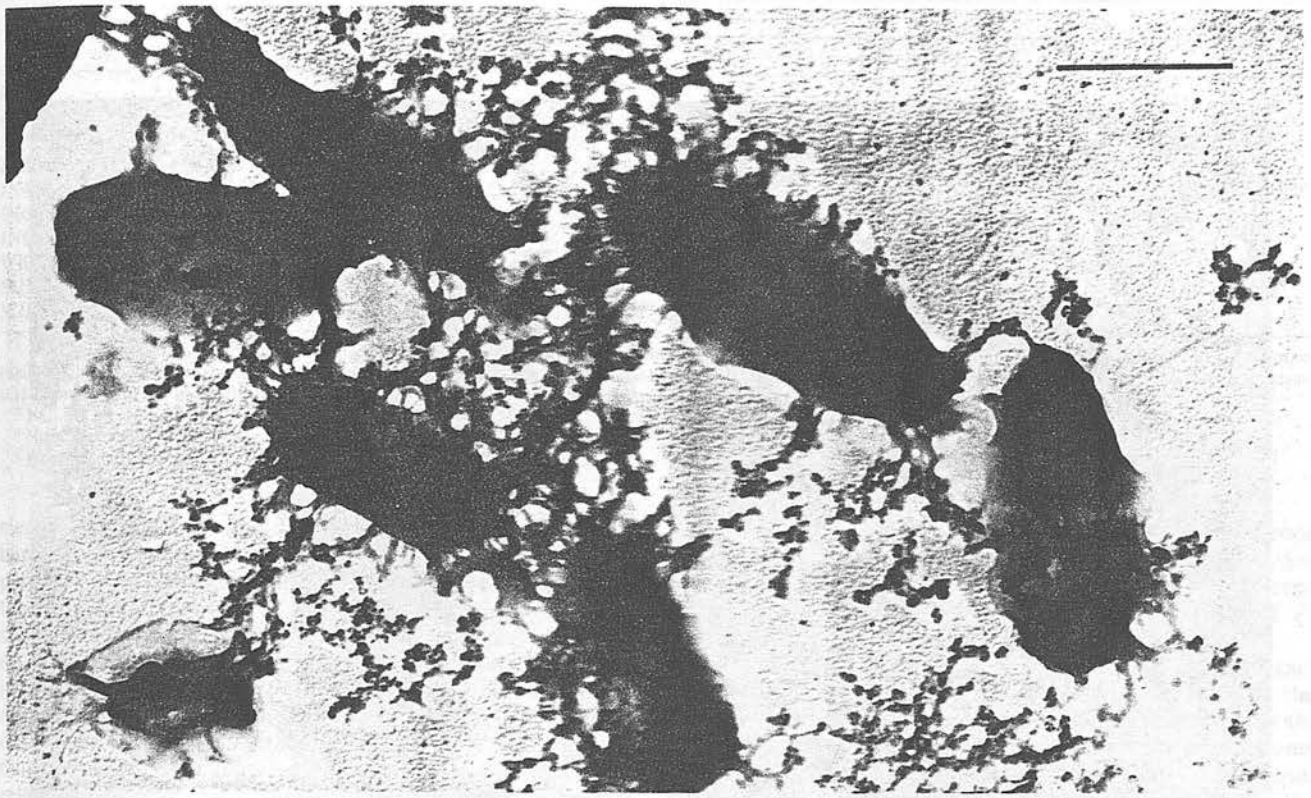

FIG. 1. Electron micrograph of *P. aeruginosa* in association with purified porcine gastric mucin (0.05 μg/ml) spread in BAC monolayer stained with uranyl acetate and subjected to unidirectional shadowing with platinum. Bar, 1 μm.

added to CF mucin and monolayers prepared as described above. In contrast to *P. aeruginosa*, only an occasional bacterial cell was observed in these monolayers, and those bacteria observed were not attached to mucin (Fig. 2D). In these control monolayers with nonadherent bacteria, the mucin appeared as lightly stained linear filaments similar in appearance to mucin in the absence of bacteria.

Chemotactic responses to *P. aeruginosa* to CF mucin and mucin components. To confirm the efficacy of the capillary chemotaxis assay in our hands, the concentration curve for serine as an attractant for *P. aeruginosa* PAO1 was determined. The chemotactic response of PAO1 was found to be similar to that reported by Moulton and Montie (25): a peak response occurred at 10^{-3} M serine.

When the chemotactic responses of nine strains of *P. aeruginosa* towards purified CF mucin were measured (Fig. 3), a meaningful response (i.e., >2.0) was observed in all strains examined, except for the nonmotile mutant M2Fla$^-$ and the nonchemotactic mutant WR-5.

The chemotactic response of *P. aeruginosa*, towards the eight major amino acids present in mucin, namely, alanine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine, and threonine, at concentrations of 10^{-1} to 10^{-5} M, was then measured. For most of the amino acids examined, considerable differences in the relevant chemotactic response of each of the strains examined were observed (Table 1). Chemoattraction to serine, alanine, glycine, proline, and threonine was particularly evident; with most of the amino acids, the peak chemotactic response occurred at a concentration of 10^{-3} M.

Chemotaxis to mucin-associated sugars. Chemotaxis assays were performed to assess the potential of the major sugar components of mucin, namely, L-fucose, D-galactose, GalNAc, GlcNAc, and NANA, to act as chemoattractants for *P. aeruginosa*. The relative chemotactic responses for these strains (Table 2) reflected the results of the previous chemotaxis assays using amino acids and emphasized a strain-dependent response to L-fucose, D-galactose, GalNAc, and GlcNAc. No strain showed a chemotactic response to NANA.

DISCUSSION

Previous studies of *P. aeruginosa* have shown its ability to adhere to tracheal epithelial cells (7, 29, 30), and it has been suggested that adhesion is the first stage in the establishment of respiratory tract infection in susceptible patients (31). In healthy individuals this adhesion probably has a protective role, enabling adherent or entangled bacteria to be removed by mucociliary clearance. In the lungs of CF patients, however, mucociliary clearance is impaired, and it is arguable that adherence of *P. aeruginosa* to CF mucin could result in potentially detrimental colonization of the mucosal surface layers. Scanning and transmission electron microscopy of CF lung tissue, done either postmortem or pre- or post-heart-lung transplantation, show that the bacteria are associated with sheets of surface secretions and not the microvillus surface of epithelial cells or deeper tissues (18; P. K. Jeffrey, personal communication).

Vishwanath and Ramphal (37) used a microdilution plate assay to demonstrate adherence of *P. aeruginosa* to wells coated with tracheobronchial mucin. In the present study we have produced further evidence of the mucinophilic properties of *P. aeruginosa*. Novel application of transmission

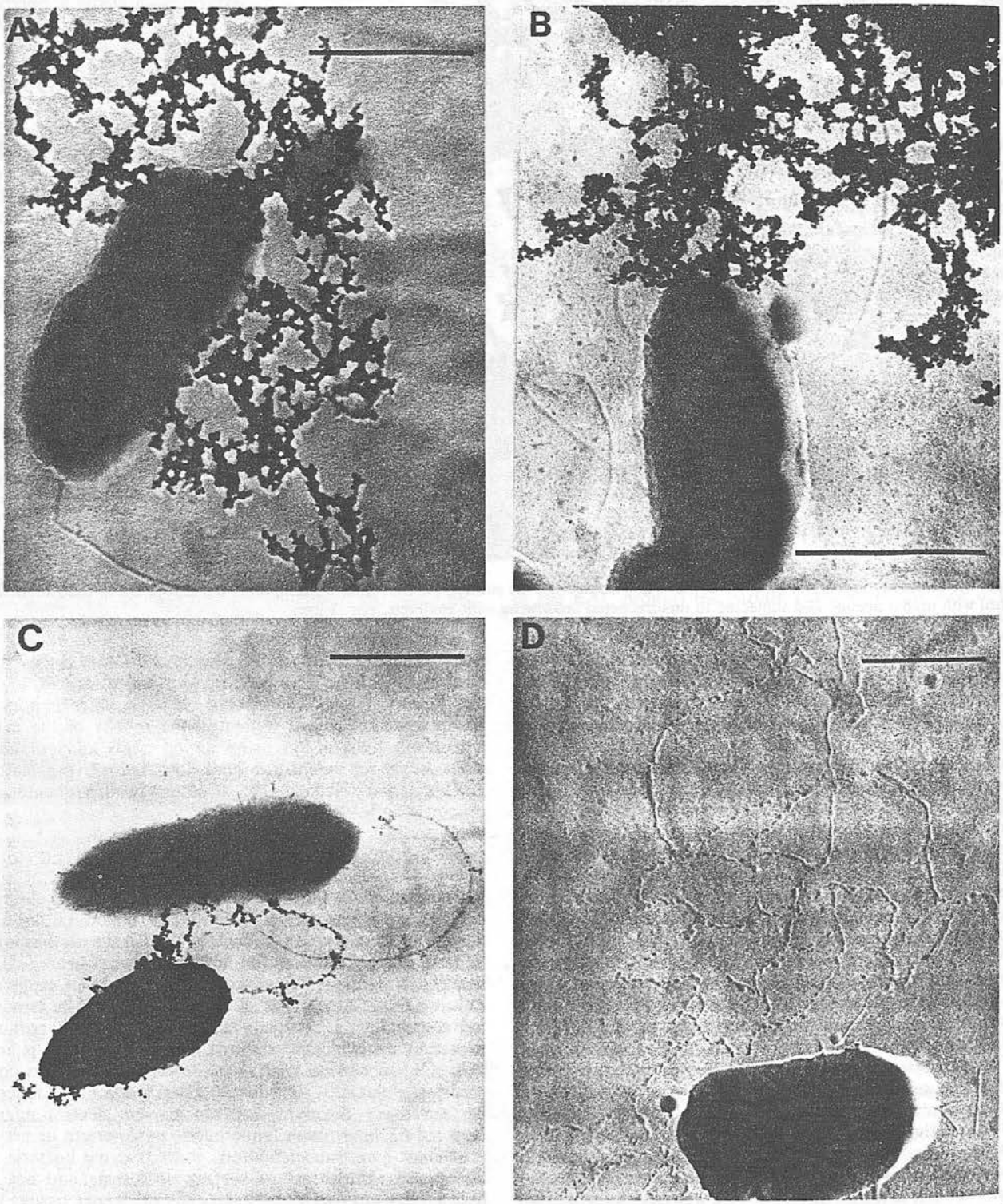

FIG. 2. (A through C) Electron micrographs of *P. aeruginosa* in association with purified tracheobronchial mucin from a CF patient showing adhesion of mucin aggregates to bacterial surfaces. (D) One of the few bacterial cells observed when *E. coli* K-12 was investigated in mucin monolayers. No direct mucin-bacterium association is evident, and the mucin appears as lightly stained linear filaments. Monolayers were stained with uranyl acetate and subjected to unidirectional shadowing with platinum. Bars, 1 μm.

TABLE 1. Chemotactic responses of *P. aeruginosa* strains toward mucin-associated amino acids

Strain	Chemotactic response[a] toward:							
	Ala	Asp	Cys	Glu	Gly	Pro	Ser	Thr
PAO1	2.6	NC[b]	NC	2.3	2.5	3.7	6.1	NC
J1375	4.3	2.5	NC	4.9	4.2	4.8	4.0	3.0
J1385	7.2	5.0	2.2	6.5	4.7	5.2	4.7	2.9
JN8	12.4 (10^{-2})	NC	3.3	2.6 (10^{-2})	14.4 (10^{-2})	10.0	19.3	6.6
JN47	5.0	NC	NC	NC	5.0	2.6 (10^{-5})	5.5 (10^{-5})	7.2 (10^{-2})
JN61	8.2	3.1	NC	4.0	5.9	7.1	3.9 (10^{-4})	4.4
JN62	5.5 (10^{-2})	2.1 (10^{-2})	NC	2.7 (10^{-2})	8.1 (10^{-1})	3.7	2.9 (10^{-4})	3.7
WR-5[c]	NC	NC	NC	NC	NC	NC	NC	NC
M2Fla$^-$[d]	NC	NC	NC	NC	NC	NC	NC	NC
M2Rev	3.3	NC	NC	NC	3.6	3.8	3.0	2.3

[a] The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing an amino acid to accumulation of bacteria in a control capillary containing no chemoattractant. A response value that is >2.0 is considered meaningful (25). The highest relative chemotactic response for each amino acid (representing the mean from duplicate assays) was at the 10^{-3} M concentration unless otherwise indicated (in parentheses).
[b] NC, No chemotaxis (i.e., <2.0).
[c] Nonchemotactic control.
[d] Nonflagellate control.

electron microscopy and BAC monolayers demonstrated bacterial entanglement and adherence to porcine gastric and purified CF mucin. In addition, quantitative measurement of the chemotactic response of strains of *P. aeruginosa* showed chemotaxis towards purified CF mucin and the major mucin-associated amino acids and sugars. The observation that individual strains of *E. coli* K-12, *Proteus mirabilis*, and *K. aerogenes* did not adhere to mucin agrees with the previous observations of Vishwanath and Ramphal (37) and suggests that the association between *P. aeruginosa* and mucin is relatively specific. In addition to mucin, other adhesion receptors for *P. aeruginosa* may include the glycosphingolipid gangliotetraosylceramide (asialo GM1), which occurs in substantial amounts in lung tissue (19).

The observation that both nonmotile and nonchemotactic mutants of *P. aeruginosa* also showed attachment to mucin indicates that adhesion is not dependent on motility or chemotaxis. The presence of mucin aggregates attached to individual cells of *P. aeruginosa* suggests that the bacterium-mucin interaction is strong enough to overcome the sheer forces as the mucin monolayer is formed. In the presence of *P. aeruginosa*, porcine and CF mucin appeared in a highly aggregated form rather than the lightly stained filamentous form observed in the case of mucin alone or in the presence of nonadherent bacteria, e.g., *E. coli*. This observation suggests that mucin molecules are degraded or undergo substantial conformational change in the presence of *P. aeruginosa*.

If initial colonization of the CF-affected respiratory tract by nonmucoid *P. aeruginosa* involves adherence to the epithelial surface or to tracheobronchial mucin or to both, chemotaxis of bacteria to mucin and mucin components would enhance the potential for bacterium-host interaction. It could be speculated that, in a given environment (e.g., the whirlpool source of J1385 and J1375), enhanced chemotaxis would confer an advantage on a potential colonizing strain, e.g., J1385. In support of this hypothesis, J1385 showed greater chemotaxis than J1375 toward CF mucin (Fig. 3) and toward the majority of mucin constituents (Tables 1 and 2). Mucin-bacterium interaction would be further enhanced if the mucin could also provide a source of bacterial nutrients. Ongoing studies indicate that mucin not only can act as a sole source of nitrogen and carbon for *P. aeruginosa* but also

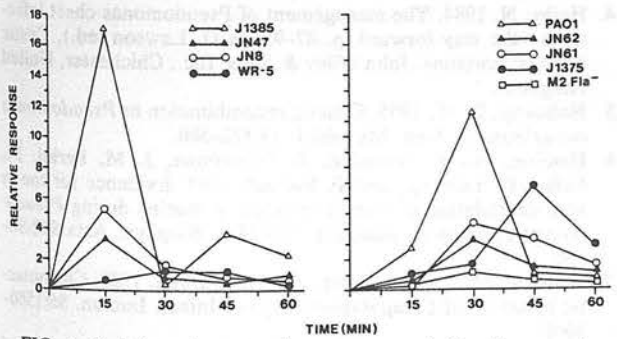

FIG. 3. Relative chemotactic responses of nine *P. aeruginosa* strains towards CF tracheobronchial mucin (100 μg/ml) from a patient with CF. The assay was performed over 60 min. Each point represents the ratio of the mean accumulation of bacterial CFU in duplicate capillaries containing mucin to the accumulation of bacterial CFU in control capillaries containing CM alone. A response greater than 2 is considered to indicate chemotaxis. *P. aeruginosa* M2Fla$^-$ (nonmotile) and WR-5 (nonchemotactic) were included as controls.

TABLE 2. Chemotactic responses of *P. aeruginosa* strains toward mucin-associated sugars

Strain	Chemotactic response[a] toward:			
	L-Fucose	D-Galactose	GalNAc	GlcNAc
PAO1	2.5 (10^{-1})	15.1 (10^{-1})	NC[b]	2.3 (10^{-4})
J1375	2.8 (10^{-2})	NC	NC	3.0 (10^{-5})
J1385	5.8 (10^{-4})	3.8 (10^{-3})	3.4 (10^{-4})	4.5 (10^{-4})
JN8	5.4 (10^{-1})	3.3 (10^{-3})	4.2 (10^{-1})	4.3 (10^{-2})
JN47	2.8 (10^{-3})	3.0 (10^{-3})	2.8 (10^{-4})	2.8 (10^{-2})
JN61	NC	2.2 (10^{-1})	2.3 (10^{-1})	NC
JN62	NC	5.6 (10^{-5})	NC	NC
WR-5[c]	NC	NC	NC	NC
M2Fla$^-$[d]	NC	NC	NC	NC
M2Rev	3.1 (10^{-4})	NC	4.6 (10^{-4})	2.4 (10^{-2})

[a] The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing a sugar to accumulation of bacteria in a control capillary containing no chemoattractant. A response value that was >2.0 was considered meaningful (25). Responses at five concentrations, from 10^{-1} to 10^{-5} M, were investigated. The highest relative chemotactic response for each sugar (representing the mean from duplicate assays) and the respective concentration at which it was obtained (in parentheses) are shown. There was no chemotaxis toward NANA detected.
[b] NC, No chemotaxis (i.e., <2.0).
[c] Nonchemotactic control.
[d] Nonflagellate control.

supports copious biosynthesis of alginate in mucoid *P. aeruginosa* (12; J. R. W. Govan, unpublished data).

There have been relatively few reports describing the chemotactic properties of *P. aeruginosa*. It has been shown, however, that the degree of chemotaxis towards arginine and serine is strain dependent (22). Motility has been shown to be an important virulence factor in burn infections due to *P. aeruginosa* (8), and an early antibody response to pseudomonas flagellar antigens is observed in patients with CF (35). To our knowledge there are no previous reports which demonstrate and compare the chemotactic responses of different strains of *P. aeruginosa* to mucin and mucin constituents.

The chemotactic responses to mucin and the major amino acids and sugars of mucin (32) (Fig. 3; Tables 1 and 2) clearly demonstrate a strain-specific chemotactic response of *P. aeruginosa* to different attractants. The different peak values obtained with individual strains could result from variation in the number of chemoreceptors present or in the affinities of those chemoreceptors. In the environment of the CF lung, proteolysis of mucin by proteases from *P. aeruginosa* and neutrophils (28) could explain how mucin fragments and components would be readily available in vivo to act as chemoattractants and nutritional sources for *P. aeruginosa*. The exposure and release of individual amino acids by proteolytic fragmentations of mucin may arguably influence the degree of chemotactic responses of individual strains. The presence of proteolytic-resistant glycopeptide regions within the mucin may reduce the release of serine, proline, threonine, and alanine, which are predominant in these regions. These and other amino acids, however, may be made available because of proteolysis of other proteins and glycoproteins, such as immunoglobulins and lactotransferrin, with which respiratory mucin is associated in vivo (16). Interestingly, NANA, which has been reported to be an adhesin for *P. aeruginosa* (29), did not act as a chemoattractant for any of the strains tested. NANA is a relatively strong acid in biological systems; however, the failure of NANA to act as a chemoattractant cannot be explained by a low pH value. Although the buffered preparations of NANA at 10^{-1} M did have low pHs, the other preparations examined (10^{-2} to 10^{-5} M) had neutral pHs.

Interactions between bacteria and host mucins are important in bacterial colonization of mucosal surfaces (4, 6, 20, 26). Chemotaxis of *Vibrio cholera* towards the intestinal mucosa (9) and chemotaxis of *Campylobacter jejuni* to porcine intestinal mucin (17) lend support to the hypothesis that chemotaxis towards mucins is an important bacterial virulence mechanism. From our results it seems reasonable to speculate that initial colonization of the respiratory tract in CF patients by nonmucoid *P. aeruginosa* could be assisted by chemotaxis towards, entanglement in, and adhesion to the mucin-rich mucosal surface. The chemotactic and adherence properties of *P. aeruginosa* towards purified glycopeptide components of CF and non-CF mucins is under investigation. Meanwhile, our results suggest that the mucin monolayers prepared by the BAC technique might be useful in studies of the interaction of other bacterial pathogens with respiratory and gastrointestinal mucins.

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Pseudomonas aeruginosa flagellar antibodies in serum, saliva and sputum from patients with cystic fibrosis

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Flagellar preparations from *Pseudomonas aeruginosa* strains were characterized and used in ELISA and immunoblot studies to detect anti-*P. aeruginosa* flagellar antibodies in sera, saliva and sputum from patients with cystic fibrosis (CF). Serum anti-flagellar IgG antibodies were detected, particularly in those CF patients intermittently or chronically colonized by *P. aeruginosa*. Antibodies to both type-a and -b flagella were detected; however, in some patients a pronounced antibody response to only one of the flagellar types was evident. Raised levels of anti-flagellar antibodies in intermittently and non-*P. aeruginosa* colonized patients may represent an early antibody response to *P. aeruginosa* colonization of the CF respiratory tract and implicate instigation of early anti-pseudomonal antibiotic therapy.

Keywords: *Pseudomonas aeruginosa*, flagella, antibody, cystic fibrosis.

Introduction

Respiratory colonization by mucoid *P. aeruginosa* presents the major microbial challenge in patients with CF[1]. Motility and chemotaxis have been shown to be important virulence determinants for non-mucoid *P. aeruginosa*[2–4] in the colonization of burn wounds. Recently, we have demonstrated chemotaxis of *P. aeruginosa* towards respiratory mucins and this property may well play a key role in the initial colonization of the CF respiratory tract[5]. Bacterial flagella are potent immunogens evoking both humoral and cellular immune responses[6]. *Pseudomonas aeruginosa* flagella may be classified into two main types, a and b[7]. The heterologous a-types can be further subdivided into five partial antigens (a_0, a_1, a_2, a_3, a_4) with molecular weights ranging from 45,000 to 52,000[8]. All a-types have the a_0 antigen with usually one or more of the additional subtypes. The b-types comprise an homologous group with a molecular weight of 53,000[8]. Anti-flagellar antibodies are able to induce phagocytosis and killing of motile strains of *P. aeruginosa*[9,10] and show protection in animal model experiments[11–13].

Serum IgG anti-*P. aeruginosa* flagellar antibodies have previously been demonstrated in patients with CF[14,15]. The aim of this study was to investigate the antibody response to

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P. aeruginosa flagella in serum, saliva and sputum samples obtained from CF patients chronically, intermittently or non-colonized by the organism. The possible significance of anti-*P. aeruginosa* flagellar antibodies is discussed.

Materials and methods

Patients

Serum, saliva and sputum specimens were obtained from patients with CF attending the paediatric and adult CF clinics in Edinburgh. The CF patients (age range, 1–27 years) included patients chronically colonized with *P. aeruginosa* as well as patients intermittently or non-colonized with the organism. Serum samples were stored at −20°C, and saliva and sputum specimens at −70°C. Prior to storage, sputa were spun at 13,000 g for 15 min and the sol phase retained for analysis.

Bacterial strains and growth conditions

Pseudomonas aeruginosa strains included the well characterized genetic strain PAO1, and three non-mucoid strains J1385, JN61 and JN62 isolated from the sputum of patients with CF. For flagellar antigen preparation 2-litre flasks containing 1 litre of nutrient broth with 0·5% (w/v) yeast extract (NYB) were inoculated with 2 ml of an overnight preculture grown in NYB. The flasks were incubated in an orbital incubator (120 r.p.m.) at 37°C for 16 h.

Preparation of flagella

Overnight bacterial cultures were harvested by centrifugation (10,000 g, 15 min, 4°C) and washed once in phosphate buffered saline (PBS). Each bacterial pellet was suspended in 20 ml of PBS and blended with a commercial blender for 2 min. Bacterial cells were removed by up to 12 cycles of centrifugation (10,000 g, 15 min, 4°C). Removal of cells was monitored at intervals by microscopy and culture of the supernatant. The cell-free supernatant was centrifuged at 100,000 g for 1·5 h at 4°C and the resultant pellet washed twice in distilled water. The pellet was then resuspended in 0·5 ml of distilled water, dialyzed overnight against distilled water at 4°C, and stored at −20°C. The amount of protein in each sample was determined by the method of Lowry[16] with bovine serum albumin as standard. The final flagellar preparations were visualized by electron microscopy after staining with 2% phosphotungstic acid. Flagella were classified into a and b flagellar types based on their migration in polyacrylamide gels, and ELISA and immunoblot analysis with rabbit anti-flagellar type-a and -b antisera.

Absorption of specimens

To avoid immunological detection of anti-LPS antibodies[17] sera, saliva and sputa were mixed with an equal volume of LPS (1 mg ml^{-1}) extracted from *P. aeruginosa* by the method of Westphal & Luderitz[18]. Prior to analyses in ELISA and immunoblot studies, samples were incubated for 60 min at 37°C followed by overnight incubation at 4°C. The LPS used for absorption of LPS antibodies was from the strain used to prepare the corresponding flagella. Removal of LPS antibodies was confirmed by ELISA against each extracted LPS.

ELISA

Diluents and buffers used in the ELISA were essentially as described previously except sodium azide was omitted[19]. Indirect ELISA was used to determine the presence of IgA and IgG anti-*P. aeruginosa* flagellar antibodies in CF sera, saliva and sputa. Flagellar antigens of both a- and b-types were diluted in coating buffer to a concentration of 1 μg ml^{-1} and added to polystyrene microwell strips (Polysorb, Nunc) at 100 μl per well. Strips were coated overnight at room temperature and washed four times with wash buffer. Strips were then post-coated with post-coat buffer (containing 5% bovine serum albumin) at 100 μl per well overnight at room temperature. After being washed four times with wash buffer, strips were stored at −20°C until used.

Pre-absorbed sera were diluted 1:200, and pre-absorbed saliva and sputa 1:100 in dilution buffer and added to flagella coated microstrips at 100 μl per well in duplicate. Strips were incubated at 37°C for 90 min before washing four times with wash buffer. Peroxidase-conjugated sheep anti-human IgA and IgG (ICN Biomedicals) were each diluted 1:1000 and added to the relevant strips at 100 μl per well. Strips were incubated for a further 90 min at 37°C. Strips were washed four times and rinsed with distilled water before substrate at 100 μl per well was added. Substrate consisted of 0·1 M sodium acetate/citrate buffer (pH 6·0) containing 1% tetramethyl-benzidine and 0·015% (vol/vol) hydrogen peroxide. Strips were incubated for 60 min at room temperature and reactions stopped by adding 2 M sulphuric acid at 25 μl per well. The optical density (OD) of wells was read at 450 nm on an automated microplate reader (Titretek Multiskan, Flow Laboratories). Final results were expressed after subtraction of the OD of negative control wells (coated only with BSA post-coat) for each serum, saliva and sputum sample.

SDS-PAGE

Flagellar preparations were analysed by SDS-PAGE with 12% acrylamide gels and the buffer system of Laemmli[20]. Flagellar protein samples (10 μg protein per loading track) were detected with Coomassie blue R250 stain as described by Hancock & Poxton[21].

Immunoblotting

For immunoblotting, flagella separated in acrylamide gels were electrophoretically transferred to nitrocellulose membranes (0·2 μm Schleicher & Schuell) by the method of Towbin *et al.*[22] The flagella were then probed with absorbed sera, saliva and sputa. Anti-flagellar antibodies in these specimens were visualized by anti-human IgA and IgG horse-radish peroxidase conjugates (ICN Biomedicals) and subsequent incubation with substrate.

Results

Flagellar preparations isolated from the three clinical *P. aeruginosa* strains were characterized by molecular weight determination in SDS-PAGE and by ELISA and immunoblot analysis with anti-flagellar type-a and -b anti-sera. Flagella isolated from PAO1, classified as flagellar type-b[8], were used as a standard. Flagella from *P. aeruginosa* strains JN61 (type-a) and J1385 (type-b) (Figure 1) were subsequently used to screen anti-*P. aeruginosa* flagellar antibodies in patients with CF.

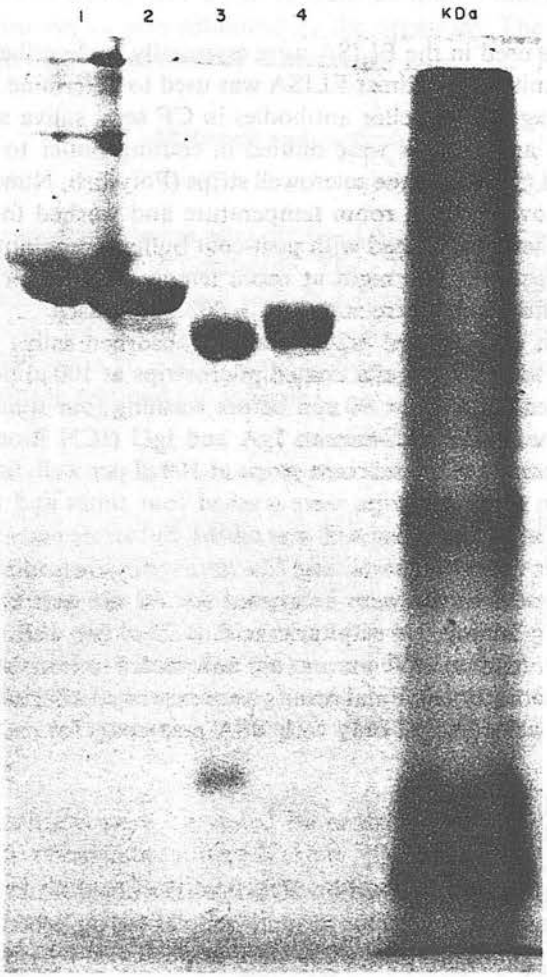

Figure 1. SDS-PAGE of flagella preparations from *P. aeruginosa* strains PAO1 (Track 1), J1385 (Track 2), JN61 (Track 3) and JN62 (Track 4) were separated using 12% w/v acrylamide gels and visualized with Coomassie blue stain. Molecular weights of protein standards are indicated.

A range of levels of anti-flagellar antibodies were demonstrated in the sera, saliva and sputum from patients with CF. Anti-flagellar IgG antibodies were found in sera from patients with CF and in sera from healthy controls (Figure 2). Elevated levels of anti-flagellar IgG antibodies above the control range were found in CF patients intermittently or chronically colonized with *P. aeruginosa*. However, a number of CF patients classed as non-*P. aeruginosa* colonized on the basis of sputum bacteriology also had increased levels of serum anti-flagellar IgG antibodies. For many of the CF patients a similar IgG antibody response to both a-type and b-type flagella was observed. A pronounced antibody response to either a- or b-type flagella was also observed for some CF patients. The difference in absorbance readings between chronically colonized patients and non-*P. aeruginosa* colonized CF patients or healthy controls was very highly significant ($P < 0{\cdot}001$, by the Student t test) for type-a and -b flagella; the

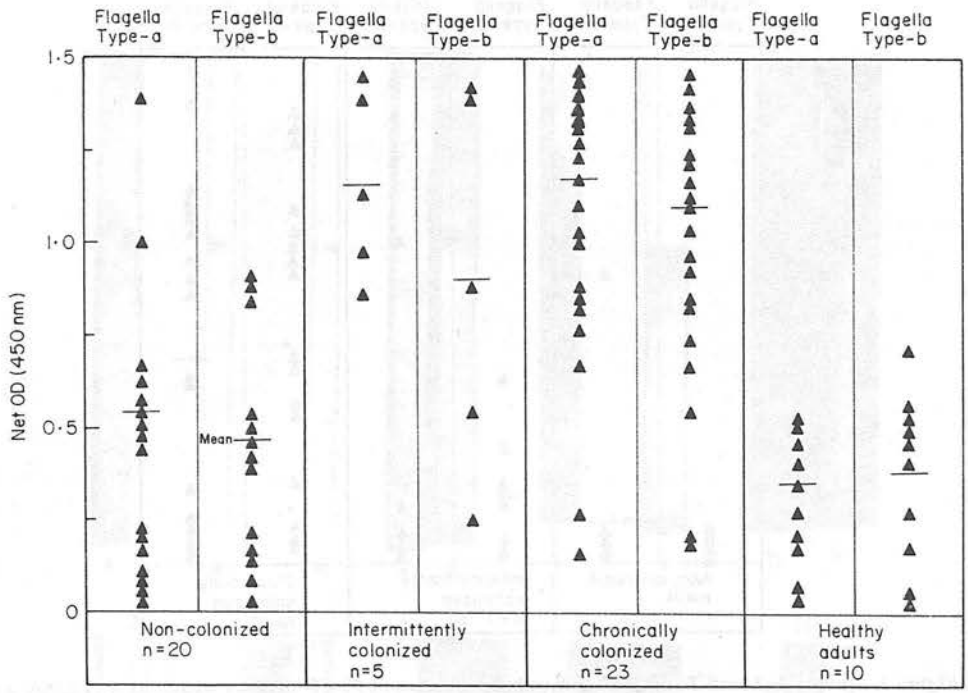

Figure 2. Serum IgG anti-*P. aeruginosa* flagellar antibodies in 48 CF patients and 10 non-CF individuals measured by ELISA with flagellar type-a and type-b as coating antigens. A total of 23 CF patients chronically colonized with *P. aeruginosa*, five intermittently colonized, 20 non-colonized CF patients and 10 healthy adults are included.

difference in OD readings between intermittently colonized CF patients and healthy controls was very highly significant ($P<0{\cdot}001$) for type-a flagella and highly significant ($P<0{\cdot}01$) for type-b flagella; the difference between intermittently and non-*P. aeruginosa* colonized patients was highly significant ($P<0{\cdot}01$) for type-a flagella and significant for type-b flagella.

Anti-flagellar IgA antibodies were found in the saliva and sputum from a number of patients with CF who were chronically or intermittently colonized with *P. aeruginosa* (Figures 3 and 4). With the exception of one individual, anti-flagellar IgA antibodies were not found in saliva or sputum from CF patients with no previous history of *P. aeruginosa* colonization. In some patients a similar IgA response to both flagella types was noted whilst in others a preponderance of IgA antibodies to either a- or b-type flagella was observed. The difference in OD readings between chronically colonized patients and non-*P. aeruginosa* colonized patients was very highly significant ($P<0{\cdot}001$); in the case of saliva the difference was significant ($P<0{\cdot}05$). The difference between intermittently colonized and non-*P. aeruginosa* colonized patients for sputum anti-flagellar type-a and -b IgA antibodies was highly significant ($P<0{\cdot}01$); the difference for the saliva results was not significant.

Immunoblot analysis of sera, saliva and sputa from patients with CF against *P. aeruginosa* flagella type-a and -b was performed and is shown in Figures 5 and 6. These immunoblot studies reflected the results obtained in ELISA. Positive blots with a response to either one or both *P. aeruginosa* flagella types were obtained with sera, saliva or sputa which showed elevated anti-flagella IgA/IgG antibodies in ELISA studies.

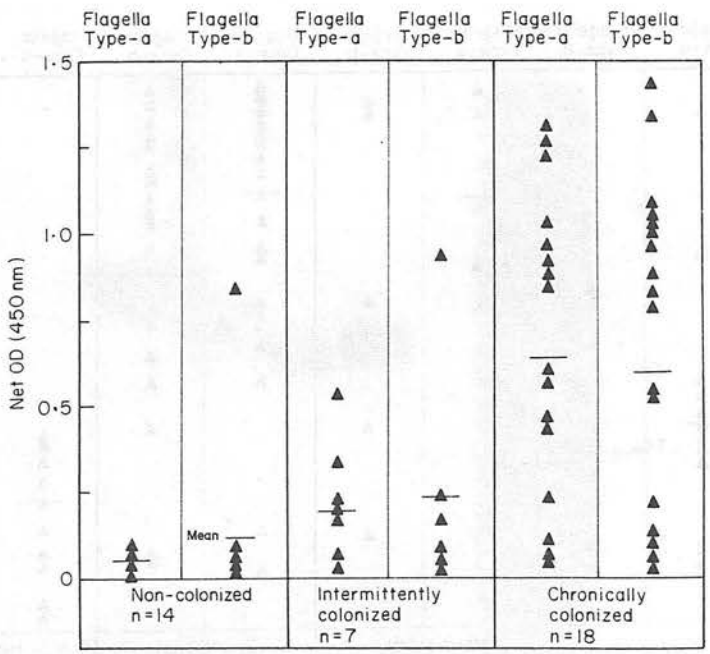

Figure 3. Sputum IgA anti-*P. aeruginosa* flagellar antibodies in 39 CF patients measured by ELISA with flagellar type-a and -b as coating antigens. A total of 18 CF patients chronically colonized with *P. aeruginosa*, seven intermittently colonized patients and 14 non-colonized CF patients are included.

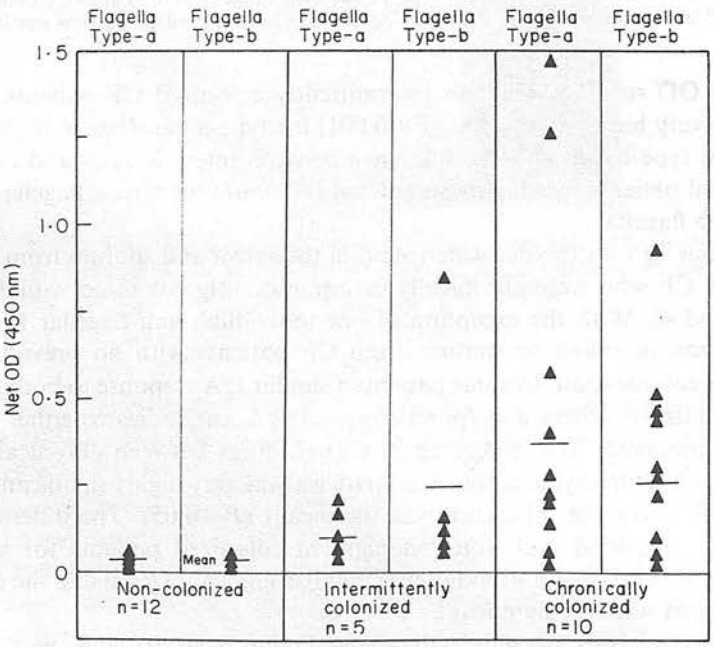

Figure 4. Saliva IgA anti-*P. aeruginosa* flagellar antibodies in 27 CF patients measured by ELISA with flagellar type-a and type-b as coating antigens. A total of 10 CF patients chronically colonized with *P. aeruginosa*, five intermittently colonized patients and 12 non-colonized CF patients are included.

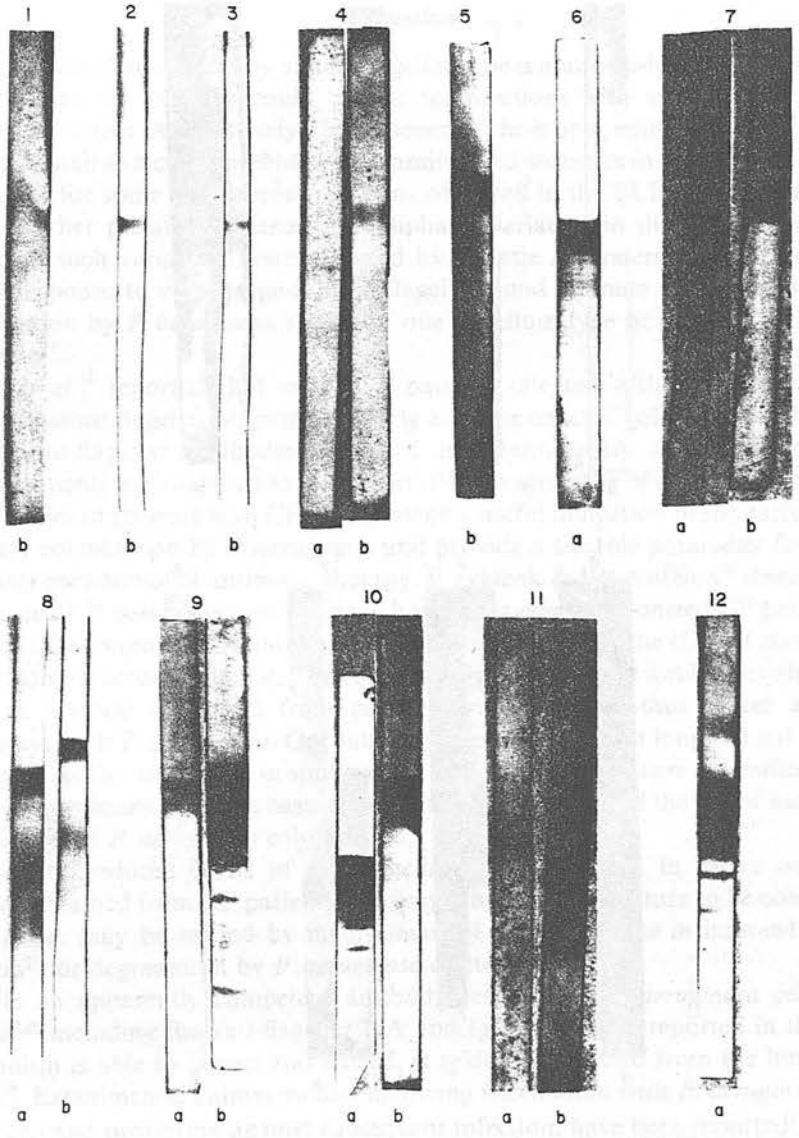

Figure 5. Strip immunoblots of isolated *P. aeruginosa* flagella (type-a and/or type-b) reacted with serum from patients with CF and analysed for the presence of anti-flagellar IgG antibodies. Strip 1, healthy control; Strip 2, CF non-col.; Strip 3, CF non-col.; Strip 4, CF col.; Strip 5, CF col.; Strip 6, CF int. col.; Strip 7, CF int. col.; Strip 8, CF int. col.; Strip 9, chron. col.; Strip 10, chron. col.; Strip 11, chron. col.; Strip 12, chron. col.

(CF non-col. = CF patient non-*P. aeruginosa* colonized; CF col. = CF patient colonized with non-mucoid *P. aeruginosa*; CF int. col. = CF patient intermittently colonized with non-mucoid *P. aeruginosa*; CF chron. col. = CF patient chronically colonized with mucoid *P. aeruginosa*).

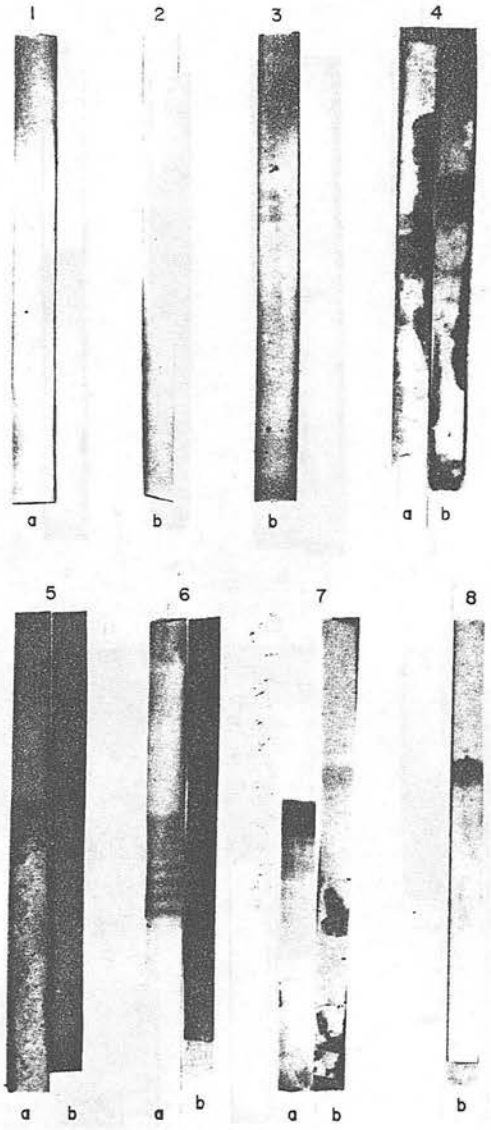

Figure 6. Strip immunoblots of isolated *P. aeruginosa* flagella (type-a and/or type-b) reacted with saliva or sputum from patients with CF and analysed for the presence of anti-flagellar IgA antibodies. Strip 1, CF non-col. (Sputum); Strip 2, CF int. col. (Sputum); Strip 3, CF int. col. (Saliva); Strip 4, CF int. col. (Sputum); Strip 5, CF int. col. (Sputum); Strip 6, chron. col. (Sputum); Strip 7, chron. col. (Sputum); Strip 8, chron. col. (Saliva).

(CF non-col. = CF patient non-*P. aeruginosa* colonized; CF int. col. = CF patient intermittently colonized with non-mucoid *P. aeruginosa*; CF chron. col. = CF patient chronically colonized with mucoid *P. aeruginosa*).

