THE *BURKHOLDERIA CEPACIA* COMPLEX: A CLINICAL AND BIOTECHNOLOGICAL PARADOX

Sazini Nzula

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ABBREVIATIONS

ABC	Adenosine 5' triphosphate –binding cassette
ABPA	Allergic bronchopulmonary aspergillosis
aGM	asialoganglioside receptor
AMP	Ampicillin
Ara4N	4-amino-deoxyarabinose
ASF	Airway surface fluid
ATCC	American type culture collection
ATP	Adenosine 5' triphosphate
BCESM	*Burkholderia cepacia* epidemic strain marker
BCSM	*Burkholderia cepacia* selection medium
bp	Base pair
Br	Bromine
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CAB	Columbia agar base
Cat	Catalase
CAT	Chloramphenicol acetyltransferase
*cbl*A	Cable pilus type A
CD	Cluster of differentiation
Cefp	Cefpirome
Ceft	Ceftazidime
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cfu	Colony forming units
CGD	Chronic granulomatous disease
Chlo	Chloramphenicol
Cipr	Ciprofloxacin
Cl	Chlorine
CR	Complement receptor
Da	Daltons
DHFR	Dihrdrofolate reductase
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphates
EDTA	Ethylenediaminotetraacetic acid
Ela	Elastase
EnaC	Epithelial sodium channel
ET12	Electrophoresis type 12

ETC	Extracellular toxic complex
Fl	Fluorine
G	Guanosine
GC	Gas chromatography
Genv	Genomovar
Grep	Grepafloxacin
H	Hydrogen
hBD	Human ß-defensin
HMQ	2-(2-heptenyl)-3-methyl-4-quinolinol
I	Iodine
IPTG	Isopropylthio-ß-D-galactosidase
IS	Insertion sequence
kb	Kilobases
KDO	3-deoxyoct-2-ulosonic acid
2KGA	2-keto-D-gluconic acid
LB	Luria broth
Lipa	Lipase
LMG	Laboritorium voor Microbiologie Gent
LPS	lipopolysaccharide
MA	Minimal agar
Mb	Megabases
Mela	Melanin
MIC	Minimum inhibitory concentration
MMP	Minimal medium plus polymyxin
Mg	Magnesium
mob	Mobilisation
mRNA	Messenger RNA
MTBE	Methyl-tertary Butyl Ether
Na	Sodium
NBD	Nucleotide binding domain
NBYE	Nutrient broth plus yeast extract
NMQ	2-(2-nonenyl)-3-methyl-4-quinolonol
NO	Nitric oxide
NTCC	National collection of typable cultures
ORCC	Outwardly rectified chloride channel

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PLC	Phospholipase C
PKA	Phosphokinase A
Pol	Polymyxin B
Pro	Protease
psi	Pounds per square inch
R	Regulatory
rDNA	Ribosomal deoxyribunucleic acid
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium docecyl sulphate polyacrylamide gel electrophoresis
SE	Sodium chloride ethylenediaminotetraacetic acid
STB	Sample treatment buffer
TBE	Tris borate ethylenediaminotetraacetic
TCA	Tricarboxylic acid
TE	Tris (hydroxymethyl)-aminomethane ethylenediaminotetraacetic acid
Tet	Tetracycline
TMD	Transmembrane domain
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF	Tumour necrosis factor
TNM	Tris sodium chloride magnesium sulphate
Tob	Tobramycin
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
tra	Transfer
Tri	Trimethoprim
TSA	Tryptone soya agar
TSBA	Tryptone soya broth agar
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactosidase

Abstract

First described in 1950 as a phytopathogen, *Burkholderia cepacia* possesses inherent resistance to most classes of antibiotics and extraordinary metabolic abilities. In the last five years, polyphasic taxonomic studies have revealed considerable diversity within clinical and environmental isolates of *B. cepacia*. Presently, the *B. cepacia* complex comprises at least seven genomovars, a term that describes distinct genomic species without a recognisable phenotype. Phenotypic characteristics distinguishing several genomovars have now been identified and the genomovars renamed. Thus, the *B. cepacia* complex currently consists of *B. cepacia* genomovar I, *B multivorans* (formerly genomovar II), *B. cepacia* genomovar III, *B. stabilis* (formerly genomovar IV), *B. vietnamiensis* (formerly genomovar V), *B. cepacia* genomovar VI and *B. ambifaria* (*B. cepacia* genomovar VII). During the 1980s members of the *B. cepacia* complex emerged as life-threatening pathogens of immunocompromised individuals, particularly patients with cystic fibrosis. Ironically, at the same time, the organisms' antifungal properties led to increasing agricultural interest in the *B. cepacia* complex as biopesticides to improve crop yields. The aims of this thesis were to investigate the biological properties of both clinical and environmental isolates of the *B. cepacia* complex, with particular relevance to their pathogenicity and potential biotechnological exploitation.

B. vietnamiensis strains were more sensitive to ceftazidime and chloramphenicol than strains from the other genomovars, and environmental *B. cepacia* genomovar III strains

were more sensitive to ciprofloxacin and chloramphenicol than clinical isolates of the same genomovar. Although resistance to antibiotics is not uniform across all the subgroups of the *B. cepacia* complex, the antibiotic-sensitive strains can readily mutate to high levels of resistance. With the exception of catalase and melanin that were only produced by clinical strains, other putative virulence factors were detected in both clinical and environmental isolates. Certain factors including genetic markers for epidemic spread, were also detected in candidate biopesticide strains. The phytopathogenicity of clinical and environmental isolates was also found to be similar.

Lack of knowledge regarding the fate of the *B. cepacia* complex strains introduced to the environment is a major obstruction to the organisms' commercial exploitation. Of major concern is the possibility of genetic exchange between different *B. cepacia* complex strains and between the *B. cepacia* complex and other soil microflora. Natural transformation of *B. cepacia* complex strains was demonstrated with DNA from the well-characterised epidemic lineage ET12, represented by the Edinburgh isolate J2315. The transformed bacteria included candidate biopesticide strains. The exchange of genes was also observed through *B. cepacia* transducing phages NS1 and NS2, recently identified in this thesis. Interestingly, the host spectrum of both phages spanned environmental and clinical isolates, several sub-populations within the *B. cepacia* complex and candidate biopesticide strains. The host range of the phages also extended to *P. aeruginosa*. Some *B. cepacia* complex strains were also sensitive to well-characterised *P. aeruginosa* transducing phages. Thus, not only is there potential for *B. cepacia* DNA being exchanged between different sub-populations of the *B. cepacia*

complex but also to *P. aeruginosa.* Sensitivity of *B. cepacia* complex strains to *P. aeruginosa* transducing phages could also facilitate the importation of foreign genes.

The similarity of putative *B. cepacia* complex virulence factors produced by clinical, environmental and candidate biopesticide strains, as well as the natural exchange of genes between all subgroups suggests that caution should be exercised on the commercial application of members of the *B. cepacia* as biopesticides.

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DECLARATION

All of the experiments and procedures in this thesis were carried out by the author unless otherwise stated.

CHAPTER 1

Introduction

1.1 *Burkholderia cepacia*

Burkholderia cepacia, originally named *Pseudomonas cepacia*, was first described in 1950 as a phytopathogen causing 'sour skin', a bacterial rot which attacks the outer fleshy scales of damaged onion bulbs (Burkholder, 1950). Between 1951 and 1965, several clinical bacterial isolates designated as Eugonic oxidiser 1 (King, 1964), later renamed *Pseudomonas kingii* (Jonsson, 1970), were submitted to the National Communicable Disease Centre. In 1966, a group of bacteria isolated from soil, water and the hospital environments was described and named *Pseudomonas multivorans* (eater of everything), to emphasize the organisms' extraordinary nutritional versatility (Stanier, Palleroni, *et al*, 1966). In 1970, it was established that *P. cepacia* and *P. multivorans* were synonyms for the same group of bacteria (Ballard, Palleroni, *et al*, 1970). A few years later, *P. kingii* and *P. cepacia* were recognised as synonymous (Snell, Hill, *et al*, 1972).

1.1.1. General characteristics

B. cepacia is a Gram negative, aerobic, non-spore-forming bacillus. It is motile by means of one or more polar flagella, typically oxidase and catalase positive, may

produce various non-fluorescent pigments and poly-ß-hydroxyalkanoates can be accumulated as intracellular reserve material. It has an optimum growth temperature of 30-35°C, a maximum growth temperature of 41°C and does not grow at 4°C (Burkholder, 1950, Palleroni, 1984).

1.1.2. Taxonomy of the *B. cepacia* complex

In the early 1970s, a taxonomic study of the aerobic pseudomonads revealed that the organisms formally known as *P. cepacia, Pseudomonas marginata* and *Pseudomonas caryophylii* shared many phenotypic characteristics with *Pseudomonas pseudomallei* and *Pseudomonas mallei*. In addition, DNA-DNA hybridisation showed varying degrees of genetic homology among all the five species which was not present with the other pseudomonads tested, including *Pseudomonas aeruginosa* (Ballard, Palleroni, *et al,* 1970). This study resulted in these five species being provisionally named 'the pseudomallei group'. Much later, bacterial whole cell protein profiles showed that members of the rRNA group II pseudomonads, of which 'the pseudomallei group' is a part, were distinct from other non-fluorescent and fluorescent pseudomonads (Li and Hayward, 1994).

In 1992, a new genus *Burkholderia* was formed from the rRNA homology group II of the genus *Pseudomonas* with *B. cepacia* as the type species, and NCTC 10743 (ATCC

24516) as the type strain (Yabuuchi, Kosako, *et al*, 1992). The new genus contained seven species *Burkholderia cepacia*, *Burkholderia mallei, Burkholderia pseudomallei, Burkholderia caryophylli, Burkholderia gladioli, Burkholderia pickettii* and *Burkholderia solanacearum.*

Within collections of isolates presumptively identified as *B. cepacia*, five major subgroups were later identified (Vandamme, Holmes, *et al*, 1997) and named genomovars I-V; the term denotes a group of organisms which are genotypically distinct but cannot be differentiated phenotypically (Ursing, Rossello-Mora, *et al* 1995). A group of nitrogen-fixing bacteria isolated from the rhizosphere of rice cultivated in Vietnam was shown to constitute a single cluster together with two strains of clinical origin (Gillis, Van, et al, 1995). The name *Burkholderia vietnamiensis* was proposed for this, the fifth genomovar of *B. cepacia.* Genomovar II was later proposed to constitute a new species *Burkholderia multivorans* (Vandamme, Holmes, *et al*, 1997) and genomovar IV was re-named *Burkholderia stabilis* (Vandamme, Mahenthiralingam, *et al*, 2000). More recent studies have identifed the additive genomovars VI and VII (Dr P. Vandamme, personal communication). At present, all seven genomovars constitute the *B. cepacia* complex. For the purpose of this thesis the terms *B. cepacia* and *B. cepacia* complex are synonymous. *B. cepacia* genomovars II, IV and V will be referred to as *B. multivorans, B. stabilis* and *B. vietnamiensis* respectively.

While the taxonomy of *B. cepacia* is an interesting and challenging subject in its own respect, it was the life-threatening infections caused by *B. cepacia*, which primarily drew attention to this group of organisms.

1.1.3. Early cases of *B. cepacia* infections

The first report of *B. cepacia* infection was in 1966 when a *Pseudomonas* sp. was identified as the cause of several cases of post-operative urinary tract infection in a UK hospital (Mitchell and Hayward, 1966). The organism was reported as being unusual in utilising an exceptionally wide range of carbohydrates. When epidemiological investigations were conducted, the source of infection was traced to the disinfectant, hibitane, which was used to disinfect the water tank which was then filled with the sterile water used for bladder irrigation (Mitchell and Hayward, 1966).

This initial report was followed by sporadic reports of nosocomial *B. cepacia* infections. *B. cepacia* was isolated from wounds (Bassett, Stokes, *et al*, 1970), the urinary and respiratory tracts, blood (Ederer and Matsen, 1972), and the heart (Noriega, Rubinstein, *et al*, 1975). In all these reports, the infection was traced to seemingly unlikely sources such as contaminated antiseptics and disinfectants (Bassett, Stokes, *et al*, 1970), benzylchloride solution in commercially packaged urinary catheter kits (Ederer and Matsen, 1972) and distilled water, (Gelbart, Reinhardt, *et al*, 1976). A slightly different cause of infection reported during this period was contamination of human serum albumin during manufacture, which later led to bacteraemia (Steere, Tenney, *et al*, 1977).

In summary, during the 60s and 70s infections caused by *B. cepacia* were sporadic and could be generally traced to the use of contaminated solutions. It was only in the 1980s that *B. cepacia* emerged as a major cause for concern in individuals with cystic fibrosis.

1.2. Cystic fibrosis

Originally described in 1938 as cystic fibrosis of the pancreas, cystic fibrosis (CF) has subsequently emerged to be the most common, potentially lethal, autosomal recessive disease in Caucasians. In most human populations, CF affects between 1in 2 000 and 1 in 4 500 individuals (Koch and Høiby, 1993). The incidence is much higher in some populations such as the Alberta Hutterites where it is 1 in 313 (Fujiwara, Morgan, *et al*, 1989), the South West African Afrikaner population where it is 1in 622 (Super, 1975) and 1in 895 in the French-Canadian population of the Saguanay-Lac St. Jean region (Rozen, Schwartz, *et al,* 1990). The unusually high incidences of CF in these populations are thought to be due to a founder effect in which the closely knit communities grew from a small number of ancestors, some of whom were carriers, followed by subsequent isolation of the population.

1.2.1. Clinical features of CF

The clinical features of CF are dominated by involvement of the respiratory tract and in approximately 50% of CF patients, the diagnosis is first considered because of pulmonary symptoms (Fitzsimmons, 1993). The symptoms include airway obstruction by thick, sticky mucus, chronic cough and sputum production, persistent chest radiograph abnormalities and persistent colonisation and/or infection with a narrow spectrum of typical CF pathogens (Collins, 1992; Rosenstein and Cutting, 1998).

Involvement of the gastrointestinal tract is also a common occurrence in most CF patients. Approximately 18% of newborns with CF have a form of intestinal obstruction called meconium ileus (Fitzsimmons, 1993). In addition, approximately 85% of CF patients, show pancreatic insufficiency as a result of obstruction of the pancreatic ducts and subsequent scarring, leading to destruction of the organ's exocrine function (Collins, 1992; Fitzsimmons, 1993). Without the intake of dietary supplements, a large proportion of ingested food would be lost in the stools with the loss of fat being especially striking (Andersen, 1938).

The discovery that there is a high concentration of Na^+, K^+ and Cl^- ions in the sweat of CF patients (DiSant'Agnese, Darling, *et al*, 1953) led to the development of a sweat test (Gibson and Cooke, 1959). 'The sweat test' remained the standard of confirmation or exclusion of the diagnosis of CF before the advent of genetic testing. This increased amount of salts in the sweat is not decreased by a low-salt diet (DiSant'Agnese, Darling, *et al*, 1953).

Reproductive fitness in females with CF is reduced and infertility is almost universal in males. Infertility of adult males with CF is usually due to absence of the *vas deferans* (Kaplan, Shwachman, *et al*, 1968) with most patients having aspermia, although

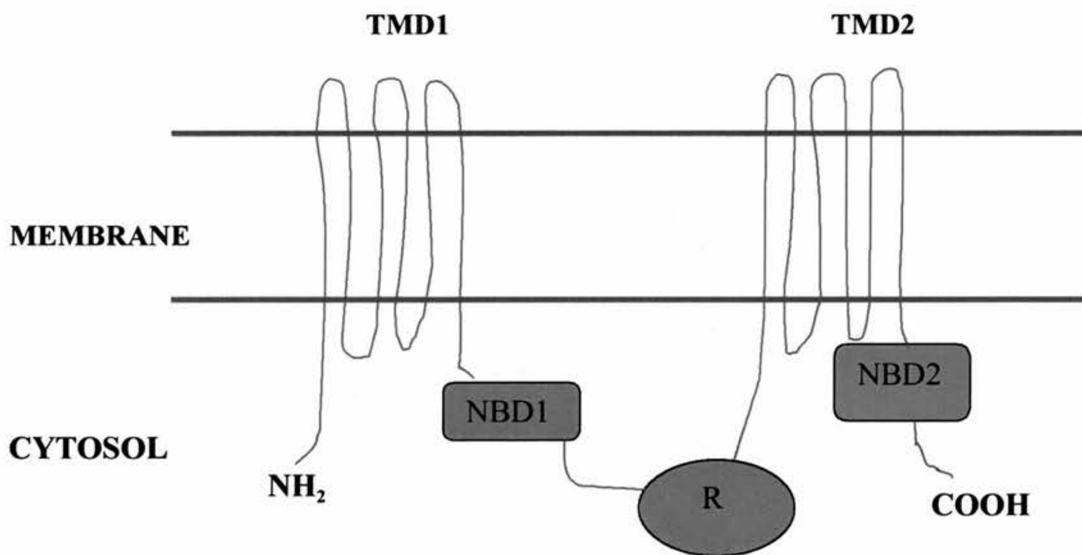
spermatogenesis has been shown to occur. Other factors contributing to infertility include a small semen volume which is acidic, testes that are diminished in size and a reduced epididymus (Kaplan, Shwachman, *et al*, 1968). In contrast, women with CF frequently give birth to healthy children although their fertility is reduced compared to healthy females.

1.2.2. The CF gene and its product

A major advance in understanding CF occurred in 1989 when the CF gene was identified and cloned through the use of chromosome walking, jumping and complementary DNA hybridisation (Kerem, Rommens, *et al*, 1989, Rommens, Ianuzzi, *et al*, 1989, Riordan, Rommens, *et al*, 1989). The 250kb of genomic DNA was found to encode a protein of 480 amino acids which was named the cystic fibrosis transmembrane conductance regulator (CFTR).

On the basis of its predicted amino acid sequence, a potential structure for CFTR was proposed (Riordan, Rommens, *et al*, 1989). The predictions were mainly based on the striking homology of the CFTR primary sequence to a family of proteins referred to as the traffic adenosine triphosphatases (ATPases), or the ABC (ATP-binding cassette) family (Hyde, Emsley, *et al*; 1990). This family now includes over thirty proteins, some of which are not associated with transport, sharing approximately 30% sequence

identification over a cassette of about 200 amino acids (Hyde, Emsley, *et al.* 1990). Structurally, this family of proteins has one or two hydrophobic transmembrane domains (TMDs), each usually involving six loops that span the membrane and one or two nucleotide-binding domains (NBDs) that bind and cleave ATP (Figure 1.1) (Hyde, Emsley, et al. 1990, Collins, 1992). In additions to the two TMDs and two NBDs, CFTR contains a unique site called the R (regulatory) domain (Riordan, Rommens, *et al*, 1989). The R domain is rich in serine residues that were predicted to be phosphokinase A (PKA)-mediated phosphorylation sites (Riordan, Rommens, *et al*, 1989).



TMD, transmembrane domain; NBD, nucleotide-binding domain; R, regulatory domain

Fig 1.1. Schematic representation of the structure of CFTR (Welsh, Anderson, *et al*, 1992)

1.2.3. Expression of CFTR

Immunocytochemistry and *in situ* hybridisation studies revealed that CFTR is abundant in the epithelial cells lining the pancreas, sweat ducts, salivary glands, lungs, intestinal crypts, vas deferans and uterus (Crawford, Maloney, *et al*, 1991, Trezise and Buchwald, 1991). Surprisingly, the levels of CFTR expression are relatively low in the epithelium lining the respiratory tract (Crawford, Maloney, *et al*, 1991, Trezise and Buchwald, 1991), whereas kidney tubules (Crawford, Maloney, *et al*, 1991), and the serus tubules of sub-mucosal glands show high expression of localised CFTR (Engelhardt and Wilson, 1992). Expression is restricted to apical rather than basolateral regions of epithelial cells and at least a portion of CFTR is associated with the plasma membrane (Crawford, Maloney, *et al*, 1991).

1.2.4. Mutations associated with CF

The first of the CF mutations to be described was a deletion of three adjacent base pairs leading to absence of the amino acid phenylalanine at position 508 in the first NBD (Riordan, Rommens, *et al*, 1989). This mutation, ΔF508, accounts for approximately 70% of all CF mutations but its relative frequency varies between different ethnic or geographic groups. ΔF508 is closely associated with pancreatic insufficiency, while patients with one or two other mutations often have normal pancreatic function (Kristidis, Bozon, *et al*, 1992).

Since a single mutation is responsible for about 70% of mutant CF chromosomes, it would be expected that the total number of mutations would be very small. However, since 1989, more than 600 mutations have been identified in the CFTR gene although only five have a world frequency of higher than 1% (Estivill, Bancells, *et al*, 1997). These are ΔF508 (66%), G542X (2.6%), N1303K (1.6%), G551D (1.5%), and W1282X (1.0%) (Estivill, Bancells, *et al*, 1997). The three most common CF mutations ΔF508, G542X and N1303K are present in most countries and regions of Europe (Estivill, Bancells, *et al*, 1997), suggesting an ancient origin and spread of these mutations. The different mutations affect the transport of several ions including Cl^- and Na^+ across the epithelium.

Four general mechanisms have been proposed to explain how the different CFTR mutations affect chloride transport across the epithelium and these are reviewed by Welsh and Smith, 1993.

- Class 1 mutations affect protein production by encoding truncated or aberrant forms of protein. They constitute nonsense, frameshift and splice mutations such as G542X and R553X and are associated with pancreatic insufficiency.

- Class 2 mutations, the most common of all CFTR mutations, are processing mutations and produce protein that is incompletely glycosylated and retained within the endoplasmic reticulum. They include ΔF508 and are associated with severe pancreatic insufficiency.

- Class 3 mutations encode proteins that are defective in channel regulation. The defect may be mild as in G551S in which case the patients would be pancreatic sufficient or it can result in complete loss of regulation as in G551D (Cutting, Kasch, *et al*, 1990) resulting in pancreatic insufficiency.

- Class 4 mutations such as R117H affect the conduction properties of the channel and usually occur in the trans-membrane domains (TMDs) (Welsh and Smith1993, Ma and Davies, 1998).

1.2.5. Functions of CFTR

An indication of the function of CFTR came firstly from observing which tissues were affected in CF and secondly from its localisation to the apical region of epithelia (Crawford, Maloney, *et al*, 1991). It was postulated that CF involved an alteration of the fluid in the epithelial tissue and this was supported by the observation that the sweat from CF patients has an abnormal concentration of Cl^- (DiSant'Agnese, Darling, *et al*, 1953).

Although expression of normal CFTR in cultured CF airway epithelial cells was shown to correct the Cl^- channel defect (Rich, Anderson, *et al*, 1990), it was not initially clear whether CFTR was a Cl^- channel or a regulator of a channel. On the basis of understanding of ABC transporters, it was initially believed that CFTR could not itself be a chloride channel (Crawford, Maloney, *et al*, 1991), for reasons that included the fact that ABC transporters accumulate substrates against concentration gradients, requiring ATP hydrolysis but chloride channels do not

Evidence that CFTR is a chloride channel was provided by an experiment in mice in which the cAMP-activated Cl^- secretory response was found to be absent from CFTR (-/-) mice as compared to littermate controls (Clarke, Grubb, *et al*, 1992). These findings not only indicated that CFTR functions as a cAMP-regulated Cl^- conductance pathway in murine epithelia but also that mice do not express an alternative cAMP-mediated Cl^- conductance that might protect them from the effects of CFTR gene disruption. Further evidence came from an experiment showing that when a basic amino acid, lysine 95 or 335, in the TMD was altered to an acidic one, the anion selectivity of cAMP-regulated channels containing either endogenous or recombinant CFTR was altered from Br>Cl>I>Fl to I>Br>Cl>Fl (Anderson, Gregory *et al*, 1991). This showed that CFTR is a chloride channel rather than a channel regulator and that either lysine 95 or 335 determine anion selectivity. However, the data did not exclude the possibility that CFTR could have other functions. Shortly afterwards Bear and co-workers, purified

and reconstituted CFTR from a cell line. When it was incorporated, the purified CFTR exhibited regulated Cl^- channel activity, providing conclusive evidence that the protein itself is the channel (Bear, Li, *et al*, 1992).

Having established that CFTR is indeed a chloride channel, the next challenge was to understand how it functions. In proposing the structure of CFTR, Riordan and co-workers, had noted that the R domain is rich in serine residues which they predicted to be sites of phosphokinase A (PKA)-mediated phosphorylation (Riordan, Rommens, *et al*, 1989). Two years later, it was shown that four serine residues 660,737,795 and 813 on the R domain are phosphorylated by PKA (Cheng, Rich, *et al*, 1991) and mutagenesis of any one of these sites does not affect Cl^- channel activity. In fact, phosphorylation at serine 660 alone is sufficient for the regulation of Cl^- channel activity. The fact that CFTR is poly phosphorylated on the R domain by PKA led to the suggestion that phosphorylation could open the channel by providing an electrostatic force to repel the R domain away from the membrane and prevent it from otherwise plugging the pore (Welsh, Anderson, et al. 1992). While this hypothesis provided a role for the R domain in CFTR, it did not provide an explanation for the role of NBDs.

The role for NBDs was provided by a group of workers who demonstrated that nucleoside triphosphates were required to open the CFTR Cl^- channel (Anderson, Berger

et al, 1991). Using two different cell types, this group found that once phosphorylated by cAMP-dependent PKA, the channels required cytosolic ATP to open. Phosphorylated channels were reversibly activated by several hydrolysable nucleotides but not with non-hydrolysable analogues or Mg^{2+} free ATP. Their studies on CFTR mutants indicated that ATP controls channel activity independent of the R domain and suggested that hydrolysis of ATP at NBD1 may be sufficient for channel opening. They concluded that nucleoside triphosphates regulate the CFTR Cl^- channel and that this regulation most likely occurs through the NBDs and may require hydrolysis since only hydrolysable nucleotides activate the channel Fl (Anderson, Gregory *et al*, 1991). The discovery that ATP opens CFTR indicated that there may be at least two mechanisms to regulate that channel, the first of which involves PKA-dependent phosphorylation of the R domain (Cheng, Rich, *et al*, 1991) and the second involving interaction of nucleotides with the NBDs Fl (Anderson, Gregory *et al*, 1991). In wild type CFTR, both processes are required to open the channel and neither one alone is sufficient.

In summary, CFTR is a Cl^- channel located in the apical cell membrane. The channel is activated by PKA-mediated phosphorylation of the serine residues on the R domain and ATP hydrolysis in the NBDs. Phosphorylation on the R domain causes channel opening by increasing negative charges which repel the R domain away from the membrane (Cheng, Rich, *et al*, 1991; Wilkinson, Strong, *et al*, 1997). Parts of the TMDs constitute the pore and the R domain functions as a channel inhibitor until it is phosphorylated by

PKA. The closed state of the molecule can be secured by dephosphorylation of the R domain (Ma and Davies, 1998).

CFTR has other functions in addition to being a chloride channel. It may also partly regulate acidification of intracellular organelles by pumping H^+ ions (Barasch, Kiss, et al, 1991) and partly control cell endocytosis and exocytosis (Moss, 1995). It also controls the efflux of ATP and concomitant regulation of the outwardly rectified chloride channel (ORCC) and cAMP-dependent negative regulator of the epithelial Na^+ channel (EnaC) (Mickle and Cutting, 1998).

Correction of the phenotypic defect by expression of CFTR in cultured CF epithelial cells (Rich, Anderson, *et al*, 1990), not only demonstrated a causal relationship between mutations in the CFTR gene and defective Cl^- transport but also suggested the feasibility of a therapeutic approach based on correcting the underlying defect. There are now major human trials in progress using a variety of viral and non-viral vectors to test the safety and efficiency of CF gene therapy (Rosenfeld, Yoshimura *et al*, 1992, Caplen, Alton, *et al*, 1995).

1.2.6. Effects of CFTR dysfunction on the respiratory system

The normal mucociliary clearance system that cleanses the bronchopulmonary epithelium of inhaled particles depends on the upward directional flow of a mucus layer positioned at the tips of cilia that move freely in an underlying watery layer (the airway surface fluid, ASF) (Mortesen, Hansen, *et al*, 1993). In conditions such as CF, the mucociliary clearance system fails to function efficiently.

Two models that attempt to explain the failure of CF airways to clear potential pathogens by mucociliary clearance now been described. The first, proposed in 1988 (Boucher, Cotton, *et al*, 1988), is called the isotonic model of CFTR dysfunction.

Isotonic model

This model assumes that in people without CF the airway epithelium is a volume buffer that controls the height of the ASF. If fluid is lost from the ASF by evaporation or coughing, water flows across the epithelium into the ASF to restore the ASF volume. The height of the ASF is critical for the normal mucociliary clearance of the bronchopulmonary system. If the level is too high, then the tips of cilia fail to reach the bottom of the mucus layer and are unable to move it. If on the other hand, the height is too low then the cilia become enmeshed in the mucus and fail to function. The volume of the ASF is controlled by the concentration of ions and by osmosis.

The respiratory epithelium of CF patients has a high potential difference due to increased Na^+ absorption in addition to lower Cl^- permeability (Boucher, Stutts, *et al*, 1986; Clarke, Grubb, *et al*, 1992). It is thought that in CF, the major apical Na^+ channel is open for an abnormally large fraction of time, moving Na^+ ions back into the epithelial cells to compensate for the inefficient or absent CFTR which can not pump chloride out of the epithelial cells into the ASF. Excessive absorption of Na^+ and Cl^- results in depletion of water on airway surfaces since the osmotic potential of the ASF is not high enough to attract water by osmosis. The lowered volume of the ASF means the cilia get tangled in mucus leading to diminished immunity in the CF lung. Thus, according to this model, the airway epithelium is a volume buffer and controls the height of the ASF, which is critical for normal functioning of cilia.

Hypotonic model

The second model of CFTR dysfunction is called the hypotonic model and was proposed by workers at the University of Iowa College of Medicine (Smith, Travis, *et al*, 1996). This model suggests that the ASF in non-CF airways has a low, hypotonic salt concentration and that a low NaCl concentration allows defensin-mediated antimicrobial action to protect the airways. This model is supported by Goldman and co-workers who demonstrated that a novel antimicrobial peptide called human β-defensin 1(hßD1), which is expressed in human epithelial cells, confers salt-dependent antimicrobial

activity in ASF (Goldman, Anderson, *et al*, 1997) which is reduced or eliminated in high salt concentrations.

A hypotonic salt gradient is formed by pumping sodium ions from the ASF into the epithelium, leading to the passive flow of chloride ions back into the epithelium through CFTR. In people with CF, this passive flow of chloride ions through CFTR can not occur, the hypotonic gradient can not be formed, thus leading to elevated salt levels in the ASF which inactivates the antimicrobial defensins in the ASF and lowers the immunity of the CF lung. Thus, the hypotonic model proposes that non-CF airways have a hypotonic salt concentration which allows naturally occurring antimicrobial peptides to contribute to the defense of the host against bacterial pathogens.

Despite these plausible models for the role of CFTR dysfunction on the respiratory system, CF lung disease is also greatly influenced by other factors, in particular, the influence of life-threatening pulmonary infections caused by a surprisingly small spectrum of bacterial pathogens (Mickle and Cutting, 1998). Pulmonary function can be extremely variable even in individuals with the same CFTR genotype. Although studies on monozygotic and dizygotic twins indicate that there are genetic factors that influence the severity of pulmonary disease (Santis, Osborne, *et al*, 1992), the influence of bacterial pathogens probably exerts the greatest influence.

1.2.7. Microbiology of CF

Most morbidity and mortality in CF is associated with progressive pulmonary deterioration caused by recurrent exacerbation of chronic endobronchial bacterial infection. Surprisingly, the bacteriology of the infected CF airways shows a unique specificity of colonising pathogens with a predictable progression of colonisation. In younger patients, infections with *Staphylococcus aureus* and *Haemophilus influenzae* predominate while chronic *P. aeruginosa* infection is typically delayed until early adolescence. Evidence suggests that the mechanisms leading to bacterial attachment to upper airways, and early colonisation, depend on adhesins on the surface of bacteria (Ramphal, 1990).

Recent research has provided an explanation for the persistence of CF pathogens in the respiratory tract. The common CF pathogens *P. aeruginosa* and *S. aureus* are killed when added to the apical surface of normal airway epithelia but multiply on CF epithelia (Smith, Travis, *et al*, 1996). Although the bactericidal activity is present in the ASF of both normal and CF epithelia, bacterial killing requires a low NaCl concentration and since CF ASF is suggested to have a high NaCl concentration, CF epithelia fail to kill bacteria. The ASF from normal cells also kills other organisms and hence has broad-spectrum bactericidal activity.

This bactericidal activity secreted into the ASF is dependent on a low salt concentration and has been identified as the antimicrobial peptide human β-defensin 1 (hBD-1) (Goldman, Anderson, *et al*, 1997). The activity of defensins in the ASF forms the basis of the hypotonic model of CF dysfunction discussed previously.

1.2.7.1. *Staphylococcus aureus*

S. aureus was the first organism to be recognised as a cause of chronic lung infections in young CF patients and in the 1930s staphylococcal infection was considered to be the major cause of mortality (Anderson,1938). *S. aureus* is associated with both chronic and sporadic infections in CF and patient-to-patient spread can occur (Goering, Bauernfeind, *et al*, 1990).

The exact mechanisms through which *S. aureus* causes infection in the CF lung are unclear. The organism produces various products some of which, for example, the enterotoxins, have been identified as causing disease. However, the majority of products, which include coagulase, catalase and haemolysins, have no clear role in human infections (Cohen, 1986). Products such as teichoic acid and exopolysaccharide may play a role in adherence to the respiratory epithelium in CF patients (Aly and Levit, 1987).

In the CF lung, chronic airway infection is typically exacerbated and characterised by an inflammatory hyper-secretion of 'sticky' tracheobronchial mucus; in non-CF individuals 'normal' mucus acts as a barrier and removal system for bacterial pathogens. In chronic bacterial lung infection however, inflammation and excess mucus may contribute to the inefficiency of mucociliary clearance and be responsible for the persistence of bacterial infection. *S. aureus* possesses surface proteins that bind to human mucins, the major constituents of mucus (Trivier, Houdret, *et al*, 1997). It is believed that this ability to bind human mucins could explain the early colonisation of the CF respiratory tract by *S. aureus*.

1.2.7.2. *Haemophilus influenzae*

The role of *H. influenzae* in CF lung disease is currently unresolved and controversial. This is because although non-capsulated strains of *H. influenzae* colonise the lower respiratory tracts of patients with CF, they may also be found in the lower respiratory tracts of non-CF children (Høiby, 1976). In children under 12 years of age with CF, *H. influenzae* may be the most frequently isolated organism from patient sputum as a pure culture (Rayner, Hiller, *et al*, 1990). However, increased isolation of *H. influenzae* often precedes the development of acute exacerbations in CF patients and clinical improvement coincides with a reduction in the isolation rate after antimicrobial treatment (Rayner, Hiller, *et al*, 1990). In addition, studies on the use of C-reactive

protein as an indicator of pulmonary inflammation provide evidence that the highest levels of C-reactive protein coincide with acute exacerbation and culture of *H. influenzae* (Glass, Hayward, *et al*, 1988). Generally, the organism is eradicated by treatment with amoxycillin or fluoroquinolones such as ciprofloxacin (Pressler, Szaff, *et al*, 1984; Rayner, Hiller, *et al*, 1990), and thus does not present the same problems of chronic infection experienced with *S. aureus* and *P. aeruginosa*.

1.2.7.3. *Pseudomonas aeruginosa*

Infection with *P. aeruginosa* exceeds that of all other pathogens with up to 90% of adult CF patients eventually becoming infected. Initially, the *P. aeruginosa* infecting the lower respiratory tract exhibits a non-mucoid colonial phenotype; chronic infection coincides with the production of bacterial alginate and the notorious mucoid phenotype (Koch and Høiby, 1993). A major reason for the persistence of *P. aeruginosa* in CF airways is growth in micro-colonies embedded in an alginate matrix or biofilm. As the infection becomes established, the host produces specific but ineffective anti-pseudomonal antibodies leading to bronchopulmonary inflammation and obstruction. This immune-mediated inflammation leads to further tissue damage, loss of lung tissue and eventually pulmonary failure (Koch and Høiby, 1993). Inflammatory cells, mainly neutrophil granulocytes, accumulate and degenerate releasing tissue-damaging elastase

and DNA, which also increases the viscosity of the secretions thus making them more difficult for removal by cilia.

A possible explanation for the presence of *P. aeruginosa* in CF airways derives from CFTR's role as a pH regulator within the Golgi complex. Mutations in CFTR result in decreased acidification of the trans-Golgi complex (Barasch and Al-Awqati, 1993). Sialylation of proteins requires an acidic pH and dysfunction or absence of CFTR leads to undersialylation. Apical surfaces of CF epithelial cells have undersialylated proteins and a higher concentration of asialoganglioside 1 (aGM_1) than membranes with wild type CFTR (Imundo, Barasch, *et al*, 1995).

It has been suggested that the tetrasaccharide moiety of aGM_1 is a receptor for *S. aureus* and the *P. aeruginosa* pilin (Saiman and Prince, 1993, Imundo, Barasch, *et al*, 1995). Thus increased aGM_1 in the apical membrane of CF epithelia makes it a likely contributor to the pathogenesis of bacterial infections (Imundo, Barasch, *et al*, 1995).

1.2.7.4. Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) caused by *Aspergillus fumigatus* was first described in patients with CF in 1965 (Mearns, Young, *et al*, 1965). In many

patients, the proliferation of *Aspergillus* in the lung does not appear to cause any symptoms but in some individuals with CF, it may lead to significant lung disease in which tissue damage due to the immune reaction of the host occurs (Hiller, 1990). Although most patients make good recoveries either spontaneously (Simmonds, Littlewood, *et al*, 1990) or with steroid treatment (Maguire, Moriarty, *et al*, 1988), recurrences are quite common (Brueton, Ormerod, *et al*, 1980; Maguire, Moriarty, *et al*, 1988).

1.2.7.5. Emerging CF pathogens

The microbiology of CF pulmonary infection has changed with the increased life expectancy of patients. Of note is the increased recovery of antibiotic-resistant organisms such as *Stenotrophomonas maltophilia* and *Alcaligenes xylosoxidans* (Burns, Emerson, *et al*, 1998; Peltroche-Llacsahuanga and Hasse, 1998). At present, the pathogenic role of these opportunistic pathogens remains unclear. Other emergent potential pathogens include *Ralstonia pickettii* and *Bordetella hinzii* (usually associated with poultry) which has been reported as causing pulmonary exacerbation in a CF patient over an extended period (Funke, Hess, *et al*, 1996). Although the role of these organisms in CF lung disease is currently controversial, they possess sufficient potential to emerge as the next new threat to CF patients. LPS from these organisms upregulates

the expression of proinflammatory cytokines to a level comparable with *B. cepacia* (Hutchison, Bonell, *et al*, 2000).

1.3. *Burkholderia cepacia* in cystic fibrosis

In the 1980s, several CF centres reported an increase in the numbers of patients infected with *B. cepacia*, with a few centres reporting an incidence as high as 20% (Isles, Maclusky, *et al*, 1984; Thomassen, Demko, *et al*, 1985). Most isolates were found to be resistant to the commonly used antibiotics with the exception of chloramphenicol and trimethoprim-sulphamethoxazole (Isles, Maclusky, *et al*, 1984). In addition, *B. cepacia* often persisted in the CF lung despite the use of antibiotics previously shown to have *in vitro* activity (Gold, 1983). *B. cepacia* infection was thought to be associated with hospitalisation since one study found that 56% of patients developed chronic infection either during or immediately after a hospitalisation (Thomassen, Demko, *et al*, 1985).

The clinical outcomes of *B. cepacia* infection were found to be different from symptoms previously experienced with other CF pathogens. Three distinct clinical patterns were identified among CF patients infected with *B. cepacia*; chronic asymptomatic carriage, progressive deterioration over many months, and a rapid, usually fatal deterioration in previously mildly affected patients (Isles, Maclusky, *et al*, 1984; Thomassen, Demko, *et al*, 1985). Patients colonised with *B. cepacia* showed significantly greater impairment of pulmonary function than those colonised with *P. aeruginosa* or with neither organism. Several patients with moderate pre-existing lung disease developed a fulminant *B. cepacia* pneumonia which was sometimes accompanied by septicaemia and died

rapidly (Isles, Maclusky, *et al*, 1984; Thomassen, Demko, *et al*, 1985). This rapid deterioration became known as the 'cepacia syndrome'. Some patients who asymptomatically harboured *B. cepacia* for several years ultimately experienced a rapid decline characterised by fever and frequent hospitalisation prior to death (Thomassen, Demko, *et al*, 1985). Females in good clinical condition were found to be more likely than males to experience a decline in clinical condition, thus *B. cepacia* worsened the already poor prognosis for females with CF (Thomassen, Demko, *et al*, 1985).

An early epidemiologic investigation showed that the risk of colonisation of CF patients with *B. cepacia* increased with; increased severity of underlying CF, increased age, having a sibling with CF who was colonised with *B. cepacia* and previous hospitalisation (Tablan, Martone, *et al*, 1987); however, the source and mode of transmission remained unclear. Since *B. cepacia* infection was thought to be associated with hospitalisation, some CF centres introduced infection control measures in order to abate the spread of infection. These measures included the physical separation of hospitalised patients colonised with *B. cepacia* from non-colonised patients, education of staff and families of patients with CF regarding precautionary measures and holding separate summer camps and facilities for the colonised and non-colonised individuals (Thomassen, Demko, *et al*, 1985; Smith, Smith, *et al*, 1992) or barrier nursing for all CF patients irrespective of microbiological findings (Millar- Jones, Paull, *et al*, 1992). These infection control practices were considered draconian and unnecessary by some

patients and CF centres but unarguably led to a general decline in the numbers of newly infected patients (Thomassen, Demko, *et al*, 1985; Whiteford, Wilkinson, *et al*, 1995). The ensuing decline in the numbers of newly infected individuals suggested that patient-to-patient transmission, either directly or indirectly, was an important mode of transmission. This speculation was confirmed by later studies (LiPuma, Dasen, *et al*, 1990; Cazzola, Amalfitano, *et al*, 1996). Infection control measures did not, however, totally prevent acquisition of *B. cepacia* as some centres still had new cases which were thought to be caused by environmental acquisition (Smith, Smith, *et al*, 1992).

Although it had not yet been unequivocally established whether or not *B. cepacia* caused pulmonary damage or merely acted as a clinical marker of progressive lung damage, studies continued to demonstrate its transmissibility among CF patients. In addition to showing that 20% of non-colonised patients acquired *B. cepacia* from a chronically colonised individual after four weeks of exposure, LiPuma and co workers reported that *B. cepacia* colonisation could be inapparent for several months after acquisition of the organism (LiPuma, Marksaustin, *et al*, 1994). This was supported by another group that showed as much as a four-fold increase in antibody response to *B. cepacia* before the first culture of the organism from sputum (Govan, Brown, *et al*, 1993). However, neither group could ascertain whether or not such inapparently colonised individuals posed a threat to non-colonised patients.

A study showing the acquisition of *B. cepacia* at summer camps was carried out in 1994 and showed that the prevalence of sputum conversion increased with the number of infected campers and the duration of the camp (Pegues, Carson, *et al*, 1994). Transmission of *B. cepacia* through social contact outwith the CF clinic and summer camps was demonstrated in the UK by the transmission of a single strain within and between several regional CF centres (Govan, Brown, *et al*, 1993); the precise nature of social contacts facilitating transmission was not determined but attendance at a weekly exercise class held in a school gymnasium was strongly suspected.

The observation of *B. cepacia* transmission during or immediately after hospitalisation was not reported by all centres. Some studies reported no evidence of *B. cepacia* transmission in the hospital setting although colonised and non-colonised patients interacted freely (Hardy, McGowan, *et al*, 1986; Simmonds, Conway, *et al*, 1990), and the sputum cultures of the non-colonised group remained free of *B. cepacia* at three and six months after discharge (Hardy, McGowan, *et al*, 1986). These varied results led to considerable controversy on the need and value of separating *B. cepacia* colonised from non-colonised patients. The differences in findings were explained when data suggested that not all isolates of *B. cepacia* were equally transmissible between CF patients. A highly transmissible clonal lineage (the gymnasium isolate referred to previously)

expressing giant cable pili was identified as being responsible for epidemics in the North American and British CF centres (Govan, 1995; Sun, Jiang, *et al*, 1995). At present, there is consensus that not only does *B. cepacia* infection occasionally cause a rapid decline in clinical condition but some strains appear to be particularly adept at transmitting from person-to-person. Evidence from national CF databases also emphasize the pathogenic role of *B. cepacia*; life expectancy has been measured as more than 50 years for CF patients free of *P. aeruginosa* and *B. cepacia*, reducing to 29 years for *P. aeruginosa* and to 21 years for *B. cepacia*-positive patients (LiPuma, 1998).

