STUDIES ON THE NATURE OF *Burkholderia cepacia* IN CYSTIC FIBROSIS

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SUMMARY

Burkholderia cepacia is an opportunistic pathogen that colonises of the lungs of cystic fibrosis (CF) patients, with a frequently fatal outcome. Antibiotic resistance is common and highly transmissible epidemic strains have been described in the UK.

37 *B. cepacia* isolates from clinical and botanical sources were characterised via metabolic capabilities, antibiotic sensitivity, fatty acid methyl ester (FAME) profiles, restriction digest analysis of chromosomal DNA by pulsed-gel electrophoresis (PFGE) (with the use of two separate restriction enzymes) and outer membrane protein (OMP) profiles. This revealed isolates of the UK CF epidemic strain to form a distinct group with a specific OMP profile. Cluster analysis of PFGE and FAME profiles revealed the species *Burkholderia gladioli* and *Burkholderia vietnamiensis* to be more closely related to each other and to laboratory strains of *B. cepacia* than to the CF epidemic strain considered a member of the latter species. The epidemic strain of *B. cepacia* may therefore be worthy of species definition in its own right. All the strains studied showed a high level of resistance to antibiotics, including the carbapenems.

Considering this, carbapenemase production by isolates of *B. cepacia* was investigated. A metallo-β-lactamase from a clinical strain of *B. cepacia* was isolated and partially purified of using Cibacron blue F3GA-coupled agarose. The resulting preparation showed a single band of β-lactamase activity (pI 8.45) after analytical isoelectric focusing. The enzyme was particularly effective in the hydrolysis of imipenem. Meropenem, biapenem, cephaloridine, ceftazidime, benzylpenicillin, ampicillin and carbenicillin were hydrolysed at a lower rate. An unusual inhibition profile was noted. Inhibition by the metal ion chelators ethylene diamine tetra acetic acid and *o*-phenanthroline was reversed by addition of zinc, indicating a metallo-enzyme, whilst >90% inhibition was attainable with 0.1mM concentrations of tazobactam and clavulanic acid. A study of 8 other clinical isolates showed an enzyme of pI 8.45 to be present and inducible by imipenem in each case. This enzyme was assigned PCM-I (*Pseudomonas cepacia* metalloenzyme I).

Key words: metallo-β-lactamase; pulsed-field gel electrophoresis; fatty acid, carbapenem, carbapenemase

To my family,
especially my Grandmother Lydia, who was
100 years of age almost to the day I submitted this thesis.

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CONTENTS

LIST OF FIGURES.

LIST OF TABLES.

ABBREVIATIONS.

It is expected that the reader will be familiar with common chemical notation such as the formulae of simple compounds (i.e. NaCl = sodium chloride); consequently these are not included in the list below. Likewise, widely-used scientific abbreviations (i.e. CF = cystic fibrosis; DNA = deoxyribonucleic acid) and commonly-used units (i.e. mm = millimetres) are also excluded from this list. For further information on fatty acid nomenclature, the reader is referred to the literature (Sasser, 1990; Welch, 1991). The following is an alphabetical list of less common abbreviations used in this thesis:

ACE	Angiotensin converting enzyme.
AP-PCR	Arbitrarily-primed polymerase chain reaction.
BCA	Bicinchoninic acid.
BLIP	β-lactamase inhibitory protein.
BSAC	British Society for Antimicrobial Chemotherapy.
CAPS	3[cyclohexylamino]-propane sulphonic acid.
CFTR	Cystic fibrosis transmembrane conductance regulator.
CGD	Chronic granulomatous disease.
CHEF	Clamped homogeneous electric field.
DEAE	Diethyl amino ethyl.
EGTA	Ethylene glycol-*bis*-(β-amino-ethyl ether) N,N'-tetra-acetic acid.
EPS	Exocellular polysaccharide.
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction.
FAME	Fatty acid methyl ester.
FPLC	Fast protein liquid chromatography.
GC	Gas liquid chromatography.
IEF	Isoelectric focusing.
IgG	Immunoglobulin G.
kb	Kilobases.
kDa	Kilodaltons.
LDC	Lysine decarboxylase.
LPS	Lipopolysaccharide.
Mb	Megabases.
MEE	Multilocus enzyme electrophoresis.
MIC	Minimum inhibitory concentration.
MIS	Microbial Identification System.
MOPS	3-[N-morpholino] propanesulphonic acid.

OF	Oxidation / fermentation.
OFPBL	Oxidation / fermentation / polymyxin B / Bacitracin / Lactose medium.
OM	Outer membrane.
OMP	Outer membrane protein.
OP	*o*-phenanthroline.
PBP	Penicillin-binding protein.
PC	*Pseudomonas cepacia* medium.
pCMB	*p*-chloromercuribenzoate.
PCR	Polymerase chain reaction.
PFGE	Pulsed-field gel electrophoresis.
pI	Isoelectric point.
PMS	Pyrolysis mass spectrometry.
PMSF	Phenylmethyl sulphonyl fluoride
psi	Pounds per square inch.
Sarkosyl	Sodium N-lauroyl sarcosinate.
SFM	Société Française de Microbiologie.
SKB	SmithKline Beecham.
Tris	Tris-(hydroxymethyl) aminomethane.
UPGMA	Unweighted pair-group mean of arithmetic averages.

1 Introduction.

1.1 Cystic Fibrosis: A Brief Overview.

Cystic fibrosis (CF) is the most common fatal genetic disorder of the white population. The term "cystic fibrosis of the pancreas" was first devised in 1938 (Anderson, 1938). This was due to the characteristic pancreatic lesions consistently identified post-mortem. The disease has been extensively reviewed in the literature (Marelich & Cross, 1996).

CF is an autosomal recessive trait present in approximately 1 in 2500 Caucasian neonates, with a carrier rate of about 1 in 25 (Corey & Farewell, 1996). These frequencies amongst other races are significantly lower (Gilligan, 1991). In 75% of cases, the defect is due to a three base pair deletion on the long arm of chromosome 7 leading to the omission of a phenylalanine residue and subsequent production of a defective cyclic-AMP regulated chloride channel (Shah *et al.*, 1996). This is the cystic fibrosis transmembrane conductance regulator, CFTR (Marelich & Cross, 1996). Defective ion transport across cell membranes arises from this, resulting in the production of abnormally viscous exocrine secretions including pulmonary and gastrointestinal mucus (Shah *et al.*, 1996).

This increase in secretion viscosity is reflected in the two greatest causes of morbidity in this group: malnutrition due to pancreatic secretory insufficiency (Corey & Farewell, 1996) and airway (as opposed to lung parenchyma) infection (Gilligan, 1991). These symptoms, together with the detection of a marked elevation in sweat chloride concentration, form the basis of the current definitive diagnostic technique (Corey & Farewell, 1996).

Treatment for CF patients has since 1964 centred on three main principles: nutrition, airway clearance and the treatment of infection (Matthews *et al.*, 1964). The means by which these are achieved has varied over the intervening 22 years, but the principles are still significant (Marelich & Cross, 1996). Treatment has succeeded in raising the median life expectancy of CF patients from a few years in the 1940s to approximately 30 years of age in the 1990s (Shah *et al.*, 1996). The prognosis is slightly worse in female CF patients and this appears to have a multifactorial basis (Corey & Farewell, 1996).

The treatment of pulmonary symptoms in CF patients is of particular importance: respiratory tract disease, associated with increased bacterial colonisation and infection, is responsible for over 90% of the morbidity and mortality in this group (Hodson & Warner, 1992). The precise way in which the physiological changes observable in CF predispose to pulmonary infection has not been elucidated (Hodson & Warner, 1992). It has been reasonably proposed that the plugging of airways with viscous, tenacious mucus is largely responsible for predisposing the CF lung to bacterial colonisation. This feature can be observed form early infancy in CF patients; the lungs of neonates with CF being histologically normal (Warner, 1992). Furthermore, a recent study has shown the apical membrane of epithelial cells in CF to be undersialylated. This has been proposed as a contributory factor in the pathogenesis of airway infection (Imundo *et al.*, 1995).