Discussion

The observation of similar levels of anti-flagellar type-a and type-b antibodies observed in many of the CF patients could be due to infections with at least two different *P. aeruginosa* strains. Alternatively, the presence of the b or a_o epitope in all 17 flagellar serotypes[7] as well as a common N-terminal amino acid sequence in the flagellin protein[10] may account for some of the cross-reactions observed in the ELISA and immunoblot studies. Another possible explanation is diphasic variation in the flagellar antigens[23] although no such variations were reported by Montie & Anderson[24]. A pronounced antibody response to either type-a or -b flagella found in some CF patients indicate either infection by *P. aeruginosa* strains of one flagellum type or perhaps by only one single strain[25].

Shand *et al.*[14] reported that many CF patients infected with *P. aeruginosa* show antibodies against flagellar preparations early after the onset of colonization. The raised levels of anti-flagellar antibodies observed in intermittently and sometimes non-colonized patients adds support to this report. ELISA screening of anti-flagellar IgA and IgG antibodies in patients with CF may provide a useful indication of the early onset of pulmonary colonization by *P. aeruginosa* and provide a suitable parameter for instigation of anti-pseudomonal antibiotic therapy. Przyklenk & Bauernfeind[26] demonstrated the presence of *P. aeruginosa* antibodies in non-*P. aeruginosa* colonized CF patients and concluded that secretory IgA antibodies correlated better with the clinical status of the patients than the serum response. The anti-*P. aeruginosa* flagellar antibodies observed in saliva and sputum specimens from patients with CF may thus reflect a current exacerbation with *P. aeruginosa*. Our future studies will focus on longitudinal monitoring of anti-flagellar antibodies in sputum and saliva from CF patients classified as non-*P. aeruginosa* colonized (on the basis of sputum bacteriology) and the use of such studies for prediction of *P. aeruginosa* colonization.

Absence or reduced levels of anti-flagellar IgA antibodies in saliva or sputum specimens obtained from CF patients, confirmed by bacterial culture to be colonized by *P. aeruginosa*, may be caused by involvement of the antibodies in immune complex formation[27], or degradation by *P. aeruginosa* elastase[28].

Despite an apparently competent antibody response to *P. aeruginosa* cell surface antigens[29,30] including the anti-flagellar IgA and IgG antibodies reported in this study, the organism is able to persist and indeed, is seldom eradicated from the lungs of CF patients[31]. Experimental animal models involving vaccination with *P. aeruginosa* flagellar antigens and protection against subsequent infection, have been reported[11–13]. Anti-flagellar antibodies may act by simple inhibition of the motility of *P. aeruginosa*[10,32] or by opsonophagocytosis of the organism[9,10]. The role of mucosal immunity and the protective capacity of IgA antibodies is not well understood. In patients with CF an antibody response to flagella is likely to be manifested at an early stage of colonization/infection since motility, chemotaxis and expression of flagellar appendages appears to be less important during chronic infection[33].

Since motility of *P. aeruginosa* may be important in early lung colonization of patients with CF, vaccination with a bivalent vaccine consisting of a- and b-type flagella or passive treatment with flagellar antibodies[10] would appear rational, but as yet unproven, strategies for prevention of colonization. Vaccination of patients already harbouring *Pseudomonas* may exacerbate pulmonary deterioration[34] as a result of immune complex mediated tissue damage[35]. However, strategies to improve pulmonary clearance via the

muco-ciliary escalator and the presence of pre-existing mucosal antibodies induced by immunization, may combined, present a more formidable barrier to initial colonization by *P. aeruginosa*.

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Role of the Far-Upstream Sites of the *algD* Promoter and the *algR* and *rpoN* Genes in Environmental Modulation of Mucoidy in *Pseudomonas aeruginosa*

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The role of several regulatory elements in environmental modulation of mucoidy in *Pseudomonas aeruginosa* was studied. Transcriptional activation of *algD*, necessary for the mucoid phenotype, was found to depend on FUS, the newly identified far-upstream sites of the *algD* promoter. The FUS were delimited to a region spanning nucleotides −432 to −332 relative to the *algD* mRNA start site. Insertional inactivation of *algR* in PAO568 abolished the *algD* promoter response to nitrogen availability and greatly diminished but did not completely eliminate reactivity to changes in salt concentration. Insertional inactivation of *rpoN* (*ntrA*) in PAO568 did not affect *algR* and *algD* transcription.

Mucoid colony morphology is an important virulence determinant (14) and a convenient marker for analysis of the mechanisms governing adaptation of *Pseudomonas aeruginosa* (10) to the conditions in the lungs of cystic fibrosis patients (3, 27). Several genes have been shown to play a role in the emergence of mucoid strains (*muc* and *algST* [12, 13, 20]), in alginate synthesis (such as the key biosynthetic gene *algD* [6]), and in transcriptional regulation of the *algD* promoter (*algP*, *algQ*, and *algR* [7, 9, 19]). The *algR* gene product is homologous (5) to the regulators from a class of transcriptional factors collectively termed environmentally responsive two-component systems (24). Using a series of isogenic mucoid derivatives of the standard genetic strain PAO, we have recently shown that *algD* promoter activity can be modulated by changing the nitrogen source and osmolarity of the medium (8). A survey of over 50 mucoid isolates from cystic fibrosis patients confirmed that a substantial fraction of clinical strains responded phenotypically to these factors. (D. W. Martin, J. R. W. Govan, and V. Deretic, unpublished results).

A well-known site for analysis of the regulation of the alginate system at the transcriptional level is the *algD* promoter (7, 9, 10, 18, 19). This promoter is strongly activated in mucoid cells (7). To investigate the mechanisms governing environmental regulation of mucoidy in response to different stimuli, we mapped the physical sites on the *algD* promoter that were required for these responses. A question asked was whether the same or different sites of the *algD* promoter would be required for the actions of different environmental signals. In most of the transcriptional fusion studies reported so far, the entire 1.2-kb *Hin*dIII-*Eco*RI fragment (18) or even larger DNA fragments (1, 7) with the *algD* mRNA start site have been used. In this work, we produced a series of consecutive deletions (4) from the distal (*Hin*dIII) end of this fragment relative to the mRNA start site and cloned them in front of the *xylE* gene on the transcriptional fusion vector pVDX18 (18). The constructs obtained were conjugated (18) into strain PAO568.

The nucleotide sequence of most of the 1.2-kb *Hin*dIII-*Eco*RI fragment containing the *algD* promoter from the PAO chromosome was determined by the chain termination method (25) (Fig. 1) (GenBank accession number M37205). When the clones of consecutive deletions placed upstream of the *xylE* reporter gene were assayed (18) in PAO568 for activity on *Pseudomonas* isolation agar, a region far upstream from the *algD* mRNA start site (beginning at least 432 bp upstream from the mRNA initiation site) was found to be essential for activation of the *algD* promoter (Fig. 1). Next, the same set of transcriptional fusions was assayed for their activation in response to nitrogen limitation and increases in sodium chloride concentrations. Strain PAO568 has been previously shown to undergo a 20- to 30- fold increase in *algD* transcription in response to the shift from ammonia- to nitrate-based media (8). Similar, dose-dependent, effects have been observed in response to increasing NaCl concentrations (8). These factors were tested by using the same set of deletion mutants (see Fig. 1 for the precise locations of the 5′ ends of the deletions). In both cases, the same region beginning at least 432 bp upstream of the mRNA start site was found to be required for full activation (Fig. 2). Further reduction in activity was observed when an additional 100 bp was removed, essentially reducing the *algD* promoter activity to 20% of its full capacity. Deletion of the region to −116 from the mRNA start site additionally lowered the residual induction level to below 10% of the activity of the full-size promoter. Thus, a region located unusually far upstream from the mRNA 5′ end for a procaryotic promoter was found critical for activation of the *algD* transcription. These findings indicate the presence of sequences that act at a distance, resembling enhancerlike elements similar to those previously identified in the *glnA* and *nif* promoters (2, 23). However, the sites in *algD* are naturally positioned further upstream than the locations of the *glnA* and *nif* upstream elements (2, 23). Moreover, the same region (termed FUS, for far upstream sites) was required for full activation in response to both types of environmental factors studied (nitrogen source and salt concentration). This finding sug-

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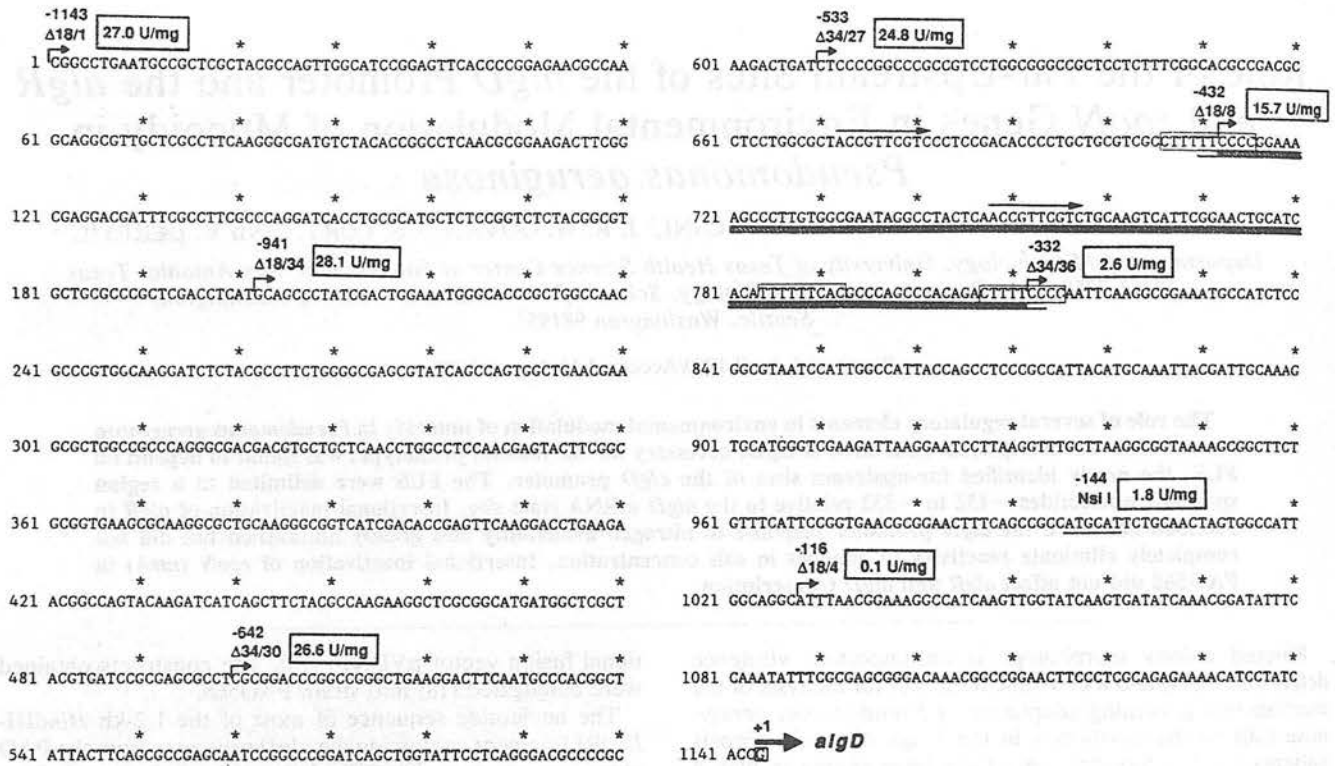

FIG. 1. Nucleotide sequence of the DNA region upstream of the *algD* mRNA start site. The 1,143-bp sequence of a region from the *Hin*dIII-*Eco*RI DNA fragment containing the *algD* promoter from *P. aeruginosa* PAO is shown. Nucleotides are numbered from the beginning of the sequence. Additional numbering (negative values) is given relative to the *algD* mRNA start site (+1, marked with a thick arrow). Bent arrows indicate the 5′ endpoints of the deletion products (designations of deletions are given above the arrows) used in transcriptional fusion studies. The numbers in boxes represent typical values of catechol 2,3-dioxygenase when the deletions were fused to a promoterless *xylE* gene on the transcription fusion vector pVDX18 (18) and the strains were grown on *Pseudomonas* isolation agar. One unit is defined as the amount of enzyme that can degrade 1 μmol of catechol in 1 min at room temperature. The region encompassed by deletions Δ18/8 and Δ34/36 is underlined to indicate the minimal region spanned by the FUS. Three polypyrimidinic blocks (see text) are boxed.

gested the existence of identical or closely positioned sites where a putative activator or activators acted to assist transduction of two disparate environmental signals into transcriptional activation events.

In support of the view that FUS may bind soluble factors is the finding that removal of FUS relieves the chromosomal *algD* gene from the multicopy inhibition by the plasmid-borne *algD* promoter sequences present in *trans* (data not shown). This effect was previously observed with the full-size *algD* promoter (8) cloned on a relatively high-copy-number (IncQ) transcriptional fusion vector, pVDX18 (19), also used in this study. Inspection of the sequences located around the threshold point where the most significant drop in activity was observed did not reveal striking homologies with the known binding sites of other activator proteins. However, several regions (not shown) displaying marginal similarities to the VirG-, OmpR-, NtrC, and catabolite gene activator protein-binding sites (11, 16, 21, 23) and three relatively long polypyrimidine tracts (Fig. 1) were observed.

Although the *algR* gene has been shown to be essential for the mucoid phenotype and transcription of *algD*, its direct involvement in the induction of *algD* in response to environmental stimuli in *P. aeruginosa* has not been previously demonstrated. It was also of interest to determine whether different environmental cues would require *algR* for their action or whether this gene could be eventually bypassed at the transcriptional level. To do so, we examined the effects of insertional inactivation of *algR* in PAO568 on the response of this strain to changes in salt concentration and nitrogen source. Strain CDM1/1 is a derivative of PAO568 that has the chromosomal *algR* gene insertionally inactivated (22). When an *algD-xylE* fusion with the full-size *algD* promoter was used to test the expression levels in CDM1/1, *algD* activity was abolished and, in the case of testing promoter induction in response to nitrogen availability, was no longer observable (Table 1). Thus, *algR* was essential for the transmission of signals generated by growth on nitrate in place of ammonia into activation of the *algD* promoter.

Although the overall levels of *algD* promoter activity were dramatically reduced, induction with salt was still observable in the strain CDM1/1 (Table 1). This residual increase of activity was very low, at least 30-fold less in its absolute level than what had been estimated to be necessary for cells to assume a mucoid phenotype (8), but was nevertheless detectable. It is also worth noting that this residual response of the full-size *algD* promoter fusion to the addition of salt in the inactivated *algR* mutant strain CDM1/1 is reminiscent of the similar response seen upon removal of the FUS in the parental strain PAO568 (*algR*$^+$) (Fig. 2). It therefore appears that either removal of FUS from the *algD* promoter or inactivation of *algR* on the chromosome had similar effects on the absolute levels of *algD* promoter activity, while both conditions permitted a very low but still detectable induction by the increased concentration of salt (severalfold). This effect therefore appears to be independent of *algR* or FUS and may be a part of a more generic promoter response to

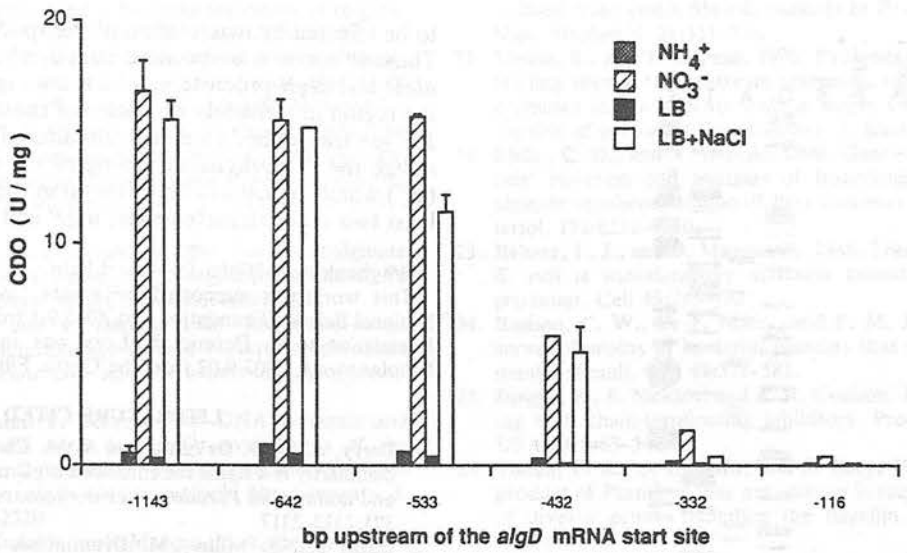

FIG. 2. Demonstration that FUS are required for full induction of the *algD* promoter in response to two different environmental signals. The deletion products of *algD* promoter (see Fig. 1) fused to a reporter gene, *xylE*, were introduced in *P. aeruginosa* PAO568 (*muc-2*), and promoter activity was measured on different media. Catechol 2,3-dioxygenase (CDO) activity is expressed as units per milligram of total cellular protein (see legend to Fig. 1) for cells grown on nitrogen-rich (▩) and nitrogen-poor (▨) media and on LB (■) and LB supplemented with 0.3 M NaCl (□). Bars indicate standard errors; where bars are absent, standard error values were beyond the resolution of the graph. The endpoints of deletions used to make fusions with *xylE* are given in negative numbers relative to their distance from the *algD* mRNA start site.

osmolarity changes via alterations in DNA supercoiling or possibly through other DNA-binding factors. These observations may also help explain reports of an extremely low-level (over 1,000 times lower than the lowest activities in *P. aeruginosa* reported here [1]) but reportedly detectable induction of the *algD* promoter on a plasmid in *Escherichia coli* grown in the presence of salt (1).

Since the *algD* and *algR* promoters are responsive to nitrogen limitation (8), and recent mapping of the *algD* and *algR* promoters indicated the presence of putative sequences resembling σ^{54}-utilized promoters (9), we wanted to determine whether *algD* and *algR* transcription was dependent on the gene product of *rpoN*, encoding the *P. aeruginosa* analog of the *E. coli* σ^{54} alternative sigma factor (15). We compared *algD* promoter transcription rates in the well-characterized strain PAO568 with the intact *rpoN* gene and with an inactivated gene. The *rpoN* gene was insertionally inactivated by the procedure described in the original report of the isolation and characterization of the *P aeruginosa rpoN* gene (15). As expected, the resulting *rpoN* derivative PAO568-N1 displayed a strong glutamine requirement when grown on minimal media. On *Pseudomonas* isolation agar, this strain was phenotypically mucoid. In addition, the strain responded to the addition of salt on LB by assuming a typical mucoid phenotype. When we measured activity of the *algD-xylE* fusion in PAO568-N1, no reduction in *algD* promoter activity was observed (Table 1). In addition, the induction levels obtained in this strain were the same or slightly higher in PAO568-N1 than in the parental strain PAO568. This finding indicated that *rpoN* was not required for *algD* transcription. In addition, *algR* was most likely significantly transcribed in this strain, since its transcription was required for the activation of *algD* (9). To assess this possibility, we assayed *algR* transcription in an *rpoN* background. Although measurable, the transcriptional levels of *algR* are in general significantly lower than the levels of *algD* transcription (9). For this reason, we determined the levels of *algR* transcription by direct measurement of *algR* mRNA, using S1 nuclease protection analysis. The levels of *algR* transcription were similar in PAO568 (*rpoN*$^+$) and its *rpoN* mutant derivative PAO568-N1 (Fig. 3). The 5′ ends of the transcripts matched the previously mapped *algR* mRNA start site (9). Transcription from this promoter is strongly regulated, and the mRNA initiation site is identical in mucoid PAO derivatives and in cystic fibrosis isolates (8, 9). The bands corresponding to the 5′ ends of the *algR* mRNAs were identical in both the *rpoN* and *rpoN*$^+$ backgrounds, which ruled out the possibility that this transcript was

TABLE 1. Effects of insertional inactivation of *algR* and *rpoN* on the induction of the *algD* promoter in response to environmental stimuli

Strain[a]	Relevant properties	CDO activity (U/mg)[b]			
		LB	LB + NaCl	NH_4^+	NO_3^-
PAO568	*muc-2*	7.3	23.8	1.8	8.1
CDM1/1	*muc-2 algR*	0.06	0.24	0.07	0.09
PAO568-N1	*muc-2 rpoN*	3.3	30.6	ND[c]	ND

[a] Strains PAO568 and CDM1/1 harbored the *algD-xylE* transcriptional fusion plasmid pCMDG1 (a modified pPAOM3 conferring gentamicin resistance); strain PAO568-N1 contained the *algD-xylE* transcriptional fusion plasmid pPAOM3 (18). All fusions contained the identical 1.2-kb *Hin*dIII-*Eco*RI fragment of *algD*.

[b] Catechol 2,3-dioxygenase (CDO) activity was determined in sonic extracts of cells grown under conditions as shown: LB, plain LB medium; LB + NaCl, LB supplemented with 0.3 M NaCl; NH_4^+, minimal medium (8) with 0.2% ammonium sulfate as the nitrogen source; NO_3^-, minimal medium (8) with 0.2% potassium nitrate as the nitrogen source. Extract preparation, enzyme assay, and definition of units were described previously (8). Standard error was below 30% (see also Fig. 2).

[c] ND, Not determined; PAO568-N1 could not grow on the minimal medium used to test the effects of nitrogen availability unless glutamine was supplemented, a condition known to interfere with the transcription of *algD* in the parental strain PAO568 (8).

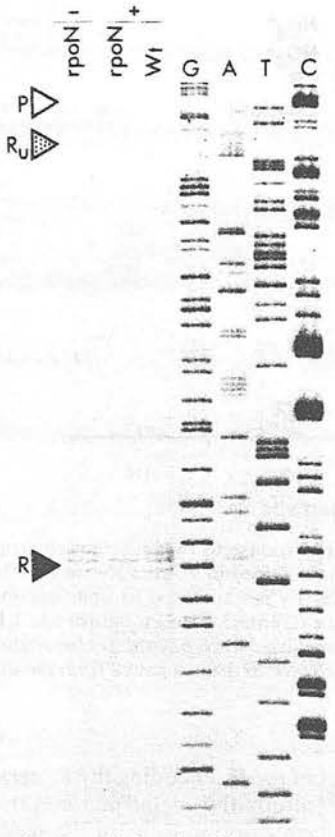

FIG. 3. S1 nuclease analysis of *algR* transcription in the *rpoN* background. Two isogenic strains, PAO568 (Wt) and its *rpoN* derivative PAO568-N1 (rpoN) were grown in LB without (−) or with (+) the addition of 0.3 M NaCl. A 100-μg sample of RNA was used for hybridization with the uniformly labeled single-stranded probe, treated with S1 nuclease, and run on a sequencing gel alongside the *algR* sequencing ladder (GATC). P, Position of the untreated *algR* probe; R_u, position of a transcript from an *algR* upstream promoter (22); R, position of the band corresponding to the previously mapped *algR* mRNA start site (9).

dependent on the *rpoN* gene product (Fig. 3). These results are in agreement with the previously published observations for *rpoN* mutants of several mucoid cystic fibrosis isolates which did not change their phenotypes (26) and are in a sharp contrast with a recent study by Kimbara and Chakrabarty, who reported that inactivation of *rpoN* abolishes transcription of *algD* and *algR* (17). The latter experiments were performed by measuring activities of transcriptional fusions on plasmids in a nonmucoid *P. aeruginosa* strain, PAK. On the basis of the results presented here, including the direct determinations of the mRNA start sites in the *rpoN* background (Fig. 3; data for *algD* not shown), it appears that σ^{54} is not involved in the high-level transcription of *algD* and *algR*. It is also important to note that there is another *algR*-specific transcript initiating at a site further upstream of the *algR* proximal promoter. The initiation site of this transcript is at least 80 bp upstream of the previously mapped *algR* promoter. Transcription from this promoter is constitutive (22) and, unlike transcription from the *algR* proximal promoter, is not dependent on the mucoid status of the cell. However, even the possible inclusion of this promoter in the transcriptional fusion studies cannot help explain the results of Kimbara and Chakrabarty (17), since transcription from this upstream promoter does not appear to be affected by inactivation of the *rpoN* gene (Fig. 3, R_u). Thus, it remains to be determined whether the regulated *algD* and *algR* promoters, which are highly homologous in the region immediately upstream of the mRNA start site (9, 10), are transcribed by a different alternative sigma factor or utilize the *P. aeruginosa* analog of the major sigma factor (σ^{70}) which appears to be involved in the transcription of at least two other alginate genes, *algP* and *algQ* (19).

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REVIEWS IN MEDICAL MICROBIOLOGY

The microbiology and therapy of cystic fibrosis lung infections

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Summary. The literature dealing with the microbiology and therapy of respiratory infections in cystic fibrosis (CF) patients is extensive and contains much controversy. There is general agreement on some topics e.g. the major role played by respiratory pathogens, and by mucoid *Pseudomonas aeruginosa* in particular, in progressive lung disease. There remain, however, considerable doubts on specific mechanisms of pathogenesis; the importance of synergistic interplay between individual pathogens; and the most effective antibiotics, their mode of delivery, dosage, treatment duration and clinical assessment. Our aim is to provide a general overview of the microbiology and antimicrobial therapy of CF, to address topics of major interest or controversy and to attempt to delineate areas for future research.

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal inherited disorder among Caucasians with an incidence of approximately 1 in 2500 live births and a carrier frequency calculated as 1 in 20. The disease is typically characterised by high electrolyte concentrations in sweat, pancreatic insufficiency and production of abnormally viscid bronchial secretions leading to chronic progressive pulmonary disease, exacerbated by microbial infections. When CF was first described in the 1940s patients seldom survived infancy. Today, the prognosis has improved considerably and many patients survive to adulthood. The last decade has seen major advances including increasingly potent antimicrobial agents, heart-lung transplants and location of the CF gene on chromosome 7. The pathophysiology of the disease remains obscure but accumulating evidence indicates a defective Cl^- ion transport in CF epithelial cells.

The CF respiratory mucosa presents a different environment to potential microbial pathogens from that in non-CF patients. As a direct or indirect consequence of the basic defect, mucociliary clearance is impaired by the viscid dehydrated nature of the mucosal secretions. The volume and viscosity of the bronchial mucus are also enhanced by additional factors including the presence of DNA from resident bacteria and from inflammatory immune cells, and raised concentrations of electrolytes in particular Na^+ and Ca^{2+}. Thus the major determinant responsible for morbidity and mortality in CF patients is the outcome of a biological jigsaw puzzle comprising the effects of the basic defect, the therapeutic stratagems and the pathological sequelae of progressive lung disease and respiratory infections (Fig. 1).

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MICROBIOLOGY OF LUNG DISEASE IN CYSTIC FIBROSIS

'The microbiology of lung disease in patients with CF is a sub speciality unto itself'.[1] As Stutman and Marks so aptly describe, the microbiological problems associated with the management of CF patients do not easily bear generalisation but can be summarised as follows: i) Infections are localised in the lungs and in particular the major and minor airways rather than the alveoli; localised infections at non-pulmonary sites or systemic infections are rare. ii) Pulmonary infections are associated with insidious and eventually chronic colonisation by bacterial pathogens, and episodes of acute debilitating exacerbations due to both bacteria and viruses, superimposed upon progress-

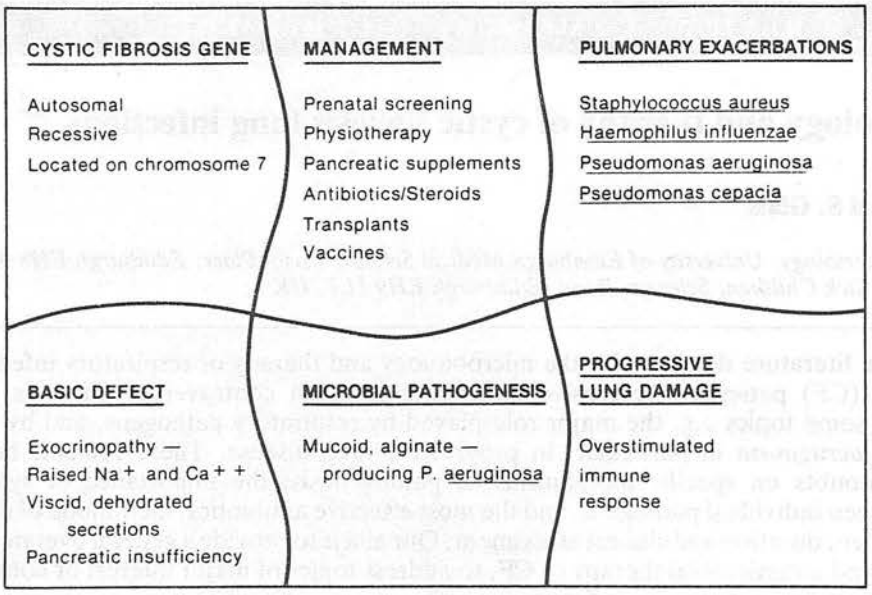

Fig.1 The biological jigsaw puzzle of CF. Factors which contribute to morbidity and mortality in CF patients include: the genetic defect, the pathophysiology of pancreatic and bronchial secretions, respiratory infections, the adverse stimulation of pulmonary immune responses and the success of therapeutic interventions.

ive lung disease. iii) The spectrum of bacteria, viruses and fungi associated with respiratory infection in CF patients is comparatively restricted. The respiratory pathogens most commonly isolated include *Staphylococcus aureus, Haemophilus influenzae* and *P. aeruginosa*. iv) The improved longevity of CF patients has revealed that susceptibility of individual CF patients to particular bacterial pathogens is usually age-related and microbial colonisation tends to follow a sequence during a patient's lifetime. In general, the sequence begins with *Staph. aureus* colonisation in infancy followed by *H. influenzae* in the early years with the highest incidence of *P. aeruginosa* occurring in adolescence.

Staph. aureus

In the first bacteriological study of CF lung disease[2] postmortem cultures of lung tissue from 14 CF patients aged 1 to 40 months, yielded *Staph. aureus* from 12 patients and single isolations of *H. influenzae* and *P. aeruginosa* (*Bacillus pyocyaneus*); as a portent of future microbiological developments the single isolate of *P. aeruginosa* was from one the oldest patients who had died aged 3 years. The predominance of *Staph. aureus* in early studies led to a widespread conviction that this species was not only the major pathogen in CF but that colonisation by *Staph. aureus* was an important precursor of subsequent colonization by *H. influenzae* and *P. aeruginosa*.

Staph. aureus remains an important cause of respiratory distress and progressive damage in CF patients; the pathogenesis of such infections is not well understood but the organism exhibits a range of putative virulence factors including adhesins, coagulase, leucocidins and toxins.

H. influenzae

Respiratory exacerbations in CF patients due to non-capsulated, non-group B *H. influenzae* are probably underdiagnosed and their pathogenic significance underestimated in CF lung disease. Contributory factors are first the difficulty in differentiating colonisation with this uniquely human respiratory commensal from active infection, and second, the problems of culturing *H. influenzae* from sputum which may simultaneously harbour *Staph. aureus* and *P. aeruginosa*.

Laboratory identification of *H. influenzae* requires careful quantitative culture of homogenised and diluted sputum on an appropriate

medium (e.g. chocolate blood agar incorporating bacitracin) and incubation in an atmosphere of 5 to 10% CO_2. Detection of *H. influenzae* at $>10^6$ CFU/ml together with associated clinical and haematological parameters may be used to discount commensal oropharyngeal carriage. A non-cultural method involving immunoperoxidase-staining with monoclonal antibody directed against an outer membrane protein of *H. influenzae* has been developed and can be applied reliably for detection of *H. influenzae* in sputum.[3] Clear evidence of a pathogenic role for *H. influenzae* in episodes of exacerbation in CF have come from studies on the use of C-reactive protein as an indicator of pulmonary inflammation.[4] Some of the highest concentrations of C-reactive protein observed (>400 mg/l) were in association with acute exacerbations and culture of *H. influenzae* at 10^8 CFU/ml sputum. It has been suggested that initial colonisation of the lower respiratory tract by *H. influenzae* includes adhesion of non-capsulated bacteria to respiratory mucus and that pulmonary damage and further invasion is subsequently mediated directly by anti-ciliary activity of secreted bacterial factors and lipopolysaccharide, and indirectly, by induction of an overactive inflammatory response.[5]

P. aeruginosa

Respiratory colonisation by *P. aeruginosa*, in particular by variants exhibiting a mucoid phenotype, is widely accepted as the major microbial challenge in CF lung disease. Thus the complex association between this adaptable opportunistic pathogen and the CF lung environment merits detailed discussion.

Non-mucoid *P. aeruginosa* are troublesome pathogens causing localised or systemic infections in compromised hosts; such infections are often intractable to therapy and, in the case of septicemia, are associated with high mortality. In contrast, there is clear evidence that non-mucoid *P. aeruginosa* are of little clinical significance in CF respiratory infections, but probably play a critical role as a microbial reservoir to allow the emergence and selection of more adaptable phenotypes associated with progressive lung disease.

Non-respiratory infections caused by *P. aeruginosa* including septicemia are rarely encountered in CF patients. The isolation of mucoid *P. aeruginosa*, however, from sputum is almost diagnostic of CF in adolescents with chronic pulmonary disease and the emergence of the mucoid phenotype following initial asymptomatic colonisation with non-mucoid *P. aeruginosa* has acquired an ominous prognostic status. Once the mucoid form emerges in the lungs of CF patients the colonisation strain of *P. aeruginosa* is seldom permanently eradicated. In addition to the mucoid phenotype, *P. aeruginosa* isolates from CF patients often exhibit other unusual adaptations to the lung environment including serum sensitivity, loss of the O antigen of lipopolysaccharide and hypersusceptibility (at least in vitro) to a range of antibiotics including beta-lactams, quinolones and trimethoprim but not aminoglycosides.[6] *P. aeruginosa* is characteristically intrinsically resistant to many antibiotics; thus the hypersusceptible phenotype may seem contradictory and even suicidal. Hypersusceptibility is regulated by at least two chromosomal genes *bls* and *tps*;[7] the phenotype appears to be restricted to isolates from respiratory infections[8] but is not restricted to CF and can be found in non-CF patients with chronic airways diseases.[6,9]

In CF patients, the incidence of *P. aeruginosa* typically increases with age and most CF centres have experienced an inexorable rise in isolations over the last four decades to a level of 70 to 80% in adolescent patients. There are, however, significant differences in isolation rates reported from individual CF centres.[10,11] The relatively low rates of less than 30% reported from clinics in Melbourne and Edinburgh contrasts with higher rates elsewhere. It is likely that the factors underlying these differences are multifactorial and probably include the age distribution of the CF population and the antibiotic policies employed.

Another important issue which has been relatively neglected is why some CF patients become colonised by *P. aeruginosa* whilst other patients of the same age and attending the same centre remain free from colonisation for long periods. Two possible explanations merit discussion. First, some strains of *P. aeruginosa* may be more able to colonise the CF lung than others and hence the exposure to such 'colonising' strains increases the risk of colonisation. Second, some CF patients may be more susceptible to microbial colonisation because of additional genetic or environmental factors. In an attempt to address the first of these possibilities we need to consider the application of suitable methods for characterising individual strains of *P. aeruginosa* and the evidence available from epidemiological studies.

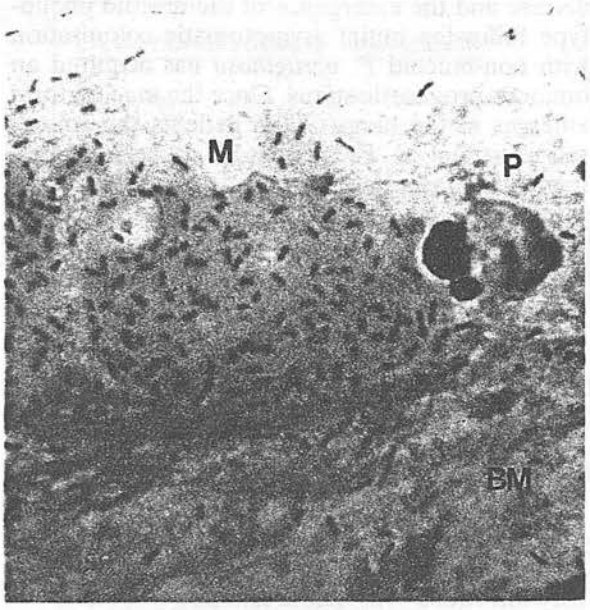

Fig.2 Gram-stained sputum from a CF patient harbouring mucoid *P. aeruginosa*. The bacteria can be seen in a gelatinous microcolony (M) which is attached to the bronchial mucosa (BM) and is significantly larger than the adjacent phagocyte (P).

Typing of P. aeruginosa — the relationship between non-mucoid and mucoid forms and the epidemiology of P. aeruginosa in CF

Serotyping is not a suitable method for characterising CF isolates of *P. aeruginosa* due to the difficulties presented by the copious bacterial exopolysaccharide and the high incidence of polyagglutinating strains with altered lipopolysaccharide structure.[12] However, the application of alternative typing methods with good discriminatory potential has provided important information on the natural history of *P. aeruginosa* in CF lung disease. Early evidence suggested that mucoid *P. aeruginosa* appeared to be derived from the original colonising strain rather than arise by acquisition from an external source. When the two forms were isolated from the same sputum they usually belonged to the same pyocin type.[13] Subsequently, ongoing longitudinal studies in the authors' laboratory using a revised pyocin typing system[6,14] and by Ogle and colleagues in Denver using a DNA-based technique incorporating Southern hybridisations[15] have confirmed the clonal relationship between the original non-mucoid colonising strain and subsequent phenotypic variants. The application of these typing systems, alone or in combination, has also provided additional epidemiological data relevant to CF management. First, cross-infection between patients attending the same CF centre is not common, with the exception of CF siblings. Second, the majority of CF patients remain colonised with a single strain of *P. aeruginosa* for long periods. New epidemiological data suggests that a subpopulation of *P. aeruginosa* might have a predilection or an enhanced ability to colonise the lungs of patients with chronic airways disease. This could assist in the identification of bacterial and host colonising factors.[16,17]

These ongoing epidemiological studies have important implications for the management of CF respiratory disease, in particular the policy in some CF centres to segregate patients colonised with *P. aeruginosa* from patients who are pseudomonas-free. It could be argued that if careful epidemiological monitoring excludes the occurrence of cross-infection, then a policy of segregation is unnecessary and indeed probably adds further anxiety for patient and clinician in this already stressful disease.

Growth of P. aeruginosa in the CF lung

In contrast to acute pulmonary infections in non-CF patients in which *P. aeruginosa* are observed, by microscopy, to be dispersed throughout the sputum, the mucoid *P. aeruginosa* present in CF sputum are often observed as microcolonies or biofilms adhering to the bronchial mucosa and posing formidable targets to phagocytic cells (Fig.2). It is possible to use the concept of a microcolony mode of growth, proposed originally in a wider environmental context,[18] to construct a model (Fig.3) for growth of mucoid *P. aeruginosa* on, or within, the CF bronchial mucosa. The model takes account of the unusual phenotypic properties expressed by CF isolates and the survival of the pathogen despite a hostile environment and aggressive antimicrobial therapy. The microcolony-frustrated phagocyte model and the mechanism of immune complex mediated tissue damage in CF during chronic *P. aeruginosa* colonisation have been discussed extensively elsewhere[6,19] and take into account the unusual clinical observations of the association of mucoid *P. aeruginosa* and the CF patient. First, the beneficial effect of anti-pseudomonal therapy

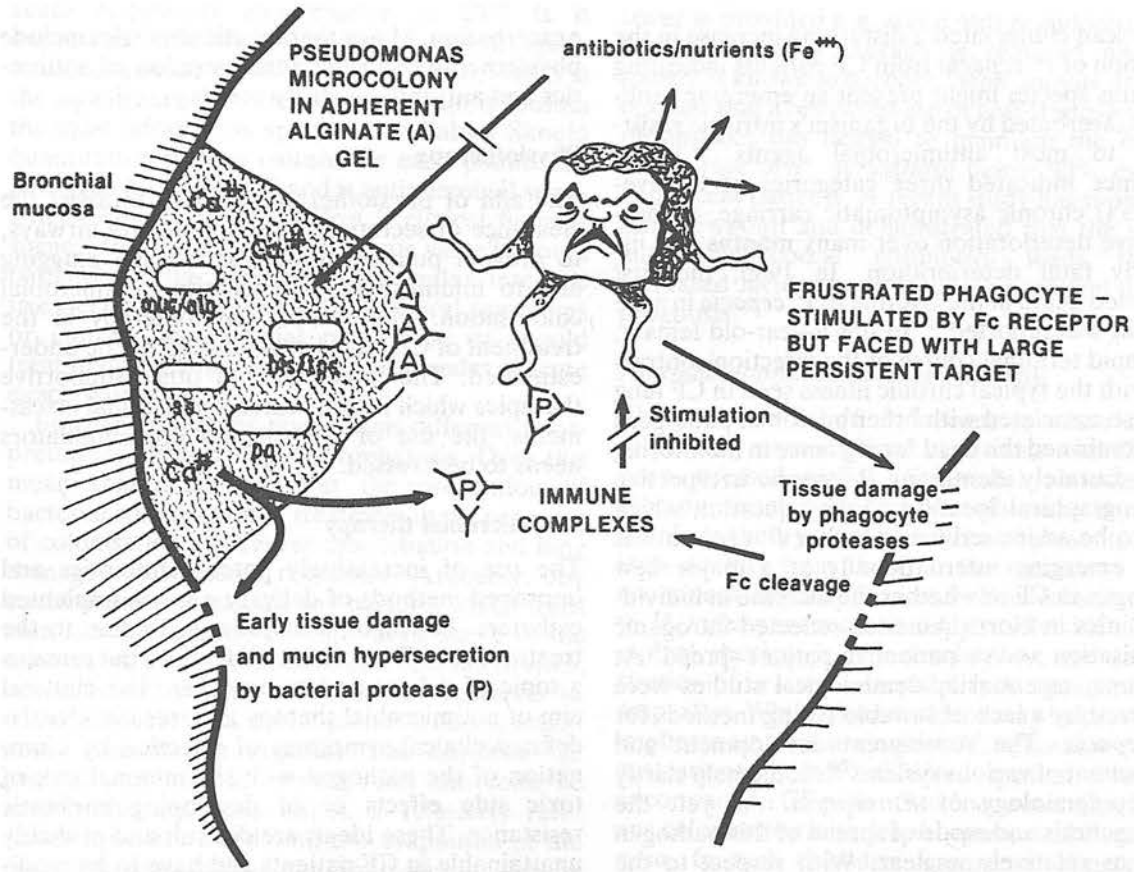

Fig.3 Microcolony mode for survival and pathogenesis of mucoid *P. aeruginosa* within a CF lung includes: attachment of bacterial alginate to bronchial mucosa and containment of bacteria exhibiting hypersusceptibility (*bls, tps*), serum sensitivity (ss) and polyagglutinating antigen (pa) within a Ca^{2+} dependent microgel composed of alginate, DNA and tracheobronchial mucin. Other properties of the bacterial microcolony consistent with in vitro and in vivo observations include, a physical and electrolyte barrier to antibiotics and reduced nutrient availability particularly with respect to iron, slow release of bacterial proteases (P) and rhamnolipids and a damaging immune-mediated response exacerbated by the size, antigenicity and persistence of the alginate matrix (A) and the indirect action of immune complexes associated with pseudomonas antigens. The model also incorporates direct tissue damage by neutrophil elastase and proposals to explain intermittent exacerbations based on Fc cleavage of immune complexes by proteases and inhibition of further phagocyte stimulation.[19] (from ref. 6).

despite failure to eradicate or even reduce the bacterial load; second, the periods of remission observed despite the presence of large numbers of bacteria; third, the progressive pulmonary damage which occurs in the presence of antibodies specific for alginate and other bacterial virulence factors; fourth, the adverse effects of vaccination when carried out in the presence of pseudomonas colonisation and fifth, the manner in which steroid therapy, a regimen which in other compromised patients is a classic precursor of opportunistic infection can paradoxically produce clinical benefit in CF patients.[20] Other important immunological factors include the demonstration of fragmented IgG immunoglobulins in CF serum and bronchial lavage fluid which would reduce opsonophagocytosis[21] and the failure of alveolar macrophages to prevent initial colonisation and subsequently the action of neutrophil elastase to cause damage both directly to lung parenchyma and indirectly through inhibition of lymphocyte response and further recruitment of elastase-releasing polymorphonuclear leukocytes.[22]

P. cepacia

In the early 1980s, reports from several North

American clinics cited a disturbing increase in the isolation of *P. cepacia* from CF patients indicating that this species might present an emerging problem exacerbated by the organism's intrinsic resistance to most antimicrobial agents. Clinical evidence indicated three categories of involvement: i) chronic asymptomatic carriage, ii) progressive deterioration over many months and iii) rapidly fatal deterioration. In 1986, the first recorded death in the UK due to *P. cepacia* in a CF patient was reported.[23] In this 9-year-old female, the rapid terminal course of the infection contrasted with the typical chronic illness seen in CF lung disease associated with other microbial pathogens and confirmed the need for vigilance in monitoring and accurately identifying *P. cepacia* irrespective of geographical location. A key question which had to be addressed was whether *P. cepacia* was truly emerging internationally as a major new pathogen in CF or whether the increase in individual clinics in North America reflected iatrogenic colonisation and/or patient to patient spread. At the time, essential epidemiological studies were hindered by a lack of suitable typing methods for *P. cepacia*. The subsequent development and assessment of various systems[24] should help clarify the epidemiology of *P. cepacia*. As yet, the pathogenesis and mode of spread of this pathogen remains relatively unclear. With respect to the incidence of *P. cepacia* in the UK, an ongoing longitudinal study in two CF centres commenced in 1986 has shown a stable incidence of less than 6% in all age groups (authors, unpublished).