The emergence of *B. cepacia* as a CF pathogen, with the accompanying controversy on its epidemiology, prevalence and pathogenic role, is closely mirrored by the current situation regarding 'new' organisms such as *Stenotrophomonas maltophilia*. Whether or not these organisms will be shown to cause future major problems in CF lung disease remains to be seen.

It has been reported that *Burkholderia gladioli*, another organism which, like *B. cepacia*, is primarily a plant pathogen, was isolated from the respiratory tracts of CF patients attending different centres in North America (Christenson, Welch, *et al*, 1989), although no deleterious effects on the condition of the patients were observed. A more intriguing report was the revelation that multi-resistant isolates responsible for high mortality,

morbidity and transmission in UK CF patients which had been previously identified as *B. cepacia* actually possessed characteristics of both *B. cepacia* and *B. gladioli* (Simpson, Finlay, *et al*, 1994). Later studies revealed that some of these strains belonged to *B. cepacia* genomovar III and included the highly transmissible ET12 lineage (Vandamme, Hlomes, *et al*, 1997). Another report of a *B. galdioli* isolate causing a cross-infection problem, rapid decline in respiratory status and fulminant bacteremia in CF patients (Wilsher, Kolbe, *et al*, 1997), was also later shown to have been a misidentification of a *B. cepacia* genomovar III isolate (Clode, Matherell, *et al,* 1999). This problem of misidentification casts some doubt on the role of *B. gladioli* in CF infections. However, there are still numerous reports of *B. gladioli* infections in different patient groups including patients with CF (Khan, Gordon, *et al*, 1996; Graves, Robin, *et al*, 1997; Jones, Stanbridge, *et al*, 2001).

In contrast to the debatable contribution of *B. gladioli*, the contribution of *B. cepacia* to CF lung disease has now been unequivocally established. What remains to be elucidated are the organism's virulence factors and the bacteria/host interactions responsible for pulmonary damage.

1.4. Pathogenicity and Virulence of *B. cepacia*

In contrast to the clearly defined virulence factors of *P. aeruginosa*, the virulence determinants of *B. cepacia* are yet to be established. However, several cellular and extracellular components have been identified as putative virulence factors.

1.4.1. Protease

The reported frequency of protease production among isolates of the *B. cepacia* complex is not consistent. Some workers report that most strains can produce extracellular proteases (McKevitt and Woods, 1984; Nakazawa, Yamada, *et al*, 1987; Gessner and Mortensen, 1990; Gilligan, 1991), whereas other groups have indicated that rhizosphere isolates are deficient in proteases (Bevivino, Tabacchioni, *et al*, 1994). Differences between the proteases of *B. cepacia* and *P. aeruginosa* have also been reported with the former being unable to degrade elastin (McKevitt and Woods, 1984).

The *B. cepacia* protease PSCP, is a zinc metalloprotease of 34kDa, with an optimum pH of 6.0 and temperature of 45°C (McKevitt, Bajaksouzian, *et al*, 1989). Sera from mice immunised with the protease show a rise in antibody to the enzyme (McKevitt, Bajaksouzian, *et al*; 1989, Kooi, 1994), and heat inactivation increases the

immunogenicity of the protease. PSCP produces bronchopneumonia when instilled intratracheally into rat lungs and production of anti-PSCP antibodies does not protect against lung damage produced by the same strain of *B. cepacia* from which the enzyme was derived.

Interestingly, antibodies to *B. cepacia* PSCP cross-react with a *B. pseudomallei* extracellular protease and the *V. cholerae* HA1 protease, as well as the elastase and alkaline phosphatase from. *P. aeruginosa* (McKevitt, Bajaksouzian, *et al*, 1989; Kooi, 1994).

1.4.2. Lipase

Extracellular lipases probably play a physiological role in bacteria by hydrolyzing exogenous triglycerides to produce free fatty acids which are used for energy generation (Lonon, Woods, *et al*, 1988). In addition, lipases, particularly phospholipases, are known to play an important role in the virulence of some bacterial pathogens. Examples in Gram-positive bacteria include the lipase produced by *S. aureus*, which possibly enhances the organism's colonisation of the skin by hydrolyzing the lipids on the human epithelial surface (Willet, 1976) and the α-toxin of *Clostridium perfringens*. Gram-negative organisms, including *V. cholerae* and various *Pseudomonas* species, also produce lipases, the most notable of which is phospholipase C (PLC) (Esselman and Liu, 1961). This enzyme is a lecithinase which catalyzes the hydrolysis of

phosphatidylcholine, a phospholipid found in the membrane of animal cells, into phosphocholine and diacylglycerol (Esselman and Liu, 1961). Evidence suggests that this enzyme is a virulence factor in pulmonary infections caused by *P. aeruginosa* (Southern, Mays, *et al*, 1970).

The lipolytic activity of the organism later to be designated as *P. cepacia,* was first described in 1941 (Starr and Burkholder, 1941). Since then, various workers have described different types of lipases including esterases and lecithinase (Lonon, Woods, *et al*, 1988; Gessner and Mortensen, 1990; Vasil, Krieg, *et al*, 1990; Lonon and Hooke, 1991). One study found that 60% of *B. cepacia* CF isolates tested produce a lecithinase which is not PLC (Lonon, Woods, *et al*, 1988) and had a molecular weight of 25 000. At least one strain of *B. cepacia* (Pc224c) has been shown to produce PLC activity which is non hemolytic, heat labile, not a lecithinase and is inhibited by inorganic phosphate (Lonon and Hooke, 1991).

One of the ways in which lipases may contribute to bacterial virulence is through their reduction of the phagocytic activity of macrophages (Straus, Lonon, *et al*, 1992). Thus lipase may be an important virulence determinant by allowing *B. cepacia* to evade the mammalian host defence system. This inhibitory effect is lost when the lipase is inactivated by heat. *B. cepacia* lipase appears to exert its antiphagocytic effect on

pulmonary rat alveolar macrophages by affecting their cell surface (Straus, Lonon, *et al*, 1992).

1.4.3. Haemolysin

Haemolysin is considered a virulence factor for several different bacteria. About 4% of *B. cepacia* strains produce a heat-labile haemolysin that is most active against human erythrocytes (Nakazawa, Yamada, *et al*,1987). In contrast to *P. aeruginosa*, production of haemolysin by *B. cepacia* strains does not correlate with phospholipase (PLC) activity. Recently, an interesting 'haemolysin' of *B. cepacia* was purified and found to cause cause apoptosis and degranulation of human neutrophils, (Hutchison, Poxton, *et al*, 1998), in addition to causing lysis of human erythrocytes.

1.4.4. Siderophores

Siderophores are iron-chelating compounds which are excreted by bacteria in iron-limiting conditions to solubilize, transport and store iron (Stephan, Freund, *et al*, 1999). Siderophores stimulate bacterial growth in iron-deficient media by enabling the cells to compete with host iron-binding proteins, thus enhancing the pathogenic potential of an organism. Several families of siderophores have been described in *B. cepacia* including pyochelin (Sokol, 1986), cepabactin (Meyer, Hohnadel, *et al*, 1989), salicylic acid

(Visca, Cierco, *et al*, 1999) and ornibactins (Stephan, Freund, *et al*, 1999). Salycylic acid is not only a precursor of pyochelin synthesis but is an endogenous siderophore for some *B. cepacia, P. aeruginosa* and *P. fluorescens* isolates (Visca, Cierco, *et al*, 1999). Azurechelin has been identified as one of the siderophores produced by *B. cepacia* (Bukovits, Mohr, *et al*, 1982) but has recently been shown to be identical to salicylic acid (Visca, Cierco, *et al*, 1999).

There is evidence to suggest that pyochelin production by clinical strains of *B. cepacia* is associated with increased morbidity and mortality of CF patients (Sokol, 1986). Most strains (81%) causing mild to moderate infections did not produce pyochelin, whereas the isolates from 'serious' infections were pyochelin-positive. It has been suggested that the CF pathogens *B. cepacia* and *P. aeruginosa* are 'co-operative' in their pyochelin usage, that is, if one does not produce pyochelin, it could use that of the other (Sokol, 1986), a process which renders both organisms excellent iron chelators.

1.4.5. Evasion of the immune system

In recent years, there has been growing evidence to suggest that *B. cepacia*'s ability to cause persistent and sometimes invasive infections may be due, at least in part, to its ability to invade and survive intracellularly in humans. Burns and co-workers showed in 1996 that a CF isolate of *B. cepacia* could invade and survive in cultured respiratory

epithelial cells more efficiently than an environmental isolate (Burns, Jonas, *et al*, 1996). So far, there is no evidence that *B. cepacia* causes destruction of respiratory epithelial cells. Rather, organisms enter the eukaryotic cells where they remain, protected from antibiotics and host defenses. The cells may also transcytose the epithelium and cause bacteraemia in a sub-population of CF patients.

Several genomovars of *B. cepacia*, of both clinical and environmental origin, can survive and replicate for several days within different strains of the free-living amoebae *Acanthamoeba* (Marolda, Hauröda, *et al*, 1999). In addition, extracellular *B. cepacia* can grow saprophytically on the by-products released by the amoeba and *Acanthamoeba* strains periodically release membrane-bound vesicles containing bacteria (Marolda, Hauröda, *et al*, 1999).

Following demonstration of the survival of *B. cepacia* in respiratory epithelial cells and amoeba, several groups have reported the invasion of macrophages (Tipper, Ingham, *et al*, 1998; Saini, Galsworthy, *et al*, 1999; Martin, and Mohr, 2000). Although clinical and environmental isolates can invade macrophages and epithelial cells with the same frequency (Martin, and Mohr, 2000), in contrast to clinical isolates, environmental isolates were unable to replicate intracellularly (Tipper, Ingham, *et al*, 1998; Martin, and Mohr, 2000). Intracellular survival of *B. cepacia* in macrophages was found to occur

despite the fact that the macrophages were activated and releasing superoxides (Saini, Galsworthy, *et al*, 1999). These repeated cycles of phagocytosis and macrophage activation without bacterial killing promote a deleterious inflammatory response causing tissue destruction and decay of the lung. Thus intracellular survival and replication of *B. cepacia* may contribute to the virulence potential of pathogenic strains and may play a role in the pathogenesis of CF lung infection.

Intracellularity of *B. cepacia* also provides possible explanations for the organism's survival in the environment in amoebae, transmission, persistence in the lungs, macrophages, neutrophils and epithelial cells despite the use of antibiotics with demonstrated *in vitro* activity, and induction of inflammation leading to CF lung disease.

1.4.6. Lipopolysaccharide (LPS)

LPS is a well-known bacterial virulence factor on account of its potent immuno-stimulatory effects on granulocytes, macrophages and lymphocytes (Reitschel, Kirikae, *et al*, 1993). Whereas, environmental isolates of *B. cepacia* only possess smooth LPS, clinical isolates possess either rough or smooth LPS, (Evans, Poxton, *et al*, 1999) which is cross-reactive amongst different *B. cepacia* and with other *Burkholderia* species, such as *B. gladioli*. Several studies indicate that *B. cepacia* LPS contributes to the organism's pathogenicity in CF lung disease. In endotoxicity assays, LPS from both clinical and

environmental isolates of *B. cepacia* was more endotoxic than LPS from *P. aeruginosa* (Shaw, Poxton, *et al,* 1995). *B. cepacia* and *B. gladioli* LPS produces at least a nine fold higher activity in tumour necrosis factor (TNF) induction compared to both a CF isolate of *P. aeruginosa* and *P. aeruginosa* PA01 (Shaw, Poxton, *et al,* 1995), indicating that *B. cepacia* LPS has greater potential than *P. aeruginosa* to cause sustained immune-mediated damage in the lung. TNFα is a pro-inflammatory cytokine that plays a key role in the regulation and secretion of other cytokines and thus amplifying and diversifying the immune response (Manthey and Vogel, 1992). It was suggested that the stimulation of TNF by *B. cepacia* LPS may contribute to destructive pulmonary inflammation since *B. cepacia* stimulates a pronounced inflammatory response in CF mice (Davidson, Dorin, *et al*, 1995) and in CF patients as measured by levels of neutrophil elastase (Elborn, Dodd, *et al*, 1994).

B. cepacia LPS is also a more potent priming agent of neutrophil respiratory burst than *P. aeruginosa* LPS (Hughes, Stewart, *et al*, 1997). It is suggested that neutrophils exposed to *B. cepacia* LPS may be involved in the recruitment of other neutrophils to the lung or the LPS may prime neutrophils to increase the release of tissue-damaging enzymes and reactive oxygen species from activated neutrophils.

1.3.7. Pili and Flagella

Binding of a potential pathogen to the host's epithelium or mucosal surfaces is often critical for the subsequent establishment of an infection. Analogous to *P. aeruginosa, B. cepacia* has polar pili and flagella but there is no homology between the two organisms' pilin structural genes, and *B. cepacia* is resistant to *P. aeruginosa* pilus-specific phages (Saiman, Cacalano, *et al*, 1990). The binding capacity of the two organisms also differs with *P. aeruginosa* binding to epithelial cell monolayers up to ten times more than *B. cepacia* (Saiman, Cacalano, *et al*, 1990). Supernatants of *P. aeruginosa* increase the binding of *B. cepacia*, suggesting that *P. aeruginosa* exoproducts alter the epithelial cell surface and promote *B. cepacia* adherence. Interestingly, mannose-binding lectins on the epithelium surface bind to *B. cepacia*, and lead to complement activation, more strongly than they bind to *P. aeruginosa* (Davies, Neth, *et al,* 2000). Thus, CF patients with mannose-binding lectin deficiency are at a particular risk of *B. cepacia* infection.

In *B. cepacia* isolates, the density of surface pili correlates with mucin-binding capacity (Sajjan and Forstner, 1992); the major mucin-binding component being a 22kDa doublet. Significantly, binding of *B. cepacia* to mucin is highest for isolates from CF patients with advanced lung disease (Sajjan and Forstner, 1992). Since mucin covers the epithelial surface of the respiratory tract and is poorly cleared in CF, the 22kDa pilin

adhesin for mucin could be a potential colonisation and/or virulence factor in *B. cepacia* infections.

1.5. Resistance of *B. cepacia* to antibiotics

Treatment of *B. cepacia* infections is difficult because of the organism's high intrinsic resistance to most of the common antibacterial agents (Fass and Barnishan, 1980). Although meropenem is more effective than other antibiotics (Lewin, Doherty, *et al*, 1993) therapeutic choices to treat most *B. cepacia* infections are chloramphenicol, fluorinated quinolones, ceftazidime and co-trimoxazole (Blumer, Stern, *et al*, 1985, Moore and Hancock, 1986). *B. cepacia*'s susceptibility to various antimicrobial agents varies widely between different isolates, including isolates with the same ribotype pattern (Pitt, Kafmann, *et al,* 1996). Treatment of *B. cepacia* infections is further complicated by failure of antibiotics with demonstrated *in vitro* activity to have a significant effect in patients with severe infections (Gold, Jin, *et al*, 1983; Isles, Maclusky, *et al*, 1984). A number of possible explanations for this occurrence have been suggested, including the failure of sufficient concentrations of antibiotic to penetrate the bronchial mucosa and impaired phagocytic activity within the CF lung (Thomassen, Boxerbaum, *et al,* 1979). Once effective antibiotics have been identified, the development of further antibiotic resistance during therapy and the induction of cross-resistance to other unrelated antimicrobial agents, adds to the already difficult task of managing *B. cepacia* infections.

1.5.1. Cross-resistance between unrelated classes of antibiotics

Several workers have observed that strains of *B. cepacia* show cross-resistance between classes of unrelated antimicrobial compounds. Multiple drug resistance that crosses drug class lines often involves a permeability change. Rajyaguru and Muszynski (1997) found that chloramphenicol resistant mutants of *B. cepacia* also expressed cross-resistance to trimethoprim/sulphamethoxazole, nalidixic acid, ciprofloxacin, oxolinic acid, ceftazidime and rifampicin. Cross-resistance seemed to be mediated by bacterial LPS, as new higher molecular weight bands were seen in the resistant strains. In addition to changes in their LPS profile, resistant mutants had lost a major outer membrane protein in the 39-47 kDa range and also expressed additional outer membrane proteins at 20-21kDa or 75-77kDa (Rajyaguru and Muszynski, 1997).

Cross-resistance is not unique to *B. cepacia* and has also been reported in other Gram-negative bacteria including *Klebsiella pneumoniae* (Sanders, Sanders, *et al*, 1984). Mutants of a strain of *K. pneumoniae* showing cross-resistance between quinolones and ß-lactams which is associated with changes in outer membrane proteins have been reported. Changes in outer membrane proteins are also thought to be responsible for ß-lactam/ aminoglycoside resistance in *Serratia marcescens* (Sanders, Sanders, *et al*, 1984).

1.5.2. Role of porins

Gram-negative bacteria have an outer membrane that acts as a permeability barrier and offers various degrees of intrinsic resistance to antimicrobial agents. The natural level of resistance created by the outer membrane can be increased by genetic or physiological alterations that lower the permeability of this membrane (Nikaido, 1989). In clinical isolates, this may be the result of stepwise mutations and may be reversible when selective pressure of growth in antibiotic-containing medium is removed (Burns and Clark, 1992).

Hydrophilic solutes most often cross the outer membrane through porins which are water-filled transmembrane channels that allow hydrophilic solutes to penetrate the membrane, subject to molecular size limits (Rosenbusch, 1974; Parr, Moore, *et al*, 1987). They are usually present as trimers, that are strongly but non-covalently linked to the underlying peptidoglycan (Rosenbusch, 1974). The tertiary and quartenary structures of porins are the ones that determine their function with neither lipids nor lipopolysaccharides playing a part (Parr, Poole, *et al*, 1986). Most porins form channels that function as sieves but a few that demonstrate substrate specificity have been identified (Burns and Clark, 1992).

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ß-lactams predominantly diffuse through the porins of *E. coli* in order to reach their target sites (Yoshimura, Nikaido, 1985). Other small hydrophilic agents, such as tetracyclines, aminoglycosides and chloramphenicol, would also be expected to use the same pathway. Porin-deficient mutants have lower permeability and thus greater resistance to hydrophilic antibiotics (Harder, Nikaido, *et al*, 1981). One of the reasons for β-lactam resistance in strains of *E. coli* is their greatly diminished level of the porin ompF (Harder, Nikaido, *et al*, 1981). However, lower outer membrane permeability is not itself sufficient to result in increased resistance to antibiotics. A secondary mechanism such as an inactivating enzyme may act in association with the impaired uptake (Harder, Nikaido, *et al*, 1981).

The sizes and permeability of porins differ between different bacteria. The outer membrane permeability coefficient of *B. cepacia* PC715j is about ten times less than that for an *E. coli* control strain (Parr, Moore, *et al*, 1987), suggesting that *B. cepacia* has a substantial outer membrane hydrophilic permeability barrier. The *B. cepacia* porin is also 8-10 times smaller than that of *E. coli*, which might explain the much lower permeability of *B. cepacia* outer membrane to the β-lactam nitrocefin (Parr, Moore, *et al*, 1987). Parr and co-workers identified an 81kDa porin complex in the outer membrane that dissociates on heating to form two proteins of 27 and 36kDa. The 27kDa porin was found to be absent or partially lost in two CF isolates which showed decreased β-lactam permeability and susceptibility (Aronoff, 1988). Thus, it has been suggested

that the 27kDa outer membrane protein is either a major porin or a major protein component of the porin complex in *B. cepacia* (Aronoff, 1988). Decreased expression of this protein leads to decreased β-lactam diffusion across the outer membrane leading to high level resistance to these antibiotics.

Since hydrophobic solutes cannot diffuse through porin channels rapidly, many Gram- negative bacteria are resistant to a number of hydrophobic antibiotics and dyes which are effective against Gram-positive bacteria (Nikaido, 1976). These antibiotics include the macrolides, novobiocin, rifamycin SV and actinomycin D.

1.5.3. Active efflux

Decreased drug accumulation may result from impermeability or from active drug efflux. A study by Burns and co-workers showed that the resistance of *B. cepacia* to chloramphenicol, trimethoprim and ciprofloxacin was due to active drug efflux. They identified and sub-cloned a fragment coding for a 50kDa outer membrane protein (Burns, Wadsworth, *et al*, 1996). The nucleotide sequence identified showed homology to a portion of the multiple antibiotic resistance efflux operon *mex*A-*mex*B-*opr*M from *P. aeruginosa* (Poole, Krebs, *et al*, 1993). Thus, in addition to restricting the entry of antibiotics, *B. cepacia* porins can also actively remove the antibiotics accumulated within the bacterial cell.

1.5.4. Cationic antibiotics:- aminoglycosides and polymyxin B

Many Gram-negative bacteria are susceptible to β-lactams, aminoglycosides, polymyxin B, colistin and tetracyclines, which are relatively small and hydrophilic and should therefore readily diffuse through porins. *B. cepacia* is an exception to this general trend in that it is inherently resistant to aminoglycosides and polymyxin B, in part because the compounds can not permeabilize the organism's outer membrane, (Fass and Barnishan, 1980, Moore and Hancock, 1986). A possible explanation for this is that the LPS of *B. cepacia* is arranged in the outer membrane in a way that masks the negative charges found on the LPS molecule, thus making them unavailable for binding to polycationic antibiotics (Moore and Hancock, 1986).

Polypeptides (polymyxin B, colistin) and aminoglycosides are absorbed by Gram-negative bacteria through a self-promoted uptake (Moore and Hancock, 1986). This mechanism has been described in *P. aeruginosa* in which these compounds aid their own entry by disrupting the outer membrane structure (Rajyaguru and Muszynski, 1997). The crucial step is the interaction of the antibiotic with anionic sites at the bacterial surface. The inability of cationic antibiotics to affect the permeability of *B. cepacia* suggests that its LPS composition, structure and or function are distinctly different from those of *P. aeruginosa* and *E. coli* (Rajyaguru and Muszynski, 1997). This difference in LPS structure is suggested by *B. cepacia* LPS being more endotoxic

than LPS from *P. aeruginosa* (Shaw, Poxton *et al*, 1995). The LPS of *B. cepacia* is low in anions such as phosphates and 3-deoxyoct-2-ulosonic acid (KDO) (Cox and Wilkinson, 1991) which means its cation-binding capacity is limited compared to other Gram-negative bacteria. In addition, the LPS of *B. cepacia* has 4-amino-deoxyarabinose (Ara4N), a four amino sugar, as a substituent for lipid A (Cox and Wilkinson, 1991) and this substitution is believed to further lower the affinity of the LPS for cationic antibiotics.

1.5.5. Effect of Salicylates

Decreased porin-mediated outer membrane permeability is the most common antibiotic resistance mechanism in *B. cepacia*. In some Gram negative bacteria, this form of resistance can be induced by growth in the presence of weak acids such as salicylates, which suppress porin synthesis (Foulds, Murray, *et al*, 1989). *E. coli* grown in 1-5mM sodium salicylate show increased resistance to ampicillin, cephalexin cephaloridine, chloramphenicol, nalidixic acid, tetracycline and cephalothin, (Rosner, 1985, Foulds, Murray, *et al*, 1989). Since these antibiotics differ in their structure, targets and modes of action, it is believed that the resistance is caused by an effect of the weak acids on antibiotic uptake (Foulds, Murray, *et al*, 1989). When *B. cepacia* is grown in the presence of salicylate, the MICs of chloramphenicol, trimethoprim and ciprofloxacin increase but that of ceftazidime remains the same (Burns and Clark, 1992).

Growth of *B. cepacia* in 10mM sodium salicylate results in the loss of a minor outer membrane protein of molecular weight 40 000 termed OpcS (Burns and Clark, 1992), or decreased expression of the porin protein OmpF (Kunin, Hua, *et al*, 1995). Outer membrane penetration of chloramphenicol is decreased while that of a β-lactam indicator compound, PADAC, is not. Thus, OpcS may function as a selective antibiotic- permeable porin which can be suppressed by growth in salicylate. The effect of salicylates on antibiotic resistance is reversible (Rosner, 1985), and its role in *B. cepacia* infections is unknown.

1.5.6. Resistance to β-lactams

Increased resistance of bacteria to β-lactams may occur through mechanisms that decrease the permeation of the outer membrane by the antibiotics or by increased antibiotic hydrolysis by the β-lactamases found in the periplasmic space (Prince, Wood, *et al*, 1988). Most CF and non-CF isolates of *B. cepacia* contain a chromosomal β-lactamase that focuses on isoelectric gels in the pH 7.9-8.1 range (Aronoff, 1988).

In *B. cepacia* strain 249, two separate β-lactamases have been reported; a penicillinase, *pen*A, responsible for about 80% of the total β-lactamase activity of the strain (Prince, Wood, *et al*, 1988) and a second enzyme with primarily cephalosporinase activity

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(Lessie and Gaffney, 1986). The β-lactamase of this strain is highly inducible by penicillin G and is dependent on its continued presence. The *pen*A β-lactamase has a molecular weight of 33 500 and is unique amongst the β-lactamases in its association with a metabolic pathway that allows *B. cepacia* to metabolise penicillin (Beckman and Lessie, 1979). All wild type strains of *B. cepacia* examined can use penicillin G as a sole source of carbon and energy (Beckman and Lessie, 1979), however, they fail to exhibit significant growth in the benzylpenicillin derivatives ampicillin and carbenicillin even though they are resistant to these antibiotics. Extracts of *B. cepacia* 249 grown in penicillin G contain high levels of β-lactamase activity, although the activity itself is not sufficient for growth in penicillin G, as seen in *P. aeruginosa* strains which have similar β-lactamase activities (Beckman and Lessie, 1979). In *B. cepacia* 249, expression and induction of *penA* is regulated by *penR* and resistance to imipenem is dependent on the presence of penR (Trépanier, Prince, *et al.* 1997).

Since overall permeability of *B. cepacia* and *P. aeruginosa* to β-lactam compounds has been found to be very similar (Parr, Moore, *et al*, 1987), the ability to rapidly induce β-lactamase expression may be an important selective advantage for *B. cepacia*. Pen A β-lactamase differs significantly by physical properties, activity, and induction kinetics and seems to be unrelated to the *amp* C-type β-lactamases of enterobacteria or *P. aeruginosa* (Bergstrom, Olsson, *et al*, 1982).

Incubation of *B. cepacia* in an atmosphere of air and 5% carbon dioxide is reported to result in the activity of β-lactams being either lost or decreased but not eliminated (Corkill, Deveney, *et al*, 1994). This effect is due to the fact that the production of β-lactamases most active against first and second generation cephalosporins, increases at least ten-fold in the presence of carbon dioxide. Interestingly, production of β-lactamases showing greater activity toward third generation cephalosporins increases 180-fold when imipenem another β-lactam, is present in the growth medium (Corkill, Deveney, *et al*, 1994). This implies that the presence of different β-lactams does not always result in synergy but may actually reduce the effectiveness of individual antibiotics.

1.5.7. Chloramphenicol Resistance

Chloramphenicol is a potent inhibitor of bacterial protein synthesis and acts by interacting with the 50S subunit of bacterial ribosomes. Resistance to chloramphenicol is usually conferred by chloramphenicol inactivation by 3-o-acetylation catalysed by the intracellular enzyme acetyltransferase (CAT) (Shaw and Brodsky, 1968). In *P. aeruginosa* and some other Gram-negative bacteria, a plasmid-specific permeability barrier has been suggested as a mechanism of resistance not conferred by CAT (Gaffney, Cundliffe, *et al*, 1981; Kono and O'Hara, 1976). This barrier is probably located on the cytoplasmic membrane, thus preventing the drug from reaching its intracellular target

and is possibly a protein that changes the structure of the membrane (Gaffney, Cundliffe, *et al*, 1981). In *P. aeruginosa,* 50-85% of chloramphenicol resistant strains produce CAT (Nitzan and Rushansky, 1981). Decreased permeability is probably the mechanism of chloramphenicol resistance in *B. cepacia*, since no CAT or ribosomal resistance has been detected (Burns, Hedin, *et al*, 1989; Rajyaguru and Muszynski, 1997). In the strains studied, Burns and co-workers found that there were no structural alterations in outer membrane proteins (including porins) nor were there any changes in LPS (Burns, Hedin, *et al*, 1989). They concluded that the most likely mechanism is structural or functional differences that are present in intact outer membranes.

1.5.8. Resistance to trimethoprim

Trimethoprim acts on the bacterial dihydrofolate enzyme and thus acts as an inhibitor of folate utilisation (Bushby and Hitchings, 1968). Sulphonamides act by inhibiting the biosynthesis of folic acid in the target organisms. A combination of trimethoprim and sulphonamides such as sulphamethoxazole may be bactericidal where individual drugs only produce bacterial stasis (Bushby and Hitchings, 1968). Resistance to trimethoprim or the combination of trimethoprim/sulphamethoxazole has been documented as being due to the production of a different dihydrofolate reductase which is either chromosomally or plasmid encoded (Amyes, 1986). The dihydrofolate reductase (DHFR) isolated from a trimethoprim-susceptible strain of *B. cepacia* is more sensitive

to trimethoprim than that from a trimethoprim-resistant strain, suggesting that the two enzymes are different (Burns, Lien, *et al*, 1989). It has been suggested that the differences between DHFR isolated from the susceptible and resistant strain are due to a modification of the active site of the enzyme (Burns, Lien, *et al*, 1989).

1.6. Biotechnological applications of *B. cepacia*

1.6.1. *B. cepacia* as a biopesticide

Intensive mono-culture of crops often leads to a decrease in crop yields and further loss from root rot diseases may occur. One of the reasons for the increased incidence of plant crop diseases is the influence of phytopathogenic fungal and bacterial populations specific to the crop (Curl and Truelove, 1986). Chemical pesticides have been the main weapons in controlling soil-borne pathogens. In recent years, ecological damage resulting from the pollution of soil and ground water and pathogen resistance resulting from the use of chemical compounds, have prompted research into alternative approaches such as biological control. In addition, the use of pesticides to control crop diseases is increasingly threatened by consumer preferences and regulatory issues concerning pesticide residues in food.

Biological control is based on plant-mediated interactions that occur between pathogens and roots and between pathogens and antagonistic microorganisms in the rhizosphere and rhizoplane (Cook and Baker, 1983). The use of natural antagonists lessens agricultural dependence on synthetic agrochemical such as herbicides, insecticides and fungicides. As oppossed to inundative chemical applications to control post-harvest diseases, biocontrol agents can colonise plant tissues and lead to plant exudates that protect sites within the plant. One focus of biological control is the use of antagonists

and a major group of natural antagonists currently being investigated is phyloplane and rhizosphere bacteria.

Microorganisms that grow in the rhizosphere are ideal for use as biological control agents since the rhizosphere provides the front-line defense for roots against attack by bacterial fungal pathogens. Such pathogens encounter antagonism from rhizosphere organisms before and during primary infection and also during secondary spread of the root. In the last few years several examples of bacteria capable of providing substantial disease control in the field have been reported. These recent successes in biological control result in part from a greater understanding of the rhizosphere and from selection of bacterial strains adapted to colonisation of the rhizosphere. Bacterial biopesticides can improve plant growth by suppressing either major or minor pathogens.

The first successful biological control agent was *Agrobacterium radiobacter* which controls crown gall caused by *Agrobacterium tumefaciens* (Kerr, 1972). *Bacillus* spp were next to be studied intensely and tested on a wide variety of plant species for their ability to control diseases. *Bacillus* spp are appealing as biocontrol agents because they produce endospores that are tolerant to heat and desiccation. Currently, *Pseudomonas* spp. and related organisms such as *B. cepacia* are receiving much attention as biocontrol agents.

B. cepacia isolated from crop plants has been demonstrated as a biological control agent of fungal diseases of commercially-important food crops such as apples and pears (Janisiewiez and Roitman, 1998), corn (Jayaswal, Fernandez, *et al*, 1990, Hebbar, Martel, *et al*, 1998), peas (Parke, 1990), sunflowers (Hebbar, Berge, *et al*, 1991), adzukibean (Hasegawa, Kondo, *et al*, 1991), and wheat. *B. cepacia* has also been used to suppress several soil-borne plant pathogens when applied as a seed coating or root dip (Homma, Sato *et al*, 1989, Parke, 1990). Some diseases such as *Aphanomyces* root rot and *Pythium* damping off of peas cause seeds to rot before they germinate or seedlings to be killed before or shortly after emergence (Parke, 1990; Parke and King, 1991), hence the importance of seed treatment.

In addition to protecting food crops, *B. cepacia* also protects commercially important plants such as protea (a flowering African shrub) against *Phytophthora cinnamomi* which causes root and collar rot, leading to a major limitation of the cultivation of proteas (Turnbull, Ogle, *et al*, 1991). In addition, compared to other closely related organisms, *B. cepacia* has been found to be particularly effective in controlling *Rhizoctonia solani* stem rot of poisenttia (a decorative plant associated with Christmas), (Cartwright, 1995).

In several tests, *B. cepacia* has performed comparably, over a wide range of temperatures, with standard fungicide treatments such as captan and metalaxyl (Parke, 1990; Parke and King, 1991), and exceeded these fungicides in efficiency (Parke and King, 1991).

Whilst most research on *B. cepacia* as a biocontrol agent has focussed on suppression of fungal pathogens, few studies have been performed to measure the effect of *B. cepacia* on pathogenic nematodes. However, one group has reported that *B. cepacia* and *B. gladioli* were the most predominant of Gram negative bacteria isolated from the rhizospheres of different plants, which were antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes (Kloepper, Rodriguez-Kabana, *et al*, 1992).

Various mechanisms have been described to account for the biopesticide activity of *B. cepacia.*

i) Production of antimicrobial agents

One of the reported mechanisms by which *B. cepacia* suppresses pathogens is through the production of antibiotics such as pyrrolnitrin (3-chloro-4-(2-nitro-3-chlorophenyl)-pyrrole (Janisiewiez and Roitman, 1998; Homma, Sato *et al*, 1989; Burkehead, Schisler, *et al*, 1994) and pseudane derivatives 2-(2-heptenyl)-3-methyl-4-quinolinol (HMQ) and 2-(2-nonenyl)-3-methyl-4-quinolonol (NMQ) (Homma, Sato *et al*, 1989).

These antibiotics have higher antifungal activity than antibacterial activity (Homma, Sato *et al*, 1989). Pyrrolnitrin, aminopyrrolnitrin and monochloroaminopyrrolnitrin were isolated from a *B. cepacia* strain J82rif and protected sunflowers from the sunflower wilt fungus *Sclerotinia sclerotiorum* (McLoughlin, Quinn, *et al*, 1992). *Sclerotinia* wilt is one of the most important diseases of sunflowers as no effective chemical control currently exists. The fungus survives in the soil as resistant sclerotia and attacks the roots, resulting in root rot basal stem canker and plant wilt (McLoughlin, Quinn, *et al*, 1992).

An antimicrobial agent produced by *B. cepacia* B5, active against the bacterial wilt disease pathogen *Pseudomonas solanacearum* affecting tobacco and other plants in the solanaceae family, was identified as 2-keto-D-gluconic acid (2KGA) (Aoki, Uehara, *et al*, 1991). Since the pathogen can survive for long periods in the soil without the host plant, *B. cepacia* was added to a contaminated soil suspension, together with either wheat bran or glucose as nutrient additives. *Pseudomonas* spp, *Acetobacter* spp. and related organisms such as *B. cepacia* can produce 2KGA from glucose (Isono, Nakanishi, *et al*, 1968), which is thought to be the mechanism of bacterial wilt disease suppression by *B. cepacia* B5. *B. cepacia* B5 has been cultured on a large scale and after long-term preservation, was still able to suppress disease in field tests (Aoki, Uehara, *et al*, 1991) which makes it suitable for commercial application.

B. cepacia also suppresses pathogenic fungi by producing the enzyme β-1,3-glucanase (laminarase), (Friedlander, Inbar, *et al,* 1993) which degrades the fungal cell wall component β-1,3-glucan. This enzyme is inducible and is excreted into the growth medium only when other carbon sources are unavailable. A β-1,3-glucanase-producing *B. cepacia* was isolated on a synthetic medium with laminarin as the sole carbon source. Enzyme induction by different fungal cell walls as sole carbon sources in synthetic medium was found to correlate with the biological control of the respective fungi by *B. cepacia* (Friedlander, Inbar, *et al,* 1993).

ii) Production of siderophores

Production of siderophores, which are low molecular weight, high affinity iron (III) chelators that transport iron into the bacterial cell, allows *B. cepacia* biopesticides to grow in iron-deficient conditions where other organisms would be unable to flourish (De Freitas, Germida, *et al*, 1991).

iii) Competition

Bacterial antagonists with a broad spectrum of antibiosis, the ability to colonise plant tissue rapidly and to survive for long periods in large numbers are potential candidates

for microbial inoculation of seed or plants. These agents also compete with the pathogen in diseased tissues. Substrate competition for nutrients supplied by the root and seed exudates and niche exclusion have been suggested as a possible mechanism by which *B. cepacia* suppresses pathogens (Parke, 1990).

Bacterial antagonists have an advantage over pathogenic fungi due to their rapid multiplication and higher populations in the rhizosphere (Herbbar, Berge, *et al*, 1991). *B. cepacia* strain 526 which suppresses maize soil-borne diseases colonises the roots and rapidly multiplies on the maize roots and rhizosphere (Herbbar, Davey, *et al*, 1992). Another strain which protects maize from soil-borne diseases, *B. cepacia* 64, has high pectinase activity which enables it to penetrate maize root mucilage while strains with low pectinase activity are unable to do so (Herbbar, Davey, *et al*, 1992).

iv) Chemotaxis

Once inoculated into the soil the ability to migrate towards the root system by chemotaxis would be greatly advantageous to a biocontrol strain. Evidence shows that *B. cepacia* migrates rapidly to root surfaces from the point of inoculation (Herbbar, Davey, *et al*, 1992)

In conclusion, the use of microorganisms such as *B. cepacia* for the control of diseases appears to be an appealing option when compared to more noxious chemical pesticides which persist in the environment and whose residues remain in harvested foods, sometimes with harmful effects. On the other hand, biological control agents have not been unequivocally shown to have no effect on the normal microbial soil flora. Furthermore, organisms such as *B. cepacia* could have potentially devastating effects on susceptible human populations, in particular, individuals with CF and other immunodeficiencies.

Conflicting evidence exists on the effects of introduced biocontrol agents on the normal environmental microbial flora. When the biocontrol agent *B. cepacia* AMMDR1 was applied to the rhizospheres of four pea cultivars, the total *B. cepacia* population fell to levels present before the application, within about six weeks (King and Parke, 1993). However, the population was elevated for several weeks after application.

Furthermore, experiments conducted to determine whether coating soybean seeds with the biological control agent *Bacillus cereus* UW85 affected the bacterial community in the rhizosphere of soybeans revealed that the communities of rhizosphere bacteria that developed on non-treated plants and on plants grown from seeds treated with the biocontrol agent were sometimes significantly different (Gilbert, Parke, *et al,* 1993).

This population distinction occurred even when the introduced strain did not persist as a common member of the bacterial community. The results from this study illustrate the potential impact of introducing large populations of a single strain of bacteria on the indigenous rhizosphere flora.

Although *B. cepacia* was first described 50 years ago, much remains to be learned about virtually every aspect of its biology including its pathogenicity, taxonomy, ecology and epidemiology. This lack of adequate information makes it impossible to adequately evaluate the commercial application of *B. cepacia* as a biological control agent. In recent years, concern has been expressed on the numerous patents being sought for the commercial application of different *B. cepacia* strains. The regulations regarding the use of biopesticides and bioremediation products, in both North America and the United Kingdom, have been criticised as outdated and inadequate for the evaluation of highly complex organisms such as the *B. cepacia* complex (LiPuma and Mahenthiralingham, 1998; Govan and Vandamme, 1998).

Although a great deal has been mentioned about 'clinical' and 'environmental' isolates of the *B. cepacia* complex, available taxonomic data indicates that there is no clear distinction between clinical and environmental or indeed, between pathogenic and non-pathogenic strains of the *B. cepacia* complex. Interestingly, new evidence has shown

that clinical and environmental isolates of the *B. cepacia* complex can be clonally related. Genotypic and phenotypic evidence of a clonal relationship between an American strain isolated from onion (ATCC 25416) and a British strain isolated from a CF patient (C1853) was obtained recently (Govan, Balendreau, *et al* 1998; Govan and Vandamme, 1998).

As previously discussed in section 1.3, transmissible strains of the *B. cepacia* complex have caused major epidemics in British and North American CF centres. Since genetic changes conferring transmissibility in the *B. cepacia* complex are thought to occur at random (Holmes, Govan, *et al*, 1998) biopesticide strains could potentially become highly transmissible. The large, complex and highly plastic genome of the *B. cepacia* complex (Cheng and Lessie, 1994; Rodley, Romling, *et al*, 1995) has been cited as making the organisms highly suited to rapid adaptation to environmental changes (Holmes, Govan, *et al*, 1998). In addition, large-scale commercial application of *B. cepacia* complex strains to the environment could promote genetic exchange with normal soil microflora and thus lead to the emergence of pathogenic organisms with the *B. cepacia* complex's inherent resistance to several antibiotics.

In view of the concerns raised and the lack of adequate information on the *B. cepacia* complex, in its risk assessment of *B. cepacia* biopesticides, the Scientific Advisory Panel

to the Environmental Protection Agency advises caution on the use of *B. cepacia* in biological control programmes (http://www.epa.gov, 12 July 2000).

1.6.2. Application of *B. cepacia* in bioremediation of contaminated sites

Industrial chemicals, manufacturing wastes, herbicides and pesticides often contain synthetic organic chemicals such as chlorinated aromatics. These compounds are mineralised very slowly or not at all because natural microbial flora fail to degrade them totally in order to derive their carbon and energy from the process. A few of these compounds are toxic and some, although not toxic at concentrations found in soil and water, are subject to biomagnification (Alexander, 1981).

The presence of recalcitrant compounds in water, land and crops is now deemed a serious environmental pollution and health problem by the general public. In addition to contaminating their sources, recalcitrant compounds can be transported some distance from their source of introduction.

One of the major recalcitrant compounds found in most industrialised nations is 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), a defoliating herbicide which is used to control various broad-leaf weeds and also as a growth regulator to delay colouration of lemons, increase the size of citrus fruits and decrease deciduous fruit drop (Grant, 1979, Kilbane, Chaterjee, *et al*, 1982; Danganan, Ye, *et al*, 1994). Not only does 2,4,5-T persist in the environment, it is also suspected of being genotoxic. An investigation of the rate of birth malformations found a statistically significant association between the rate of spray of

2,4,5-T and talipes (congenital deformity of the soles of feet), though no association with malformations of the central nervous system was found (Hanify, Metcalf, *et al*, 1981).

Before 1982, no known microorganisms capable of utilising 2,4,5-T as a source of carbon and energy had been described (Alexander, 1981). In 1982, however, pure culture of *B. cepacia* AC1100 capable of growing on 2,4,5-T was obtained from a mixed culture where individual strains were incapable of utilising the compound as a sole carbon source (Kilbane, Chaterjee, *et al*, 1982). Laboratory experiments demonstrated that *B. cepacia* AC1100 can significantly decontaminate soils heavily contaminated with 2,4,5-T. The organism not only degrades the herbicide 2,4,5-T but also oxides a number of chlorophenols that comprise major constituents of different toxic chemical dump sites (Kilbane, Chaterjee, *et al*, 1982). The cluster of genes involved in the metabolism of 2,4,5-T by *B. cepacia* AC1100 has been identified and cloned, revealing it to be unique and not related to other identified gene clusters (Daubarus, Saido, *et al*, 1995).

Application of *B. cepacia* AC1100 to contaminated sites is unlikely to lead to serious ecological disturbance as it rapidly disappears after exhaustion of 2,4,5-T (Kilbane, Chaterjee, *et al*, 1982). Contaminated soil incapable of supporting the growth of plants is able to do so after treatment with *B. cepacia* AC1100. Although the quality of plants in these reclaimed sites is not as good as that of plants growing in virgin soils, treatment of

contaminated sites with an appropriate microorganism virtually allows total restoration of the original soil conditions.

B. cepacia has been successfully used in the bioremediation of sites contaminated by several other recalcitrant compounds including trichloroethylene a consituent of the volatile organics used in industry for degreasing (Shields and Reagin, 1992), and polycyclic aromatics hydrocarbons, found amongst other things, in oil and wood preservation agents (Juhasz, Britz, *et al*, 1997). Application of *B. cepacia* to sites contaminated by various recalcitrant compounds, results in the detoxification of the parent compound to products that are no longer hazardous to human health or the environment. *B. cepacia*'s unusual metabolic versatility means that its exploitation in bioremediation is likely to increase.

1.7. Mechanisms of genetic exchange and genome alteration

Release of large amounts of *B. cepacia* into the environment as biopesticides has the potential to facilitate transfer of genetic information to other soil organisms and/ *B. cepacia* strains of clinical interest. Although there is currently no experimental evidence showing that this exchange does indeed occur, it has not been unequivocally shown that it cannot occur. Based on currently available knowledge therefore, genetic exchange in soil between different strains of *B. cepacia* and also with other members of the normal soil flora should be considered as possible.