1.1.1 Microbiology of Cystic Fibrosis Lung Infections.

As outlined above, various histopathologic changes in the lungs of CF patients appear to facilitate microbial colonisation. This can include bacteria, fungi and viruses. However, a surprisingly narrow variety of species are most frequently involved. The predominant viral pathogen is the respiratory syncitial virus (RSV). *Aspergillus* spp. are the prevailing fungal pathogens. These infective agents may have a role in the degeneration of CF lung function which is not fully appreciated (Gilligan, 1991). The major bacterial pathogens include *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia* (formerly *Pseudomonas*) *cepacia*. Less common bacterial pathogens include *Mycobacterium* spp., *Stenotrophomonas* (formerly *Xanthomonas*) *maltophilia* and *Legionella pneumophila* (Gilligan, 1991; Govan & Nelson, 1993).

Bacterial infection in the CF lung frequently, but not without exception, follows a recognisable pattern which is well documented (Govan & Nelson, 1992). Typically, *S. aureus* colonisation occurs in infancy and *H. influenzae* infections in early childhood. Chronic colonisation with *P. aeruginosa* occurs at approximately 8 years of age (Roussey *et al.*, 1994). Mucoid, alginate-producing strains of this latter pathogen are recognised as a significant contributor to the deterioration of pulmonary function in CF patients.

The advent of colonisation with each of these species is postulated to evoke pulmonary damage via the consequences of both the infection itself, and the inflammatory-immune response of the host to that species. This damage to the already physiologically compromised CF lungs may permit colonisation by the next species in the chronological

list above (Marelich & Cross, 1996). However, the treatment of *S. aureus* infection is not without controversy: some studies have indicated that prolonged antistaphylococcal therapy may yield an increased incidence of *H. influenzae* and *P. aeruginosa* and result in a poor prognosis (Govan & Nelson, 1992).

Pulmonary infection in CF currently occurs in a cycle of alternating relative exacerbation and remission, with each cycle resulting in a further decrease in lung function. Typical progress of lung disease in CF is outlined in figure 1.1 Paradoxically, a small minority of CF patients appear less susceptible to pulmonary infection and subsequent decline in lung function. This group enjoys a better prognosis than the rest of the CF population (Gilligan, 1991).

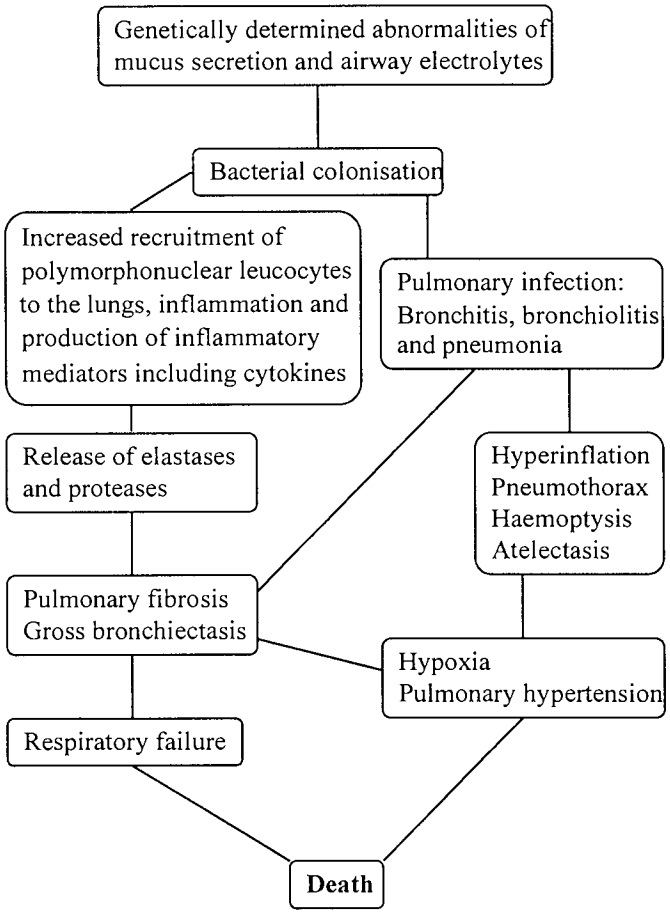


Figure 1.1 Progress of lung disease in Cystic Fibrosis.
(Adapted from Hodson & Warner, (1992).)

Additionally, some patients become colonised with *B. cepacia*, a rare pathogen in humans that is mostly nosocomial in origin and usually colonises those with a compromised immune status. Those in early adulthood appear particularly vulnerable. The outcome of such colonisation is variable, ranging from chronic asymptomatic carriage in the lungs to *B. cepacia* syndrome, a rapid and ultimately fatal decline in clinical status via necrotising pneumonia and septicaemia (Govan & Nelson, 1992; Isles *et al.*, 1984).

1.2 *Burkholderia cepacia.*

1.2.1 Taxonomy of the Genus *Burkholderia.*

Pseudomonas spp. consists of phylogenetically unrelated groups of proteobacteria which share a comprehensive metabolic capacity (Rodley, Römling & Tümmler, 1995). These were classified by analysis of a relatively small number of strains into 5 groups by homologies within their rRNA sequences (Palleroni *et al.*, 1973). These groups are shown in figure 1.2. Group I contains the fluorescent pseudomonads, amongst which are *P. aeruginosa* and *Pseudomonas fluorescens*. This group is occasionally referred to as *Pseudomonas* sensu stricto as they are considered to be ultimately the "genuine" pseudomonads (Urakami *et al.*, 1994; Li *et al.*, 1993). Group II contains species noted for their extreme metabolic flexibility and phytopathogenic capability. Group III and Group IV contain further groups of (largely environmental) pseudomonads. Group V contains species that have since been transferred to *Stenotrophomonas* spp. and *Xanthomonas* spp. Detailed descriptions of these groups and the species within them can be found in the literature (Palleroni, 1984).

It is was suggested over 20 years ago that each of the homology groups still remaining within *Pseudomonas* spp. may in the future be transferred to a novel genus (Palleroni *et al.*, 1973). Since this suggestion, many novel genera have been proposed, including *Comamonas*, *Stenotrophomonas*, *Sphingomonas*, *Acidovorax*, *Telluria*, *Brevundimonas*, *Hydrogenophaga*, *Brevundimonas* and *Burkholderia* (Ochi, 1995). The redesignations assigned to date may well prove to be only the first steps in a long-overdue rationalisation of the genus *Pseudomonas*.