Other pathogens in CF lung disease

The role of other (non-bacterial) pathogens in pulmonary exacerbations has been confirmed in several studies and the extent to which these agents contribute to exacerbations and progressive lung damage should not be underestimated. These pathogens include chlamydia and mycoplasmas, respiratory syncytial and influenza viruses and *Aspergillus fumigatus*.[32–34]

THERAPY OF RESPIRATORY INFECTIONS IN CF

Progressive lung disease exacerbated by intermittent debilitating respiratory infections is the main threat to both the quality of life and life expectancy in CF patients. Treatment is aimed at reducing or preventing pulmonary colonisation by microbial pathogens and at episodes of acute respiratory exacerbation. Major therapeutic strategies include physiotherapy and the administration of antibiotics and anti-inflammatory agents.

Physiotherapy

The aim of physiotherapy in CF is to assist the clearance of secretions from pulmonary airways, to prevent pulmonary obstruction and plugging and to minimise the opportunity for microbial colonisation. The role of physiotherapy in the treatment of CF lung disease should not be underestimated. The importance of other supportive therapies which include careful nutritional assessments, the use of oxygen and bronchodilators needs to be stressed.

Antimicrobial therapy

The use of increasingly potent antibiotics and improved methods of delivery e.g. via implanted catheters, has made a major contribution to the treatment of CF respiratory infections but remains a topic of debate and controversy. The classical aim of antimicrobial therapy is to resolve clearly-defined clinical symptoms of infection by elimination of the pathogen with the minimal risk of toxic side effects or of developing antibiotic resistance. These ideals are difficult and probably unattainable in CF patients and have to be modified to include clinical benefits which include maintenance of weight and lung function.[25]

There is little agreement on indications for optimum time of use, the choice of antibiotic, dosage schedules, duration of treatment and the method of delivery e.g. intravenous, oral or inhaled. Carefully controlled studies of the use and assessment of antibiotics in CF, either for the treatment of acute exacerbation or prophylaxis are difficult due to several factors. First, lack of clear parameters on which to base objective clinical and microbiological judgements; second, lack of a clear definition as to what constitutes an exacerbation; third, doubt on how antibiotic treatment affects acute infection as distinct from progressive lung disease; fourth, the complex and changing status of disease progression in individual patients; fifth, the pharmacokinetics involved in the distribution of antibiotics in CF patients and sixth, a lack of detailed understanding of the mechanisms of pathogenesis underlying infections with different microbial pathogens. Typical questions which must be addressed include: what constitutes clinical improvement following an

acute respiratory exacerbation in CF? Is it improved pulmonary function, weight gain, reduced sputum purulence and volume? What is the significance of bacterial culture and is sputum the most informative specimen available? Should quantitative sputum culture for each pulmonary pathogen be attempted and is antimicrobial sensitivity testing worth the effort if clinical findings suggest that it is of little prognostic value? Should patients receive antibiotics at regular intervals irrespective of the presence of clinical symptoms or culture of microbial pathogens, or should treatment be restricted to proven episodes of acute exacerbation?

Further difficulties result from different interpretations of terms e.g. i) Prophylaxis. Does this mean treatment aimed at the prevention of bacterial colonisation or treatment in the presence of colonisation to prevent exacerbation and lung damage? ii) Bacterial resistance. Arguably, one could define as resistant any bacteria which resist elimination from the CF lung irrespective of the agent used, or the value of MIC measured in vitro, or the concentration of antibiotic achieved in bronchial secretions. In CF patients, classic resistance, whereby an organism has acquired the genetic potential to destroy the antibiotic or modify the antibiotic target, is relatively rare. Instead, we are faced with the adaptation of the pathogen to withstand the antibiotic in vivo by various physiological methods including reduced permeability. In addition, in view of the difficulty of achieving high concentrations of antibiotics in CF bronchial secretions and particularly within areas of consolidation and abscess, the relevance of in vitro measurements of activity may be misleading.[25,26]

This review cannot solve the controversy nor provide the definitive antibiotic regimen but will attempt to discuss and clarify some of the 'grey areas' of antimicrobial therapy.

Anti-staphylococcal therapy

The antibiotic policy used against this pathogen differs in different treatment centres. Continuous long-term anti-staphylococcal therapy from first diagnosis in infancy to the age of 2 years or more is a key stratagem of CF management in some centres. In other centres, including those in Edinburgh, the policy is to treat only acute, bacteriologically-proven exacerbations due to *Staph. aureus*, although occasionally short term prophylactic cover is provided e.g. when anti-pseudomonal or anti-haemophilus therapy is required and the patient is known to harbour *Staph. aureus*. It is our opinion that a restricted use of anti-staphylococcal prophylaxis has helped to maintain the colonisation rate of *P. aeruginosa* below 40% even in adolescent patients. A recent study has supported this viewpoint and demonstrated that the use of anti-staphylococcal antibiotics leads to an increased incidence of *P. aeruginosa* and a poor prognosis.[27]

Anti-haemophilus influenzae therapy

The potential for *H. influenzae* to cause severe respiratory exacerbations in patients with CF has been stressed earlier. Successful antimicrobial therapy directed against *H. influenzae* is not as simple as theory would predict and presents problems distinct from those in the treatment of *P. aeruginosa* and *Staph. aureus*. Exacerbations due to *H. influenzae* are not easily resolved if antimicrobial therapy is based solely on the demonstration of in vitro susceptibility to agents such as ampicillin. Whilst clear evidence may be present to implicate ampicillin-sensitive *H. influenzae* at concentrations of $>10^6$ CFU/ml sputum it is not uncommon in CF patients to encounter simultaneous colonisation with *Staph. aureus* and *P. aeruginosa*. In such episodes, apparently appropriate therapy with a beta-lactam antibiotic may be antagonised by the other oganisms present. Even if other beta-lactamase-producing bacteria are absent, it is our experience that a more successful and reliable antimicrobial therapy against *H. influenzae* is by the use of ceftazidime. This third generation cephalosporin is not generally considered as an appropriate anti-haemophilus agent, nevertheless, where prior therapy of sensitive *H. influenzae* has failed, the use of ceftazidime invariably results in eradication of the pathogen from sputum and remission of respiratory symptoms within 2 or 3 days. The use of ceftazidime alone or in combination with an aminoglycoside or flucloxacillin is also worth consideration when large numbers of *H. influenzae* are cultured together with significant numbers of *Staph. aureus* or *P. aeruginosa*.

Anti-pseudomonal therapy

The development of anti-pseudomonal agents has been a major advance in CF management. For many years, high dose therapy with carbenicillin,

ticarcillin or azlocillin combined with an aminoglycoside (tobramycin or gentamicin), was first line treatment for exacerbations due to *P. aeruginosa*. Newer third generation cephalosporins, particularly ceftazidime, have shown monotherapy to be equally effective and similar results have been observed with aztreonam and the 4-quinolone ciprofloxacin, the first anti-pseudomonal agent available in oral form.

Despite the clinical efficacy of monotherapy and little clear evidence in favour of combined therapy, there remains a tendency to combine two anti-pseudomonal agents based on a theoretical rationale that combined therapy will reduce the emergence of multi-resistant strains. In some centres there is also a reluctance to use potent new anti-pseudomonal agents because of the risk of resistance. The validity of this long-term aim is debatable since in the short-term, sensitive strains are seldom, if ever, eradicated and as a consequence it could be argued that bacterial adaptation or resistance is a less serious consideration in treatment of CF patients. In practice, antibiotic resistance in *P. aeruginosa* is often temporary and reverts in most instances within weeks or months after treatment is stopped. Eventually, however, after repeated courses of antibiotic treatment which are a feature of CF therapy, there is a gradual decrease in bacterial susceptibility at least on the basis of in vitro tests. Do such 'resistant' strains provide an insuperable hazard in CF or are they less virulent? Resistant strains usually grow very slowly, produce small colonies and lose the ability to produce important virulence factors such as elastase.[6]

The use of nebulisers to deliver antibiotic aerosols to the bacterial targets within the lung is perhaps the longest standing therapeutic strategy in CF management. From the first descriptions of the use of nebulised penicillin in 1946 by Sant' Agnese and colleagues,[2] the benefits and disadvantages of aerosolised antibiotics in CF therapy has taken up a considerable proportion of the CF literature and conference debate. Provided suitably powerful nebulisers are used, there is undoubted evidence from controlled studies that this form of antibiotic administration results in improved lung function and reduces the number of acute hospital admissions in some CF patients.[28]

An important and strategically different use of nebulisers has been concerned with the attempts to prevent or control early colonisation by *P. aeruginosa* through the use of nebulised colomycin at the first isolation of non-mucoid *P. aeruginosa*.[29]

The results of these therapeutic studies serve to emphasise the real difficulties in CF in devising strategies which are suitable for all patients irrespective of age, disease status, the bacterial target, or in social terms the patient's degree of access to a major CF treatment centre.

Prospects for vaccination against *P. aeruginosa*

A long-term trial of a pseudomonas lipopolysaccharide-based vaccine in CF patients who were already colonised with mucoid *P. aeruginosa* was discontinued when it became clear that vaccinated patients were deteriorating more quickly than non-vaccinated patients.[30] Nevertheless, the development of a vaccine would be the ideal stratagem to prevent colonisation by *P. aeruginosa*. A successful vaccine would have to confer long-lasting local protection at the mucosal surfaces of the respiratory tract. Elucidation of the optimum bacterial immunogen(s) will require clearer understanding of the factors involved in asymptomatic colonisation and will probably include a combination of adhesins, alginate, elastase and cell-associated factors. Such a vaccine would be delivered to an already compromised host and thus presents a considerable challenge. The special requirements of vaccination to accommodate the unusual pathogenesis of *P. aeruginosa* in CF and current progress in the development of vaccines based on different strategies have been discussed elsewhere.[6,31]

Assessment of therapy

One of the problems associated with assessing the benefits of antibiotic therapy in CF patients is that clinicians and microbiologists caring for the CF patients are faced with the problem of what objective parameters are available to measure the severity of exacerbation and the efficacy of antimicrobial therapy.

In CF patients, the relatively clear clinical and microbiological parameters observed in acute respiratory infections in non-CF patients are not present. Cough, sputum production, fever, changes in chest X-ray and the presence of significant numbers of microbial pathogens (which are eradicated or reduced by appropriate antibiotic therapy) may not always be present. In each

patient or episode the course of exacerbation can be variable. Some patients will have increased cough and sputum with fever and X-ray changes; most patients will have decreased appetite and associated weight loss, malaise and decreased pulmonary function. However, a significant number of patients show no acute changes in chest X-ray which may already show significant pathology. Microbiological evidence, even if based on quantitative cultures from homogenised sputum, may also be unhelpful, particularly in patients who have harboured mucoid *P. aeruginosa* or *Staph. aureus* for several years or have undergone repeated courses of the same antibiotic. In these patients large numbers of bacteria ($>10^7$/ml sputum) may persist in the absence of clinical symptoms or following clinical response to antibiotic therapy.

When patients are hospitalised for treatment of an acute exacerbation the efficacy of antibiotic treatment is even more difficult to assess as other therapeutic factors associated with patient care (professional physiotherapy, dietary control, rest etc) undoubtedly assist recovery. Weight gain and pulmonary function provide objective measurements for short-term and long-term assessment respectively but these parameters do not always show significant improvement and may be unreliable. A final judgement on the degree of clinical recovery may have to rely on the patients response to 'How well are you?' or to various signs of clinical health e.g. exercise tolerance.

Serial measurements of acute-phase reactants such as C-reactive protein which can provide an objective haematological parameter of inflammatory response may assist this difficult problem in CF management.[4] Studies have shown that in the absence of exacerbation, C-reactive protein concentrations in CF patients are within normal limits (<10 mg/l). A raised concentration however is present at the onset of exacerbation and falls rapidly to normal values during antibiotic treatment and the resolution of clinical symptoms. C-reactive protein levels also fall when patients are treated with intravenous antibiotics at home thus confirming the contribution of antimicrobial therapy in the management of exacerbation. These results suggest that serial assays of acute-phase proteins provide a useful objective parameter in assessment of antibiotic therapy in CF patients, as an indicator of the response of the patient rather than the pathogen to antibiotic therapy.

FUTURE CONSIDERATIONS

Understanding of the natural history of respiratory infection in patients with CF and developments in antimicrobial therapy have made a major impact on morbidity and mortality. It is hoped that the benefits provided by the development of heart-lung transplants are not limited by the emergence of new problems of infection and patient care as CF patients assume a different immunosuppressed status and the risk of systemic infection. Other controversies remain unsolved. The role of nebulised antibiotics and prophylactic therapy against *Staph. aureus* are the subjects of ongoing trials.

To date, most pseudomonas orientated research in CF has been directed towards the pathogenesis and management of exacerbations caused by mucoid *P. aeruginosa*. There is, however, increasing awareness of the importance of early asymptomatic colonisation by non-mucoid *P. aeruginosa*. There is a need to address the question: why are *P. aeruginosa* so able to colonise the CF respiratory tract prior to the emergence of mucoid variants and to identify the bacterial and host factors involved. Recent evidence suggests that the gastrointestinial tract is not a significant reservoir of *P. aeruginosa* prior to pulmonary colonisation.[35]

Alginate biosynthesis by *P. aeruginosa* is recognised as a major factor in chronic pulmonary colonisation of CF patients. It is now appreciated that this unusual exopolysaccharide is produced at low levels in non-mucoid *P. aeruginosa* but 'switched on' to higher levels in mucoid variants. The observation by Deretic et al[36] that the *algR* gene which regulates mannose dehydrogenase, a key enzyme in alginate synthesis, belongs to a recently recognised class of environmentally responsive bacterial regulatory genes controlling bacterial reactions to osmolarity, nitrogen and phosphate limitation, is an important development and suggests that conditions within the CF lung may control some aspects of alginate biosynthesis in vivo.

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Pseudomonas cepacia: an appraisal for CF

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ABSTRACT

Bacteria can survive and multiply just about anywhere you care to think of. Although microscopic — you could line up 2,000 bacteria across a pin head — they are prolific growers and are capable of existing as enormous populations, sometimes equivalent to the spectators filling two million large sports stadia! The human lung is normally free of bacteria. Unfortunately, several species of bacteria have successfully developed means to avoid the normally efficient lung defences. Presently, there is concern in CF over the role of *Pseudomonas cepacia*. The precise role of this bacterium as a pathogen (a microbe capable of causing disease), and the manner by which it is acquired and transmitted are presently unclear. This lack of knowledge raises several important issues in particular the segregation of CF patients for social and therapeutic purposes.

General microbiology of CF

The CF lung presents a unique environment to potential microbial pathogens. In non-CF individuals, bacteria inhaled into the airways during respiration are directed onto mucus lining these airways and then moved upwards by the wave-like movement of underlying hair-like cilia; these bacteria are then swallowed and destroyed by the acidic contents of the stomach. Bacterial invaders able to overcome this mucociliary clearance are also faced with scavenger cells called phagocytes, which eat and destroy bacteria, and by specific immunological mechanisms including anti-bacterial antibodies.

As a consequence of the CF gene defect, mucociliary clearance of bacteria from the lung is impaired by the viscid dehydrated nature of the airway secretions. Furthermore, mucoid variants of *Pseudomonas aeruginosa* thwart the phagocytic cells by production of a gel-like substance called alginate, within which bacteria are protected from phagocytes and antibiotics. Thus the ability of bacteria to colonise the lungs of CF patients is the outcome of a biological jigsaw puzzle comprising the effects of the CF gene defect, the severity of underlying lung disease and the ability of individual bacterial species to overcome normally highly effective lung defences. As a consequence, relatively few bacterial species, *Staphylococcus aureus, Haemophilus influenzae* and *P. aeruginosa* are associated with pulmonary infections in CF.

In the early 1980s, reports from some North American CF centres cited a disturbing increase in the findings of *Pseudomonas cepacia* in sputa from CF patients, primarily young adults. The presence of *P. cepacia* in as many as 20 per cent of patients caused concern, not only because of the apparent increase in the number of colonized patients but also because of the clinical consequences. In some CF patients, long-term infection was associated with slowly declining lung function; in a few patients, rapidly fatal lung infection was observed. Although these reports are a cause for concern, it should be appreciated that a third group of CF patients colonized with *P. cepacia* had no detectable change in their clinical status. Because of the potentially serious consequences of colonization with *P. cepacia*, the Cystic Fibrosis Research Trust has set up a working party to investigate the problem in the United Kingdom and to address some of the social and clinical issues raised. The purpose of this article is to provide a brief description of *P. cepacia* and to discuss some of the issues which have arisen concerning the transmission and treatment of this relatively little known pathogen.

Involvement of *Pseudomonas cepacia* in CF

P. cepacia was first described in 1950 as a plant pathogen, in particular as the cause of soft rot of onion bulbs. The species is widespread in soil and water, and has a striking ability to grow in disinfectants and antiseptics.

P. cepacia is naturally resistant to most antibiotics in common use and can even utilise penicillin and herbicides as nutrients! Human infections with *P. cepacia* are rare, tending to occur only in patients with impaired defences or by acquisition in hospital from contaminated equipment. In recent years, perhaps due to evolving treatment procedures and increased life expectancy, young CF adults are particularly susceptible to *P. cepacia* colonization. Because of the characteristic resistance of *P. cepacia* to antibiotics, the management of *P. cepacia* colonization is a particular challenge and thus emphasis has been placed on preventing acquisition. The mechanism of transmission of *P. cepacia* and the risk of acquisition for individual CF patients remains unclear and is the subject of much debate. There is evidence, however, that the risk of *P. cepacia* colonization may be increased by (i) existing severe lung damage, (ii) having a sibling with CF who is also colonized, (iii) increasing age, (iv) hospitalization during the previous six months, and, in some studies, (v) treatment with aminoglycoside antibiotics.

Until recently, major problems with *P. cepacia* have been lack of (1) selective media to culture the organism from respiratory secretions and environmental surfaces; in a multicentre study reported in 1987, only 36 (32 per cent) of 115 laboratories cultured *P. cepacia* from sputum specimens containing the organism and (2) methods for typing ('fingerprinting') isolates to distinguish one strain from another.

Transmission of *P. cepacia*

Three factors suggest that person-to-person spread may be the primary means of transmission for *P. cepacia:* (i) the increased risk for patients with a colonized sibling, (ii) failure in some outbreaks to recover *P. cepacia* during extensive surveys of respiratory equipment, such as nebulisers or PEP masks, and hospital surfaces or to demonstrate a link between environ-

mental and human isolates, and (iii) the clustering of cases within some clinics together with the suggestion that colonization rates are reduced if colonized patients are isolated from non-colonized patients.

Attempts to demonstrate person-to-person transmission have yielded tantalising clues, but the extent of transmissibility among CF patients remains unclear. It is interesting to compare the mode of transmission of *P. cepacia* with that of *P. aeruginosa*; with *P. aeruginosa*, transmission between CF patients attending the same clinic is rare but sibling-sibling transmission is well documented. This suggests that acquisition requires close and continuous contact or alternatively, prolonged exposure to a common reservoir within the home environment. With the exception of sibling-sibling spread, direct evidence that *P. cepacia* is transmitted from person to person, either in hospital or in other settings where CF patients congregate, has been difficult to obtain. One of the reasons for this ignorance is the lack of knowledge regarding the properties and behaviour of this relatively little-known bacterium.

It is likely that *P. cepacia* can be acquired from environmental surfaces; this has been suggested for *P. cepacia* infections in non-CF patients. In some CF centres the organism has been located in the hospital environment suggesting that hospital spread may occur; in other centres, no potential transmission sources have been identified. Retrospective studies have indicated that colonized patients are more likely to have been hospitalized or exposed to individuals with *P. cepacia* infection within the three to six month period prior to their first positive culture. Based on this evidence, and fearing transmission between patients, some CF clinics have instituted procedures to separate colonized patients from non-colonized patients. Other investigators, however, have found no evidence of transmission of *P. cepacia* amongst hospitalized patients who shared rooms, socialized, exercised and ate meals together, and concluded that separation of patients may not be necessary.

The only convincing study to demonstrate person-to-person transmission of *P. cepacia* between CF patients was published in the *Lancet* in November 1990. LiPuma and colleagues relied on a selective culture medium and on a DNA-based typing system, known as ribotype analysis, to show that colonized patients attending the same CF centre harboured the same strain. Evidence was also produced for person-to-person transmission between two CF patients from different CF centres attending a summer educational camp. The two patients involved were in close contact for several days. However, none of the other 14 young adult CF patients acquired *P. cepacia* despite exposure to the colonized patient for a longer period of time. It was concluded that, in addition to social contact, other unknown factors may influence the risk of transmission, and that unnecessary contact between colonized and non-colonized CF patients should be avoided. This last comment illustrates the challenge to be faced in adopting reasonable procedures to reduce the risk of *P. cepacia* colonization. Given the already considerable problems which CF poses for patients and those involved in patient care, to what limits should a segregation policy be taken? Some CF clinics, employing no segregation policy, have not encountered a single case of *P. cepacia* in the last decade.

Pathogen or marker?

Evidence from case-controlled studies points to an association between *P. cepacia* colonization and poorer lung function. This statement, however, is too sweeping and needs careful consideration. More precisely, some studies have suggested that persistent colonization and a poor outcome are associated with severe lung disease at the onset of *P. cepacia* colonization and the acquisition of multi-resistant strains. Patients with normal or mildly impaired lung function who were colonized by *P. cepacia* which were reasonably sensitive to antibiotics remained stable and some patients showed only transient colonization. The pathogenic significance of multi-resistant *P. cepacia* requires further study; in our experience, patients may be colonized by multi-resistant *P. cepacia* and show little or no deterioration in lung function.

A major issue concerning *P. cepacia* colonization in CF is whether the organism has an active role in progressive lung disease, or merely acts as a marker of pre-existing lung damage. There is little direct evidence to suggest that *P. cepacia* is a true pathogen. Studies show that *P. cepacia* lacks many of the toxins and other bacterial factors associated with tissue damage. Data from animal studies also support the argument that *P. cepacia* is nothing more than a colonizer.

However, there is some indirect evidence to suggest that the organism is a true pathogen and may cause tissue damage by over-stimulation of the immune response, in a manner similar to that observed for *P. aeruginosa*. Recent studies in Edinburgh have shown that as the period of colonization progresses there is a rise in the level of anti-*P. cepacia* antibodies; this immunological evidence suggests true infection rather than colonization and the possibility of immune-mediated tissue damage. What is certain is that research is necessary to determine the exact role of *P. cepacia* in lung disease both in terms of the virulence properties of individual strains and of the clinical and immunological response in individual CF patients.

Anti-*P. cepacia* therapy

It seems probable that the characteristic resistance of *P. cepacia* to most available anti-microbial agents plays an important role in the emergence and persistence of *P. cepacia* in the lungs of CF patients. Even the potent anti-pseudomonal agents, ceftazidime and ciprofloxacin which show good activity against some strains of *P. cepacia* in laboratory tests, produce variable results in clinical practice. Novel approaches to the treatment of *P. cepacia* infections and the continued development of anti-microbial agents may result in improved therapeutic regimens. Several anti-microbials under investigation show promising activity against the organism. One approach to therapy is the use of amiloride which reduces the viscosity of bronchial secretions and improves sputum clearance. The use of such agents as aerosols and in combination with tobramycin is one experimental treatment which might prove useful and encourage the development of other novel therapeutic strategies. Meanwhile, a major strategy for the control of this organism must be a better understanding of the manner by which *P. cepacia* establishes itself in the CF lung and how it is transmitted between CF patients.

Final comments

What conclusions, if any, can be drawn from this wealth of conflicting data? Is *P. cepacia* really a new pathogen for CF patients and if so why has it only emerged as a challenge during the last decade?

It is tempting to attribute the increased prevalence of the organism to factors associated with patient care, for example the selective pressure of prolonged oral or nebulised antibiotic therapy. It is also possible that the increase is apparent rather than real and that in some centres this is the result of increased laboratory awareness and the recent use of selective culture media. Associated with improved culture techniques is the availability of multi-test systems to aid the identification of the organism.

The precise mode of transmission of *P. cepacia* between CF patients and the manner by which patients initially become colonized remains unclear. Based on the wide distribution of the organism in the environment, CF patients probably encounter the organism frequently. Controlled prospective studies to determine the key elements in *P. cepacia* transmission and to define more precisely the risk associated with various types of exposure will be difficult. As a starting point, however, we need answers to the following questions:

1. What properties of *P. cepacia* contribute to pulmonary colonization and do strains differ in virulence?

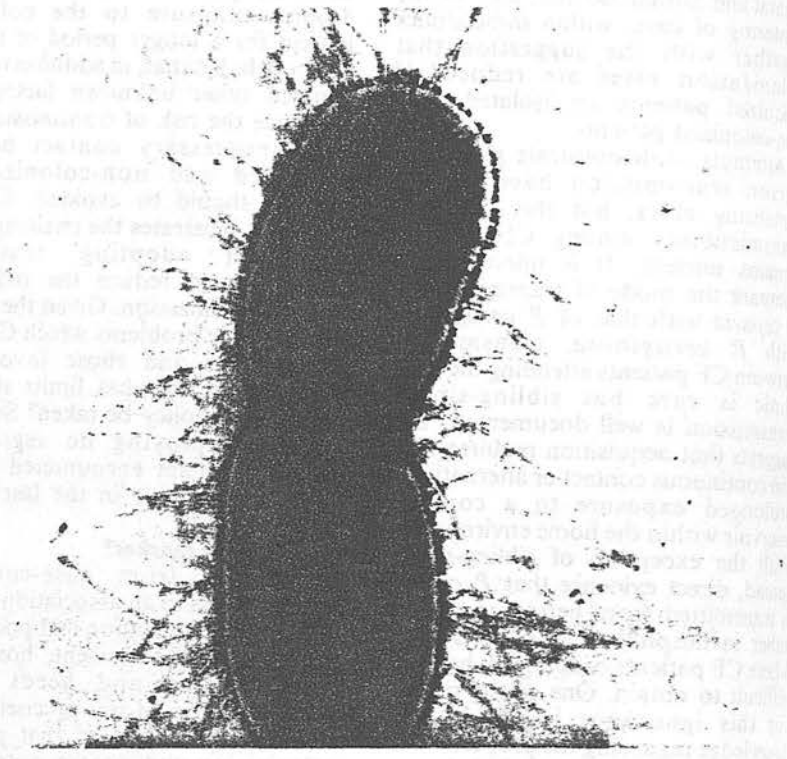
2. Do patients colonized with *P. cepacia* contaminate their environment and, if so, how long do the organisms survive after deposition on surfaces?

3. What is the risk of person-to-person transmission? Is this risk enhanced by an increased pool of colonized patients within a CF centre and by transient carriage of the organism on the hands of medical staff?

4. What are the therapy-related risk factors for non-colonized patients to acquire *P. cepacia*.

5. Finally, and perhaps most difficult to answer, to what extent should social contact between colonized and non-colonized patients be controlled?

Inevitably, attempts to answer these questions will raise important social and ethical issues affecting the management of respiratory infections in CF patients.



Pseudomonas cepacia *viewed by an electron-microscope (magnification 25,000 times). The photograph shows the organism in the process of dividing into two separate cells. The slender, thread-like structures projecting from the cell surface are called pili, and may aid attachment of the organism to surfaces.*

ROLE OF THE CYSTIC FIBROSIS NURSE SPECIALIST

ONE-DAY MEETING AT BAFTA, LONDON ON 21 NOVEMBER 1991

Chairman:
Dr. T. J. David, Senior Lecturer in Child Health, Honorary Consultant Paediatrician, Booth Hall Children's Hospital, Manchester.

Programme:
10.15am Registration.

MORNING SESSION
Chairman's Introduction.

The role of the CF nurse specialist — a general review by June Dyer, Bristol.

The CF nurse specialist and home intravenous antibiotics (1) training and supervision by Teresa Robinson, Leeds.

The CF nurse specialist and home intravenous antibiotics (2) cannulae, long lines, implantable devices by Lucy Wilde, Leicester.

Lunch

AFTERNOON SESSION
The role of the CF nurse specialist in terminal care and bereavement by Jenny Cottrell, Liverpool.

The CF nurse specialist and neo-natal screening by Kathleen Nicholson, Cambridge.

Tea.

The CF nurse specialist and heart-lung transplantation by Fran Duncan, London.

The benefits of having a CF nurse specialist by Dr. Jim Littlewood, Leeds.

Close of meeting.

Limited places only.

Closing date for registration 1 November 1991.

Please note that this meeting is for nurses and doctors only.

To register, write to:
CF Meeting, PO Box 6, Hampton, Middlesex, TW12 2HE.
For further enquiries telephone: 081-990 2972.

Role of the Cystic Fibrosis Nurse Specialist, 21 November 1991

I would like to register for the meeting ☐

I have enclosed a cheque for £3.00 (made payable to the 'CF Research Trust') ☐

Name .....

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Greenwood, Stack and Penther
Medical Microbiology 14E

29

Pseudomonas and non-fermenters

Opportunistic infection; cystic fibrosis; melioidosis

J. R. W. Govan

The genus *Pseudomonas* comprises more than 200 species, mostly saprophytes found widely in soil, water and other moist environments. A few species are pathogenic for plants, insects and animals. *Pseudomonas aeruginosa* is the species most commonly associated with human disease but *Ps. mallei* and *Ps. pseudomallei* are also important pathogens in some parts of the world. Several other species of pseudomonas and a number of other glucose non-fermenters are occasionally isolated from human clinical specimens as opportunistic pathogens. The reasons for the pre-eminent status of *Ps. aeruginosa* as an opportunistic pathogen lie in its adaptability, its innate resistance to many antibiotics and disinfectants, its varied armoury of putative virulence factors, and in an increasing supply of patients compromised by age, underlying disease or immunosuppressive therapy.

PSEUDOMONAS AERUGINOSA

DESCRIPTION

Ps. aeruginosa is a Gram-negative bacillus, non-sporing, non-capsulate, and usually motile by virtue of one or two polar flagella (Fig. 29.1). It is a strict aerobe but can grow anaerobically if nitrate is available. The organism grows readily on a wide variety of culture media over a wide temperature range and emits a sweet grape-like odour that is easily recognized. Most strains of *Ps. aeruginosa* produce diffusible pigments; typically, the colony and surrounding medium is greenish blue due to production of a soluble phenazine pigment, *pyocyanin*, and the yellow-green fluorescent pigment *pyoverdin*, which acts as the major siderophore; additional pigments include *pyorubrin* (red) and *melanin* (brown). Some 10–15% of *Ps. aeruginosa* strains readily produce pigment only when grown on pigment-enhancing media. Individual colonies of *Ps. aeruginosa* can occur as five distinct types ranging from dwarf colonies to large mucoid colonies; the most common colonial

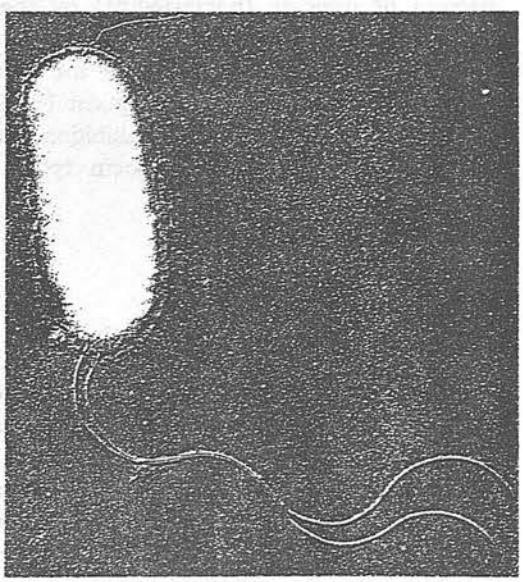

Fig. 29.1 Shadowed electron micrograph of *Ps. aeruginosa* showing two polar flagella and a single polar pilus.

form is relatively large, low-convex with an irregular surface, and edge that is translucent and an oblong shape with the long axis parallel to the line of inoculum.

Ps. aeruginosa differs from members of the Enterobacteriaceae by deriving energy from carbohydrates by an oxidative rather than fermentative metabolism. In carbohydrate fermentation tests, *Ps. aeruginosa* appears inactive and only glucose is utilized. However, all strains give a positive oxidase reaction and this is a useful preliminary test for non-pigmented strains. Although a few Gram-negative bacilli are also oxidase-positive, none reacts as swiftly as *Ps. aeruginosa*, which gives a positive reaction within 30 s.

For epidemiological purposes, or to confirm the clonal relationship between isolates, *Ps. aeruginosa* can be characterized on the basis of: O and H antigens; sensitivity to sets of bacteriophages; bacteriocin typing; and DNA probes prepared from fragments of the exotoxin A or pilin genes. At present, serotyping and pyocin typing are generally accepted as the most readily usable and reliable methods. Seventeen serotypes based on heat-stable somatic (O) antigens are recognized but, in practice, four or five serotypes account for the majority of strains. Pyocin typing depends on the production of pyocins (bacteriocins) by the strain under investigation; pyocin types of *Ps. aeruginosa* are designated on the basis of the inhibition patterns observed when tested against 13 indicator strains and on the size of the inhibition zones. In this manner at least 105 pyocin types of *Ps. aeruginosa* are recognized.

PATHOGENESIS

Ps. aeruginosa can infect almost any external site or organ. In the community, infections caused by *Ps. aeruginosa* are mostly mild and superficial, e.g. otitis externa and varicose ulcers; such conditions are often chronic, but not disabling. Recreational and occupational conditions associated with susceptibility to pseudomonas infections include jacuzzi or whirlpool rash (an acute self-limiting folliculitis) and industrial eye injuries, which may lead to panophthalmitis.

In hospitalized patients, pseudomonas infections are more common, more severe and more varied. Infection is usually localized, as in catheter-related urinary tract infection, in infected ulcers, bed sores or burns, and in eye infections. In patients compromised by age, or immunosuppressing diseases such as leukaemia, and in those treated with immunosuppressive drugs or corticosteroids, pseudomonas infections frequently become generalized and the organism may be cultured from the blood or from many organs of the body post-mortem. *Ps. aeruginosa* is not a major cause of Gram-negative septicaemia or necrotizing pneumonia but is associated with high mortality in these conditions. The lungs of children with cystic fibrosis are very susceptible to infection with *Ps. aeruginosa*. In these patients, asymptomatic pulmonary colonization with typical non-mucoid forms of *Ps. aeruginosa* eventually leads to the emergence of mucoid variants (Fig. 29.2); debilitating episodes of pulmonary exacerbation due to mucoid *Ps. aeruginosa* are the major cause of morbidity and mortality in cystic fibrosis.

Most strains of *Ps. aeruginosa* produce two exotoxins, exotoxin A and exo-enzyme S, and a variety of cytotoxic substances including proteases, phospholipase, pyocyanin and rhamnolipids; an alginate-like exopolysaccharide is responsible for the mucoid phenotype (Fig. 29.3). The importance of these putative virulence factors depends upon the site and nature of infection: proteases play a key role in corneal ulceration; exotoxin and proteases are important in burn infection; and phospholipases, proteases and alginate are associated with chronic pulmonary colonization.

LABORATORY DIAGNOSIS

Ps. aeruginosa grows on most common culture media. If specific investigation for *Ps. aeruginosa* is desired, material should be cultured on a medium as *Pseudomonas* isolation agar (Difco), which is both selective and enhances the production of the characteristic blue-green pigment pyocyanin. If *Ps. aeruginosa* is sought in aqueous environments or soil, then enrichment media containing acetamide as the sole carbon and nitrogen source

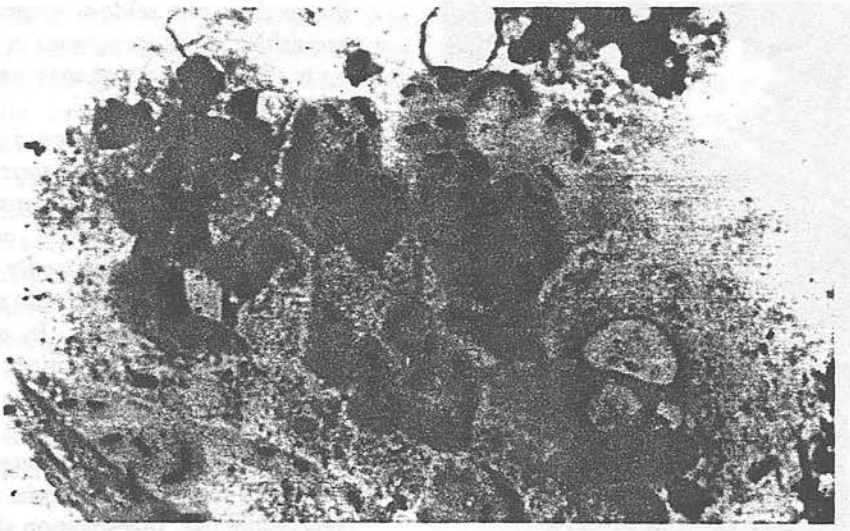

Fig. 29.2 Gram-stained sputum from patient harbouring mucoid *Ps. aeruginosa*. Note spawn-like microcolony and adjacent phagocytes.

may be used before culture on *Pseudomonas* isolation agar. Resultant colonies are usually easily identified. About 10% of *Ps. aeruginosa* isolates do not produce readily detectable pigment even on suitable media; in such cases an oxidase test should be performed and, if positive, further identification should be carried out with an appropriate multitest system. If the result is equivocal, production of pyocins is a useful confirmation of identity. Except in patients with chronic obstructive airway diseases, tests for serum antibodies have no place in diagnosis.

TREATMENT

Strains of *Ps. aeruginosa* are normally intrinsically resistant to most commonly employed antimicrobial agents. Before the introduction of the first antipseudomonal β-lactam agent, carbenicillin, in the 1960s, pseudomonas infections were usually treated with polymyxins, agents that exhibit considerable toxicity. With the development of more potent β-lactam compounds, such as ticarcillin and azlocillin, and the discovery of antipseudomonal aminoglycosides like gentamicin and tobramycin, treatment with a combination of an aminoglycoside and a penicillin was commonly adopted. Such a combination possesses the potential advantage of antibacterial synergy, although there is no convincing clinical evidence of the superiority of combined therapy, and the newer, broad-spectrum β-lactam agents such as ceftazidime and imipenem are often used alone.

Each of the agents mentioned so far has to be administered parenterally, but the fluoroquinolones of the ciprofloxacin type can also be given by mouth and this may be an advantage if therapy has to be prolonged. Ciprofloxacin exhibits good activity against *Ps. aeruginosa* and penetrates well into most tissues. It has been successfully used in pseudomonas infection, but resistance sometimes emerges during therapy.

Passive vaccination may be useful for the treatment of septicaemia and burn infections due to *Ps. aeruginosa* which are associated with high mortality; active vaccination to prevent pulmonary colonization would be highly desirable for patients with cystic fibrosis and a polysaccharide-based vaccine is under clinical trial at the time of writing.

Ps. aeruginosa is resistant to, and may multiply in, many of the disinfectants and antiseptics commonly used in hospitals. It can be a troublesome contaminant in pharmaceutical preparations and may cause ophthalmitis following the faulty chemical 'sterilization' of contact lenses.

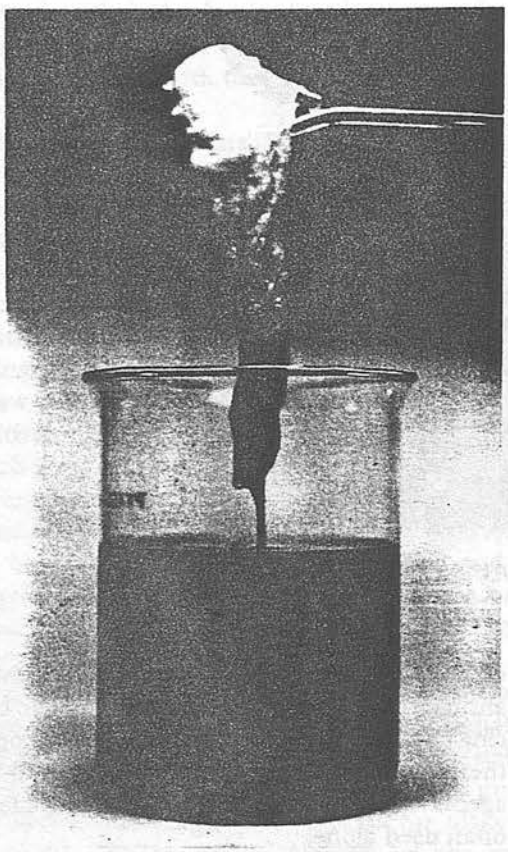

Fig. 29.3 Alcohol extraction of alginate from mucoid *Ps. aeruginosa*.

EPIDEMIOLOGY

Ps. aeruginosa can be isolated from a wide variety of environmental sources and is a competent and hardy saprophyte. The ability of the species to persist and multiply, particularly in moist environments, and on moist equipment (e.g. humidifiers) in hospital wards, bathrooms and kitchens, is of particular importance in cross-infection control. Consumption of salad vegetables contaminated with pseudomonas is a potential risk for immunocompromised patients in intensive care units.

Most hospital-acquired infections with *Ps. aeruginosa* originate from exogenous sources but some patients suffer endogenous infection, particularly of the urinary tract. Healthy carriers of *Ps. aeruginosa* usually harbour strains in the gastro-intestinal tract, but in the open community the carriage rate seldom exceeds 10%. In contrast, acquisition of *Ps. aeruginosa* in a hospital is rapid and up to 30% of patients may excrete the organisms within 2 of admission.

Epidemics of pseudomonas infection in newborn and young infants in maternity units and paediatric wards are not uncommon. The ability of *Ps. aeruginosa* to survive, and occasionally to flourish, in many supposedly antiseptic or disinfectant solutions explains the method of spread in some of these episodes. In one large maternity unit 71 cases of infection, including gastroenteritis and eye infections, suddenly occurred among infants in a period of 5 weeks. Only babies that were artificially fed were affected and *Ps. aeruginosa* was cultured from milk feeds immediately before use. Investigation showed that although the milk and the bottles had been adequately sterilized, rubber bungs used to close the bottles had been stored in a solution of antiseptic. The epidemic strain of *Ps. aeruginosa* was isolated from the stored bungs. Heat sterilization of the fully prepared and treated milk feeds terminated the epidemic.

Burned patients are another category especially at risk, from *Ps. aeruginosa*; the presence of the organism in ward air, dust and in eschar shed from the burns suggests that infection can be airborne. However, contact spread has been demonstrated and is probably now more important than the airborne route with the use of effective topical agents for prophylaxis and treatment of burn and wound infection. Transmission may occur directly via the hands of medical staff, or indirectly via contaminated apparatus. Severely burned patients and those with chest injuries who require artificial ventilation are very susceptible to *Ps. aeruginosa*; pulmonary infection not infrequently precedes septicaemia, which is associated with high mortality. Modern intensive care equipment can be difficult to clean and there is little doubt that infection can be spread by this source.

Eye infection may result from contaminated contact lenses, or from installation of pseudomonas-contaminated medicament during ophthalmic procedures. Similarly, in the early days of North Sea oil exploration, diving operations had occasionally to be aborted because of outbreaks of trouble-

some ear infection due to *Ps. aeruginosa*. The organism thrives in the conditions of high humidity and poor sanitation associated with prolonged saturation diving, even in the helium-rich atmosphere of the diving bell. Moreover, the low-level pain normally associated with otitis externa is particularly intense under conditions of saturation diving and is exacerbated by the need for lengthy periods of decompression. The warm, moist and aerated conditions under which *Ps. aeruginosa* thrives are ideally met in poorly maintained whirlpools or jacuzzis. An irritating folliculitis known as *jacuzzi rash* is a typical example of an opportunistic pseudomonas infection that may be acquired from such a source.

CONTROL

Prevention is easier than cure; once *Ps. aeruginosa* has gained access to the hospital environment, or has established infection, it is notoriously difficult to eradicate. Four guide-lines to control infection are offered in the knowledge that is not always easy to put them into practice:

1. Patients at a high risk of acquiring infection with *Ps. aeruginosa* (e.g. a patient being evaluated for renal or heart–lung transplantation) should not be admitted to a ward where cases of pseudomonas infection are present.
2. A patient infected with *Ps. aeruginosa* should, if at all possible, be isolated until the infection has been eradicated.
3. Strict infection control measures should be followed by all medical and nursing staff handling patients. All instruments and apparatus, dressings, etc. must be not only clean but sterile. Antimicrobial and other therapeutic substances and solutions must be free from bacteria; a particular danger exists when multidose ointments, creams or eye drops are used to treat several individuals over a period. Initially, the preparation may be sterile but contamination can easily occur between uses, and *Ps. aeruginosa* can multiply readily at a range of temperatures in many medicaments.
4. In hospital units, episodes of cross-infection due to a single strain of *Ps. aeruginosa* may occur as sporadic infections in individual patients over a considerable period of months or years. For this reason, if facilities are available, it is advantageous to monitor all clinically relevant isolates of *Ps. aeruginosa* by a suitable typing system to identify epidemic strains.