Three principal ways by which bacteria can exchange genetic information are transduction, conjugation and transformation.

1.7.1. Transduction and properties of bacteriophages

Genetic transduction is the transfer from one cell to another of non-viral genetic material within a viral coat. Two types of transduction have been identified, generalised and specialised, and these are mediated, respectively, by generalised transducing particles which carry a fragment of host DNA and specialised transducing particles which contain both host and viral particles as a single entity. The significant role played by bacteriophage in different bacteria can not be over-emphasized. Phage typing is one of the most reliable

methods currently used for classifying important pathogens such as *Salmonella typhi* (Hickman-Brenner, Farmer, 1983), *Vibrio cholerae* (Guidolin and Manning, 1987), *Mycobacterium tuberculosis* (Jones, Good , *et al*, 1982) and *Klebsiella pneumoniae* (Rennie, Nord, *et al*, 1978).

Bacteriophages have been shown to be responsible for the transfer of virulence factors in several bacterial pathogens. In *V. cholerae* bacteriophages are not only important for typing but have been associated with virulence (Parker, Richardson, *et al*, 1970), induction of toxinogenesis (Siddiqui and Bhattacharyya, 1987), biotype transition (Mitra, 1989) and possibly also the evolution of epidemic strains (Mooi, and Bik, 1997). Thus, there is now overwhelming evidence to support the concept that choleraphages play a major role in the horizontal transmission of genetic information in *V. cholerae*. In other pathogenic genera, lysogenic phages have been implicated in the transmission of neurotoxigenicity from toxigenic *Clostridium butyricum* strains to a harmless *C. botulinum* type E-like strain (Zhou Sugiyama, *et al*, 1993).

The genes for two of the three pyrogenic exotoxins produced by *Streptococcus pyogenes* are bacteriophage encoded (Kapur, Nelson, *et al*, 1992). Two distinct alleles of the gene s*pe*C, which codes for the streptococcal pyrogenic toxin C, are present in natural populations of *S. pyogenes* with each allele occurring in clones that are well differentiated in overall chromosomal character (Kapur, Nelson, *et al*, 1992). It is believed that this toxin

allele-clone distribution pattern may be due to episodes of horizontal gene transfer and recombination occuring by transduction. In *E.coli*, a nonlysogenic, nontoxigenic strain can be converted to a lysogenic and heat labile enterotoxin-producing strain by infection with a temperate phage (Takeda and Murphy, 1978).

Recent studies indicate that broad-host-range bacteriophages are more common than had been thought previously (Jensen, Schrader, *et al* 1998). In their studies, Jensen and co-workers found that for two bacteriophage collections, 90% of the isolated phages had a broad host range. Infection with these broad-host-range bacteriophages was not limited by dependency on the presence of a specific plasmid and the phages were capable of infecting a broad range of hosts.

Although plasmids are commonly implicated in the transmission of antibiotic resistance, some evidence suggests that bacteriophages can also be responsible for the spread of antibiotic resistance. The resistance of *Pseudomonas aeruginosa* to single antibiotics such as imipenem (Blahová, Králiková, *et al*, 1998), and multiple antibiotic resistance (Knothe, Lebek, *et al*, 1981, Blahová, Králiková, *et al*, 1998, Blahová, Králiková, *et al*, 1999) can be transferred through transduction by wild-type as well as generalised transducing phages.

Most studies on genetic exchange in soil are conducted on sterile soil with no competition from the indigenous microbial flora. However, generalised transduction by phage P1 does occur in *E. coli* maintained in non-sterile soil (Germida and Khachatourians, 1987). When microorganisms are introduced to the environment in large numbers, horizontal gene transfer has the potential to occur not only in the soil but also on plant surfaces. Phage F116-mediates gene transfer among *P. aeruginosa* strains on the same leaf surface (Kidambi, Ripp, *et al*, 1994). In conditions of high density planting, such as might occur in intensive farming, transduction has been observed between bacterial strains on adjacent plants.

As early as 1930, d'Hérelle, one of the discoverers of bacteriophages suggested their use as therapeutic agents for treatment of infectious disease. Despite this early enthusiasm, the use of bacteriophages as antibacterial agents was all but abandoned with the discovery of antibiotics in the 1940s. The use of bacteriophages as treatment agents actually dates back to 1921 when it was initiated by Bruynoghe and Maisin in the treatment of staphylococcal skin infections (Bruynoghe and Maisin, 1921). The process' initial lack of success, due to misunderstanding of phage biology, coupled with the discovery of antibiotics, led to very little research in subsequent years. Recently, the rise in antibiotic resistance in major bacterial pathogens and the emergence in clinical practice of inherently multiple antibiotic resistant organisms such as *B. cepacia,* has led to increased investigation into the application of phages as treatment of life-threatening bacterial infections. Current opinion

is that bacteriophage therapy is likely to be focused on veterinary practice, in the first instance, perhaps paving the way for its later use in human infections (Dixon, 1987).

The range of phage therapy applications is wide and this therapeutic strategy has found particular favour in Eastern Europe. Phage therapy has been applied in the treatment of infections induced by *Staphylococcus*, *Klebsiella*, *Escherichia*, *Proteus* and *Pseudomonas* as well as infections in the digestive sytem caused by *Salmonella* and *Shigella* (Slopek, Durlakowa, *et al*, 1983). Although oral and localised treatment seems to be the standard method of phage delivery (Slopek, Durlakowa, *et al*, 1983), even when oral treatment has to be abandoned because of nausea and vomiting, direct application of phage to infected sites has been enough to produce healing (Cislo, Dabrowski, *et al*, 1987).

In conclusion, bacteriophages are not only useful for typing bacterial strains, but they function as vectors for the transmission of genetic material and show potential for use in the treatment of bacterial infections, a property which might be particularly relevant to the treatment of infections caused by emerging multiresistant pathogens such as *B. cepacia*.

1.7.2. Conjugation

Conjugation accounts for most horizontal gene transfer between phylogenetically distant

prokaryotes. The prime requirement for conjugation, as a system of genetic exchange, is usually a plasmid that will promote chromosome transfer such as the F plasmid of *E.coli* K12. Although such plasmids have been difficult to detect in other organisms, clinical strains of *P. aeruginosa* frequently carry plasmids capable of acting as fertility factors (Dean, Royle, *et al*, 1979; Holloway, 1998). Drug resistance plasmids have also been shown to promote chromosome transfer (Sugino and Hirota, 1962). Of even greater interest to the issue of genetic exchange are those plasmids that replicate in a range of unrelated Gram-negative bacteria and can be transferred freely among different genera (Lowbury, Kidson, *et al*, 1969). Conjugal transfer of broad-host-range, incompatibility group P (IncP-type) plasmids between Gram-negative bacteria is well known (Schäfer, Kalinowski, *et al*, 1990), and is reviewed by Thomas and Smith (1987). These and similar groups of plasmids such as IncC, IncN, IncQ and IncW are self-transmissible at high frequency. Incompatibility plasmids are those that share either replication control or partitioning function and thus compete for stable inheritance (Thomas and Smith, 1987). The host range of IncP type plasmids includes several Gram-negative bacteria, in fact, *Bacteroides* is the only Gram-negative genus that has been documented as being non-permissive for IncP plasmid maintenance (Guiney, Hasegawa, *et al*, 1984). It is not just plasmids that can be transferred by conjugation, conjugative transposons have been isolated from a number of Gram-positive and Gram-negative bacteria (Torres, Korman, *et al*, 1991, Bertram, Strätz, *et al*, 1991). Conjugative transposons have broad host range and transpose into the genomes of a wide variety of Gram-positive and some Gram-negative hosts.

For the transfer of plasmids, stable cell to cell contact is required between bacterial cells in the initial stages of conjugation. The donor cell contains the plasmid that has a transfer (*tra*) locus that encodes the fertility pilus which attaches to the recipient cell and generates a conjugal pore through which the DNA will enter. The donor cell's plasmid also encodes mobilisation (*mob*) genes which promote the transfer of DNA through interaction with another region called the origin of transfer (*oriT*) (Buchanan-Wollaston, Passiatore, *et al*, 1987). During bacterial conjugation a linear single strand of DNA is transferred from a donor to a recipient cell where it is regenerated into a double-stranded DNA molecule. Successful conjugation requires the plasmid to replicate either autonomously or to be integrated into the replicon of the new host.

In a study of the survival, plasmid transfer and impact of *Pseudomonas fluorescens* introduced into soil, it was found that although the introduced donor and recipient strains showed a progressive decline in numbers in non-sterile soil, the strains still caused stimulation of total numbers of heterotrophic bacteria and selected physiological groups (Kozdroj, 1997). Conjugation between the donor and recipient cell was observed as early as within the first three days of the strains being introduced to the soil. Thus although bacteria introduced to the soil may show limited survival, their potential for conjugation and impact on soil microflora may still cause an ecological risk.

Plasmids have been isolated from several *B. cepacia* strains (Gonzalez and Vidaver, 1979) but in most cases, no phenotypic characteristics are assigned to them. From their work, Gonzalez and Vidaver postulated that tetracycline resistance in *B. cepacia* may be plasmid-determined. A different group isolated a plasmid pJW2 which conferred tetracycline resistance to a clinical isolate of *B. cepacia* (Williams, Yeggy, *et al*, 1979). The same group also identified a plasmid pJW3 which was responsible for resistance of *B. cepacia* to ampicillin. Later work identified a 32 MDa IncP plasmid Rms45 in a clinical isolate *B. cepacia* GN11131, which mediated resistance to streptomycin and mercury (Hirai, Iyobe, *et al*, 1982). Interestingly, this plasmid could be transferred not only to other *B. cepacia* isolates by transformation but also to *P. aeruginosa* and *E .coli,* as well as by conjugation between isogenic strains of *P. aeruginosa* and *E. coli*.

1.7.3. Transformation

Genetic transformation is a process by which a cell takes up naked DNA from the surrounding medium and incorporates it to acquire an altered genotype that is heritable. Bacteria and possibly some yeasts are the only organisms which can undergo natural transformation (Smith, Danner, *et al*, 1981). Cells with this ability for DNA uptake are described as competent; such cells express specialised proteins that allow them to bind and take up large pieces of DNA from their environment. In most microorganisms, natural genetic competence is regulated, that is, expression of the proteins that form the DNA-

uptake complex is switched on and off. Chromosomal and plasmid DNA released into the environment after cell lysis is stabilised against nucleases degradation by adsorption to particulate matter (Aardema, Lorenz, *et al*, 1983, Lorenz and Wackernagel, 1987) and this protection from degradation depends on the type of particulate matter involved (Lorenz and Wackernagel, 1987). DNA taken up during natural competence can efficiently replace homologous regions of the chromosome (Solomon and grossman, 1996) and thus add to the genetic plasticity of the host.

Although very little research on transformation in *B. cepacia* has been carried out, *B. cepacia* plasmid DNA has been shown not only to transform other *B. cepacia* strains but also to transform the major CF pathogen and soil inhabitant *P. aeruginosa* (Hirai, Iyobe, et al, 1982, Sabaté, Villanueva, *et al*, 1994). Thus, available evidence from *in vitro* experiments indicates that *B. cepacia* complex strains could exchange genes between themselves and with strains belonging to different genera, through transformation.

In conclusion, *B. cepacia*, an opportunistic pathogen of life-threatening and transmission in compromised hosts such as CF patients, could be introduced into the environment in bulk quantities as a biopesticide and bioremediation agent. *B. cepacia* also has a large, complex and highly plastic genome (Cheng and Lessie, 1994; Rodley, Romling, *et al*, 1995). Exchange of genetic material between the introduced *B. cepacia* complex isolates and soil flora is a probable event with as yet unknown consequences.

Aims of thesis

The *B. cepacia* complex, representing at least seven newly described genomovars (*B. cepacia* genomovar I, *B. multivorans*, *B. cepacia* genomovar III, *B. stabilis*, *B. vietnamiensis*, *B. cepacia* genomovar VI and *B. ambifaria*) comprises nutritionally adaptable, multiresistant, soil and water organisms. These organisms have a large genome with several different transposable elements. The transposable elements impart a considerable potential for re-arrangement of the *B. cepacia* complex genome. Although previously known as a plant pathogen, *B. cepacia* complex bacteria are now recognised as a major cause of fatal pulmonary infections and septicaemia in immuno-compromised individuals, in particular, patients with cystic fibrosis and chronic granulomatous disease. Highly transmissible strains responsible for major epidemics have been associated with fatal or life-limiting infections in both CF and non-CF patients. The potential threat of the *B. cepacia* complex to CF patients is particularly well recognised. In ironic contrast, agricultural microbiologists are seeking commercial licenses to exploit the antifungal and nutritional adaptability of these bacteria as biopesticides and agents for soil bioremediation. At present, potential hazards of introducing large quantities of the *B. cepacia* complex to the environment on the human population and on the normal microbial flora cannot be assessed accurately, due to lack of adequate information on the pathogenicity, taxonomy, ecology and epidemiology of these challenging and complex bacteria. The aims of this thesis are four-fold and focus on the pathogenicity and virulence markers of the *B. cepacia*

complex and the potential threat that biotechnological *B. cepacia* complex strains pose for humans.

1. A study of the general characteristics and biological properties of the *B. cepacia* complex, with emphasis on candidate biocontrol strains and strains of clinical interest.
2. Investigation of the prevalance of the different genomovars of the *B. cepacia* complex in the natural environment. Despite *B. cepacia* genomovar III being isolated with the highest frequency from the respiratory tracts of CF patients, prior to this thesis there were no reports of the isolation of genomovar III from natural environments.
3. Evaluation of the putative virulence and epidemic strain markers of *B. cepacia* and comparison of the potential pathogenicity of environmental and candidate biopesticide strains with that of clinical strains.
4. Investigation of potential mechanisms of genetic exchange and alteration, firstly within the *B. cepacia* complex and secondly, between *B. cepacia* and other organisms. Of particular interest, would be genetic exchange between candidate biopesticide strains, strains isolated from the environment and strains of clinical origin.

CHAPTER 2

Materials and Methods

2.1. MATERIALS

2.1.1. Bacterial strains

The bacterial strains used are shown in Tables 2.1 and 2.2, and were obtained from the Cystic Fibrosis Laboratory Collection (Department of Medical Microbiology, University of Edinburgh) and included strains from the recently created *B. cepacia* strain panel (Mahenthiralingam, Coenye, *et al*, 2000). Information on the taxonomy of the strains was obtained from Dr. P. A. R. Vandamme, (Laboratorium voor Microbiologie, Universiteit Gent, Belgium).

2.1.2. Chemicals and Media

Unless otherwise stated all the media were provided by Oxoid Ltd., Basingstoke, Hampshire, England and all the chemicals by Sigma-Aldrich Company Ltd., Dorset, England. All media were prepared in distilled water and sterilised by autoclaving at 121°C 15 psi for 15 min.

Columbia agar base (CAB): 39g L^{-1}

Iso-sensitest agar: 31.4g L^{-1}

Nutrient broth and Yeast extract (NBYE): Nutrient broth No.2 plus 0.5% yeast extract (Difco Laboratories, Detroit, Michigan, U.S.A.)

Nutrient agar buffered (NAB): Nutrient agar buffered with 0.45g/L KH_2PO_4 and 2.39g/L $Na_2HPO_4.12H_2O$

Burkholderia cepacia medium (BCSM): (MAST Diagnostics, Bootle, Merseyside) 32.5g L^{-1} were sterilised and cooled to approximately 60°C before adding one selactab (MAST) per 100ml of agar. The final concentration of antibiotics was 100µg ml^{-1} of ticarcillin and 300 units ml^{-1} of polymyxin B.

Tryptone soya agar (TSA**):** 40g L^{-1}

MacConkey Agar(without salt): 47g L^{-1}

Pseudomonas agar F (Difco): 38g L^{-1} with 1% glycerol added prior to sterilisation

Minimal salts NH_4Cl 20g, NH_4NO_3 4g, Na_2SO_4 anhydrous 8g, K_2PO_4 anhydrous 12g, KH_2 PO_4 4g, Mg SO_4 $7H_2O$ 0.4g, salts were dissolved in distilled waterin the order indicated and the volume made up to 1000ml

Minimal agar (MA) made up by mixing: 300ml sterile bacteriological agar No 1 solution, 100ml minimal salts and 4ml 20% glucose solution

Phosphate buffered saline(PBS): one tablet dissolved in 100ml of water and sterilised.

Saline: 0.85% NaCl (w/v), sterilised.

Skimmed milk: Skimmed milk powder 10% (w/v) sterilised by autoclaving at 121°C 15 psi for 5 min.

Table 2.1: *Burkholderia cepacia* complex isolates used in this thesis

Strain	Source and Location	Reference
B. cepacia		
genomovar I		
CEP 509	CF, Australia	Mahenthiralingam *et al*, 2000
C1963	CF, Edinburgh, UK	Maxwell, 2000
ATCC 17759	Soil, Trinidad	Mahenthiralingam *et al*, 2000
ATCC 25416	Onion, USA	Mahenthiralingam *et al*, 2000
J2535	Rotting bark, Edinburgh, UK	Butler *et al*,1995
J2540	Soil, Edinburgh, UK	Vandamme *et al*, 1997
J2552	Soil, Edinburgh, UK	Butler *et al*,1995
LMG 17997	UTI, Sweden	Mahenthiralingam *et al*, 2000
B. multivorans		
CF-A1-1	CF epidemic, UK	Mahenthiralingam *et al*, 2000
C1052	CF, Edinburgh, UK	Maxwell, 2000
C1524	CF, Edinburgh, UK	This thesis
C1572	CF, Manchester, UK	This thesis
C1576	CF, epidemic Glasgow, UK	Mahenthiralingam *et al*, 2000
C1652	CF, Cardiff, UK	This thesis
C1670	CF, Glasgow, UK	This thesis
C1857	CF, Dundee, UK	This thesis
C1911	CF, Edinburgh, UK	This thesis
C2775	CF, Aberdeen, UK	Nzula *et al*, 2000
C5393	CF, Canada	Mahenthiralingam *et al*, 2000
LMG 13010	CF, Belgium	Mahenthiralingam *et al*, 2000
JTC	CGD, USA	Mahenthiralingam *et al*, 2000
C1962	Pus from brain abscess, UK	Hobson *et al*, 1995

Table 2.1. cont.

Strain	Source and Location	Reference:
ATCC 17616	Soil, USA	Mahenthiralingam *et al*, 2000
J2395	Hospital plant and soil, UK	This thesis
J2866	Rhizosphere, Edinburgh, UK	This thesis
J2867	Rhizosphere, Edinburgh, UK	This thesis
J2868	Rhizosphere, USA	This thesis
249-2	Laboratory, USA	Mahenthiralingam *et al*, 2000
B. cepacia **genomovar III**		
BC7	CF epidemic, Canada	Mahenthiralingam *et al*, 2000
CEP 511	CF epidemic, Australia	Mahenthiralingam *et al*, 2000
C517	CF, Edinburgh, UK	This thesis
C1335	CF, Edinburgh, UK	This thesis
C1394	CF, epidemic, Manchester, UK	Mahenthiralingam *et al*, 2000
C1632	CF, epidemic, Newcastle, UK	Maxwell, 2000
C2374	CF, Edinburgh, UK	This thesis
C5424	CF, epidemic, Canada	Mahenthiralingam *et al*, 2000
C6433	CF, epidemic, Canada	Mahenthiralingam *et al*, 2000
K56-2	CF, epidemic, Canada	Mahenthiralingam *et al*, 2000
PC184	CF, epidemic, USA	Mahenthiralingam *et al*, 2000
J415	CF, Edinburgh, UK	Mahenthiralingam *et al*, 2000
715j	CF, USA	Abe, Nakazawa, 1996
J2315	CF, epidemic, Edinburgh, UK	Mahenthiralingam *et al*, 2000
ATCC 17765	UTI, UK	Mahenthiralingam *et al*, 2000
C1773	Blood culture, Papworth, UK	Maxwell, 2000

Table 2.1 cont.

Strain	Source and Location	Reference:
***B. cepacia* genomovar III**		
R2817	Rice rhizosphere, Philippines	This thesis
R2827	Rice rhizosphere, Philippines	This thesis
R8351	Wheat rhizosphere, Australia	This thesis
R8571	Rice rhizosphere, Philippines	This thesis
R8574	Rice rhizosphere, Philippines	This thesis
R8580	Rice rhizosphere, Philippines	This thesis
R8605	Rice hizosphere, Philippines	This thesis
R9235	Rice rhizosphere, Philippines	This thesis
R9239	Rice rhizosphere, Philippines	This thesis
R9243	Rice rhizosphere, Philippines	This thesis
R9335	Wheat rhizosphere, Australia	This thesis
R9342	Wheat rhizosphere, Australia	This thesis

Table 2.1 cont.

Strain	Source and Location	Reference:
B. stabilis		
LMG 14294	CF, Belgium	Mahenthiralingam *et al*, 2000
C7322	CF, Canada	Mahenthiralingam *et al*, 2000
LMG 18888	Blood, Belgium	Mahenthiralingam *et al*, 2000
LMG 14086	Respirator, UK	Mahenthiralingam *et al*, 2000
B. vietnamiensis		
LMG 6988 (C2972)	Blood, Sweden	Vandamme *et al*, 1997
LMG 6999 (C2973)	Neck abscess, Sweden	Nzula *et al*, 2000
FC 441	CGD	Mahenthiralingam *et al*, 2000
C1704	CF, Edinburgh, UK	This thesis
LMG16230 (C2977)	CF, Belgium	This thesis
LMG16234 (C2979)	CF, Sweden	This thesis
LMG 16232	CF, Sweden	Mahenthiralingam *et al*, 2000
PC 259	CF, USA	Mahenthiralingam *et al*, 2000
ATCC 53266	Corn roots, USA	Nzula *et al*, 2000
ATCC 53267	Corn roots, USA	Nzula *et al*, 2000
ATCC 29424	Soil, USA	Nzula *et al*, 2000
LMG10823 (C2974)	Soil, Indonesia	This thesis
LMG10824 (C2975)	Rice root, Indonesia	This thesis
LMG10929 (C2976)	Rice, Indonesia	Gillis *et al*, 1995
AMMD*	Pea rhizosphere, USA	King, Parke, 1993
LMG 10929	Rice, Vietnam	Mahenthiralingam *et al*, 2000
J1697	CDC, Atlanta	This thesis
J1705	CDC, Atlanta	This thesis
Indefinite		
C1511 I-III-IV	CF, Manchester, UK	Maxwell, 2000
C1763 I-III-IV	CF, Manchester, UK	This thesis
C2008 I-III	CF, Liverpool, UK	This thesis
C2349 I-III	CF, Glasgow, UK	This thesis
ATCC 25608	Incision wound, USA	This thesis
J2891	Cornfield, USA	This thesis
C3039	Rice, Canada	This thesis
C3041	Rice, Canada	This thesis
ATCC 10856	Onion, USA	This thesis

* now *B. ambifaria* (*B. cepacia* genomovar VII)

Table 2.2. Other bacterial isolates used in this thesis

Strain	Description
Pseudomonas aeruginosa	
PAO1	Non-CF clinical isolate, classic laboratory strain
NTCC 10662	Control strain for antibiotic sensitivity testing
Escherichia coli	
HB101	Contains the plasmid pLof with a Hg resistance marker, commonly used for large-scale plasmid production
JM 109	Commonly used strain that will support growth of vectors carrying amber mutations.
Burkholderia gladioli	
ATCC 10247	Isolated from Gladiolus, USA

2.1.3. Maintenance of bacteria

Bacteria were cultured on CAB overnight at 37°C and a bacterial suspension made in skimmed milk was stored at -70°C.

2.1.4. Media for the isolation *of B. cepacia* from the rhizosphere

Malka minimal media

Malka minimal media (Robert-Gero Poiret, *et al*, 1970) comprised the following reagents dissolved in distilled water;

Solution A: $Na_2 HPO_4$, 73.4g L^{-1} and KH_2PO_4, 32.4g L^{-1} and 2-3 drops of $CHCl_3$ added, pH 7.0.

Solution B: Mg_2SO_4, 20.5g L^{-1} and 2-3 drops of $CHCl_3$ added.

Solution C: 1M mono-sodium L-glutamate, filter sterilised

Solution D: $FeSO_4$ $7H_2O$ 1.83g $L^{-1}$and one drop of concentrated HCl added

Solution E: $(NH_4)_2SO_4$ 50g L^{-1}, filter sterilised

The sterile solutions were mixed by adding; 8ml solutions A, B, C, E, 0.4ml solution D, 2ml of sterile 50% glucose, 400ml sterile distilled water and 300μg ml^{-1} of filter sterilised polymyxin B sulphate (Sigma). 10ml of the mixture was dispensed into sterile universal tubes.

Arginine -glucose media

Arginine-glucose medium (Stewart, 1971) was prepared as follows;

The indicator mixture was made up by adding 0.03 g cresol red (BDH, Merk Ltd., Dorset, England) and 0.02g bromothymol blue (BDH) to 1ml of 1N NaOH and 99ml of distilled water and heating on a hot plate for 10 minutes to dissolve the dyes.

30ml of indicator mixture were added to1.0g Nutrient broth No 2, 0.5g L-arginine (Oxoid), 5.0g Glucose (Sigma), 4.0g Agar No 3 and 459.9ml distilled water. The pH was adjusted to 7.4 before the medium was sterilised, allowed to cool and 10ml dispensed into sterile glass test tubes. Once the media had set on a slope it was stored at

4°C for up to two weeks.

PCAT medium

PCAT medium (Burbage and Sasser, 1982) was prepared as follows:

0.2g $MgSO_4$. $7H_2O$ and 2g azelaic acid (ICN Biomedicals Ltd., Oxfordshire, England) were added to 0.5L of distilled water was heated while stirring using a magnetic stirrer in a large flask with until the later had dissolved. Once the solution had cooled the following were added, 0.2g tryptamine ((ICN) dissolved in a large drop of ethanol), 4g KH_2PO_4, 4g K_2PO_4, 0.02g yeast extract 15g agar No1. The volume of the medium was then made up to 1L with distilled water. The pH was adjusted to 5.7 before sterilisation. A filter sterilised alcoholic solution of cycloheximide (Sigma) 200mg L^{-1} was added before the plates were poured. The medium was kept at 4°C and used within 48h.

2.1.5. Buffers for pulsed field gel electrophoresis

SE buffer:

4.38g of NaCl and 9.30g of EDTA Na_2 were dissolved in 900ml distilled water, the pH adjusted to 7.5, and after the volume had been made up to 1L, the buffer was filter-sterilised and stored at 4°C.

Lysis buffer:

1 g N-lauroylsarcorsine and 18.6g EDTA Na_2 were added to 80ml distilled water and approximately 10ml of 10M NaOH to dissolve the EDTA. After adjusting the pH to 9.5

and the volume being made up to 100ml, the buffer was filter-sterilised and stored at room temperature.

TE buffer:

1.21g Tris (hydroxymethyl)-aminomethane (Bio-Rad, Laboratories Ltd., Hertfordshire, England) and 3.72g EDTA were dissolved in 900ml of distilled water. After adjusting the pH to 7.5 and the volume being made up to one litre, the buffer was filter-sterilised and stored at 4°C.

2.1.6. Media for the selection of transformed isolates

LB media for the selection of *E. coli* JM109 cells transformed with pGEM containing the *dsb* fragment was made up as follows:

3g of Agar No1 and 4g of LB broth (Gibco BRL, Life Technologies Ltd., Paisley, Scotland) were dissolved in 200ml distilled water and sterilised. When the agar had cooled to approximately 60°C, filter-sterilised ampicillin was added to a concentration of 100mg L^{-1}; then 40μL of IPTG (Promega UK, Southampton, England) was added from a 1g stock solution and 200μL of X-gal (Promega) was added from a 50mg ml^{-1} stock solution. After mixing thoroughly and gently, 20ml was poured into a sterile petri dish and allowed to set.

2.1.7.Media and reagents for cellular fatty acid analysis

Trypticase soy broth agar (TSBA) for growing aerobic bacteria for cellular fatty acid analysis was prepared by combining the following reagents in a Duran bottle.

30g Trypticase soy broth (BBL Microbiology Systems, Becton Dickinson European HQ, Erembodegem, Belgium), 15g Granulated agar (Difco) and 1L of distilled water.

The mixture was stirred to dissolve the reagents and autoclaved for 15 min at 121°C. After cooling in a water bath to 60°C, the agar was dispensed aseptically into sterile petri dishes, allowed to solidify and the plate kept at 4°C until required.

Reagents for fatty acid analysis were supplied by members of staff at the Laboritorium voor Microbiologie, Universiteit Gent.

Saponification reagent: The saponification reagent consisted of the following;

45g NaOH (Merck Ltd., Dorset, England), 150ml Methanol and 150ml de-ionised water. The NaOH pellets were added to water and methanol while stirring and the stirring continued until the pellets had dissolved.

Methylation reagent: 325 ml 6.0N HCl and 275ml Methanol (Merck)

The HCl was added to the methanol while stirring.

Extraction Solvent: 200ml Hexane (Merck) and 200ml

Methyl-tertiary Butyl Ether, MTBE, (Merck). The MTBE was added to hexane while stirring

Base wash: 10.8g NaOH (Merck) and 900ml deionised distilled water. The NaOH pellets were added to water while stirring and the stirring continued until the pellets had dissolved.

2.2. METHODS

2.2.1. Isolation of *Burkholderia cepacia* from the environment

B. cepacia was isolated from 10g of soil collected around the rhizosphere of plants found in different locations within the United Kingdom.

2.2.1.1. Isolation of *B. cepacia* by antibiotic selection

B. cepacia was isolated by modification of the Butler method (Butler *et al*, 1995). 10g of soil were suspended in 10ml of Malka minimal media and incubated at 37°C for five and 12 days. 100µl of each soil suspension was then spread on the surface of BCSM agar plates using a sterile glass spreader and the plates incubated at 30°C for 48h. Each different colonial morphotype was picked and sub-cultured on CAB until pure cultures were obtained. Suspensions of the pure cultures were made in 0.85% saline and then inoculated, using a multi-point inoculator (A400 Multipoint inoculator, Denley, Billinghurst, Surrey), onto CAB, which was incubated at 4, 37 and 41°C. The same cultures were also inoculated onto MacConkey agar and *Pseudomonas* agar F in order to

identify and exclude lactose fermenting and fluorescent organisms respectively. The remaining cultures were tested for oxidase production and those that gave positive results were sub-cultured onto arginine-glucose medium. The results were read after 24, 48 and 120h incubation at 30°C. All isolates that gave a yellow slope with the bottom of the tube remaining green and maintained this pattern even after five days incubation, were presumptively identified as *B. cepacia* and were sub-cultured onto CAB. After overnight incubation at 30°C, the isolates were identified using the API 20NE (bio Mériex, Marcy l'Etoile, France) according to the manufacturer's instructions.

2.2.1.2. Isolation of *B. cepacia* using PCAT medium

10g of soil were suspended in 10ml in 0.85% saline and left at ambient temperature for three hours. 100µl of each soil suspension was spread on the surface PCAT medium agar plates using a glass spreader and the plates incubated at 30°C for 48h. Each different colony was picked and sub-cultured on CAB until pure cultures were obtained. The pure cultures were subsequently treated as described for the pure cultures in 2.2.1.1.

2.2.2. Determination of MIC

The agar dilution method for MIC determination was used following guidelines outlined in a guide to sensitivity testing, (British Society for Antimicobial Chemotherapy, 1991). Antibiotics dissolved in the appropriate solutions were filter-sterilised before being

added to cooled Iso-Sensitest agar. The antibiotic plates were stored overnight at 4°C before use. Overnight cultures of test organisms were diluted and inoculated onto the antibiotic plates using a multipoint inoculator, giving a final inoculum of 10^4 cfu for each spot. The plates were incubated overnight at 37°C in air or in air with 5% CO_2 as appropriate. MIC was recorded as the lowest concentration of antimicrobial inhibiting the visible growth of each test organism after overnight incubation. The tests were repeated three times with *P. aeruginosa* NCTCC 10662 as the control organism.

2.2.3. Bacteriocin typing

Bacteriocin typing was carried out using previously described methods (Govan, Harris, 1985). Six producer and eight indicator *B. cepacia* isolates were grown in CAB plates overnight at 30°C. Suspensions of each isolate to be tested and each producer strain, also grown overnight in CAB plates, were made in 0.9 ml of 0.85% sterile saline and mixed by vortexing before inoculating plates containing 10ml of tryptone soy agar using a multi-point inoculator. The plates were incubated at 30°C for 5.5 h. Suspensions of indicator and test strains were made in Wasserman tubes containing 2.5ml nutrient broth and incubated at 30°C for 4h. After incubation, the plates inoculated with producer and test strains were placed over filter paper soaked in chloroform for 15 min in a fume hood. The plates were then exposed to air for a further 15 min. 100µl of broth culture containing indicator or test strains was mixed with 2.5ml of overlay agar, mixed and poured over the plates inoculated with test strains or producer strains respectively. After

the agar had set, the plates were incubated overnight at 30°C and results interpreted as previously described (Govan and Harris, 1995). The overlay agar was made by adding 1g bacto-peptone and 0.3g agar to 100ml distilled water, sterilised and 2.5ml aliquots dispensed in sterile Wasserman tubes and kept warm until required.

2.2.4. Pulsed–field gel electrophoresis

Overnight cultures of each isolate in NBYE were centrifuged at 3 200 x g for 10min and the pellet re-suspended in SE buffer. The inoculum was standardised to OD 590 of 1.5 and plugs of 1 % low melting agarose (Bio-Rad) were cast in a plug mould which had previously been wiped in 70% ethanol.

Lysis of plugs

The set plugs were transferred to sterile bijou bottles with 2ml of lysis buffer containing 0.05% Triton-X 100 before incubating overnight at 56°C in a water-bath. Lysis buffer was replaced by three changes of an equal volume of TE buffer with incubation at 4°C for 30min with each change.

Restriction of DNA in agarose

Restriction of DNA in agarose was carried out on a 2.5mm x 9mm portion in a sterile Eppendorf tube. 90µl of sterile distilled water was mixed with 10µl 10x reaction buffer2 (Gibco BRL) and left at 4°C for 30min. The buffer was replaced with 90µl of sterile

distilled water, 10μl 10x reaction buffer2, 2μl of 10mgml^{-1} bovine serum albumin, 2μl 0.1M dithiothriotol and 2μl *Xba*I (Gibco BRL), mixed and incubated overnight in a water-bath at 37°C.

Separation of DNA fragments

The enzyme mixture was replaced with TE buffer before being used. A 1% pulse field agarose gel was prepared in 0.5 x Tris Borate EDTA (TBE) buffer, allowed to set and the plugs loaded. The plugs were sealed using some molten agarose left over from the gel. Pulsed-field gel electrophoresis was carried out in a CHEF-DR II tank (Bio-Rad) using 2L of 0.5%TBE. The gel was run for 20 h at a constant temperature of 14°C and voltage of 200V, with pulse times of 2.9 to 35.4s and a ratio of 1.0.

Following electrophoresis, the gel was stained for 15min in 1μgml^{-1} solution of ethidium bromide (Bio-Rad), destained twice using distilled water and shaking for 15min and the bands viewed under a UV transilluminator. Photographs were taken using a polaroid camera.

2.2.5. Production of extracellular enzymes

Overnight broth cultures of *B. cepacia* isolates and *P. aeruginosa* PAO1in NBYE were washed in saline, diluted to a concentration of 10^8 cfu ml^{-1} and then inoculated onto appropriate substrates (see following sections). The plates were incubated at 37°C and

observed for clearing of the media around the bacterial growth after 72hours.

Production of Caseinase

Four different media were used for the detection of caseinase. These were made up by mixing equal quantities of each sterile medium with 10ml of sterile skimmed milk (Oxoid), 10% w/v. The basic media included: a 3% w/v solution of agar bacteriological (Agar No1), tryptone soya agar, tryptone soya agr with 5% peptone (w/v) (Difco) and LB Broth (Gibco BRL) with 3% w/v solution of agar bacteriological (Agar No1).

Production of Elastase

Elastase production was determined on media containing 2% (w/v) Agar No 1 which was sterilised and allowed to cool before adding 1% Elastin-congo red (ICN), which had been decontaminated by placing under a UV lamp for 15min. 20ml medium was dispensed into sterile petri dishes and allowed to set at room temperature before use. Elastase production was observed as a zone of clearing around the bacterial growth.

Production of lipase

Production of lipase was tested on CAB with 5% egg yolk emulsion added to the cool, sterile agar. The plates were inoculated with a multipoint inoculator, incubated at 37°C for 48h and examined for a pearly sheen around the bacterial growth.

Production of catalase

Production of catalase was tested by placing one or two bacterial colonies on a drop of hydrogen peroxide on top of a microscope slide and observing for the production of effervescence within 20 seconds.

2.2.6. Pathogenicity of *B. cepacia* and *B. gladioli* on onions and potatoes.

Peeled white and red onions and washed baking potatoes, all of British origin, were sliced into rings and placed into sterile petri dishes lined with Kimberly Clarke wipes soaked in sterile distilled water. The rings were inoculated with 1ml of an overnight culture of *B. cepacia* J2315, AMMD or *B. gladioli* ATCC 10247 giving a final concentration of 10^8 cfu and incubated at 30°C for five days. Controls were inoculated with an equal volume of sterile distilled water before incubation for a similar period. After five days the plates were examined for tissue maceration and discolouration.

2.2.7. Isolation and propagation of *B. cepacia* bacteriophages

Overnight NYBE cultures of 38 *B. cepacia* isolates were harvested at 3 200 x g for 30 min and the supernatants filtered using 0.2μm syringe filters (Gelman Sciences, Ann Arbor, U.S.A.). The supernatants were screened for the presence of phage by spotting onto bacterial lawns of the same panel of isolates, using a multipoint inoculator.

A single phage plaque on an agar plate was removed by stabbing into the medium using a sterile glass pipette. This was transferred to 1.5ml of phage buffer, centrifuged at 13 000 x g and the supernatant collected. Stock phage preparations were made by filtration as stated above.

High-titre phage preparations were made as follows: 100µl of a phage preparation were added to 2.5ml molten soft nutrient agar, previously seeded with 100µl of an overnight culture of the propagating strain. The mixture was then overlayed on CAB plates and allowed to set. After 18 h at 37°C, overlays showing semi-confluent lysis were transferred into 10 ml phage buffer (10mM-Tris/HCl, pH 8.0,10mM $MgCl_2$) (Matsumoto, Itoh, *et al*, 1986). The lysate was then vortexed, centrifuged at 3 200 x g for 30 min and the supernatant filtered. Phage titres were determined as plaque-forming units (pfu ml^{-1}) in soft agar overlays. Stock preparations were maintained at 4°C.

2.2.8. Isolation of antibiotic resistant bacterial mutants

Spontaneous bacterial mutants were isolated by spreading 100 µl of overnight broth cultures of *B. cepacia* (approximately 10^8 cfu ml^{-1}), on the surface of iso-sensitest agar containing antibiotic at concentrations ranging from the basic MIC to 32 x the MIC. After incubation at 37°C in air for 48h, mutational frequencies were calculated from results obtained with the highest concentration of antibiotic on which resistant mutants

were detected. The mutants were cultured again on the medium containing the same concentration of antibiotic and maintained in skimmed milk at -70 ^{0}C.

2.2.9. Host range of *B. cepacia* phages

Stock phage solutions, diluted in phage buffer to approximately 10^{8} pfu ml^{-1} were spotted on to lawns of *B. cepacia* and *P. aeruginosa* using a multipoint inoculator and examined for lytic activity after 24 h at 37°C.

2.2.10. Extraction of phage DNA

Phage DNA was extracted using a previously described method (Matsumoto, Itoh, *et al*, 1986). Genome size of unrestricted DNA was estimated by PFGE (initial and final pulse time of 5s and 20s, 170V at 14ºC for 19h) incorporating a λ ladder DNA PFGE standard kit (New England Biolabs, Hitchin, Hertfordshire,UK).

2.2.11. Extraction of LPS

B. cepacia LPS was extracted using the aqueous phenol method of Westphal and Luderitz (1954) as modified by Hancock and Poxton (1988). Bacteria were grown for 48 hours in NBYE at 37ºC in an orbital incubator (Gallenkamp, Loughborough, Leics)

at 120 rpm. The cells were harvested by centrifugation at 10 000 x g for 15min and washed twice in PBS. The pellet was incubated overnight at -70ºC before being lyophilised over 48 hours (Edward Modylo freeze dryer, Edwards High Vacuum Ltd., Surrey, UK). Dried cells were resuspended to 5% w/v in pyrogen-free water and then placed in a 67ºC water-bath in a fume cupboard. An equal volume of 90% phenol was also heated to 67ºC before being mixed with the cell suspension and holding at the same temperature for 15 min with occasional mixing. The mixture was cooled in an ice bath and then centrifuged at 10 000 x g for 15min at 4ºC in sterile pyrogen-free glass tubes. The upper aqueous layer containing the LPS was transferred to dialysis tubing and dialysed overnight against running tap water and then for 4h with two changes of pyrogen-free water. After ultracentrifugation at 100 000 x g for 3h at 4ºC, the pellet was resuspended in a minimal volume of pyrogen-free water using a syringe and needle and then frozen and lyophilised as described above. The LPS was stored at -20ºC.

2.2.12. Neutralisation of phage activity by LPS

The effect of pre-incubation with LPS on phage activity was determined using a modification of the method described by Govan (Govan, 1974). Phage preparation (0.5ml), containing approximately 10^{3} pfu ml^{-1} was mixed with 0.5 ml of bacterial LPS (20 mg ml^{-1} solution in distilled pyrogen-free water) and incubated at 37ºC for 1 h. Controls comprised phage and buffer without LPS. The experiment was repeated and the results calculated as the means of three experiments.

2.2.13. Transduction

Transduction experiments were carried out using the method described by Krishnapillai (Krishnapillai, 1971). Phages NS1 and NS2 were propagated on the ceftazidime resistant mutants of three *B. cepacia* strains with MICs of 8μg ml^{-1}. Phage lysate (0.5ml) was added to a PBS suspension of the sensitive *B. cepacia* parent strains ATCC 53265, ATCC 29424 and C2973 at a multiplicity of 1 and left for 20 min at 37°C to allow phage adsorption. The cells were harvested at 3 200 x g for 30 min, resuspended in TNM buffer (Tris 0.01M pH 7.4, NaCl 0.15M, $MgSO_4$ 0.01M) (Krishnapillai, 1971) and 100μl spread on CAB containing ceftazidime at 8 ug ml^{-1}. Transductants were recovered after incubation at 37°C for 72h. Controls comprised recipient strains plus TNM buffer alone.

2.2.14. Electron microscopy

Phage lysates were centrifuged at 3 200 x g to remove cell debris, and then at 100 000 x g for 1h. The phage pellet was resuspended in 1M ammonium acetate. After negative staining with 2% (w/v) potassium phosphotungstate solution (pH 7.0), phage morphology was observed with a transmission electron microscope (Hitachi type 12A operating at 60 K).

2.2.15. Extraction of genomic DNA for *dsb* polymerase chain reaction (PCR)

Genomic DNA was extracted using standard methods (Sambrook, Fritsch, *et al*,1998).

2.2.16. Amplification of the *dsb* gene by PCR

Amplification of the *dsb* gene was carried out using the following primers:

Forward primer (dsb^+): d AACTCGAAACGGACGACGAC

Reverse primer(dsb^-): d CCTTGAACATGCCAGGGAAC

All amplifications were carried out in a 50μl volume containing 10μl of reaction buffer (Promega UK, Southampton, England), 5μl of a template DNA sample, 4U of *Taq* DNA polymerase (Promega), 0.13 mM each 2'-deoxynucleoside 5'-triphosphate (Promega), 0.5μM each nucleotide primer and 2.5μl dimethyl sulfoxide (DMSO). The reactions were performed on a DNA thermal cycler (*Gene E*, Techne, Cambridge, UK) for 35 cycles, each cycle consisting of 1min 10s at 94°C for denaturation, 1 min at 62°C for annealing and 1min 30s at 72°C for polymerisation.

10 % gel loading buffer, containing 0.05% bromophenol blue, 0.05% xylene cyanol and 25% glycerol, was added to the PCR products and 20 μl loaded to a gel. A 100bp ladder (New England Biolabs, Beverley, MA, U.S.A.) was used to estimate the size of the PCR product. Gel electrophoresis was carried out on a 1.5% agarose gel in 0.5 x TBE buffer at 50V for 3h. A band of 750bp was presumed to be the *dsb* PCR product. Purity and concentration of the PCR product was determined using optical density measurements (An absorbance of 1 at 260nm is equivalent to $50\mu g ml^{-1}$) (Sambrook, Fritsch, *et al*,

1998).

Sequencing of the *dsb* fragment was performed at the Department of Haematology, The Edinburgh Royal Infirmary with the same primers as those used for PCR.