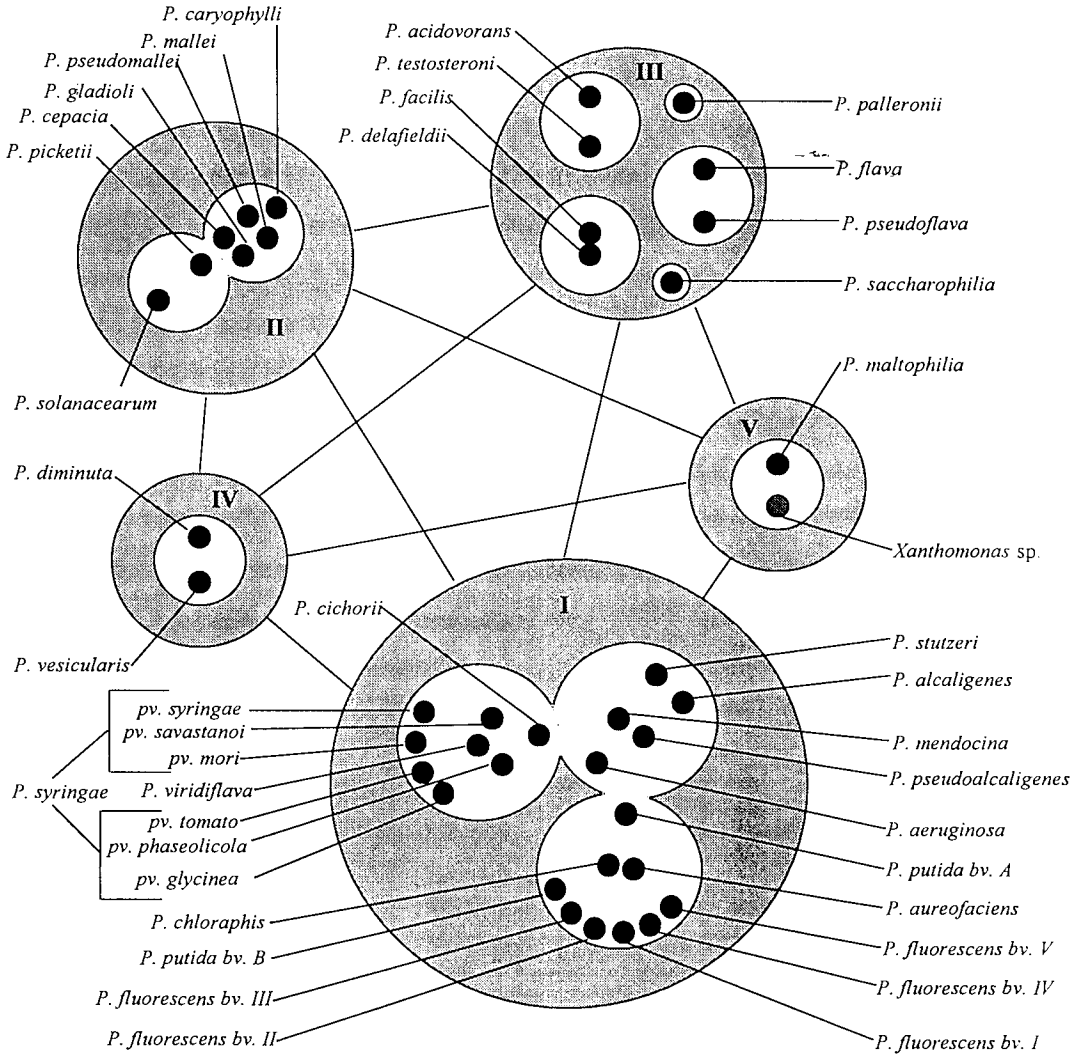


Figure 1.2 Some significant *Pseudomonas* species, biovars and pathovars, arranged according to rRNA and DNA homologies. The shaded circles represent rRNA homology groups and within these the white circles represent DNA homology groups. The Roman numerals refer to the rRNA homology groups as summarised in the text. This shows the variety of species that have at some time been classified under this broad genus, many of which have since been assigned newly created genera. (Adapted from Palleroni *et al.*, (1973).)
pv = pathovar *bv* = biovar

A study of the 16S rRNA sequences, DNA-DNA homology, lipid and fatty acid characteristics of seven members of *Pseudomonas* spp. RNA homology group II led to a proposal for the establishment of a new genus, *Burkholderia*, of which *B. cepacia* was proposed as the type species (Yabuuchi *et al.*, 1992). It is usually accepted that a valid proposal of a new combination be made via *International Journal of Systematic*

Bacteriology (Swings & Hayward, 1990) and the combinations proposed by Yabuuchi *et al.* were subsequently validated by publication in that source (Anonymous, 1993).

The original proposal of the genus *Burkholderia* included only one laboratory strain from each candidate species (Yabuuchi *et al.*, 1992). However, sufficient data exists in the literature to support this reclassification (Stead, 1992; Li *et al.*, 1993).

The seven members initially described (*B. cepacia, Burkholderia gladioli, Burkholderia pseudomallei, Burkholderia mallei, Burkholderia solanacearum, Burkholderia pickettii and Burkholderia caryophylli*) have since been joined by the new combinations *Burkholderia plantarii* and *Burkholderia glumae* (Urakami *et al.*, 1994), *Burkholderia cocovenenans* (Zhao *et al.*, 1995), *Burkholderia andropogonis* (Gillis *et al.*, 1995). These were all formerly members of the genus *Pseudomonas*. The new species *Burkholderia vandii* (Urakami *et al.*, 1994) and *Burkholderia vietnamiensis* (Gillis *et al.*, 1995) have also been proposed. Figure 1.3 shows the dendrogram from this latter study. At the time of writing, this is the most comprehensive taxonomic study on the genus *Burkholderia* in the literature.

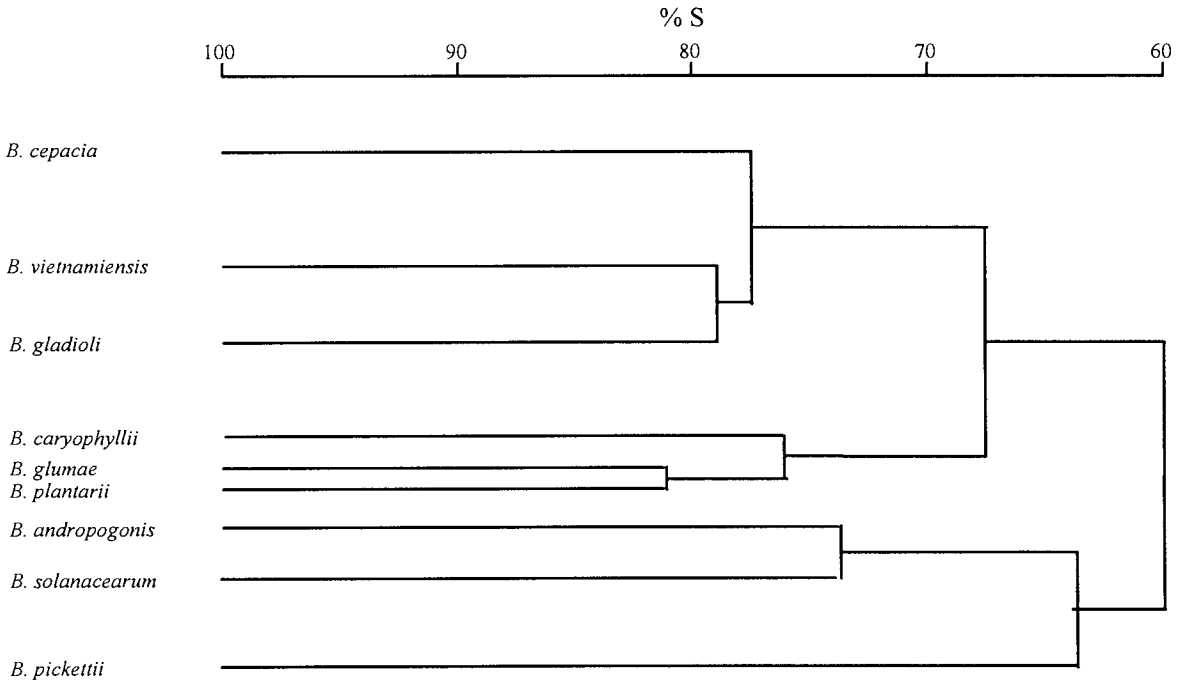


Figure 1.3 Dendrogram obtained from an unweighted average pair group cluster analysis of Dice similarity coefficients of strains from the genus *Burkholderia*. (Adapted from Gillis *et al.*, (1995).)

Doubt has been thrown on the inclusion in this genus of *B. pickettii* and *B. solanacearum*: these may represent one or more separate genera (Gillis *et al.*, 1995). It is notable that these two species are known to occupy a different DNA homology group (figure 1.2) and were in fact not originally included in rRNA homology group II (Palleroni, 1984).