PSEUDOMONAS MALLEI AND *PS. PSEUDOMALLEI*

These organisms were previously assigned to various genera, including *Bacillus* and *Loefflerella*, but are now considered to belong to the genus *Pseudomonas*. *Ps. mallei* is an animal parasite occasionally transmitted to man whereas *Ps. pseudomallei* is a free-living organism that can infect an animal or human host. Laboratory-acquired infection with these organisms is a hazard; both species are categorized as category A pathogens and must be handled with great care and under strictly designated conditions.

Both species are easily cultured. *Ps. pseudomallei* produces a characteristic wrinkled colonial appearance after several days of growth on nutrient agar; fresh cultures emit a characteristic pungent odour of putrefaction. Both organisms are oxidase-positive but neither species produces diffusible pigment. *Ps. mallei* is invariably non-motile.

GLANDERS

Glanders is a disease of horses caused by *Ps. mallei*. It is nowadays restricted to Asia, Africa and the Middle East. Guinea-pigs are highly susceptible to infection; if an inoculation is made intraperitoneally into a male guinea-pig, the tunica vaginalis is rapidly invaded by the bacilli and swelling of the testes results (*Straus reaction*). The infected animals die within a few days of inoculation and generalized lesions can be seen at necropsy.

Glanders, in the natural host, is associated with a profuse catarrhal discharge from the nose, and the nasal septum shows nodule formation; later in the disease the nodules break down with the production of irregular ulcers. When infection occurs in superficial lymph vessels and nodes following infection through the skin — e.g. via abrasions caused by a rubbing harness — the

clinical term *farcy* is used to describe the infection. The lymph vessels show irregular thickening, become corded and are termed *farcy pipes*. Humans may become infected via skin abrasions or wounds which come into contact with the discharges of a sick animal.

MELIOIDOSIS

Melioidosis is a tropical disease of animals and humans that is endemic in South-East Asia and northern Australia. The causative organism, *Ps. pseudomallei*, is found in soil and surface water in rice paddies and monsoon drains; the isolation rates are highest during the rainy season and in still rather than flowing water. Human infection is mainly acquired cutaneously through skin abrasions or by inhalation of contaminated particles. In humans the clinical manifestations range from a subclinical infection, diagnosed by the presence of specific antibodies, to a benign pulmonary infection that may resemble tuberculosis, or to a fulminating septicaemia with a mortality rate of 80–90%. Virtually every organ can be affected and hence melioidosis has been called the 'great imitator' of every infectious disease. Melioidosis commonly presents as pyrexia and, in endemic areas, serological testing for *Ps. pseudomallei* in important is the evaluation of pyrexia of unknown origin. *Ps. pseudomallei* can survive intracellularly within elements of the reticuloendothelial system and this ability may account for latency and the emergence of symptoms many years after exposure. Suppurative parotitis is a characteristic presentation of melioidosis in children.

Early diagnosis and appropriate antibiotic therapy are key factor in the successful management of melioidosis. The organism may be observed, usually in very small numbers, as small, bipolar-stained Gram-negative bacilli in exudates and may be isolated from sputum, urine, pus or blood. Enzyme-linked immunosorbent assay (ELISA) for the detection of specific IgG and IgM antibody to *Ps. pseudomallei*, as well as an indirect haemagglutination test for serum immunoglobulin antibody, are useful serological screening tests in the investigation of subclinical melioidosis. Combinations of antimicrobial agents such as tetracycline and chloramphenicol have been standard therapy in the treatment of melioidosis; prolonged treatment is necessary to avoid relapse. The ability of *Ps. pseudomallei* to survive and multiply in phagocytes may be the cause of the difficulty of treating melioidosis in spite of the fact that antibiotics are effective against the organism in vitro and the frequent recurrences when the duration of treatment is not long enough. Of the newer, ceftazidime is especially effective in vitro.

GLUCOSE NON-FERMENTERS

A small but increasing percentage of clinically relevant Gram-negative bacilli belong to pseudomonas species other than those already discussed. This group includes *Ps. cepacia*, *Ps. maltophilia*, *Ps. putida*, *Ps. fluorescens* and *Ps. stutzeri*. In addition, there is a group of bacteria that are commonly referred to as glucose-non-fermenters that are taxonomically distinct from the carbohydrate-fermenting enterobacteriaceae and the oxidative members of the genus *Pseudomonas*. The clinical relevance of non-fermenters is based on their role as opportunistic pathogens in hospital-acquired infections and their intrinsic resistance to many antimicrobial agents. Glucose non-fermenters comprise a heterogeneous group of species and none requires specific cultural conditions. Unequivocal identification may be difficult as most species are relatively inert in the biochemical tests used in identification of Gram-negative bacteria. Tests for motility and oxidase production are useful and identification is assisted by computer-based probabilistic methods or multitest identification systems.

SPECIES OTHER THAN *PSEUDOMONAS*

Eikenella corrodens is a commensal of mucosal surfaces which may cause a range of infections, in particular endocarditis, meningitis, pneumonia and infections of wounds and various soft tissues.

Flavobacterium meningosepticum is a saprophyte whose natural habitat is soil and moist environments, including nebulizers; it may cause opportunistic nosocomial infections, particularly in

infants. As the name suggests, this species is associated with meningitis and has been responsible for high mortality in epidemic outbreaks.

Moraxella lacunata and *M. phenylpyruvica* are commensals of mucosal membranes but may give rise to opportunistic infections.

Acinetobacter calcoaceticus is a saprophytic species found in soil and aquatic environments, including sewage, and occasionally as a commensal of moist areas of human skin. The most important hospital-acquired infections associated with the two subspecies *A. calcoaceticus* ssp. *anitratus* and *A. calcoaceticus* ssp. *lwoffi* include pneumonia and other infections of the upper respiratory tract.

Two other genera of Gram-negative bacteria, *Alcaligenes* and *Achromobacter*, are often included among non-fermenters. They may be confused with *Pseudomonas* species. Like other non-fermenters, they are saprophytes found in moist environments in nature and in the hospital environment, and are associated with a range of hospital-acquired opportunistic infections, including septicaemia and ear discharges.

RECOMMENDED READING

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Production and characterisation of mouse monoclonal antibodies reactive with the lipopolysaccharide core of *Pseudomonas aeruginosa*

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Summary. Monoclonal antibodies (MAbs) to the core antigen region of lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* were produced from mice immunised with whole cells of heat-killed rough mutants of *Pseudomonas aeruginosa* expressing partial or complete core LPS. MAbs were screened in an enzyme-linked immunosorbent assay (ELISA) against three different antigen cocktails: S-form LPS from three *P. aeruginosa* strains, R-form LPS from six *P. aeruginosa* strains and, as a negative control, R-form LPS from *Salmonella typhimurium* and *Escherichia coli*. Selected MAbs were subsequently screened against a range of extracted LPS and whole cells from both reference strains and clinical isolates of *P. aeruginosa*. The antibodies were also screened in ELISA against whole-cell antigens from other *Pseudomonas* spp. as well as strains of *Haemophilus influenzae*, *Neisseria subflava* and *Staphylococcus aureus*. Five MAbs reacting with the core component of *P. aeruginosa* LPS were finally selected. Two of these, MAbs 360.7 and 304.1.4, were particularly reactive in immunoblots against unsubstituted core LPS, including that from O-antigenic serotypes of *P. aeruginosa*. The MAbs also reacted with some of the other *Pseudomonas* spp., but not with *P. cepacia* or *Xanthomonas* (*Pseudomonas*) *maltophilia*. Cross-reactivity with whole cells from other bacterial species was minimal or not observed. Reactivity of MAbs with some *Staph. aureus* strains was observed, and binding to the protein A component was implicated. The reactivity of the MAbs was investigated further by flow cytometry and immunogold electronmicroscopy. The suitability of the MAbs for an immunological assay for detection of *P. aeruginosa* in respiratory secretions from CF patients is discussed.

Introduction

Pseudomonas aeruginosa is an opportunist pathogen *par excellence*, responsible for a diverse range of infections in patients compromised by chemotherapy, injury, surgical procedures or underlying disease, and by the inherited disease cystic fibrosis (CF). Pulmonary colonisation of patients with CF by *P. aeruginosa* can be diagnosed by bacteriological culture and detection of pseudomonas-specific antibodies.[1–3] However, in the early stages of asymptomatic colonisation such techniques may be unhelpful.

There has been increasing interest in the development of immunoassays which can detect infective agents directly in clinical specimens and be adapted to the rapid screening of a large number of specimens.[4–6] The development of an immunoassay based on a monoclonal antibody (MAb) for the early detection of *P. aeruginosa* antigens in saliva and sputum specimens from CF patients was considered a potentially useful diagnostic technique.

P. aeruginosa is serologically heterogeneous and contains at least 20 different heat-stable O-antigens.[7] The aim of the current study was to produce and characterise MAbs specific for *P. aeruginosa* and then assess their suitability for the detection of any of the distinct serotypes which may colonise individual CF patients. *P. aeruginosa* produces several intra- and extra-cellular antigens. Amongst these, lipopolysaccharide (LPS) is highly immunogenic and readily released from bacterial cells.[8,9] Therefore, the common core region of LPS was considered to be an appropriate antigen for the production of anti-*P. aeruginosa* MAbs for use in an immunoassay to detect *P. aeruginosa* in clinical specimens from CF patients.

Materials and methods

Bacterial strains

P. aeruginosa R-mutants defective in the production

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*This paper is dedicated to the memory of Lisel Micklem.

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of LPS (PAC556, PAC557, PAC605, PAC608, PAC609 and PAC611) were obtained from Professor P. M. Meadow, University College, London.[10] Standard serotype strains of *P. aeruginosa* were obtained from Dr R. J. Jones, University of Liverpool. The standard genetic strain *P. aeruginosa* PAO1 was also included in the study. Strains of *P. acidovorans* NCIB 9681, *P. fluorescens* NCIB 10525, *P. mendocina* NCIB 10541, *P. pseudoalkaligenes* NCIB 9946, *P. putida* NCIB 1007, *P. stutzeri* NCIB 9040 and *P. testosteroni* NCIB 8893 were supplied by the National Collection of Industrial Bacteria. *P. cepacia* strains J1685, J1695, J1705, J1715, J1725, J1735, J1765 and J1775 were obtained from the Center for Communicable Disease Control, Atlanta, GA, USA.[11] Bacteria denoted by the prefix JN were isolated from sputum of patients with CF and included strains of *P. aeruginosa*, *P. cepacia*, *Xanthomonas* (*Pseudomonas*) *maltophilia*, *Haemophilus influenzae*, *H. parainfluenzae*, *Neisseria subflava*, *Moraxella* (*Branhamella*) *catarrhalis* and *Staphylococcus aureus*. Enterobacterial strains used as controls included Ra mutants *Escherichia coli* R1 and *Salmonella typhimurium* 1542, and a strain of *Proteus mirabilis*.

Immunisation procedure and production of MAbs

P. aeruginosa was grown in nutrient broth containing yeast extract 0·5% w/v (NYB), washed twice with phosphate-buffered saline (PBS, pH 7·2) and resuspended to a density of 10^8 cells/ml. Five female BALB/c mice were immunised by intravenous (i.v.) injection with 10^7 heat-killed (100 °C for 5 min) cells. Each mouse received 0·1 ml of cells from *P. aeruginosa* PAC608 (full core mutant) on days 1, 2, 3, 8, 9 and 10 followed by cells from *P. aeruginosa* PAC605 (partial core mutant) on days 22, 23 and 24. Blood was taken from the tail vein of each mouse on days 1 (pre-inoculation), 20, 31 and 38, and the antibody response was measured by ELISA (see below). A mouse with serum showing a strong, cross-reactive antibody response to *P. aeruginosa* LPS antigens was selected for fusion. The mouse was given a booster injection of 0·1 ml of heat-killed cells of PAC608 (10^7 cells), i.v., and was killed 3 days later. Mouse spleen cells were fused with cells of the NS-1 plasmacytoma cell line[12] by the method of Oi and Herzenberg.[13] Hybridomas producing anti-*P. aeruginosa* LPS antibodies were screened by ELISA (see below). Antibody-positive hybrids were cloned by limiting dilution.

Preparation of LPS

Extraction of LPS from bacterial strains expressing the S-form LPS phenotype was based on the aqueous phenol method of Westphal and Luderitz.[14] The aqueous phenol, chloroform, petroleum ether method of Galanos *et al.*,[15] incorporating the diethyl ether precipitation of LPS described by Qureshi *et al.*[16] (as described by Hancock and Poxton[17]), was used to prepare LPS from bacteria expressing the R-form LPS.

Characterisation of MAbs

Isotype determination. The class and subclass of MAbs in culture supernates were determined by a mouse MAb isotyping kit (RPN29: Amersham International plc).

Enzyme-linked immunosorbent assay (ELISA). An ELISA was used (a) to detect the antibody response of mice to vaccination; (b) to screen hybridomas for the production of anti-*P. aeruginosa* antibody; and (c) to test the reactivity of MAbs against other bacterial strains. A primary ELISA screen consisting of three different LPS cocktails was used for an initial assessment of the reactivity of antibodies in sera from mouse-tail samples and in hybridoma supernates. Extracted LPS antigens were complexed with polymyxin as described by Scott and Barclay[18] and antigen cocktails were formed by the addition of equimolar proportions of individual complexes. Cocktail-A consisted of O-antigenic S-form LPS from three serotype strains of *P. aeruginosa* (O1, O2 and O11); cocktail-B consisted of core R-LPS from six LPS R-mutants (PAC556, PAC557, PAC605, PAC608, PAC609 and PAC611); cocktail-C (negative control) consisted of LPS from *S. typhimurium* 1542 and *E. coli* R1. Hybridomas producing a strong antibody response directed to one or both of the *P. aeruginosa* LPS cocktails were selected and the antibodies were subsequently tested in a secondary ELISA consisting of the individual LPS antigens contained in the LPS cocktails of the primary screen. Hybridomas producing antibodies of interest were further screened in ELISA against a comprehensive range of antigens which included additional LPS-polymyxin complexes, individual pseudomonas vaccine antigens PVA-1–16 which comprise the polyvalent pseudomonas extract vaccine (PEV),[19] and whole-cell bacterial antigens. All antigens for ELISA studies were diluted in carbonate-bicarbonate coating buffer (pH 9·6) and added to the wells of polystyrene microplate 8-well strips (Nunc, 'polysorb') at 100 μl/well. LPS-polymyxin complexes were diluted 1 in 50 in coating buffer and vaccine antigens were coated at 1 μg/ml. Bacterial whole cells (grown overnight in NYB and washed twice with PBS) were resuspended to a density of 10^7 cells/ml in coating buffer and added to the microtitration plate wells, and the plates were centrifuged at 1400 *g* for 5 min to sediment bacteria to the wells. After washing with wash buffer (PBS containing Tween 20 0·05% v/v and sodium azide 0·02% w/v), all plates were post-coated with post-coat buffer (PBS containing bovine serum albumin, BSA, 5% w/v) at 100 μl/well. After being washed four times with wash buffer, plates were stored at −20 °C until use.

Primary antibody (sera from mouse tail samples diluted 1 in 100, or supernate from hybridomas diluted 1 in 5) was added (100 μl) to each well. Antibody diluent was PBS with Tween 20 0·05% v/v, BSA,

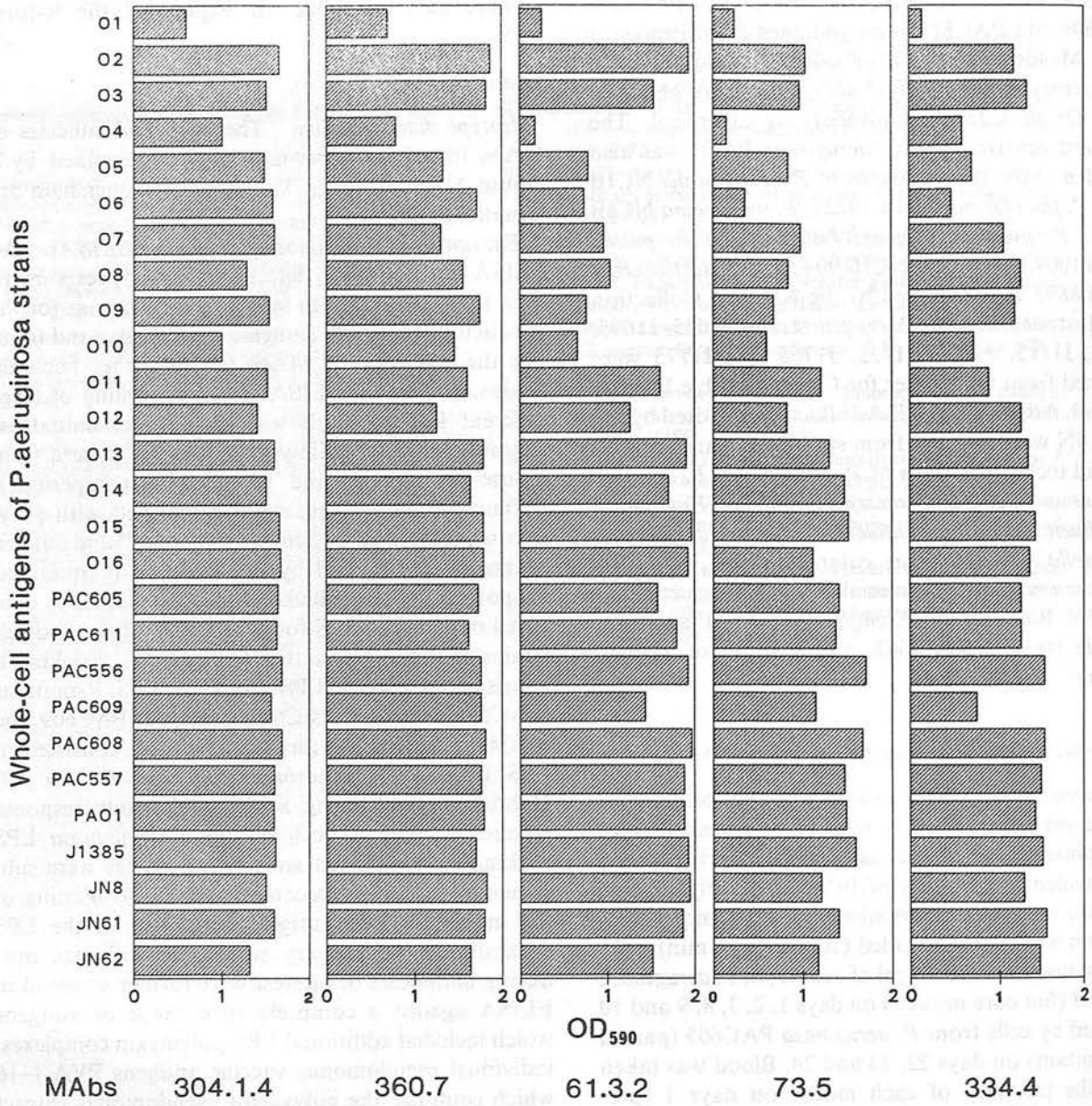

Fig. 1. Reactivity in ELISA of anti-LPS MAbs against *P. aeruginosa* whole cells of standard serotype strains O1–O16, R-LPS mutants, PAC605–PAC557, the standard genetic strain PA01 and clinical isolates from patients with CF, J1385–JN62.

0·5% w/v, polyethylene glycol 6000 4% w/v and sodium azide 0·02% w/v. After incubation at 37 °C for 90 min, plates were washed four times with wash buffer. Urease-conjugated anti-mouse Ig (Sera Lab) was diluted 1 in 500 in diluent, and then 100 μl was added to each well. The plates were incubated for a further 90 min at 37 °C. Thereafter they were washed and rinsed, and 100 μl of urease substrate (Sera Lab) was added to each well. Finally, the plates were incubated for 60 min at room temperature and the optical density (OD) of wells was read at 590 nm in a Titertek Multiscan. Results were expressed after subtraction of the corresponding OD of negative control wells (coated only with BSA) for each antibody sample.

Immunoblotting. LPS was prepared by the proteinase K method of Hitchcock and Brown[20] and separated on polyacrylamide 14% gels with the buffer system of Laemmli[21] but without sodium dodecyl sulphate in the stacking and separating gel buffers. Separated antigens were transferred to nitrocellulose membranes (Schleicher and Schuell, pore size 0·2 μm) by the method of Towbin *et al.*[22] and processed by the method of Hancock and Poxton.[17] Antigens were probed with hybridoma culture supernate diluted 1 in 5 for 3 h at room temperature and the immune complexes were detected with anti-mouse Ig horseradish peroxidase conjugate (ICN Biomedicals) and HRP colour reagent (BioRad).

Flow cytometry. The binding of MAbs (hybridoma supernates) to whole bacteria was analysed by the flow cytometry method described by Nelson *et al.*[23]

Immunogold electronmicroscopy. *P. aeruginosa* strains were grown overnight in NYB, and harvested by centrifugation at 10000 *g* for 10 min. Cells for thin sections and the subsequent immunoassay were prepared as described by Hancock and Poxton.[17] MAb supernates and the anti-mouse IgG immunogold conjugate (10 nm particle size, Sigma) were diluted 1 in 10 in antibody diluent which was the same as for the

Table I. Cross-reactivity in ELISA of anti-LPS MAbs against bacterial whole cells

Bacterial species tested	Number of strains tested	Percentage antibody binding relative to *P. aeruginosa**				
		304.14	360.7	61.32	73.5	334.4
P. aeruginosa	21	100	100	100	100	100
P. mendocina	2	56	89	48	100	44
P. fluorescens	3	78	84	47	69	65
P. pseudoalkaligenes	1	56	66	13	95	87
P. acidovorans	1	83	85	0	35	85
P. putida	4	86	73	64	100	100
P. stutzeri	1	77	87	30	86	78
P. testosteroni	1	0	0	0	93	0
P. cepacia	10	0	0	0	0	0
X. maltophilia	7	0	7	12	0	0
H. influenzae	5	0	0	0	0	0
H. parainfluenzae	2	0	6	5	0	0
N. subflava	2	0	0	0	0	0
M.(B.) catarrhalis	2	0	0	0	0	0
Staph. aureus	4	32	29	33	50	38
E. coli R1	1	0	0	0	0	0
S. typhimurium 1542	1	0	0	0	0	0
Pr. mirabilis	1	0	0	0	0	0

* Percentage antibody binding values represent the mean OD_{590} relative to the mean OD_{590} calculated for *P. aeruginosa*.

ELISA studies. Sections were examined before and after staining with aqueous uranyl acetate, in a Hitachi 12A electronmicroscope at 75 kV.

Protein A assay. The ELISA for detection of protein A was performed as described by Warnes *et al.*[24]

Results

A total of 530 hybridoma supernates were screened in ELISA; 42 hybridomas produced an ELISA OD of > 0·5 in the initial screening against the *P. aeruginosa* R- and S-LPS cocktails. Antibody-positive hybrids were cloned and five hybridomas that produced specific antibody were characterised further.

Isotype determination

One MAb (73.5) was classified as IgG_1, two MAbs (304.1.4 and 360.7) as IgG_{2b} and two MAbs as IgG_3 (61.32 and 334.4).

Reaction in ELISA

The five MAbs selected for study produced a positive response against all the *P. aeruginosa* whole-cell antigens used in the screening (fig. 1). A strong reaction (ELISA OD > 1·0) against most of the *P. aeruginosa* whole-cell antigens was particularly marked for MAbs 304.1.4 and 360.7. The MAbs also showed reactions with whole cells from other *Pseudomonas* spp. (table I). All five MAbs reacted with the whole cells of *P. fluorescens*, *P. mendocina*, *P. pseudoalkaligenes*, *P. putida* and *P. stutzeri*. All the MAbs except 61.3.2 produced a positive reaction with *P. acidovorans*, but only MAb 73.5 produced a detectable reaction with *P. testosteroni*. MAb 304.1.4 failed to show a detectable reaction with any of the *P. cepacia* or *X. maltophilia* strains, whereas MAbs 73.5 and 334.4 produced weak reactions with individual *X. maltophilia* strains. MAbs 360.7 and 61.3.2 also failed to produce a positive reaction with any of the *P. cepacia* strains, although weak reactions were obtained with some of the *X. maltophilia* strains. None of the MAbs reacted with whole cells of *H. influenzae*, *H. parainfluenzae*, *E. coli* R1, *S. typhimurium* 1542, *Pr. mirabilis*, *N. subflava* and *M.(B) catarrhalis*. Positive reactions were observed with *Staph. aureus* strains JN91 and JN92 which were subsequently identified as strong producers (> 25 ng/ml) of protein A.

Confirmation of the cross-reactive nature of the five MAbs with all serotypes of *P. aeruginosa* were also provided by the positive response of each MAb to the cell-wall vaccine antigens (PVA), serotypes O1–O16 (fig. 2). The strongest reactions were again observed with MAbs 304.1.4 and 360.7. The MAbs also reacted with each of the extracted *P. aeruginosa* LPS antigens. In ELISA, the response of the five MAbs to core R-LPS from the PAC mutants tended to be lower towards the shortest core structure PAC605 and the complete core structures PAC557 and PAC608. The antibodies did not react with the enterobacterial R-LPS antigens from *E. coli* R1 and *S. typhimurium* 1542.

Immunoblotting

The immunoblot of MAb 360.7 is shown in fig. 3. A positive reactive band corresponding to the low-M_r core glycolipid of the *P. aeruginosa* LPS preparations was observed. The apparent absence of bands corresponding to the high-M_r LPS of serotype strains indicated that the MAbs were unable to recognise core LPS substituted with O-antigenic side chains. However, a very faint ladder pattern corresponding to the high-M_r LPS of serotypes O4 and O6 (tracks 4 and 6

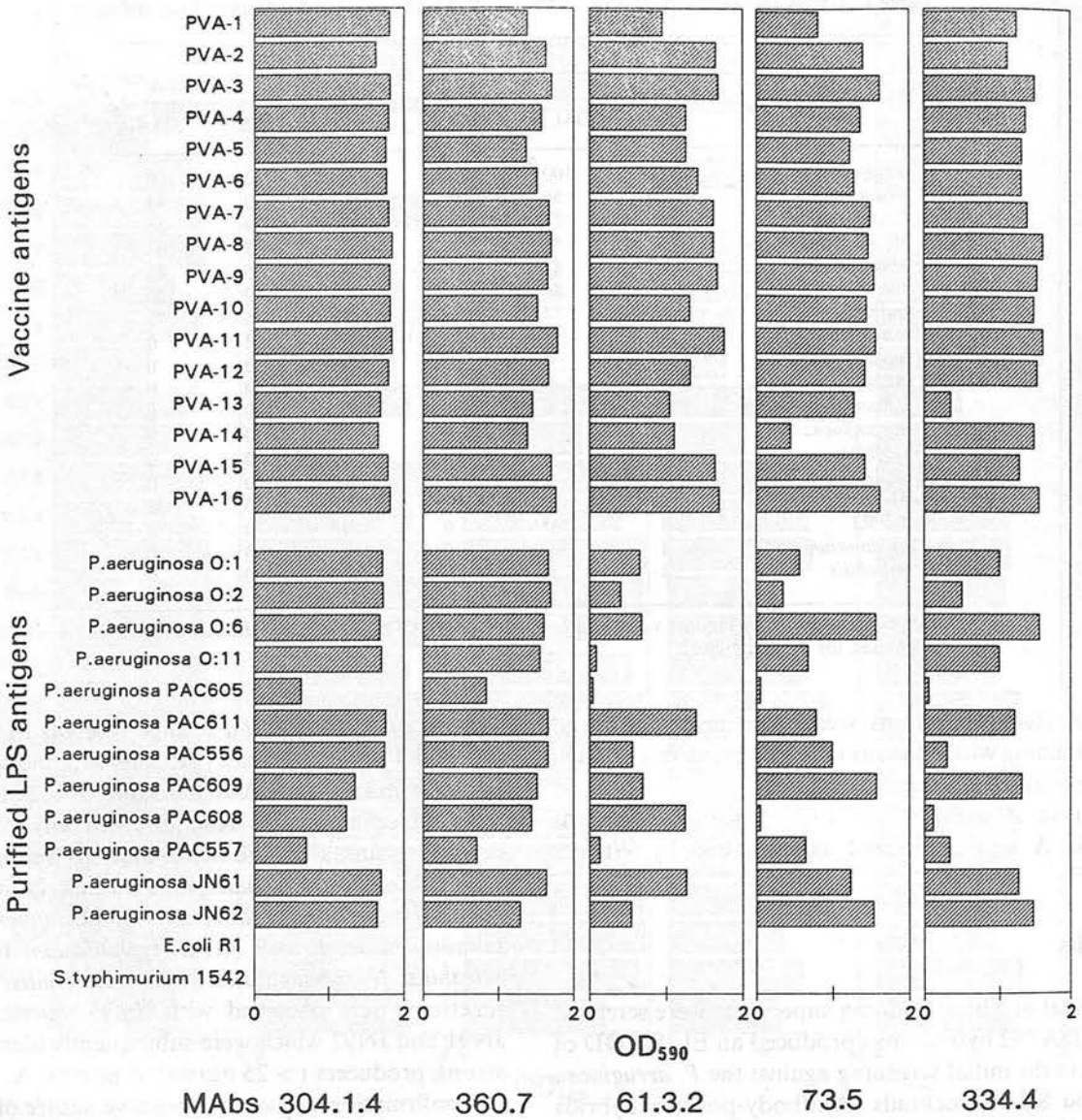

Fig. 2. Reactivity in ELISA of anti-LPS MAbs against *P. aeruginosa* S-LPS O-serotype vaccine components (PVA-1–16), extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O1–O11) and R-LPS (PAC605–PAC557), LPS from two *P. aeruginosa* strains (JN61 and JN62) from patients with CF, and LPS from two Enterobacteriaceae strains (negative controls).

respectively) was observed with MAb 360.7. The ladder patterns are barely visible on the photograph shown. The immunoblots obtained with MAbs 61.3.2, 73.5 and 334.4 were all weaker than the immunoblots obtained with MAbs 304.1.4 and 360.7. However, a positive reaction with the core components of most of the different LPS preparations was observed (data not shown).

Flow cytometry on whole bacteria

The binding of MAbs 304.1.4, 360.7 and 334.4 to whole *P. aeruginosa* cells was analysed by flow cytometry. The highest percentage values of bacteria exhibiting positive fluorescence were observed for the LPS R-mutants that expressed core or partial core structures. The MAbs also reacted with *P. aeruginosa* whole cells expressing O-antigen LPS. No reactivity was observed with whole cells of *P. cepacia* or the enterobacterial R-mutants *E. coli* R1 and *S. typhimurium* 1542 (table II).

Immunogold electronmicroscopy

Immunogold electronmicroscopy was also used to assess the binding of MAbs to *P. aeruginosa* cells processed for thin-sections. Fig. 4a and b shows the binding of MAb 360.7 to *P. aeruginosa* PAC611, which expresses only a partial core LPS, and to *P. aeruginosa* serotype O1. There was negligible binding of the MAbs to cells of *P. cepacia* (data not shown).

Discussion

The aim of this investigation was to prepare MAbs suitable for an immunoassay for the detection of non-mucoid strains of *P. aeruginosa*. MAbs recognising *P. aeruginosa* core LPS unsubstituted by O-serotype antigens were isolated and characterised. The MAbs were shown to be cross-reactive with core LPS antigens belonging to a variety of reference and clinical *P. aeruginosa* strains. The pan-reactive core-specific response of the MAbs, particularly MAbs 304.1.4 and

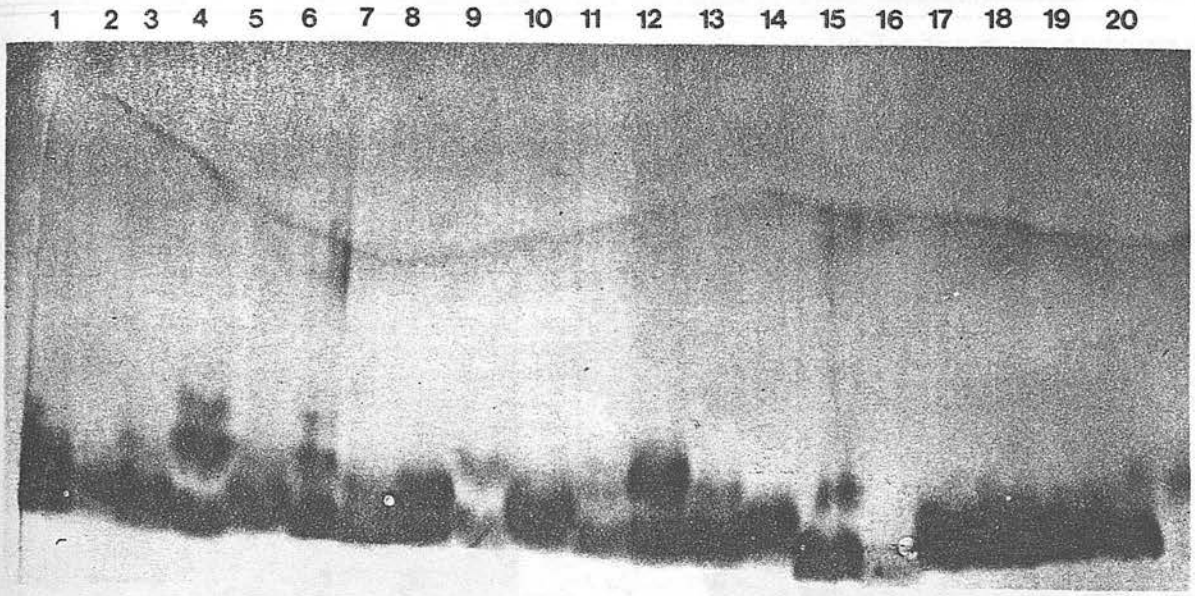

Fig. 3. Immunoblot of proteinase K whole-cell digests of 20 *P. aeruginosa* strains separated by SDS-PAGE followed by transfer to nitrocellulose paper and probed with MAb 360.7 (supernate diluted 1 in 5). The immunoblot profiles against *P. aeruginosa* serotype strains O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, O13, O15, and *P. aeruginosa* LPS R-mutants PAC611, PAC605, PAC609, PAC557 and PAC608 are shown in tracks 1–20 respectively.

Table II. Reactivity in flow cytometry of anti-LPS MAbs against whole bacterial cells.

Strain tested	Percentage reactivity* of MAb		
	304.1.4	360.7	334.4
LPS-defective mutants			
P. aeruginosa PAC605	71	80	75
P. aeruginosa PAC611	20	50	18
P. aeruginosa PAC556	40	73	40
P. aeruginosa PAC609	13	64	52
P. aeruginosa PAC608	24	87	60
P. aeruginosa PAC557	21	39	40
Serotype strains			
P. aeruginosa 01	28	31	27
P. aeruginosa 02	30	45	31
P. aeruginosa 06	30	52	28
P. aeruginosa 09	37	51	41
P. aeruginosa 011	32	31	29
Negative controls			
P. cepacia JN93	0	0	0
E. coli R1	0	0	0
S. typhimurium 1542	0	0	0

*Values represent percentage of bacteria exhibiting positive fluorescence above background levels.

360.7, supports the idea of the presence of a common antigenic structure in the core region of *P. aeruginosa* LPS. The inner core region of *P. aeruginosa* LPS is believed to be homogeneous and also common to most gram-negative bacteria, whereas the outer core region is considered to be heterogeneous[25,26] and different from that of other gram-negative genera.[27] Failure of the MAbs to react with LPS from *E. coli* R1 or *S. typhimurium* 1542, both of which express an Ra core LPS chemotype (complete core), or with whole cells of enterobacteria demonstrate that the MAbs generated recognise core epitopes in *P. aeruginosa* LPS which are not present in these selected strains of Enterobacteriaceae.

Failure of the MAbs to react with the ladder pattern of core LPS substituted with O-antigen, as shown by the immunoblot analysis, suggested that the epitope recognised was present at the distal end of the unsubstituted core oligosaccharide which became unavailable when O-polysaccharide was linked. The fact that the MAbs showed strong reactions in ELISA and flow cytometry with *P. aeruginosa* whole cells expressing S-LPS antigen indicated that there was sufficient unsubstituted core glycolipid to allow binding by the MAbs. This may be a reflection of the low capping frequency of *P. aeruginosa* LPS (the percentage of core LPS constituents covered with O-antigen) leaving a large percentage of core determinants exposed at the cell surface.[25,27,28] In contrast, however, this low level of covering with O-polymer

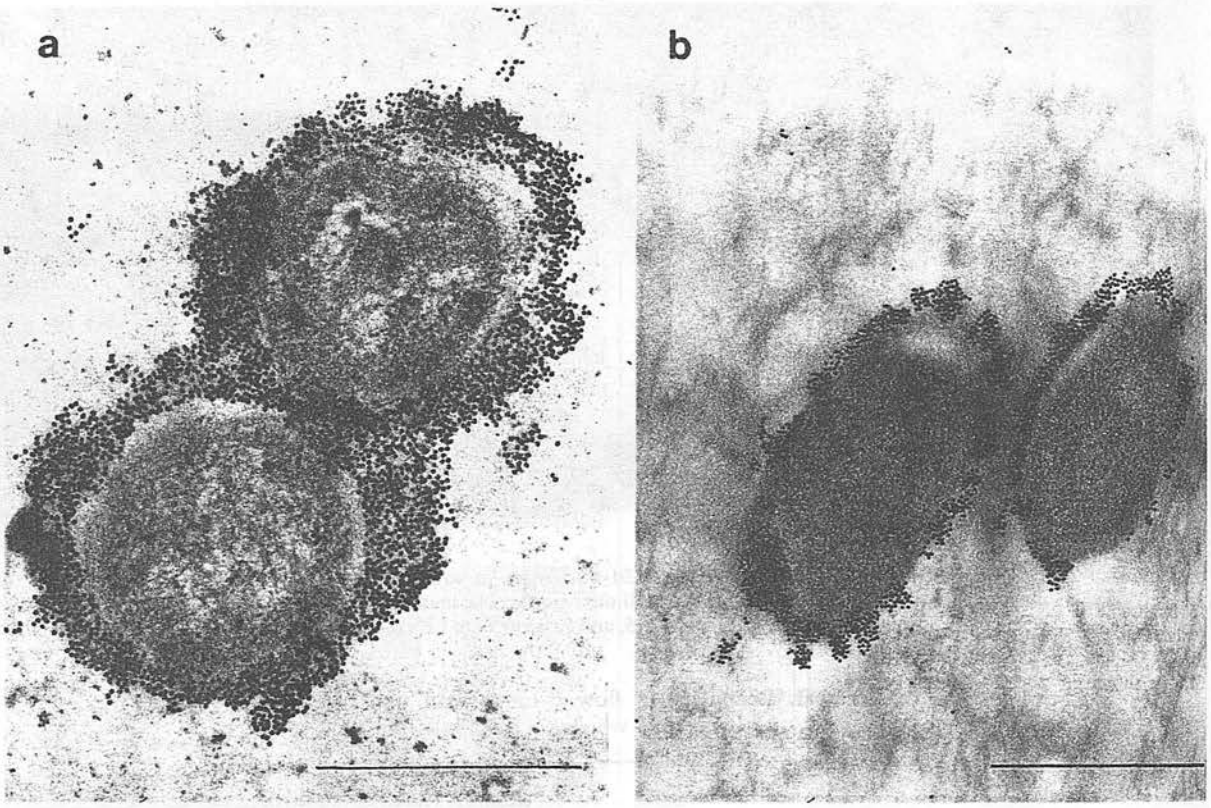

Fig. 4. Electronmicrographs of thin sections from cells of *P. aeruginosa* serotype O1 (**a**) and *P. aeruginosa* PAC611 (**b**) prepared by reaction with MAb 360.7, followed by anti-mouse IgG-gold conjugate. Bars, 1 μm.

was able to inhibit the binding of the rough-core specific MAbs characterised by Sadoff *et al.*[29] MAbs 304.1.4, 360.7 and 334.4 produced a reaction in ELISA, immunoblotting and flow cytometry with all the core mutants which indicated binding to a common epitope.

The MAbs investigated in this study were shown to react with various forms of *P. aeruginosa* LPS, including extracted LPS preparations, cell-wall extracts and whole bacterial cells, including those immobilised on microtitration-plate wells and those prepared for flow cytometry.

Cross-reactivity of the MAbs with whole cells representing a number of other *Pseudomonas* spp. indicates that these species contain core LPS similar in composition and structure to *P. aeruginosa*. Alternatively, their LPS may be structurally dissimilar, but serologically cross-reactive with that of *P. aeruginosa*. The absence of a significant response to (formerly *Pseudomonas*) *X. maltophilia* confirms the observations of Neal and Wilkinson[30] who reported similarities between the LPS of *P. maltophilia* and that of some *Xanthomonas* spp. The lack of a significant response to *P. cepacia* may also reflect some of the reported differences in the structure of *P. cepacia* LPS.[31]

MAbs against *P. aeruginosa* core LPS and reactive with some[26] or all serotypes have been reported previously.[32] The MAbs described in this study are LPS-specific, pan-reactive with all *P. aeruginosa* regardless of serotype and, therefore, potentially useful for the detection of any of the serotype strains which may colonise individual CF patients. Recent studies have focused on the incorporation of the MAbs into a rapid immunological assay for the early diagnosis of asymptomatic colonisation of the CF respiratory tract. False positive reaction caused by the detection of *H. influenzae*, *X. maltophilia*, *P. cepacia*, *Neisseria* spp. and *M.(B.) catarrhalis*, any of which may be found in CF respiratory secretions, are unlikely, given the fact that the MAbs failed to react significantly with whole cells of these organisms. However, the MAbs demonstrated reactivity with strong protein A-producing *Staph. aureus* cells which was probably caused by the interaction of protein A with the Fc portion of antibodies. Since *Staph. aureus* is commonly found in respiratory secretions of patients with CF, any false positive reactions may have to be eliminated from an immunoassay for the detection of *P. aeruginosa* by pre-incubation with an Ig of a different non-cross-reactive species.

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Isolation of a Mucoid Alginate-Producing *Pseudomonas aeruginosa* Strain from the Equine Guttural Pouch

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The isolation and characterization of a mucoid, alginate-producing strain of *Pseudomonas aeruginosa* from a nonhuman host, namely, in chondroids from an equine guttural pouch, is reported for the first time. Pure cultures of *P. aeruginosa* 12534 were isolated from a 17-month-old pony mare with a history of chronic bilateral mucopurulent nasal discharge from the right guttural pouch. Transmission electron microscopy of chondroids showed mucoid *P. aeruginosa* growing as microcolonies within a matrix of extracellular material. On the basis of expression of the mucoid phenotype under different growth conditions, *P. aeruginosa* 12534 belongs to group 1 and resembles other isolates carrying the *muc-23* mutation. The bulk of the extracellular material was characterized as being alginate by chemical and ^{1}H nuclear magnetic resonance analyses, which showed that it had a composition similar to that produced by isolates of *P. aeruginosa* from human patients with cystic fibrosis.

Pseudomonas aeruginosa is a gram-negative, oxidase-positive bacterium that usually appears as nonmucoid colonies when cultured on agar media. However, there is also a mucoid colony phenotype of *P. aeruginosa* which is characterized by overproduction and secretion of copious quantities of the extracellular polysaccharide alginate (11, 29, 30). The mucoid phenotype is due to the influence of *muc* and other mutations which affect the regulation and biosynthesis of alginate in response to environmental stimuli (8, 14). Much of the alginate-biosynthetic pathway has been elucidated (25), and many of the genes have been mapped onto the *P. aeruginosa* PAO chromosome (23, 27). Also, Southern blotting and hybridization experiments have established that both mucoid and nonmucoid isolates of *P. aeruginosa* have chromosomal genes that encode enzymes of alginate biosynthesis. It seems reasonable to conclude from these studies that the genetic capability for alginate biosynthesis is probably present in all strains of *P. aeruginosa* and in certain other closely related *Pseudomonas* species.

Mucoid *P. aeruginosa* was first described by Sonnenschein (33). Subsequent reports of these unusual pseudomonads were sporadic and superficial (4, 6), and most early isolates of mucoid *P. aeruginosa* reported in the literature had been obtained from human sputum or urine. More recently, rare mucoid isolates have been reported from blood (1) and ears (24). Mucoid variants of other pseudomonads, including *P. fluorescens*, *P. putida*, and *P. mendocina*, have been isolated in vitro by using an antibiotic-based selection technique (16), and alginate has also been detected in the plant pathogen *P. syringae* (10, 28).

During the last 2 decades, mucoid *P. aeruginosa* has come to be particularly associated with chronic pulmonary colonization in the lungs of patients with cystic fibrosis or other chronic obstructive airway diseases (13, 19). The initial colonization is with a nonmucoid pseudomonas, but subsequently mucoid variants emerge and eventually predominate. Once established in the lungs, mucoid *P. aeruginosa* is seldom if ever eradicated, even by use of aggressive antibiotic therapy (12, 16).