Ligation of PCR product and cloning vector

pGEM (Promega) was chosen as the cloning vector. In order to determine the amount of insert DNA required for the vector, the following formula was used:

$$\text{ng of insert} = \frac{\text{ng of vector x kb size of insert x vector: insert ratio}}{\text{kb size of vector}}$$

where; the vector: insert ratio is 3:1

size of insert is 742bp = 0.72kb

size of vector is 3000bp = 3kb

Ligations were set up as shown in Table 2.3 and left at 4°C overnight.

Table 2.3. Ligation of product and PCR product reaction mixes

Product	Reaction	Positive control	Back control
10 x ligase buffer	1μL	1μL	1μL
pGEM	1μL	1μL	1μL
PCR product	1μL	none	none
Control insert DNA	none	2μL	none
T4 DNA ligase	1μL	1μL	1μL
Sterile water	6μL	5μL	7μL

2.2.17. Transforming *E. coli* cells

50μL of competent *E. coli* JM109 cells was dispensed into three sterile Eppendorf tubes and 2μL of ligation mix added with a control of just *E. coli* cells. After leaving the cells undisturbed on ice for 30 min, the cells were heat shocked by being placed in a 42°C waterbath for 50s and immediately placed on ice for 5min. 950μL of SOB (Sambrook, Fritsch, *et al*, 1998) was added to each tube and mixed gently before the tubes were incubated at 37°C with gently shaking at 100rpm for 1.5h. Serial tenfold dilutions of the culture were made in sterile 0.85% saline and 100μL spread on LB selection agar plates with 100mgL^{-1} ampicillin, IPTG (Promega) and X-gal (Promega) and incubated overnight at 37°C.

Plates were removed from the incubator and kept at 4°C to aid clearer differentiation between colonies. Single colonies that were less intensely blue than the others were transferred into a sterile Wasserman tube containing 1.5 ml of SOB with $100mgL^{-1}$ ampicillin and incubated with vigorous shaking. Mini preparations of plasmid DNA were made from 1ml of each culture using standard methods (Sambrook, Fritsch, *et al*, 1998) and 0.5ml of each culture was reserved.

A restriction mix for each plasmid extraction was set up to remove the *dsb* fragment from pGEM using the restriction enzyme *Eco* RI (Promega) according to the manufacturer's instructions.

The restricted DNA was separated by electrophoresis on a 1.5% agarose gel in 0.5 x TBE at 60v for approximately 3h. The gel was stained and examined for a band of 742bp. When such a band was detected, the reserved 0.5ml of SOB culture was streaked onto LB agar plates containing $100mgL^{-1}$ ampicillin. Plasmid mini preparations were made, digested and separated by agarose gel electrophoresis as already described.

Large-scale preparation of plasmid DNA was carried out using standard methods (Sambrook, Fritsch, *et al*, 1998). 500ml of sterile LB with $100mgL^{-1}$ ampicillin was inoculated with the isolate harbouring the 742bp band and incubated with shaking at 37°C. The plasmid DNA was purified by precipitation with polyethylene glycol as outlined by (Sambrook, Fritsch, *et al*, 1998).

The purified plasmid DNA was restricted with *Eco*RI and separated by agarose gel electrophoresis as already described.

E. coli HB101 containing the plasmid pLof Hg was recovered from storage in glycerol at -70°C, streaked onto CAB plates and incubated overnight at 37°C. Large-scale preparation of plasmid DNA was carried out as already described and restricted with *Sfi*I (New England Biolabs) according to the manufacturer's instructions.

This digest was incubated at 50°C for 2h leaving a 3' overhang. The restricted DNA was separated on a 0.8% gel in 0.5x TBE at 70v for 2h. The gel was stained in ethidium bromide and a band of about 4kb was excised.

2.2.18. Recovering DNA from agarose gel

The excised band was placed into a sterile 0.5ml PCR tube with a hole at the bottom made with a hypodermic needle. The PCR tube had 80μL of glass beads (Sigma, diameter 212-300 microns) and was held inside a sterile Eppendorf tube. The two tubes were left overnight at -20°C and centrifuged at 13 000 x g for 30min. Ethanol precipitation of the DNA in the liquid that collected at the bottom of the Eppendorf tube was carried out using standard methods (Sambrook, Fritsch, *et al*, 1998).

2.2.19. Creation of blunt ends.

Blunt ends were created in pSN1 using the enzyme *Bbs*I (New England Biolabs), as indicated by the manufacturer. Incubation for 2h at 37°C resulted in a 5' overhang. 1μl of T4 DNA polymerase and 0.5μL dNTP mix (100μM of each dNTP) was added to the restriction mix. This was incubated at 37°C for 5min and the reaction stopped by adding 2μL of sterile 0.5M EDTA.

A reaction mix for pLof was set up by placing 2 μL plasmid DNA (with 3' overhangs resulting from digestion with *Sfi*I) to a strelile PCR tube. 1μL of T4 DNA polymerase (Promega), 0.5μL dNTP mix (100μM of each dNTP) and 46.5μl buffer E (Promega) was added. The mixture was incubated at 37°C for 5min and the reaction stopped by adding 2μL of sterile 0.5M EDTA.

The DNA was purified by two phenol / chloroform extractions followed by ethanol precipitations.

2.2.20. Ligating blunt ends

A ligation mix consisting of blunted pSN1 DNA, pLofHg DNA and ligase buffer (Promega) was set up. This was mixed gently and heated at 45°C for five minutes, cooled on ice for two minutes before adding 2μL T4 DNA ligase (Promega), mixing and

incubating at 16°C for 72h.

2.2.21. Transformation of *B. cepacia* J2315 by electroporation

B. cepacia J2315 was grown in 100ml of LB at 37°C in an orbital incubator to an optical density of 0.6 at λ 595. The cells were centrifuged at 3 200 x g for ten minutes and the pellet was re-suspended in sterile ice-cold 10% glycerol in half the original volume of the media. The process was repeated five times using ice-cold apparatus and solutions each time.

Precipitated DNA from the ligation of pSN1 and pLof was added to 50μL of cells and electroporation carried out on a (Equibo, Easyjetplus) electroporator using the following parameters;
Voltage: 250V, capacity: 25 uF, resistance: 201Ω.

The cells were immediately added to 1ml of cold LB and left to stand on ice for 15min before being incubated at 37°C with gently shaking (100 rpm) for two hours. Ten-fold dilutions were made and 100μL was spread on the surface of BCSM containing $18mgL^{-1}$ of $HgCl_2$. The plates were incubated at 37°C for 72h.

Genomic DNA was extracted and stored at -20°C. After staining in ethidium bromide, a band of about 3kb was excised and the DNA recovered.

2.2.22. Natural transformation of the *B. cepacia* complex

Natural transformation of the *B. cepacia* complex was carried out using modifications of a previously described method (Juni, 1974). *B. cepacia* J2315 transformed with pLof containing the mercury resistance gene was cultured on BCSM plates with $18mgL^{-1}$ mercury chloride. Crude DNA for transformation was prepared by suspending two loopfuls of the transformed *B. cepacia* J2315 in 0.5ml sterile lysing solution (0.05% SDS in 0.15M NaCl and 0.015M trisodium citrate). The suspension was heated at 70ºC for 1h, cooled to room temperature and stored at -20ºC until required.

A grid of squares was marked on the bottom of a CAB plate and each square labeled to correspond with the recipient *B. cepacia* strains in which the *dsb* gene had been identified. A small amount of cell paste from each strain, cultured in either CAB or minimal agar was placed on the corresponding square. A loopful of the crude DNA extract was mixed with the cell paste and spread over an area of approximately 5-8mm. The loop was sterilised between different strain to avoid cross-contamination. A loopful of crude DNA alone was also spread to verify sterility. An equal amount of cell paste was spread on a different CAB plate without the crude DNA extract to serve as a negative control and the plates were incubated overnight at 30ºC.

After overnight incubation, the DNA extract was checked for sterility. A small amount of cell paste was removed from each square of the test and control plates and streaked uniformly on a sector of BCSM plates with $18mgL^{-1}$ mercury chloride after which the

plates were incubated at 30°C for 48h. The assay was repeated three times each, with the recipient strains propagated on both CAB and minimal agar.

2.2.23. Isolation of Plasmids

Overnight cultures of *B. cepacia* in NBYE were centrifuged at 3200 x g for 10 min and the pellet washed in 5ml 10mM Tris pH 8.0. After centrifugation under the same conditions, the pellet was dispensed in 5.4 ml TE buffer pH 8.0 and 0.21ml of 10mg ml^{-1} lysozyme (Sigma) in TE buffer was added and the bottles incubated in a water-bath at 37°C for 1h. 0.3ml of 20% SDS, pre-incubated at 55°C, was added and the mixture incubated for 15min in a 55°C water-bath. After incubation, the contents were vortexed at high speed for up to two minutes in order to shear the DNA. The solution was denatured by adding 0.2 ml of freshly made 3N NaOH and left for ten minutes at room temperature with occasional mixing. 0.5ml of 2M Tris pH 7.0 was added, the preparation mixed and 1.5ml of 5M NaCl added. The mixture was then treated with an 8ml of salt-saturated phenol (Sigma), centrifuged at 13 000 x g and the top aqueous phase removed and treated with an equal volume of choroform: isoamyl alcohol (24:1). After centrifugation, the top phase was again removed and placed into sterile universal bottles. To the top phase was added 6ml of chloroform. The top phase was again removed, 12ml of ethanol added and the tubes placed at -70°C for 1h. The contents were then centrifuged at 3 200 x g for 30 min. The top phase of the ethanol was removed leaving approximately 1.5ml containing the pellet. This was transferred to an

Ependorff tube, centrifuged at 13 000 x g for 10 min and after removing the supernatant, the pellet was re-suspended in 150μl TE buffer pH 8.0.

2.2.24.Cellular fatty acid analysis

Inoculating plates and harvesting

TSA plates were inoculated from a pure bacterial culture, rotating the plate 90° and sterilising the loop between each of the four quadrants as illustrated in Fig 2.1.

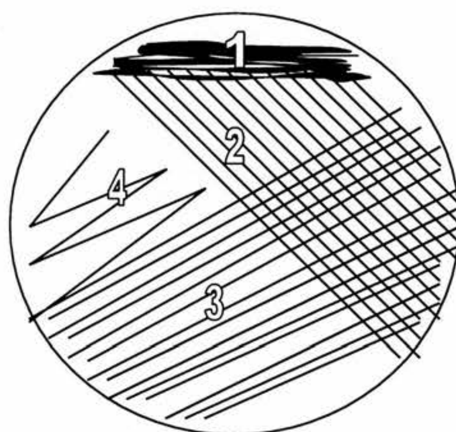


Fig 2.1 Inoculating of TSBA plate for cellular fatty acid analysis.

After incubating at 28°C for 24 hours, the cells were harvested from the overlap of the second and third quadrant by scraping the surface of the culture medium using a sterile loop. The cells in this area are in late log phase and yield the most stable fatty acid compositions. The harvested cells were wiped onto the bottom of a clean, dry 13mm x 100mm screw cap culture tube.

Saponification

1.0ml of the saponification reagent was added to each tube in the batch and a control tube with no cells in it. The tubes were tightly sealed with a clean teflon-lined screw cap, vortexed for 5-10s and placed in boiling water. After 5 minutes the tubes were removed from the boiling water, vortexed for 5-10 s and returned to the boiling water. After 30 minutes of saponification, the tubes were cooled in water at room temperature. (The saponification reagent is a strong methanolic base and when combined with heat, it kills and lyses the cells and their fatty acids are cleaved from the cell lipids and converted to their sodium salts).

Methylation

Methylation converts the sodium salts of fatty acids to fatty acid methyl esters, which increases the volatility of the fatty acids for the gas chromatography (GC) analysis. When the tubes had cooled to room temperature, 2.0 ml of the methylation reagent was added to each tube. The tubes were heated at 80°C in a water bath for 10min and cooled to room temperature before extraction of the fatty acid methyl esters could be carried out.

Extraction

During this step, the fatty acid methyl esters are extracted from the acidic aqueous phase and transferred to an organic phase with a liquid-liquid extraction procedure. This was carried out by adding 1.25ml of the extraction solvent to each tube and tightly sealing it

before placing it on a shaking machine (Janke & Kunkel HS250) for 10 min at 75 rev min^{-1}. The lower, aqueous phase was removed and discarded using a clean Pasteur pipette from each sample.

Base wash

A mild base solution is required to remove the free fatty acids and residual reagents from the organic extract. It is essential to remove the residual reagents as they damage the chromatographic system. 3.0 ml of the base wash was added to each tube and the tubes placed on a shaking machine for 5 min. Whenever necessary, a few drops of saturated NaCl / water solution were added and the tubes rotated gently, while being held vertically, between the palms and left to settle for a few minutes to aid in breaking the emulsion. Using a clean Pasteur pipette for each sample, the upper, organic phase was removed from each bottle and transferred to a labeled, clean GC sample bottle. A cap was immediately placed on the bottle to prevent evaporation. The caps were then crimped onto the sample bottles and loaded onto the automatic sampler behind the calibration mix.

Calibration was carried out by a member of staff at the Laboratorium voor Microbiologie, Universiteit Gent, after loading the injector turret with a fresh bottle of hexane/MTBE wash solvent and two empty waste bottles.

Details of bottles loaded into the sample tray were entered into the computer's sample table and the computer prompted to analyse the samples.

A detailed description of the GC equipment is given in the Appendix.

2.2.25. Preparation of whole-cell bacterial protein extracts

A loopful of bacteria grown for 48h at 28°C in nutrient agar buffered (NAB) was transferred to a sterile Eppendorf tube and washed by resuspending in 900µL NaPBS buffer followed by centrifugation at 1000 rpm for 5 min on a bench centrifuge (Eppendorf centrifuge 5417C). The supernatant was discarded and 900µL sample treatment buffer (STB) followed by 100µL 20% SDS added to the pellet. After mixing thoroughly, the mixture was heated at 95°C in a dry bath for 10 minutes (Thermolyne, Type 16 500 Dri bath). The tubes were cooled on ice and the suspension treated with ultrason at an output of 50W (Labsonic 2000, needle probe tip 127mm long 4mm diameter) for 15s in order to break down DNA and polysaccharides. The suspension was then centrifuged at 10 000rpm for 8 min and the supernatant, containing the protein extract poured into a sterile Eppendorf tube. The extract was stored at -20°C until required.

2.2.26. SDS-PAGE analysis of whole-cell bacterial protein extracts

Separation gel

A 12% separation gel, 1.5mm thick was prepared using stock solutions shown in the Appendix.

The gel was made up by mixing the following constituents in a flask with a stirrer: 10ml separating gel buffer, 16.0ml monomer solution, 0.4ml of 10% SDS and 13.4ml double distilled water. The mixture was warmed to 25°C for about 5min before adding

20.0µl TEMED and 0.14ml of 10% freshly opened and made up $(NH_4)_2S_2O_8$.

After mixing thoroughly, the mixture was poured between the assembled glass plates in casting stand, to a height of about 12.5cm from the base. The gel was overlaid with 2ml of water saturated isobutanol to maintain an anaerobic condition and to obtain a flat surface and then submerged in a water bath at 25ºC for two hours. After polymerisation, the water-saturated isobutanol was discarded and the gel surface rinsed twice with distilled water and twice with double distilled water. The gel was overlaid with 1.6ml of 1/4 diluted separating gel buffer containing 0.1% SDS and left to polymerise overnight at 19ºC. The overlaying ¼ diluted separating gel buffer was discarded, the gel surface rinsed twice in double distilled water and any excess liquid drained.

Stacking gel

A 5% stacking gel, 1.5mm thick was prepared using stock solutions, shown in the Appendix.

A stacking gel was made up by mixing the following constituents in a 50ml Erlenmeyer flask with a stirrer: 2.5ml stacking gel buffer, 1.7ml monomer solution, 0.1ml of 10% SDS and 5.7ml double distilled water. The mixture was warmed at 25°C in a water bath for about 5min before adding 12.5µl TEMED and 0.05ml of 10% freshly opened and made up $(NH_4)_2S_2O_8$. After mixing thoroughly, the stacking gel solution was poured on top of the polymerised separating gel, a 1.5mm teflon comb inserted between the plates and the gel left to polymerise for at least an hour.

Sample application and electrophoresis of SDS-solubilised proteins

Four slots of the reference strain *Psychrobacter immobilis* LMG 1125 were loaded in each gel and one slot with a low molecular weight marker (see Appendix). The final volume in each slot was adjusted to 10μl with tracking dye (see Appendix).

Once loaded the upper buffer reservoir was attached on top of the plates which were then removed from the casting stand and immersed into the lower buffer reservoir containing running buffer (see Appendix). The running buffer was used for a maximum of three times. Freshly prepared upper tank buffer was poured into the upper chamber and the gel run at a constant current of 6.0mA overnight until the tracking dye reached a level of about 9.5cm from the top of the separating gel.

Gel fixing, staining and destaining

The proteins were fixed and denatured in fresh 3% Tricarboxylic acid (TCA) solution with gentle shaking for 15min. The fixing solution was discarded and staining was carried out for 1 hour in fresh Serva R blue staining solution (see appendix), with gentle shaking at room temperature. After the proteins were stained, the staining solution was discarded and fresh destaining solution added to the gel and left on a gently shaking machine for 20 min. The step was repeated three times before the gel was ready for drying.

Gel drying gel and photography

Uncoated cellulose acetate sheets (type 325 P23 UCB) were prepared for gel drying by soaking in a water bath at about 40°C. The gel apparatus was placed on the gel drier (Hoeffer Scientific Instruments, drygel jr model SE540) in the following order from top to bottom;

filter paper
uncoated cellulose acetate sheet,
gel
uncoated cellulose acetate sheet
filter paper and
permeable plastic sheet of dryer.

The gel was dried under vacuum for 2hours. Gels were photographed in the department and photographic positive prints standardised.

Processing of dried of gels

The dried gels were scanned using a LKB Bromma 2202 Ultroscan Laser Densitometer and the software used was Gel Compar, Comparative Analysis of Electrophoresis Patterns, Version 2.0, Data Capture Program 1992. The data was re-scaled using Gel Compar, Comparative Analysis of Electrophoresis Patterns Version 2.0, Data Input and Conversion Program, 1992. The spectra were then converted to protein bands using Gel Compar Normalisation SDS-PAGE 3.1 and the bands aligned to the reference strain *Psychrobacter immobilis* LMG 1125 pattern in the database. The data was then

analysed using Gel Compar, Comparative Analysis of Electrophoresis Patterns, Version 4.2, 1992-1997, and compared to the laboratory's SDS-PAGE data base.

2.2.27. PCR for the identification of *B. cepacia*

Alkaline lysis extraction of DNA

DNA used in PCR for the taxonomic identification of bacterial isolates was extracted by alkaline lysis. 20µl of lysis buffer was added to 1or 2 bacterial colonies in a sterile Eppendorf tube and heated on a heating block for15min at 95°C. The heated suspension was centrifuged briefly before adding 180µl of double distilled water. After centrifugation at 13 000 rpm on a bench centrifuge for 5min, the suspension was stored at -20°C.

PCR for the identification of the B. cepacia complex

PCR for the identification of rhizosphere isolates was carried out using template DNA extracted by alkaline lysis. All PCR reagents were purchased from Qiagen Inc. Canada. All amplifications were carried out in a 25µl volume containing 5µl Qiagen solution Q, 1 U *Taq* DNA polymerase, 2µl template DNA, 15µl of each primer and 250µM of each deoxynucleoside triphosphate. The reactions were performed on a DNA thermal cycler.

Analysis of the16S rDNA genes

Primer pairs UNI2 and UNI5 (universal 16S rDNA primers) (Mahenthiralingam, Bischof, *et al*, 2000), were used to amplify a 1020bp fragment. The PCR reaction was initiated with denaturation at 94ºC for 2 min, followed by 30 cycles each consisting of 30s at 94ºC for denaturation, 45s at 60ºC for annealing and 60s at 72ºC for polymerisation. This was followed by a final polymerisation step at 72ºC for 10 min. In order to produce discriminatory restriction fragment length polymorphisms (RFLPs) the PCR product was restricted with *DdeI* (New England Biolabs) as indicated by the manufacturer. The product was separated on a 2% agarose gel in 1 x TBE at 70V.

Analysis of recA

Genomovar-specific *recA* primers (Mahenthiralingam, Bischof, *et al*, 2000), were used for the identification of the *B. cepacia* genomovars I and III.

BCRG11 and BCRG12 (*B. cepacia* genomovar I) (Mahenthiralingam, Bischof, *et al*, 2000) were used to amplify a 492bp product. Amplification conditions were the same as those for 16S rDNA, with annealing being carried out at 62ºC.

BCRG3A1 and BCRG3A2 (*B. cepacia* genomovar III-A), BCRG3B1 and BCRGB2 (*B. cepacia* genomovar III-B) (Mahenthiralingam, Bischof, *et al*, 2000) were used to amplify 378 and 781 bp products respectively. Amplification conditions were the same as those for 16S rDNA, with annealing at 62ºC.

2.2.28. Amplification of epidemic strain markers

Two markers of epidemic *B. cepacia* strains, cblA (Sun, Jiang, *et al*, 1995) and BCESM (Mahenthiralingam, Simpson, *et al*, 1997) were amplified using previously reported primers and cycling conditions.

CHAPTER 3

The *Burkholderia cepacia* complex

Pseudomonas cepacia was originally described at the agent of 'soft rot', a bacterial rot that occurs in damaged onion bulbs (Burkholder, 1950). Advances in taxonomy led to the creation of the genus *Burkholderia* in 1992, with *Burkholderia (Pseudomonas) cepacia* as the type strain (Yabuuchi, Kosako, *et al*, 1992). More recent taxonomic studies involving integrated genotypic and phenotypic analysis of a large collection of *Burkholderia* strains revealed that *B. cepacia* is not a single species but rather, consists of at least seven distinct genomic species, called genomovars a term introduced to denote phenotypically similar but genotypically distinct groups of strains (Ursing, Rossello-Mora, *et al*, 1995). Initially there were no distinguishing phenotypic characteristics between the *Burkholderia* genomovars but phenotypic characteristics peculiar to genomovars II, IV and V were subsequently identified. This led to these groups being renamed *B. multivorans*, *B. stabilis* and *B. vietnamiensis*, respectively (Vandamme, Holmes, *et al*, 1997). The remaining *B. cepacia* genomovars, together with the renamed species, now constitute the *B. cepacia* complex. In recent years, this group of organisms has gained considerable interest as opportunistic pathogens, biopesticides and bioremediation agents.

3.1. Colonial morphology and pigmentation

The colonial morphotypes of members of the *B. cepacia* complex vary considerably in size and shape but the majority are circular with smooth edges and range from less than 1mm to 4mm in diameter. Although most strains are non-pigmented, and do not produce exopolysaccharide on CAB, a variety of pigments are produced across the *B. cepacia* complex, as depicted in Fig 3.1. C1963, a CF isolate, is the only strain studied in this thesis that produces exopolysaccharide on CAB. However, some strains are able to produce exopolysaccharide when cultured on the richer S medium (Richau, Leitao, *et al*, 2000) and incubated for longer periods (see section 3.2). A few strains, such as the well-characterised epidemic genomovar III strain J2315, have a dry colonial appearance and produce a brown melanin-like pigment when grown on CAB at 37ºC. A few strains produce a yellow pigment and the environmental *B. cepacia* genomovar I strain J2535, was found to produce a red/magenta pigment when grown in CAB at 30ºC.

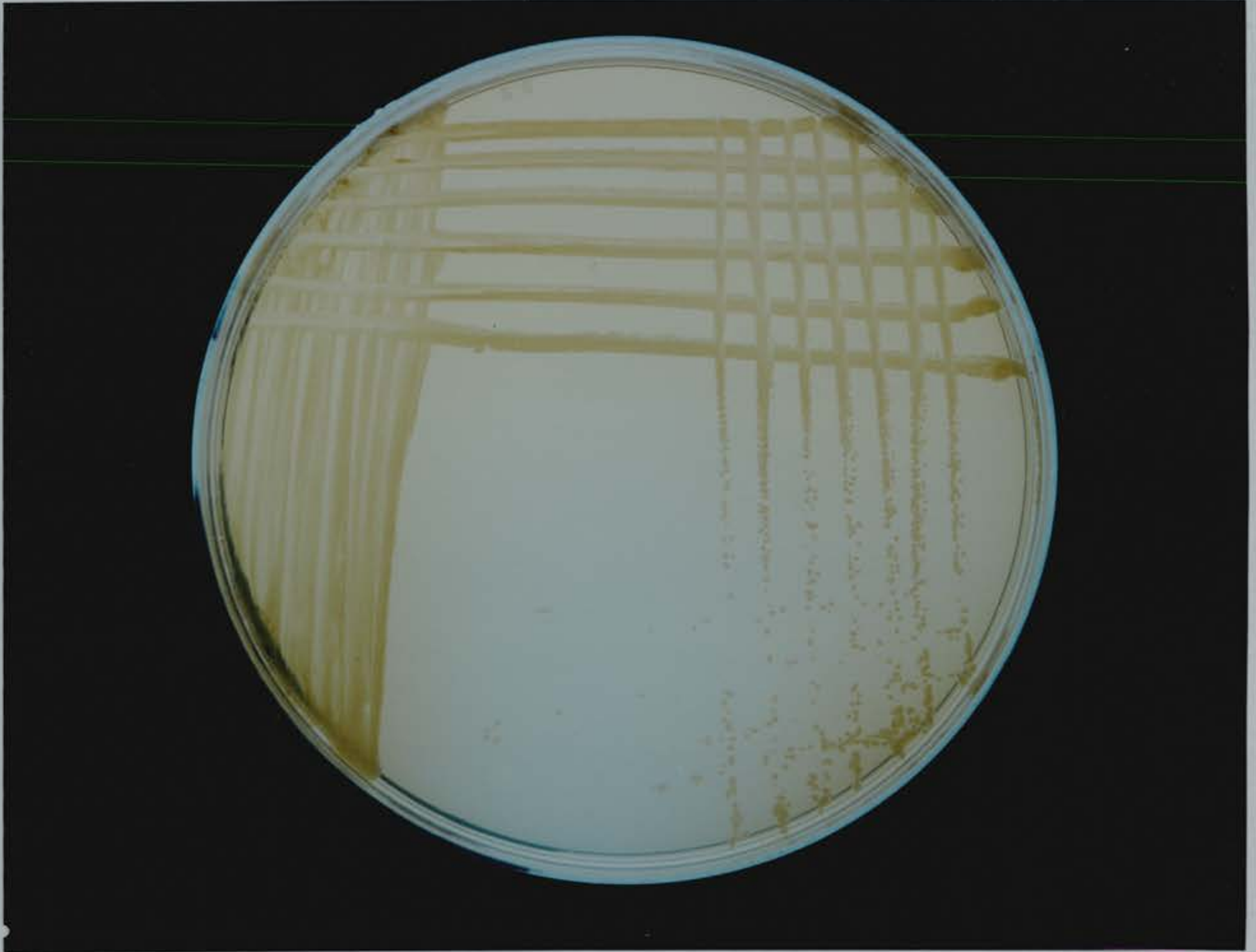


Fig 3.1.a. *B. cepacia* J2315 showing moderate production of brown melanin-like pigment and dry colonies when grown on CAB at 37ºC for 24 h.

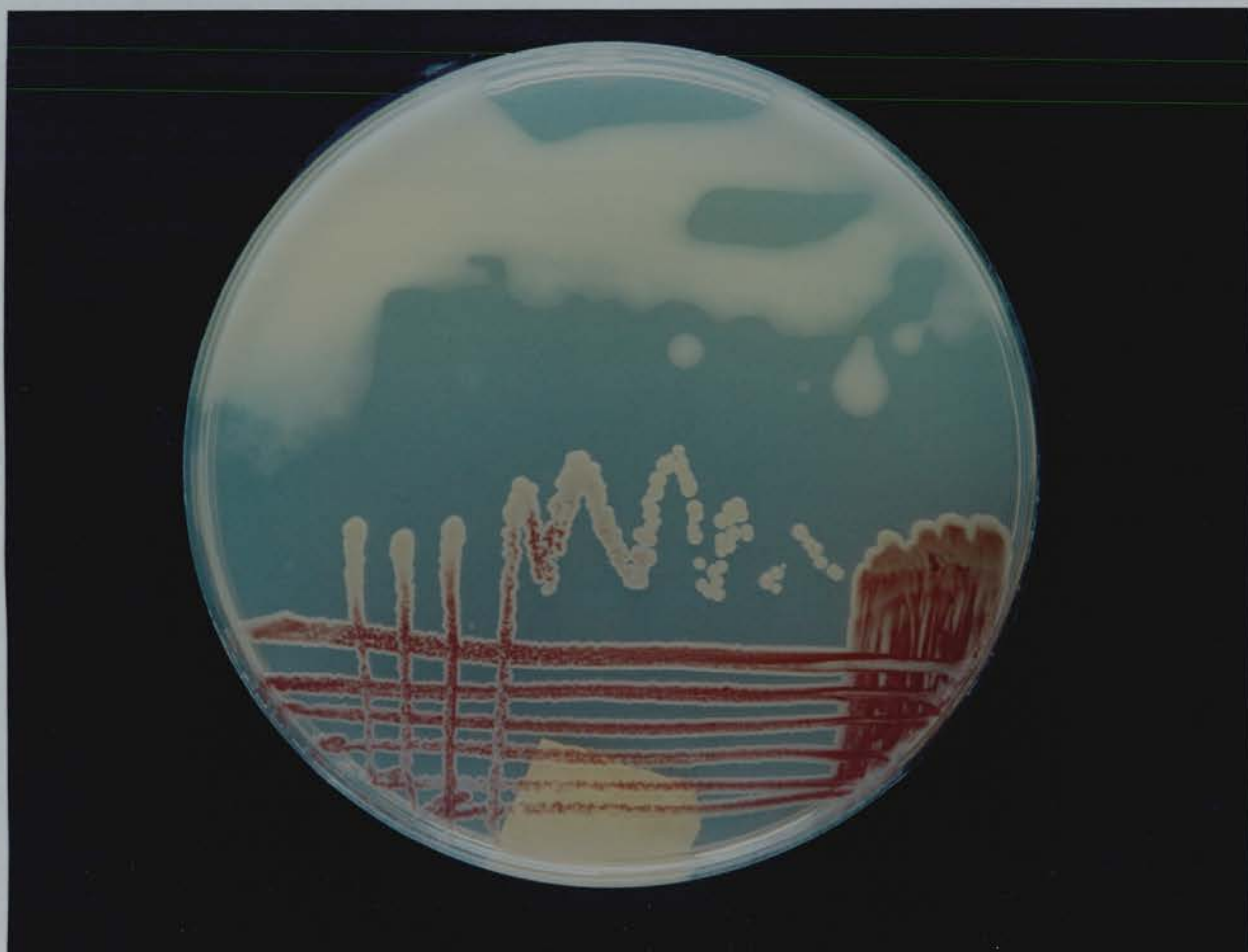
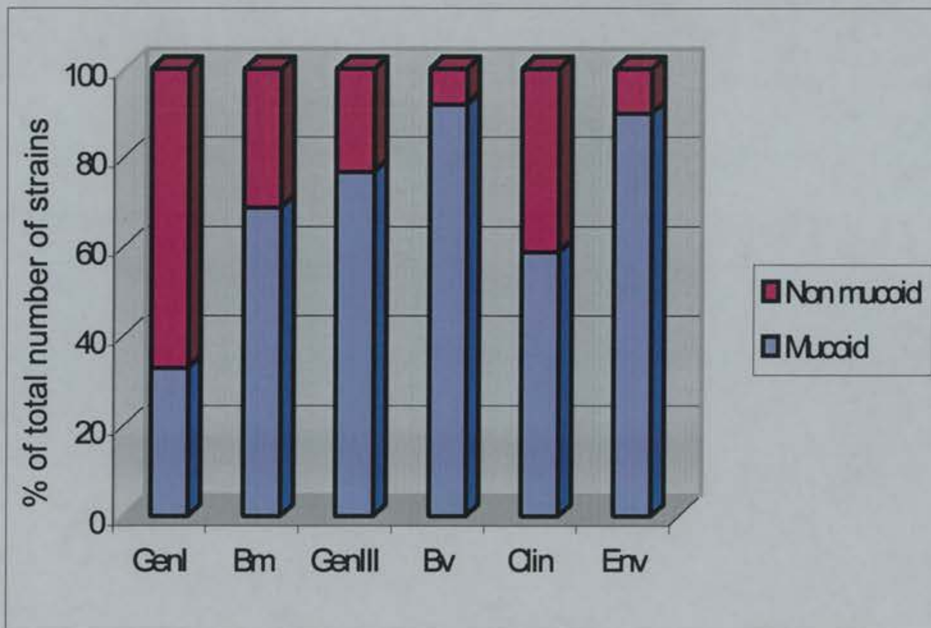


Fig 3.1.b. *B. cepacia* C1963 and J2535 (both genomovar I) showing mucoidy and red pigmentation, respectively when grown on CAB at 30ºC for 24 h.

Fig. 3.1.c. *B. cepacia* ATCC 25416 (genomovar I) and strain AMMD (*B. vietnamiensis*) illustrating yellow pigmentation and non-pigmentation respectively after growth on CAB at 37ºC for 24 h.

3.2. Exopolysaccharide production in the *B. cepacia* complex

In this thesis most *B. cepacia* strains did not produce exopolysaccaride after growth on CAB. However, when the same strains were grown on S medium (Richau, Leitao, *et al*, 2000), more strains were found to produce exopolysaccharide. The strains studied consisted of three *B. cepacia* genomovar I, 13 *B. multivorans*, 26 *B. cepacia* genomovar III, 12 *B. vietnamiensis*; with 29 of the strains isolated from clinical specimens and 25 from the environment. Fig 3.2 illustrates the production of exopolysaccharide by members of the *B. cepacia* complex on S medium after incubation for five days at 30°C.



Gen I, *B. cepacia* genomovar I; Bm, *B. multivorans*; Gen III, *B. cepacia* genomovar III; Bv, *B. vietnamiensis*; Clin, clinical strains; Env, environmental strains.

Fig. 3.2. Distribution of exopolysaccharide production on S medium by clinical and environmental isolates of the *B. cepacia* complex

3.3. Phenotypic and genotypic typing of *B. cepacia* complex isolates

3.3.1. Bacteriocin typing

Bacteriocin typing was used to investigate the heterogeneity of isolates belonging to the *B. cepacia* complex. The technique employed was previously described by Govan and Harris (1985). The distribution of cepaciacin types found in a collection of 45 clinical and environmental strains of the *B. cepacia* complex is shown in Table 3.1.

Table 3.1. Distribution of cepaciacin types of 45 clinical and environmental strains of the *B. cepacia* complex.

Cepaciacin type	No. of strains	% total
S1/P0	1	2
S3/P0	8	18
S3/P6	1	2
S8/P0	1	2
S12/P0	4	9
S13/P0	3	7
S16/P0	4	9
S22/P0	11	24
S24/P0	2	4
unclassifiable	10	22

3.3.2. Pulsed-field gel electrophoresis (PFGE)

The phenotypic heterogeneity in bacteriocin production and sensitivity demonstrated by bacteriocin typing was confirmed when genotypic fingerprinting of the same collection

of *B. cepacia* complex isolates was performed by PFGE. Taken together, the results of bacteriocin typing and PFGE confirmed that the *B. cepacia* isolates included in this study were distinct strains i.e. non-clonal

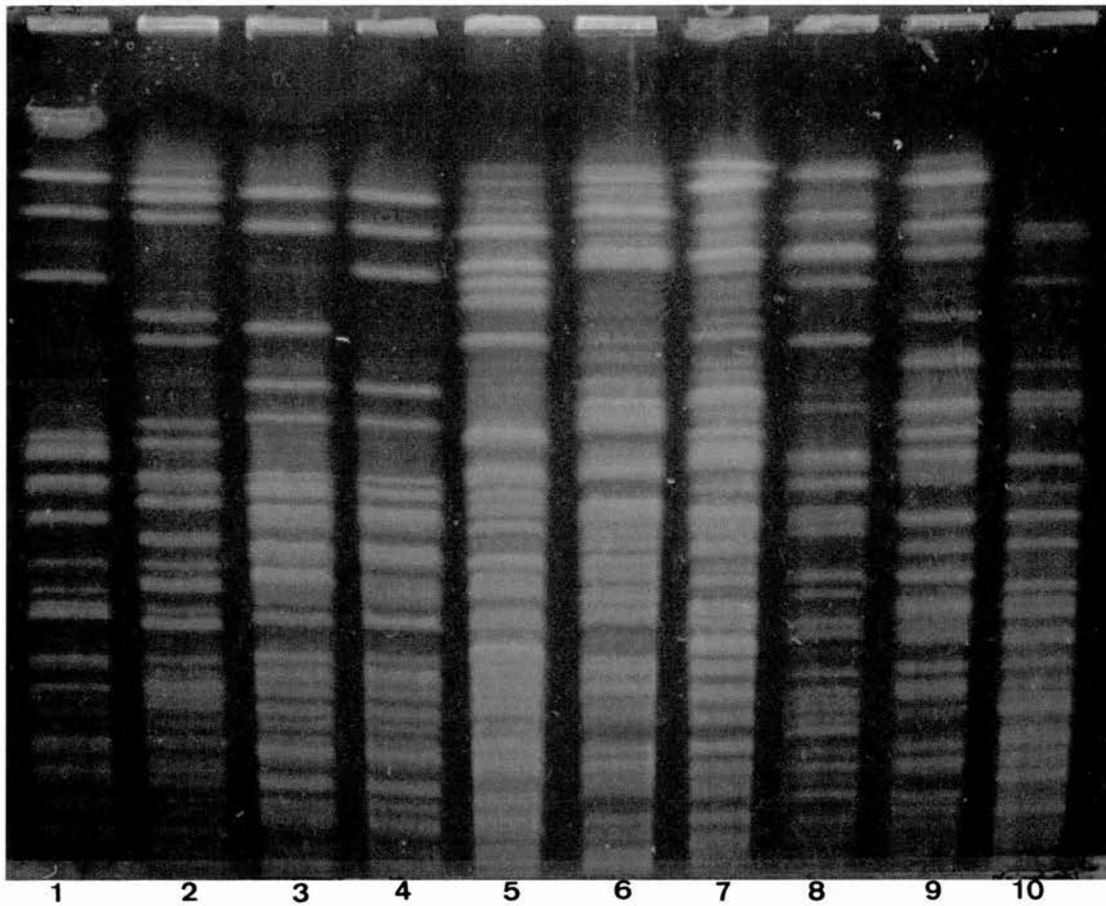


Fig 3.3.a) PFGE profiles of environmental and clinical isolates of the *B. cepacia* complex. Lanes 1 to 10, J1705, C1704, ATCC 53266, ATCC 53267, ATCC 29424, C2972, C2973, C2974, C2975, C2976, respectively

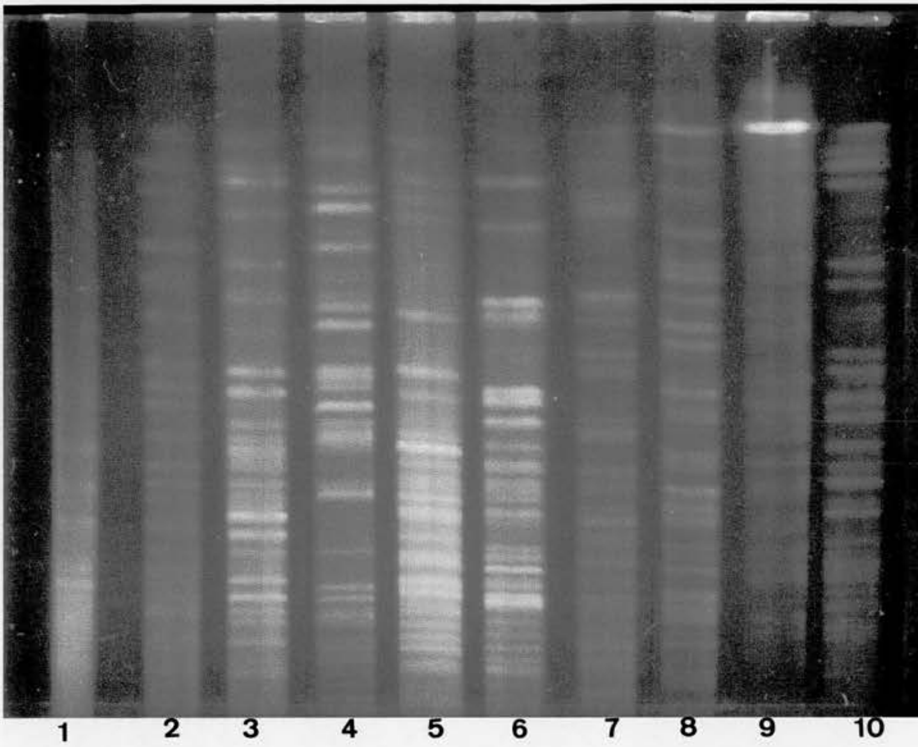


Fig 3.3.b) PFGE profiles of environmental and clinical isolates of the *B. cepacia* complex. Lanes 1 to 10, C1052, C1572, C1652, C1857, C1911, C2775, 715j, ATCC 17616, J2502, AMMD, respectively.

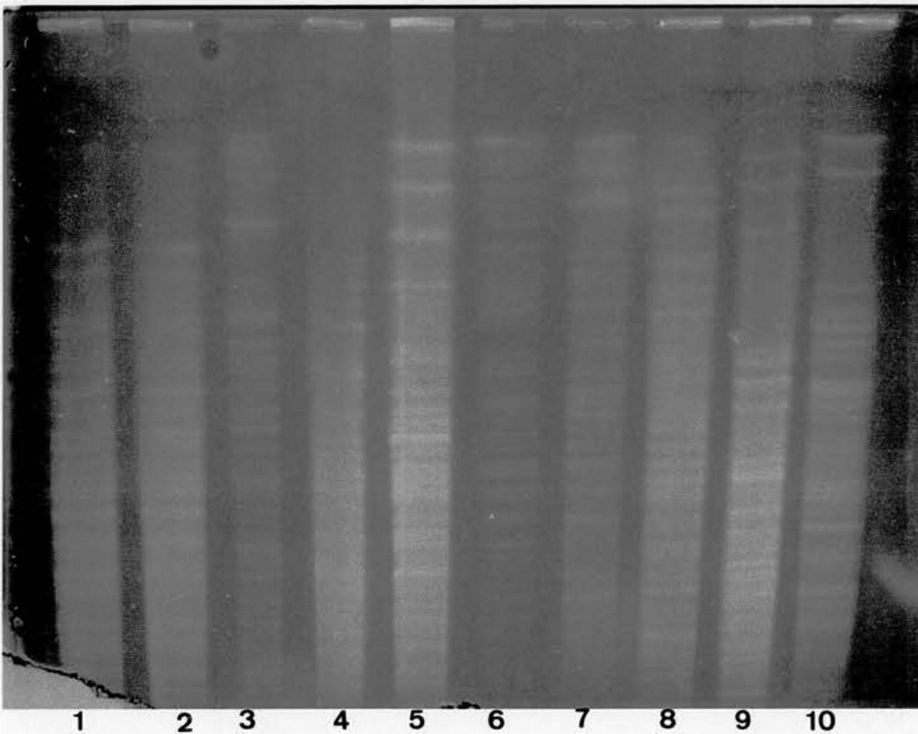


Fig 3.3.c) PFGE profiles of environmental and clinical isolates of the *B. cepacia* complex. Lanes 1 to 10, C517, C1335, C1511, C1524, C1670, C1773, C2349, C2374, J1697, C3041, respectively.