1.2.2 *Burkholderia cepacia.*

B. cepacia is a Gram-negative, motile, aerobic rod (Holmes, 1986). It was first identified as the phytopathogen involved in onion rot (Burkholder, 1950). A variety of pigments have been observed (Richard *et al.*, 1981). The name *B. cepacia* (via *P. cepacia*) is currently considered synonymous with the now defunct *P. multivorans* and *P. kingii* (Palleroni & Holmes, 1981). Doubt has recently been cast on the validity of this (Yohalem & Lorbeer, 1994a). *B. cepacia* has proved to be somewhat less prevalent in the environment than previously considered (Butler *et al.*, 1995; Mortensen, Fisher & LiPuma, 1995).

As *P. cepacia*, the name was first validly published in 1950 (Burkholder, 1950) but inexplicably lost its validity via omission from the Approved List of Bacterial Names in 1980 (Skerman, McGowan & Sneath, 1980). The name was revived the following year (Palleroni & Holmes, 1981).

B. cepacia has been found in two separate studies to bear three chromosomal replicons of between 3.65 and 0.9 Mb in size and a total genome size of approximately 8 Mb Cheng & Lessie, 1994; Rodley *et al.*, 1995). Other studies have shown that it is not obligatorily clonal (i.e. it is capable of extensive genetic rearrangement) and that environmental populations display extensive genetic mixing (Wise, Shimkets & McArthur, 1995). The carriage of a large quantity of genetic material is phenotypically expressed in part by a remarkable nutritional versatility, as has been noted from the earliest descriptions of the species (Burkholder, 1950; Stanier, Palleroni & Doudoroff, 1966). This, and its ability to antagonise a range of plant pathogenic fungi has led to the proposal for the use of genetically manipulated forms as biocontrol agents (Oedjijono, Line & Dragar, 1993).

B. cepacia possesses an innate resistance to disinfectants and antibiotics, thus making it a potentially dangerous organism (Holmes, 1986). It can grow in distilled or deionised water (Gelbart, Reinhardt & Greenlee, 1976), utilise penicillin G as a carbon source (Prince *et al.*, 1988), and has been isolated from the water system of a space shuttle orbiter due to growth in the disinfectants used (Pyle, Watters & McFeters, 1994). Thus it can be seen that eradicating *B. cepacia* is extremely difficult under many circumstances.

1.2.3 Clinical Significance of *Burkholderia cepacia.*

B. cepacia is an opportunistic pathogen in humans, mainly in the immunosuppressed. Reports exist in the literature of infections against a background of chronic granulomatous disease (CGD) (Lacy *et al.*, 1993) and, perhaps unsurprisingly, human immunodeficiency virus (HIV) infection (Verghese *et al.*, 1994). *B. cepacia* has also been reported as causing a septicaemia in patients with burns (Brauner *et al.*, 1985), as infecting wounds (Bassett, Stokes & Thomas, 1970) and as causing brain abscesses and temporal bone infection secondary to chronic suppurative otitis media with no other pre-existing medical conditions of note (Hobson, Gould & Govan, 1995). Nosocomial outbreaks have been recorded subsequent to the inadvertent contamination of various reusable apparatus, such as electronic ventilator temperature probes (Spencer, 1995).

In CF, pulmonary infection with *B. cepacia* can result in clinical outcomes ranging from asymptomatic carriage to a rapid fatal deterioration as mentioned above. It has also been reported as infecting the temporal bone subsequent to causing otitis media (Dettelbach, Hirsch & Weissman, 1994). When lung function in CF has deteriorated to end-stage, there is little option but to perform double lung or heart lung transplantation, which is invariably accompanied by immunosuppressive therapy to prevent host rejection of the transplant (Whitehead *et al.*, 1991). However, in those patients colonised with *B. cepacia*, there is the added risk of a potentially untreatable infective complication (Noyes *et al.*, 1994). Lung transplantation in CF patients carries an increased risk potential but can improve quality of life and survival time (Egan *et al.*, 1995). From the above, it can be seen that colonisation with *B. cepacia* constitutes an additional risk. However, transplantation does not appear to carry a risk of new *B. cepacia* infection arising (Steinbach *et al.*, 1994). It has been suggested that carriage of *B. cepacia* should be considered an adverse risk factor on a relative basis only

and the decision to transplant be made with respect to co-existing risk factors (Egan *et al.*, 1994).

Treatment of infection with *B. cepacia* is lengthy and costly (Hobson *et al.*, 1995). A satisfactory outcome is not always reached. *B. cepacia* is an exceedingly durable and adaptable organism and the speculation of events should it adapt to infect immunocompetent individuals is worrying (Hobson *et al.*, 1995).

1.2.4 *Burkholderia cepacia*: Virulence Factors.

The subject of virulence factors in *B. cepacia* was recently reviewed in the literature (Nelson *et al.*, 1994). Some factors of major significance are summarised here. *B. cepacia* has been shown to be less virulent that *P. aeruginosa*, but still appears capable of colonising patients with little inter-species assistance (Nelson *et al.*, 1994).

1.2.4.1 Adherence.

Studies with CF and non-CF respiratory tract cell cultures have demonstrated no significant difference in the binding capabilities of *B. cepacia* to either. The same study showed a correlation between the absence of pili and a reduced frequency of cell adhesion (Cervin *et al.*, 1994). Pili are also of significance in the ability of *B. cepacia* to bind CF mucus glycoproteins (Sajjan *et al.*, 1995). The deduction of the nucleotide sequence of the major subunit pilin gene, the structure of the 17.3kDa aggregate of the assembled fibres, and the identification of the associated mucin-binding adhesin have all been recent developments (Sajjan *et al.*, 1995). This showed the pili of *B. cepacia* to be of a novel morphology lacking in homology with the corresponding structure from *P. aeruginosa* (Sajjan *et al.*, 1995). The adhesin was shown to be present along the length of the mesh-like cabled pilin fibres, maximising mucus network binding potential (Sajjan *et al.*, 1992). The presence of such cable pili has been proposed as an epidemiological marker but further work is required to substantiate this (Sajjan *et al.*, 1995). Furthermore, the same group has demonstrated the presence of 5 morphologically discrete pilus types in *B. cepacia* (Goldstein *et al.*, 1995). This led to the proposal that selection-driven horizontal transfer of pilus operons occurs, permitting the acquisition of the most environmentally appropriate structure (Goldstein *et al.*, 1995).

Additionally, a second adhesion receptor, thought to be lipid in nature, has been reported. This seems to function as the major cell surface binding receptor in sparsely-piliated and non-piliated strains (Sylvester, Sajjan & Forstner, 1996).

1.2.4.2 Other Cell Surface Characteristics.

The apparent molecular masses of the major outer membrane proteins (OMP) of *B. cepacia* are 36kDa, 24.5kDa, 17kDa and 14.5kDa (Gotoh *et al.*, 1994). Of these, the first and last are heat-modifiable (Anwar *et al.*, 1983). An 81kDa diffusion pore protein in the outer membrane of *B. cepacia* has been shown to dissociate into two subunits: a major 36kDa protein as mentioned above and a minor 27kDa component (Gotoh *et al.*, 1994). The major OMP components vary according to available nutrients (Anwar *et al.*, 1983).

Previous reports have concluded that the outer membrane of *B. cepacia* is an important contributor to its innate antibiotic resistance, providing a permeability barrier to the entry of many classes of antimicrobial (Moore & Hancock, 1986). However, a recent major development has been the identification of an outer membrane lipoprotein that appears to have a significant role in multiple antibiotic resistance and behaves as an antibiotic efflux pump (Burns *et al.*, 1996). This discovery may alter many previously held beliefs pertaining to the antibiotic resistance of this species.