Despite the isolation of *P. aeruginosa* from a wide range of environmental niches and various tissue sites in infected hosts, no natural niche for mucoid *P. aeruginosa* has been reported. Isolation of mucoid strains of *P. aeruginosa* from patients with cystic fibrosis is relatively commonplace, but to our knowledge, no cases of mucoid *P. aeruginosa* infections in horses or other animals have previously been published. We now report the isolation and characterization of a mucoid strain of *P. aeruginosa* present in chondroids from the equine guttural pouch.

MATERIALS AND METHODS

Source of bacterial isolate. *P. aeruginosa* 12534 was isolated from a 17-month-old pony mare admitted to Glasgow University Veterinary School with a 2-month history of chronic bilateral mucopurulent nasal discharge emanating from the right guttural pouch, an expansion of the Eustachian tube unique to the family *Equidae*.

Identification. Isolate 12534 was confirmed as *P. aeruginosa* by its positive oxidase reaction (21), oxidative utilization of glucose (20), and production of pyocyanin on Pseudomonas Isolation Agar (PIA; Difco Laboratories, Detroit, Mich.); identification was confirmed by the API 20NE system (API-bioMérieux, Vercieu, France).

Electron microscopy. Electron microscopy was based on the ruthenium red staining method of Springer and Roth (34). Chondroid tissue was fixed in a mixture of 1 ml of ruthenium red (1.5 mg/ml) in distilled water, 1 ml of 3.6% (vol/vol) cacodylate buffer, pH 6.5, and kept at 0°C for 1 h. After being washed in 0.07 M cacodylate buffer, the tissue was suspended in a mixture of 1 ml of ruthenium red (1.5 mg/ml), 1 ml of 4% (wt/vol) osmium tetroxide, and 1 ml of 0.2 M cacodylate buffer and incubated for 3 h at 27°C. The tissue was washed once in 0.07 M cacodylate buffer and dehydrated in successive 15-min steps in 25, 50, 75, and 90% (vol/vol) ethanol and then in absolute ethanol for two 1-h periods. Propylene oxide was added for two 10-min periods, and the tissue was then kept in a propylene oxide-Epon

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Araldite (1:1, vol/vol) mixture. The tissue was embedded in fresh Epon Araldite in disposable capsules and maintained at 60°C for 48 h. Sections were cut and placed on rhodium-coated grids. The thin sections were stained with saturated uranyl acetate in 75% ethanol in the dark for 30 min, washed once in 75% ethanol and three times in distilled water, blotted dry, and examined in a Hitachi HU12A electron microscope operating at 75 kV.

Extraction and purification of polysaccharide. *P. aeruginosa* 12534 was cultured on plates of PIA. The growth from three plates incubated at 37°C for 72 h was removed with a glass rod, the extracellular material was separated from bacterial cells by centrifugation, and the supernatant was dialyzed and freeze-dried (31). The crude freeze-dried material was analyzed for total carbohydrate, uronic acid, and protein prior to further purification. The crude freeze-dried extract was redissolved in a minimum of distilled water, and cetylpyridinium chloride was added to give a final concentration of 2% (wt/vol) to precipitate the alginate. The alginate-cetylpyridinium chloride complex was collected by centrifugation (5,000 × *g* for 10 min at 4°C), and the alginate was redissolved by slow addition of solid NaCl (final concentration, 1 M) (26). Further purification was achieved by addition of an equal volume of propan-2-ol to precipitate the alginate, which was redissolved in water. The propan-2-ol precipitation procedure was repeated once, and the samples were freeze-dried. Protein was assayed by the method of Bradford (3) with bovine serum albumin as the standard. Total carbohydrate was assayed with phenol-sulfuric acid reagent (9) and uronic acids with carbazole-borate reagent (2) using D-mannuronic 6,3-lactone as the standard. The O-acetyl content of the alginate sample was assayed by the method of Buscher et al. (5) with β-D-glucose penta-acetate as the standard.

Proton nuclear magnetic resonance spectroscopy. Samples (5 mg) of freeze-dried alginate, the viscosity of which had been reduced by minimal hydrolysis in HCl (30 min, 100°C, pH 2.9), followed by neutralization with NaOH, were added to EDTA (3 mg) and dissolved in D_2O (0.5 ml) at pD 7. Spectra were obtained at 90°C with a Bruker WM 360 nuclear magnetic resonance spectrometer operating in the Fourier transform mode. A 180-τ-90 pulse sequence (τ, ~3 s) with a recycle time of 5 s was used to eliminate the solvent peak (HDO). The ^1H chemical shifts were expressed in parts per million downfield from the internal standard of sodium 4,4-dimethyl-4-silapentane sulfonate. The assignment of peaks was based on previous work (17) and confirmed by the use of algal alginate block structures of known composition (18).

RESULTS AND DISCUSSION

Etiology of the equine infection. Initial culture of the mucopurulent nasal discharge from the right guttural pouch of the pony mare yielded a profuse growth of *Streptococcus zooepidemicus*, an organism considered to be significant in pyemic infections of the equine respiratory tract. The presence of other organisms was not reported at this stage, although appropriate culture methods, which, for example, would have revealed *P. aeruginosa*, were employed. No other abnormality or disease of the respiratory system was noted, nor was the case history indicative of other concomitant respiratory disease. A tentative diagnosis of guttural pouch empyema was made.

Treatment was carried out by flushing the pouch daily for 14 days with 1 liter of sterile saline containing 1 g of ampicillin by means of an indwelling Foley catheter. Trimethoprim-sulfamethoxazole (co-trimoxazole) was administered in the food as a supplementary treatment. The discharge disappeared 2 weeks after initiation of treatment, when multiple chondroids (smooth, pebblelike structures formed from inspissated pus in chronic guttural pouch empyema) were first seen in radiographs of the guttural pouch and later on endoscopy. It is not known whether the chondroids had been present from the time of initial examination, but we suspect that they were and that they became visible to radiographic inspection only because of the reduction in pus production as a result of antibiotic therapy.

The chondroids were removed surgically. During the procedure, a swab was taken from the pouch by the surgeon and a profuse axenic growth of mucoid *P. aeruginosa* was isolated. Similar colonies were isolated from the chondroids and from the postoperative wound, which healed uneventfully. No nonmucoid colonies of *P. aeruginosa* were detected in cultures from the chondroids, guttural pouch, or wound.

Lavage of the pouch continued for 1 week postoperatively. Further radiographs and endoscopy confirmed that the guttural pouch was normal in appearance and, as nasal discharge was no longer present, therapy was discontinued and the animal was returned to its owner. A few weeks later, the discharge reappeared and the animal was destroyed without further investigation or referral to the Veterinary School.

Characterization of bacterial isolates. To the naked eye, the chondroids appeared as pebblelike structures measuring up to 3 cm in length (Fig. 1) and enclosed a matrix of cartilage and pus. From a homogenate of the chondroids, mucoid *P. aeruginosa* was isolated in pure culture at a concentration of 10^7 CFU/g of chondroid; the strain was designated 12534. Large mucoid colonies (Fig. 1) were obtained after 24 h of culture on PIA at 37°C. Isolates obtained from the chondroids were characterized by a simple classification system for mucoid *P. aeruginosa* based on expression of the mucoid phenotype on certain agar-based media (8). Strain 12534 and other already defined isolates of *P. aeruginosa* appeared mucoid when grown on PIA and nutrient, blood, deoxycholate citrate, and minimal agars and classified as belonging to group 1 on this basis.

Recently, we reported that the mucoid phenotype is influenced by the presence of *muc* mutations which affect transcription of alginate regulatory and biosynthetic genes in response to environmental stimuli (8). In this respect, strain 12534 and the other isolates resembled the *muc-23* mutation present in *P. aeruginosa* PA-579 in that in both strains mucoidy is suppressed by high osmolarity (0.3 M NaCl) but increased by the presence of nitrate as the nitrogen source and unaffected by phosphate and sulfate availability.

The antibiotic susceptibilities of the mucoid *P. aeruginosa* isolate and the *S. zooepidemicus* isolate were determined for a wide range of antibiotics by using a disk assay system (Table 1). Of the antibiotics tested, practical veterinary use in the United Kingdom is restricted to penicillin, trimethoprim-sulfamethoxazole, and streptomycin. The in vitro sensitivity of *S. zooepidemicus* is consistent with the clinical observation that treatment with ampicillin followed by trimethoprim-sulfamethoxazole eliminated the nasal discharge. The mucoid *P. aeruginosa* was resistant to this combination of antibiotics, and subsequent determination of the MICs of ampicillin (>128 μg/ml), trimethoprim (4 μg/ml), and sulfamethoxazole (76 μg/ml) supported the clinical observations.

Electron microscopy studies. Transmission electron mi-

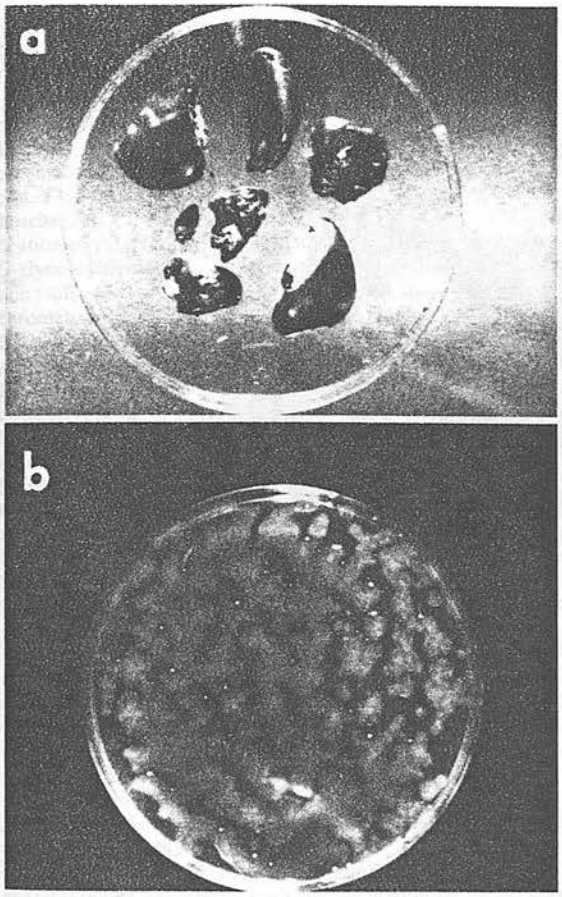

FIG. 1. Pebblelike chondroids (a) from an equine guttural pouch and mucoid *P. aeruginosa* (b) cultured from chondroid tissue.

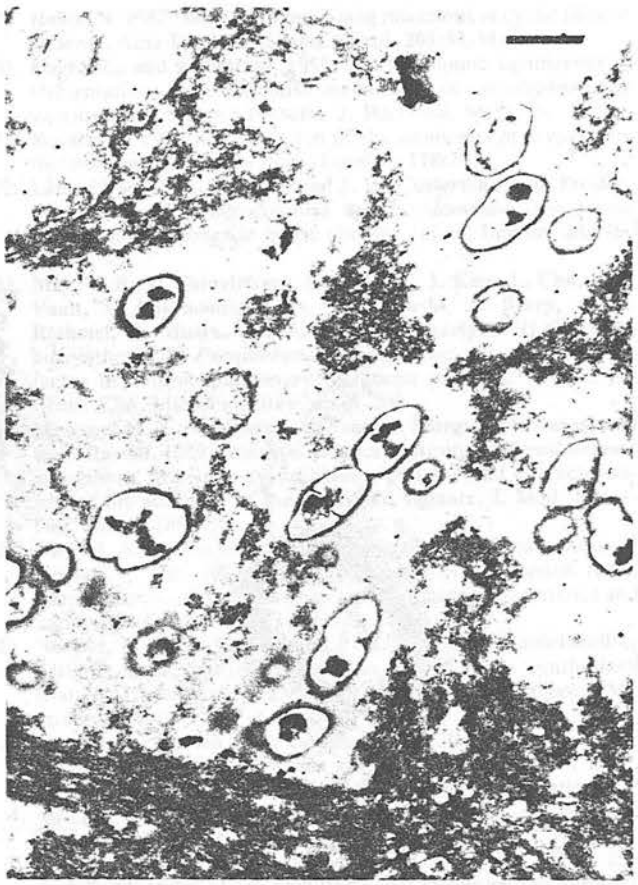

FIG. 2. Electron micrograph of a thin section of chondroid tissue stained with ruthenium red showing dividing bacterial cells surrounded by capsular material and growing within cohesive microcolonies. Bar, 1 μm.

croscopy of ruthenium red-stained thin sections prepared from the chondroids showed the presence of dividing bacilli surrounded by capsular material and growing within cohesive microcolonies (Fig. 2) or as individual cells having a gram-negative cell-like envelope and surrounded by extracellular material (Fig. 3). This is directly comparable to the situation in the lungs of patients with cystic fibrosis, in which mucoid *P. aeruginosa* organisms grow as microcolonies or as biofilms attached to the bronchial mucosa (16, 19, 22).

TABLE 1. Antibiotic susceptibilties of *S. zooepidemicus* and mucoid *P. aeruginosa* isolates

Antibiotic	Amt of antibiotic/disk[a]	Susceptibility[b]	
		S. zooepidemicus	*P. aeruginosa*
Ampicillin	10	+	−
Chloramphenicol	10	+	±
Gentamicin	10		+
Lincomycin	2	+	−
Oxytetracycline	30	+	+
Penicillin G	10	+	−
Polymyxin	300		+
Sulfamethazole-trimethoprim	23.75, 1.25	+	−
Streptomycin	10	−	−

[a] Amounts of antibiotics in disks are given in micrograms, except for those of penicillin and polymyxin, which are given in units.
[b] Antibiotic susceptibilities were determined by using a standard agar plate method with antibiotic-impregnated filter paper disks.

Characterization of extracellular material. Growth of strain 12534 on PIA for 72 h typically resulted in a yield of 225 mg of crude freeze-dried water-soluble extract per three plates. The crude material was composed of 59.3% carbohydrate, 39.2% uronic acid, and 1.8% protein. Following precipitation of the extracellular material with cetylpyridinium chloride, an alginate fraction was obtained in which >85% of the carbohydrate was uronic acid. Analysis of this material by ^{1}H nuclear magnetic resonance showed that in common with alginates from other *Pseudomonas* spp. (31, 32), there was no discernible polyguluronate block structure. Nearest-neighbor frequency analysis of the various doublet frequencies showed that the alginate was composed of 40% polymannuronate and 60% mixed-block structure, giving a mannuronate-to-guluronate ratio of 2.33. The ratio of O-acetyl groups to total uronic acid was found to be 0.76, as determined by chemical assay. Assuming that the O-acetyl groups are associated exclusively with mannuronate residues (7), this value would give a mannuronate-to-O-acetyl ratio of 1.09, indicating that a proportion of the mannuronate residues are di-O-acetylated, presumably in the 2 and 3 positions on the sugar ring (32). The analytical data show that strain 12534 produces alginate with a composition similar to that produced by isolates of *P. aeruginosa* from patients with cystic fibrosis and from other sources (31, 32).

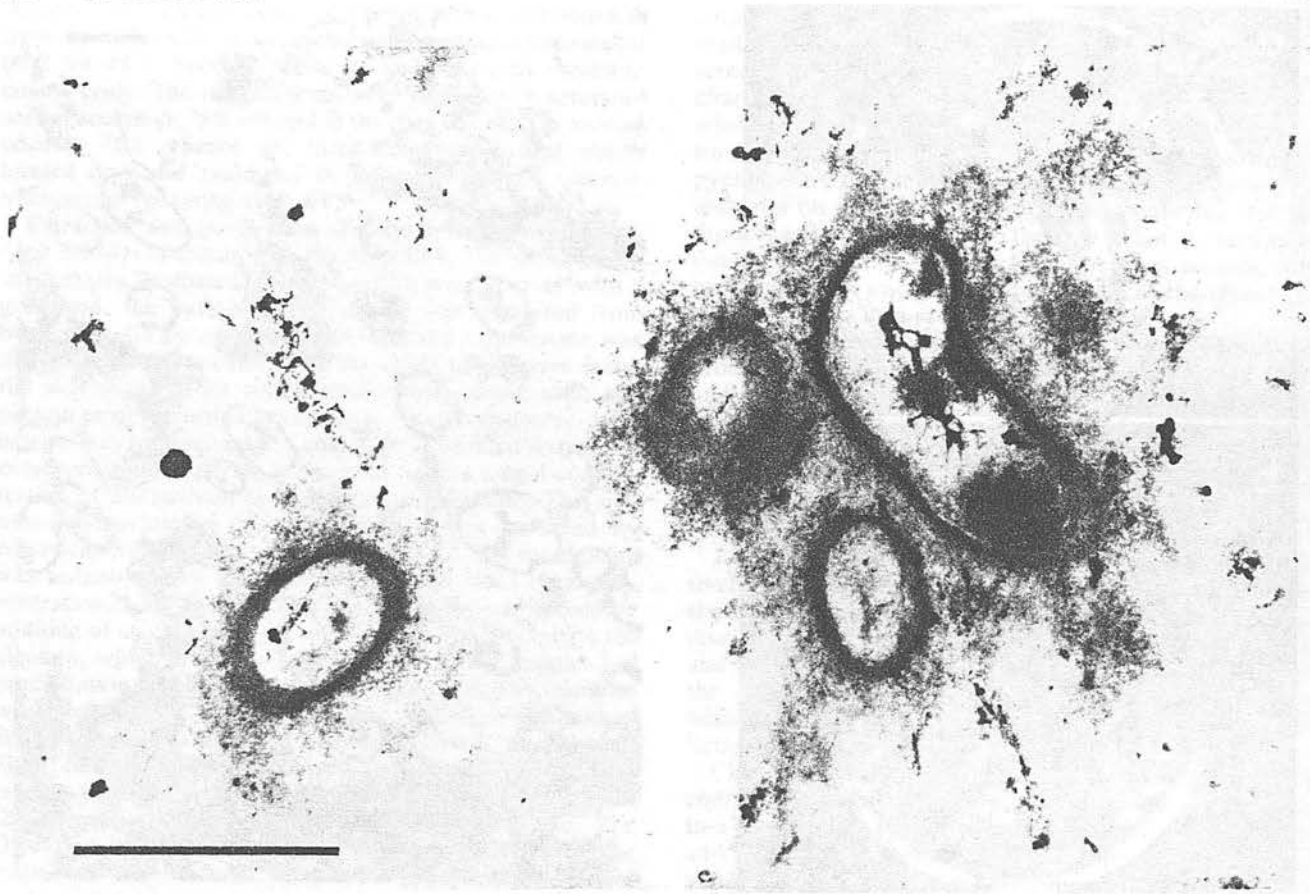

FIG. 3. Electron micrograph of a thin section of chondroid tissue stained with ruthenium red showing gram-negative bacteriumlike cells surrounded by fibrous extracellular material. Bar, 1 μm.

Implications for equine medicine. *S. zooepidemicus* empyema of the guttural pouch is a frequent end stage of equine strangles, a horse form of streptococcal sore throat which is initiated by *S. equi* and occurs in young horses. However, the presence of mucoid *P. aeruginosa* within the chondroids indicates that this organism is also implicated in chronic equine respiratory disorders and was probably trapped during the formation of these structures. The case history shows that the presence of *P. aeruginosa* in the pus was not apparent until the initial streptococcal infection had been eliminated by treatment with antibiotics. Thus, the emergence of mucoid *P. aeruginosa* following antibiotic treatment for equine *Streptococcus* infections is analogous to the microbial etiology of cystic fibrosis in humans, in which previous antibiotic therapy for infection with *Staphylococcus aureus* and/or *Haemophilus influenzae* is associated with subsequent colonization and secondary infection with *P. aeruginosa* (16). It is well documented that in cystic fibrosis, infection with *P. aeruginosa* is associated with chronicity. In the present report, it is unclear whether *S. zooepidemicus* or *P. aeruginosa* was the primary colonizer. The clinical etiology of a chronic infection and the heavy colonization with *P. aeruginosa* in the chondroids so soon after clearance of *S. zooepidemicus* by antibiotic therapy indicate that the *P. aeruginosa* infection was well established by the time of admission of the pony for treatment. Also, it is important to note that the clinical symptoms persisted after eradication of the *S. zooepidemicus* infection and therefore *P. aeruginosa* is probably a genuine causative agent of chronic respiratory infection in horses.

This report describes the first isolation of mucoid *P. aeruginosa* from an equine source. However, the pathological significance of mucoid *P. aeruginosa* in veterinary medicine is unknown, although other pseudomonads such as *P. diminuta* and *P. maltophila* can be associated with chronic respiratory diseases in horses, and in scanning electron microscopy of guttural pouch epithelium we have observed bacterial rods identified as pseudomonads together with *S. zooepidemicus* (29a). These studies on *P. aeruginosa* 12534 confirm the association of the mucoid phenotype with chronic disease in a nonhuman host and provide an interesting and unusual biological parallel between equine microbiology and human pulmonary microbiology.

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Detection of *Pseudomonas aeruginosa* in sputum from cystic fibrosis patients by the polymerase chain reaction

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A DNA amplification procedure using heat stable *Taq* polymerase and the polymerase chain reaction is described for the detection of *Pseudomonas aeruginosa* in specimens from cystic fibrosis patients. A set of primers was selected on the basis of the nucleotide sequence of the *algD* gene encoding GDP mannose dehydrogenase, a major enzyme in the biosynthesis of alginate by *P. aeruginosa*. Using this set of primers in conjunction with the polymerase chain reaction, *P. aeruginosa* could be specifically detected, with a sensitivity approximating 10 bacteria, in sputum harbouring large numbers of other respiratory pathogens, including *Staphylococcus aureus* and *Haemophilus influenzae*. These results suggest that amplification of specific sequences within the *algD* gene by the polymerase chain reaction may provide a highly sensitive and specific tool for the detection of *P. aeruginosa* in the early stages of pulmonary colonization.

KEYWORDS: Pseudomonas aeruginosa, polymerase chain reaction, cystic fibrosis

INTRODUCTION

Pulmonary colonization with mucoid *Pseudomonas aeruginosa* is a major cause of morbidity and mortality in patients with cystic fibrosis (CF). Initial asymptomatic and often intermittent colonization of the upper respiratory tract with a non-mucoid strain of *P. aeruginosa* usually precedes chronic colonization with mucoid variants of the original strain.[1,2] In CF patients, detection of *P. aeruginosa* in the early stages of pulmonary colonization would increase the opportunity for early therapeutic intervention and could assist in the identification of primary colonization sites. At present, detection of pulmonary colonization with *P. aeruginosa* is based on quantitative culture of organisms from homogenized sputa, or indirectly by detection of pseudomonas-specific antibodies using enzyme-linked immunosorbent assay.[3,4]

Since the polymerase chain reaction (PCR) has been used successfully to identify other bacteria, we attempted to develop a rapid and sensitive method for detecting *P. aeruginosa*. This approach might serve as an alternative to other methods for the detection of non-mucoid and mucoid forms of *P. aeruginosa*, particularly in the early stages of pulmonary colonization.

In prokaryotes, alginate biosynthesis is restricted to *Azotobacter vinelandii*, an organism not associated with human colonization, and to *P. aeruginosa* and a few other pseudomonas species.[5–7] We chose the *algD* gene as the conserved region of the *P. aeruginosa* genome to be amplified, since it codes for GDP mannose dehydrogenase, an enzyme essential for alginate biosynthesis:[8] *algD* is present in non-mucoid and mucoid *P. aeruginosa* and is not found in higher organisms.

MATERIALS AND METHODS

Bacteria

The bacterial strains used are listed in Table 1.[9–14]

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Strains of *P. aeruginosa* comprised the well-characterised genetic strain PAO1 and its isogenic mucoid variants PAO568, and PAO579 and the mucoid CF isolate 492a. In addition to the bacterial species listed in Table 1, 12 non-mucoid isolates of *P. aeruginosa* (six CF isolates and six non-CF isolates) and 10 strains of *P. cepacia* were investigated.

Sputum culture

Expectorated sputum was obtained after chest physiotherapy, and after liquefaction with Sputolysin (Behring Diagnostics, La Jolla, California, USA) and suitable dilution (10^2, 10^4) in sterile physiological saline, quantitative culture for respiratory pathogens was performed by plating out 0·1 ml volumes onto blood agar, lysed blood agar incorporating 0·5% bacitracin and *Pseudomonas* isolation agar (Difco Laboratories, Detroit, Michigan, USA) and incubating aerobically at 37°C for 48 h. Isolates were identified by standard procedures.[2]

Polymerase chain reaction

Genomic DNA from either bacterial suspensions or Sputolysin-treated sputum samples was obtained by phenol-chloroform extraction and ethanol precipitation. Briefly, 10 µl of sample was diluted to 100 µl with 0·1 M Tris-Cl pH 7·5 and added to an equal volume of phenol/chloroform, heated at 37°C for 5 min and centrifuged for 3 min at 10,000 rev/min (Eppendorf microfuge). The aqueous layer was extracted twice with an equal volume of chloroform and DNA was precipitated by addition of 1/10th volume of 3 M NaCl and 3 volumes of ethanol. The pellet was resuspended in 10 µl of sterile water and amplified by the polymerase chain reaction.[15] The primers shown in Table 2 were designed using the *algD* sequence data of Deretic *et al.*[8] and obtained from Oswel DNA Service (Department of Chemistry, University of Edinburgh). The nesting procedure, which allows for greater sensitivity, was performed using the internal primers Pa2 and Pa4 to amplify 2 µl of the PCR product from an initial round of PCR with

Table 1. Bacterial species and strains used in this study

Species	Strain and reference	
P. aeruginosa	PAO1	(9)
P. aeruginosa	PAO568*	(9)
P. aeruginosa	PAO579*	(9)
P. aeruginosa	CF492a*	(9)
P. fluorescens	NCIMB 10525 strain 12	(10)
P. putida	NCIMB 10007 strain C1-B	(10)
P. testosteroni	NCIMB 8893 strain 79	(10)
P. acidovorans	NCIMB 9681 type strain	(10)
P. alkaligenes	NCIMB 9946 type strain	(10)
P. mendocina	NCIMB 10541 type strain CH-50	(11)
P. diminuta	NCIMB 9393 type strain	(12)
P. stutzeri	NCIMB 9040	(13)
Xanthomonas maltophilia	NCIMB 9203 type strain	(14)
Staphylococcus aureus	Clinical isolate	
Streptococcus pyogenes	Clinical isolate	
Haemophilus influenzae	Clinical isolate	

* Mucoid. NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen U.K.

Table 2. Sequences used for the oligonucleotide primers

Primer sequence	Homologous to *algD* position*
Pa1 5′ GACAGGTTGAGCTTGTGG	1297–1280
Pa2 5′ CGAACTGGACAAGCAGAC	1067–1084
Pa3 5′ GAATTCCTCCGCGAGAGC	1005–1022
Pa4 5′ GCAGATCACGTCCATCAC	1274–1257

* Sequence from Deretic *et al.*[8]

primers Pa1 and Pa3. The appropriate primer pair (50 pmol each) and DNA were mixed in 100 µl of 10 mM Tris-Cl pH 8·3, 50 mM KCl, 2·5 mM MgCl, 10% DMSO and amplified through 30 cycles of 91°C (30 s), 40°C or 50°C (30 s) and 72°C (1 min) with 2 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Hemel Hempstead, England) using an automated Thermal-Cycler (Perkin-Elmer/Cetus). After the 30th cycle, incubation continued at 72°C for 10 min to complete elongation of the PCR products. Ten per cent of the reaction product was run on a 2% agarose gel and stained with ethidium bromide to visualize the amplified DNA.

To show that DNA prepared directly from small amounts of bacteria can be amplified, the results obtained from serial 10-fold dilutions of a culture *P. aeruginosa* PAO1 are shown (Fig. 2b). On the original gel it was possible to see faint bands in lanes containing PCR product from reactions with 30 and three organisms (lanes 10 and 1, respectively). Again no signal was visible in the negative controls. These results show that nested PCR is sufficiently sensitive and specific to detect very small numbers of *P. aeruginosa*.

To demonstrate that the procedures could detect *P. aeruginosa* in clinical material, DNA was extracted from 10 µl of sputolysin-treated sputum from patients with CF known to be colonized by respiratory pathogens, and amplified by the nested PCR as previously described. The results shown in Fig. 3 indicated that the PCR procedure can specifically detect small numbers of mucoid and non-mucoid *P. aeruginosa* in sputum against a background of other bacterial species.

RESULTS

For maximal sensitivity and specificity it was found necessary to adopt the 'nested primer' technique (Materials and Methods and Fig. 1). An initial pair of primers (Pa1 and Pa3) were used in 30 cycles of PCR to generate a product of 292 bp from a relatively large number of genomic templates (3×10^5, Fig. 1). Increasing the number of cycles allowed the detection of a smaller number of templates (3×10^3), but only by using 2% of the original reaction product in a second round of PCR with the internal ('nested') primers Pa2 and Pa4 (Table 2) could as few as 30 genomes be detected (Fig. 1). The identity of the PCR product was confirmed initially by digestion with restriction enzymes predicted to cut the amplified DNA, and subsequently by DNA sequencing (data not shown). No signal was visible when no template DNA was included in the reactions: the extreme sensitivity of PCR requires that such negative controls be included in every experiment.

The sensitivity and specificity of the technique are illustrated in Fig. 2. First, genomic DNA of known concentration, from the *P. aeruginosa* strain PAO1, was serially diluted to give the number of templates shown and amplified as described above. No signal was visible using less than three copies of *P. aeruginosa* DNA as template or with 10^5 copies of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Haemophilus influenzae* or human DNA (Fig. 2a). The 13 non-mucoid and three mucoid *P. aeruginosa* investigated each gave a positive reaction (data not shown). In contrast, no signal was visible using as template 10^5 copies of 10 strains of *P. cepacia* and the various

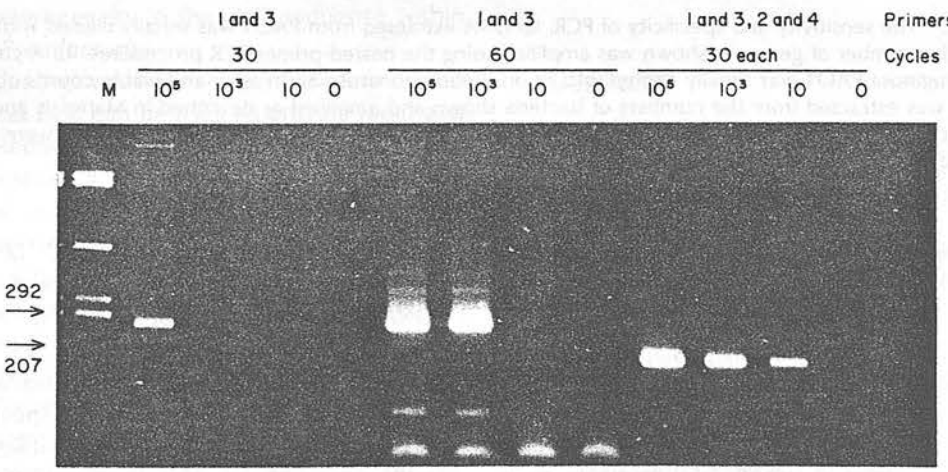

Fig. 1. The sensitivity of nested-primer PCR. DNA extracted from *P. aeruginosa* strain PAO1 was diluted in water to give the number of genomes shown and amplified with the primer pairs indicated, as described in Materials and Methods. During the PCR, reactions with primers Pa1 and Pa3 were annealed at 40°C to maximize sensitivity, those with primers Pa2 and Pa4 were annealed at 50°C to maximize specificity. The 292 bp and 207 bp products are arrowed. Lane M contains DNA size markers (PBR322 DNA digested with *Taq* 1).

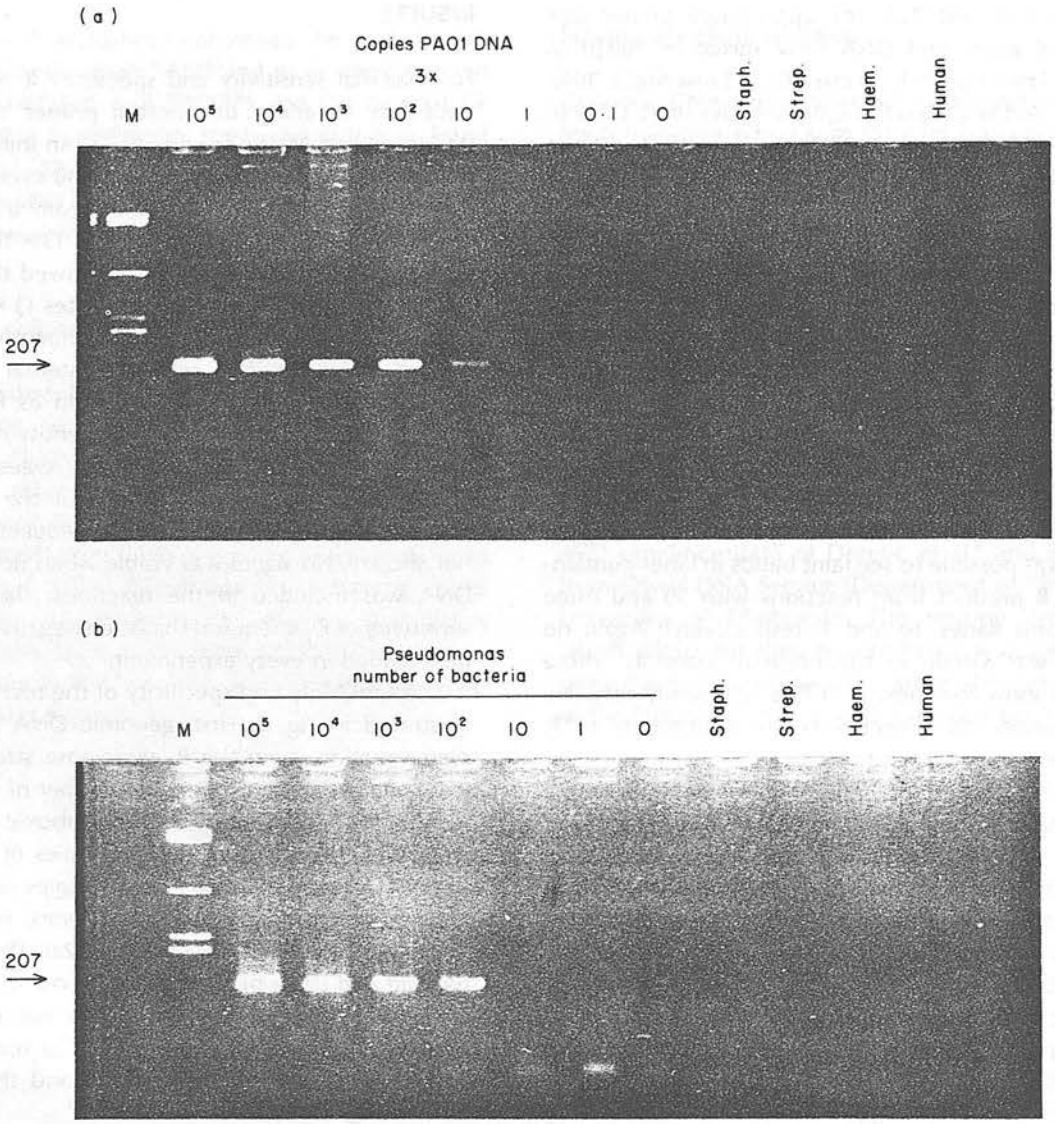

Fig. 2. The sensitivity and specificity of PCR. (a) DNA extracted from PAO1 was serially diluted with water and the number of genomes shown was amplified using the nested-primer PCR procedures. (b) A culture of *P. aeruginosa* PAO1 was serially diluted, plated on *Pseudomonas* isolation agar, and viable counts obtained. DNA was extracted from the numbers of bacteria shown and amplified as described in Materials and Methods. Copies of genomic DNA, 3×10^5, from *S. aureus*, *S. pyogenes*, *H. influenzae* and man were similarly amplified in each experiment as controls.

other species of *Pseudomonas* listed in Table 1 (data not shown).

DISCUSSION

The data presented in this study show that PCR provides a technique as specific and probably more sensitive than the assays explored so far. Quantitative bacterial culture from homogenized sputum by the method described can detect 2×10^2 colony-forming units (cfu) ml^{-1}. Amplification of small amounts of genomic DNA from bacterial cultures indicated that as little as three copies of PAO DNA could be detected. Three copies of pseudomonas genomic DNA is equivalent to approximately 0·01 pg. Investigation of samples of sputum from CF patients containing *P. aeruginosa* and other respiratory bacteria demonstrated good sensitivity and specificity since as little as 10^3 cfu of *P. aeruginosa*, identified by bacterial culture, provided a clear signal despite the presence of large numbers of other bacteria.

As a diagnostic tool, serology does not necessarily reflect active colonization. In the present study sputum samples culture-negative for *P. aeruginosa* but providing a clear signal following PCR may indicate a

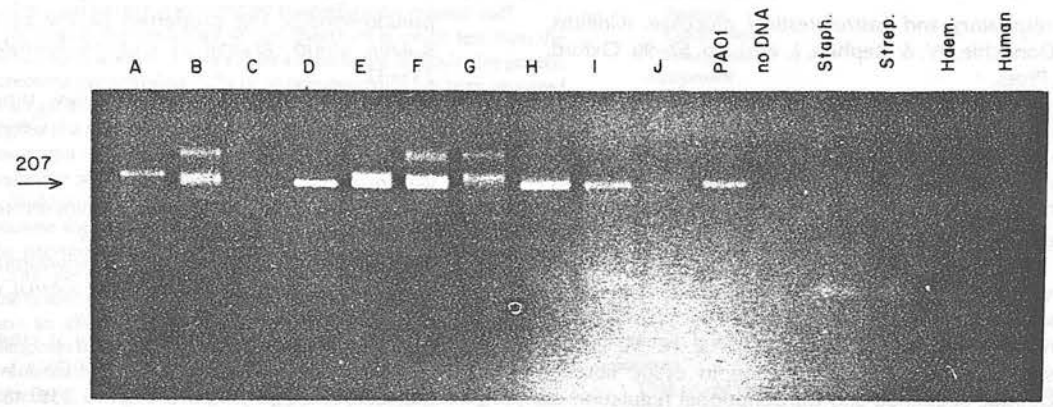

Fig. 3. Specific detection of *P. aeruginosa* in the presence of other bacterial species. DNA was extracted from a series of sputum samples and amplified as described in Materials and Methods. By bacterial culture, mucoid *P. aeruginosa* was detected in samples A, B, E, F, G and J (at 3×10^7 ml^{-1} to 1×10^8 ml^{-1}); non-mucoid *P. aeruginosa* in sample D (at 1×10^3 ml^{-1}); *S. aureus* in samples C, D, H, and I (at 4×10^6 ml^{-1} to 3×10^7 ml^{-1}): and *H. influenzae* in samples D, H and I (at 2×10^5 ml^{-1} to 4×10^7 ml^{-1}). DNA amplification was not observed in sputum sample C which produced 10 cfu of *S. aureus* but no *P. aeruginosa*. Controls are as described in the legend to Fig. 2.

high sensitivity rather than false-positive reactions. This explanation was supported by the negative PCR results obtained with sputa obtained from non-CF patients and previously shown to be culture-free for *P. aeruginosa* (data not shown).

It was interesting, and unexpected, to observe that the strains of *P. putida*, *P. fluorescens* and *P. mendocina* examined in the study did not produce a signal with the *alg*D primers employed; previous studies had shown these strains to be capable of alginate biosynthesis.[6,16] The negative PCR result for these pseudomonas species illustrates the specificity of the chosen *alg*D primers for identification of *P. aeruginosa* and suggests heterogeneity in the *alg*D sequence within the genus *Pseudomonas*.

In conclusion, we have presented data which suggests that PCR can be used to provide rapid and specific detection of small numbers of *P. aeruginosa* in sputa against a background of other bacterial species. The use of *alg*D primers resulted in an assay which is apparently specific for *P. aeruginosa* and sufficiently sensitive, first: to indicate the absence of *P. aeruginosa* colonization which would be useful in the application of vaccination therapy, and second: to detect the initial stages of colonization and the early and beneficial institution of antipseudomonas chemotherapy.[17]

It also seems reasonable to speculate on the potential use of PCR to detect other bacteria in the sputa of CF patients. *Pseudomonas aeruginosa* may overgrow *H. influenza* and other potential pathogens in culture. As a consequence, non-capsulated, non-group B *H. influenzae* are probably underdiagnosed and their pathogenic significance underestimated in CF lung disease.[2] Another bacterial candidate for detection by PCR is *P. cepacia*, a species which has emerged as an important pathogen among CF patients and whose pathogenicity and epidemiology merit further study.[2,18]

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understanding that individual results would not be available, and that some couples may have misunderstood the test and assumed that a negative result was truly negative for both partners. Furthermore, the value of information about carrier status for siblings of carriers had not been mentioned to patients—they were given information about carrier testing in an explanatory leaflet rather than having non-directive counselling by trained staff.

Although the frequency of non-paternity is much less than the 10% widely quoted, it may still be a substantial difficulty for genetic screening programmes.[1] 2% or so non-paternity has been reported amongst the parents of CF children[4] but non-paternity rates may be higher in a population where the risk of CF is not perceived as high and might not be seen by the mother to justify the complications of assigning paternity explicitly.

Wald wishes to spare carriers whose partners test negative anxiety because they "cannot be reassured" by a residual risk higher than the pre-test risk (risk of affected fetus is 1/500 in contrast with 1/2500 pre-test). It is quite possible that women may perceive this risk as low, be glad to be able to inform their family, and, if they do have an affected child, be less upset than if they had been given an inappropriately low risk result by couple screening.

It is only ethical to withhold information about carrier status if the benefits of doing so are rigorously proven. Genetic testing requires non-directive counselling by trained staff to allow individuals to make a truly informed choice.

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Pseudomonas cepacia in inpatients with cystic fibrosis

SIR,—*Pseudomonas cepacia* is now recognised as an important pathogen in patients with cystic fibrosis (CF).[1-3] Patients colonised with this organism seem to be at increased risk of pulmonary exacerbations and death.[4] *Ps cepacia* tends to be resistant to antibiotics and disinfectants, and pulmonary infection is very difficult to treat and to prevent, because modes of transmission and factors involved in pulmonary colonisation are not established. A 1984 study[1] in CF patients suggested nosocomial transmission but a link with epidemic pulmonary colonisation was not confirmed. A longitudinal study[4] suggested a nosocomial source for colonisation but *Ps cepacia* was not cultured from solutions or environmental surfaces. Ribotyping of isolates has suggested person-to-person transmission of *Ps cepacia* in CF, and the failure of other investigators to recover *Ps cepacia* from the hospital environment was cited in support of that view.[5]

We have used ribotyping and bacteriocin typing[6] to characterise *Ps cepacia* isolated from environmental surfaces in a hospital room occupied by 2 CF patients. The 2 women (X and Y, aged 26 and 27) had been admitted with exacerbations of bronchiectasis and they had shared a two-bed room for two weeks and used the same shower. Both were known to be colonised by *Ps cepacia*, but with different strains.

Selective medium[7] was used for culture studies. Swabs were first moistened in minimal broth containing polymyxin (300 U/ml). After primary inoculation swabs were incubated at 30°C for 48 h and turbid broths were subcultured. Culture plates were incubated at 30°C and checked for growth at 24 h intervals up to 72 h. *Ps cepacia* was identified biochemically (API 20 NE system; API-bioMérieux).

Ps cepacia bacteriocin type S3/P0:ribotype A was cultured from the sputum of patient X and isolates with similar profiles were also cultured from a drinking cup used by patient X and from gloves worn by a doctor who had handled her sputum container. *Ps cepacia* was not cultured from gloves or cup before use. S21 P0:B (the profile of *Ps cepacia* colonising patient Y) was cultured from the drain of the shower:

Source	Culture
Patients X and Y	
Sputum	+
Cough plates	–
Nose, hands	–
Equipment	
Gloves (before:after use)	– +
Drinking cup (before:after use)	– +
Stethoscope, nebuliser, vitalograph	–
Environmental surfaces	
Sink drains, taps, and basins	–
Shower head	–
Shower drain	+
Vase-water	+
Plant soil	+
Table surfaces	–

Isolates from water in a vase of flowers and from soil in a plantpot differed in typing profile from the organisms colonising the patients.

This study demonstrates both the value of selective media for isolation of *Ps cepacia* from environmental sites and surfaces and the potential for indirect transmission of *Ps cepacia* between patients. LiPuma et al[5] favoured person-to-person transmission and concluded that unnecessary contact between colonised and non-colonised CF patients should be avoided. Others doubt if ordinary social contact between colonised and non-colonised patients is harmful.[2] We found that CF patients can contaminate environmental surfaces with *Ps cepacia*, confirming that indirect transmission is possible. We did not isolate *Ps cepacia* from a CF patient in the room next to patients X and Y who had frequent social contact with them. Our findings suggest the need for reasonable precautions in the management of colonised patients. What is meant by "reasonable" in this context has yet to be defined, and until the precise mode of transmission of this opportunist pathogen is known a policy of segregating colonised from non-colonised CF patients must remain controversial.

We thank Dr Ty Pitt for arranging the ribotyping.