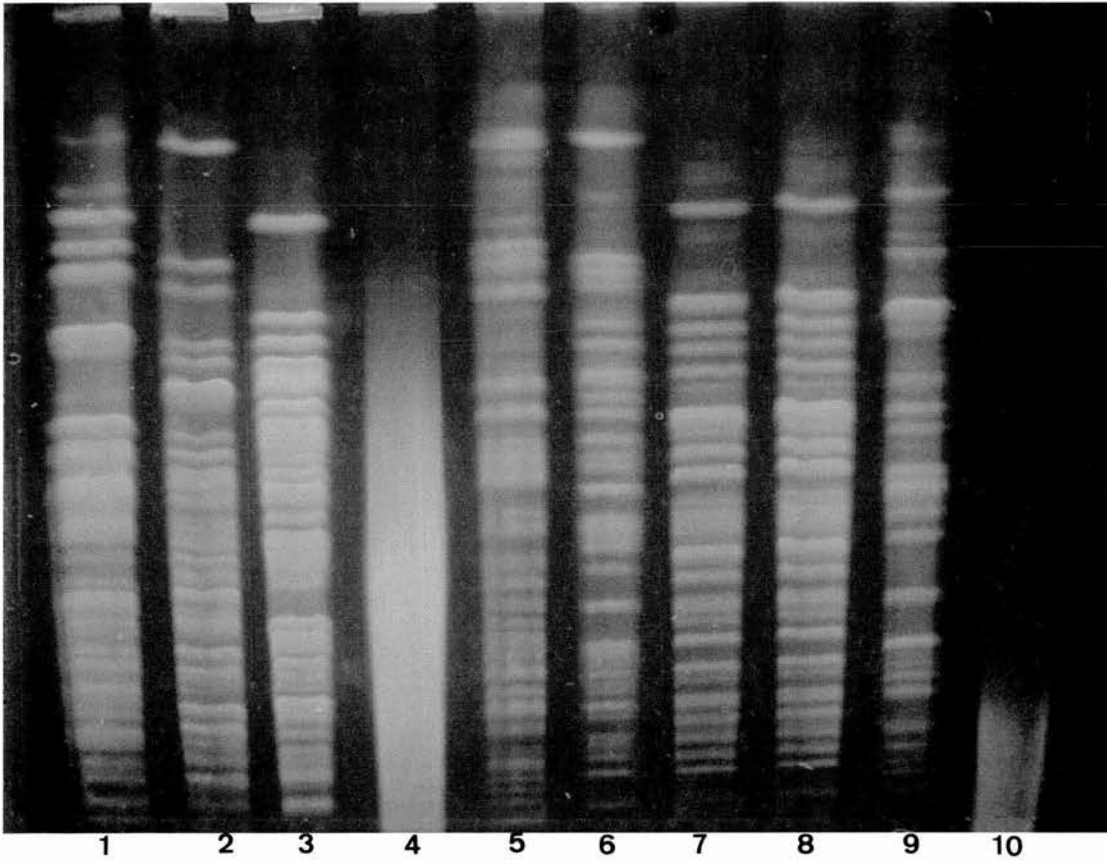


Fig 3.3.d) PFGE profiles of environmental and clinical isolates of the *B. cepacia* complex. Lanes 1 to 10, C1963, J2540, J2535, J2552, C2008, J2395, J2866, J2867, J2868, J2891, respectively.

3.4. Discussion

3.4.1. Colonial morphology and pigmentation

Diversity between and within the genomovars is one of the distinguishing features of the *B. cepacia* complex. In this study, differences in colony morphology and pigment production were observed between strains in the same genomovar. Although strains ATCC 25416, C1963 and J2535, all belong to *B. cepacia* genomovar I they have distinct appearances on CAB medium. Strain C1963 is non-pigmented and mucoid on CAB medium whereas strain ATCC 25416 produces a yellow pigment, and J2535 produces a red/margenta pigment on CAB. Very few isolates were found to produce a mucoid colonial phenotype. Mucoidy of strain C1963 on CAB medium was found to be temperature dependent, with copious amounts of exopolysaccharide produced at 30°C and very little, if any, at 37°C.

The optimum growth temperature of the *B. cepacia* complex isolates examined was found to be 30-37°C with a maximum of 41°C and most strains grew well at 37°C. However, strain J2535 was found to be non-culturable when a frozen sample was thawed and incubated at 37°C. This critical dependence on temperature, although not common, could lead to failure to culture from frozen stocks of other members of the *B. cepacia* complex. Interestingly, a similar phenomenon has been found in some *B. cepacia* strains when stocks are incubated at 37°C after storage at 4°C (Pitt and Govan 1993).

3.4.2. Production of exopolyssacharide by the *B. cepacia* complex

The role of exopolysaccharide production in increasing the pathogenicity of invading organisms has been reviewed by (Costeron, Cheng, *et al.* 1987). These authours suggest that the exopolysaccharide allows bacteria to survive, grow and the colonies to persist within a protective matrix which acts as a barrier to reduce the penetration of antibiotics and protects the bacteria from the host's defence mechanisms, in particular, engulfment by phagocytes. The role of mucoidy is strikingly demonstrated in the case of *P. aeruginosa* infection of CF lungs. The *P. aeruginosa* strains infecting CF patients are non-mucoid and with persistence, become highly mucoid due to the production of the exopolysaccharide, alginate (Koch and Høiby, 1993). In the patients, this transformation to a mucoid colonial morphotype coincides with chronic infection and the inability to eradicate the pathogen by antibiotic therapy (Govan and Deretic, 1996).

Mucoid *B. cepacia* isolates are considered to be extremely rare in both environmental and clinical isolates (Govan and Deretic, 1996). However, in this study, when cultured on S medium, the majority strains (75%) of the *B. cepacia* complex produced exopolysaccharide compared to only one isolate when the strains were cultured on CAB. Similar results with S medium have recently been reported, (Richau, Leitao, *et al,* 2000). In this study, Richau and colleagues found that 70% of the isolates cultured on S medium produced exopolysaccharide. Interestingly, mucoidy on S medium requires incubation at 30°C, the same temperature at which the *B. cepacia* genomovar I strain C1963 is mucoid on CAB. Maximal exopolysaccharide production in *B. cepacia* has

been reported to occur in media containing 2% (w/v) glucose and 0.4 M NaCl and grown at 35°C (Allison and Glodsbrough, 1994). Thus, exopolysaccharide production by *B. cepacia* depends not only on the composition of the growth medium but also on the incubation temperature.

Unlike the case of mucoid *P. aeruginosa*, the exopolysaccharide produced by the *B. cepacia* complex is not alginate but a heteropolysaccharide composed of glucose, mannose, rhamnose, glucuronic acid and galactose (Allison and Glodsbrough, 1994). However, this polysaccharide, like alginate, restricts the diffusion of different antibiotics and can interact with host and other bacterial polymers to form a heterodisperse polysaccharide gel (Allison, 1992).

In this study, *B. cepacia* genomovar I was the only genomovar examined with a greater proportion of non-mucoid (67%) to mucoid strains (33%). However, the relatively small number of strains examined in this genomovar could influence these proportions. Interestingly, a much larger proportion of environmental isolates (90%) were mucoid compared to clinical isolates (59%). This result would appear to contrast with the predomonance of mucoid *P. aeruginosa* in clinical isolates from CF patients. It should be noted, however, that the experimental conditions used did not mimic conditions in the CF lung. The correlation between production of exopolysaccharide and medium osmolarity (Allison and Glodsbrough, 1994) is comparable to the high concentrations of

Na^+ and Cl^- ions in the CF lung. Presently, it is not known whether *B. cepacia* exopolysaccharide assists in the organisms' proliferation and persistence in the lung.

3.4.3. Phenotypic and genotypic typing of *B. cepacia* complex isolates

The use of bacteriocin typing to characterise members of the *B. cepacia* complex was found to be restricted; 22% of the isolates investigated could not be classified. Of the typable isolates, 24% belonged to the same cepaciacin type S22/P0, this type also included strains from different genomovars and those of clinical as well as environmental origin. PFGE proved to be a more discriminating technique than bacteriocin typing. PFGE revealed distinct profiles for each of the forty-five isolates and confirmed a lack of clonality between isolates included in this study. A minor caveat is that PFGE profiles for the strains J2552 and J2891 could not be generated despite the use of different methods and various restriction enzymes. This phenomenon has been encountered in a minority of *B. cepacia* strains and is thought to be due to the production of DNAses by these organisms (Dr. C. J. Doherty, personal communication).

CHAPTER 4

Resistance of the *B. cepacia* complex to antibiotics

4.1. Antibiotic susceptibility of the *B. cepacia* complex

The sensitivity of the *B. cepacia* complex to various antibiotics was determined by the agar dilution method following standard guidelines (British Society for Antimicrobial Chemotherapy, 1991). *P. aeruginosa* NTCC 10662 was used as the reference strain; there is currently no antibiotic sensitivity reference strain for the *B. cepacia* complex.

Although inherent resistance to multiple antibiotics is often reported to be one of the typical characteristics of the *B. cepacia* complex, the data summarised in Tables 4.1 and 4.2 shows that resistance is not uniform across the group. In this study, clinical strains tended to be more resistant to antibiotics, probably because of previous exposures to the same antimicrobial drugs. Surprisingly, the inhibitory effect of chloramphenicol was dependent on genomovar status; for example, *B. vietnamiensis* strains were more sensitive to ceftazidime and chloramphenicol than strains from other genomovars, regardless of their source. Ciprofloxacin was found to be the most effective inhibitory agent and all strains examined were resistant to polymyxin and tetracycline

Table 4.1a MICs (mg/L) of different antibiotics against *B. cepacia* genomovar I and *B. multivorans* (genomovar II)

Strain	Genv	Ceft	Chlo	Cipr	Tob	Pol	Tet	Tri
ATCC17759	I	4	8	0.12	32	>128	64	4
ATCC25416	I	4	16	4	64	>128	64	2
LMG17997	I	2	8	2	16	>128	64	2
CEP 509	I	8	16	4	128	>128	>128	4
C1963	I	2	16	1	64	>128	>128	64
J2535	I	1	8	1	16	>128	64	4
J2540	I	0.12	4	0.25	8	>128	>128	0.5
J2552	I	2	16	1	16	>128	2	0.5
ATCC17616	II	4	8	0.5	32	>128	8	2
LMG13010	II	2	32	2	16	>128	8	2
CF-A1-1	II	4	16	4	64	>128	16	4
C1962	II	0.5	8	2	128	>128	8	2
C5393	II	8	128	4	32	>128	16	32
C1576	II	>128	32	8	>128	>128	16	4
C1572	II	8	32	4	>128	>128	64	4
C1857	II	0.5	16	2	>128	>128	16	2
C1911	II	2	16	2	64	>128	8	2
C2775	II	8	16	16	16	>128	16	2
C1632	II	8	32	2	>128	>128	128	8
JTC	II	4	16	2	64	>128	4	2
249-2	II	2	8	0.12	16	>128	2	0.5
Breakpoint	-	2	8	4	1	4	1	2
NTCC 10662	-	1	128	0.12	1	1	16	32

Genv, genomovar; Ceft, ceftazidime; Chlo, chloramphenicol; Cipr, ciprofloxacin; Tob, tobramycin; Pol, polymyxin; Tet, tetracycline; Tri, trimethoprim.

Table 4.1b MICs (mg/L) of different antibiotics against clinical and environmental strains of *B. cepacia* genomovar III

Strain	Genv	Ceft	Chlo	Cipr	Tob	Pol	Tet	Tri
ATCC17765	III	4	16	2	64	>128	32	2
BC7	III	2	16	0.12	8	>128	128	0.25
CEP 511	III	2	16	32	128	>128	64	32
C1394	III	1	16	2	>128	>128	>128	64
C5424	III	2	8	0.12	8	>128	128	0.25
C6433	III	2	16	32	32	>128	>128	32
C517	III	0.5	64	2	32	>128	32	64
C1335	III	0.5	64	2	128	>128	32	64
C1773	III	1	16	>128	128	>128	128	2
C2374	III	8	16	>128	64	>128	64	32
J2315	III	8	16	4	>128	>128	128	64
J415	III	4	64	2	64	>128	>128	4
K56-2	III	4	16	4	128	>128	32	32
PC 184	III	0.12	4	16	8	>128	64	0.5
R2817	III	2	4	0.25	4	>128	32	1
R2827	III	4	16	0.25	16	>128	64	16
R8351	III	1	8	0.25	8	>128	32	1
R8571	III	4	4	0.25	8	>128	64	4
R8574	III	4	8	0.25	16	>128	64	1
R8580	III	4	8	0.25	16	>128	64	1
R8605	III	4	16	0.25	16	>128	64	1
R9235	III	32	32	0.25	16	>128	64	16
R9239	III	2	8	0.25	8	>128	64	1
R9243	III	2	8	0.25	8	>128	64	4
R9235	III	2	8	0.25	16	>128	64	1
R9342	III	2	8	0.25	16	>128	128	2
Breakpoint	-	2	8	4	1	4	1	2
NTCC10662	-	1	128	0.12	1	1	16	32

Genv, genomovar; Ceft, ceftazidime; Chlo, chloramphenicol; Cipr, ciprofloxacin; Tob, tobramycin; Pol, polymyxin; Tet, tetracycline; Tri, trimethoprim; 'R', environmental isolates.

Table 4.1c MICs (mg/L) of different antibiotics against *B. stabilis* (genomovar IV) and *B. vietnamiensis* (genomovar V)

Strain	Gen	Ceft	Chl	Cipr	Tob	Pol	Tet	Tri
C7322	IV	2	16	4	64	>128	>128	32
LMG18888	IV	2	16	0.12	8	>128	64	0.25
LMG14294	IV	8	4	64	8	>128	64	32
LMG14086	IV	8	16	0.12	8	>128	64	2
LMG10929	V	2	4	0.12	16	>128	2	32
LMG16232	V	2	8	1	16	>128	8	4
ATCC53266	V	1	8	0.5	32	>128	64	1
ATCC53277	V	2	8	1	16	>128	32	2
C1704	V	1	8	1	32	>128	8	2
C2972	V	4	32	2	32	>128	16	8
C2973	V	0.5	8	0.5	1	>128	2	1
C2974	V	0.5	8	0.5	16	>128	4	2
C2975	V	1	8	0.5	16	>128	4	2
C2977	V	0.5	8	0.5	0.5	>128	1	2
C2979	V	2	8	1	32	>128	4	32
FC 441	V	2	8	0.12	16	>128	4	2
J1697	V	1	16	0.5	4	>128	4	1
J1705	V	0.5	8	0.5	2	>128	2	2
AMMD	V	0.5	8	0.25	16	>128	64	0.5
PC 259	V	2	8	0.12	16	>128	8	32
Breakpoint	-	2	8	4	1	4	1	2
NTCC10662	-	1	128	0.12	1	1	16	32

Gen, genomovar; Ceft, ceftazidime; Chlo, chloramphenicol; Cipr, ciprofloxacin; Tob, tobramycin; Pol, polymyxin; Tet, tetracycline; Tri, trimethoprim.

Table 4.2.a. Susceptibility of *B. cepacia* genomovar I and *B. multivorans* (genomovar II) to different antibiotics

Strain	Genv	Ceft	Chlo	Cipr	Tob	Tet	Tri
ATCC17759	I	R	S	S	R	R	R
ATCC25416	I	R	R	S	R	R	S
LMG17997	I	S	S	S	R	R	S
CEP 509	I	R	R	S	R	R	R
C1963	I	S	R	S	R	R	R
J2535	I	S	S	S	R	R	R
J2540	I	S	S	S	R	R	S
J2552	I	S	R	S	R	R	S
ATCC17616	II	R	S	S	R	R	S
LMG13010	II	S	R	S	R	R	S
CF-A1-1	II	R	R	S	R	R	R
C1962	II	S	S	S	R	R	S
C5393	II	R	R	S	R	R	R
C1576	II	R	R	R	R	R	R
C1572	II	R	R	S	R	R	R
C1857	II	S	R	S	R	R	S
C1911	II	S	R	S	R	R	S
C2775	II	R	R	R	R	R	S
C1632	II	R	R	S	R	R	R
JTC	II	R	R	S	R	R	S
249-2	II	S	S	S	R	R	S
NTCC 10662	-	S	R	S	S	R	R

Genv, genomovar; Ceft, ceftazidime; Chlo, chloramphenicol; Cipr, ciprofloxacin; Tob, tobramycin; Pol, polymyxin; Tet, tetracycline; Tri, trimethoprim; S, sensitive; R, resistant.

Table 4.2.b. Susceptibility of clinical and environmental strains of *B. cepacia* genomovar III to different antibiotics against

Strain	Genv	Ceft	Chlo	Cipr	Tob	Tet	Tri
ATCC17765	III	R	R	S	R	R	S
BC7	III	S	R	S	R	R	S
CEP 511	III	S	R	R	R	R	R
C1394	III	S	R	S	R	R	R
C5424	III	S	S	S	R	R	S
C6433	III	S	R	R	R	R	R
C517	III	S	R	S	R	R	R
C1335	III	S	R	S	R	R	R
C1773	III	S	R	R	R	R	S
C2374	III	R	R	R	R	R	R
J2315	III	R	R	S	R	R	R
J415	III	R	R	S	R	R	R
K56-2	III	R	R	S	R	R	R
PC 184	III	S	S	R	R	R	S
R2817	III	S	S	S	R	R	S
R2827	III	R	R	S	R	R	R
R8351	III	S	S	S	R	R	S
R8571	III	R	S	S	R	R	R
R8574	III	R	S	S	R	R	S
R8580	III	R	S	S	R	R	S
R8605	III	R	R	S	R	R	S
R9235	III	R	R	S	R	R	R
R9239	III	S	S	S	R	R	S
R9243	III	S	S	S	R	R	R
R9335	III	S	S	S	R	R	S
R9342	III	S	S	S	R	R	S
NTCC10662	-	S	R	S	S	R	R

Genv, genomovar; Ceft, ceftazidime; Chlo, chloramphenicol; Cipr, ciprofloxacin; Tob, tobramycin; Pol, polymyxin; Tet, tetracycline; Tri, trimethoprim; S, sensitive; R, resistant.

Table 4.2.c. Susceptibility of *B. stabilis* (genomovar IV) and *B. vietnamiensis* (genomovar V) to different antibiotics

Strain	Genv	Ceft	Chlo	Cipr	Tob	Tet	Tri
C7322	IV	S	R	S	R	R	R
LMG18888	IV	S	R	S	R	R	S
LMG14294	IV	R	S	R	R	R	R
LMG14086	IV	R	R	S	R	R	S
LMG10929	V	S	S	S	R	R	R
LMG16232	V	S	S	S	R	R	R
ATCC53266	V	S	S	S	R	R	S
ATCC53277	V	S	S	S	R	R	S
C1704	V	R	S	S	R	R	S
C2972	V	R	R	S	R	R	R
C2973	V	S	S	S	S	R	S
C2974	V	S	S	S	R	R	S
C2975	V	S	S	S	R	R	S
C2976	V	S	S	S	R	R	S
C2977	V	S	S	S	S	S	S
C2979	V	S	S	S	R	R	R
FC 441	V	S	S	S	R	R	S
J1697	V	S	R	S	R	R	S
J1705	V	S	S	S	R	R	S
AMMD	V	S	S	S	R	R	S
PC 259	V	S	S	S	R	R	R
NTCC10662	-	S	R	S	S	R	R

Genv, genomovar; Ceft, ceftazidime; Chlo, chloramphenicol; Cipr, ciprofloxacin; Tob, tobramycin; Pol, polymyxin; Tet, tetracycline; Tri, trimethoprim; S, sensitive; R, resistant.

4.2. Effect of carbon dioxide on the susceptibility of the *B. cepacia* complex to different antibiotics

Alteration of growth conditions, particularly incubation in a microaerophilic atmosphere has been reported to alter bacterial sensitivities to antibiotics. The MICs of antibiotics for representative study strains were determined by the agar dilution method in the presence of air and 5% CO_2 and the results are shown on Table 4.3. In this study, incubation in 5% CO_2 was found to have no effect to *B. cepacia*'s sensitivity to selected antibiotics.

Table 4.3. Effect of CO_2 on the antibiotic susceptibility of the *B. cepacia* complex (MICs, mg/L)

Strain	Ceft	Ceft CO_2	Cefp	Cefp CO_2	Cipr	Cipr CO_2	Tri	Tri CO_2	Chlo	Chlo CO_2
C1963	2	2	8	8	1	2	64	64	16	16
J2535	1	1	1	1	1	1	4	2	8	16
J2552	2	4	2	4	1	2	0.5	1	16	16
C1572	8	16	64	>128	4	8	4	4	32	8
C1576	>128	>128	128	>128	8	8	4	4	32	32
C1857	0.5	1	8	16	2	4	2	2	16	16
C1911	2	1	2	8	2	2	2	2	16	8
C2775	8	8	8	8	16	8	2	4	4	4
J2395	2	2	8	16	8	2	4	4	8	16
C517	0.5	0.5	8	8	2	8	64	64	64	64
C1335	0.5	1	8	8	2	8	64	64	64	64
C1394	1	1	8	8	2	4	64	64	16	16
C1773	1	4	16	8	>128	>128	2	4	16	16
C2374	8	8	>128	>128	>128	>128	32	64	16	8
J2315	4	8	128	>128	4	8	32	64	16	16
C1704	1	1	16	16	1	2	2	4	8	8
J1697	1	1	2	2	0.5	0.5	1	4	16	16
J1705	0.5	1	1	2	0.5	1	2	4	8	8
J2742	0.5	1	2	2	0.25	1	0.5	1	8	8
NCTC 10662	1	1	2	2	0.12	1	32	64	128	128

Ceft, ceftazidime; Cefp, cefpirome; Cipr, ciprofloxacin; Tri, trimethoprim; Chlo, chloramphenicol.

4.3. Antibiotic-resistant mutants of the *B. cepacia* complex

One of the major difficulties in managing *B. cepacia* infections is that once effective antibiotics have been identified, further antibiotic resistance often develops during therapy. A series of experiments were performed to determine the rate of acquisition of resistance to different antibiotics. *B. cepacia* complex strains repeatedly cultured in increasing amounts of ceftazidime, ciprofloxacin and chloramphenicol, were considered to be antibiotic-resistant mutants if the MIC increased at least six-fold. Using this method, resistant mutants were selected against ceftazidime and ciprofloxacin but not against chloramphenicol. A total of four *B. cepacia* genomovar I, nine *B. multivorans* (genomovar II), seven *B. cepacia* genomovar III and 14 *B. vietnamiensis* (genomovar V) were tested.

Table 4.4. Selection of antibiotic resistant mutants of the *B. cepacia* complex

Genomovar	% mutants selected against		
	ceftazidime	ciprofloxacin	chloramphenicol
I	50	75	0
II	56	44	0
III	29	0	0
V	36	86	0

4.3.1. Antibiotic susceptibility of antibiotic-resistant mutants

B. cepacia strains that develop further resistance during therapy may show cross-resistance between classes of unrelated antimicrobial compounds. In order to determine the degree of cross-resistance to different classes of antibiotics the sensitivity of ceftazidime-resistant mutants, obtained in the previous section, to ampicillin, cefpirome, ciproflaxacin and chloramphenicol was determined using the agar dilution method. The sensitivity of ciproflocaxin-resistant mutants to ofloxacin, grepafloxacin, ceftazidime and chloramphenicol was also determined. Table 4.5 shows that the ceftazidime-resistant mutants only showed resistance to other β–lactam antibiotics and not to unrelated antibiotics.

Table 4.5. Antibiotic susceptibility (MICs, mg/L) of ceftazidime-resistant mutants

Strain	Ceft	Amp	Cefp	Cipr	Chlo
ATCC 29424	1	4	1	0.5	8
ATCC 29424b	8	128	1	0.25	8
ATCC 53266	1	64	4	0.25	8
ATCC 53266b	16	128	64	0.25	8
ATCC53267	2	8	1	0.5	8
ATCC53267b	16	16	8	0.5	4
C2973	0.5	16	4	0.25	16
C2973b	8	0.12	0.5	0.25	8
C517	0.5	16	4	2	64
C517b	3	32	8	2	64
C1911	2	64	2	2	16
C1911b	16	128	16	1	16
J2540	0.12	0.5	2	0.25	4
J2540b	6	16	8	0.25	4
J2742	0.5	16	0.5	0.12	8
J2742b	8	128	64	0.12	8

'b', ceftazidime-resistant mutant; Ceft, ceftazidime; Amp, ampicillin; Cefp, cefpirome; Cipr, ciprofloxacin,; Chlo, chloramphenicol.

Table 4.6 shows that, like the ceftazidime-resistant mutants, the ciprofloxacin-resistant mutants in this study had additional resistance to other quinolones and not to unrelated classes of antimicrobial agents.

Table 4.6. Antibiotic susceptibility (MICs, mg/L) of ciprofloxacin-resistant mutants

Strain	Cipr	Oflo	Ceft	Grep	Chlo
CEP509	4	16	8	4	32
CEP509c	32	32	16	16	128
ATCC 17616	0.5	2	4	1	32
ATCC 17616c	8	128	8	64	64
LMG 18888	0.12	8	2	4	32
LMG 18888c	2	64	4	8	32
LMG 14086	0.12	2	8	0.5	32
LMG 14086c	8	16	8	1	32
PC 259	0.12	8	2	8	64
PC 259c	8	16	2	16	64
C2973	0.5	16	0.5	4	64
C2973c	16	16	1	16	128
J1705	0.5	32	0.5	2	8
J1705c	8	64	1	2	8
J2742	0.25	4	0.5	1	8
J2742c	2	8	0.5	2	16

'c', ciprofloxacin-resistant mutant; Cipr, ciprofloxacin; Oflo, ofloxacin,; Ceft, ceftazidime; Grep, grepafloxacin; Chlo, chloramphenicol.

4.4. Discussion

4.4.1. Antibiotic susceptibility of the *B. cepacia* complex

Inherent resistance to different classes of antibiotics is one of the characteristics of the *B. cepacia* complex. In this study, the antibiotics used for susceptibility- testing were chosen to represent the major classes of antibiotics, as well as agents that are routinely used for the treatment of infections caused by pseudomonads. Polymyxins are cationic polypeptides and although five classes have been identified, polymyxins A, B, C, D and E only polymyxin B and E are available for clinical use; polymyxins A,C, and D are considered too toxic for human use. Polymyxin E is commonly called colistin and has a similar antibacterial spectrum to polymyxin B. In general, the polymyxins are active against Gram-negative bacteria such as *P. aeruginosa* and *H. influenzae,* but have little or no activity against Gram-positive organisms. The mode of action of polymyxins is bactericidal through interference with the structure and function of their outer cytoplasmic membranes.

Although colomycin (colistin) is effectively used for the treatment of *P. aeruginosa* and other Gram-negative bacilli infections (Littewood, Koch, *et al*, 2000), Table 4.1 shows that all study strains belonging to the *B. cepacia* complex are resistant to polymyxin B. (Preliminary tests indicated that similar sensitivities were obtained for both colistin and polymyxin B, hence the latter was subsequently used in this study). These results are

similar to those obtained by Fass and Barnishan (1980), who found all the *B. cepacia* strains to have colistin MICs of greater than 64 mg/L. It should be noted, however, that the 1980 study could not take account of the genomovar status of the *B. cepacia* strains examined. Resistance to polymyxin appears to be inherent in all members of the *B. cepacia* complex and accounts for its value as the selective agent in *Burkholderia cepacia* medium, including the highly effective medium produced by Mast Diagnostics which 300u/ml of polymyxin B.

Tobramycin is an aminoglycoside, a group of basic compounds containing amino sugars and an amino cyclitol structure. Tobramycin is one of the first-line antibiotics used in the treatment of *P. aeruginosa* infections as well as infections caused by other Gram-negative bacilli. Like other aminoglycosides, tobramycin acts by suppressing bacterial growth through the inhibition of protein synthesis. Aminoglycosides bind to the 30S subunit of bacterial ribosomes leading to misreading of mRNA codons as well as detachment of ribosomes from the mRNA. Out of the 68 strains tested in this study, only 2 (3%) were sensitive to tobramycin. Interestingly, both of the sensitive strains were *B. vietnamiensis*. The remaining 66 strains were resistant to tobramycin although the level of resistance was varied and generally less than that exhibited towards polymyxin. For the study strains, tobramycin MICs ranged from 0.5->128 mg/L and 38 (56%) had MICs of less than 32 mg/L. This wide range of MICs is similar to that obtained by Rajyaguru and Muszynski (1997), namely, 0.06->32 mg/L. However, this data differs slightly from previously described results (Fass and Barnishan, 1980) in which a MIC range of 32 ->64 mg/L, when only 10 strains were examined.

Cationic antibiotics such as polymyxins and aminoglycosides are absorbed by Gram-negative bacteria through a self-promoted uptake that disrupts the organism's outer membrane structure (Rajyaguru and Muszynski, 1997). Cationic antibiotics bind to anionic sites at the bacterial surface and alter the permeability of the outer membrane. It has been shown that members of the *B. cepacia* complex are inherently resistant to polymyxins and aminoglycosides in part because these compounds can not permeabilize the organisms' outer membranes (Moore and Hancock, 1986). It has also been suggested that the inability of cationic antibiotics to permeabilise the *B. cepacia* complex outer membrane is due to the bacteria having an LPS composition, structure or function that is different from that of other Gram-negative organisms (Rajyaguru and Muszynski, 1997). Evidence for this hypothesis includes the fact that the *B. cepacia* complex contains much lower levels of anions, such as phosphates, compared to other Gram-negative bacteria (Cox and Wilkinson, 1991). Lower level of anions would lead to a lowered affinity of the LPS for cationic antibiotics.

Since first isolated in 1944, several tetracycline compounds have been identified. All tetracyclines have a similar molecular structure, consisting of four benzene rings and also exhibit a similar spectrum of antibacterial activity. Tetracyclines are active against most Gram-positive bacteria and some Gram-negatives; the latter includes *H. influenzae* and *Bordetella pertussis*. Tetracyclines are another group of antibiotics that inhibit bacterial protein synthesis though binding to the 30S ribosomal. The use of tetracyclines

in clinical practice has gradually decreased owing to the prevalence of resistance and the development of more effective alternative antibiotics. In this study, all but one of the *B. cepacia* complex strains showed resistance to tetracycline; the exception was C2977, a *B. vietnamiensis* strain that was also sensitive to all the antibiotics except polymyxin B. In the case of tetracycline, a wide range of MICs, 1-128 mg/L, covering both susceptibility and resistance to the antibiotic was observed. This data differs from previously reported results in which all the *B. cepacia* strains were resistant to tetracyline, with MIC ranges of 8->64 mg/L (Fass and Barnishan, 1980) and 16-512 mg/L (Pitt, Kaufmann, *et al*, 1996). It should be noted, however, that the MIC range obtained in this study, which includes sensitivity to tetracycline, is due to a single sensitive strain.

Similar to the aminoglycosides and polymyxins, tetracyclines have a relatively small molecular weight and are hydrophilic and cationic. In the case of the *B. cepacia* complex, resistance to tetracycline could be partly due to the inability of the antibiotic to insert into and permeabilise the bacterial outer membrane. Plasmid-encoded resistance to tetracycline is also widespread.

Chloramphenicol was the first broad spectrum antibiotic to be discovered and is also a potent inhibitor of bacterial protein synthesis. In this study, the MICs of the strains studied ranged from 4-64 mg/L and 50% of the strains were resistant to chloramphenicol. Interestingly, in the early 80s, chloramphenicol was reported to be

one of the few antibiotics exhibiting activity against '*B. cepacia'* (Isles, Maclusky, *et al*, 1984). In recent years its use has declined. Resistance to chloramphenicol is frequently due to the production of the enzyme chloramphenicol acetyltransferase (CAT) that acetylates and inactivates the drug. In *B. cepacia* complex decreased permeability is thought to be the mechanism of resistance as no production of CAT or ribosomal resistance was detected in a high level resistance strain (Burns, Hedin, *et al*, 1989). This mechanism of resistance has been described in other Gram-negative bacteria.

Trimethoprim interrupts bacterial purine synthesis by interfering with the enzyme dihydrofolic acid reductase (DHFR), an essential enzyme in DNA synthesis. Since trimethoprim acts on the same metabolic pathway as sulphonamides, combination of these two drugs as trimethoprim-sulfamethoxazole results in synergy. Trimethoprim MICs observed in this study ranged from 0.25-64 mg/L and thus the *B. cepacia* strains tested in this study were more sensitive than those studied by Pitt and co-workers (1996), who recorded a MIC range of 2-512 mg/L. Historically, trimethoprim-sulfamethoxazole and chloramphenicol were considered to be the most effective antibiotics for the treatment of *B. cepacia* infections (Isles, Maclusky, *et al*, 1984) even though they have rather limited antimicrobial activity. Newer, more effective antibiotics have now surpassed them both.

Ciprofloxacin is a quinolone and like other quinolones inhibits bacterial DNA synthesis by targeting bacterial topoisomerases or gyrases. The target for ciprofloxacin is the A

subunit of DNA gyrase. In this study, most strains tested (88%) were sensitive to ciprofloxacin although the range of MICs observed, 0.12-64 mg/L, was very wide. This wide MIC range is similar to previously reported results (Lewin, Doherty, *et al*, 1993), who reported a range of < 0.06-128mg/L.

Ceftazidime is a third generation cephalosporin and inhibits bacterial cell growth. Although the majority of strains tested in this study (62%) were sensitive to ceftazidime, the proportion was much less than that observed for ciprofloxacin. Ceftazidime is currently very effective in treating *B. cepacia* infections (Blumer, Stern, *et al*, 1985, Lu, Chang, *et al*, 1997), despite resistance to β-lactams being common and well documented. In most bacterial pathogens, increased resistance to β- lactams occurs through decreased permeability or the production of β-lactamases. It has been reported that *B. cepacia* complex strains produce a unique and highly inducible β- lactamase, PenA, an enzyme that is associated with a metabolic pathway enabling some *B. cepacia* to use penicillin G as a sole source of carbon and energy (Beckman and Lessie 1979, Prince, Wood *et. al*, 1988). To my knowledge, no other bacterial pathogens have been shown to have the ability to metabolise an antibiotic in this way.

4.4.2. Antibiotic susceptibility of different genomovars within the *B. cepacia* complex

In this study, all *B. cepacia* complex strains were shown to be resistant to polymyxin B; in contrast, sensitivity of all the strains studied to a single antibiotic was not detected.

Interestingly, susceptibility of a collection of recently identified environmental isolates of *B. cepacia* genomovar III to ceftazidime and trimethoprim did not differ significantly to that of the clinical strains belonging to genomovar III. However, the environmental isolates of genomovar III proved to be more sensitive to ciprofloxacin and chloramphenicol than the clinial strains, X^2=5.3, DF=1, $0.05 > p > 0.02$ and X^2=9.8, DF=1, $0.01 > p > 0.001$, respectively. To date, *B. cepacia* genomovar III strains have been isolated most frequently from CF patients and, in some cases, are associated with a rapid decline and a fulminant, necrotising pneumonia that is often referred to as the 'cepacia syndrome'. The similarity in susceptibility of clinical and environmental isolates of genomovar III to trimethoprim, a previously routinely used antibiotic, and ceftazidime that is currently one of the few antibiotics effective against the *B. cepacia* complex is remarkable. Clinical isolates are typically thought to be more resistant to antibiotics used in clinical practice than environmental isolates because of previous exposures to the drugs.

Susceptibility of *B. cepacia* complex strains to choramphenicol was also dependent on the genomovar status, X^2=15.3, DF=4, 0.01>p>0.001 and the 17 *B. vietnamiensis* strains tested were found to be more sensitive to chloramphenicol than strains belonging to other genomovars, X^2=14.8, DF=1, $p < 0.001$. The increased susceptibility of *B. vietnamiensis* strains to chloramphenicol might be explained by a subtle difference in binding of chloramphenicol to the 70S ribosomes of this genomovar. This could be due to differences in the binding sites or other factors associated with the binding of the antibiotic. The amino acid sequences of target sites of representative strains from the different genomovars would have to be compared to ascertain this. Interestingly, *B. vietnamiensis* strains, of both clinical and environmental origin, were also more sensitive to ceftazidime than strains from genomovars I-IV, X^2=6.73, DF=1, 0.01>p>0.001.

4.4.3. Effect of carbon dioxide on antibiotic sensitivity

The MICs of representative *B. cepacia* strains in air and carbon dioxide were determined for the ß-lactams ceftazidime and cefpirome. In addition, trimethoprim, ciprofloxacin and chloramphenicol were also tested. Increased resistance to ß-lactams in carbon dioxide, when observed, was not more than a two-fold increase in MIC and much less than indicated previously (Corkill, Deveney, *et al*, 1994). Similarly, this study found that incubation of *B. cepacia* strains in 5% CO_2 had no effect on their susceptibilities to trimethoprim, ciprofloxacin or chloramphenicol. In the present study, the increases in MIC in the presence of carbon dioxide were erratic and not related to the genomovar

status or clinical or environmental source. Corkill *et al* (1994) reported that inhibition of *B. cepacia* by ß-lactam antibiotics decreased in an atmosphere of air with 5% CO_2 compared to air alone, and this reduced susceptibility was attributed to increased expression of ß-lactamase activity in a CO_2 –enriched atmosphere.

In the present study, carbon dioxide was found to have no effect on the susceptibility of *P. aeruginosa* to ß-lactams, confirming previous observations (Corkill, Deveney, *et al*, 1994). However, incubation in 5% CO_2 led to an eight-fold increase in *P. aeruginosa*'s MIC in ciprofloxacin; this antibiotic was not tested by Corkill and co-workers. The decreased susceptibility of *P. aeruginosa* to ciprofloxacin after incubation in carbon dioxide contrasts with Traub and Leonhard (1995) who found,with the exception of aminoglycosides, no appreciable alteration of MICs of most antimicrobial drugs for different bacterial pathogens, including *P. aeruginosa*, after incubation in 3% and 5% CO_2.

4.4.4. Sensitivities of the antibiotic-resistant mutants

B. cepacia complex strains are inherently resistant to different classes of antibiotics. In addition, once effective antibiotics have been identified, further antibiotic resistance often develops during therapy. Ideally, biopesticide strains should be sensitive to the antibiotics used in clinical practice and not have the potential for becoming resistant. *B. vietnamiensis* strains are favoured for biopesticide use because of their low incidence of isolation from CF patients. This study found *B. vietnamiensis* strains more sensitive to ceftazidime and chloramphenicol than strains from other genomovars. Sensitivity to

antibiotics used in clinical practice would seem to imply that *B. vietnamiensis* strains are safe for large-scale commercial application since if acquired by CF patients, they could be eradicated by antibiotic treatment. Strains that are naturally sensitive to antibiotics could acquire resistance by mutation or horizontal transfer of resistance genes. This study was carried out to determine the incidence of mutation conferring resistance to antibiotics in the *B. cepacia* complex.

Ceftazidime and ciprofloxacin resistant mutants were generated in strains from *B. cepacia* genomovar I, *B. mulivorans*, *B. cepacia* genomova III and *B. vietnamiensis*. Mutants were detected from frequencies of 10^{-6} to as low as 10^{-10}. Similar quinolone and β–lactam resistant mutational frequencies of 10^{-7} to 10^{-8} were reported previously (Sanders, Sanders, *et al*, 1984). Interestingly, in contrast to other antibiotics, no chloramphenicol-resistant mutants (i.e. with a six-fold increase in MIC) were generated. *B. vietnamiensis* isolates showed the greatest increases of MICs for ceftazidime and ciprofloxacin. Thus, although *B. vietnamiensis* strains were shown to be more sensitive to ceftazidime than strains from the other genomovars, they also showed the greatest potential for mutations to high resistance. If acquired by CF patients and other immuno-compromised individuals, therefore, *B. vietnamiensis* infections could be difficult to eradicate owing to the organisms' mutations to high resistance to the antibiotics used in clinical practice.

Some of the ceftazidime-resistant mutants had increased MIC for the other ß-lactam antibiotics, cefpirome and ampicillin, but no corresponding increases were observed for ciprofloxacin and chloramphenicol. Similarly, the ciprofloxacin resistant mutants also had increased resistance to other quinolones ofloxacin and grepafloxacin but no increased resistance to chloramphenicol or ceftazidime. Hence, it appeared that the resistance generated in *B. cepacia* only affected antibiotics belonging to the same class, possibly implying that the antibiotics binding sites were altered rather than porin-associated decreased permeability, which would have resulted in cross-resistance involving unrelated antibiotics.

The use of antibiotics is often linked to the selection of resistant mutants since the antibiotics increase the frequency of genetic variation, resulting in resistance. Strong mutator genes promote genetic diversity which is probably the driving force in the evolution of antibiotic resistance under antibiotic pressure (Taddei, May, *et al*, 1997). Pathogenic bacteria are associated with a high frequency of strains with enhanced rates of mutations, i.e. hypermutable strains (LeClerc, Li, *et al*, 1996). Isolates of *E. coli* and *Salmonella enterica* implicated in food-related outbreaks were found to contain a higher incidence of hypermutable strains, compared to non-pathogenic strains (LeClerc, Li, *et al*, 1996).

Recent studies suggest that the adaptation of bacteria to a heterogeneous and changing environment such as the CF lungs, that are progressively deteriorating, constantly exposed to different antibiotics and the host's immunity, promotes the selection of

hypermutable strains (Oliver, Cantón, *et al*, 2000). *P. aeruginosa* strains isolated from CF patients, unlike those obtained from patients with acute infections, have a high frequency of mutator strains (Oliver, Cantón, *et al*, 2000). Interestingly, the same authors found the high mutator *P. aeruginosa* strains to be at least twice as resistant to several antibiotics as the non-mutator isolates. It is believed that the high proportion of mutator *P. aeruginosa* isolates from CF patients suggests that rapid adaptation is required by bacterial populations to survive in the lungs of these patients.

Thus, according to these recent theories, since the *B. cepacia* complex consists of emerging pathogens, and in the CF lungs, they inhabit a heterogeneous, changing environment, it is possible that this group of organisms contains a high incidence of hypermutable strains. High mutator *P. aeruginosa* isolates from CF patients had mutation frequencies of $x10^{-6}$ (Oliver, Cantón, *et al*, 2000). In the present study, some *B. cepacia* strains had resistant mutant frequencies to ceftazidime and ciprofloxacin of $x10^{-6}$, which, according to Oliver and co-workers, indicates hypermutable strains.

This study provides preliminary data that, like the other major CF pathogen *P. aeruginosa*, *B. cepacia* complex strains could have a high frequency of strains with enhanced rates of mutations. A more detailed study on *B. cepacia*'s mutation rates would have to be carried out to ascertain this. There is also a need to determine whether or not *B. cepacia*'s high resistance to several antibiotics is associated with hypermutation, as in *P. aeruginosa*.

In conclusion, although certain members of the *B. cepacia* complex such as *B. vietnamiensis* and the environmental isolates of genomovar III are more sensitive to antibiotics, when these strains were challenged with antibiotics routinely used in the treatment of CF patients, they showed increased resistance that was more marked than that observed for the less sensitive strains. Thus strains cannot be regarded as posing a lower risk of causing difficult-to-clear infections, since they all have the potential to become resistant to antibiotics. Since the *B. cepacia* complex comprises emerging pathogens in the changeable environment of CF lungs, it is expected that these organisms have a high incidence of hypermutable strains, which in some organisms, are associated with increased levels of resistance to antibiotics. To my knowledge, this is the first 'proper' examination of antibiotic susceptibilities of the *B. cepacia* complex as opposed to *B. cepacia*. However the data on *B. stabilis* (*B. cepacia* genomovar IV) might not reflect the accurate antibiotic susceptibilities of the species since only a few organisms were examined.

CHAPTER 5

The *B. cepacia* complex: virulence factors & epidemic markers

As described in some detail in the introduction, although *B. cepacia* was originally described as a phytophathogen, it has now emerged as a life-threatening opportunistic pathogen in immuno-compromised humans. Ironically, members of the *B. cepacia* complex have also raised considerable biotechnological interest as candidate biopesticides and bioremediators. The issue of human hazards posed by *B. cepacia* has become increasingly contentious. It has been argued that since the majority of candidate biopesticides were originally isolated from natural environments they present a relatively low risk to humans. Similarly agricultural microbiologists and biotechnologists have argued that clinical isolates of *B. cepacia* are distinctive from environmental *B. cepacia* and, for example, lack phytopathogenicity (Bevivino, Tabacchioni *et al*, 1994).

Members of the *B. cepacia* complex have been recognised as important pathogens in patients with cystic fibrosis since the early 1980s. Treatment of CF patients colonised with *B. cepacia* is complicated not only by the organism's intrinsic resistance to commonly used antipseudomonal antibiotics but also by its ability to spread from patient-to-patient, both in hospitals and through social contact. The prognosis for CF patients harbouring *B. cepacia* is variable, ranging from asymptomatic carriage through

a gradual decline to a rapid, fatal necrotising pneumonia that is sometimes accompanied by septicaemia (Isles, Maclusky, *et al*, 1984). The nature of the virulence factors and host/ pathogen interactions that contribute to the pathogenicity of the *B. cepacia* complex remain largely unkown. Several putative virulence factors have been proposed, some of which will be investigated in this section.

5.1. Phytopathogenicity of the *B. cepacia* complex and *B. gladioli*

In order to determine whether or not there are differences in the phytopathogenicity of clinical and biopesticide *B. cepacia* strains, the pathogenicity of an epidemic CF strain, *B. cepacia* J2315, a candidate biopesticide strain *B. vietnamiensis* AMMD and a *B. gladioli* ATCC 10247 was investigated on British white and red onions, and baking potatoes. In this study, phytopathogenicity was found to be dependent on the host plant tested.