B. cepacia lipopolysaccharide (LPS) shows an endotoxic response and cytokine response greater than that demonstrable with the corresponding component from *P. aeruginosa* (Shaw, Poxton & Govan, 1995). Earlier studies had previously shown an exocellular toxic complex resembling LPS to cause considerable pathological changes to the lungs when introduced into the lungs of rats (Straus *et al.*, 1988). The cytokine response has been proposed as a contributing factor in the processes that cause lung damage associated with *P. aeruginosa* colonisation in CF (Greally *et al.*, 1993). This may assist in explaining the precipitous decline in clinical status of some CF patients colonised with *B. cepacia*. Additionally, there appears to be a preponderance of *B. cepacia* isolates in CF with rough LPS as opposed to smooth in some centres (Govan *et al.*, 1993). Antibodies for *B. cepacia* LPS (IgG in serum and IgA in sputum) may be useful in a diagnostic sense due to an apparent inter-strain core LPS heterogeneity and no cross-reactivity with the corresponding component of *P. aeruginosa* (Nelson *et al.*, 1993). However, this response appears incapable of eliminating the colonising organism from the CF lung (Nelson *et al.*, 1993).

Components of the cell surface have been proposed as targets for immunotherapy (Burnie *et al.*, 1995). Possession of immunoglobulin G (IgG) antibody against a 30kDa porin protein has been suggested as providing a significantly better prognosis in *B. cepacia* colonisation of the CF lung, whereas other anti-OMP IgGs were not necessarily so (Burnie *et al.*, 1995). Other studies have shown that much of the IgG response to *B. cepacia* OMP is specific (Lacy *et al.*, 1995). Anti-30kDa-*B. cepacia*-OMP immunotherapy has been suggested as potentially beneficial in CF. However, whether this is the case remains to be seen.

1.2.4.3 Siderophore Production.

Siderophore production permits bacterial sequestration of iron from host-produced iron chelating proteins such as lactoferrin and transferrin. The necessity of this in the establishment and maintenance of host colonisation is widely accepted. *B. cepacia* produces four known chelators of iron. These include pyochelin, azurechelin (the most widespread amongst strains of this species) and cepabactin (Nelson *et al.*, 1994). Pyochelin from *B. cepacia* is identical to the pyochelin produced by *P. aeruginosa* (Sokol, 1986). Azurechelin has been identified as salicylic acid (Visca *et al.*, 1993). Recently, the production of ornibactins (peptide structures lacking a chromophore) was demonstrated in some *B. cepacia* strains (Meyer *et al.*, 1995). Further studies have indicated the presence of an hydroxamate-like siderophore distinct from the cepabactins and similar to the ornibactins in clinical isolates of *B. cepacia* (Tabacchioni *et al.*, 1995).

1.2.4.4 Lipase Production.

A study of 48 CF isolates of *B. cepacia* found 69% to produce lipase (McKevitt & Woods, 1984). Lipase production was subsequently shown to have no direct cytotoxic effect on HeLa cells *in vitro*, nor any toxicity after intravenous administration to healthy mice (Lonon, Woods & Straus, 1988). Further investigation demonstrated the potential of *B. cepacia* lipase to inhibit phagocytic function in rat alveolar macrophages (Straus, Lonon & Hutson, 1992). This, and the potential for lipase-mediated disruption of lung processes, such as the degradation of phospholipid lung surfactant, indicates a potentially significant role for *B. cepacia* lipase in virulence.

Other studies have shown that CF strains of *B. cepacia* have considerable lipolytic activity in general, but are often lacking in C14 lipase activity (Gessner & Mortensen, 1990). The significance of this is unclear.

1.2.4.5 Other Exocellular Products.

Caseinase, gelatinase, alginase, mucinase, albuminase, trypsin, chymotrypsin, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-fucosidase have all been detected from *B. cepacia*. Of these enzymes, CF isolates appear to produce alginase and trypsin to a significantly lesser extent than *B. cepacia* at large (Gessner & Mortensen, 1990).

Haemolysin is produced by some strains, but studies have produced quite variable results. For example, McKevitt and Woods found only 1 haemolytic strain in 48 investigated (McKevitt & Woods, 1984), whereas Gessner and Mortensen found evidence of some haemolytic activity in 26% of 98 CF isolates of *B. cepacia* using human erythrocytes (Gessner & Mortensen, 1990). No toxin A or exoenzyme S activity has been observed with *B. cepacia* (McKevitt & Woods, 1984). Isolates of *B. cepacia* are reported as showing no direct cytotoxicity to *in vitro* cell preparations (Gessner & Mortensen, 1990).

Exocellular polysaccharide (EPS) production *in vitro* by clinical strains of *B. cepacia* isolated from CF patients does not appear to correlate with ability to colonise the respiratory tract (Nelson *et al.*, 1994). However, studies with laboratory strains of *B. cepacia* have shown significant variations in EPS production with growth conditions (Allison & Goldsbrough, 1994). EPS can form a diffusion barrier and confer an apparent resistance to the local bacterial population. Various chemical characteristics of EPS, such as rhamnose content, acetate content and molecular weight, can affect the rheological properties of the polymer produced. This can have a bearing on the efficiency of the EPS in conferring protection against antibiotics (Allison & Goldsbrough, 1994).

Addition of cell-free exocellular material from strains of *P. aeruginosa* to a culture of *B. cepacia* showed a rise in siderophore, lipase and protease production in proportion to the quantity (McKenney, Brown & Allison, 1995). The occurrence of these particular virulence factors may be of unknown importance in CF lung colonisation with *B. cepacia*, but it raises the possibility of interspecies effects of a virulence-potentiating manner in the CF lung.

1.2.4.6 β-Lactamases.

β-lactamases have been isolated from *B. cepacia,* but these are primarily inducible penicillinases (Hirai *et al*, 1980; Prince *et al*, 1988). Reports have suggested that *B. cepacia* can hydrolyse the carbapenem antibiotic imipenem in a manner reversible by the addition of the penem β-lactamase inhibitor BRL 42715. This suggests that resistance to carbapenems may at least in part be due to the production of a carbapenemase. This was also the case with meropenem (Simpson, Hunter & Govan, 1995). Further investigation of this phenomenon may be useful in ensuring the successful use of carbapenems against *B. cepacia.*

1.2.4.7 Differences Between Botanical and Clinical Isolates.

Tests have shown that isolates from botanical and clinical sources differ markedly in their ability to produce certain virulence factors, such as siderophore synthesis and protease production. Differences in uroepithelial cell adhesion were also demonstrated (Bevivino *et al.*, 1994). Further molecular studies by the same group have demonstrated an apparent divergence between botanical and clinical isolates (Tabacchioni *et al.*, 1995). Thus it was proposed that botanical isolates of *B. cepacia* may have a very limited capability to behave pathogenically in humans (Bevivino *et al.*, 1994). However, these studies only included four isolates (two of both clinical and botanical origin). As the virulence factors studied have no defined role in CF lung infections as yet, it would seem imprudent to suggest the use of *B. cepacia* as a biocontrol agent at the present time (Butler *et al.*, 1995). Indeed, some epidemiological studies have concluded that despite compelling evidence for person-to-person transmission of *B. cepacia*, the environmental reservoir of *B. cepacia* beyond the clinical environment still plays an important role in the infection of the CF population (Cazzola *et al.*, 1996). Furthermore, pulmonary exposure of healthy mice to a genetically engineered *B. cepacia* strain has been shown to result in the persistence of that strain for between 2 days and 1 week, varying between physiological sites. During this time a considerable impact on the intestinal flora was noted. This may have a deleterious affect on the protective capacity of the intestinal microbe population, causing an indirect negative effect on health on top of any direct pathogenic capabilities (George *et al.*, 1993).

Additionally, a study of 59 strains of *B. cepacia* from a variety of clinical and botanical sources by multilocus isoenzyme diversity attributed less than 2% of the observed genetic diversity to differences between the clinical and environmental subpopulations (Yohalem & Lorbeer, 1994a). Phytopathogens were absent from the clinical subpopulation (the converse was not necessarily true). The study failed to resolve electrophoretic type clustering observed in terms of strain origin and therefore reasonably concluded that the present species concept of *B. cepacia* is too broad (Yohalem & Lorbeer, 1994a). It would appear that no consensus will be forthcoming on this point until this species has been investigated taxonomically in more detail.