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Antithrombin III and arterial disease

SIR,—Dr Meade and colleagues (Oct 5, p 850), in their prospective investigation of the relation between antithrombin III and subsequent death from arterial disease, find that there are more deaths from arterial disease in the lower and upper thirds of the antithrombin III distribution than in the middle third. We examined the distribution of fatal and non-fatal well documented atherothrombotic events in 953 patients with pre-existing coronary, cerebral, or peripheral ischaemic disease followed for 24 months in

EDITORIALS

Pseudomonas cepacia—more than a harmless commensal?

Fears of *Pseudomonas cepacia* cross-infection and the clinical outcome of colonisation have caused patients with cystic fibrosis (CF) and their doctors to take drastic measures. What scientific evidence underlies their actions?

Until lately, the bacterial pathogens largely responsible for pulmonary exacerbations in CF patients were *Staphylococcus aureus, Haemophilus influenzae*, and *Pseudomonas aeruginosa*. In the early 1980s there were reports from North American clinics of a disturbing increase in the prevalence of *P cepacia* from CF sputa.[1,2] Between 1986 and 1989, surveillance studies in the UK indicated a maximum prevalence of 7%,[3-5] but isolations have now risen in some CF centres to equal the 40% prevalence noted in the latest report from a North American centre, the Hospital for Sick Children in Toronto, Canada.[6] The increased isolations cannot be attributed solely to the use of selective culture media and reliable identification systems.

Clinically, *P cepacia* colonisation can be symptomless or it can be associated with slowly declining lung function. More alarming, some colonised patients who previously have been mildly affected by their disease unexpectedly succumb to an accelerated and fatal deterioration in pulmonary function with fever, necrotising pneumonia, and in some cases septicaemia; this syndrome is not observed with other CF pathogens and females previously in good clinical condition seem to be at special risk.[2] In early studies, *P cepacia* colonisation was largely restricted to young adults; colonisation in children remains low, but even so the first reported death in the UK associated with *P cepacia* was in an eight-year-old CF patient.[7]

P cepacia, an environmentally ubiquitous plant pathogen, takes its name from its role as the cause of soft rot of onions. Paradoxically, this organism also produces antibiotic compounds that play an important part in the biological control of plant infections such as tobacco wilt and fungal spoilage of fruit.[8] *P cepacia* is nutritionally versatile—it can grow in disinfectants and antiseptics and can even use penicillin G as a nutrient. The pathogen is naturally resistant to most antibiotics, and, even if individual strains show in-vitro susceptibility to an antibiotic, there is little clinical response and so the patient is denied effective therapy.

Human infections with *P cepacia* are uncommon and tend to occur in immunocompromised patients and in those who have acquired the organism in hospital from contaminated equipment.[9] Although CF patients are often heavily infected with *P cepacia*, with sputum colony counts of 10^8 CFU/ml, a direct pathogenic role for the organism is not proven. *P cepacia* is virtually non-pathogenic in healthy human beings and does not synthesise alginate or elastase, key virulence determinants associated with *P aeruginosa* colonisation in CF patients.[5] Some strains of *P cepacia* synthesise iron-chelating siderophores[10] and exhibit variable degrees of binding to human respiratory mucin;[6] these observations led to speculation that such factors contribute to morbidity and mortality and may explain why some strains colonise patients transiently whereas other strains, once acquired, are never lost. Similarly, variations in virulence may explain the clinical spectrum observed in CF patients. The role of host factors in determining clinical outcome is probably important. The manner by which the organism evades host immunity remains to be determined; synergy with other pathogens is a possibility. With the exception of a non-protective IgG antibody response to *P cepacia* surface antigens,[11] little is known about the host immune response and pathophysiology of *P cepacia* infection.

The reasons for the susceptibility of CF adults to *P cepacia* colonisation are unclear. A case-control study[12] showed that, apart from increasing age, colonisation was associated with severity of underlying disease, a colonised sibling, and previous hospital admission. Moreover, colonisation was associated with increased morbidity. Inhaled colistin—to which the organism is invariably resistant—is another possible risk factor; however, there is little evidence to support this hypothesis and the role of other antibiotics is unclear.[12]

Cross-infection with *P cepacia* is the cause of widespread concern. An epidemiological study[13]

mentioned previous failures to recover *P cepacia* from extensive surveillance cultures of respiratory equipment and environmental surfaces, and the researchers concluded that direct person-to-person transmission of the organism might be the primary means by which transmission occurs. A subsequent prospective study[14] with reliable selective media and typing systems showed that colonised patients can contaminate their environment—ie, indirect transmission might occur via contaminated surfaces. Although there is no scientific evidence to confirm that skin contact, respiratory aerosols, sharing food, kissing, or other forms of intimacy increase the risk of acquiring *P cepacia*, doctors who care for CF patients have come round to the view that kissing and having a colonised sibling probably present special risks.

There are calls for policies to reduce the risk of acquisition of *P cepacia* and to reassure non-colonised patients. Segregation of colonised from non-colonised patients effectively creates "microbiological outcasts". The social effects of segregation on older patients are devastating since their lives often revolve around CF contacts. Meanwhile, non-colonised patients become increasingly anxious about "getting cepacia". Finally, segregation imposes a considerable burden on medical staff in large clinics and needs to be accompanied by advice that social contacts should also be restricted.[15]

Attitudes to *P cepacia* are evolving rapidly and it is difficult to give firm guidance about the risks and consequences of colonisation. However, several centres in Europe and North America segregate colonised from non-colonised patients, and a British CF association has produced interim guidelines to reduce the risk of cross-infection.[16] An indication of the impact of these recommendations can be appreciated from this section alone: "*P cepacia* colonised CF individuals should avoid kissing and intimacy with non-colonised CF individuals. They should also refrain from sleeping, exercising or performing physiotherapy in the same room as non-colonised CF individuals. Contact between colonised CF individuals and CF children should be avoided. On current evidence, colonised CF individuals should probably not attend CF conference/holiday camps." For patients and all concerned in their care, the stigma and problems of *P cepacia* colonisation are very hard to accept. Nevertheless, the risks cannot be ignored.

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Heart disease: in the beginning

In Norway in the 1970s, Forsdhal was puzzled by the geographical distribution of cardiovascular mortality.[1] Why were there substantial regional differences when the standard and mode of living was reasonably uniform throughout the country? Could these differences be related, he wondered, not to present circumstances but to poverty or deprivation in early life? More recently, Rose has pointed out that well-established risk factors for coronary heart disease—cigarette smoking, high serum cholesterol, and high blood pressure—have very limited ability to predict disease in adults.[2] Could childhood influences explain this gap in our understanding of aetiology? Epidemiologists, notably Barker and colleagues in the UK Medical Research Council Environmental Epidemiology Unit at Southampton University, have taken up the challenge by exploring Forsdahl's hypothesis on childhood deprivation.[3]

In a series of studies based mainly on national statistics in the UK, the USA, and Norway, adult cardiovascular mortality rates were shown to correlate with indicators of childhood deprivation in earlier years—ie, with infant mortality[1,4–7] and short adult and childhood height.[8,9] There are also correlations with measures suggestive of intrauterine deprivation such as maternal and neonatal mortality.[10,11] Not content with these statistical comparisons, Barker et al identified, by means of painstaking detective work, two populations that they were able to follow-up retrospectively.[12,13] In 5654 men born in Hertfordshire between 1911 and 1932, they found that the death rate from ischaemic heart disease was related inversely to weight at one year and was higher in men who had weighed less than 5·5 lb (2·5 kg) at birth.[12] Furthermore, indicators of early deprivation were associated in the same cohort with cardiovascular risk factors in adult life—blood pressure, plasma

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Microbiology of lung infection in cystic fibrosis

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This review is dedicated to the memory of Janis McCulloch who it was a privilege to know as a friend and whose courage and fight for the highest quality of life achievable will continue to be an inspiration.

Bronchopulmonary infection in cystic fibrosis (CF) patients is associated with chronic progressive lung disease and episodes of acute exacerbation. Infection is predominantly caused by bacteria, although infections with viruses, mycoplasma and fungi may play undervalued roles. Bacteria commonly isolated from CF sputum include *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa*. Colonisation of the airways by mucoid, alginate-producing variants of *P. aeruginosa* is recognised as a major cause of pulmonary deterioration. In addition, there is now considerable concern relating to the clinical consequences of colonisation and cross-infection with *P. cepacia*. This review discusses the microbiology of CF focussing on the pathogenesis and epidemiology of *P. aeruginosa* and *P. cepacia*.

Despite the development of modern antibiotics and vaccines, we are still faced with many debilitating and fatal infections which demonstrate the adaptability of microbial pathogens. The pathophysiology and treatment of bronchopulmonary infections in patients with cystic fibrosis (CF) present one of the most daunting modern challenges to patient, clinician and scientist.

This review will summarise present understanding of the complex association and adaptation of bacteria to the lungs of patients with CF, the laboratory detection and epidemiology of the most important pathogens, mechanisms of pathogenicity and developments in the action and assessment of antibiotics against chronic bronchopulmonary infection. Other respiratory problems including antibiotic regimens are discussed

in pages 931–948. Aspects of CF microbiology which are generally accepted or have been discussed extensively in previous reviews[1–4] will be dealt with briefly.

MICROBIOLOGY OF CF

The abnormal host defence mechanisms in the airways of CF patients resulting from expression of the CF gene and the resulting susceptibility to chronic pulmonary colonisation do not submit readily to generalisations either in explaining the mechanisms involved in colonization by individual bacterial species, the pathophysiology of repeated exacerbations or the most appropriate strategies for antimicrobial therapy. In general, however, the microbiological problems of CF can be summarised as follows:

1. Although the lungs of neonates with CF are histologically normal[5] susceptibility to pulmonary infections is soon apparent. Infections are localised in the lungs and in particular in the major and minor airways rather than the alveoli; localised infections at non-pulmonary sites or systemic infections are rare.

2. Pulmonary infections are associated with insidious and eventually chronic colonisation by bacterial pathogens, and episodes of acute debilitating exacerbations due to bacteria, viruses and occasionally fungi, superimposed upon progressive lung disease.

3. The spectrum of bacteria, viruses and fungi associated with respiratory infection in CF patients is comparatively restricted. The respiratory pathogens most commonly isolated include *S. aureus*, non-capsulate *H. influenzae* and *P. aeruginosa*; predominant viruses include RSV whilst *Aspergillus* spp tend to predominate amongst the fungi.

4. In association with the improved life expectancy of CF patients and developments in therapeutic regimens (intermittent and often prolonged use of potent antimicrobial agents and steroids) so the spectrum of opportunistic microbes has expanded; today, in some CF centres there is concern on the increasing incidence of *P. cepacia* and mycobacteria particularly in older CF patients. Longitudinal studies of individual CF patients diagnosed at birth have revealed that susceptibility to particular pathogens is usually age-related; microbial colonisation tends to follow a sequence during a patient's lifetime. In general, but not invariably, the sequence begins with *S. aureus* colonisation in infancy followed by *H. influenzae* in the early years, *P. aeruginosa* in early adolescence with the highest incidence of *P. cepacia* and mycobacteria in the late teens.

MICROBIAL PATHOGENS

S. aureus

The first organism recognised to cause chronic lung infections in the majority of young CF patients was *S. aureus*; in the pre-antibiotic era, few CF patients survived beyond infancy and staphylococcal infection was the major cause of mortality.[5,6] The predominance of *S. aureus* in early studies led to a widespread conviction that this species was not only the major pathogen in CF patients but that colonisation by this organism played a key role in causing tissue damage which 'primed' the lung for subsequent colonisation by *H. influenzae* and *P. aeruginosa*. The consequence of this hypothesis was the development in many CF centres of antimicrobial policies which included long term or intermittent antistaphylococcal therapy on diagnosis of CF. The policy of long term, prophylactic antistaphylococcal therapy has remained the subject of controversy almost since the advent of the antibiotic era. Proponents of the policy suggest that such treatment prolongs protection against inevitable colonisation with other pathogens, in particular *P. aeruginosa*; in contrast, retrospective studies point to an association between prolonged antistaphylococcal therapy and increased incidence of *H. influenzae* and *P. aeruginosa* and a poor prognosis.[7, 9] In Edinburgh, the policy is to treat exacerbations associated with high sputum carriage of *S. aureus* (>10^7 cfu/ml), although occasionally short term prophylactic cover is provided, eg when anti-pseudomonal or anti-haemophilus agents are required and the patient is known to harbour *S. aureus*. This restricted use of anti-staphylococcal prophylaxis may be one factor to explain the relatively low incidence of 30% colonisation with *P. aeruginosa* even in adolescent CF patients.[10] The efficacy and consequences of long-term antistaphylococcal prophylaxis in CF patients is presently the subject of several longitudinal, placebo-controlled studies.

Despite the controversy of prophylactic antistaphylococcal therapy, there is little doubt that this organism is an important cause of respiratory distress and progressive lung damage during the life of a CF patient. *S. aureus* produce teichoic acid and exopolysaccharide which appear to play a role in adherence to respiratory epithelium in CF patients.[11,12] The precise role, if any, of other virulence factors including coagulase, leucocidin, protein A, catalase, hyaluronidase, haemolysins and exotoxins in pulmonary colonisation, evasion of immune clearance systems and direct or indirect lung damage remains unclear.

P. aeruginosa

Early bacteriological investigations of CF patients showed that colonisation with *P. aeruginosa* played a minor role to *S. aureus*. Under its original species epithet *P. pyocyanea* the organism was recovered from only one of the 14 post mortem studies reported by Di Sant'Agnese in 1946.[6] With the development of antistaphylococcal antibiotics and the increased population of older CF patients, *P. aeruginosa*, emerged as the most important bacterial pathogen responsible for lung disease in CF patients.[1–4]

P. aeruginosa is well-known as an opportunistic pathogen par excellence, causing a wide range of infections in compromised patients. This reputation stems from an innate resistance to most commonly used antibiotics and disinfectants and the ability to produce a range of potential virulence determinants. Important features associated with pseudomonas colonisation in CF patients is firstly that colonisation remains restricted to the lung and secondly that the organism undergoes considerable phenotypic adaptation within the CF lung environment.[13] The most striking phenotypic characteristic of the organism, is the emergence of mucoid variants due to copious production of the exopolysaccharide, alginate.[13,14] During the last decade, the debilitating pathological sequelae of chronic pulmonary colonisation and repeated exacerbations due to mucoid *P. aeruginosa* and the involvement of alginate in a complex process of adhesion, protection against immune clearance, and indirect tissue damage has been the subject of considerable research. Recent understanding of the molecular regulation of alginate biosynthesis in *P. aeruginosa* has also provided a fascinating insight into the influence of bacterial mutation and the lung environment on the adaptation of *P. aeruginosa* to the CF lung.[13,15–17] An attempt will now be made to review the 'state of the art' on these topics.

Colonisation with *P. aeruginosa*

In the 1970s, pyocin typing studies suggested that mucoid *P. aeruginosa* were derived from the original non-mucoid colonising strain rather than as *de facto* variants acquired by CF patients from exogenous sources.[18] Subsequently, the use of an improved pyocin typing method[19] and a DNA-based typing system using restriction fragments derived from the exotoxin A gene[20] confirmed this hypothesis in one of the few detailed accounts of the transition to the mucoid phenotype following waterborne acquisition of a non-mucoid *P. aeruginosa* by 2 CF patients[13] (Fig. 1).

Colonisation of the CF airways by *P. aeruginosa* is usually with nonmucoid forms. However, since the interval between primary coloni-

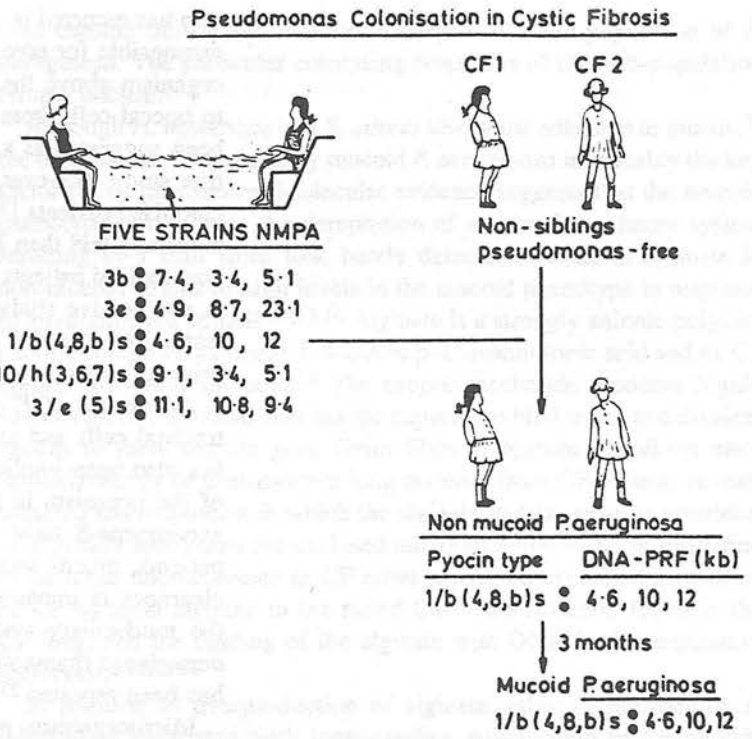

Fig. 1 Water-borne acquisition of *P. aeruginosa* and transition to the mucoid phenotype. One week after using a hydrotherapy pool, two previously non-colonised CF patients were found to be sputum positive for the same strain of *P. aeruginosa*: pyocin type 1/b (4,8,b)s, DNA probe type 4.6, 10, 12. Water sampled from the pool contained the colonising strain together with four other strains of *P. aeruginosa*. Three months later, a mucoid variant of the colonising strain was isolated from one of the patients. For further explanation *see* text and References 1, 13, 30.

sation and the emergence of mucoid variants can be less than three months,[13] mucoid strains may be observed on primary bacteriological investigation. In most cases, chronic colonisation occurs with a single strain of *P. aeruginosa* which undergoes phenotypic variation over a period of time; in some patients, however, transient carriage of more than one strain of *P. aeruginosa* occurs.

The mechanism and identity of the sites of early colonisation with *P. aeruginosa* remain obscure. It has been suggested that colonisation of the upper respiratory tract may precede aspiration of the organism into the lower airways.[21] Reports of upper respiratory tract colonisation sites include maxillary sinuses, tongue, buccal mucosa, saliva and dental plaque.[21–23] Following heart-lung transplantation, pulmonary colonisa-

tion has recurred in some patients with the same strain of *P. aeruginosa* responsible for pretransplant colonisation suggesting a reservoir of the organism above the surgical anastomosis. Adhesion of *P. aeruginosa* to buccal cells from CF patients can be demonstrated in vitro and has been suggested as a possible site for initial colonisation.[23] A prospective study, however, failed to confirm colonisation of buccal cells in colonised patients.[13] Although gastrointestinal carriage of *P. aeruginosa* is usually less than 10% in healthy humans, it increases significantly in hospitalised patients and is often a reservoir for opportunistic infections. A prospective study, however, showed no evidence that the gastrointestinal tract of CF patients is a significant reservoir of *P. aeruginosa* prior to pulmonary colonisation.[24]

Adherence of *P. aeruginosa* to other respiratory surfaces including tracheal cells and mucins, the major component of respiratory mucus, has also been implicated in pulmonary colonisation and establishment of the organism in the CF respiratory tract; alginate, pili, lectins and exoenzyme-S have been suggested as bacterial adhesins.[25,26] In CF patients, mucus secretions are dehydrated and viscid and mucociliary clearance is impaired; viral and mycoplasmal infections may damage the mucociliary system in CF and inactivation of ciliary beat by *P. aeruginosa* rhamnolipid, pyocyanin and proteases and by *H. influenzae* has been reported.[27,28]

Microorganisms may become entrapped in respiratory mucus because of its stickiness or through specific interactions between receptors in mucin and surface components of bacteria. In vitro assay systems have demonstrated adherence of *P. aeruginosa* to respiratory mucins,[29,30] as well as chemotaxis of the organism towards mucin and its constitutive amino acid and sugar components.[30] The particular affinity of *P. aeruginosa* for respiratory mucins may explain the propensity of the organism to colonise and persist in the mucosal secretions of CF patients.

Further support for this hypothesis comes from the enhanced chemotactic and mucinophilic properties of the water-borne colonising strain described earlier[13,30] and from the studies of CF lung tissue using scanning electron microscopy which show the association of *P. aeruginosa* with surface mucous secretions rather than with the underlying epithelia.[31] Binding of *P. aeruginosa* to mucin may also protect the organism from opsonophagocytic killing, and may contribute to its persistence in the CF respiratory tract.[32] Finally, it is often considered that CF patients are colonised by any strain of *P. aeruginosa*. This may not be true. In addition to the evidence that mucinophilic properties may enhance the colonising potential of individual strains, a retrospective study of the pyocin type distribution of *P. aeruginosa* for CF and non-CF patients showed that strains colonising CF patients and non-CF patients

with chronic obstructive diseases belonged to a sub-population of *P. aeruginosa*. The particular colonising properties of this sub-population remain unclear.[33]

Although *H. influenzae* and *S. aureus* also show adhesion to mucus,[34] the biosynthesis of alginate by mucoid *P. aeruginosa* is probably the key factor for its persistence. Molecular evidence suggests that the mucoid phenotype results from the derepresion of a normal regulatory system resulting in a shift from low, barely detectable levels of alginate in non-mucoid strains to high levels in the mucoid phenotype in response to environmental stimuli.[13,15,16] Alginate is a strongly anionic polysaccharide composed of linear 1–4 linked β–D-mannuronic acid and its C5 epimer α-L-guluronic acid.[14] The exopolysaccharide produces highly viscid aqueous solutions and has the capacity to bind water and divalent cations to form flexible gels. Gram films of sputum and direct electronmicroscopy of post-mortem lung material from CF patients reveals bacterial microcolonies in which the alginate matrix occupies considerably greater space than the enclosed microbes.[2,3,13,35,36] The formation of bacterial microcolonies in CF airways may be explained by gelling of the bacterial alginate in the raised Ca^{2+} concentration found in the CF lung, and the binding of the alginate with DNA in the respiratory secretions.[2,13,14,36]

In addition to overproduction of alginate, other phenotypes of *P. aeruginosa* associated with long-standing colonisation in CF include loss of surface O-specific lipopolysaccharide, polyagglutination with O-specific sera, sensitivity to the bactericidal action of normal human serum and enhanced susceptibility (at least in vitro) to a range of antibiotics.[2,13] Factors which may contribute to the intractibility of mucoid *P. aeruginosa* to antibiotic therapy include: difficulty in achieving bactericidal concentrations in the airways, antagonism of β-lactam and aminoglycoside antibiotics by the high electrolyte content of the alginate gel, and the protection afforded by abscesses and bronchial plugs formed by the association of alginate with bronchial secretions.[36,37] Protection by the microcolony mode may account for the apparently suicidal characteristics of serum sensitivity and hypersensitivity to antibiotics observed in many CF isolates.[13] In addition the term 'frustrated phagocyte'[2,38] has been applied to phagocytes, particularly neutrophils, which stimulated by immune complexes and immunoglobulins, and faced with microcolonies measuring up to 60μm, release elastase or toxic oxygen radicals and contribute to the considerable immune mediated damage observed in CF lungs[39] (*see* Warner, this issue).

Prevention of chronic *P. aeruginosa* by early antibiotic treatment

Epidemiological and genetic evidence suggests that non-mucoid *P. aeruginosa* play a key role as a microbial reservoir to allow the emergence and selection of mucoid and other phenotypes associated with chronic colonisation and progressive lung disease.[13] Molecular studies indicate that the mucoid phenotype is due to spontaneous *muc* mutations in the colonising strain which regulate alginate biosynthesis in a positive or negative manner in response to environmental stimuli, for example, increased osmolarity.[13,15,16] Thus it would seem rational to attempt to prevent the initial stages of asymptomatic colonisation by non-mucoid strains. The major strategems include prevention of acquisition either directly by person-to-person transmission or indirectly from contamintated environmental sources or therapeutic intervention by antibiotic treatment and vaccination.

Transmission of P. aeruginosa

It is unrealistic to totally protect non-colonised CF patients from such a ubiquitous saprophyte as *P. aeruginosa*. However, the availability of reliable and discriminating typing methods including pyocin typing,[19] analysis with DNA probes[20] and genomic fingerprinting (using endonucleases and pulse field electrophoresis)[40] assists epidemiological surveillance. The choice of typing method depends on the facilities available and the presence of reference laboratories. Recently, the results of a comparative study of simple methods such as serotyping, phage typing, and pyocin typing compared to the more demanding DNA-based methods including DNA probe typing and genome fingerprinting showed that the results of genome fingerprinting and DNA probe typing showed the best correlation followed by pyocin typing. The discriminatory effect of genome fingerprinting was higher than that of DNA probe typing, and the former was found to be the best single method for epidemiological investigations of polyagglutinable *P. aeruginosa* from CF patients.[41] Surveillance is recommended to identify cross-infection within individual clinics or between patients attending different clinics who may be in social contact. It may be advisable to re-examine hygiene and institute controversial procedures, such as segregation of colonised from non-colonised patients. Some CF clinics maintain a policy of segregation at all times, despite the considerable financial and social implications. During the last 15 years, surveillance studies of Edinburgh patients as agreed with those of other centres, namely that with the well-documented exception of CF siblings, cross-infection with *P. aeruginosa* is relatively rare, each patient being colonised by a distinct strain of *P. aeruginosa*. Although an individual

patient may be transiently colonised by additional strains, the original coloniser is usually maintained for many years. Thus in Edinburgh clinics, a policy of segregating patients colonised with *P. aeruginosa* has not been implemented.

Prevention by early antibiotic treatment

Several studies have shown that temporary eradication of *P. aeruginosa* can be achieved by antibiotic therapy during the early stages of colonisation.[42,43] Recently, a controlled prospective study has shown that chronic colonisation with *P. aeruginosa* can be prevented in cystic fibrosis by early institution of a therapeutic regimen comprising oral ciprofloxacin and aerosol inhalations of colistin twice daily for 3 weeks, whenever *P. aeruginosa* is isolated from routine sputum cultures.[44]

Vaccination against P. aeruginosa

The prospect of a successful vaccine to prevent colonisation by *P. aeruginosa*, has been a major aim in the management of CF patients. The special requirements of vaccination to accommodate the pathogenesis of *P. aeruginosa* in CF have been discussed in previous reviews.[13,45] Briefly, a successful vaccine would have to: (a) confer long-lasting local protection at the mucosal surfaces of the respiratory tract; (b) take account of immune complexes as a major cause of tissue damage; (c) be based on the most appropriate bacterial antigen from the various virulence determinants known to be involved in colonisation and pathogenesis; and (d) be administered at the most suitable age and microbial status to avoid side-effects and achieve maximum benefit.

In the 1980s, a long term trial of a pseudomonas lipopolysaccharide-based vaccine in CF patients who were already colonised with mucoid *P. aeruginosa* was discontinued when it became clear that vaccinated patients were deteriorating more quickly than non-vaccinated patients. In 1987, an interesting retrospective study of antibody status in colonised and non-colonised CF patients identified alginate-specific opsonophagocytic killing antibodies which appeared to protect against *P. aeruginosa* in a subset of older CF patients.[45] Recently, a study has been completed in young non-colonised CF patients using a conjugate vaccine based on a *P. aeruginosa* octavalent O-conjugate polysaccharide-toxin.[46] Opsonic and toxin A-neutralising antibodies to all vaccine antigens were significantly raised after vaccination and remained so for 12 months. After vaccination, there was no significant change in clinical status, and adverse reactions were mild and self limiting. The protective effect of these promising vaccines is under investigation.

P. cepacia

In the early 1980s, several North American clinics reported a disturbing increase in the isolation of *P. cepacia*,[47,48] a relatively little known phytopathogen first isolated in 1950 as the cause of bulb rot in onions. Human infections with *P. cepacia* are rare, tending to occur in hospitalised patients with reduced immune status who acquire the organism from contaminated equipment or disinfectant solutions.[49] The species is naturally resistant to most antibiotics and can even utilise penicillin G as a nutrient! Young CF adults appear to be particularly susceptible to *P. cepacia* colonisation. Clinical evidence indicates three categories of involvement:

1. Chronic asymptomatic carriage,
2. Progressive deterioration over many months, and
3. Rapidly fatal deterioration[47,48]—septicemia, a rare occurrence in CF has also been recorded.

In 1986, the first recorded death in the UK associated with *P. cepacia* was reported.[50] In this young 9-year-old female, the rapid terminal course of the infection contrasted with the typical prolonged deterioration seen in CF lung disease associated with other microbial pathogens and emphasized the need for vigilance in monitoring and accurately identifying the organism in all centres.

Epidemiology of *P. cepacia* colonisation

Retrospective case-controlled studies[51] suggest that the risk of *P. cepacia* colonisation may be increased by: (a) existing severe lung disease; (b) having a sibling with CF who is also colonised; (c) increasing age, hospitalisation during the previous 6 months; and, in some studies, (d) treatment with aminoglycoside antibiotics. In the absence of suitable methods to characterise individual strains, it was unclear whether *P. cepacia* was emerging *de facto* as an inevitable coloniser of CF patients or whether the increase in individual clinics reflected cross-infection. Another problem concerned the ability of laboratories to successfully isolate and identify *P. cepacia*. For example, in a quality control exercise, only 36 of 115 laboratories isolated *P. cepacia* from simulated sputum specimens seeded with the organism; whereas 14 of 15 laboratories using *P. cepacia* selective media isolated the organism.[52] The development of selective culture media for *P. cepacia*[4,53] has allowed more accurate estimates of the incidence of *P. cepacia* in CF patients, contamination of hospital staff, equipment and environmental surfaces and nature of environmental habitats. The development of typing systems to characterise individual strains has also assisted epidemiological studies of cross-infection and routes of transmission in CF patients. Recent

evidence based on ribotyping and bacteriocin typing suggests that *P. cepacia* may be transmitted from patient-to-patient[54] and that colonised patients can contaminate the hospital environment and thus increase the risk of indirect acquisition.[55] There is still much debate, however, whether *P. cepacia* is primarily transmitted by person-to-person spread, or indirectly via contaminated equipment or solutions. Evidence for the former route includes: (a) the increased risk for patients with a colonised sibling; (b) failure in some outbreaks to recover *P. cepacia* despite extensive surveys of respiratory equipment or to demonstrate a link between environmental and human isolates; and (c) the clustering of cases within some clinics and the observation that colonisation rates are reduced if colonised patients are isolated from non-colonised patients. The second factor could be explained by the use of inappropriate culture media and the previous lack of typing systems to compare individual isolates. LiPuma et al used selective culture medium and ribotyping to show that colonised patients attending the same CF centre harboured the same strain, and to provide evidence of person-to person transmission between two CF patients from different CF centres during attendance at an educational camp.[54] Although the evidence supported person-to person transmission, LiPuma and colleagues did not include environmental surveillance for *P. cepacia* which could have implicated indirect transmission from contaminated environmental sources. In addition, the study gave only tantalising clues as to the degree of personal contact required for transmission. The two patients involved were in contact for only 6 days; in contrast, none of the other 14 young adult CF patients who remained in contact with the original colonised patient for a further 4 weeks acquired *P. cepacia*. Additional evidence for a transmission risk comes from an ongoing investigation of cross-infection in patients attending different UK clinics; ongoing epidemiological studies suggest that the risk of cross-infection during a CF clinic is low and that close and prolonged social contact probably presents a greater risk (Govan and Nelson, unpublished results)

Epidemiological evidence from several CF centres suggests that the transmissibility of *P. cepacia* is greater than that of *P. aeruginosa*. Thus it could be argued that segregation of colonised from non-colonised patients should be considered to reduce the risk of *P. cepacia* colonisation. Segregation, however, may not be achievable in some clinics and the social and ethical consequences of such a policy cannot be taken lightly; for example, what are the merits of segregating young adults within a CF clinic if they frequently 'socialize' outside the clinic or may even be married to a non-colonised CF patient! In conclusion, much remains to be clarified concerning the colonising factors, epidemiology and clinical significance of *P. cepacia*. Debate on the issues outlined in Table 1 will

continue for some time and hopefully be assisted by data from ongoing multicentered studies.

Table 1 Major issues associated with *P. cepacia* colonisation in CF

1	What properties of *P. cepacia* contribute to pulmonary colonisation and do individual strains differ in virulence?
2	To what extent does the clinical outcome in an individual CF patient depend upon bacterial and host factors?
3	To what extent do immunoglobulins produced against *P. cepacia* protect against, or contribute to, pulmonary damage?
4	Do patients colonised with *P. cepacia* contaminate their enviroment and, if so, how long do the organisms survive after deposition on surfaces?
5	What is the risk of person-to-person transmission? Is this risk enhanced by an increased pool of colonised patients within a CF centre and by carriage of the organism on the hands of patients or hospital staff?
6	What are the therapy-related risk factors for non-colonised patients to acquire *P. cepacia*? For example, does the use of steroids or particular antibiotic regimens increase the risk of acquisition?
7	To what degree should social contact between colonised and non-colonised patients be controlled?
8	The previous question has important social and financial implications; for example, should summer camps or young adult conferences be avoided or restricted to non-colonised patients. Since the latter policy is conditional on reliable culture and identification of *P. cepacia* how far should steps be taken to ensure the use of agreed laboratory protocols?

OTHER MICROBIAL PATHOGENS

Although, *S. aureus, P. aeruginosa* and *P. cepacia* have the major impact on morbidity and mortality in CF patients, the extent to which other microbial pathogens contribute to exacerbations, inter-species synergy and progressive lung damage should not be underestimated.[1,2,4]

Respiratory exacerbations due to non-capsulate, non-typable *H. influenzae* are probably underestimated and underdiagnosed in CF patients. Contributing factors include difficulties in distinguishing coloni-

sation by this respiratory commensal from infection and culturing the relatively fastidious *H. influenzae* from sputum in the presence of large numbers of other organisms. In our experience, some of the highest concentrations (>200mg/l) of the inflammatory marker, C-reactive protein, have been associated with *H. influenzae* exacerbations.[2,56] The isolation of various other microbes from CF sputa, including *Xanthomonas maltophilia*, is probably of little clinical significance unless it is present at concentrations >10 ^{6} cfu/ml and associated with clinical symptoms including significant serum concentrations of inflammatory markers.

Vaccination against influenza and other respiratory viruses is to be recommended in CF patients. In the diagnostic laboratory, the potential involvement of chlamydia, mycoplasmas, *Candida albicans* and in particular *Mycobacteria* spp requires vigilance. Amongst the pathogenic fungi, *Aspergillus fumigatus* may be responsible for noninvasive allergic bronchopulmonary aspergillosis (ABPA) for which corticosteroids are the treatment of choice.[57,58] A positive test for Aspergillus precipitins is supportive evidence but diagnosis of ABPA is usually based on characteristic clinical findings including bronchoconstriction, pulmonary infiltration, eosinophilia, increased serum IgE levels, skin test reactivity and serum precipitins to *A. fumigatus*.[58] The prevalence of ABPA in CF has been reported to be between 0.5 and 11% and occurs primarily in young adult patients.[15]

LABORATORY INVESTIGATION OF CF PATHOGENS

Culture

Quantitative culture of potential pathogens from the sputum of CF patients is useful to provide information on the load of bacteria within the airways and on the efficacy of antimicrobial therapy. The homogenisation and dilution involved in quantitative culture, also aids recovery of relatively fastidious pathogens such as *H. influenzae* which may be obscured by overgrowth of other organisms, particularly mucoid *P. aeruginosa*. Developments in non-cultural techniques for *H. influenzae* include immunoperoxidase-staining with monoclonal antibody directed against a bacterial outer membrane protein[59] and the use of the polymerase chain reaction (PCR).[60] Clinical improvements including weight gain, improved pulmonary function and reduction in serum acute phase proteins can be associated with a reduction, but seldom eradication of pathogens whilst the patient is undergoing antibiotic therapy.[2,9,10,56,61,62] The clinical value of quantitative culture, however, declines as colonisation becomes prolonged. In these patients, rising

concentrations of C-reactive protein in excess of 10mg/l are useful in assessing the efficacy of antibiotic therapy.[2,56,62]

Appropriate selective media for culture of *S. aureus* include blood agar and mannitol-salt agar. The latter medium is particularly recommended in association with long-term use of trimethoprim-sulfamethoxazole which results in thymidine-dependent *Staphylococci* exhibiting abnormal colonial morphology.[4] Despite intensive antimicrobial pressure, methicillin-resistant *S. aureus* are infrequently isolated from CF patients. Reliable selective media for culture of *H. influenzae* include the use of chocolate blood agar supplemented with bacitracin (10 units/ml) and incubated anaerobically or in an atmosphere of 10% CO_2.[2]

Suitable selective media for *P. cepacia* include a polymyxin-ticarcillin PC medium[4] (Mast Laboratories, UK)—a positive culture, however, must be confirmed as *P. cepacia* using an appropriate system (such as API 2ONE) since some strains of *X. maltophilia* and *Comamonas* (formerly *Pseudomonas acidovorans*) will also grow on this medium (Govan, unpublished observations). The selective medium of choice for *P. aeruginosa* is Difco Pseudomonas medium—if this medium is used for quantitative culture of sputum it should be appreciated that the presence of hypersusceptible mutants can reduce the numbers of bacteria recovered.

Diagnosis of *P. aeruginosa* based on immunological and polymerase chain reaction methodology

CF patients mount a specific antibody response against cellular and extracellular antigens of *P. aeruginosa*. Pseudomonal antibodies have been detected by a variety of immunological methods including crossed immunoelectrophoresis,[63] enzyme-linked immunosorbent assay[64,65] and immunoblotting.[66] An increasing antibody response against *P. aeruginosa* is useful to diagnose progression from superficial colonisation to invasive infection and provides an insight into immune-mediated tissue damage associated with immune-complexes.[39,63,64] In some, but not all studies, it has been reported that antibodies can be detected in serum before sputum bacteriology becomes positive for *P. aeruginosa*.[67] From accumulated evidence it seems reasonable to suggest that detection of raised anti-*P. aeruginosa* antibodies, whether in the presence or absence of detectable organisms in sputum, should encourage instigation of anti-pseudomonal therapy in an attempt to improve patient prognosis.

In addition to bacteriological culture and measurement of *Pseudomonas* specific antibodies, rapid detection of the organism may be achieved at concentrations of $>10^2$ cfu/ml sputum by monoclonal an-

tibody based immunoassays[68] and by detection and amplification of nucleic acid sequences specific to the *alg*D gene using the polymerase chain reaction.[69]

Antibiotic susceptibility testing

Susceptibility testing of *S. aureus* and *H. influenzae* isolates by standard methods is relatively uncontroversial though therapy with appropriate agents seldom eradicates the pathogen during an exacerbation. In contrast the value and mode of testing of *P. aeruginosa* is controversial because of multiple colonial morphotypes with different susceptibilities and the presence of hypersusceptible mutants which may be recovered from a single sputum specimen. Thus testing of a single colony is not representative. Testing multiple colonial representatives is of value only in studies of the heterogeneity of the *Pseudomonas* population and is clearly not practical in routine testing of antibiotic susceptibility. It has been shown that testing of multiple morphotypes together is a relatively accurate and inexpensive compromise to testing each morphotype individually.[70]

A simplistic view of antibiotic susceptibility and resistance in a pathogen is that it provides a forecast of the outcome of antimicrobial therapy. In such a scenario, susceptibility is good news and resistance a bad omen. The situation in CF microbiology is, however, more complex. Comparison of antimicrobial action in the form of laboratory tests with the protective milieu of the CF airways obviously introduces many considerations. There is abundant evidence that irrespective of the results of susceptibility testing and, in older patients in the absence of demonstrable reduction in the bacterial load, antibiotic treatment of exacerbations generally results in improved lung function and a reduction in inflammatory markers. It is interesting to note that the expression of pseudomonal virulence determinants including elastase and alginate are significantly reduced in the presence of sub-lethal concentration of certain antibiotics. For example, the use of a *alg*D. *xyl*E transcriptional fusion[15] has shown that mannose dehydrogenase, an essential enzyme in alginate biosynthesis, is switched off by ciprofloxacin at 1/20th of the MIC value. Related studies of *P. aeruginosa* isolates, which have developed resistance to antimicrobial agents during therapy, also indicate that some resistance mechanisms are associated with reduced bacterial virulence (Govan, unpublished observations). In conclusion, although the eradication of bacterial pathogens is a primary aim, the clinical benefits of antimicrobial therapy in the absence of bacterial eradication, and the mechanisms responsible, are worth consideration.

CONCLUSION

In the 1980s, a major impact on reducing morbidity and mortality arising from bronchopulmonary colonisation in CF patients followed an improved understanding of the factors involved in microbial colonisation of the CF lung and an increasing awareness that whilst eradication of pathogens in CF is seldom achieved, antibiotics may produce clinical benefit by suppressing biosynthesis of important virulence determinants.

Whilst the 1990s will undoubtedly see exciting developments in gene therapy of CF, continued research in the microbiology of CF infections is essential. Important topics will include: (a) the outcome of prophylactic antibiotic usage against *S. aureus*; (b) clarification of the epidemiology and pathogenic potential of *P. cepacia*; (c) careful vigilance for previously rare pathogens, in particular, *Mycobacteria* spp in older CF patients; (d) clinical trials of vaccines against *P. aeruginosa*; (e) improved diagnostic procedures for *P. aeruginosa* based on monoclonal antibodies and PCR technology; and (f) clarification at a molecular level of the mechanism by which alginate regulatory genes in *P. aeruginosa* respond to environmental stimuli in the CF lung and global control of key virulence determinants.

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Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: The role of mutations in *muc* loci

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1. SUMMARY

Mucoid alginate-producing mutants of *Pseudomonas aeruginosa* are major pathogens in debilitating chronic pulmonary infections in patients with cystic fibrosis. The mucoid phenotype results from alginate biosynthesis whose genes are arranged in at least three chromosomal loci. Structural genes are located at the 34-min region and regulatory genes at 9 min. A third cluster at the 70 min region contains *muc* mutations which affect transcription of a key structural gene, *algD*, in response to environmental stimuli. Control of mucoidy includes bacterial signal transduction systems, histone-like elements controlling nucleoid structure and, possibly, factors affecting superhelicity. Thus, the control of mucoidy in *P. aeruginosa* has become one of the focal systems for analysis of how bacterial pathogens adapt to the host environment.

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2. INTRODUCTION

An important characteristic of pseudomonads is the ability of some species to synthesise alginate, a family of unbranched (1-4)-linked polysaccharides comprised of β-D-mannuronate and its C5-epimer α-L-guluronate [1]. Alginates have commercial importance as stabilisers and gelling agents and, in medical microbiology, as determinants of bacterial virulence [1,2]. This paper will focus on the use of genetic and molecular approaches to clarify the regulation of alginate biosynthesis in mucoid variants of *P. aeruginosa* (MPA) which are major pulmonary pathogens in patients with cystic fibrosis (CF).

3. MUCOID *P. AERUGINOSA*

In *P. aeruginosa* alginate biosynthesis is usually repressed, hence typical isolates are nonmucoid when cultured on agar media; in contrast, alginate-producing variants produce a mucoid phenotype. Although MPA were described as early as 1927 (ref. 3; *Bacterium pyocyaneum mu-*

cosum), present interest in the phenotype originates from the 1960s as a result of studies by Doggett and colleagues demonstrating the association of MPA with the CF lung [4]. Although there is general acceptance that MPA result from in vivo conversion following initial colonisation by nonmucoid *P. aeruginosa* (NMPA) there has been scanty published evidence to support this hypothesis until the report of an epidemiological study which documented the in vivo transition to the mucoid phenotype in two CF patients following water-borne acquisition of the same NMPA strain [5]. The association of MPA with the pathophysiology of CF lung infection is also established including the role of alginate as an adhesin for respiratory epithelia [6] and in the formation of bronchial plugs and bacterial microcolonies which 'frustrate' phagocytosis and antibiotic therapy leading to immune-mediated lung damage [2,7]. Interestingly, a recent isolation of MPA from an equine pharynx has confirmed the pulmonary predilection of the phenotype and provides an unusual parallel between veterinary and human microbiology [8].