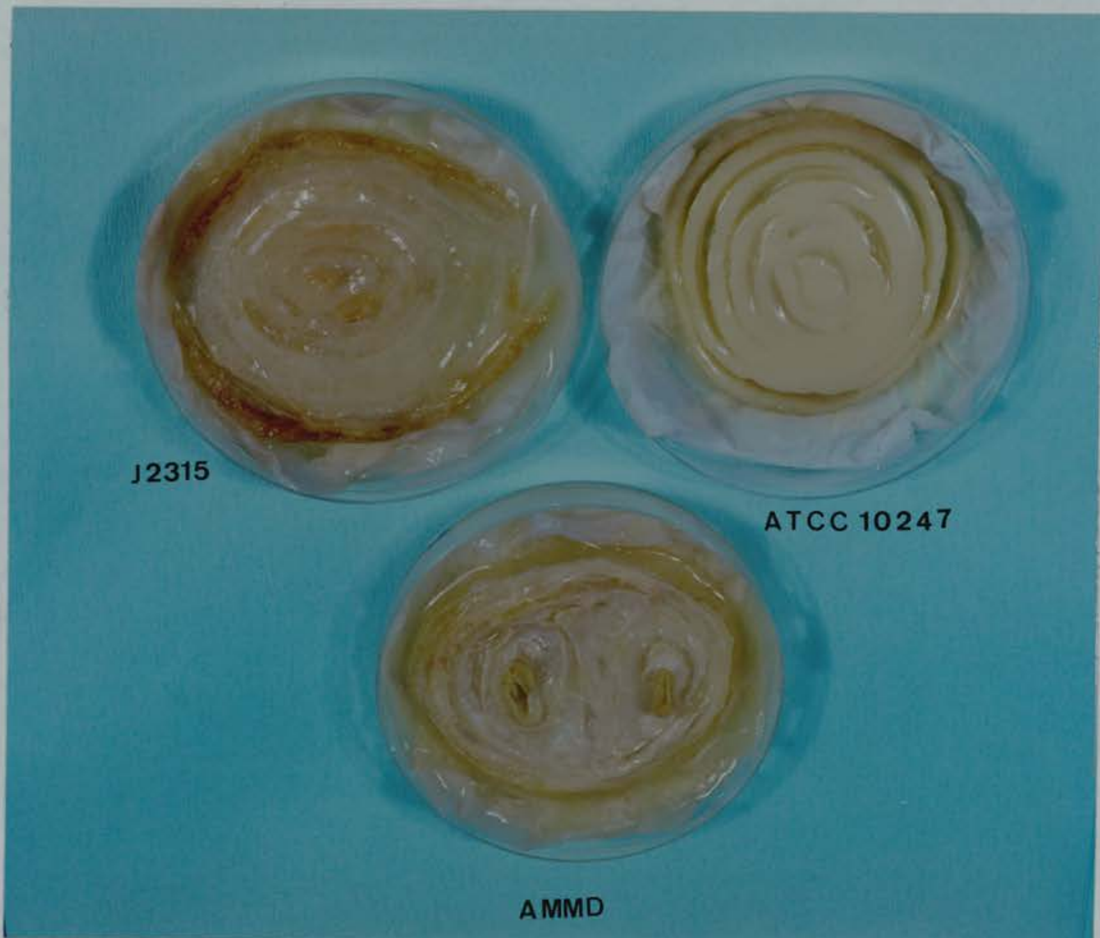
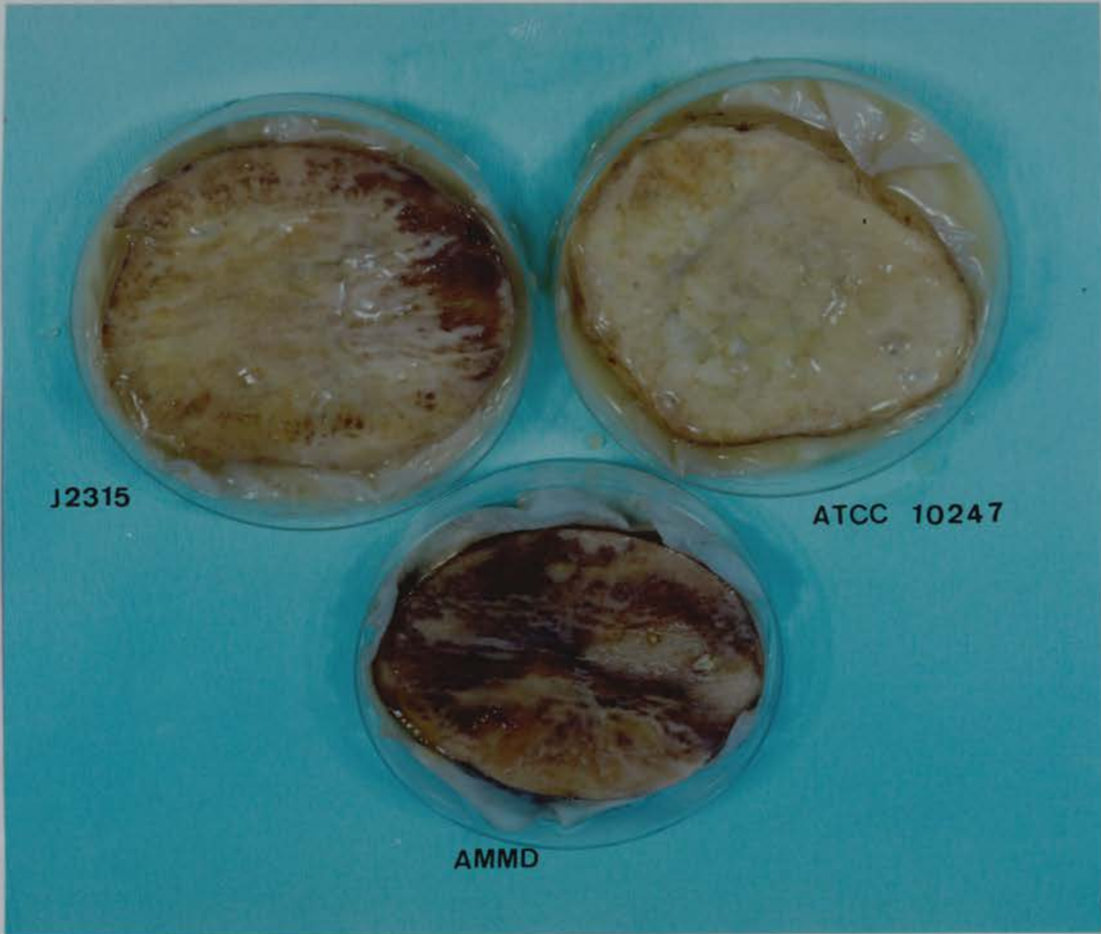


Fig 5.1.a) Phytopathogenicity of the *B. cepacia* complex (J2315, AMMD) and *B. gladioli* (ATCC 10247) on white onions.

Fig 5.1.b) Phytopathogenicity of the *B. cepacia* complex (J2315, AMMD) and *B. gladioli* (ATCC 10247) on red onions



5.1.c) Phytopathogenicity of the *B. cepacia* complex (J2315, AMMD) and *B. gladioli* (ATCC 10247) on potatoes.

5.2. Putative virulence factors and epidemic markers of the *B. cepacia* complex

Several putative virulence markers have been identified in the *B. cepacia* complex. In addition, two epidemic strain markers *cbl*A, encoding a major adhesin, cable pilus and the genomic marker, BCESM (*Burkholderia cepacia* epidemic strain marker) have also been associated with some epidemic strains. Most previous studies of epidemic markers were performed prior to or during the taxonomic re-organisation of the *B. cepacia* complex. In this study, the distribution of elastase, lipase, melanin, catalase and the regulatory dsb gene, together with cblA and BCESM was investigated in 71 isolates representing the major sub-populations which comprise the *B. cepacia* complex. These isolates included the 30 strains that make up the *B. cepacia* strain panel. The results are summarised in Table 5.1 and Fig 5.2. Overall, the distribution of putative virulence and epidemic markers varied considerably both in incidence across the whole *B. cepacia* complex and within genomovars. The significance of these results is discussed separately in section 5.3.

Table 5.1.Putative virulence factors of the *B. cepacia* complex

Strain	Ela	Lipa	*dsb*	Prot	Cat	Mela	BCESM	*cbl A*
***B. cepacia* genomovar I**								
ATCC 17759	-	+	-	+	-	-	-	-
ATCC 25416	-	+	+	+	-	-	-	-
LMG 17997	-	+	-	+	-	+	-	-
CEP 509	-	+	-	-	-	+	-	-
C1963	-	+	-	+	-	-	-	-
J2535	-	+	+	+	-	-	-	-
J2540	-	+	-	+	-	-	-	+
J2552	-	+	-	+	-	-	NT	NT
B. multivorans								
ATCC 17616	-	-	-	+	-	-	-	-
LMG 13010	-	+	-	-	+	-	-	-
CF-A1-1	-	+	-	-	+	-	-	-
C1596	-	+	-	+	+	-	NT	NT
C1572	+	-	-	-	+	-	NT	NT
C1576	-	-	-	+	+	-	-	-
C1652	-	-	-	-	+	-	-	-
C1857	-	+	-	+	+	-	-	-
C1911	-	-	-	+	+	-	-	-
C2775	-	+	-	+	+	-	-	-
C5393	-	+	-	-	+	+	-	-
C1962	-	+	-	-	+	-	-	-

Ela, elastase; Lipa, lipase; Prot, protease; Mela, melanin; BCESM, *Burkholderia cepacia* epidemic strain marker; *cbl* A, cable pilus type A; NT, not tested; +, present; -, absent.

Table 5.1. cont.

Strain	Ela	Lipa	*dsb*	Prot	Cat	Mela	BCESM	*cbl A*
JTC	-	+	-	+	+	-	-	-
J2395	-	+	-	+	+	-	-	-
J2866	-	+	-	-	+	-	-	-
J2867	-	+	-	-	+	-	-	-
J2868	-	+	-	-	+	-	-	-
249-2	-	-	-	-	-	-	-	-
***B. cepacia* genomovar III**								
ATCC 17765	-	-	-	-	-	-	+	-
BC7	-	+	+	-	+	+	+	+
C517	-	-	-	-	+	-	-	-
C1335	-	-	-	+	-	-	-	-
C1394	-	-	+	+	-	-	-	+
C1632	-	-	-	+	+	-	-	-
C2374	-	+	-	+	-	-	NT	NT
C5424	-	+	+	+	+	+	+	+
C6433	-	+	-	+	+	-	+	-
C1773	-	+	-	-	+	-	NT	NT
K56-2	-	+	+	+	-	-	+	+
PC184	-	+	+	+	+	-	+	-
715j	-	+	+	+	-	-	NT	NT
J2315	-	+	+	+	+	+	+	+
CEP 511	-	+	+	+	+	+	+	-

Ela, elastase; Lipa, lipase; Prot, protease; Mela, melanin; BCESM, *Burkholderia cepacia* epidemic strain marker; *cbl* A, cable pilus type A; NT, not tested; +, present; -, absent.

Table 5.1 cont.

Strain	Ela	Lipa	*dsb*	Prot	Cat	Mela	BCESM	*cbl A*
B. cepacia								
genomovar III								
R2817	-	+	NT	-	-	-	-	-
R2827	-	-	NT	-	-	-	-	-
R8351	-	NT	NT	-	-	-	-	-
R8571	-	-	NT	-	-	-	-	-
R8574	-	-	NT	-	-	-	-	-
R8580	-	-	NT	-	-	-	-	-
R8605	-	+	NT	-	-	-	-	-
R9235	-	-	NT	-	-	-	-	-
R9239	+	-	NT	-	-	-	-	-
R9243	+	-	NT	-	-	-	-	-
R9338	-	NT	NT	-	-	-	-	-
R9342	-	NT	NT	-	-	-	-	-
C3039	+	+	-	+	-	-	-	-
C3041	-	+	+	+	-	-	+	-
Indefinite								
C1511 I-III-IV	-	+	NT	+	-	-	NT	NT
C2008 I-III	-	+	NT	+	-	-	NT	NT
C2349 I-III	-	+	NT	+	-	-	NT	NT

Ela, elastase; Lipa, lipase; Prot, protease; Mela, melanin; BCESM, *Burkholderia cepacia* epidemic strain marker; *cbl* A, cable pilus type A; NT, not tested, R****', environmental genomovar III; +, present; -, absent.

Table 5.1 cont.

Strain	Ela	Lipa	*dsb*	Prot	Cat	Mela	BCESM	*cbl A*
B. stabilis								
LMG 18888	-	+	-	+	-	-	-	-
LMG 14086	-	+	-	+	-	-	-	-
LMG 14294	-	+	-	+	-	-	-	-
C7322	-	+	-	-	-	-	-	-
B. vietnamiensis								
ATCC 53266	-	+	-	+	-	-	-	-
ATCC 53267	-	+	-	+	+	-	-	-
ATCC 29424	-	+	-	+	-	-	-	
LMG 16232	-	+	-	+	-	-	-	-
LMG 10929	-	+	-	-	-	-	-	-
C2972	-	-	-	-	-	-	-	-
C2973	-	+	-	+	-	-	-	-
C1704	-	-	-	+	-	-	-	-
C2977	-	+	-	-	-	-	-	-
C2979	-	+	-	+	-	-	-	-
C2974	-	+	-	-	-	-	-	-
C2975	-	+	-	+	-	-	-	-
C2976	-	+	-	-	-	-	-	-
FC 441	-	+	-	-	-	-	-	-
PC 259	-	+	-	-	-	-	-	-
AMMD	-	+	-	+	-	-	-	-
J1697	-	+	-	+	-	-	NT	NT
J1705	-	+	-	+	-	-	NT	NT

Ela, elastase; Lipa, lipase; Prot, protease; Mela, melanin; BCESM, *Burkholderia cepacia* epidemic strain marker; *cbl* A, cable pilus type A; NT, not tested; +, present; -, absent.

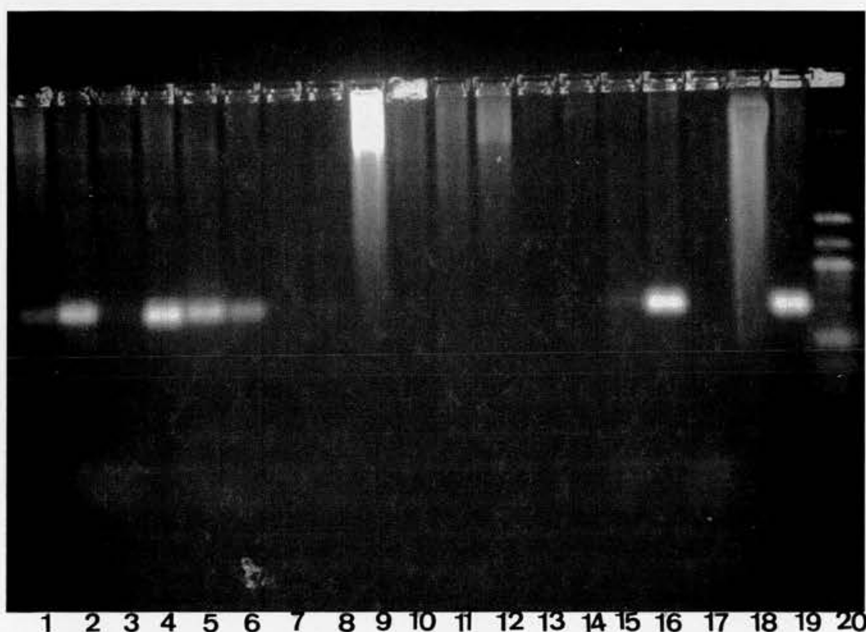


Fig 5.2.a) Distribution of the *dsb* gene in the *B. cepacia* complex strain panel
Lanes 1 to 20, CEP 511, PC184, C6433, C5424, K56-2, BC7, 249-2, ATCC 17616, JTC, CF-A1-1, LMG 13010, C5393, C1962, , C1576, ATCC 17759, ATCC 25416, LMG 17997, CEP 509, pSN1, 100 bp ladder, respectively.

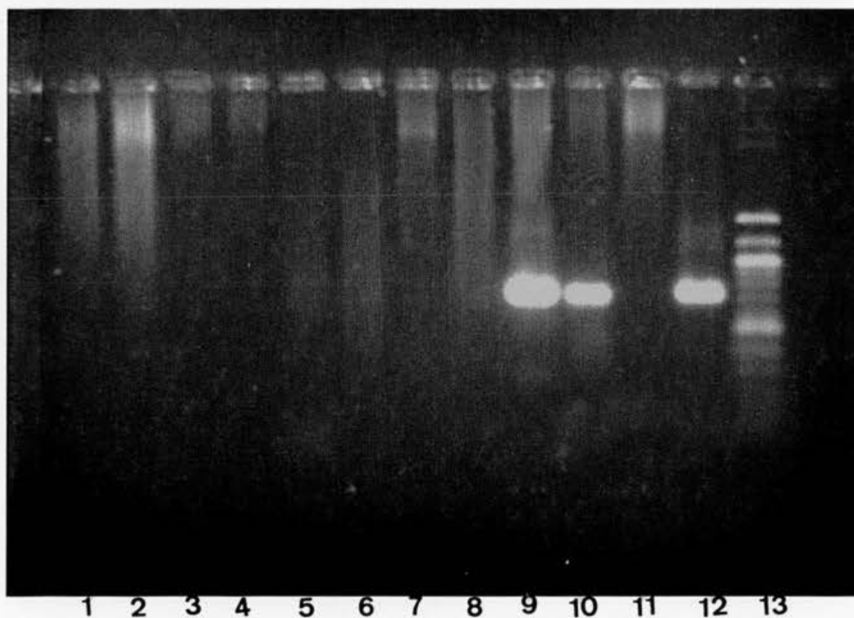


Fig 5.2.b) Distribution of the *dsb* gene in the *B. cepacia* complex strain panel
Lanes 1-13, LMG 16232, LMG 10929, FC 441, PC 259, LMG 10486, LMG 18888, C7322, LMG 14294, ATCC 17765, J2315, C1394, J415, pSN1, 100 bp ladder, respectively.

5.3. Discussion

5.3.1. Phytopathogenicity of the *B. cepacia* complex

Investigation of phytopathogenicity with members of the *B. cepacia* complex was informative and suggested differences to some currently held opinions. For example, these preliminary studies showed that the clinical *B. cepacia* strain J2315 and the rhizosphere strain *B. vietnamiensis* AMMD both caused maceration of British white onions; furthermore, there was no difference in the extent or nature of the maceration. This result contrasts with previous reports, (Gonzalez and Vidaver, 1979), in which *B. cepacia* strains isolated from plants or the rhizosphere had greater plant pathogenicity than strains of clinical origin. An explanation for this discrepancy could lie in the type of onion used and the low number of strains examined.

In British red onions, strain AMMD caused a slimy soft rot, similar to the type of rotting observed on the white onion, whereas the clinical strain J2315 failed to cause any rotting. This onion-specific difference in phytopathogenicity is interesting and suggests that the pathogenicity of the *B. cepacia* complex for onions may be dependent on the variety of onion used, as well as on the individual strains examined. This dependence of *B. cepacia* complex strains' pathogenicity on the variety of onion tested was recently reported by Sfalanga and co-workers (1999); who found that the *B. cepacia* complex strain NCPPB5782 caused complete maceration of the cv. *Bianca agostana* onion slices but had no effect on the cv. Dorata di Parma slices.

B. gladioli is taxonomically closely related to the *B. cepacia* complex and routinely used identification methods such as the API 20NE are unable to distinguish between these two groups of organisms. Inoculation of both types of onions with a strain of *B. gladioli* resulted in the accumulation of a slimy layer on the surface of the onion slices and not the typical soft rot caused by *B. cepacia.* It is possible that *B. gladioli* does not cause soft rot on onions although more studies would have to be carried out to verify this observation.

Since the *B. cepacia* complex could be used as a biopesticide on a wide variety of plants, pathogenicity on potatoes was also tested. *B. gladioli* caused a complete softening of the potato slice, *B. cepacia* J2315 caused less rotting and *B. vietnamiensis* AMMD caused no rotting of the potato slice. When the phytopathogenicity of the *B. cepacia* complex was assessed on potato slices therefore, the representative clinical strain was more pathogenic than the environmental isolate from the rhizosphere. This result again confirms that phytopathogenicity was not restricted to environmental isolate tested and that the property was possessed by a well-documented clinical strain.

In conclusion, phytopathogenicity of the *B. cepacia* complex may not be restricted to the origin of the strain, whether clinical or rhizosphere, but rather, be dependent on the host plant and the individual strain of the *B. cepacia* complex, regardless of its origin.

5.3.2. Production of proteases by the *B. cepacia* complex

In this study, a minority (6%) of the *B. cepacia* complex strains studied produced proteolytic activity which degraded elastin and the strains producing elastase included both environmental and clinical isolates. This low prevalence together with the comprehensive collection investigated could explain why a previous study on *B. cepacia* isolates from CF patients reported that none of the isolates studied could degrade elastin (McKevitt, Woods, *et al*, 1984). Elastin forms the major constituent of elastic tissue fibres and these include the walls of the alveoli of the lungs. Results from this and previous studies indicate that elastase production is not a widespread property within the *B. cepacia* complex and is probably not one of the virulence factors responsible for CF lung disease.

In this study, most strains of the *B. cepacia* complex (57%) produced protease as assessed by their ability to digest skimmed milk. Significantly, the proteolytic strains were of both clinical and environmental origin and spanned all five genomovars studied. This widespread distribution contrasts markedly with (Bevivino Tabacchioni, *et al*, 1994), who reported that only clinical strains produced protease. However,in the Bevivino *et al* study, only four strains were included and azoalbumin was used as the substrate and not skimmed milk. A similar study that also used skimmed milk as a substrate reported that the majority of strains studied could degrade casein (McKevitt, Woods, *et al*, 1984). However, this study found that the ability to detect casein degradation varied with the medium used. Few strains were able to degrade casein in all

four media used. Thus, estimates of protease production need to be assessed carefully and may be underestimated if an unsuitable medium is used for the assay. A striking result from the proteolytic assays performed during this thesis was that none of the recently identified rhizosphere *B. cepacia* genomovar III strains showed the ability to degrade casein. This is an interesting result and needs to be investigated in more detail.

In an attempt to probe protease production further, the presence of the *dsb* gene, whose product is required for the formation of *B. cepacia's* metalloprotease (Abe and Nakazawa, 1996), was investigated. The *dsb* gene was detected by PCR in 11 (17%) of the 65 strains studied and of those strains possessing the gene, eight (73%) belonged to *B. cepacia* genomovar III. All the genomovar III strains were isolated from CF patients. Interestingly, no less than seven of the eight genomovar III strains with the *dsb* gene have caused epidemics in different CF communities. It should be noted however that three isolates J2315, BC7 and K56-2 are clonally related representatives of the epidemic ET12 lineage, isolated from different geographical regions (Mahenthiralingam, Coenye, *et al*, 2000). No information was available on the epidemic potential of the remaining *B. cepacia* strain 715j. Interestingly, the *dsb* gene was not detected in strain C1632, a *B. cepacia* genomovar III isolate that caused an epidemic in Newcastle, UK (Simpson, Finlay et al, 1994). These results could mean that either the observed association is purely coincidental or that the *dsb* gene is unstable and C1632 has subsequently lost its copy.

Two other strains with the *dsb* gene, J2535 and ATCC 25416 belong to *B. cepacia*

genomovar I and were isolated from rotting bark and onion, respectively (Butler, Doherty, *et al*, 1995; Mahenthiralingam, Coenye, *et al*, 2000). The remaining strain, C3041, was one of three candidate biopesticide strains that were subsequently withdrawn because they were perceived to be of a human risk owing to possession of 'virulence' characteristic including the BCESM, or because they were found to belong to genomovar III.

Although the role of proteases in the pathogenicity of the *B. cepacia* complex has not been unequivocally established, intratracheal instillation of a purified metalloprotease produced by the *B. cepacia* genomovar III strain 715j into rat lungs, produces bronchopneumonia (McKevitt, Bajaksouzian, *et al*, 1989). In conclusion, as far as the virulence potential of proteases in the *B. cepacia* complex is concerned, the jury is still out.

5.3.3. Lipase production in the *B. cepacia* complex

In this study, production of lipase was found to be common within the *B. cepacia* complex and these results are similar to previous observations (McKevitt, Woods, *et al*, 1984; Gessner and Mortensen, 1990). The egg yolk plate assay is commonly used for the detection of lecithinase, an enzyme which catalyzes the hydrolysis of lecithin (phosphatidylcholine) resulting in the liberation of phosphorous and choline as well as the precipitation of fat, which gives rise to the reaction's characteristic opalescence

(Esselmannm and Liu, 1961). Lecithin is a phospholipid found in the membranes of animal cells.

Egg yolk is a complex substrate consisting of a mixture of compounds and a more direct measurement of lecithinase activity would be to use a single substrate such as *p*-nitrophenylphosphorylcholine. Previous research has shown that *B. cepacia* strains producing a typical egg yolk reaction may not produce lecithinase activity on *p*-nitrophenylphosphorylcholine (Lonon, Woods, *et al*, 1988), indicating that the lipase activity on egg yolk agar could be due to an enzyme other than lecithinase. Despite these sometimes misleading results, egg yolk agar is still routinely used for the detection of lecithinase in other organisms such as *Clostridium perfringens*. Egg yolk agar has the advantages of being cheap and readily available.

Earlier studies found all lecithinase producing *B. cepacia* strains to be haemolytic (Esselmann and Liu, 1961) but more recent studies have found no direct correlation between the amount of lecithinase and beta haemolytic activity (Nakazawa, Yamada, *et al,* 1987; Vasil, Krieg, *et al,* 1990). However, all haemolytic strains have been shown to produce lecithinase (Nakazawa, Yamada, *et al*, 1987; Vasil, Krieg, *et al*, 1990).

A possible role of *B. cepacia* lipase as a virulence factor was provided by the demonstration that rat pulmonary alveolar macrophages have a reduced capacity for phagocytosis when incubated with *B. cepacia* lipase (Straus, Lonon, *et al* , 1992). These researchers found that *B. cepacia* lipase acted on and changed the surface of

macrophages and led to a reduction in their ability to internalise the organism. Thus *B. cepacia* lipase could allow the organism to evade the immune system by avoiding phagocytosis by macrophages.

5.3.4. LPS and virulence of the *B. cepacia* complex

Although this study did not include examination of the LPS chemotypes of the *B. cepacia* complex, LPS is a well known virulence factor due to its potent immuno-stimulatory effects on macrophages, granulocytes and B-lymphocytes (Rietschel, Kirikae, *et al*, 1993). Several studies indicate that *B. cepacia* LPS contributes to the organisms' pathogenesis in CF lung disease and suggest an explanation for the 'cepacia syndrome', its unique association with *B. cepacia* colonisation and absence from patients colonised with other pathogens. Most strains of the *B. cepacia* complex express smooth LPS, although some genomovar III strains can also express rough LPS (Evans, Poxton, *et al*, 1999) but virulence is not related to LPS chemotype.

Various strains of *B. cepacia* produce an extracellular toxic complex (ETC) composed of carbohydrate, LPS and protein, which is lethal to mice and produces extensive lung damage in rats (Straus, Lonon, *et al*, 1989). The most toxic ETC preparations are those with the highest concentration of LPS, and purified LPS has the same toxicity on mice as the complex, an indication that the LPS portion of the ETC probably confers toxicity to the complex.

More evidence for the toxicity of *B. cepacia* LPS was provided by (Shaw, Poxton, *et al*, 1995), who demonstrated that *B. cepacia* LPS is not only endotoxic but can also induce high levels of tumour necrosis factor (TNF). Interestingly, this study found that *B. cepacia* LPS induced approximatley nine times as much TNF compared to *P. aeruginosa* LPS. This indicates that although *P. aeruginosa* is the leading CF pathogen affecting up to 90% of patients, *B. cepacia* has a greater potential to cause and sustain immune-mediated damage in the lung. The same researchers also found that environmental isolates of *B. cepacia* had a high capacity to stimulate TNF, although at much lower levels than clinical isolates. LPS preparations from both clinical and environmental strains of the *B. cepacia* complex were at least four times more endotoxic than preparations from *P. aeruginosa* strains. Stimulation of TNF by *B. cepacia* LPS was recently confirmed by Zughaier and co-workers (Zughaier, Ryley, *et al*, 1999b), who also found *B. cepacia* LPS to have greater inducing activity than LPS from *P. aeruginosa* and even *S. maltophilia*.

Involvement of *B. cepacia* LPS in the immune system was further demonstrated when it was shown to increase CD11b, a component of the complement receptor CR3 which is expressed on the surfaces of macrophages and is involved in various neutrophil activities including activation (Hughes, Stewart, *et al*, 1997). LPS from both clinical and environmental *B. cepacia* induced a marked increase in CD11b expression whereas LPS from *P. aeruginosa* had very little effect (Hughes, Stewart, *et al*, 1997). Similarly, LPS from *B. cepacia* was able to prime intracellular production of H_2O_2 in neutrophils to

greater levels than for *P. aeruginosa*. A significant observation was that LPS preparations of the environmental *B. cepacia* genomovar I strain J2552 primed neurophil respiratory burst activity to the greatest degree.

Two groups of researchers have proposed a hypothesis for the involvement of *B. cepacia* LPS in the 'cepacia syndrome'. Hughes *et al* (1997) and Zughaier *et al* (1999b) have suggested that *B. cepacia* LPS-mediated stimulation of neutrophils and macrophages may be greater in certain individuals, thus increasing the rate of inflammatory damage and sometimes permitting the systemic spread of *B. cepacia* and thus resulting in the 'cepacia syndrome'. The greater immuno-stimulatory effect of *B. cepacia* LPS compared to that of other CF pathogens (Shaw, Poxton, *et al*, 1995 ; Hughes, Stewart, *et al*, 1997; Zughaier, Ryley, *et al*, 1999b; Hutchinson, Bonell, *et al* , 2000) could also explain the exclusive observance of 'cepacia syndrome' in patients colonised with *B. cepacia*.

5.3.5.Catalase and melanin production in the *B. cepacia* complex

Catalases are widely distributed in nature and perform diversified functions. Arguably, the most beneficial catalase for humans is the catalase-peroxidase encoded by the *katG* gene of *Mycobacteria tuberculosis* (Heym, Zhang, *et al*, 1993). The catalase-peroxidase of *M. tuberculosis* mediates the organism's susceptibility to isoniazid, one of the most effective agents in the treatment of tuberculosis (Zhang, Heym, *et al* 1992). Mutation of the *katG* gene leads to resistance to isoniazid.

Most bacterial species produce catalases that confer obvious advantages to themselves rather than to humans. In this study of members of the *B. cepacia* complex, catalase production was only observed in clinical strains and not in environmental ones. These potentially important results are consistent with a previous report (Gessner and Mortensen, 1990) that found significant differences in the production of catalase by *B. cepacia* isolates from CF patients compared to environmental and non-CF controls.

Stimulated neutrophils and macrophages reduce molecular oxygen to highly reactive and toxic oxygen species that include the superoxide O_2^- and hydrogen peroxide H_2O_2. The superoxide is unstable and reacts to form hydrogen peroxide (Hassett and Cohen, 1989). Catalases convert hydrogen peroxide to harmless water and molecular oxygen and can thus play an important role in bacterial virulence by combating the respiratory burst of human phagocytes. The environmental *B. cepacia* genomovar I strain J2552, shown in this study not to produce catalase, is efficiently killed by hydrogen peroxide (Smith, Green, *et al*, 1999). In contrast *B. cepacia* J2315 which produces catalase is resistant to killing by hydrogen peroxide alone (Smith, Green, *et al*, 1999). Thus, strain J2315 is resistant to killing by hydrogen peroxide because the catalase it produces converts hydrogen peroxide to harmless water and molecular oxygen. In contrast, the environmental isolate J2552 is rapidly killed by hydrogen peroxide since it lacks the catalase required to inactive it.

Owing to their nature as stable free radicals, melanins from various sources typically demonstrate potent scavenger activity against O_2^- (Sichel, Corsaro, *et al*, 1991). More recently, melanin from the epidemic *B. cepacia* ET12 lineage has been shown to act as an efficient scavenger of superoxide radicals produced during the respiratory burst of monocytes (Zughaier, Ryley, *et al*, 1999a). These researchers suggest that melanin-producing strains of *B. cepacia* derive protection from its free-radical scavenging properties, which could aid the colonisation and transmission of these strains.

An interesting and potentially important result from this survey of *B. cepacia* isolates was that melanin-producing strains were of clinical origin a result which mirrors that found with catalase production. Furthermore, most strains that produced melanin also produced catalase. Interestingly, two of the three clinical genomovar I strains, which produced no catalase, produced melanin and thus still exhibit an antioxidant mechanism.

In conclusion, whilst *B. cepacia* LPS is a potent immuno-stimulant, the organisms could remain protected from the reactive oxygen species of the respiratory burst by the antioxidant enzyme catalase and by the scavenging properties of melanin. While continual shedding of LPS activates phagocytes and induces a potent inflammatory response, protection of *B. cepacia* from the host's defense system contributes to the organism's persistence in the CF lung.

5.3.6. Distribution of genes encoding putative transmissibility factors in the *B. cepacia* complex

The ability of some *B. cepacia* isolates to cause cross-infection among CF patients is now well known and documented (LiPuma, Dasen, *et al*, 1990; Govan, Brown, *et al*, 1993), and CF patients colonised with *B. cepacia* are routinely treated separately from non-colonised patients in order to minimise the risk of cross-infection. Since *B. cepacia* can also be transmitted by social contact (Govan, Brown, *et al*, 1993; Pegues, Carson, *et al*, 1994; Cazzola, Amalfitano, *et al*, 1996), colonised and noncolonised patients are advised to avoid social contacts and comply with other draconian segregation policies.

To date, three genetic markers have been described in strains responsible for patient-to-patient spread. The first of these markers to be described was the *cbl A* gene, encoding giant cable pili that bind to CF mucin and airway epithelial cells (Sajjan, Corey, *et al*, 1992; Sajjan and Forstner, 1992; Sajjan and Forstner, 1993; Sajjan, Sun, *et al*, 1995; Sun, Jiang, *et al*, 1995). Two other markers, a hybrid of two insertion sequences, the IS402-IS1356 element (Tyler, Rozee, *et al*, 1996) and a 1.4kb fragment designated the *B. cepacia* epidemic strain marker (BCESM) (Mahenthiralingam, Simpson, *et al*, 1997), have also been associated with epidemic strains but no known function.

Distribution of the *cblA* and BCESM markers was investigated in this study. Four of the eight *B. cepacia* genomovar III epidemic isolates possessed both markers, two had only the BCESM and one had only the *cbl A* marker. C1632, the Newcastle, UK, epidemic

strain possessed neither of these two markers. Notably, this was also the only epidemic *B. cepacia* genomovar III strain in which the *dsb* gene, encoding a protease assembly protein, was not detected. The BCESM marker was also detected in strain ATCC 17765, a *B. cepacia* genomovar III strain that caused a urinary tract infection in a UK non-CF patient.

Neither *cbl*A nor BCESM markers was detected in C1576, a *B. multivorans* strain responsible for a large epidemic in Glasgow. Absence of the BCESM from epidemic strains has been reported previously (Mahenthiralingam, Simpson, *et al*, 1997) and it was suggested that this region may be subject to some instability. Of interest from an ecological and evolution view, the *cbl A* marker was also detected in J2540, a *B. cepacia* genomovar I strain isolated from soil.

Taken together, these results suggest that genetic markers are useful in the identification of potentially epidemic strains but should be used with caution since no presently known factor can be used to unequivocally designate a strain as either epidemic or non-epidemic. A recent report (Clode, Kaufmann, *et al,* 2000) suggests that only patients colonised with strains in which epidemic markers are identified by PCR should be segregated from other CF patients. In view of current knowledge such a recommendation and practice could prove presumptuous and indeed dangerous, since strains such the Glasgow epidemic strain, C1576 (Whiteford, Wilkinson, *et al*, 1995; Mahenthiralingham, Coenye, *et al*, 2000) possess none of the known epidemic markers.

CHAPTER 6

B. cepacia genomovar III: Origins

Although all reported genomovars of the *B. cepacia* complex have been isolated from CF patients (Vandamme, Holmes, *et al*, 1997), the majority of these isolates, and most epidemic strains, are associated with *B. cepacia* genomovar III and *B. multivorans* (Vandamme, Holmes, *et al*, 1997; Mahenthiralingam, Coenye, *et al*, 2000; Mahenthiralingam, Bischof, *et al*, 2000). The high prevalence of *B. cepacia* genomovar III isolates in clinical material and their association with life-threatening and epidemic spread, has led to this genomovar being considered an unacceptable risk to humans and thus strains of this genomovar are excluded from consideration as candidate biopesticides.

One of the puzzling aspects of genomovar III is that despite being isolated with the greatest frequency from CF patients, until recently, there were no reports of its isolation from the environment. Several theories were put forward to explain this anomaly. One theory was that CF patients are initially colonised by other *B. cepacia* genomovars strains which evolve *in vivo* into *B. cepacia* genomovar III strains. However, the evolutionary distances between the different *B. cepacia* genomovars obtained from taxonomic studies indicate that such evolutionary transformation is highly unlikely (Dr. P. Vandamme, personal communication). Another possibility is that the lack of isolation

of *B. cepacia* genomovar III from the environment is due to the use of unsuitable selection culture conditions. For example, isolation methods used by medical microbiologists commonly use antibiotic selection (Butler, Doherty , *et al*, 1995) which could suppress the growth of some soil organisms. Another possible reason for lack of genomovar III isolation from environmental sites is that the environmental sites surveyed might not include the natural habitats of these organisms. For these reasons, during this study an attempt was made to isolate *B. cepacia* from the rhizosphere of plant species using several cultivation methods.

6.1. Isolation of *B. cepacia* from the rhizosphere

In preliminary experiments, forty-five rhizosphere sites were examined for *B. cepacia*. In addition, five pond sediments were also examined because of a previous publication concerning *B. cepacia* in such environments (Wise, Shimkets, *et al*, 1995). The selection media used were PCAT medium (Burbage and Sasser,1982) and Malka minimal medium (Robert-Gero, Poiret, *et al*, 1970; Butler, Doherty, *et al*, 1995) which contains 300u/ml polymyxin B. Oxidase positive, non-fluorescent, non-lactose fermenters were screened on arginine-glucose medium (Stewart, 1971; Butler, Doherty, *et al*, 1995) and presumptively identified as '*B. cepacia'*. Further identification was performed using the API 20 NE system. From these studies, 21 '*B. cepacia'* isolates from eleven different sites were obtained and these are indicated in Table 6.1. Only one '*B. cepacia'* isolate was cultured from pond sediment.

Table6.1: *B. cepacia* complex isolates cultured from pond sediment and the rhizosphere, identified using the API 20NE test

Isolated by PCAT only	Isolated by MMP	Isolated by both methods
J2925	J2859	J2927[b]
J2952[p]	J2939	J2928[b]
J2953	J2940	J2941
	J2942[a]	J2944[b]
	J2943[a]	J2945[b]
	J2951	J2946[b]
	J2954	J2947[c]
	J2955	J2848[c]
		J2949[b]
		J2950[b]

[p]Pond sediment isolate; MMP, minimal media with polymyxin; [a,b,c] Identical PFGE profiles.

6.2. Taxonomic identification of *B. cepacia* isolates

Modern bacterial classification is based on a polyphasic taxonomic approach that utilises phenotypic tests such as whole-cell protein and fatty acid analysis, as well as genotypic tests. This study used a polyphasic taxonomic approach for the identification of the environmental isolates presumptively identified as members of the *B. cepacia* complex.

6.2.1. Identification of isolates using fatty acid analysis

Although bacteria alter the fatty acid composition of their lipids to maintain membrane fluidity, in constant growth conditions the qualitative and quantitative analysis of fatty acid composition is a useful tool for the classification and identification of organisms. Thus, fatty acid composition was used as the preliminary step for the classification of the environmental isolates. Although most environmental isolates presumptively identified by API 20NE as *B. cepacia,* were confirmed by fatty acid analysis, Table 6.2 shows that some discrepancies between these two methods were observed in a minority of the strains.

Table 6.2: Identification of environmental isolates based on cellular fatty acid analysis

Isolate	**Identification**	**Probability**
J2859	*Sphingobacterium*	0.770
J2925	*Pseudomonas putida*	0.793
J2927	*B. cepacia*	0.626
J2928	*B. cepacia*	0.669
J2939	*B. glathei*	0.527
J2940	*B. cepacia*	0.315
J2941	*B. cepacia*	0.361
J2942	*B. cepacia*	0.662
J2943	*B. cepacia*	0.655
J2944	*B. cepacia*	0.616
J2945	*B. cepacia*	0.477
J2946	*B. cepacia*	0.581
J2947	*B. cepacia*	0.664
J2948	*B. cepacia*	0.735
J2949	*B. cepacia*	0.584
J2950	NT	NT
J2951	*B. cepacia*	0.553
J2952	*Salmonella*	0.167
J2953	*Salmonella*	0.418
J2954	*B. glathei*	0.313
J2955	*B. glathei*	0.552

NT not tested. The probability was determined by computer; which compared the fatty acid composition of the test isolate to that of bacterial standards stored in the database.

6.2.2. Identification of isolates using SDS-PAGE

In the next stage of polyphasic identification, whole-cell bacterial protein extracts were prepared and used to classify the environmenal isolates. Standard growth conditions were used to enable comparisons between organisms, as the composition of whole-cell proteins also varies with growth conditions. Fig 6.1 shows that a few isolates had identical SDS-PAGE profiles.

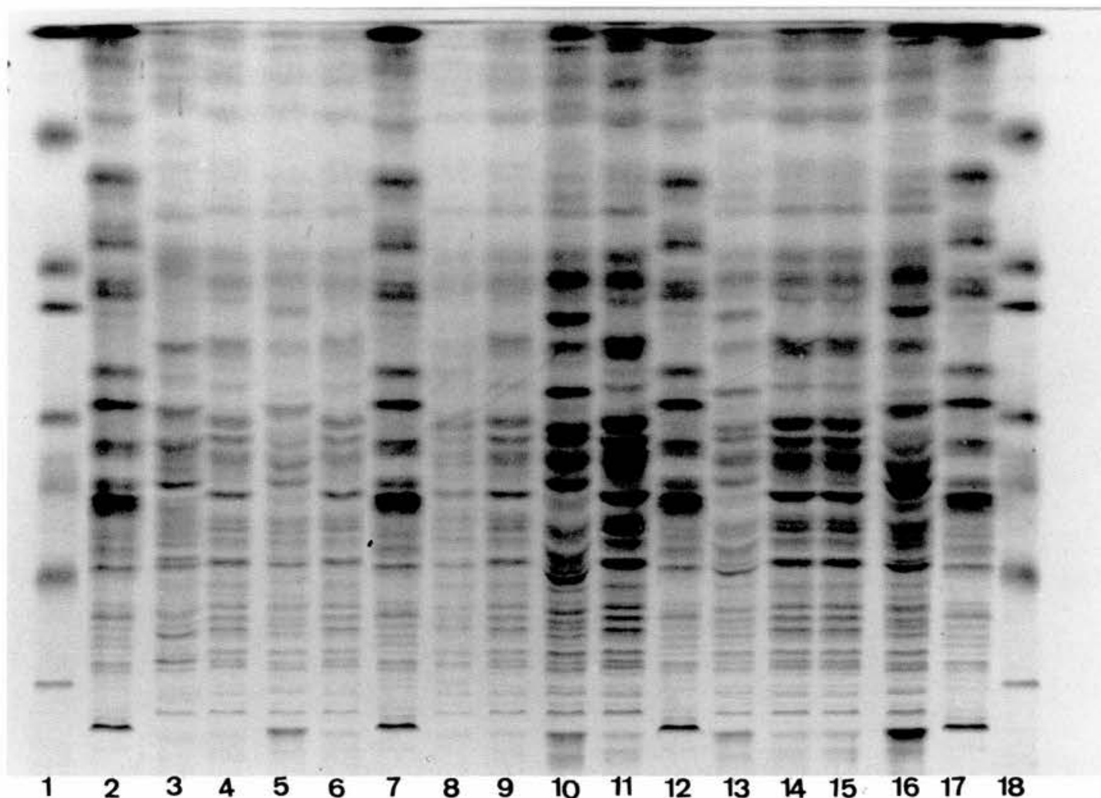


Fig.6.1.a). SDS-PAGE Profiles: Lanes 1 to 18; Marker, reference strain LMG 1125, J2948, J2927, J2928, J2943, LMG 1125, J2950, J2942, J2944, J2951, LMG 1125, J2949, J2942, J2941, J2940, LMG 1125, Marker, respectively.