1.2.5 *Burkholderia cepacia* in Cystic Fibrosis.

An ominous trend amongst CF patients over the last two decades or so has been the increased incidence of *B. cepacia* infection. Since the first reported isolation from a CF patient in Philadelphia in 1977, *B. cepacia* has emerged as an opportunistic pathogen in the CF lung (Stableforth & Smith, 1994). By the mid-1980s, colonisation had reached 40% in some North American clinics (Govan & Nelson, 1993), and studies had confirmed the increased mortality risk arising from colonisation with *B. cepacia* (Stableforth & Smith, 1994).

The first reported death of a UK CF patient from *B. cepacia* was in 1986 (Glass & Govan, 1986). The carriage frequency of this opportunistic pathogen amongst the CF population in the UK has also peaked at approximately 40% in many affected centres (Govan & Nelson, 1993). The variability in response to this infection has been mentioned above. Different centres report between 20% and 40% of those infected to experience *B. cepacia* syndrome (Stableforth & Smith, 1994; Spencer, 1995).

The involvement of *B. cepacia* and *P. aeruginosa* in CF lung infections has been reviewed in recent months by Govan and Deretic (Govan & Deretic, 1996).

1.2.5.1 Epidemiological considerations.

Due to an apparent inability to rid the CF lung of *B. cepacia* colonisation in many cases, prevention of infection must comprise a major component of the battle against this organism. The sources of infection and their contribution to *B. cepacia* carriage in the CF

community are therefore of significant interest. Retrospective case control studies have indicated several factors that increase the likelihood of *B. cepacia* infection in CF. These include the severity of pulmonary disease, increasing age, the presence of an infected sibling and hospitalisation within the last 6 months (Gilligan, 1991). The latter two points, taken together, point towards person-to-person transmission of *B. cepacia* infections in CF.

Molecular analyses of *B. cepacia* isolates in CF have been largely responsible for the substantiation of epidemiological theories. It is now widely accepted that the transmission of *B. cepacia* from patient to patient is extremely important in CF (Govan *et al*, 1993). Techniques such as multilocus enzyme electrophoresis (MEE) (John *et al.*, 1994), pyrolysis mass spectrometry (PMS) and whole cell sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Corkill *et al.*, 1994b) have proved of use in the study of *B. cepacia* epidemiology in CF. However, the greatest weight of data have been provided by genetic typing techniques such as ribotyping and various methods of pulsed-field gel electrophoresis (PFGE). Data from both these techniques largely support the theory of person-to-person transmission of *B. cepacia.*

1.2.5.1.1 Pulsed-Field Gel Electrophoresis.

PFGE electrophoretically separates the DNA fragments that result from the digestion of chromosomal DNA using a carefully selected restriction (cutting) enzyme. This produces a pattern of bands in an agarose gel that, when visualised using ethidium bromide and ultraviolet (UV) light, and analysed, is reproducibly characteristic of a single bacterial strain within a species.

The maximum DNA fragment size separable by conventional linear-current forms of electrophoresis is about 50 kb. This limit is raised by the introduction of a pulse or direction change in the applied electric field, a procedure which at the molecular level results in successive reorientations in the DNA molecule during its passage through an agarose gel. The speed of reorientation is proportional to DNA fragment size, and this provides the basis for the orderly separation of fragments (Kaufmann & Pitt, 1994).

Various methods permitting the application of a reorienting pulse have been developed. Arguably the greatest improvement of PFGE was achieved by the development of the contour-clamped homogeneous electric field (CHEF) technique. This uses electrostatic principles to apply the correct voltages required to generate an homogeneous electric field with three pairs of electrodes set at angles of 120° to each other. This permits the separation of molecules of up to 7000 kb (Chu, Vollrath & Davis, 1986).

The choice of restriction enzyme is critical to the success of PFGE. A suitable enzyme should produce a range of fragment sizes that can be separated successfully. The fragments produced should be of a number that makes analysis practical. Thus, an enzyme that cuts fairly rarely in the genome of interest is essential. Literature searches may well yield the identity of usable enzymes, but otherwise a process of trial and error in selection will result.

In addition, PFGE requires high-quality non-sheared DNA for analysis: standard extraction techniques produce DNA fragments in the order of 500 kb. This is insufficient for PFGE. Suitable DNA can be obtained by first immobilising a suitable quantity of cells in an agarose block of sufficient concentration to permit the free passage of preparative solutions whilst preventing DNA shearing and maintaining sufficient mechanical strength for ease of handling. Such agarose blocks, containing prepared DNA, can be used as stocks. Suitably-sized slivers can be taken from "master blocks" and subjected to PFGE.

Important variable factors in the running of a CHEF gel involve temperature control, the concentration of agarose employed, pulse times, field strength and total running time. Successful marriage of these variables is essential. The effects of each feature mentioned have been adequately described in the literature (Kaufmann & Pitt, 1994). Briefly, temperature must be constant and low to ensure optimum resolution and a constant fragment mobility; field strength must be optimised to permit rapid DNA fragment separation whilst maintaining an acceptable resolution and agarose concentration must be optimised to provide maximal band clarity with minimal adverse effect on fragment mobility. Pulse times must be varied to permit optimal fragment separation (longer pulse times generally favour the movement of fragments up to a larger size) and "ramped" (i.e. increased in time during a run) in a manner that maximises resolution.

The banding patterns thus produced are commonly subjected to pairwise comparison and the results displayed numerically in terms of a coefficient of relatedness (such as a Euclidean distance) or graphically in a dendrogram constructed via comparison of such coefficients (Pitt, 1994) and the result has both epidemiologic and taxonomic applications (Pitt *et al.*, 1996; Grothues & Tümmler, 1991). Guidelines have been proposed for the interpretation of the DNA restriction patterns yielded by PFGE (Tenover *et al.*, 1995).

1.2.5.1.2 Ribotyping.

Ribotyping is based on the analysis of the section of the bacterial genome that contains the ribosomal ribonucleic acid (rRNA) genes, the *rrn* operon. This portion of the bacterial genome contains structural information that appears to be highly conserved in the eubacterial kingdom (Bingen, Denamur & Elion, 1994).

The *rrn* operon is a polycistronic transcription unit coding for the 3 rRNA species (23S, 16S and 5S) present in equimolar quantities in the 70S bacterial ribosome These are co-transcribed as a 30S rRNA precursor that matures into the 23S, 16S and 5S rRNA molecules. It is unique insofar as it is present in multiple copies in the bacterial genome: *B. cepacia* ATCC 25416, for example, possesses 6 *rrn* operons within its genome (Rodley *et al.*, 1995). The potential discriminating power of ribotyping rises in proportion to the number of *rrn* operons present in the genome. Such genes are arranged with the section coding for 16S rRNA and the section coding for 5S rRNA towards the 5' and 3' ends respectively, with that coding for 23S rRNA towards the centre. Between these coding regions lie non-coding intergenic spacer segments. The whole structure is in the order of 7kbp in size (Bingen, Denamur & Elion, 1994).

The 16S to 23S intergenic spacer region and the 3' flanking regions are of particular importance as they display great heterogeneity due to reciprocal recombination in the course of evolution. They contain several defunct transfer ribonucleic acid (tRNA) genes and several direct repeat sequences. The 16S and 23S rRNA sequences vary little amongst prokaryotes, with a considerable homology between various bacterial species. Indeed, it was noted that ^{32}P-labelled *E. coli* rRNA, used as a probe in a range of different Gram-positive and Gram-negative species, reacted even with species phylogenetically remote to *E. coli*. Also, frequent cutting restriction enzymes such as *Eco*RI could be expected to cut

in such a way that different lengths of low-interspecies-homology intergenic spacer are left attached to lengths of high-interspecies-homology rRNA coding sequence. For these reasons, it was proposed that *E. coli* rRNA has uses as a universal probe to study restriction patterns for taxonomic purposes (Grimont & Grimont, 1986).