3.1. Muc mutations

Following the in vitro isolation of mucoid variants of *P. aeruginosa* PAO using a selection technique based on enhanced antibiotic resistance [9], mutations associated with conjugal transfer of the mucoid phenotype to *P. aeruginosa* PAO derivatives were identified and designated *muc* [10]. These early genetic studies revealed a cluster of *muc* mutations which showed linkage with the chromosomal markers *cys-59* and *hisI* located at the 10 min region. Further progress was frustrated, however, by anomalous linkage data based on the location of *cys-59/hisI* and other markers in the region. The problem was solved on recalibration of the PAO map in 1987 and relocation of *cys-59/hisI* to the 70 min region [11]. The revised locus for *cys-59/hisI* and the availability of additional markers in this relatively 'dark region' of the chromosome revived interest in *muc* mutations and led to the demonstration of two distinct *muc* loci on the PAO chromosome; the first contained *muc-23* located between *hisI* and *proB* in the 69 min region and a second cluster contained the phenotypically distinct mutations *muc-2* and *muc-22* which were co-transducible with *pru-354* and lay between *pru-354* and *hisI* at the 67 min region [12,13]. Although these genetic studies had been carried out on PAO mucoid strains which were phenotypically similar to MPA from CF patients there was no assurance that such mutants bore any relationship to MPA from CF patients. The possibility that PAO mucoid strains might be genetically distinct from MPA isolated from CF patients was dispelled by the demonstration that a *muc* locus in the CF isolate *P. aeruginosa 492c* could be mapped by a R68.45-mediated interstrain cross with PAO 1042 and was located at the 67 min region between *pur-70* and *hisI* [12,13]. In a second approach, the locus responsible for mucoidy in the CF isolate *P. aeruginosa* 3739 (*muc*-3739) and four other CF isolates was transferred by means of pM060-mediated conjugation to PAO and shown to map in the same region as *muc-2*, *muc-22* and *muc-492c* [14]. Additional evidence of the similarity in *muc* mutations in PAO and clinical isolates came from construction of an R-prime *pru*$^+$ plasmid pJF4 and the demonstration that pJF4 could 'switch off' alginate biosynthesis in PAO strains containing *muc-2* or *muc-22* and *muc* mutations in four of five clinical MPA isolates tested [13]. Failure of pJF4 to complement *muc-23* and a phenotypically similar mutation in the CF isolate *P. aeruginosa* 492a (*muc-492a*) confirmed previous linkage data which suggested that these *muc* mutations represented a second *muc* locus between *hisI* and *proB* [12,13].

By the mid 1980s, the application of molecular studies had led to a rapid expansion in knowledge of alginate regulation including the location of other alginate gene clusters. Structural genes involved in the alginate biosynthetic pathway, represented by *algD*, encoding the key alginate biosynthetic enzyme, GDP mannose dehydrogenase, were located at the 34-min region [15]. Transcription of algD was shown to be dependent on algR, a regulatory locus mapping at the 9-min region [16]. Subsequently, a link between *algR*, *algD* and *muc* mutations was confirmed by evidence that *muc* mutations modified transcription of *algD* either positively or negatively when stim-

ulated by raised osmolarity or nitrogen limitation and that the modification was dependent upon individual *muc* alleles [17]. The key role of *muc* mutations in the environmental regulation of mucoidy explains previous observations which indicated that, although increased osmolarity was associated with increased alginate production in some MPA, this stimulus alone was not sufficient to induce mucoidy in NMPA. Further studies indicated that *muc* mutations also influence other pseudomonas virulence determinants. Use of PAO568 (*muc-2*) [10,12] which displays increased *algD* transcription and mucoidy in increasing concentrations of NaCl [17], indicated that *muc-2* is involved in a see-saw expression pattern of *algD* and the elastase stuctural gene *lasB* suggesting that the *lasB* gene and the alginate system are co-ordinately regulated with *muc-2* acting as a global regulator [18].

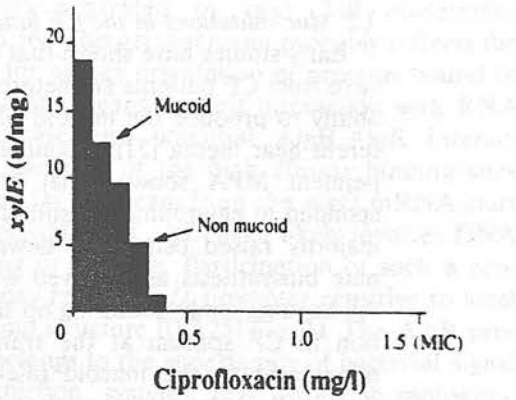

Fig. 1. Suppression of *algD* transcription and mucoidy in *P. aeruginosa* in the presence of ciprofloxacin. Measured by transcriptional fusion assay with an *algD – xylE* fusion [17]. The MIC of ciprofloxacin is 1.5 mg/l and the mucoid phenotype is associated with levels of reporter gene activity > 5 units.

Physiological changes in response to environmental stimuli such as osmolarity and anaerobicity can be regulated at transcriptional level by alterations in DNA superhelicity in *Escherichia coli* and *Salmonella typhimurium* [19]. Similarly, there is circumstantial evidence that alginate biosynthesis may be regulated by DNA supercoiling or protein-DNA binding controlled by a gene product of the *muc* region. Firstly, the use of an *algD.xylE* reporter system [17] indicated that the gyrase inhibitor, ciprofloxacin, caused *algD* to be switched off at concentrations as low as 5% of the minimum inhibitory concentration (Fig. 1). Secondly, individual *muc* mutations are associated with different susceptibility to gyrase inhibitors but not other classes of antibiotics (Table 1). In addition, transcription of *algD* depends on another unusual genetic element, *algP*, which encodes a product whose carboxy-terminal domain is similar to carboxy-terminal tails of histone H1-like DNA binding proteins which are emerging as another class of common regulators of virulence in pathogenic bacteria [20]. The earlier observation that R-prime plasmids containing wild-type *muc* genes were capable of complementing *muc* mutations in trans to produce a non-mucoid phenotype suggested that the product of *muc* genes may be a cytoplasmic regulator.

Alternatively, since *muc* affects *algR*, a transcriptional regulator of alginate biosynthetic genes, *muc* could participate in the sensory processes being locked in the 'on' position leading to constitutive expression of alginate and the mucoid phenotype [17].

Table 1

MICs for isogenic derivatives of *P. aeruginosa* PAO containing environmentally responsive *muc* mutations

Strain/ genotype	*algD* response to raised osmolarity *	MICs (mg/l)				
		Ceftazidime (increments 0.1 mg/l)	Tobramycin (0.1)	Ciprofloxacin (0.1)	Nalidixic acid (10)	Novobiocin (100)
568 *muc2*	Increased	2.0	1.0	0.7	120	500
579 *muc23*	Reduced	2.0	1.0	0.3	50	< 100
595 *muc95*	Increased	2.0	1.0	0.2	30	500

* Measured by transcriptional fusion assay with *algD – xylE* fusion in presence of 0.3 M NaCl [see ref. 17].

3.2. Muc mutations in the CF lung

Early studies have shown that MPA isolated in vitro from CF patients are heterogeneous in their ability to produce the mucoid phenotype on different agar media [21]. Examination of 50 independent MPA showed that most isolates responded to environmental stimuli and that in the majority raised osmolarity down-regulated alginate biosynthesis as observed with *muc-23* [17]. An ongoing study, focussing on the MPA population in CF sputum at the transition from the nonmucoid to the mucoid phenotype indicates that in all seven patients studied to date the MPA population is heterogeneous in its environmental response (authors' unpublished results). Heterogeneity at this early stage of colonisation suggests that the CF lung provides a variety of environmental niches in which appropriately responsive individual *muc* mutations emerge from the original non-mucoid colonisers rather than the emergence of a single mutation which undergoes subsequent adaptation.

The evidence that a variety of *muc* mutations affect regulation of alginate biosynthesis in response to environmental stimuli both in vitro and in vivo presents a clear indication of the complexity of the alginate regulatory system and the hazards of using studies of individual MPA to generalise on the nature of the mucoid phenotype. For example, the extensive use of *P. aeruginosa* 8821 and its derivatives [22] has contributed significantly to knowledge of the molecular biology and enzymology of alginate biosynthesis. Strain 8821 exhibits enhanced mucoidy in the presence of raised osmolarity and supported the attractive hypothesis that elevated salt concentrations in CF lungs may favour alginate biosynthesis. This type of environmental response, however, is shown by a minority of CF isolates; a more common response is an absence of any significant response to this factor or loss of mucoidy when MPA are grown on media with high osmolarity [17]. Similarly, in an elegant review of their gene replacement studies [23], Ohman and Goldberg de-

Table 2

Genes and gene products involved in control of mucoidy in *Pseudomonas aeruginosa*

Genes		Gene products and associated function
muc AlgU	Sites of mutations conducive to mucoid phenotype; several genes involved.	Possible site of *muc* mutations; 27 kDa; AlgU homologous to a sigma factor required for sporulation in *B. subtilis*.
algD GMDH	Transcriptionally activated in mucoid cells; promoter contains three binding sites (RB) for AlgR forming a lopsided palindrome; RB1 and RB2 located far upstream from the mRNA start site.	GDPmannose dehydrogenase; NAD-linked 4 electron transfer dehydrogenase; overproduction is necessary to channel sugar intermediates into alginate.
algR AlgR	A response regulator from the superfamily of signal transduction systems; transcribed from two promoters; the proximal promoter activated in mucoid cells.	Binds to the RB1, RB2 and RB3 sites within the *algD* promoter; undergoes phosphorylation; transduces environmental signals to activate *algD* (e.g. growth on nitrate).
algB AlgB	Another response regulator affecting high levels of alginate production.	Exact site of action currently unknown.
algP Hp1	A gene participating in the control of *algD* and mucoidy; Contains multiple direct repeats which can undergo clonal variation.	Alternative names AlgP and AlgR3; C-terminal domain similar to tails of histones H1 from eukaryotes; Putative function-bending or looping.
algQ AlgQ	Acting synergistically with *algP*, both *algQ* and *algP* are constitutively transcribed.	Possible GTP binding protein; proposed to phosphorylate an unidentified 31 kDa polypeptide.
algST NadB	Proposed site for conversion to mucoidy in a CF isolate; mapping near *muc* genes.	Aspartate oxidase; involved in NAD synthesis; adjacent to *algT*.

scribed the use of the CF isolate FRD1 to demonstrate a genetic 'master switch' which they termed *algS*. The genetic data suggested that *algS* controls the expression of a gene whose product acts in trans and that this product controls the expression of distant alginate genes. This hypothesis indicates a close similarity in function between *muc* mutations and *algS* and the possibility that *algS* represents a particular *muc* allele. Evidence for the latter explanation has been provided by examination of the effect of environmental stimuli on mucoidy in FRD1 which indicated that FRD1 belongs to group 2 MPA which represent a minority of CF isolates [21] and in its response to nitrate and osmolarity resembles isolates carrying the *muc-2* mutation [17].

3.3. Genetics of mucoidy

After a relatively long hiatus following the initial mapping of *muc* mutations, the genetics of mucoidy in *P. aeruginosa* has recently received significant attention. As a result of the work of several groups [20,22,23], many previously unsuspected details of the regulation of alginate production have been unravelled. Table 2 illustrates how control of mucoidy includes bacterial signal transduction systems (such as AlgR and AlgB), elements controlling nucleoid structure Hpl, alternative name AlgP [24], and, possibly, factors affecting superhelicity (see Fig. 1 and Table 1). Interestingly, similar types of regulatory systems control virulence determinants in many other bacterial pathogens. Thus, the control of mucoidy in *Pseudomonas* has become one of the focal systems for analysis of how bacterial pathogens adapt to the host environment.

Two extensively studied check points in the control of mucoidy are: (i) transcriptional activation of *algD* [15], the gene encoding GDPmannose dehydrogenase (GMDH) [15]; and (ii) its regulation by *algR*. Transcriptional activation of *algD* is a sine qua non for the mucoid phenotype. GMDH catalyses double oxidation of GDPmannose into alginate precursors, and channels sugar intermediates into the production of the exopolysaccharide. Activation of *algD* is absolutely dependent on AlgR. AlgR binds to the *algD* promoter in an unusual fashion, to sites comprising a lopsided palindrome with symmetrical sequences separated by over 340 nucleotides [25,26,36]. This arrangememt probably reflects the need for proper orientation of proteins bound to DNA with regard to their interaction with RNA polymerase and potential AlgR-AlgR interactions. Because of the high affinity binding sites located far upstream from the *algD* mRNA start site, activation of *algD* most likely involves DNA looping or bending. Participation of such a process may render *algD* promoter sensitive to local nucleoid structure ([17,25] Fig. 1). The AlgR protein belongs to the superfamily of bacterial signal transduction systems [27]. AlgR homologues, termed response regulators, are known to undergo a phosphorylation-dependent modulation of their activity [28]. This process relies in many instances on a second protein, commonly a histidine kinase, which, in response to environmental signals, undergoes ATP-dependent autophosphorylation followed by a phosphotransfer reaction to response regulators such as AlgR. This type of regulatory signalling is the preponderant mode of how bacteria control their central metabolic functions and complex phenotypes such as expression of virulence factors. A typical second component interacting with AlgR has not been identified. It has been proposed that *algQ* [16] encodes the second component, but when this gene was sequenced [29,30] its predicted gene product did not show similarity to any known histidine protein kinase. A recent study with crude protein extracts and partially enriched proteins led to a proposal that AlgQ may act as a novel GTP-dependent kinase that phosphorylates a polypeptide of a similar electrophoretic mobility as AlgR [31]. However, the use of appropriate controls such as *algR* mutants and monoclonal antibodies against AlgR demonstrated that this polypeptide was not AlgR [32]. Furthermore, it has been recently demonstrated [32] that AlgR can be phosphorylated by typical histidine protein kinases using heterologous kinases such as CheA, normally involved in chemotoaxis, and BvgS, a central regulator of virulence in *Bordetella pertussis*. Thus a kinase (sensor) typical of two component signal transduction systems, may exist in the alginate system.

By analogy with other systems, phosphorylation is expected to activate AlgR. If this is true, an exciting possibility, originally proposed by Deretic et al. [20], is to develop inhibitors specific for bacterial signal transduction. It is expected that development of such potential drugs may provide a new line of antimicrobial agents for use in the treatment of MPA infections in CF patients.

Interestingly, small molecular mass phosphodonors such as acetyl phosphate and carbamoyl phosphate (participants in intermediary metabolism), have been recently shown to interact with AlgR, possibly phosphorylating it or interfering with the phosphorylation process [32]. Similar reactions have been reported for several other response regulators such as CheY, another protein controlling chemotaxis [33], and PhoB, the central regulator of phosphate metabolism [34]. Carbamoyl phosphate is an important intermediate in pyrimidine and arginine synthesis. In *P. aeruginosa*, which is generally considered to be a non-fermenter, it can be used for substrate level ATP synthesis from arginine under anaerobic conditions [35]. Acetyl phosphate is related to the synthesis of acetyl-CoA, a key substrate for the TCA cycle, and is an important intermediate in utilization of pyruvate, glucose and acetate as well as in ATP synthesis [35].

Exciting new questions emerge: Do these intermediates affect regulation of mucoidy? Are intracellular levels of these compounds related to environmental conditions that affect mucoidy? Are these molecules a new 'currency' for phosphate exchange and phosphorylation? Can direct participation of small molecular mass phosphodonors help explain the perplexingly large number of environmental factors which affect mucoidy? Are *muc* mutations or the function of *muc* genes related to or affected by levels of these compounds?

More than ever before, it is becoming critical to understand the nature of genetic elements such as *muc* which are responsible for or participate in conversion to mucoidy. The existence of mapped mutations [10,12,14] is playing an invaluable role in facilitating their cloning and molecular characterisation. In collaboration with Dr. Bruce Holloway, several genes that affect mucoidy which map in the region known to contain *muc* mutations are currently being analysed. An analysis of one of the loci, mapping near *muc-2* or identical to it, indicates the presence of two genes (*algU* and *algV*). The *algU* gene encodes a polypeptide of 27 kDa (P27). A downstream gene encodes a polypeptide of 20 kDa (P20). The *algU* gene has been sequenced, and the primary structure of its predicted gene product indicates that this could be an alternative sigma factor (D.W. Martin, B.W. Holloway and V. Deretic, in preparation). Its homologue is a sigma factor participating in the early stages of sporulation in *Bacillus subtilis*. This suggests that mucoidy may represent a developmental process. Sporulation in *B. subtilis* also involves signal transduction systems, such as AlgR homologues SpoOA and SpoOF [27], and there may be several more parallels between the two systems. It is of interest that alginate plays a role in another developmental process, viz. in encystment of *Azotobater vinelandii* [12].

4. CONCLUDING REMARKS

In conclusion, the current cloning and molecular analysis of *muc* mutations may help uncover what is now considered to be a holy grail sought by many scientists in CF microbiology. Once reasons and mechanisms for the emergence of the mucoid phenotype of *P. aeruginosa* in CF patients are clarified and the nature of *muc* genes and mutations unveiled, it may be possible to intervene with the processes conducive to mucoidy and prevent establishment of chronic *P. aeruginosa* infections in CF lungs.

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The Pyocin Sa Receptor of *Pseudomonas aeruginosa* Is Associated with Ferripyoverdin Uptake

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We have used Tn*5* mutagenesis to obtain a mutant resistant to pyocin Sa. When grown in iron-deficient succinate medium this mutant lacked an 85-kDa iron-regulated outer membrane protein (IROMP), and expression of a 75-kDa IROMP was increased compared with that in the parent strain. The mutant was deficient in pyoverdin biosynthesis and showed a 95% decrease in transport of ferripyoverdin purified from the parent strain, suggesting that the 85-kDa IROMP is the specific receptor for ferripyoverdin and pyocin Sa. The mutant compensated for the deficiency in pyoverdin biosynthesis and transport by exhibiting a fourfold increase in ferripyochelin transport. The low-level transport of ferripyoverdin in the Sa-resistant mutant, which extended to heterologous pyoverdins from other strains, suggests that *Pseudomonas aeruginosa* has a second ferripyoverdin uptake system of lower affinity and broader specificity.

The expression of a high-affinity iron uptake system is an important component in the adaptation of cells to growth conditions encountered in vivo (11). The iron-binding glycoproteins lactoferrin and transferrin ensure that the level of extracellular iron in the body available to invading organisms is far below their growth requirements (2). To overcome this nutrient deprivation, some bacteria, including *Pseudomonas aeruginosa*, derepress an iron uptake system based on low-molecular-weight iron-chelating compounds (siderophores), which compete with transferrin and lactoferrin for host iron and deliver it to the cell via interaction with an iron-regulated outer membrane protein (IROMP) receptor (11). *P. aeruginosa* produces two siderophores, pyochelin and pyoverdin. Pyochelin is produced by all strains of *P. aeruginosa* (5), and two uptake systems, involving a 14-kDa ferripyochelin binding protein in the outer membrane (OM) (22) and a 75-kDa IROMP (12), have been identified.

Studies of ferripyoverdin uptake have been complicated by the marked heterogeneity of this system. Strains of *P. aeruginosa* produce several different pyoverdins, each having the characteristic yellow-green fluorescent 2,3-diamino-6,7-dihydroxyquinoline chromophore attached to a peptide backbone varying from 6 to 10 amino acid residues (6). Three pyoverdin-mediated iron-uptake groups have been identified from amongst a series of type strains and clinical isolates, on the bases of growth promotion studies, labelled uptake studies, and amino acid composition of purified pyoverdins (3). Antibodies raised against an 80-kDa IROMP from two of the groups reacted only against strains producing the same pyoverdin group (3), and several reports have indicated heterogeneity in molecular weight and immunological cross-reactivity of ferripyoverdin receptors from different strains (16, 18, 20). These data point to significant variation in ferripyoverdin IROMP structure which is sufficient to discriminate between pyoverdins having only subtle structural differences.

In an attempt to gain further insight into the regulation and structure of the ferripyoverdin uptake system, we have reinvestigated the receptors for the colicinlike S pyocins. Three classes of pyocin, R, F, and S, have been identified on the bases of structural and chemical properties (9, 13, 19). Phage-tail-like R and F pyocins are produced by more than 90% of *P. aeruginosa* strains, and colicinlike, trypsin-sensitive S pyocins are produced by approximately 70% of isolates (8). Susceptibility to one or more classes of pyocin occurs in 100% of isolates.

The S-type pyocins are of particular interest because their lethal activity appears to be mediated via high-molecular-weight IROMPs. Iron-rich conditions decreased the absorption and lethality of pyocin S2, whereas under iron-deficient conditions, susceptibility was significantly enhanced and accompanied by the appearance of an OM protein which appeared to act as the S2 receptor (17). Pyocin S2-resistant mutants lacking a minor IROMP have been described, but competition data suggested that this IROMP did not have a role in iron uptake (17). Govan (10) identified a new pyocin Sa, produced by *P. aeruginosa* J1003, by screening for activity against a strain resistant to pyocins S1 and S2. A further survey of 1,000 strains with a mutant resistant to S1, S2, and Sa failed to demonstrate additional S pyocin receptors in this strain.

In order to characterize the Sa receptor, we screened our laboratory culture collection for other susceptible strains. Only one clinical isolate, 0:9, from more than 20 laboratory and clinical strains tested was sensitive, and Tn*5* mutagenesis (21) was used to obtain a resistant mutant. Pooled neomycin-resistant exconjugants were plated in soft agar over a surface culture of J1003 grown for 6 h at 30°C and inactivated by exposure to chloroform vapor for 15 min. After overnight incubation at 37°C, approximately 100 exconjugants were picked from the center of the zone of inhibition, but on restreaking against the producer strain, only one mutant, designated PH1, showed no growth inhibition. When discs saturated with ferripyoverdin were placed on the soft agar overlay containing the sensitive strain 0:9, distinct zones of growth were detected, indicating that ferripyoverdin could inhibit killing by pyocin Sa. Outer

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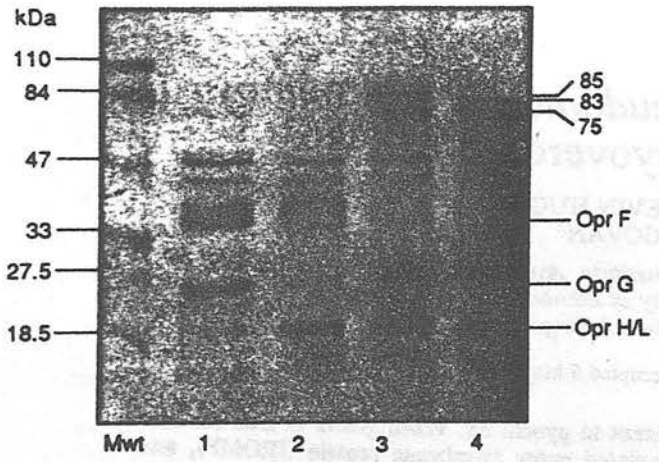

FIG. 1. SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from strains of *P. aeruginosa* grown in iron-deficient succinate medium with (lanes 1 and 2) or without (lanes 3 and 4) 50 μM $FeSO_4$. Lanes 1 and 3, 0:9; lanes 2 and 4, pyocin Sa-resistant mutant PH1.

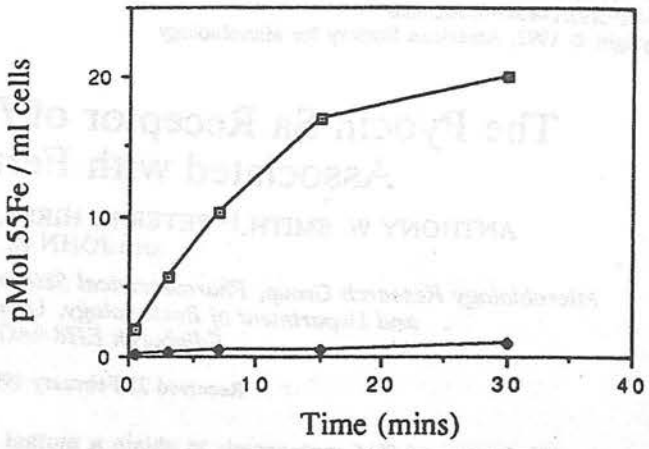

FIG. 2. Pyoverdin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* 0:9 (⊡) and PH1 (◆) grown in iron-deficient succinate medium. The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM), and 1 ml of cells at an optical density at 470 nm of 1.0. Data are representative from four experiments.

membranes were prepared by the Sarkosyl solubilization method (7) and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (14). OM protein profiles from 15-h cultures of PH1 and 0:9, grown in succinate minimal medium (15) at 37°C are shown in Fig. 1. After growth without added iron, three major IROMPs of 75, 83, and 85 kDa were induced in the OM. The Sa-resistant mutant PH1 specifically lacked an 85-kDa IROMP (lane 4), and expression of the 75-kDa IROMP was increased compared with that in 0:9. Growth in succinate medium with added iron confirmed that these proteins are iron repressible (lanes 1 and 2). The 25-kDa OM protein G (OprG) expressed by 0:9 under iron-rich growth conditions (lane 1) was partially repressed in PH1 (lane 2) by approximately 50%, as determined by densitometry. A 10-fold decrease in the pyoverdin fluorescence peak for PH1 culture supernatants was noted (not shown), indicating that a component of a pyoverdin siderophore biosynthesis-uptake operon was inactivated. In addition, PH1 was unable to grow in succinate medium supplemented with the nonutilizable chelator ethylene diamine di(*o*-hydroxyphenyl) acetic acid (400 μM; EDDHA), indicating perturbed pyoverdin production or transport.

Pyoverdin was purified from 0:9 culture supernatants, and uptake studies were performed as described by Poole et al. (18). Uptake profiles for 15-h stationary-phase cells of 0:9 and PH1 grown in iron-deficient succinate medium are shown in Fig. 2. By using a 1:50 molar ratio of ^{55}Fe to pyoverdin, the uptake rate of PH1 was 0.03 pmol of Fe per min per ml of cells compared with 0.57 pmol of Fe per min per ml of cells in 0:9, a reduction of 95%. Increasing the molar ratio of iron to pyoverdin did not affect uptake.

Ferripyochelin transport assays were undertaken to determine if the increased expression of the 75-kDa IROMP was associated with elevated transport incurred by using this siderophore, since pyochelin binding and transport have been associated with a 75-kDa IROMP in late-logarithmic or early-stationary phase cells (12). Pyochelin was purified from PH1 culture supernatants as described by Heinrichs et al. (12), and uptake assays were performed with 15-h stationary-phase cells grown in succinate medium as described by Cox (4). The initial rate of ferripyochelin uptake in PH1 was 0.4 pmol of Fe per min per ml of cells compared with 0.1 pmol of Fe per min per ml for 0:9 (Fig. 3).

In this work we have isolated a mutant resistant to *P. aeruginosa* pyocin Sa which lacks an 85-kDa IROMP and shows greatly reduced ferripyoverdin uptake. The inhibition of pyocin Sa killing by competition with ferripyoverdin strongly suggests that the 85-kDa IROMP is both the pyocin Sa receptor and a ferripyoverdin transporter. Indeed, this may be the same transporter as the 90-kDa IROMP identified by Poole et al. (18), although it remains to be determined whether this strain produces a pyoverdin molecule from the same uptake group (3) and is sensitive to pyocin Sa. Using a mutant deficient in the 90-kDa IROMP, these workers also provided evidence for a second uptake system (18). The residual uptake noted in this study also suggests a second transporter, although it was not sufficient to permit growth in the presence of EDDHA. Evidence for a second uptake system has also been found with the related fluorescent pseudomonad *P. putida* (1). The *pupA* gene coding for the

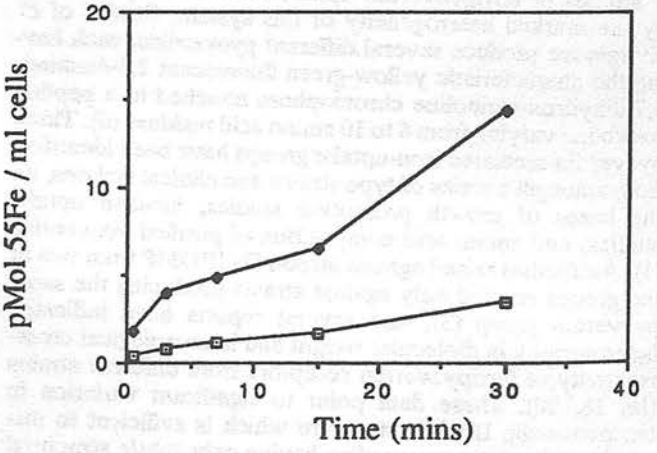

FIG. 3. Pyochelin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* 0:9 (⊡) and PH1 (◆) grown in iron-deficient succinate medium. The uptake mixture contained pyochelin (60 μM), $^{55}FeCl_3$ (115 nM), and 1 ml of cells at an optical density at 470 nm of 1.0. Data are representative from three experiments.

85-kDa pseudobactin 358 receptor was identified, and *pupA* mutants were obtained by marker exchange. These mutants still showed 30% uptake, whilst uptake of other pseudobactins was not affected. In this present study the Sa mutant showed only 5% uptake of parental siderophore. Uptake experiments with chromatographically distinct pyoverdins from other strains all showed low uptake of approximately 5% both in PH1 and in the parent strain 0:9 (data not shown). It therefore seems likely that the 85-kDa IROMP is a specific high-affinity ferripyoverdin receptor, whereas a second receptor, which has low affinity for a wider range of pyoverdins, exists.

The ferripyochelin transport assays described in this work support the finding that the 75-kDa IROMP is a second pyochelin transporter (12) and suggest that PH1 compensates for the deficiency in pyoverdin biosynthesis and transport by derepressing further the pyochelin-based system. This effect seems to be restricted to an increased ability to transport the siderophore complex, rather than to produce siderophore, since we did not detect any differences in pyochelin production between PH1 and 0:9. Similarly, with the growth conditions used in this study, the increase in ferripyochelin transport correlated with the 75-kDa IROMP transporter rather than the well-characterized 14-kDa ferripyochelin binding protein (22).

The perturbations in expression of OM proteins in PH1 were not seen only after growth in iron-depleted media. When grown in iron-rich conditions, which represses synthesis of IROMPs, expression of OprG in PH1 was reduced by approximately 50%. Whilst the role of OprG remains unclear, its expression has been shown to increase when the level of available iron is similarly increased (24), and we are now reviewing its role in iron uptake.

In summary, our data indicate that the site of interaction of pyocin Sa is a ferripyoverdin receptor and provides evidence for a second low-affinity pyoverdin uptake system. Whilst the deficiency in pyoverdin transport and synthesis could be attributed to downstream effects exerted by Tn*5* insertion into an operon (23) and the increased ability to transport ferripyochelin could be a response to the increased iron deficiency imposed by the inability to transport ferripyoverdin, the altered expression of OprG under iron-rich conditions, where pyoverdin-mediated iron transport is not thought to operate, was surprising. Moreover, the observation that the receptor for this pyocin is a critical component of the iron uptake system and yet few strains are susceptible to it lends further credence to the notion that pyoverdin biosynthesis and uptake are highly heterogeneous amongst *P. aeruginosa* strains. Sequence analysis will be required to determine the extent of the similarities of the systems. We are now cloning the site of the transposon insertion to determine whether a pyoverdin synthesis-uptake operon has been inactivated or if a larger iron-regulated operon has been identified.

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In Vitro Activities of Meropenem, PD 127391, PD 131628, Ceftazidime, Chloramphenicol, Co-Trimoxazole, and Ciprofloxacin against *Pseudomonas cepacia*

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In a study of 110 *Pseudomonas cepacia* isolates from patients without cystic fibrosis, the in vitro potencies of three new compounds, meropenem, PD 127391, and PD 131628, were comparable to those of ceftazidime and ciprofloxacin and exceeded those of chloramphenicol and co-trimoxazole. The MICs of ceftazidime, ciprofloxacin, meropenem, and the PD compounds for 90% of strains tested were ≤4 μg/ml, whereas they were 32 μg/ml for chloramphenicol and co-trimoxazole. Data for 20 isolates from patients with cystic fibrosis indicated that the isolates were less susceptible to all seven antibiotics tested, with the most active compounds being meropenem and PD 127391.

Pseudomonas cepacia, a phytopathogen first described as the cause of soft rot of onions (4), is now recognized as a significant opportunistic pathogen in patients with nosocomial infections (9, 12). Patients with cystic fibrosis (CF) appear to be particularly susceptible to pulmonary colonization with this organism (2, 13, 23, 25): CF patients colonized with *P. cepacia* experience more pulmonary exacerbations and a higher mortality than do noncolonized CF patients (26).

P. cepacia is innately resistant to a wide range of antibiotics and disinfectants including polymyxin, aminoglycosides, and traditional antipseudomonal penicillins such as ticarcillin (3, 7, 15, 21). It is not surprising, therefore, that *P. cepacia* infections are generally refractory to antibiotic therapy and pose a significant challenge in the management of patients with CF (9, 10). Historically, the most effective antibiotics for the treatment of *P. cepacia* infections were trimethoprim-sulfamethoxazole and chloramphenicol, although these agents displayed limited clinical efficacy (20, 27). More recently available antibiotics, including ceftazidime, temocillin, imipenem, and ciprofloxacin, exhibit some in vitro activity against this organism (1, 17, 26). However, initial clinical experience with some of these compounds has shown variable benefits (8).

The availability of new agents with in vitro and in vivo activities against *P. cepacia* would be of considerable importance in the management of patients with CF. Thus, we examined a new carbapenem, meropenem, and two more recently developed quinolones for their activities against a large collection of *P. cepacia* isolates, including those from patients with CF.

Meropenem (ICI 194660 or SM 7338) is a new carbapenem with an exceptionally broad spectrum of antibacterial activity (14). Unlike imipenem, which is metabolized by renal dipeptidases and requires the coadministration of cilastatin, meropenem is stable to dehydropeptidase-I, and thus, it is not necessary to coadminister it with cilastatin (6, 18). PD 127391, 7-(3-amino-1-pyrrolidinyl)-8-chloro-1-cyclopropyl-6-fluoro-1,4,dihydro-4-oxo-3-quinolone-carboxylic acid, and PD 131628, 7-(3-amino-1-pyrrolidinyl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid, are oral quinolones that are being developed by Warner-Lambert and that may possess pharmacokinetics superior to those of clinically available 4-quinolones (5, 16). The data presented here describe the in vitro activities of these compounds against *P. cepacia* compared with those of the most active antibiotics available, ceftazidime, ciprofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole (co-trimoxazole). Most previous in vitro studies of the activity of meropenem and the two PD compounds have tested only small numbers of *P. cepacia* or used collections of pseudomonads which were not identified to the species level (5, 14, 16).

In the present study, we investigated a total of 130 *P. cepacia* isolated from CF and non-CF patients in France, the United Kingdom, and the United States. All strains were identified by standard methods (11), including use of the API 2ONE system (API System, La Balme les Grottes, France) and determination of their ability to grow on a selective medium (7). The strains included in the study were further characterized by bacteriocin typing (11, 22); multiple isolates from individual patients or epidemics belonging to the same bacteriocin type were excluded from the study. The antibiotics used in the study were obtained through the indicated sources: meropenem (ICI 194660, SM 7338), ICI Pharmaceuticals, Macclesfield, United Kingdom; PD 127391 (CI 960), PD 131628 (CI 990), and chloramphenicol, Clinical Research Northern Europe, Parke-Davis Warner-Lambert, Eastleigh, United Kingdom; ceftazidime, Glaxo Pharmaceuticals Ltd., Greenford, United Kingdom; ciprofloxacin, Bayer UK Ltd., Newbury, United Kingdom; and co-trimoxazole, Wellcome, Bromley, United Kingdom. Determination of MICs was done by the guidelines of the National Committee for Clinical Laboratory Standards (19). Bacterial antibiotic susceptibility tests were performed by an agar dilution method on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, United Kingdom), with an initial inoculum of approximately 10^4 CFU per spot, by using a multipoint inoculator (model A400; Denley Instruments Ltd., Sussex, United Kingdom). The MIC was determined as the lowest

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TABLE 1. MICs of seven antimicrobial agents for 110 non-CF and 20 CF isolates of *P. cepacia*

Antimicrobial agent	Source	MIC (μg/ml)		
		Range	50%	90%
Chlorampenicol	Non-CF	1–128	16	32
	CF	2–>128	16	64
Co-trimoxazole	Non-CF	0.5–>128	4	32
	CF	4–>128	32	128
Ceftazidime	Non-CF	0.06–>128	2	4
	CF	1–>128	8	32
Ciprofloxacin	Non-CF	<0.06–128	1	2
	CF	0.25–128	8	128
Meropenem	Non-CF	<0.06–16	2	4
	CF	1–16	2	16
PD 127391	Non-CF	<0.06–16	0.5	1
	CF	0.12–32	4	16
PD 131628	Non-CF	<0.06–64	0.5	1
	CF	0.12–128	4	64

concentration of antibiotic that inhibited growth of the test organism after 48 h of incubation at 37°C.

Details of the in vitro activities of PD 127391 and PD 131628 against 110 non-CF isolates of *P. cepacia* are given in Table 1. The activities of PD 127391 and PD 131628 were compared with those of other relevant antibacterial agents. On the basis of the MICs for 50% (MIC_{50}s) and 90% (MIC_{90}s) of isolates tested, both of the new PD quinolone compounds showed in vitro activities slightly better than those of ciprofloxacin and ceftazidime and were considerably more active than either co-trimoxazole or chloramphenicol. On the basis of similar parameters, meropenem was less active than the PD compounds at the MIC_{50}s and MIC_{90}s. For none of the isolates tested were the MICs of meropenem or PD 127391 greater than 16 μg/ml, while for some of the isolates, MICs of the other antimicrobial agents tested, with the exception of PD 131628, were 128 μg/ml or greater. On the basis of the published activity of imipenem (24), meropenem and the PD compounds were more active at the MIC_{50}s and MIC_{90}s.

None of the presently available antimicrobial agents has proved to be effective in the treatment of *P. cepacia* colonization in patients with CF, although combination therapy with temocillin and an aminoglycoside has been reported to have some clinical effect even against aminoglycoside-resistant isolates (27). Hence, the development of new agents with in vitro and in vivo activities against *P. cepacia* would be an important advance in the management of patients with CF. The isolates from patients with CF examined in the present study were less susceptible than isolates from patients without CF, perhaps reflecting the frequent use of antibiotics in patients with CF. Nevertheless, on the basis of their in vitro activities, the carbapenem meropenem and the new quinolone PD 127391 provide alternative agents with in vitro activities against *P. cepacia* that surpass those of the five other compounds. If the upper limit of susceptibility for meropenem is taken as 8 μg/ml (14), with an MIC_{50} of 2 μg/ml and an MIC range of 1 to 16 μg/ml, most isolates of *P. cepacia* from patients with CF examined in the present study were susceptible. If the upper limit of susceptibility for the PD compounds is put at 2 to 4 μg/ml, as suggested for similar compounds (28), PD 127391, with an MIC_{50} of 4 μg/ml and an MIC range of 0.12 to 32 μg/ml, appears marginally better than PD 131628 and the existing quinolone standard ciprofloxacin.

It is a cause of concern that two strains of *P. cepacia* that exhibited resistance to all seven antibiotics tested were isolated from two patients with CF in Wales and Scotland. Further investigation of the prevalence of such multiresistant strains is needed to assess future problems in the management of patients with CF. The MIC data indicate that, with the exception of the two isolates from patients with CF in Wales and Scotland, meropenem and the PD compounds are highly active against isolates of *P. cepacia* that are resistant to the four other antibacterial agents investigated.

We conclude that the overall activities of meropenem and the PD compounds in in vitro susceptibility tests against a comprehensive collection of *P. cepacia* isolates indicate that these are promising new agents and that further studies of their pharmacokinetics and clinical efficacies in patients with CF are needed.

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Serum IgG and sputum IgA antibody responses to core lipopolysaccharide antigen from *Pseudomonas cepacia* in patients with cystic fibrosis

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ABSTRACT

The immunological response of cystic fibrosis (CF) patients to lipopolysaccharide (LPS) antigens of *Pseudomonas cepacia* was investigated. Enzyme-linked immunosorbent assay (ELISA) employing either *P. cepacia* whole cells or extracted core LPS from a clinical isolate of *P. cepacia* were used to measure serum IgG and sputum IgA anti-*P. cepacia* antibodies. The ELISA incorporating *P. cepacia* core LPS as the antigen differentiated 9 CF patients colonized by *P. cepacia* from 9 age and sex matched non-colonized CF patients. The rate of increase of anti-*P. cepacia* IgG antibodies after bacteriologically proven *P. cepacia* colonization varied in individual patients: in some patients the first isolation of *P. cepacia* was preceded or accompanied by a 2-4 fold rise in anti-*P. cepacia* LPS IgG titers. Absorption studies and immunoblot analysis of serum from *P. cepacia* colonized patients demonstrated that a significant component of the anti-*P. cepacia* core LPS antibodies was specific for *P. cepacia* and not reactive with the core LPS of *Pseudomonas aeruginosa*. Immunoblotting also illustrated that there may be a degree of core heterogeneity between different isolates of *P. cepacia*. Detection of *P. cepacia* LPS specific antibodies in serum (IgG) and sputum (IgA) from CF patients is recommended to assist the identification of *P. cepacia* colonization in CF patients.

INTRODUCTION

Pseudomonas cepacia is a major pulmonary pathogen in patients with cystic fibrosis (CF)[1-3]. Originally considered a phytopathogen, the organism has been isolated with increasing frequency from patients with CF[3-6] also recognised as an important aetiological agent in nosocomial infection[7,8]. Innate and inducible resistance to many anti-pseudomonal antibiotics[9,10] and the ability to survive under conditions of minimal nutrition or in the presence of certain disinfectants contribute to the role of *P. cepacia* as a formidable nosocomial pathogen[8-9]. Prevention and treatment of pulmonary infections due to *P. cepacia* present a major challenge. The association of *P. cepacia* with the CF lung is complex, and clinical sequelae include rapid fatal deterioration of pulmonary function, long term colonization accompanied by a slow decline in lung function, and chronic asymptomatic carriage.[11]

Although the humoral response of CF patients to *Pseudomonas aeruginosa* antigens has been extensively investigated[12], there are relatively few reports defining the response to *P. cepacia* antigens.[13-14] The aim of this study was to investigate the antibody response of CF patients to *P. cepacia* whole cell and extracted lipopolysaccharide (LPS) antigens. The extent to which antibodies cross-react with *P. cepacia* and *P. aeruginosa* core LPS was also analysed. An enzyme-linked immunosorbent assay (ELISA) employing core rough-LPS (R-LPS) from *P. cepacia* was developed for the study and shown to assist diagnosis of *P. cepacia* colonization in patients with CF.

MATERIALS and METHODS

Patients. Nine patients (5 female, mean age 21.6 years, age range 16-27 years) attending the Edinburgh adult CF clinic, were identified as being persistently colonized by *P. cepacia* (PC+) by serial bacteriological cultures of sputa (at least 3 consecutive samples positive). For each patient an age and sex-matched CF control was selected in whom *P. cepacia* had never been never been isolated (PC-).

Sera and sputa. Serum samples were obtained from CF patients and from healthy blood donors at the Blood Transfusion Centre, Edinburgh. All sera were stored at -20°C. Sputa used for antibody analysis were centrifuged at 10,000 x *g* for 15 min and the supernatant stored at -70°C. Control sputa, obtained from chronic bronchitic patients, were processed as for CF sputa. Whenever possible sputum and sera were obtained from CF patients at the same time and analysed as appropriate. Specimens from PC+ and PC- CF patients, including those with and without *P. aeruginosa* were subjected to detailed immunological analysis; such patients were designated I-IX (see Results).

Bacteriological analysis of sputa. Bacteriology was performed on sputa which had been homogenized in sputalysin (Calbiochem, La Jolla, CA, USA). Following appropriate dilutions, sputa was cultured quantitatively on blood agar, horse digest agar, Pseudomonas isolation agar (Difco, Detroit, Michigan, USA), and *Pseudomonas cepacia* selective medium (Mast Laboratories, Bootle). Bacteria cultured on *P. cepacia* selective media were identified biochemically by the API 20 NE system (API System, La Balme les Grottes, France).

Bacterial strains. *P. cepacia* strains J1780 (serotype 1), J1680 (serotype 2), J1705 (serotype 3), J1774 (serotype 4), J1749 (serotype 5), J1772 (serotype 6), J1690 (serotype 7), J1687 (serotype 8), J1758 (serotype 9) and J1745 (non-typeable) were obtained from the Communicable Diseases Center (Atlanta, GA, USA). The serotype was based on the serotyping scheme proposed by Heidt *et al.*[15] *P. cepacia* strains J1359 (non-typeable), SBC21 (non-typeable), SBC42 and SBC29 were isolated from the sputa of CF patients. *P. cepacia* SBC8 was an environmental strain isolated from a plant. Serotyping sera was unavailable for the typing of SBC42, SBC29 and SBC8, but were confirmed as distinct strains by the method of ribotyping.[16] *P. aeruginosa* R-mutant PAC608, defective in the production of LPS, was obtained from Professor P.M. Meadow (University College, London).

Preparation of lipopolysaccharides. The aqueous phenol, chloroform, petroleum ether method of Galanos *et al.*[17] incorporating the diethyl ether precipitation of LPS described by Qureshi *et al.*[18] (as described by Hancock and Poxton[19]) was used to prepare LPS from *P. cepacia* expressing R-form LPS. Briefly, LPS was extracted from washed, freeze-dried bacteria from an overnight culture. The LPS was then washed and purified by centrifugation at 100,000 x *g* for 4h and freeze-dried.

The proteinase K digestion of bacteria[20] was also used to prepare LPS for analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

SDS-PAGE and immunoblotting. LPS was separated on 14% polyacrylamide gels with the buffer system of Laemmli (21) (except that sodium dodecyl sulphate was omitted from the stacking and separating gel

buffers in gels used for immunoblotting). The LPS separating gels were stained with silver by the method of Tsai and Frasch.[22] For immunoblotting separated antigens were transferred to nitrocellulose membranes (pore size 0.2μm, Schleicher & Schuell, Dassel, Germany) by the method of Towbin *et al*.[23] Antigens were probed with diluted sera (1:200) and sputa (1:100) for 3h at room temperature, and the immune complexes detected with anti-human IgG and IgA horseradish peroxidase conjugates (ICN Biomedicals Ltd., High Wycombe) and HRP colour development reagent (Bio-Rad Laboratories, Richmond, USA).