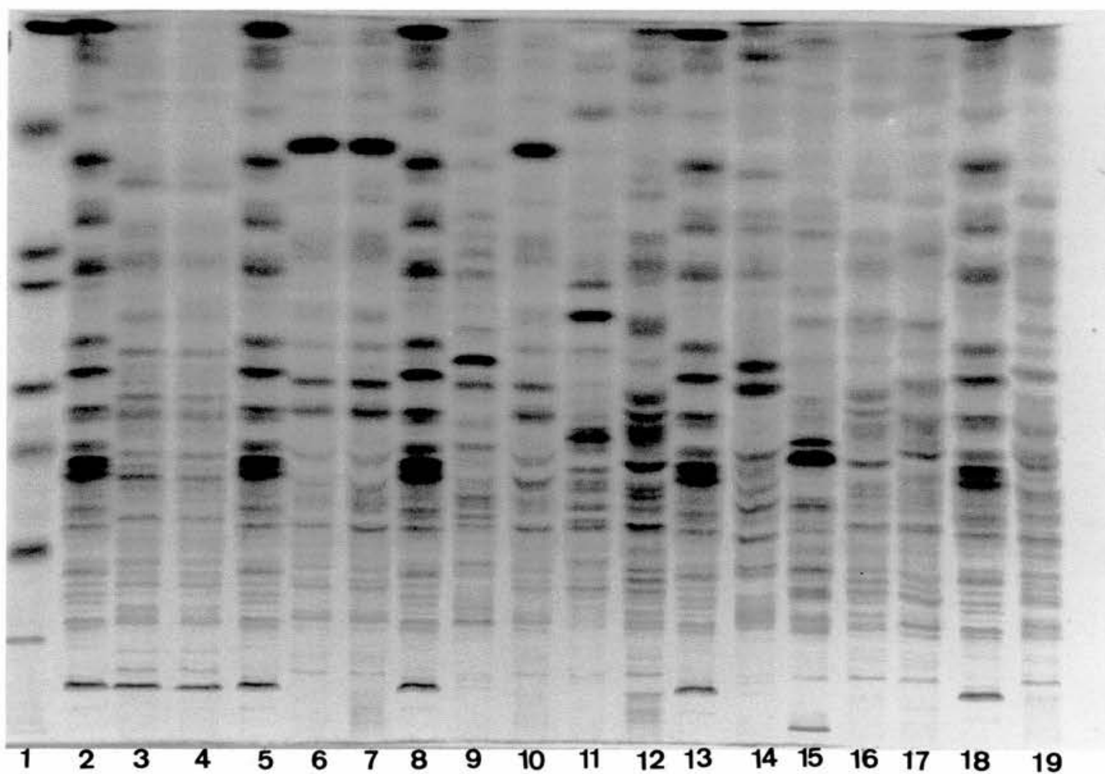


Fig.6.1.b). SDS-PAGE Profiles: Lanes1 to 19; Marker, reference strain LMG1125, E26, E27, LMG1125, J2955, J2954, LMG1125, J2953, J2939, J2925, J2945, LMG1125, J2952, J2859, J2946, J2940, LMG1125, LMG19447.

The SDS-PAGE profiles obtained for the environmental isolates were compared to standard *Burkholderia* SDS-PAGE profiles both manually and using a computer programme as detailed in the appendix. Table 6.3 shows that the isolates identified by fatty acid analysis as belonging to genera other than *Burkholderia*, were also excluded from the *B. cepacia* complex by SDS-PAGE. The remaining 17 isolates were confirmed as *B. cepacia* by SDS-PAGE and also allocated to genomovar I-III-IV, and *B. multivorans* (previously known as genomovar II) and to a group similar to the newly described *Burkholderia* species *B. graminis* (Viallard, Poirier, *et al*, 1998; Kahng, Kukor, *et al* 2000).

Table 6.3. Identification of environmental isolates using SDS-PAGE

Isolate	Identification
J2859	Not *Burkholderia*
J2925	Not *Burkholderia*
J2927	*B. cepacia* genomovar I-III-IV
J2928	*B. cepacia* genomovar I-III-IV
J2939	*B. graminis-like*
J2940	*B. graminis*-like
J2941	*B. cepacia* genomovar I-III-IV
J2942	*B. multivorans*
J2943	*B. multivorans*
J2944	*B. cepacia* genomovar I-III-IV
J2945	*B. cepacia* genomovar I-III-IV
J2946	*B. cepacia* genomovar I-III-IV
J2947	*B. multivorans*
J2948	*B. multivorans*
J2949	*B. cepacia* genomovar I-III-IV
J2950	*B. cepacia* genomovar I-III-IV
J2951	*B. cepacia* genomovar I-III-IV
J2952	Not *Burkholderia*
J2953	Not *Burkholderia*
J2954	*B. graminis-like*
J2955	*B. graminis-like*

6.2.3. Identification of isolates using 16S rDNA

The genotype of an organism is stable in all environments and identification based on parts of the genome is increasingly being employed in taxonomic studies. In this study, the 16S rDNA gene of the environment isolates was examined and the results are shown in Fig 6.2 and Table 6.4.

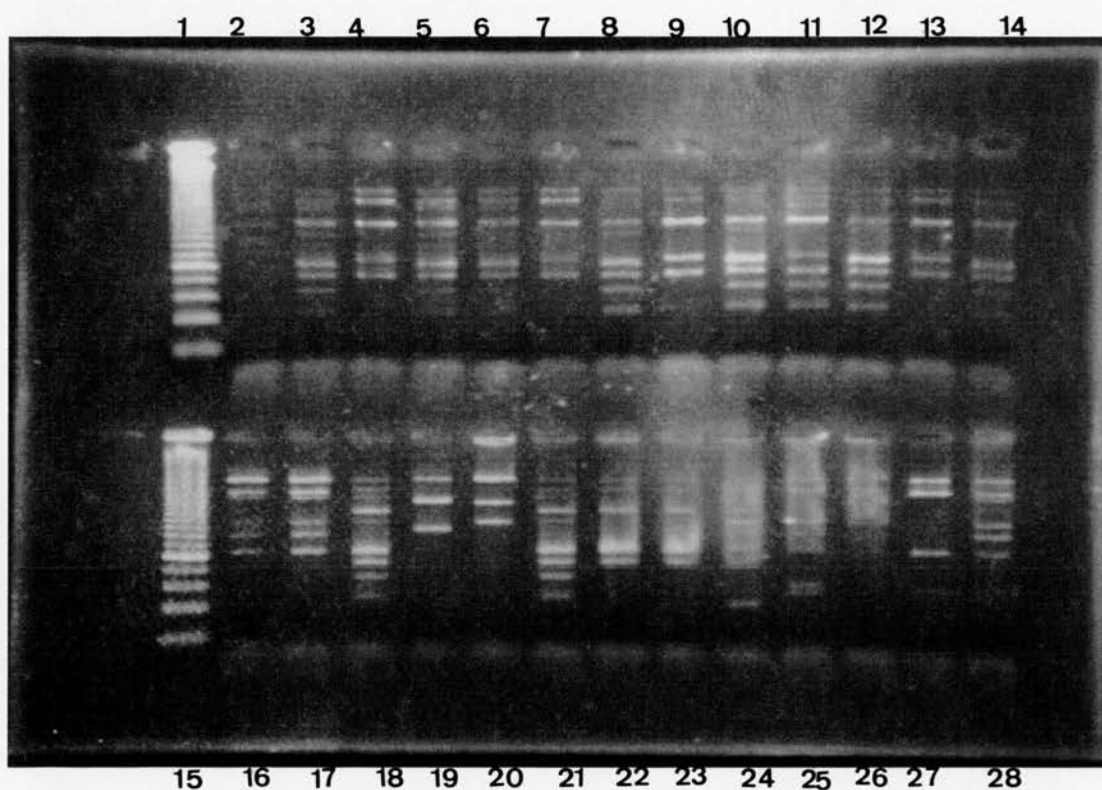


Fig 6.2. 16S rDNA Lanes 1 to 14; marker, negative control, positive control, J2948, J2927, J2928, J2943, J2950, J2942, J2944, J2951, J2949, J2947, J2941, respectively.

Lanes 15 to 28; marker, J2940, J2940, J2946, J2859, J2952, J2945, E26, E27, J2925, J2939, J2953, J2954, J2955, respectively.

The rhizosphere isolates' 16S rDNA amplified by PCR was restricted with *Dde*I and restriction fragment length polymorphism (RFLP) analysis revealed sequence polymorphisms that could identify *B. multivorans* and strains not belonging to the *B. cepacia* complex. However, *B. cepacia* genomovars I, III, IV (*B. stabilis*) could not be separated.

Table 6.4. Identification of rhizosphere isolates based on the RFLP of the 16S rDNA

Lane No.	Isolate	Identification
4	J2948	*B. multivorans*
5	J2927	*B. cepacia* genomovar I, III, IV
6	J2928	*B. cepacia* genomovar I, III, IV
7	J2943	*B. multivorans*
8	J2950	*B. cepacia* genomovar I, III, IV
9	J2942	*B. multivorans*
10	J2944	*B. cepacia* genomovar I, III, IV
11	J2951	*B. cepacia* genomovar I, III, IV
12	J2949	*B. cepacia* genomovar I, III, IV
13	J2947	*B. multivorans*
14	J2941	*B. cepacia* genomovar I, III, IV
16	J2940	Not *B. cepacia* complex
17	J2940	Not *B. cepacia* complex
18	J2946	*B. cepacia* genomovar I, III, IV
19	J2859	Not *B. cepacia* complex
20	J2952	Not *B. cepacia* complex
21	J2945	*B. cepacia* genomovar I, III, IV
24	J2925	Not *B. cepacia* complex
25	J2939	Not B. cepacia complex
26	J2953	Not *B. cepacia* complex
27	J2954	Not B. cepacia *complex*
28	J2955	Not B. cepacia *complex*

Lanes 22 & 23 were *B. multivorans* CF isolates

6.2.4. Genomovars I and III *rec*A-specific PCR

In the next stage of polyphasic taxonomic analysis, the environmental isolates identified by 16S rRNA analysis as *B. cepacia* genomovars I, III and IV were examined using *rec* A specific PCR (Mahenthiralingham, Bischof, *et al*, 2000) for *B. cepacia* genomovars I, III a and III b. All were found to be negative.

6.2.5. Further analysis of rhizosphere isolates

The negative genomovar I and III *rec*A-specific PCR results prompted a DNA-DNA hybridisation investigation of all *Burkholderia* sp isolates and the following results were obtained:

i) J2942, J2943, J2947 and J2948 were identified as *B. multivorans* (genomovar II).

ii) J2927, J2928, J2941, J2944, J2945, J2946, J2949, J2950 and J2951 were found to represent yet another genomovar within the *B. cepacia* complex; *B. cepacia* genomovar VIII

iii) J2940 was identified as *B. graminis*

iv) Three isolates, J2939, J2954 and J2955 were found to represent a novel *Burkholderia* species closely related to *B. graminis*.

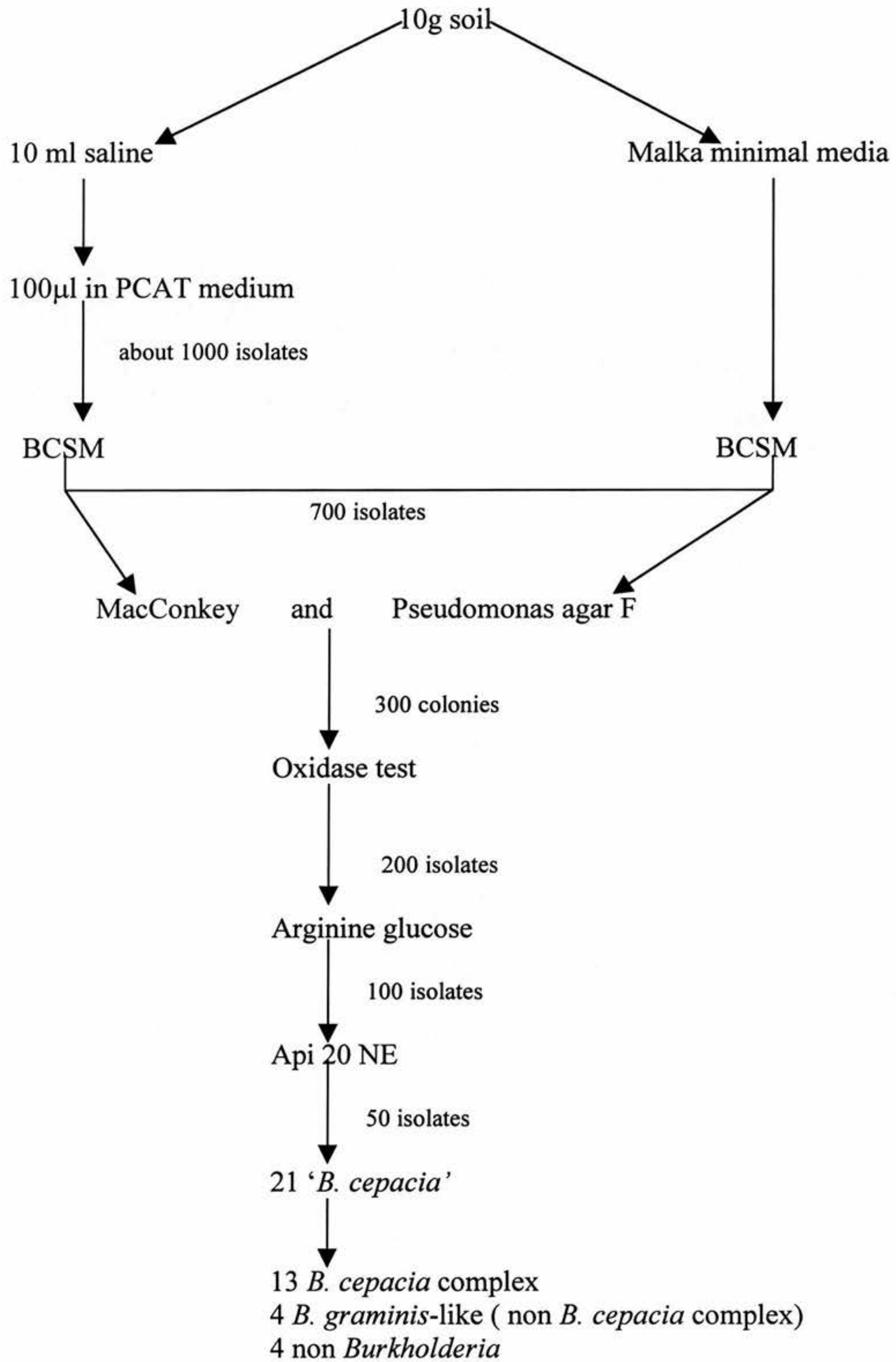


Fig 6. Schematic representation of the isolation of the *B. cepacia* complex from the environment.

6.3. Discussion

In this study, a survey of 50 sites yielded 21 *B. cepacia* isolates (42%) when identification was performed by the API 20NE system. More isolates (38%) were identified on minimum media with 300 u/ml polymyxin (MMP), than on the PCAT medium (14%) and the majority of isolates (48%) were identified using both media. Fatty acid and whole-cell protein analysis revealed that all the isolates identified on PCAT medium (J2925, J2952, J2953) and one identified on the MMP medium (J2859) did not belong to the genus *Burkholderia*. Isolates J2925 (*Pseudomonas fluorescens*), J2952 and J2953 (both *Salmonella* as identified by whole-cell fatty acid analysis) were initially identified as *B. cepacia* on the API 20NE, with good identification scores ; namely, at 99.9%, 95% and 99.9% discrimination levels, respectively. When API 20NE test was repeated, isolate J2925 was identified as *P. fluorescens*, however; J2952 and J2953 were identified as *B. cepacia* at 66.3% and 95% discrimination levels, respectively. In addition, the API 20NE identified all four *B. graminis*–like organisms as *B. cepacia*.

The API 20NE system is one of several commercial systems that are routinely used in diagnostic laboratories for the identification of *B. cepacia* from CF specimens and identification at 95% discrimination level is normally considered acceptable identification. Disturbingly, this study confirms previous reports indicating that commercial identification systems are unreliable for the identification of the *B. cepacia* complex (Kiska, Kerr, *et al*, 1996; van Pelt, Verduin, *et al*, 1999; Shelley, Spilker, *et al*,

2000). Furthermore, most commercial identification systems cannot distinguish *B. gladioli* from *B. cepacia* (Kiska, Kerr, *et al*, 1996; van Pelt, Verduin, *et al*, 1999; Shelley, Spilker, *et al*, 2000). Despite being flawed, the API 20NE test is still considered to be the best commercial system for the identification of *B. cepacia* currently available (van Pelt, Verduin, *et al*, 1999). At present, the use of DNA-based methods are considered necessary for reliable final identification of members of the *B. cepacia* complex (van Pelt, Verduin, *et al*, 1999; Shelley, Spilker, *et al*, 2000). Since culture of the *B. cepacia* complex from CF patients has profound medical, social and psychological implications, it is imperative that these organisms are identified accurately.

In this study, the PCAT medium failed to provide the high degree of selectivity for *B. cepacia* reported previously (Nacamulli, Bevivino, *et al*, 1997). Further selection tests were required for the identification of *B. cepacia* in addition to growth on both PCAT and malka mimimal medium. Isolation of the *B. cepacia* complex from the environment using these two methods was extremely laborious and time-consuming, making these methods impractical for large-scale application. In this thesis, several months were spent isolating only 13 'true' *B. cepacia* complex isolates from the environment. More practical methods of isolation urgently need to be developed before accurate assessments of the background levels of the *B. cepacia* complex in the natural environment can be made.

In this study, the isolation rate of confirmed *B. cepacia* 13 (26%) is marginally higher than the figure of 21% previously reported by (Butler, Doherty, *et al*, 1995) using

similar methods. Reports on the natural abundance of *B. cepacia* in various environments range from very low, 1% (Mortensen, Fisher, *et al,* 1995) and 4% (Mortensen, Fisher, *et al*, 1995) to reasonably frequent, 213 isolates from 11 different sites of backwater stream sediment (Wise, Shimkets, *et al*, 1995). The wide variation in isolation rates of '*B. cepacia*' from the environment is could be due to the different habitats sampled and to errors caused by simple phenotypic identification systems which are less accurate than the polyphasic system used in the present study.

Although '*B. cepacia*' has been recovered form a variety of habitats, recent evidence suggests that a greater proportion of viable bacteria can be cultured from the rhizosphere and rhizoplane than from the bulk soil (Troxler, Zala, *et al*, 1997). The different sampling techniques used in previous studies range from molecular techniques (Wise, Shimkets, *et al*, 1995) to growth on a range of selective media (Hagedorn, Gould, *et al*, 1987; Nijhuis, Maat, *et al*, 1993; Butler, Doherty, *et al*, 1995; Nacamulli, Bevivino, *et al*, 1997), and could be a major factor accounting for the discrepancies in isolation rates. Finally, physical properties of the soil such as the pH, water content and chemical composition could result in different rates of colonisation by '*B. cepacia*', and thus influence the rates of isolation.

Whole–cell fatty acid and protein analysis are used extensively and effectively in the taxonomic classification and identification of bacteria (Vancanneyt, Witt, *et al*, 1996; Vandamme, Holmes, *et al*, 1997; Vandamme, Mahenthiralingam *et al*, 2000). In this study, whole-cell fatty acid analysis was found to be effective only in identifying the

genus *Burkholderia* but was unreliable in differentiating between the different species. However, it was still found to be useful for excluding organisms that were not related to '*B. cepacia*'. This study confirmed that although SDS-PAGE is a phenotypic mechanism of taxonomic classification, the results of SDS-PAGE correlated well with 16S rDNA classification. In conclusion, polyphasic taxonomy is necessary for reliable identification of members of the *B. cepacia* complex and should now be included in taxonomic research.

As has been indicated previously, *B. cepacia* genomovar III and *B. multivorans* are recovered most frequently from CF patients and, are most commonly associated with epidemic spread and poor clinical prognosis (Vandamme, Holmes, *et al*, 1997; Mahenthiralingam, Coenye, *et al*, 2000; Mahenthiralingam, Bischof, *et al*, 2000; Shelley, Spilker, *et al*, 2000). The need for further studies on the clinical relevance of the different genomovars is well recognised (Vandamme, Holes, *et al*, 1997; http://www.epa.gov, 12 July 2000; Mahenthiralingam, Bischof, *et al* 2000). Information from such studies could be useful in predicting the clinical outcome from particular isolates. Furthermore, since most biopesticide strains being considered for commercial applications are *B. cepacia* genomovar I or *B. vietnamiensis,* information on the risks associated with different genomovars would be useful in determining the risks associated with the use of *B. cepacia* complex isolates as biopesticides.

In this study, 53% of the *Burkholderia* sp isolates obtained from the rhizosphere were found to belong to a new genomovar within the *B. cepacia* complex; genomovar VIII.

No comparisons could be made with previous studies on the isolation rates of '*B. cepacia*' from the rhizosphere since earlier studies were performed prior to the knowledge of the complex taxonomy of the *B. cepacia* complex. Interestingly, although very few environmental *B. multivorans* isolates have been described, this study found that four, (24%) of all *Burkholderia* sp isolated from the rhizosphere belonged to this species. Interestingly, three isolates were found to represent a novel *Burkholderia* species. The results from this study illustrate the complexity of the taxonomy of the *B. cepacia* complex and the detailed identification of *Burkholderia* isolates now possible through use of the polyphasic taxonomic approach. Identification methods used prior to the polyphasic taxonomic approach would have identified all the isolates obtained in this study, simply as '*B. cepacia*.' Thus, a comparison of environmental '*B. cepacia*' isolates should take into account the identification methods used.

Although no *B. cepacia* genomovar III isolates were obtained from the rhizosphere in this study, isolation of *B. cepacia* genomovar III from the rhizosphere in France, Phillipines and Italy has recently been reported (Govan, Balendreau, et al, 2000; Dr. P. Vandamme, personal communication). This new data implies that some of the *B. cepacia* genomovar III isolates recovered from CF patients could have been acquired from the natural environment. Interestingly, *B. cepacia* genomovar III and *B. vietnamiensis* have also been demonstrated recently as agents of sub-clinical mastitis in sheep (Berriatua, Ziluaga, *et al*, 2001). This report represents the first description of natural *B. cepacia* infection in domestic animals and a possible mode of transmission of the organism to susceptible human hosts. It is interesting to note that in the Berriatua *et*

al study, *B. cepacia* genomovar III strains (isolated with a high frequency from CF patients and associated with epidemic spread) and *B. vietnamiensis* (often associated with candidate biopesticide strains), were both responsible for the mastitis in sheep. Thus, the two groups of *B. cepacia* complex members that could be considered most dangerous and least dangerous, were both responsible for infection in veterinary medicine. This report casts serious doubts on the consideration of some *B. cepacia* complex members as less dangerous than others and hence safe for large-scale commercial application as biopesticides.

Knowledge of the true level of *B. cepacia* in the environment is one of the factors that the United States Environmental Protection Agency considered as essential for adequate evaluation of *B. cepacia* as a biopesticide (http://www.epa.gov, 12 June 2000). This information is required to determine the influence of introduced biopesticide strains on the natural *B. cepacia* population and to assess the risks, if any, posed by these biopesticides to susceptible humans, in comparison with the risks associated with naturally occurring background levels of *B. cepacia*. The recently detected environmental *B. cepacia* genomovar III isolates can now be included in such studies, leading to a more robust and comprehensive assessment of the relationship between environmental isolates and the potential hazards of '*B. cepacia*' biopesticides to humans.

CHAPTER 7

The *B. cepacia* complex genome and genetic exchange

The *B. cepacia* complex exhibits contrasting characteristics that have led to it being referred to as both humanity's friend and foe (Govan, Balendreau, *et al,* 2000). Typically, members of the complex have the ability to metabolise a wide variety of compounds and can even use the antibiotic, penicillin G as a sole carbon and energy source (Beckman and Lessie, 1979). This ability to degrade complex compounds has generated commercial interest in these bacteria as bioremediation agents for environmental degradation of recalcitrant xenobiotic compounds,including herbicides and defoliants such as agent orange (Grant, 1979; Kilbane, Charterjee, et al, 1982, Danganan, Ye, *et al*, 1994). Historically, members of the *B. cepacia* complex were best known as phytopathogens causing 'soft rot' in damaged onion bulbs (Burkholder, 1950). In recent years, the *B. cepacia* complex has emerged as important life-threatening opportunistic human pathogens in immuno-compromised hosts, in particular, in patients with cystic fibrosis and chronic granulomatous disease. It has been suggested that this adaptability of the *B. cepacia* complex is due the organisms' large, complex genome (Holmes, Govan et al, 1998; Govan and Vandamme, 1998; Mahenthiralingam, Coenye, *et al*, 2000).

The *B. cepacia* complex genome consists of three large circular replicons that have numerous insertion sequence elements and rRNA genes, as well as a large plasmid of about 200kb, making its total genome between 6.8 and 8.1 Mb (Cheng and Lessie, 1994, Rodley, Romling, *et al*, 1995). It has been suggested that partitioning of the genome is advantageous because it allows members of the *B. cepacia* complex to replicate rapidly despite their large genome size (Stouthammer and Kooijman, 1993).

It is estimated that *B. cepacia* complex isolates have approximately twelve different transposable elements in their genome (Gaffney and Lessie, 1987); the insertion sequences present in the plasmid are also duplicated in the chromosomes. The different transposable elements and insertion sequences mediate genomic re-arrangements (Gaffney and Lessie, 1987) and activate the expression of poorly expressed genes through both insertions and deletions (Scordilis, Ree, *et al*, 1987); this would enable the *B. cepacia* complex to make use of genetic material which otherwise would not be expressed. Transposable elements, therefore, contribute significantly to the genomic plasticity of the *B. cepacia* complex and possibly played an important role in the evolution of novel catabolic functions such as the ability to degrade recalcitrant organic compounds and penicillin G.

Interestingly, the insertion sequences themselves are probably of foreign origin, as suggested by relatively low guanosine plus cytosine (G+C) content compared to that of *B. cepacia* complex DNA (Ferrante and Lessie, 1991; Byrne and Lessie, 1994). This implies that at some point in their evolution, members of the *B. cepacia* complex

successfully sequestered and incorporated foreign DNA into their genome. Insertion sequencies not only impart unusual metabolic abilities but also transmissibility to the *B. cepacia* complex in the context of human pulmonary infection. A hybrid of two insertion sequencies IS 402-IS1356 is one of the three markers associated with the epidemic spread of *B. cepacia* complex strains within the CF community (Tyler, Rozee, *et al*, 1996).

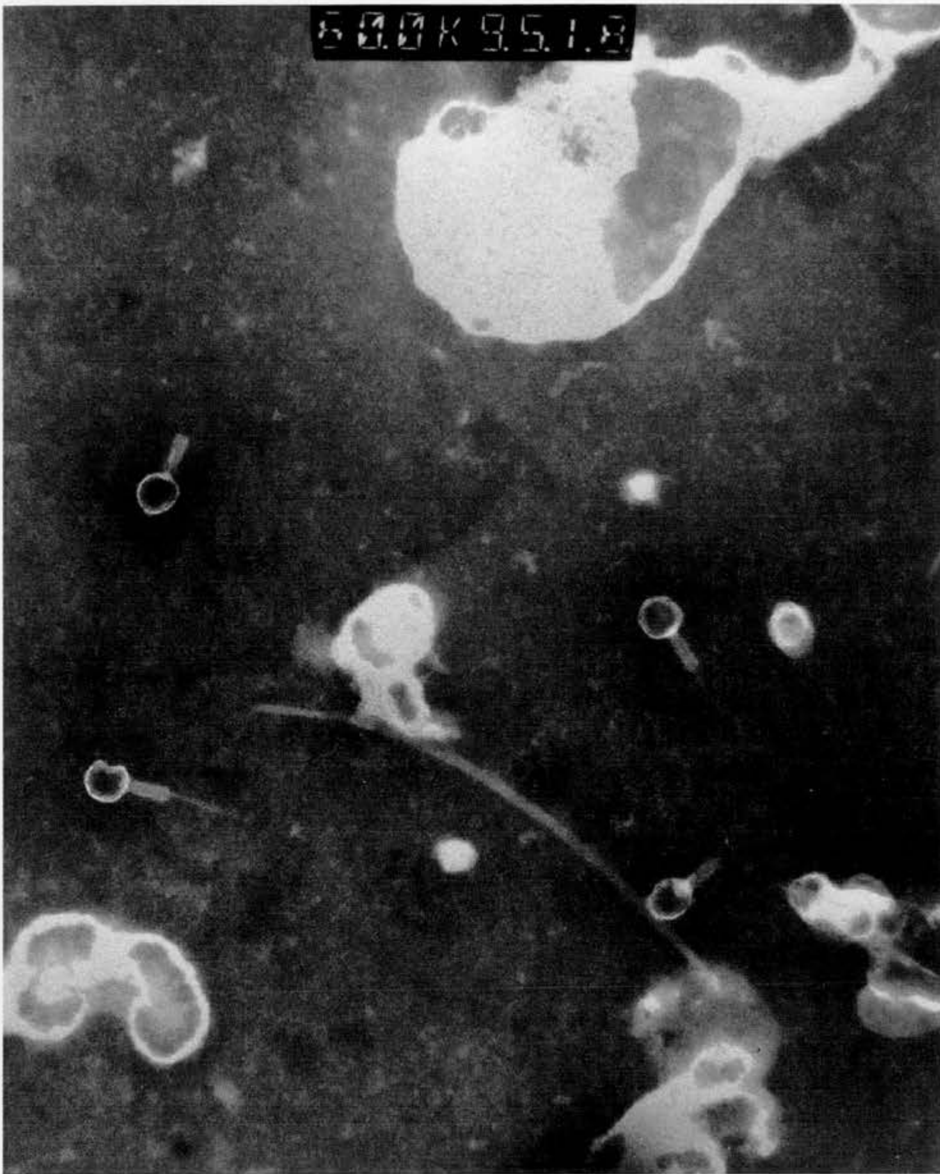
Thus, the *B. cepacia* complex presents important and diverse challenges. These inherently antibiotic-resistant organisms, with a complex and highly plastic genome, cause life-threatening infections in susceptible humans but ironically are also highly efficient and cost-effective biopesticides and bioremediation agents. Bulk release of *B. cepacia* complex biopesticides into the environment could potentially facilitate horizontal gene transfer with soil microorganisms and/or members of the *B. cepacia* complex of clinical interest. The three most common methods of bacterial gene transfer are; transduction, which is facilitated by bacteriophages; conjugation through direct cell contact and transformation, mediated by uptake of naked DNA from the organism's environment. This thesis assessed the potential for transduction, conjugation and transformation to facilitate horizontal genetic exchange across different subgroups of the *B. cepacia* complex, including clinical, environmental and candidate biopesticide strains. Bacteriophages whose host spectrum spanned all subgroups of the *B. cepacia* complex were isolated and natural transformation was demonstrated.

7.1. Bacteriophages of the *B. cepacia* complex

Some of the studies on the bacteriophages of the *B. cepacia* complex which form part of this thesis have recently been published (Nzula, Vandamme, *et al*, 2000). In summary, 38 strains of *B. cepacia* from clinical and environmental sources were tested for lysogeny by employing the same *B. cepacia* strains as indicator strains. Two strains, ATCC 29424 (genomovar V) and ATCC 17616 (genomovar II), proved to be lysogenic, providing the phages, named NS1 and NS2 respectively.

7.1.1. Virological characteristics and morphology of the phages NS1 and NS2

Plaques of NS1 and NS2 on host cell lawns were found to be about 1mm in diameter, and either clear or turbid depending on the host strain. Neither phage was inactivated by treatment with chloroform. Electron microscopy showed NS1 and NS2 to resemble T-even-like phages with a hexagonal head and a contractile tail (Fig 7.1).



50U

Fig 7.1. Electron micrograph of NS2

7.1.2 Transduction

The potential for NS1 and NS2 to act as agents of transduction was examined by propagating the bacteriophages on ceftazidime-resistant mutants of the *B. cepacia* complex strains ATCC 53266, ATCC 53267 and C2973; phage lysates were then used to infect the sensitive parent strains. Ceftazidime resistance was transduced at a frequency ranging from 1.0×10^{-8} to 7.0×10^{-6} per infected cell depending on the phage and recipient strain; these frequencies compared to spontaneous mutation frequencies which varied from undetectable to 6.8×10^{-11}. Similar results were obtained in three replicate experiments.

7.1.3 Lytic spectrum of phages NS1 and NS2 against *B. cepacia* and *P. aeruginosa.*

Table 7.1 outlines *B. cepacia* strains sensitive to one or more phages; these strains include environmental and clinical isolates of the *B. cepacia* strain panel (Mahenthiralingam, Coenye, *et al*, 2000) and representatives of the species and genomovars of the *B. cepacia* complex (Vandamme, Holmes, *et al*, 1997). When combined, the host range of NS1 and NS2 included *B. multivorans* and *B. vietnamiensis,* as well as genomovars I and III. Interestingly, J2540 was the only genomovar I strain among 18 strains tested that was sensitive to NS1 or NS2. Neither phage lysed any of the four *B. stabilis* strains tested. Only a few representatives of this group were available for investigation and these results are inconclusive, however, as the genome of this organism is highly conserved and shows little diversity based on PFGE analyses

(Vandamme, Mahenthiralingam, *et al*, 2000), it is possible that resistance to NS1 and NS2 may be a characteristic of *B. stabilis*. Of particular interest, each of the seven genomovar III clones in the *B. cepacia* strain panel, including the intercontinental epidemic lineage ET12, showed sensitivity to one or more of the phages. However, none of the recently isolated environmental *B. cepacia* genomovar III strains were sensitive to NS1 and NS2. Several biopesticide candidate strains were also found to be sensitive to NS1 and NS2; these included ATCC 53266 and ATCC 53267.

Table 7.1 Isolates of the *B. cepacia* complex sensitive to NS1 and NS2

Isolate	**Genomovar**	**NS1**	**NS2**
J2540	I	+	+
C2775	II	+	+
J2315	III	-	+
ATCC 17765	III	-	+
C1394	III	-	+
J415	III	-	+
BC7	III	-	+
K56-2	III	-	+
C6433	III	-	+
PC184	III	+	+
CEP511	III	+	+
LMG 18836	V	-	+
ATCC 53266	V	+	+
ATCC 53267	V	+	+
ATCC 29424	V	-	+
C2973	V	+	+
LMG 16232	V	+	+

+, sensitive; -, resistant

To determine if the host range of *B. cepacia* phages extended to other pseudomonads, 42 strains of *P. aeruginosa,* (14 environmental and 28 CF isolates), were examined for sensitivity to NS1 and NS2. Eight *P. aeruginosa* isolates (19%) were lysed by at least one of the phages, five by both phages (12%), one (2%) by NS1 and two (5%) by NS2 (Table 7.2). Unfortunately, the well-characterised laboratory strain PAO1, was insensitive to both phages.

Table 7.2. Isolates of *P. aeruginosa* sensitive to NS1 and NS2

Isolate	**Source**	**NS1**	**NS2**
A13	CF sputum	+	-
A14	CF sputum	-	+
A18	CF sputum	+	+
A20	CF sputum	+	+
A21	CF sputum	+	+
A31	CF sputum	-	+
J1376	Environment	+	+
J1476	Soil	+	+

+, sensitive; -, resistant

Sensitivity of *P. aeruginosa* to *B. cepacia* phages prompted an investigation of the corresponding sensitivity of *B. cepacia* isolates to *P. aeruginosa* phages. Of 38 strains of *B. cepacia* investigated, three strains one each of genomovars I, III and V were found to be sensitive to four well-characterised *P. aeruginosa* phages. These included the virulent phage E79, and the transducing phages B3, F116L and G101 (Holloway, Krishnapillai, *et al*, 1975).

7.1.4. Effect of LPS on phage activity

Sensitivity of the *B. cepacia* strains to NS1 and NS2 did not appear to correlate with previously reported rough or smooth LPS chemotypes (Simpson, Finlay, *et al*, 1994; Evans, Poxton, *et al*, 1999). However, experiments obtained during this thesis suggested that LPS receptors were responsible for sensitivity of *B. cepacia* to both phages.

Table 7.3 shows the effect of pre-incubating NS1 and NS2 with LPS before infecting sensitive strains. Depending on the phage and host strain, titres of plaque-forming units decreased by between 33 and 98% compared to controls which had been pre-incubated with buffer.

Table 7.3 Effect of LPS on phage activity

Strain	**Phage**	**Number of units**	**Plaque forming per ml**	
		Control	Phage and LPS	Decrease (%)
ATCC 29424	NS2	3620	870	76
C2973	NS1	640	430	33
C2973	NS2	270	130	52
ATCC 53267	NS1	5290	120	98
ATCC 53267	NS2	2050	90	96

7.2. *B. cepacia* complex plasmids

This study examined 29 *B. cepacia* complex strains and found that 26 (90%) harboured a small plasmid of about 23kb that could be detected by agarose gel electrophoresis. Larger plasmids were detected by PFGE; nine of the ten strains in which large plasmids were detected, contained a plasmid of 194 kb. The *B. cepacia* genomovar III strain CEP 511 contained a larger plasmid of about 242 kb (Fig 7.3).

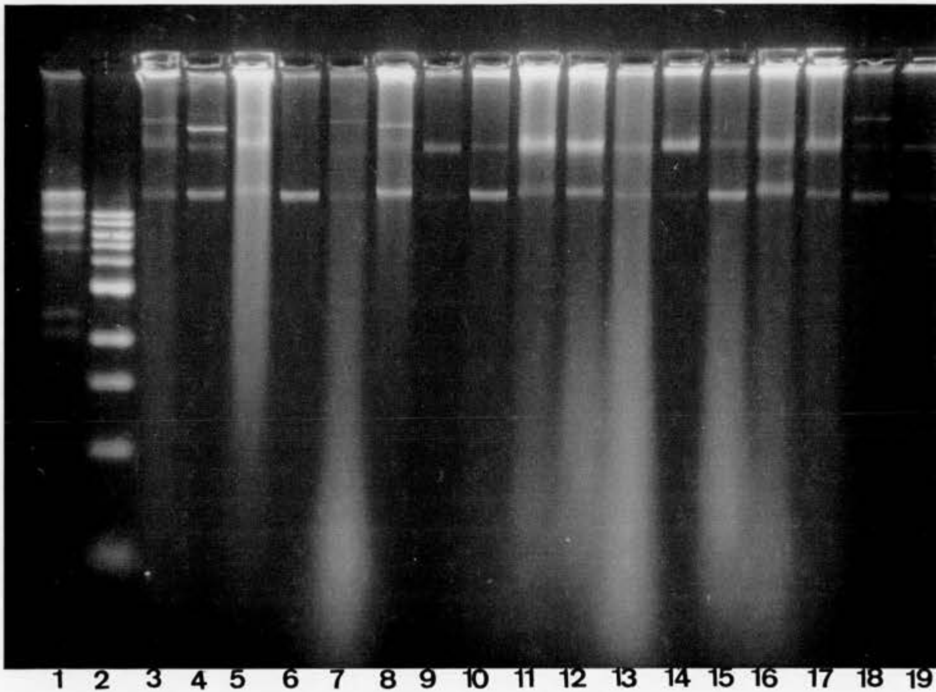


Fig 7.2.(a) Plasmids of the *B. cepacia* complex detected by agarose gel electrophoresis Lanes 1 to 19, λ *Hind* III marker, 1kb ladder, J2315, CEP 511, C1576, C5393, K56-2, AMMD, 249-2, PC184, ATCC15416, FC441, LMG 10929, C1962, ATCC 17759, J415, JTC, C5424, C1394, respectively.

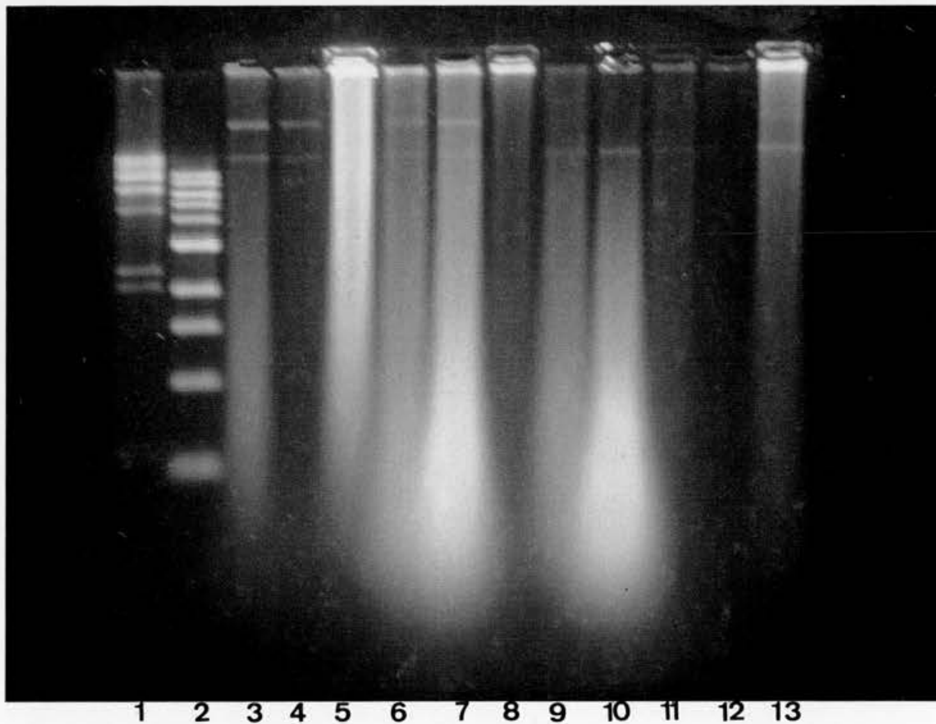


Fig 7.2.(b) Plasmids of the *B. cepacia* complex detected by agarose gel electrophoresis Lanes1 to 13 λ *Hind* III marker, 1kb ladder, LMG 14086, CF-A1-1, LMG 18888, PC 259, LMG 13010, LMG17997, BC7, ATCC 17616, LMG 16232, C6433, CEP 509, respectively.

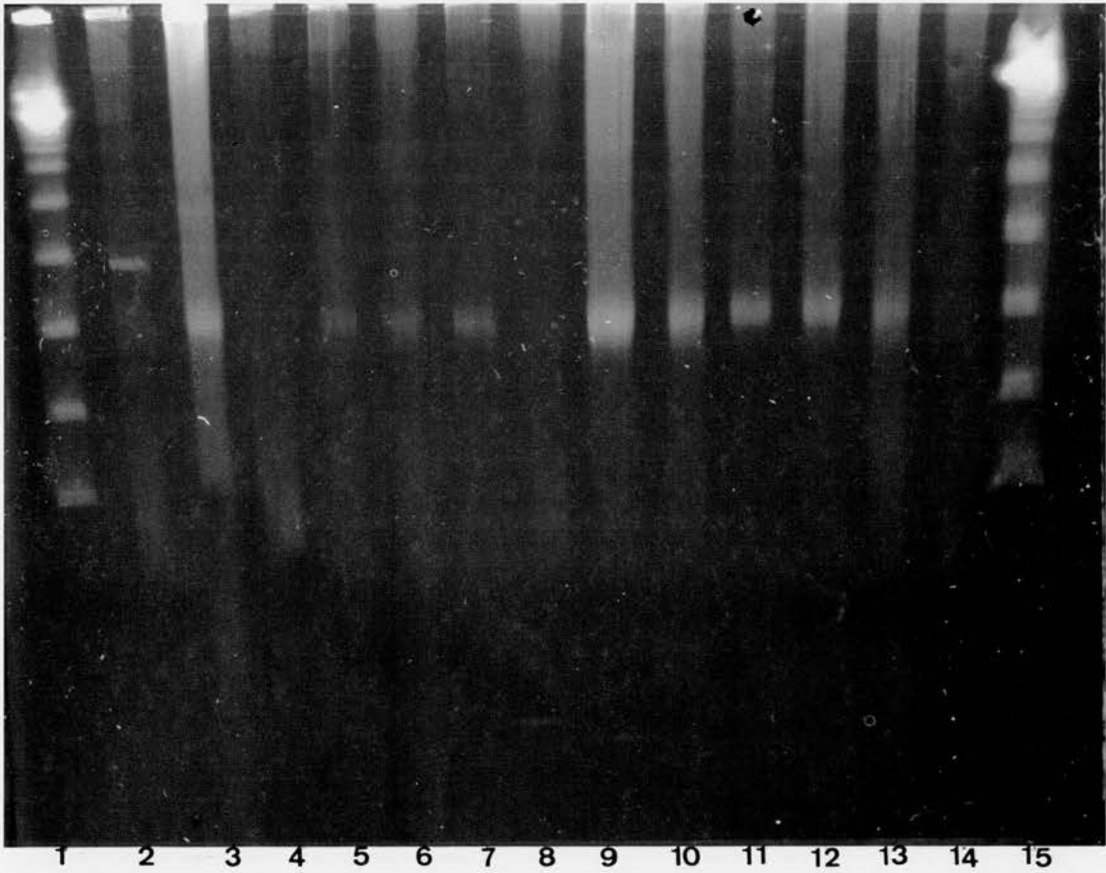


Fig 7.3 Plasmids of the *B. cepacia* complex detected by PFGE

Lanes 1 to 15, λ ladder PFG marker, CEP511, C1576, C5393, LMG 14086, CF-A1-1, 249-2, PC184, ATCC 25416, FC411, C1962, JTC, J415, J2315, λ ladder PFG marker, respectively.

7.3. Natural transformation of the *B. cepacia* complex

Ten recipient *B. cepacia* complex strains in which the *dsb* gene had been detected, were propagated on CAB. The recipient strains were transformed with DNA from a recombinant *B. cepacia* J2315 strain generated in this thesis, which harboured a mercury resistance marker ligated to its *dsb* gene. Transformed cells were able to grow in BCSM with 18 ml/L of mercury whereas the non-DNA-treated controls could not.