This universal probe concept has applications in the study of epidemiology and was subsequently developed for that use. The name ribotyping resulted from this epidemiological development. (Stull, LiPuma & Edlind, 1988). Figure 1.4 displays a schematic representation of an outline method for this technique. The banding pattern produced by ribotyping contains both species-specific and strain-specific components. (Bingen, Denamur & Elion, 1994). Thus, ribotyping has a potential role in identifying species as well as a role in strain typing studies.

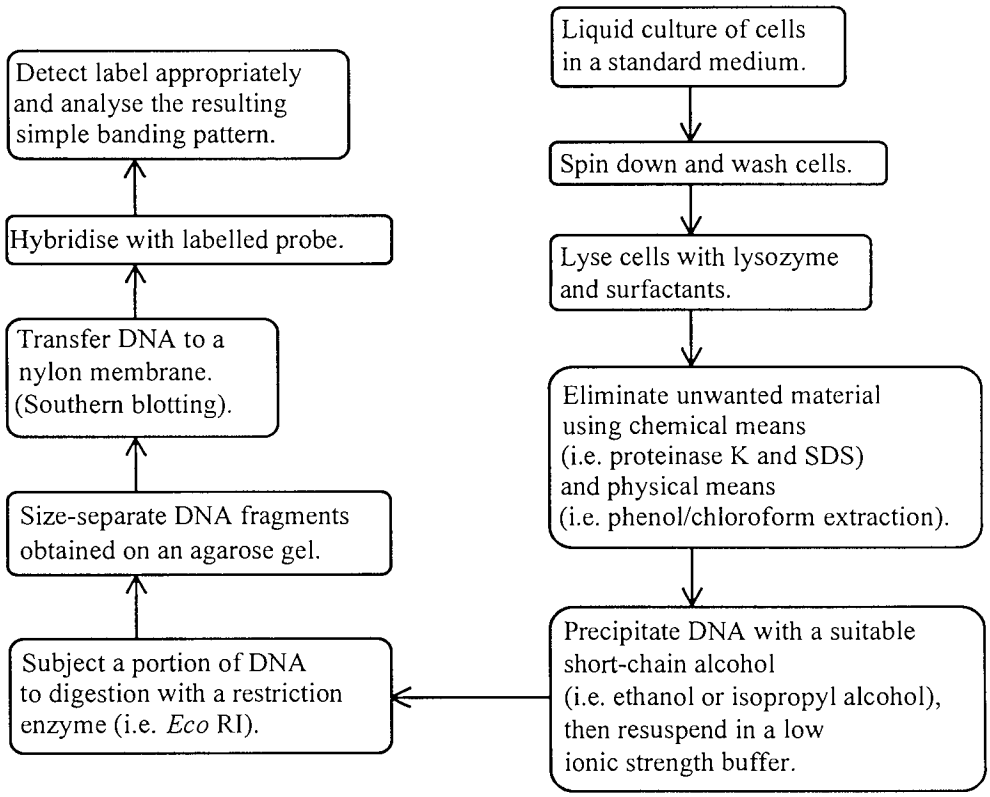


Figure 1.4 An outline schematic representation of ribotyping.

Ribotyping has been attempted in conjunction with polymerase chain reaction (PCR). PCR comprises the extension of two oligonucleotide primers by a thermostable DNA polymerase towards each other. This produces elongation of one primer complimentary to the template (genomic) DNA and this in turn produces a new template for the second primer. This results in a chain reaction that extends the double-stranded DNA product, with twice the quantity produced in each successive cycle. Sufficient product for any application is produced. Theoretically, only one template molecule is required for initiation (Lenstra, 1995). Thus, less starting material is required, a great asset when investigating a fairly slow-growing organism such as *B. cepacia*. One can make primers from the 3' end of the 16S rRNA portion of the *rrn* operon and from the 5' end of the 23S rRNA portion of the *rrn* operon. This way, one can amplify the important 16S to 23S intergenic spacer region for investigation. This is PCR-ribotyping (Dasen *et al.*, 1994).

Additional PCR-based techniques for molecular epidemiological analysis include enterobacterial repetitive intergenic consensus (ERIC)-PCR, which uses PCR primers derived from conserved, palindromic repeated sections of the bacterial genome away from the *rrn* operon and arbitrarily primed (AP)-PCR, which uses an arbitrary primer. These techniques discriminate between types by the pattern of PCR products obtained from the primers used (Liu *et al.*, 1995; Liu *et al.*, 1996).

Extended ribotyping uses one of several restriction enzymes to disassemble the genomic DNA prior to electrophoretic separation. This can increase the discriminatory power of the technique (Rozee *et al.*, 1994).

1.2.5.1.3 Comparison of Pulsed-Field Gel Electrophoresis and Ribotyping.

A broad-based study assessing the potential of various typing systems for *B. cepacia* in CF concluded that ribotyping appeared to be the most useful technique of those tested (Rabkin *et al.*, 1989). However, PFGE was not included in this comparison.

Ribotyping requires none of the careful DNA extraction that is required for PFGE. DNA is extracted by the standard technique of phenol extraction and ethanol precipitation (Sambrook, Fritsch & Maniatis, 1989). Likewise, little need for careful restriction enzyme selection is necessary. Special apparatus for separating the digested DNA is not required either: a standard flatbed electrophoresis tank will suffice. For hybridisation with a labelled rRNA probe, DNA is blotted onto nylon membrane by a well established technique

(Southern, 1975). Detection of hybridised fragments is possible via autoradiography or chemiluminescence, also established techniques. Therefore much of the apparatus and expertise required for this technique probably already exists in many laboratories. Additionally, the banding pattern that results is fairly simple to that gained from PFGE and is therefore more straightforward to interpret.

PFGE can be criticised for the time-consuming steps involved in the optimisation of the technique, such as the preparation of sample master blocks containing a suitable DNA loading and the selection of suitable restriction enzymes and running conditions. The apparatus used for gel running is quite expensive and specialised, and may not find other uses in a clinical laboratory. Additionally, sample preparation and running times are lengthy and the banding patterns gained for analysis are fairly complex.

Recently, a rapid method for the preparation of bacterial DNA for PFGE was described. This shortens the time required to prepare the master blocks to under 5 hours (Matushek, Bonten & Hayden, 1996).

A drawback of ribotyping is that it exclusively detects a section of the bacterial genome that is of dubious functional significance, ignoring the remainder. Whilst it is not impossible that this is irrelevant, PFGE analysis is much more encompassing of any bacterial genome to which it is applied and hence may be useful to detect more subtle yet significant banding pattern changes. Although time-consuming, optimisation of the technique only need be conducted in full once, and a careful search of the literature may well shorten this stage appreciably. It must also be said that whilst execution of the procedure might be lengthy, it is not especially taxing. Therefore, although PFGE is arguably more complex, the additional time investment is rewarded in the return of a more detailed result. Ribotyping would currently appear ideal for clinical use and PFGE for more detailed laboratory research.

AP-PCR and ERIC-PCR would appear to overcome the criticism levelled at ribotyping in terms of the functional relevance of the portion of the genome utilised, but still address only limited portions of the genome in question. ERIC-PCR is reproducible and seems to have slightly greater discriminatory power than ribotyping, but AP-PCR appears more difficult to reproduce (Liu *et al.*, 1995). PCR reactions must be kept scrupulously free of nucleic acid contaminants as these will be potentially just as amenable to amplification as the DNA under examination. This may lead PCR-based techniques to prove more demanding in practice than their non-PCR counterparts. That apart, PCR-

ribotyping carries a mean concordance of 98% with the standard technique and proves less labour-intensive in practice (Dasen *et al.*, 1994).

Ribotyping has been considered to possess a slightly lesser discriminatory power than PFGE (Liu *et al.*, 1995). Other studies have exploited both techniques, with PFGE used to investigate possible subdivisions of ribotypes (Smith *et al.*, 1993).