Enzyme-linked immunosorbent assay (ELISA). Antigens used for coating microplates included (a) *P. cepacia* whole cells, serotypes 1-9 and a non-typeable strain, and (b) extracted LPS from a rough isolate, *P. cepacia* J1359. For the whole cell assay, bacteria were grown overnight in nutrient broth containing 0.5% w/v yeast extract (NYB), harvested and washed twice with PBS. Cells were resuspended to a density of 10^7 cells/ml in carbonate-bicarbonate coating buffer (pH 9.6) and 100 μl added to the wells of polystyrene 'polysorb' microplates (Nunc, Roskilde, Denmark). Plates were then centrifuged at 1,365 x *g* for 5 min to sediment bacteria to the wells. In the case of the LPS antigen, extracted LPS was complexed with polymyxin as described by Scott and Barclay[24] and used at a final concentration of 10 ng/ml. LPS-polymyxin complexes were diluted in coating buffer and added to microplates at 100 μl/well. All plates were coated overnight at room temperature and washed four times with wash buffer (PBS pH 7.2, containing 0.05% w/v Tween 20 and 0.02% w/v sodium azide). All plates were then post-coated with post-coat buffer consisting of PBS containing 5% w/v bovine serum albumin (BSA), at 100 μl/well. After being washed four times with wash buffer, plates were stored at -20°C until used.

Sera and sputa were serially diluted in dilution buffer and added to coated microplates at 100 µl/well in triplicate. Antibody diluent consisted of PBS (pH 7.2) containing 0.05% v/v Tween 20, 0.5% w/v BSA, 4% w/v polyethylene glycol 6000 and 0.002% w/v sodium azide. After incubation at 37°C for 90 min plates were washed four times with wash buffer. Conjugates, including either alkaline phosphatase conjugated anti-human IgG or anti-human IgA (Sigma, Poole) were diluted in dilution buffer at 1:1000 and added at 100 µl/well. Plates were incubated for a further 90 min at 37°C and then washed and rinsed prior to addition of alkaline phosphatase substrate (Sigma) at 100 µl/well. After a 30 min incubation at room temperature the optical density (OD) of wells was read at 405 nm (alkaline phosphatase) in a Titertek Multiscan plate reader (Flow Laboratories Ltd., Irvine). Final results were expressed as the titer giving an OD >0.1 after subtraction of the OD of negative controls (wells coated only with post-coat) for each sample. Analytical variation of the ELISA, including intraplate and day to day variations, was performed with six serum samples (three determinations for each) with low (n=2), medium (n=2) and high (n=2) ELISA titers, and the coefficient of variation was calculated. The intraplate and day to day variation of the ELISA was 5.5% and 13.9% respectively.

Absorption Studies. Sera from CF patients were serially absorbed with whole cells of *P. cepacia* J1359 and *P. aeruginosa* PAC608. Overnight cultures of bacteria grown in NYB, were harvested by centrifugation, washed twice in PBS and resuspended to a density of 10^8 cells/ml. Bacterial suspensions (1 ml) were added to Eppendorf tubes and bacteria harvested by centrifugation using a microcentrifuge. Bacteria were resuspended in serum diluted 1:200 in dilution buffer, incubated for 15 min at room

temperature and recentrifuged. The supernatant was then added to another pellet of cells and the process repeated. This step was repeated three times for each absorbing bacterial strain. Serum anti-*P. cepacia* and anti-*P. aeruginosa* core LPS antibodies were assayed by ELISA and immunoblotting as described above.

Statistics. Data were not normally distributed and statistical analysis was by Wilcoxon signed rank tests.

RESULTS

Whole cell ELISA. IgG antibodies directed to *P. cepacia* whole cell antigens were detected in sera from patients with CF including both PC+ and PC- patients (data not shown). A response to each of the serotype strains was evident although the difference in antibody titers between PC+ and PC- CF patients was significant only in the case of serotype 5 and the non-typeable whole cells ($p < 0.05$).

Core R-LPS ELISA: detection of serum IgG and sputum IgA antibodies. The ELISA employing extracted LPS from an isolate expressing core R-LPS was better than the whole cell ELISA in discriminating PC+ from PC- patients (Fig. 1). In 9 CF patients persistently sputum positive for *P. cepacia*, IgG titers were significantly higher (median 1:12800, range 3200 - 51200) than in 9 age and sex matched CF PC- controls (median 400, range 200 - 3200) ($p < 0.01$ Wilcoxon signed rank test), and in 9 healthy blood donors (median 400, range 200 - 800). There was no significant difference in the IgG titers of the PC- CF patients and the healthy subjects. Seven out of nine PC- CF controls were colonized by *P. aeruginosa*, whilst six out of nine PC+ patients were colonized by *P. aeruginosa*.

The 9 PC+ CF patients also had significantly higher titers of sputum IgA antibodies reactive with core LPS of *P. cepacia* (median 1600, range 200 - 6400) than did the 9 PC- patients (median 50, range 25 - 100) and 9 chronic bronchitics (median 50, range 25 - 400) ($p < 0.01$ Wilcoxon signed rank test); there was no significant difference between the non-colonized CF patients and the chronic bronchitis (Fig. 2). None of

the chronic bronchitic patients were colonized by *P. cepacia*.

Longitudinal analysis of serum IgG and sputum IgA. Longitudinal analysis of available CF sera indicated that the rate of increase in the levels of anti-*P. cepacia* IgG antibodies after bacteriological diagnosis of the onset of *P. cepacia* colonization varies from patient to patient: in some cases, for example, patients III, IV, VI, VII, the first isolation of *P. cepacia* was preceded or accompanied by a 2-4 fold rise in anti-*P. cepacia* R-LPS IgG titers (Fig. 3).

Longitudinal analysis of sputa from five CF patients, four of whom were colonized by *P. cepacia*, demonstrated the variable IgA antibody responses to core R-LPS of *P. cepacia*. During a ten week study period the titer of sputum IgA antibodies ranged from 1:400 - 1:6400 for two of the PC+ CF patients, and from 1:800 - 1:3200 for a third PC+ patient. In the case of a fourth PC+ patient, the IgA antibody titer was maintained between 1:100 - 1:200, which was similar to that of the PC- patient included in the study. There was no correlation between the titer of sputum IgA antibodies and the numbers of *P. cepacia* isolated by bacteriological culture from sputum (data not shown), but a tendency for IgA levels to rise during hospitalization for pulmonary exacerbations.

Immunoblot analyses. Isolated core LPS from *P. cepacia* J1359 is shown in the silver stained PAGE (Fig. 4C). Reactivity of sera from two PC+ CF patients against *P. cepacia* LPS antigens are shown in the Western blots (Fig. 4A and 4B). In both cases, a positive reactive band corresponding to the low molecular weight core LPS of most but not all of the *P. cepacia* isolates was observed: reactivity with 9/10 and 6/10 core

LPS antigens was observed for patients III and VII respectively Fig. 4A and 4B). In addition, a response corresponding to higher molecular weight O-antigen subunits of LPS from SBC42, SBC29 and SBC8 was noted. The serum used for Fig. 4A was obtained from a CF patient colonized with J1359 (ribotype A), whilst the serum for Fig. 4B was obtained from a CF patient colonized with J1359 (ribotype A) and SBC42 (ribotype C). Immunoblot analysis of serum from two PC- CF patients, one of whom was colonized with *P. aeruginosa*, did not produce a visible response to any of the *P. cepacia* LPS antigens used (data not shown).

Sputum from the same PC+ CF patient as used for Fig. 4A was used for immunoblot analysis of IgA antibodies reactive with *P. cepacia* LPS antigens (Fig. 5). A strong response to the core LPS of J1359 and a weak response to the O-antigen subunits of SBC8 was observed.

Absorption studies. Serum from CF patients colonized with *P. cepacia* and/or *P. aeruginosa*, or neither, were absorbed with whole cells from *P. cepacia* strain J1359 and *P. aeruginosa* PAC608 and the sera analysed by ELISA (Fig. 6) and immunoblotting (Fig. 7). In the case of PC+ CF patients (patients I-VII, Fig. 6) antibodies reactive with *P. cepacia* core LPS were substantially removed after absorption with the corresponding *P. cepacia* whole cells, but not after absorption with *P. aeruginosa* whole cells. Sera from patients colonized with *P. aeruginosa* (patients II, VI, VII and VIII, Fig. 6) demonstrated a relatively high antibody titre to *P. aeruginosa* core LPS; these antibodies were removed after absorption with *P. aeruginosa* whole cells but not after absorption with *P. cepacia* whole cells. These absorption studies demonstrated that a significant component of the anti-*P. cepacia* core LPS antibodies was specific for *P. cepacia* and

not reactive with core LPS from *P. aeruginosa*. For example, patient I, colonized by *P. cepacia* but not by *P. aeruginosa*, had a serum IgG titer of 51200 against *P. cepacia* core LPS and a titer of 200 against *P. aeruginosa* core LPS. The antibodies against *P. cepacia* core LPS were removed after absorption with *P. cepacia* whole cells but not by the *P. aeruginosa* whole cells as shown by the ELISA (Fig. 6) and immunoblotting studies (Fig. 7). Conversely, serum from a CF patient colonized by *P. aeruginosa* but not by *P. cepacia* (patient VIII) showed a reaction with *P. aeruginosa* core LPS but not with *P. cepacia* core LPS.

DISCUSSION

Antibodies reactive with *P. cepacia* whole cells and extracted core R-LPS were demonstrated in sera and sputum from patients with CF. A core LPS preparation was used as a coating antigen since: (a) LPS represents a more defined antigenic preparation than a system based on whole cells; (b) available evidence suggests antigenic cross-reactivity between some outer-membrane protein antigens of *P. cepacia* and *P. aeruginosa*)[13,14]; (c) core LPS is believed to be a relatively conserved component of LPS[25] and therefore provides a suitable antigen for the detection of an antibody response against all serotypes of *P. cepacia*; (d) many strains of *P. cepacia* isolated from CF patients produce rough LPS (i.e. lacking O-specific side-chains)[26] (S. Butler, unpublished observations). The ELISA system based on core R-LPS from a CF clinical isolate of *P. cepacia* was found to clearly differentiate PC+ CF patients from PC- CF patients. However, inspection of Figures 1 and 2 reveal that a range of serum IgG and sputum IgA antibody titers were obtained for both PC+ and PC- CF patients and it is possible that overlap between the two groups could occur in certain cases. Thus detection of a rising antibody titer performed on two or more serum samples would appear desirable. Indeed, in the longitudinal studies some patients demonstrated a rise in the level of anti-*P. cepacia* IgG antibodies prior to or accompanying the first isolation of *P. cepacia*. An ELISA based on core R-LPS from *P. aeruginosa* has also been reported for the diagnosis of chronic *P. aeruginosa* infection in CF.[27]

Measurement of anti-*P. cepacia* antibodies to identify *P. cepacia* colonization may prove useful. Although the value of selective media for *P. cepacia* is well recognised,[1,6], problems may still be encountered,

including growth of *Xanthomonas maltophilia* and *Pseudomonas acidovorans*[4] and the fact that some clinical isolates of *P. cepacia*, on initial isolation from sputum, may take up to seven days to grow on the media. In view of the considerable concern relating to pulmonary colonization of patients with CF by *P. cepacia*[4,5,28], the ability to identify *P. cepacia* colonization early and accurately is important, especially in clinics where segregation of colonized and non-colonized patients is practised.

Recent studies[13,14] have demonstrated the presence of IgG antibodies to outer membrane antigens of *P. cepacia* in serum from CF patients colonized with *P. cepacia* and/or *P. aeruginosa*. These authors concluded that some *P. cepacia* outer membrane components may be antigenically related to those of *P. aeruginosa*. Similarly, in our study, serum antibodies reactive with *P. cepacia* whole cells were found in CF patients colonized with *P. cepacia* and/or *P. aeruginosa*. In terms of LPS antigens however, our Western blotting and absorption studies demonstrate that a significant proportion of the antibodies reactive with core LPS of *P. cepacia* were not reactive with core LPS of *P. aeruginosa*. These observations indicate differences in the structure and composition of core LPS between *P. aeruginosa* and *P. cepacia*, confirming previous findings including (a) structural differences[29] e.g., the lack of phosphorus in the core-LPS of *P. cepacia*, and (b) the inability of a monoclonal antibody reactive with *P. aeruginosa* core LPS to react with *P. cepacia*.[30] In addition, there is unlikely to be cross-reactivity with core LPS of *X. maltophilia* given the structural differences reported by Neal and Wilkinson.[31] A degree of core heterogeneity between different isolates of *P. cepacia* may exist since Western blotting demonstrated that sera from *P. cepacia* colonized patients produced a reactive band with some but not all

P. cepacia core lipopolysaccharides.

Although anti-core IgA antibodies were demonstrated in sputum from chronically colonized patients, the levels of these antibodies may have been underestimated due to the possibility of immune-complex formation and fragmentation of antibody by elastases derived from neutrophils or *P. aeruginosa*.[32,33]

Our data suggests that in CF patients colonized by *P. cepacia*, antibodies reactive with *P. cepacia* are unable to eliminate the organism from the lungs. Indeed, this study and those of Aronoff *et al.*[13,14] have shown that *P. cepacia* colonization occurs in the presence of antibodies specific for outer membrane components of the organism. However, a relatively high titer of IgG antibodies against *P. cepacia* was observed in one of the PC- patients from whom *P. cepacia* has never been isolated despite contact with members of the PC+ group. Indeed, it is interesting to speculate on the possible protective effect of antibodies in a subset of PC- CF patients.

Measurement of anti-*P. cepacia* antibodies currently forms part of a prospective longitudinal study on a larger number of CF patients from different regional centres. Such investigations will provide important information regarding the diagnostic applicability and usefulness of the antibody assay for detection of *P. cepacia* in patients with CF.

Future studies will also investigate the immunological properties of anti-*P. cepacia* antibodies from CF patients, in particular their opsonising potential.

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LEGENDS FOR FIGURES

Fig. 1. Serum IgG anti-*P. cepacia* core R-LPS antibodies in 9 CF patients persistently colonized for *P. cepacia* (titer range 3200-51200) and 9 non-colonized CF patients (titer range 200-3200) and 9 healthy controls (titer range 200-800) measured by ELISA with core R-LPS as coating antigen. CF patients were age and sex matched. Bars represent median titer values. Antibody titers for the PC+ CF patients were obtained on serum samples obtained after colonization with *P. cepacia* was confirmed by bacteriological culture.

Fig. 2. Sputum IgA anti-*P. cepacia* core R-LPS antibodies in 9 CF patients persistently colonized with *P. cepacia* (titer range 200-6400) and 9 non-colonized CF patients (titer range (25-400) and 9 chronic bronchitics (titer range 25-400) measured by ELISA with core R-LPS as coating antigen. CF patients were age and sex matched. Antibody titers for the PC+ CF patients were obtained on sputum samples obtained after colonization with *P. cepacia* was confirmed by bacteriological culture. Bars represent median titer values.

Fig. 3. Longitudinal study of serum IgG anti-*P. cepacia* LPS antibodies in 8 CF patients measured by ELISA with core R-LPS as coating antigen. Patients I, III, IV and V were colonized by *P. cepacia* only; patients II, VI and VII were colonized by both *P. cepacia* and *P. aeruginosa*; patient VIII was colonized by *P. aeruginosa* only. The arrows indicate the time when *P. cepacia* was first isolated from sputum obtained from each respective patient.

Fig. 4. Immunoblot analysis of *P. cepacia* LPS antigens reacted with sera from two CF patients colonized with *P. cepacia* and analysed for IgG anti-LPS antibodies. Serum used for immunoblots A and B were obtained from patients III and VII respectively. The *P. cepacia* LPS antigens included: lane 1, J1780 (serotype 1); lane 2, J1680 (serotype 2); lane 3, J1774 (serotype 4); lane 4, J1690 (serotype 7); lane 5, J1758 (serotype 9); lane 6, J1359 (non-typeable); lane 7, SBC21 (non-typeable); lane 8 SBC42; lane 9, SBC29; lane 10, SBC8. (c) Silver stained PAGE (14% w/v acrylamide) of isolated core LPS from *P. cepacia* J1359.

Fig. 5. Immunoblot analysis of *P. cepacia* LPS antigens reacted with sputum from a CF patient (patient III) colonized with *P. cepacia* and analysed for IgA anti-LPS antibodies. *P. cepacia* LPS antigens were the same as Fig. 4.

Fig. 6. Serum IgG anti-*P. cepacia* and anti-*P. aeruginosa* LPS antibodies in CF patients measured by ELISA with (A) core R-LPS from *P. cepacia* J1359 and (B) core R-LPS from *P. aeruginosa* PAC608 as coating antigens before and after absorptions with *P. cepacia* J1359 and *P. aeruginosa* whole cells. Patients I, III, IV and V were colonized by *P. cepacia* only; patients II, VI and VII were colonized by both *P. cepacia* and *P. aeruginosa*; patient VIII was colonized by *P. aeruginosa* only; patient IX was not colonized by either organism.

Fig. 7. Immunoblot analysis of (A) anti-*P. cepacia* and (B) anti-*P. aeruginosa* core LPS IgG antibodies in sera from five CF patients before and after a series of absorptions with *P. cepacia* J1359 and/or *P. aeruginosa* PAC608 whole cells. Unabsorbed sera, lanes 1, 5, 9, 13, 17; sera

absorbed with *P. cepacia* J1359 whole cells, lanes 2, 6, 10, 14, 18; sera absorbed with *P. aeruginosa* PAC608 whole cells, lanes 3, 7, 11, 19; and sera absorbed with both *P. cepacia* J1359 and *P. aeruginosa* PAC608 whole cells lanes 4, 8, 12, 16, 20. Patients I and IV (lanes 1-4 and 13-16 respectively) were colonized by *P. cepacia* only; patients II and III (lanes 5-8 and 9-12 respectively) were colonized by *P. cepacia* and *P. aeruginosa*; and patient VIII (lanes 17-20) was colonized by *P. aeruginosa* only.

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Microbiology of cystic fibrosis lung infections: themes and issues

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Literature covering the microbiology of lung infections in patients with cystic fibrosis encompasses an evolving spectrum of microbial pathogens, their epidemiology, pathogenesis, antibiotic therapy, vaccination and issues associated with lung transplantation[1-4]. This review will focus on a selection of these topics.

Microbial pathogens

The susceptibility of patients with CF to pulmonary infections has been recognized since the first descriptions of the disease in the 1940s. One of the striking features of this susceptibility is that relatively few microbial pathogens are involved and that the significance of individual pathogens has evolved in parallel with improved patient management and longevity.

The earliest microbiological study[5] of postmortem cultures of lung tissue from 14 patients with CF, aged 1-40 months, yielded *Staphylococcus aureus* from 12, indicating the predominant role of this organism in very young patients in the early days of antibiotic therapy. Subsequently, *Staph. aureus* and *Haemophilus influenzae* became recognized as the most prevalent pathogens in children with CF. Today, the most prevalent lung infections in patients with CF are associated with *Pseudomonas aeruginosa*; however, the spectrum of pathogens continues to evolve. The clinical significance of influenza and respiratory syncitial viruses as a cause of acute pulmonary exacerbations and *Aspergillus fumigatus* as a cause of allergic bronchopulmonary aspergillosis is well recognized. In addition, the significance of previously uncommon bacterial opportunists in patients with CF, including mycobacteria species, *Pseudomonas maltophilia* and, in particular *P. cepacia*, needs to be carefully monitored as the population of adults with CF, including transplanted patients continues to expand[1-4].

Pseudomonas aeruginosa

Immune-mediated tissue damage

Chronic colonization with mucoid variants of *P. aeruginosa* leading to intermittent episodes of pulmonary exacerbation remains the most important cause of morbidity and mortality in patients with CF.

important OK? col 2

A striking feature of pseudomonas pathogenesis in patients with CF is that infection remains localized in the lung and the role of bacterial toxins and exoenzymes appears to be restricted to the early stages of colonization by nonmucoid *P. aeruginosa*. Subsequently, mucoid, alginate producing variants of

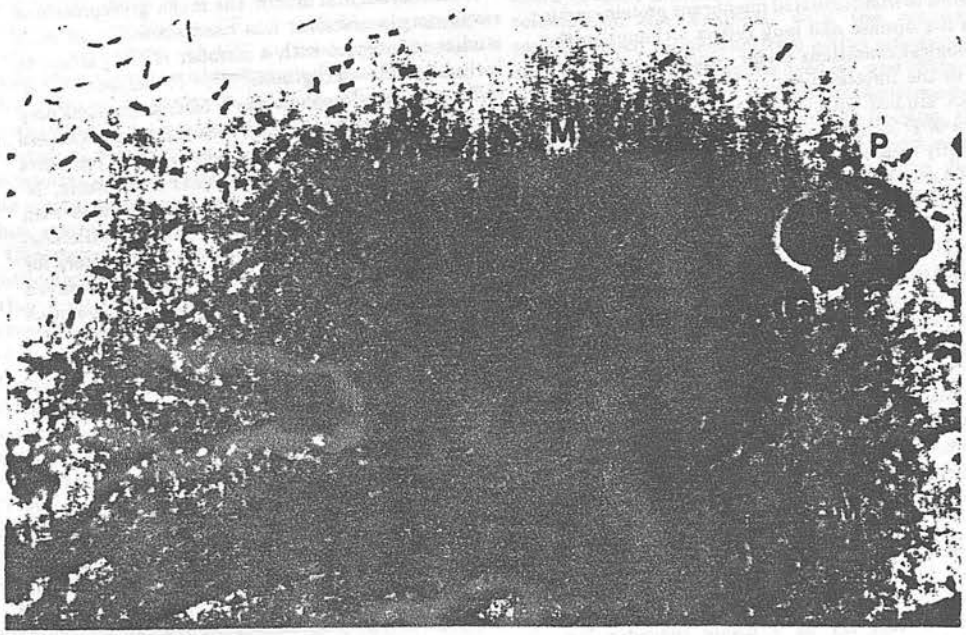

Figure 1. Gram-stained sputum from a patient with cystic fibrosis harbouring mucoid P. aeruginosa. *The bacteria can be seen in a gelatinous microcolony (M) which is attached to the bronchial mucosa (BM) and is significantly larger than the adjacent 'frustrated' phagocyte (P). (Reproduced by permission of Churchill Livingstone from Govan and Glass,* Microbiol Rev *1990[2].)*

the original colonizing strain colonize the airways within the protective environment of bacterial microcolonies (Figure 1) or extended biofilms leading to a chronically overstimulated immune response dominated by 'frustrated' phagocytic cells[6,7]. The hypothesis, originally proposed by Hoiby and colleagues[8], that lung damage primarily arises from chronic inflammation mediated by an antibody response to pseudomonas antigens and the formation of immune complexes, leucocyte proteases, myeloperoxidase and oxygen radicals, is now almost generally accepted[8-10].

What is the scientific evidence for this overstimulated immune response which underlines the unusual strategy of using corticosteroids in the treatment of a bacterial infection?

Many studies have shown that CF patients mount a vigorous antibody response against extracellular and cellular antigens of *P. aeruginosa* and that elevated levels of IgA and IgG are associated with pulmonary deterioration[6,11-14]. Use of purified *P. aeruginosa* antigens has demonstrated antibodies to alkaline protease, elastase, exotoxin A, phospholipase C and exoenzyme S[15-18]. Antibodies to cellular components including alginate[19], lipopolysaccharide[20,21], outer membrane proteins[22], pili and flagella[23,24] have also been demonstrated.

In a longitudinal study[15] the immune response of patients with CF increased after the onset of chronic *P. aeruginosa* and included elevated antibody titres to proteases and an increase in the number of precipitins to pseudomonas antigens. These results suggested that the action of elastase and alkaline protease are probably important at the onset of chronic infection, after which time they are neutralized by specific antibodies. In a related study[17], antibodies to a number of *P. aeruginosa* exoproteins were detected; serum antibodies to phospholipase C developing first, approximately 3 months after initial colonization. In contrast Shand *et al.*[25] observed that antibodies to iron-regulated membrane proteins occur late in the disease and may reflect a change in the physiological conditions under which *P. aeruginosa* grows in the infected lung.

ELISA studies with whole cells[26] or a sonicated mixture of *P. aeruginosa* antigens[27] showed that in chronically colonized patients, serum IgG titres increased with the duration of infection and correlated with a deterioration in clinical state. In a longitudinal study[27] of serum antibodies to *Pseudomonas* lipopolysaccharide, elevated anti-lipopolysaccharide IgG antibodies and precipitins correlated with poor prognosis including impaired respiratory function and severe changes on chest radiographs. IgG and IgA anti-lipopolysaccharide antibodies rose significantly at the onset of chronic infection and continued to increase to very high levels in the later stages of infection.

In some patients with CF, antibodies can be detected in serum before sputum bacteriology becomes positive for *P. aeruginosa*[21,28-30]. Although elevated titres against *P. aeruginosa* antigens may be found in a few non-colonized patients, a positive titre usually indicates significant exposure to *P. aeruginosa*[21,31]. Detection of anti-*P. aeruginosa* antibodies is perhaps particularly useful in patients unable to produce sputum, and may act as a useful indicator for instigation of early antipseudomonal treatment[1]. (See also discussion on prevention of chronic *P. aeruginosa* colonization.)

The presence of *P. aeruginosa* specific antibodies in the sputum of patients with CF has been reported by several investigators[21,23,32,33]. In the study of Przyklenk and Bauernfeind[33] patients colonized by *P. aeruginosa* had significantly higher secretory IgA antibody levels against pseudomonas lipopolysaccharide and elastase compared to non-colonized patients and increases in secretory IgA correlated with acute exacerbations attributable to *P. aeruginosa*. Recently, Nelson and colleagues[24] demonstrated the presence of anti-*P. aeruginosa* flagellar antibodies in saliva and sputum specimens from patients with CF intermittently or chronically with *P. aeruginosa*.

Bacterial adherence

A dogma of bacterial pathogenesis states that the first interaction between a potential pathogenic organism and its host entails attachment to a eukaryotic cell surface[34]. In this context, CF literature abounds with details of the adherence of microorganisms to cell surfaces and the potential for adherence in pulmonary colonization.

In vitro studies have demonstrated adherence of *P. aeruginosa* to buccal cells obtained from patients with CF and the association of adherence with decreased amounts of the cell surface glycoprotein, fibronectin and increased levels of salivary proteases[35,36]. Although CF buccal epithelial cells bind *P. aeruginosa* in vitro, the importance of this observation in vivo is unclear. Evidence for *P. aeruginosa* adherence to CF buccal cells in vivo could not be demonstrated in a prospective study[37]. In addition, immunohistopathological studies[38] and scanning electron microscopy[39] of CF lung tissue have shown that *P. aeruginosa* is almost always associated with surface mucous secretions rather than with the underlying epithelia. Furthermore, respiratory epithelial cells from patients with CF do not appear to possess specific *P. aeruginosa* adhesins[40].

Tracheobronchial mucin, the major glycoprotein of respiratory secretions, has been shown in in vitro studies to interact with a number of CF pathogens including *P. aeruginosa*[41-43], *P. cepacia*[44], *H. influenzae* and *Staph. aureus*[45]. Microorganisms may become entrapped in mucus because of its stickiness or because of specific interaction between receptors in mucin and surface components of bacteria. N-acetylneuraminic acid[42] and oligosaccharides with type 1 (GalB1-3GlcNAc) and type 2 (GalB1-4GlcNAc) units[46] have been suggested as mucin receptors for *P. aeruginosa*. The *P. aeruginosa* adhesins involved in adherence to mucin include pilus[47] and non-pilus adhesins[46,48].

Controversy remains regarding the specificity of the binding of *P. aeruginosa* to host mucin. Sajjan *et al.*[44] concluded that there was no preferential binding of *P. aeruginosa* to mucin compared with various proteins and glycoproteins and that there was no specific mucin receptors for the attachment of *P. aeruginosa* pili. Our own investigations[43] and most recent unpublished results indicate that *P. aeruginosa* binds more readily than *P. cepacia* to respiratory mucin and to other substrates and that adhesion plays an important role in colonization of patients with CF especially by highly transmissible strains.

P. aeruginosa *vaccines*

Since the majority of patients with CF have succumbed to respiratory colonization with

P. aeruginosa by early adolescence the prospect of administering a multivalent vaccine in early childhood to prevent chronic colonization is an important goal.

In the early 1980s, vaccination of patients with CF with a relatively crude vaccine proved disappointing and suggested that vaccination might predispose to more serious infections[49]. However, recent studies of a conjugate antipseudomonal vaccine have been encouraging. Ongoing evaluation of an octavalent O-polysaccharide (O-PS)-toxin A conjugate vaccine[50] has shown that adverse reactions were mild and self-limiting and that IgG antibody concentrations were raised significantly to the vaccine antigens and remained so for 12 months. Immunization of 22 patients with CF with no previous history of pseudomonas colonization produced opsonic and toxin A neutralizing antibodies and there was no significant change in clinical status. Further reports on the efficacy of this vaccine in preventing pseudomonas colonization are awaited with interest.

An alternative approach to vaccination is based on clinical data which implicated alginate-specific, opsonizing antibodies as a potential immunological protective mechanism[51]. After immunization with a mucoid exopolysaccharide antigen, mice and rats that elicited opsonizing antibody had reduced levels of infection compared with nonimmune controls after intratracheal instillation using a model of chronic infection in which the bacteria are enmeshed within agar beads. Interestingly, parallel experiments in which passive transfer of antibodies were used yielded similar results. The major barrier that prevents evaluation of the pseudomonas vaccine in patients with CF is its poor immunogenicity in humans; if this problem can be solved the results of vaccine trials of this preparation in patients with CF would be awaited with interest.

Antibiotic therapy

Antibiotic therapy in CF is probably the most controversial topic of CF microbiology. Despite aggressive therapy with increasingly potent agents, and laboratory evidence to suggest appropriate susceptibility, eradication of chronic colonization by individual pathogens is seldom achieved. It is well recognized, however, that antibiotic therapy relieves the symptoms of acute pulmonary exacerbations and thus acts as a palliative and not a cure.

Controversy associated with present forms of antibiotic therapy include the value of aerosols versus other routes of administration, the benefits and adverse effects of long-term therapy against *Staph. aureus*, the value of susceptibility testing to predict clinical outcome and the choice of individual agents or combinations to improve clinical efficacy and reduce the risk of bacterial resistance. A devil's advocate might argue that bacterial resistance is of little significance in the treatment of patients with CF since susceptibility based on minimum inhibitory concentrations (MICs) does not predict clinical outcome or a successful reduction in the load of 'susceptible' strains during therapy.

Despite these controversies, antibiotic therapy remains a cornerstone in the management of patients with CF. In this update we will focus on three recent developments.

Prevention of chronic P. aeruginosa *colonization*

Initial colonization with nonmucoid *P. aeruginosa* is usually asymptomatic but forms a key microbial reservoir for the emergence of mucoid variants and other phenotypes associated with chronic colonization[37]. In addition, since nonmucoid forms are more susceptible to antipseudomonal agents in vitro[52] and can be eradicated in vivo by antibiotic therapy[53], we have previously argued that it would be rational to initiate antipseudomonal therapy when *P. aeruginosa* was first cultured or colonization identified by ELISA or DNA-based methodology[3,37]. Recently, two important studies have shown the benefits of early treatment. Littlewood and colleagues[54] showed that nebulized colomycin, given to patients at an early stage of pseudomonas colonization, decreased the number of positive cultures when compared with periods when colomycin was not given. Subsequently, Hoiby and colleagues[55] reported the results of a controlled trial using aerosol inhalations of colomycin and oral ciprofloxacin twice daily for 3 weeks whenever *P. aeruginosa* was isolated from routine monthly sputum cultures. During the 27 months of the trial, infection with *P. aeruginosa* became chronic in significantly fewer treated than untreated subjects (14% versus 58%; $P=0.05$) and there were significantly fewer isolations in routine sputum cultures in the treated group (23% versus 41%; $P=0.0006$). Although the relative merits of colomycin and ciprofloxacin in this study are unclear, the data supports the stratagem that early institution of antipseudomonas therapy reduces the incidence of subsequent chronic colonization.

Inflammatory markers and suppression of bacterial virulence

Quantitative sputum culture may allow measurement of the decrease in bacterial load but clinical improvement can occur without reduction in the numbers of bacteria[53]. Thus, on the basis of sputum culture, there is often difficulty in distinguishing bacterial colonization (for example, with the respiratory commensal *H. influenzae*) from active infection and, accordingly, in assessing the need for, and efficacy of, antimicrobial therapy. Clinical measurements in patients with CF based on pulmonary function, chest radiographs, body weight, and haematological markers are useful but are not always instructive. Serial measurements of C-reactive protein (CRP) in CF sera are useful as an additional index of acute or impending pulmonary exacerbation and of the response of the patient (rather than the pathogen) to antibiotic therapy[56]. Other inflammatory markers including tumour necrosis factor and plasma neutrophil elastase are also informative[57,58]. CRP, however, is probably the most useful inflammatory marker in recognizing an acute respiratory exacerbation. Objective measurements can be made from whole blood, blood spots, finger blood and serum. CRP concentrations can be determined rapidly by ELISA-based immunoassays, fluometry (TDX, Abbot Laboratories) or semi-quantitatively by latex-based kits; the most reliable kit in our experience being the RapiTex CRP system (Behringwerke AG, Marburg). In the absence of pulmonary exacerbation, CRP levels in patients with CF are generally within normal limits (< 10 mg/l) but may increase 20-fold at the onset of exacerbation. During effective antibiotic therapy CRP levels begin to fall within 48 h and thus allow a rapid assessment of efficacy. The use of inflammatory markers such as CRP should be seen as an adjunct rather than replacement for other clinical parameters. Serial CRP

measurements in individual patients with CF during and between exacerbations show that individual patients have their own profile; for example, patient A may never exceed 50 mg/l during an exacerbation whilst patient B may show a more extended range from less than 10 mg/l when well to levels as high as 300 mg/l during exacerbations. Administration of steroids also needs to be taken into account in evaluating the clinical significance of CRP levels; however, levels of CRP may prove to be useful in evaluating the efficacy and dosage of steroids to achieve an anti-inflammatory effect.

Antibiotics and virulence

How do we explain the fact that antibiotic therapy can reduce concentrations of inflammatory markers and be clinically effective in the absence of bacterial eradication and often with no significant reduction in bacterial load. Do sublethal concentrations of antibiotics reduce bacterial virulence in vivo by reducing binding of *P. aeruginosa* to tracheobronchial mucin and suppressing synthesis or export of bacterial toxins and tissue-damaging enzymes? There is increasing evidence from in vitro and in vivo studies that bacterial binding to epithelial surfaces and the expression of major virulence factors for example elastase, exotoxin A and alginate is reduced in the presence of sublethal concentrations of antibiotics[37,59-61]. Recently, the use of an *alg*D-*xyl*E transcriptional fusion has provided molecular evidence that antipseudomonal agents at concentrations as low as 5% of the MIC, reduce transcription of *alg*D, the gene encoding GDPmannose dehydrogenase, the key enzyme in alginate biosynthesis and biofilm formation in the CF lung[62].

Pseudomonas cepacia

The most striking addition to the list of CF pathogens in the last decade has been the emergence of *P. cepacia* as a major threat to the management of patients with CF[63,64]. Fears of the clinical outcome of colonization and the risk of cross-infection within CF clinics have caused much concern to patients and prompted their doctors to take drastic actions. The scientific evidence underlying this concern is limited but is sufficient to warrant appropriate concern.

In the early 1980s, reports from North American clinics demonstrated a disturbing increase in the prevalence of *P. cepacia* from CF sputa[63,65]. Subsequent surveillance data indicated that the prevalence of colonization varied widely in different regional centres. The reliability of this data, however, was questionable due to the lack of selective culture media and reliable identification systems. For example, in a multicentre study of sputum seeded with *P. cepacia* only 36 (32%) of 115 laboratories detected the organism[66]. Recent experience has indicated that vigilance needs to be maintained[64]. For example, between 1986 and 1989, surveillance studies in the UK indicated a maximum prevalence of 7%; by 1992, however, isolations had risen in some centres to equal the 40% prevalence associated with a contemporary study from a North American centre[14]. In surveillance studies, the value of selective media for *P. cepacia* has been emphasized[2,3,66,67]. A positive culture, however, may require incubation for 72 h or more and, since some strains of *P. maltophilia* and *Comamonas acidovorans* will also grow on this medium, a positive culture should be identified by an appropriate multitest system such as API20NE[3].

Properties

The organism, an environmentally ubiquitous plant pathogen, takes its name from its role as the cause of soft rot of onions.

P. cepacia is nutritionally versatile - hence its previous designation as *P. multivorans* (eater of everything). It can use a wide variety of organic matter as carbon sources including disinfectants and antiseptics and most isolates are innately resistant to a wide range of antibiotic compounds. The *pen*A gene, for example, is associated with an exquisitely sensitive inducible beta lactamase which allows the organism to utilize penicillin G as a carbon source[68,69]. Even if an isolate shows in vitro susceptibility to an antibiotic, there is little clinical response; however, use of combined therapy with temocillin and an aminoglycoside has been reported to have clinical efficacy in some patients[70].

Clinical significance and transmission

P. cepacia can be asymptomatic or associated with slowly declining lung function. A major cause of anxiety however, is based on the observation that some colonized patients, who previously have been mildly affected by their disease, unexpectedly succumb to '*P. cepacia* syndrome', an accelerated and fatal deterioration in pulmonary function with fever, necrotizing pneumonia, and in some cases, septicaemia[63-65]. Additional clinical concern arises from epidemiological evidence indicating that the risk of direct or indirect transmission of the organism between patients with CF is considerably greater than that observed with *P. aeruginosa* and other CF pathogens[64,67,71-73].

Colonization and virulence

The bacterial factors responsible for colonization and tissue damage are unclear and a direct pathogenic role for *P. cepacia* has not been proven.

Adherence of *P. cepacia* to respiratory epithelial cells has been reported[44] with suggestions that a synergistic relationship between *P. aeruginosa* and *P. cepacia* may facilitate increased *P. cepacia* attachment. Whether this phenomena is important in vivo remains unclear. Colonization with *P. aeruginosa* is not a prerequisite for *P. cepacia* colonization. For example, in the Edinburgh clinics, one-third of patients with CF colonized with *P. cepacia* have no previous history of colonization with *P. aeruginosa*.

A 22-kDa pilin associated protein has been identified as a mucin-binding adhesin in *P. cepacia*[44]. Variability in the degree of binding of different *P. cepacia* isolates to respiratory mucin has led to speculation that such a factor may contribute to enhanced transmissibility of epidemic strains of *P. cepacia* and also explain why some strains are associated with transient colonization whereas other strains, once acquired, are never lost (authors' unpublished observations).

P. cepacia produce a number of potential virulence factors including lipase, protease, lecithinase and haemolysin[74-76]. There is no evidence, however, that any of these factors play a direct role in pathogenesis. Although *P. cepacia* is also capable of producing a non-alginate polysaccharide composed of hexoses and uronic acid, a survey of isolates from patients with

CF indicated no correlation between polysaccharide production and colonization of patients[77]. Straus *et al.*[78] reported that *P. cepacia* lipopolysaccharide was toxic when administered to mice intraperitoneally and suggested this cellular component may be responsible for lung tissue damage.

Immune response

Immunological studies on *P. cepacia* colonization indicate that the organism persists despite a considerable antibody response and suggests the possibility of immune-mediated lung damage.

Aronoff *et al.*[79,80] demonstrated the presence of IgG antibodies to outer membrane antigens of *P. cepacia* in serum from patients with CF colonized with *P. cepacia* and/or *P. aeruginosa*. These authors concluded that some *P. cepacia* outer membrane components may be antigenically related to those of *P. aeruginosa* and that colonization with *P. cepacia* occurs in the presence of antibodies specific for the outer membrane of the organism. Serum IgG and sputum IgA antibodies directed towards the core lipopolysaccharide of *P. cepacia* have also been described (Nelson *et al.*, submitted): these antibodies appear to be specific for *P. cepacia* and are not significantly reactive with core lipopolysaccharide of *P. aeruginosa*.

Epidemiology

The reasons for a high prevalence of *P. cepacia* in some CF centres is unclear but may include use of appropriate selective media, a relatively high proportion of older susceptible patients and regional differences in patient management and social interaction between colonized and non-colonized patients.

A case-controlled study showed that colonization was associated with increasing age, recent hospitalization and a colonized sibling[81]. Inhaled colomycin - to which the organism is invariably resistant - is another possible risk factor; however, there is little evidence to support this hypothesis and the role of other antibiotics is unclear[81,82]. At present, it seems reasonable to conclude that the increase in isolations of *P. cepacia* reflect the enhanced susceptibility of a growing population of patients with CF treated in large regional centres and the spread of highly transmissible strains.

Cross infection with *P. cepacia* is a cause of widespread concern[64] and in some centres appears to be a major cause of increased colonization (Govan *et al.*, in preparation). There is increasing evidence to suggest that transmission can occur directly from person to person[71], indirectly from environmental contamination[67] and that transmission can occur both in hospital and through social contacts[3,72]. The risk of transmission of *P. cepacia* appears to be considerably greater than that experienced with *P. aeruginosa* and there has been increasing calls for guidelines to reduce the risk acquisition. In some centres segregation of colonized from non-colonized patients is practised to varying degrees and a British CF Association has produced guidelines to reduce the risk of cross infection[83]. The social effects of segregation policies on patients who previously have been encouraged to develop social support groups are devastating and create microbiological outcasts. What is meant by social contact? At present the degree of risk must be based on available evidence which suggests that there is a low risk associated with casual encounters but a high risk associated with a colonized sibling or sexual relationships (Table 1). We urgently need to identify the major routes of *P. cepacia* transmission, bacterial colonizing factors and the pathophysiological and/or immunological factors which account for the rapid clinical deterioration in some patients.

Table 1. Transmission of P. cepacia

Social contact	*Risk of transmission*
Social gatherings	Low
Casual conversations	
Handshakes	Medium
Sharing cups, food, etc.	
Sharing rooms	
Fitness classes	
'Social' kissing	
Sibling contacts	High
Sexual relationships	

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Future prospects

In addition to the topics discussed, other aspects of CF microbiology beyond the scope of this review may bring particular dividends in the near future.

Genetic and molecular studies of *P. aeruginosa* are beginning to reveal the basis of environmental regulation of alginate biosynthesis in the CF lung and show interesting similarities with co-ordinated regulation of virulence in other bacterial pathogens. Control of alginate biosynthesis includes bacterial signal transduction systems, histone-like elements controlling nucleoid structure and, possibly, factors affecting superhelicity of bacterial DNA[62,84]. If phosphorylation activates alginate biosynthesis as in other signal transduction systems, then there is the exciting possibility of developing inhibitors specific for this process and hence provide a new type of antimicrobial agent for the treatment of mucoid *P. aeruginosa* in patients with CF[84].

Knowledge of the properties of *P. cepacia* and its association with CF patients is expanding rapidly. The development of gene probes from highly transmissible strains, studies of the host immunological response to *P. cepacia* and determination of colonization sites and infective dose may reveal the identity of putative virulence factors, provide prognostic indicators and explain why some strains are associated with transient rather than chronic colonization. At present, there remains some doubt about whether *P. cepacia* is a true pathogen or merely a marker of severe lung disease[2]. A recent case-controlled study of 13 patients with CF colonized with *P. cepacia* illustrates the paradox concerning the clinical status of the organism[85]. Individual responses to colonization by the same epidemic strain of *P. cepacia* ranged from rapid decline leading to death within 4 months of acquisition in two previously stable patients to persistence over 3 years with minimal change. In the *P. cepacia*-colonized group, initial lung function measurements (FEV_1% and VC%) were worse in non-survivors than in survivors. Colonized patients had high titres of *P. cepacia*-specific IgG

antibodies but titres did not differ between survivors and non-survivors. Similarly, survival could not be predicted from age, sex and duration of colonization. Further scientific evidence on the clinical significance and transmission of *P. cepacia* is necessary. At present, it appears that in some patients the organism is associated with accelerated decline in lung function and increased mortality and that patients with poor lung function at time of acquisition are at most risk. Based on this evidence and the undoubted transmissibility of the organism, it would seem prudent to err on the side of caution and to maintain microbiological surveillance and patient segregation especially in large regional centres. Surveillance might benefit from improvements in the culture of *P. cepacia* from CF sputum. As indicated earlier in this review, available selective media is not specific for *P. cepacia*. In addition, some strains of *P. cepacia*, particularly when cultured from environmental surfaces require prolonged incubation. Preliminary data from our own studies suggest that culture in the presence of CO_2 enhances growth of the organism.

Finally, a CF animal model would be invaluable in the investigation of many aspects of CF microbiology including bacterial/host associations and assessments of new forms of antimicrobial therapy. Thus, the recent development of a viable CF mouse[86] and a mouse model of chronic mucosal colonization by *P. aeruginosa*[87] is exciting and the results of microbiological studies of these animals are keenly awaited.

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