When the recipient strains were propagated on minimal agar before treatment with the crude DNA extract, more strains became transformable (Table 7.4). Most of the *B. cepacia* genomovar III strains studied could undergo natural transformation. Interestingly, the former candidate biopesticide C3041, and the *B. cepacia* genomovar I strain J2535, could also be transformed naturally with DNA extracted from the highly transmissible CF strain J2315.

Transformation could not be investigated in *B. cepacia* genomovar III strains 715j and CEP 511, and the *B. cepacia* genomovar I strain ATCC 25416 since they were found to be inherently resistant to mercury at concentrations of 18 mg L^{-1}. With most strains it was necessary to increase competence for transformation by previous growth in MA.

Table 7.4. Natural transformation of the *B. cepacia* complex

Growth medium	Transformed strains
Columbia agar base	C1394
Minimal agar	C1394, J2535, C3041, BC7, K56-2, C5424

7.4. Discussion

7.4.1 Bacteriophages of the *B. cepacia* complex

A literature search found only two previous reported studies on *B. cepacia* phages. NS1 and NS2 appear to be morphologically similar to the T-even phages, with hexagonal heads and contractile tails. Similar characteristics were described previously (Cihlar, Lessie, *et al*, 1978; Matsumoto, Itoh, *et al,* 1986) for the transducing phages CP1 and CP75, respectively. The molecular mass of NS1 and NS2 phage genomes was approximately 48kb, which is reasonably close to the molecular size of 52 kb reported for CP75. Unfortunately, phages CP1 and CP75 were unavailable for comparative studies.

The shared host spectrum of phages NS1 and NS2 between *B. cepacia* and *P. aeruginosa* is particularly interesting and potentially important. *P. aeruginosa* and *B. cepacia* share environmental habitats and often co-colonise airways of CF patients at high bacterial density (10^{9} cfu ml $^{-1}$). Different strains of each pathogen may also co-colonise the same patient. Phages such as NS1 and NS2, together with the classic *P. aeruginosa* transducing phages F116L, G101 and B3, would have the potential and opportunity to mediate transfer of virulence and resistance factors *in vivo* between clinical isolates of two major opportunistic pathogens and environmental isolates of the *B. cepacia* complex, including isolates that might be candidate biopesticide strains.

Acquisition of virulence factors mediated by transduction has been reported in *Vibrio cholerae* (Parker, Richardson, *et al*, 1970) and *Streptococcus pyogenes* (Kapur, Nelson, *et al*, 1992). In *V. cholerae,* it has been suggested that transduction contributes to the evolution of epidemic strains (Mooi and Bik, 1997). Although most transduction studies are carried out on auxotrophic mutants, the transduction frequencies of 1.0×10^{-8} to 7.0×10^{-6} obtained in this thesis are similar to previously published frequencies of transduction in different bacteria and phages (Krishnapillai, 1971; Matsumoto, Ito, et al, *1986*; Weiss, Capage, *et al*, 1994; Toth, Mulholland, et al, 1997).

Previous evidence for the assimilation of foreign DNA by *B. cepacia* has drawn on the significantly lower G + C content of insertion sequences when compared with native DNA (Lessie, Hendrickson, *et al*, 1996). It has also been reported that insertion sequences found in *B. cepacia* J2315, a representative of the genomovar III and epidemic lineage ET12, are identical to those found in *Burkholderia pseudomallei* (Mack and Titball, 1998). *B. pseudomallei* is the highly virulent agent of the tropical disease melioidosis and shares environmental habitats with *B. cepacia* and *P. aeruginosa.* Of equal, if not more significance, there are also recent reports of CF patients co-colonised with *B. pseudomallei* and *B. cepacia* following holidays in South East Asia. These developments are important and suggest that further studies should be performed:

1.to determine if the host range of NS1, NS2 and the *P. aeruginosa* transducing phages extends to *B. pseudomallei*, and to the closely related and highly virulent equine and human pathogen, *Burkholderia mallei*.

2. to determine whether *B. cepacia* and *P. aeruginosa* transducing phages can mediate transduction of genes associated with virulence and epidemic markers between different subpopulations of the *B. cepacia* complex and between members of the complex and other 'pseudomonads'.

The activity of NS1 and NS2 is inhibited by LPS, suggesting that LPS is a binding site for both phages. An LPS receptor would explain the sensitivity of *P. aeruginosa* to *B. cepacia* phages since Western blotting and absorption studies have indicated cross-reactivity in antibodies to the core LPS of both species (Nelson, Butler, et al, 1993). An NS2 receptor located within core LPS is also supported by previous analyses of *B. cepacia* LPS chemotypes which showed that most clinical genomovar III isolates express only core LPS (Evans, Poxton, *et al*, 1999) and our observation in this study that the majority of clinical genomovar III isolates were sensitive to NS2. The LPS chemotypes of the recently identified environmental *B. cepacia* genomovar III isolates have still to be determined. However, the resistance of these environmental isolates to NS2 could indicate that the LPS from environmental *B. cepacia* genomovar III isolates differ from that of the clinical isolates belonging to the same genomovar. Further studies are required to compare the LPS chemotypes of clinical and environmental *B. cepacia* genomovar III isolates.

An exhaustive literature search indicates that this is probably the first report of *B. cepacia* transducing phages whose host spectrum spans environmental and clinical isolates, several subpopulations within the *B. cepacia* complex and candidate biopesticide strains. It is also the first report of the sensitivity of *B. cepacia* isolates to well-characterised transducing phages of *P. aeruginosa.* Given the present difficulties in genetic manipulation of multiresistant strains of *B. cepacia*, transducing phages could provide a useful mechanism to study horizontal gene transfer in *B. cepacia* and other pseudomonads. Further studies of the bacterial receptors for NS1 and NS2 could also clarify the relationships of the LPS chemotypes of *B. cepacia* and *P. aeruginosa*.

7.4.2. *B. cepacia* plasmids

Plasmids have been found in many genera of soil bacteria and conjugative plasmids have been described as an important means of gene transfer in the environment (Reanney, Gowland, *et al*, 1983). In this study, most of the *B. cepacia* strains examined were found to harbour a plasmid of about 23kb. This common occurrence of plasmids in *B. cepacia* has been demonstrated previously (Gonzalez and Vidver, 1979), although no phenotypic function was assigned to any of these plasmids. Furthermore, these studies were performed prior to the knowledge of the complex taxonomy of what at the time was described as '*P. cepacia*'. The large plasmid of 194kb detected in this thesis is probably the same as the 200kb plasmid reported previously as a constituent of the *B. cepacia* genome (Cheng and Lessie, 1994; Rodley, Romling, *et al*, 1995). Plasmids conferring resistance to drugs such as tetracycline, ampicillin (Williams, Yeggy, *et al*,

1979) and streptomycin (Hirai, Iyobe, *et al*, 1982), have been isolated from *B. cepacia* strains. The streptomycin resistance *B. cepacia* plasmid Rms 425 can be transferred by conjugation between isogenic strains of *P. aeruginosa* and of *E. coli* (Hirai, Iyobe, *et al*, 1982). The conjugative transfer of plasmids between *B. cepacia* and *P. aeruginosa* is important as these organisms share environmental habitats and both colonise airways of CF patients.

Not only can *B. cepacia* DNA be transferred to *P. aeruginosa,* but *B. cepacia* can also accept DNA by conjugative transfer from *P. aeruginosa* and transfer it to other *B. cepacia* strains (Lennon and DeCicco, 1991). In addition, *B. cepacia* can harbour broad-host-range plasmids that can be transferred by conjugation to *P. putida* as well *as* *E. coli* and other enterobacteria (Sabaté, Villanueva, *et al*, 1994).

Conjugation is the only mechanisms of genetic exchange that has thus far been demonstrated to occur in *B. cepacia* in the natural environment. Conjugation of *B. cepacia* strains in the rhizosphere of barley and radish has been shown to occur within 24 hours of inoculation (Walter, Porteous, *et al*, 1991). The number of transconjugates in the rhizosphere peaks at about two days after planting and then start to decline (Walter, Porteous, *et al*, 1991). Thus, *B. cepacia* appears to be able to exchange genes by conjugation if present in high densities in the soil. Experiments on the use of *B. cepacia* complex strains as biopesticides indicate that there is a high density of organisms in the soil immediately after inoculation, before the levels start to decline and

stabilise (King and Parke, 1993). Thus, gene transfer through conjugation could be expected to occur during this 'time frame' of high bacterial density in the soil.

7.4.3 Natural transformation of the *B. cepacia* complex

While relatively few studies have demonstrated gene exchange in soil, transformation could occur if the DNA released from dead cells was able to transform competent recipients. Most soils contain DNAse; and thus DNA released from bacteria would not be expected to survive intact for long. However, it is possible in some ecosystems that naked DNA might be protected from degradative nucleases by adsorption to particulate material such as clay (Lorenz and Wackernagel, 1987).

In *P. stutzeri* and related *Pseudomonas* species (Carlson, Pierson, *et al*, 1983), natural transformation by homologous chromosomal DNA has been demonstrated to occur readily on plates. Carlson and co-workers found only double-stranded linear DNA to be effective, with plasmid DNA and single-stranded DNA unsuitable for transformation of *P. stutzeri*. A later study found that not only could *P. stutzeri* be transformed by plasmid and extracellular DNA but transformation could occur in a non-sterile soil (Sikorski, Graupner, *et al*, 1998). Thus, *P. stutzeri* appears to be able to exchange DNA by transformation in its natural habitat. Interestingly, *B. cepacia* plasmid DNA can not only transform other *B. cepacia* strains but it can also transform *E. coli* and other enterobacteria, as well as the major CF pathogen and soil inhabitant *P. aeruginosa*

(Hirai, Iyobe, et al, 1982; Sabaté, Villanueva, *et al*, 1994). Thus, genetic exchange through natural transformation with plasmid DNA could potentially occur between different *B. cepacia* complex strains as well as between the *B. cepacia* complex and *P. aeruginosa*.

Linear double-stranded DNA was used in this thesis to demonstrate natural transformation of the *B. cepacia* complex. Interestingly, this study found that competence of the *B. cepacia* complex increased with nutrient deprivation, as the majority of strains were only transformed when propagated on minimal agar. Similar results were reported by Carlson and co-workers (1983), who found that the competence of *P. stutzeri* increased with nutrient deprivation. Of major importance in this thesis, DNA from the highly transmissible *B. cepacia* genomovar III strain, J2315, was successfully used to transform other *B. cepacia* complex strains, including a biopesticide candidate. This observation suggests that transformation could occur in the environment, resulting in the exchange of DNA between clinical and biopesticide strains. Biopesticides strains could be present in very large quantities in the environment if granted licenses for commercial application.

Available evidence from *in vitro* experiments indicates that some exchange through transformation can occur between *B. cepacia* complex strains and between members of the complex and other genera. While laboratory experiments do not automatically translate to events in the natural environment, demonstration of natural competence in the *B. cepacia* complex indicates a potential for transformation in conducive conditions in natural environments.

In conclusion, exchange of genes between different *B. cepacia* complex strains and between the *B. cepacia* complex and other genera has been demonstrated in vitro through transformation, transduction and conjugation. In addition, genetic exchange by conjugation is known to occur in the rhizosphere, the natural habitat of the *B. cepacia* complex. It seems highly likely that large-scale bulk applications of the *B. cepacia* complex to the environment would encourage horizontal gene transfer not only between different *B. cepacia* complex strains but also with other soil flora such as *P. putida* and the major CF pathogen and soil organism, *P. aeruginosa*. At the moment, the risk of such events to CF patients is not known and more studies are urgently required to facilitate informed decision-making on the large-scale biotechnological applications of the *B. cepacia* complex. The exchange of genetic material through natural transformation between the well-characterised, epidemic *B. cepacia* genomovar III strain J2315, and a candidate biopesticide strain demonstrated in this thesis; as well as the isolation of transducing phages whose host spectrum spans clinical, environmental and candidate biopesticide *B. cepacia* complex strains, provides new and potentially important evidence of the transfer of virulence and resistance factors between pathogenic and biopesticide strains.

CHAPTER 8

General conclusions

In the last twenty years, members of the *B. cepacia* complex have emerged as life-threatening pathogens of immuno-compromised patients that occasionally infect healthy individuals. Ironically, due their potent antifungal and extraordinary metabolic properties, these bacteria also have considerable potential for commercial exploitation as agricultural biopesticides and remediators of contaminated soils. In these roles, the *B. cepacia* complex would provide an efficient, cost-effective and environmentally-friendly replacement of conventional pesticides and reduce the need for nitrogenous fertilisers by up to 20%. In an attempt to resolve these conflicting roles, and possibly provide 'safe' biological-based commercial agents, attempts have been made to identify differences between clinical and environmental isolates of the *B. cepacia* complex.

At present, the virulence factors of the *B. cepacia* complex remain ill-defined and not well understood. Intrinsic resistance to several classes of antibiotics is characteristic of the *B. cepacia* complex. For example, all *B. cepacia* complex strains are resistant to colistin (colomycin), a polymyxin antibiotic that has been effectively used for many years in the treatment of infections caused by *P. aeruginosa* and other Gram-negative bacilli (Littlewood, Koch, *et al*, 2000). Although resistance to colomyxin is universal for all *B. cepacia* complex strains, resistance to some antibiotics appears to be genomovar-dependent. This could be encouraging for the use of the *B. cepacia* complex as biopesticides, since strains for large-scale commercial application could be chosen

from genomovars that are sensitive to antibiotics. However, from the seven antibiotics studied in this thesis, genomovar-dependent sensitivity was only observed for chloramphenicol, which is no longer routinely used for the treatment of *B. cepacia* complex infections and is associated with toxicity to the bone marrow.

In the studies performed for this thesis, *B. vietnamiensis* (genomovar V) strains appeared more sensitive to chloramphenicol and ceftazidime than strains from the other genomovars. It has often been argued that *B. vietnamiensis* poses a minor threat to CF patients since the numbers isolated from the respiratory tracts of patients are relatively low and strains in this genomovar have not, so far, been associated with acute exacerbations and 'cepacia syndrome'. Thus, *B. vietnamiensis* strains have been regarded as ideal candidates for use as biopesticides. Sensitivity to ceftazidime, one of the few currently effective antibiotics for the treatment of *B. cepacia* complex infections, could provide an argument for the commercial application of *B. vietnamiensis* strains as biopesticides. However, *B. vietnamiensis* strains were not significantly sensitive to ciprofloxacin, the other routinely used antibiotic. Furthermore, sensitive strains were found to mutate readily to resist high levels of ceftazidime and ciprofloxacin. In conclusion, the sensitivity of *B. vietnamiensis* to antibiotics does not provide a sound argument for their bulk distribution into the environment.

The widespread belief that only environmental, and not clinical isolates, possess phytopathogens is potentially misleading. Both clinical and environmental strains can be pathogenic for plants depending on the plant host tested. Thus, phytopathogenicity is

not dictated by the origin of strains but rather, might be dependent on the individual strain concerned. Accumulating evidence suggests that there are no inherent differences between clinical and environmental strains that determine the phytopathogenic potential of members of the *B. cepacia* complex.

Although the mechanisms of pathogenicity involved in *B. cepacia* complex infections are unclear, several putative virulence factors have been described. Production of elastase would be advantageous for a respiratory pathogen since elastin forms the major constituent of elastic tissue fibres such as the walls of the alveoli of the lungs. However, only a minority of *B. cepacia* complex strains, of both clinical and environmental origin, produce elastase, making it an unlikely contributor to the organism's role in lung disease. Most environmental, clinical and candidate biopesticide strains produce protease. Interestingly, strains representing several sub-groups within the *B. cepacia* complex were found to harbour the *dsb* gene whose product is required for the disulphide bonds of *B. cepacia*'s metalloprotease PSCP. PSCP produces bronchopneumonia in rats, thus all sub-groups of the *B. cepacia* complex could potentially produce bronchpneumonia in suitable hosts.

The association of certain *B. cepacia* complex isolates with epidemic spread is now well recognised and three genetic markers associated with enhanced transmissibility have been identified. These 'epidemic markers', (cable pili, BCESM and IS402-IS1356) have not only been detected in clinical strains but also in strains isolated from the environment and candidate biopesticide strains. Of greater significance is the lack of

detection of any of these markers in *B. multivorans* strains that are known to have caused epidemics (Whiteford, Wilkinson, *et al*, 1995; Mahenthiralingam, Coenye, *et al*, 2000). Thus, at present, although identification of currently known epidemic markers is useful and informative, their presence does not unequivocally identify all members of the *B. cepacia* complex with enhanced transmissibility.

With the exception of the enzyme catalase and the pigment melanin that were produced only by clinical strains, most putative virulence factors of the *B. cepacia* complex were found in this thesis to be also produced by candidate biopesticide strains and environmental isolates. Thus, clinical, environmental and candidate biopesticide strains within the *B. cepacia* complex could be pathogenic to susceptible human hosts.

Until recently, the high isolation rate of *B. cepacia* genomovar III isolates from the respiratory tracts of CF patients, contrasted with their apparent absence from the natural environments, presented an interesting and important enigma. However, reports of *B. cepacia* genomovar III environmental isolates have recently started to emerge and renewed the 'biopesticide' argument of agricultural microbiologists that there is no danger in adding it to the environment when it is already present naturally. It could be also argued that sub-populations of the *B. cepacia* complex that are naturally maintained at high, stable numbers in the environment and have minimal clinical effects, would be most suitable for commercial exploitation. Such a sub-population is yet to be identified. The incidence of each of the *B. cepacia* genomovars in the environment and the clinical outcome of patients colonised by them, needs to be assessed in order to determine the

effects of introducing large quantities of the *B. cepacia* complex to the environment. In conclusion, current evidence indicates that the classification of *B. cepacia* complex strains as clinical or environmental is superficial and bears no correlation to a strain's potential pathogenicity to either humans or plants.

Undoubtedly, strains of the complex could prove to be effective and ecologically-friendly biopesticides. Furthermore, their ability to degrade an array of recalcitrant xenobiotic or toxic compounds suggests that they would also make ideal bioremediation agents. One of the major obstructions to the biotechnological applications of the *B. cepacia* complex is the lack of knowledge regarding the fate of these agents once introduced into the environment. Factors such as bacterial persistence in the environment, reproduction and mutation rate, and rate of gene transfer to other soil organisms need to be studied and understood before sound and unequivocable judgements can be made.

Genetic exchange between different *B. cepacia* complex strains and between the complex and other genera has been demonstrated through the three major mechanisms of transduction, transformation and conjugation. *B. cepacia* complex transducing bacteriophages identified during this thesis and whose host spectrum spans environmental, clinical and candidate biopesticide strains will be useful to investigate gene transfer between these host groups. *B. cepacia* complex phages whose host spectrum also includes *P. aeruginosa* (and possibly other pseudomonads) could facilitate the exchange of genes between these two soil organisms and co-colonisers of the

airways of CF patients. Furthermore, large-scale bulk applications of *B. cepacia* to the environment could provide the necessary high densities of organisms to facilitate the exchange of genes between different *B. cepacia* complex strains and between the *B. cepacia* complex and other soil organisms such as *P. aeruginosa.*

B. cepacia complex strains that are not pathogenic to humans, are incapable of transferring their genes or acquiring foreign ones and have minimal effect on soil microflora would be ideal for large-scale commercial exploitation. Such strains have yet to be identified. Although there are some animal models of CF available and they have proved useful (Dorin, Dickinson, *et al*, 1992; Dorin, Stevenson, *et al*, 1994; Davidson, Dorin, *et al*, 1995; Speert, Steen, *et al*, 1999), they are not reliable enough to provide the unequivocal evidence of 'safe biopesticides'. At present, the introduction of bulk quantities of the *B. cepacia* complex to the environment poses an unacceptable threat to humans, healthy or immuno-compromised, and might also carry some as yet unidentified risks.

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Appendix

1. Gas Chromatography

1.1. *The Microbial Identification System (MIS) Chromatographic unit for cellular fatty acid analysis.*

The MIS chromatographic unit consists of a Hewlett-Packard 5890A Gas Chromatograph (with flame ionising detector (FID)), a 3396A Integrator and a 7673A Automatic Sampler (with injector, controller and tray).

The column used with the MIS is a 25mm x 0.2 mm methyl phenyl silicone fused silica capillary column (HP 19091-102).

The MIS operates at a 100:1 split ratio i.e. 1/100 of the sample enters the capillary column while the rest is vented out the split vent.

1.1.1. *Gas flows*

Supply gas	**Flows** (approximate)
Air	400ml min^{-1}
Hydrogen	30ml min^{-1} (FID)
	55 ml min^{-1} (split vent)
	5ml min^{-1} (Septum purge), carrier gas
Nitrogen (auxilliary gas)	30ml min^{-1}

The column head pressure is approximately 9psi (retention time of solvent peak should be approximately 1.6min)

1.1.2. *Temperature program*

Injector temperature	250°C
Detector temperature	300°C
Initial temperature	170°C
Rate 1	5°C min^{-1} from 170°C to 270°C
Rate 2	30°C min^{-1} from 270°C to 310°C
Final time	2 min at 310°C
Equilibrium time	3 min at 170°C

1.2. *MIS computer system*

1.2.1. *Computer system connected to the chromatographic unit*

Hewlett-Packard Series 310 workstation with 1 megabyte RAM

40 megabytes 9153 C disk drive with 8.89cm flexible disk drive

Monochrome moniter

This unit has the following responsibilities:

Data acquisition from the chromatographic unit

Storing data on hard disk

Library search

1.2.2. *Second computer unit*

The second computer unit consists of the following:

IBM-compatible 864DX PC with 4 megabytes RAM

130 megabytes hard disk with 8.89 and 13.34 floppy drives

SVGA colour monitor

This unit has the following responsibilities:

Organisation of data on hard disk

Library search

Supporting library generation

Cluster analysis

2. SDS-PAGE Stock solutions

2.1. NaPBS buffer

0.2M $Na_2HPO_4.12H_2O$	40.5ml
0.2M $NaH_2PO_4.2H_2O$	9.5ml
NaCl	8g

Made up to 1.0L with double distilled water. pH 7.3 and stored at 4°C

2.2. *Tris-HCl buffers*

The pH together with the conductivity were adjusted with a standardised 1.72M HCl solution, titrated against NaOH 1N .

a). *Sample treatment buffer (STB)*

Tris	0.7g
C_2H_6OS	5ml
Sucrose	5g
H_2O up to	100ml

pH 6.8 and stored at -20°C

0.75g Tris, 50ml of double distilled water and 3.27 ml 1.72N HCl gives a pH of 6.80. After adding 5ml C_2H_6OS and 5g sucrose and adjusting the volume to 100ml, the solution has a conductivity of 3.87mS at 23°C.

b). *Separation gel buffer*

Tris	18.15g
H_2O up to	100ml

pH 8.8 and stored at 4°C

Mixing 18.15g Tris, 50ml double distilled water, 24.2ml 1.72N HCl and adjusting the volume to 100ml gives the solution a pH of 8.78 and a conductivity of 16.7mS at 23°C.

c). *Stacking gel buffer*

Tris	6g
H_2O up to	100ml

pH 6.8 and stored at 4°C

Mixing 6g Tris, 50ml double distilled water, 27.8ml 1.72N HCl and adjusting the volume to 100ml gives the solution a pH of 6.82 and a conductivity of 25.8mS at 24°C.

2.3. *Tris-glycine buffers*

a). Running buffer for lower buffer reservoir

Tris (BDH)	12g
Glycine (BDH)	57.5g
SDS (BDH)	4g
H_2O up to	4L

pH 8.59 and stored at room temperature

The conductivity is 624μS at 19°C.

b). *Tank buffer for upper reservoir*

Tris (BDH)	1.5g
Glycine (BDH)	7.2g
SDS 10%(BDH)	5ml
H_2O up to	500ml

Buffer is freshly prepared for each gel and has a conductivity of 624μS and pH of 8.59 at 19°C.

2.4. *Monomer solution*

Acrylamide (BDH)	29.2g
Bis acrylamide (BDH)	0.8g
H_2O up to	100ml

Solution has conductivity of 624μS, should be kept on ice and stored at 4°C.

2.5. *SDS solution*

10 or 20g sodium dodecyl sulphate is dissolved in 100ml of distilled water to make a 10 or 20% solution. The solution should be kept at room temperature as SDS precipitates below 10°C.

2.6. *Staining solution*

The staining solution is composed of:

Coomasie brilliant blue R-250	2g
Methanol	500ml
Acetic acid	100ml
Ethylene glycol	30ml
H2O up to	1L

Store at room temperature

2.7. *Destaining solution*

Methanol	500ml
Acetic acid	200ml
Ethylene glycol	60ml
H2O up to	2L

Store at room temperature

2.8. *Bromophenol blue 0.001%*

Stock solution 10mg 100ml^{-1} sample treatment buffer with 2% SDS and a 10-fold dilution in sample treatment buffer with 2% SDS is used.

2.9. *Loading dye/ Volume adjusting buffer*

STB with 2% SDS and 0.001% bromophenol blue.

Store at -20°C.

2.10. *Low molecular weight markers*

The protein molecular weight markers were all obtained from Sigma. They were mixed with STB with 20% SDS to obtain the optimal concentrations recommended by the manufacturer. The proteins used were:

β-galactosidase

bovine albumin

egg albumin

glyceraldehyde-3-phosphate dehydrogenase

carbonic anhydrase

trypsinogen

trypsinogen inhibitor

lysozyme

alfa-lactalbumin

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Sensitivity of the *Burkholderia cepacia* complex and *Pseudomonas aeruginosa* to transducing bacteriophages

Sazini Nzula [a,*], Peter Vandamme [b], John R.W. Govan [a]

[a] *Department of Medical Microbiology, The University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK*
[b] *Laboratorium voor Mikrobiologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*

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Abstract

Burkholderia cepacia is now recognised as a life-threatening pathogen among several groups of immunocompromised patients. In this context, the proposed large-scale use of these bacteria in agriculture has increased the need for a better understanding of the genetics of the species forming the *B. cepacia* complex. Until now, little information has been available on the bacteriophages of the *B. cepacia* complex. Transducing phages, named NS1 and NS2, were derived from the lysogenic *B. cepacia* strains ATCC 29424 and ATCC 17616. The frequency of transduction per phage particle ranged from 1.0×10^{-8} to 7.0×10^{-6} depending on the phage and recipient strain used. The host range of NS1 and NS2 differed but in each case included environmental and clinical isolates, and strains belonging to several species and genomovars of the *B. cepacia* complex. The host range of both phages also included *Pseudomonas aeruginosa*. Some *B. cepacia* complex isolates were sensitive to the well-characterised *P. aeruginosa* transducing phages, B3, F116L and G101. The lytic activity of NS1 and NS2 was inhibited by *B. cepacia* lipopolysaccharide suggesting that this moiety is a binding site for both phages. The molecular size of the NS1 and NS2 genomes was approximately 48 kb. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Burkholderia cepacia*; Bacteriophage; Transduction; Genomovar; *Pseudomonas aeruginosa*

1. Introduction

Burkholderia (previously *Pseudomonas*) *cepacia* was first described in 1950 as the phytopathogen responsible for bacterial rot of onion bulbs [1]. In the 1960s, seminal taxonomic studies by Stanier and colleagues [2] highlighted the organism's extraordinary metabolic ability to degrade a wide range of organic compounds. Inherent resistance to antibiotics and survival in nutritionally limited environments were also exemplified by the organism's use of penicillin G as a sole carbon source [3] and its role as a major bacterial contaminant of space shuttle water supplies [4] and bottled drinking water [5].

In the last two decades, *B. cepacia* has emerged as a life-threatening human pathogen, in particular as a cause of intractable life-threatening pulmonary infections in patients with chronic granulomatous disease and cystic fibrosis [6] or undergoing treatment in intensive care units [7].

Recently, attention has focused on the taxonomy of *B. cepacia* for reasons which will become apparent. In 1992, the new genus *Burkholderia* was introduced with *Burkholderia cepacia* named as the type species [8]. Integrated genotypic and polyphasic taxonomy [9,10] showed that isolates presumptively identified as *B. cepacia* comprise several genomic species, or genomovars. Three genomovars have been awarded species status as they can be distinguished phenotypically: genomovar II (*Burkholderia multivorans*), genomovar IV (*Burkholderia stabilis*) and genomovar V (*Burkholderia vietnamiensis*). These three species together with genomovars I, III and VI presently constitute the *B. cepacia* complex. Unless otherwise indicated, the term *B. cepacia* will be used in this report to refer to all members of the complex.

Interest in the taxonomy of *B. cepacia* arises not only from the organism's role as a plant and human pathogen but ironically from intense debate on the potential human hazards associated with plans to exploit the antifungal and

* Corresponding author. Tel.: +44 (131) 650 3165; Fax: +44 (131) 650 6653; E-mail: snzula@ed.ac.uk

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metabolic properties of these organisms as agricultural biopesticides and bioremediators [11–14]. *B. cepacia* can repress many soil-borne plant pathogens and thus improve germination and crop yields [15]. Its striking metabolic potential can also be exploited in the bioremediation of environmental sites contaminated with toxic organic pesticides and herbicides. Regulatory agencies in North America are presently faced with the task of providing Solomon-like judgements on licence applications to perform field trials of candidate biopesticide strains. Use of *B. cepacia* on an agricultural scale would involve large quantities of bacteria and, as noted in a recent report of the United States Environmental Protection Agency (http://www.epa.gov/oscpmont/sap/1999/july/finalrpt1.pdf), introduces the potential hazard of horizontal gene transfer between biopesticide strains and other members of the *B. cepacia* complex, including strains with potential to cause human infections.

Transduction is a major mechanism by which genetic exchange could be facilitated between members of the *B. cepacia* complex, furthermore, some of the difficulties in genetic manipulation of *B. cepacia* [16], including vector markers based on antibiotic resistance, could be resolved by the availability of recombination systems based on transducing phage. There are few published studies on *B. cepacia* phage, and none recent enough to take account of the major developments in *B. cepacia* taxonomy. To our knowledge, the only previous report of a *B. cepacia* transducing phage is that of Matsumoto et al. [17]. The aim of our study was to search for evidence of lysogeny within the *B. cepacia* complex and to investigate any phages isolated. We report here the biological properties of two *B. cepacia* transducing phages, NS1 and NS2; their host ranges include several groups within the *B. cepacia* complex and *Pseudomonas aeruginosa*. We also report the sensitivity of *B. cepacia* to several classic *P. aeruginosa* transducing phages.

2. Materials and methods

2.1. Bacteria and phage strains

The strains used in this study included 38 environmental and clinical isolates of the *B. cepacia* complex. Also included were the 30 well-characterised strains of the *B. cepacia* strain panel [16] which contains type strains and representatives of the major genomic species belonging to the *B. cepacia* complex [9]. Identification of all isolates was confirmed by the API 20 NE system (bioMérieux, Marcy l'Etoile, France) and their genomovar and species status identified by whole cell protein analysis and DNA:DNA hybridisation using methods previously described [9]. In addition, 42 strains of *P. aeruginosa* were screened as potential hosts for phages NS1 and NS2. To ensure a lack of clonal relationship, isolates were fingerprinted by pulsed field gel electrophoresis (PFGE, CHEF, Bio-Rad Laboratories Inc.) using protocols described previously for *B. cepacia* [18] and *P. aeruginosa* [19]. Three isolates (J2315, BC7 and K56-2) were already known to be clonal [16] but were included in the *B. cepacia* complex strain panel as geographically distinct representatives of the major epidemic lineage ET12 [6,20]. Resistant mutants of strains ATCC 53267 (ATCC 53267R), ATCC 29424 (ATCC 29424R) and C2973 (C2973R) with minimum inhibitory concentrations (MICs) of ceftazidime of 8 μg ml^{-1} were used for transduction studies. Cultures were preserved in 10% skimmed milk at −70°C.

2.2. Media

All cultures were grown in nutrient broth+0.5% yeast extract (NBYE) at 37°C in an orbital incubator at 140 rpm. Soft agar for phage experiments contained nutrient broth with 0.3% (w/v) agar.

2.3. Isolation of bacterial mutants

Spontaneous bacterial mutants were isolated by spreading 100 μl of overnight broth cultures (approximately 10^8 cfu ml^{-1}) on the surface of iso-sensitest agar (Oxoid) containing antibiotic at concentrations ranging from the MIC to 32 times the MIC. After incubation at 37°C in air for 48 h, mutational frequencies were calculated from results obtained with the highest concentration of antibiotic on which resistant mutants were detected. The mutants were cultured again on the medium containing the same concentration of antibiotic and maintained in skimmed milk at −70°C.

2.4. Isolation and propagation of bacteriophages

Overnight NBYE cultures of *B. cepacia* were harvested at $3200\times g$ for 30 min and the supernatants filtered (Millipore: pore size 0.2 μm) and screened for the presence of phage by spotting onto bacterial lawns using a multipoint inoculator.

High-titre phage preparations for transduction were prepared as follows: 100 μl of a phage preparation, containing approximately 10^5 plaque-forming units (pfu ml^{-1}), was added to 2.5 ml of molten soft nutrient agar, previously seeded with 100 μl of an overnight culture of the donor strain. The mixture was then overlaid on nutrient agar and allowed to set. After 18 h at 37°C, overlays showing semi-confluent lysis were transferred into 10 ml phage buffer (10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$) [17]. The lysate was then vortexed, centrifuged at $3200\times g$ for 30 min and the supernatant filtered. Phage titres were determined as pfu ml^{-1} by incorporating diluted filtrate in soft agar overlays using TNM buffer (Tris 0.01 M pH 7.4, NaCl 0.15 M, $MgSO_4$ 0.01 M) [21]. Stock preparations were maintained at 4°C.

2.5. Host range of phages against B. cepacia and P. aeruginosa

Stock phage solutions, diluted in phage buffer to approximately 10^8 pfu ml^{-1}, were spotted on to lawns of *B. cepacia* and *P. aeruginosa* using a multipoint inoculator and examined for lytic activity after 24 h at 37°C.

2.6. Purification of phage DNA

Phage DNA was extracted using the method described by Matsumoto et al. [17]. Genome size of unrestricted DNA was estimated by PFGE (initial and final pulse times of 5 and 20 s, 170 V at 14°C for 19 h) incorporating a λ ladder DNA PFGE standard kit (New England Biolabs, Hitchin, Hertfordshire, UK).

2.7. Extraction of lipopolysaccharide (LPS)

B. cepacia LPS was extracted using the aqueous phenol method of Westphal and Luderitz [22] as modified by Hancock and Poxton [23].

2.8. Neutralisation of phage activity by LPS

The effect of pre-incubation with LPS on phage activity was determined using a modification of the method described by Govan [24]. Phage preparation (0.5 ml), containing approximately 10^3 pfu ml^{-1}, was mixed with 0.5 ml of LPS (20 mg ml^{-1} solution in distilled pyrogen-free water) and incubated at 37°C for 1 h. Controls comprised phage and buffer without LPS. The experiment was repeated and the results were calculated as the means of three experiments.

2.9. Transduction

Transduction experiments were carried out using the method described by Krishnapillai [21]. Phages NS1 and NS2 were propagated on the ceftazidime-resistant mutants of three *B. cepacia* strains with MICs of 8 μg ml^{-1}. Phage lysate (0.5 ml) was added to a phosphate-buffered saline suspension of the sensitive parent strains ATCC 53267, ATCC 29424 and C2973 at a multiplicity of 1 and left for 20 min at 37°C to allow phage adsorption. The cells were harvested at $3200 \times g$ for 30 min, resuspended in TNM buffer and 100 μl spread on nutrient agar containing ceftazidime 8 μg ml^{-1}. Transductants were recovered after incubation at 37°C for 72 h. Controls comprised recipient strains plus TNM buffer alone.

2.10. Electron microscopy

Phage lysates were centrifuged at $3200 \times g$ to remove cell debris, and then at $100\,000 \times g$ for 1 h. The phage pellet was resuspended in 1 M ammonium acetate. After negative staining with 2% (w/v) potassium phosphotungstate solution (pH 7.0), phage morphology was observed with a transmission electron microscope (Hitachi type 12A).

3. Results

In preliminary experiments, 38 strains of *B. cepacia* from clinical and environmental sources were tested for lysogeny by employing the same *B. cepacia* strains as indicator strains. Two strains, ATCC 29424 (genomovar V) and ATCC 17616 (genomovar II), proved to be lysogenic, providing the phages named NS1 and NS2 respectively.

3.1. Virological characteristics and morphology of the phages NS1 and NS2

Plaques of NS1 and NS2 on a host cell lawn were about 1 mm in diameter and either clear or turbid depending on the host strain. Neither phage was inactivated by treatment with chloroform. Electron microscopy showed NS1 and NS2 to resemble T-even-like phages with a hexagonal head and a contractile tail (Fig. 1).

3.2. Transduction

Phages NS1 and NS2 were propagated on three ceftazidime-resistant mutants of *B. cepacia* and used to infect the sensitive parent strains. Ceftazidime resistance was transduced at a frequency ranging from 1.0×10^{-8} to 7.0×10^{-6} per infected cell depending on the phage and recipient strain; these compared to spontaneous mutation frequencies between undetectable and 6.8×10^{-11}. Similar results were obtained in three replicate experiments.

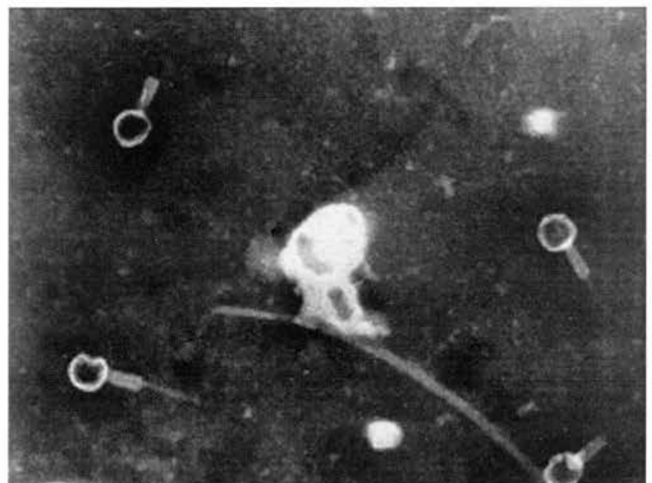

Fig. 1. Electron micrograph of NS2 (×56 000).

Table 1
Isolates of the *B. cepacia* complex sensitive to NS1 and NS2

Isolate	Genomovar	NS1	NS2	Reference
J2540	I	+	+	[9]
C2775	II	+	+	This paper
J2315	III	–	+	[16]
ATCC 17765	III	–	+	[16]
C1394	III	–	+	[16]
J415	III	–	+	[16]
BC7	III	–	+	[16]
K56-2	III	–	+	[16]
C6433	III	–	+	[16]
PC184	III	+	+	[16]
CEP511	III	+	+	[16]
LMG 18836	V	–	+	[16]
ATCC 53266	V	+	+	This paper
ATCC 53267	V	+	+	This paper
ATCC 29424	V	–	+	This paper
C2973	V	+	+	This paper
LMG 16232	V	+	+	[16]

3.3. Lytic spectrum of phages NS1 and NS2 against *B. cepacia* and *P. aeruginosa*

Table 1 illustrates *B. cepacia* strains sensitive to one or more phages; these strains include environmental and clinical isolates of the *B. cepacia* strain panel [16] and representatives of the species and genomovars of the *B. cepacia* complex [9]. When combined, the host range of NS1 and NS2 included *B. multivorans* and *B. vietnamiensis*, as well as genomovars I and III. J2540 was the only genomovar I strain among 18 strains tested that was sensitive to NS1 or NS2. Neither phage lysed any of the four *B. stabilis* strains tested. Only a few representatives of this group were available for investigation and these results are inconclusive; however, as the genome of this organism is highly conserved and shows little diversity on PFGE analyses [10], it is possible that resistance to NS1 and NS2 may be a characteristic of *B. stabilis*. Of particular interest, each of the seven genomovar III clones in the *B. cepacia* strain panel, including the intercontinental epidemic lineage ET12, showed sensitivity to one or more of the phages. Several biopesticide candidate strains were also found to be sensitive to NS1 and NS2; these included ATCC 53266 and ATCC 53267.

To determine if the host range of *B. cepacia* phages extended to other pseudomonads, 42 strains of *P. aeruginosa* were examined for sensitivity to NS1 and NS2. Eight *P. aeruginosa* isolates (19%) were lysed by at least one of the phages, five by both phages (12%), one (2%) by NS1 and two (5%) by NS2.

Sensitivity of *P. aeruginosa* to *B. cepacia* phages prompted an investigation of the corresponding sensitivity of *B. cepacia* isolates to *P. aeruginosa* phages. Of 38 strains of *B. cepacia* investigated, three strains, one each of genomovars I, III and V, were found to be sensitive to four well-characterised *P. aeruginosa* phages. These included the virulent phage E79, and the transducing phages B3, F116L and G101 [25].

Table 2
Effect of LPS on phage activity

Strain	Phage	Number of plaque-forming units per ml		
		Control	Phage and LPS	Decrease (%)
ATCC 29424	NS2	3620	870	76
C2973	NS1	640	430	33
C2973	NS2	270	130	52
ATCC 53267	NS1	5290	120	98
ATCC 53267	NS2	2050	90	96

3.4. Effect of LPS on phage activity

Table 2 shows the effect of pre-incubating NS1 and NS2 with LPS before infecting sensitive strains. Depending on the phage and host strain, titres of plaque-forming units decreased by 33–98% compared to controls which had been pre-incubated with buffer.

4. Discussion

NS1 and NS2 are morphologically similar to the T-even phages with hexagonal heads and contractile tails. Similar characteristics were described by Matsumoto et al. [17] for the transducing phage CP75. The molecular mass of NS1 and NS2 phage genomes was approximately 48 kb, which is reasonably close to the molecular size of 52 kb reported for CP75. CP75 or the lysogenic strain PCT1 were unavailable for comparative studies.

P. aeruginosa and *B. cepacia* share environmental habitats and often co-colonise airways of cystic fibrosis patients at high bacterial density (10^9 cfu ml^{-1}). Different strains of each pathogen may also co-colonise the same patient. Thus, NS1 and NS2, together with the classic *P. aeruginosa* transducing phages F116L, G101 and B3, have the potential and opportunity to mediate transfer of virulence and resistance factors between clinical isolates of two major opportunistic pathogens and environmental isolates of the *B. cepacia* complex selected as candidate biopesticide strains.

Acquisition of virulence factors mediated by transduction has been reported in *Vibrio cholerae* [26] and *Streptococcus pyogenes* [27]. In *V. cholerae,* it has been suggested that transduction contributes to the evolution of epidemic strains [28]. Evidence for the assimilation of foreign DNA by *B. cepacia* has been presented by citing the significantly lower G+C content of insertion sequences when compared with native DNA [29]. It has also been reported that insertion sequences found in *B. cepacia* J2315, a representative of the genomovar III and epidemic lineage ET12, are identical to those found in *Burkholderia pseudomallei* [30]. *B. pseudomallei* is the highly virulent agent of the tropical disease melioidosis and shares environmental habitats with

B. cepacia and *P. aeruginosa*. There are also reports of cystic fibrosis patients co-colonised with *B. pseudomallei* and *B. cepacia* on return from holiday in South East Asia. Further studies are required to determine: (1) if the host range of NS1, NS2 and the *P. aeruginosa* transducing phages extends to *B. pseudomallei* and to the closely related and highly virulent equine and human pathogen *Burkholderia mallei*; and (2) if *B. cepacia* and *P. aeruginosa* transducing phages can mediate transduction of genes associated with virulence and epidemic markers between different subpopulations of the *B. cepacia* complex and between members of the complex and other pseudomonads.

The activity of NS1 and NS2 is inhibited by LPS, suggesting that LPS is a binding site for both phages. An LPS receptor would explain the sensitivity of *P. aeruginosa* to *B. cepacia* phages since Western blotting and absorption studies have indicated cross-reactivity in antibodies to the core LPS of both species [31]. An NS2 receptor located within core LPS is also supported by previous analyses of *B. cepacia* LPS chemotypes which showed that most genomovar III isolates express only core LPS [32] and our observation in this study that the majority of genomovar III isolates were sensitive to NS2.

To our knowledge, this is the first report of *B. cepacia* transducing phages the host spectrum of which spans environmental and clinical isolates, several subpopulations within the *B. cepacia* complex and candidate biopesticide strains. It is also the first report of the sensitivity of *B. cepacia* isolates to well-characterised transducing phages of *P. aeruginosa*. Given the present difficulties in genetic manipulation of multiresistant strains of *B. cepacia*, transducing phages could provide a useful mechanism to study horizontal gene transfer in *B. cepacia* and other pseudomonads. Further studies of the bacterial receptors for NS1 and NS2 could also clarify the relationships of the LPS chemotypes of *B. cepacia* and *P. aeruginosa*.

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