1.2.5.1.4 Epidemiology of *Burkholderia cepacia* in Cystic Fibrosis.

In practice, ribotyping and PFGE tend largely to give supporting results. A great weight of data suggests that *B. cepacia* is transmitted from patient to patient in CF. Many patients at any given clinical centre harbour the same strain of *B. cepacia* (LiPuma *et al.*, 1990; Anderson *et al.*, 1991; Govan *et al.*, 1993; Ouchi *et al.*, 1995; Pitt *et al.*, 1996). This is countered by one study that found otherwise (Steinbach *et al.*, 1994).

Practical substantiation of the possibility for person-to-person transmission arises from experiences involving the introduction of segregative policies in clinical centres, which has been documented to dramatically reduce the number of newly colonised patients identified (Whiteford *et al.*, 1995). Studies have demonstrated the longevity of *B. cepacia* survival in respiratory droplets on environmental surfaces typically found in CF clinics (Drabick, 1996). Importantly, there appears to be variance in the ability of any given strain to remain viable in the clinical environment. This may have significance in the transmissibility of individual strains (Drabick, 1996). However, it appears that environmental surfaces are far from frequently contaminated even if the possibility for this exists as isolation from such surfaces has proved difficult (Gilligan, 1991). Nebulisers have also been implicated in the introduction of CF patients to *B. cepacia* (Hamill *et al.*, 1995; Hutchinson *et al.*, 1996).

Further evidence for the importance of the nosocomial environment in the passage of *B. cepacia* between CF patients is provided from analysis of air samples after occupation of clinic rooms by colonised patients. *B. cepacia* persisted in the room air for up to 45 minutes after vacation by colonised patients and bacterial count was perhaps unsurprisingly higher when patients were coughing (Humphreys *et al.*, 1994). The apparent relative absence of *B. cepacia* in the environment in general also tends to suggest infection from other patients as opposed to the environment (Butler *et al.*, 1995). Ribotyping analysis of isolates taken over time from infected patients shows that most chronically colonised CF

patients are colonised by a single strain. Such isolates' ribotypes are stable over a considerable period of time (LiPuma *et al.*, 1991).

Segregation of infected and non-infected patients in the clinical environment has proved beneficial in limiting the spread of *B. cepacia* between patients (Whiteford *et al.*, 1995). However, considerable data suggests that social contact outside the clinical environment, including presence at CF summer camps, is of greater significance in the spread of *B. cepacia* (Govan & Nelson, 1992; Corkill *et al.*, 1994b; Johnson, Tyler & Rozee, 1994). For this reason colonised CF patients are often segregated from those who are not, both in and out of the clinic. CF summer camps have been seriously curtailed and CF conferences have been conducted on a segregated basis (Walters & Smith, 1993). This has undesirable social consequences, creating a group of effective "microbiological outcasts" within a minority group (Govan & Nelson, 1993). Furthermore, such a move creates anxiety amongst non-colonised patients, who fear the day they may become "*B. cepacia*-positive" (Anonymous, 1992). However, such draconian measures have seemed to achieve their purpose as *B. cepacia* colonisation has fallen dramatically since segregation policies were first instigated (Muhdi *et al.*, 1996). It is perhaps premature to claim a complete end to the epidemic as colonisation may well recur. Given the delayed nature of isolation in some instances (LiPuma, 1994), colonisation may well have attained significant levels before the hazard is obvious.

1.2.5.1.5 *Burkholderia cepacia*: The Epidemic Strain.

Numerous different strains of *B. cepacia* have been isolated from the CF lung. However, most CF patients in most UK centres who are colonised with *B. cepacia* are colonised with one particular strain (Pitt *et al.*, 1996). This epidemic strain is found on both sides of the Atlantic and is the prevailing strain in Ontario, Canada. It was passed between the UK centres and Ontario by social contact via summer camps that formerly led to the congregation of CF sufferers from different geographical locations (Johnson *et al.*, 1994).

This strain is highly transmissible between CF patients, with even brief close contact appearing to permit passage of the strain between individuals (Govan *et al.*, 1993). The spread of this strain between patients and centres has been documented and led to a realisation of the great significance of social contact in the passage of this strain from one CF patient to another (Govan *et al.*, 1993). Whether this strain has increased virulence for

CF patients beyond its superior transmissibility is unclear (Johnson *et al.*, 1994). However, certain prominent phenotypic features are somewhat atypical of *B. cepacia* sensu stricto. Colonies appear dry when grown on solid media, due to the presence of rough LPS (LPS that possesses no polysaccharide O-antigen). This strain also appears to be deficient in C14 lipase. Additionally, a diffusible red/brown melanin-like pigment especially promoted by growth on tyrosine-supplemented media, is produced (Govan, 1993). This latter feature is atypical of *B. cepacia*, which is most commonly described as producing a yellowish or greenish nonfluorescent pigment (Saddler, 1994).

Many isolates of this epidemic strain have been recovered from the lungs of numerous CF patients in the UK. All are apparently of a similar strain type by genetic analyses (Pitt *et al.*, 1996). However, considerable stable variations in phenotypic appearance have been noted between such isolates in many studies. These include variations in antibiotic susceptibility profiles as well as biochemical differences (Pitt *et al.*, 1996; Larsen *et al.*, 1993., Ouchi *et al.*, 1995). Indeed, auxotrophic and prototrophic strains of *B. cepacia* were in one study shown to possess such similar macrorestriction profiles by PFGE analysis to prevent the designation of separate types (Barth & Pitt, 1995). Whilst reports such as these do not in themselves permit one to judge whether most prevalent strains from different countries are the same epidemic strain, they do confirm that in any given locality one strain tends to dominate. They also confirm that differences in phenotypic expression between strains of ostensibly the same genetic type are not uncommon within this species.

In the field of bacterial systematics, the current vogue is to integrate data from many different techniques to permit a consensus approach to the designation of species (Vandamme *et al.*, 1996). This approach is known as polyphasic taxonomy and is considered necessary for the delineation of taxa at all levels (Murray *et al.*, 1990). As a general rule, the species as a taxonomic unit defines a group of strains sharing a DNA-DNA relatedness of 70% or more when hybridisation experiments are conducted under prescribed thermal conditions. Taxonomy could potentially be conducted with rules such as this regarded as absolute, and yield a nomenclature of no practical use. Therefore some compromise is often necessary (Vandamme *et al.*, 1996).

Genomic analysis for the purpose of strain typing is largely structural at present, with little functional input. If a polyphasic approach is necessary for useful taxonomic rationalisation, then one could perhaps argue that the use of any one technique alone for the purpose of assigning the much finer division of strain type is likely at some time to prove unable to detect differences observable in the clinical or phenotypic pictures in a poorly defined species such as *B. cepacia*. A simple extension might be the use of more than one restriction enzyme in a PFGE study and comparison of the patterns produced by statistical means. The heterogeneous nature of the current species definition of *B. cepacia* is discussed in section 1.2.5.2.2.

The subject of strain transmissibility has led to the suggestion that only those amongst the CF population who harbour this epidemic strain may require segregation (Taylor *et al.*, 1994). Such a suggestion may prove premature, as centres globally have reported fatalities from *B. cepacia* carriage. It may merely be that the epidemic strain is the most exquisitely pathogenic and that the next most competently pathogenic strain will take its place in its absence. However, one detailed study has shown a link between transmissible CF strains and the presence of the cable pili described above (Sun *et al.*, 1995). This is a highly significant finding that may lead to a simple diagnostic routine for the detection of such strains. It is interesting that some clusters in the dendrogram arising from this study contain both environmental and clinical isolates (Sun *et al.*, 1995).

The recent availability of a *cftr* mutant mouse model of CF will permit closer investigation of *B. cepacia* infections in CF. The development of lung disease in the CF mouse similar to that observed in CF patients upon exposure to strains of *B. cepacia* has been described within the last 18 months (Davidson *et al.*, 1995).

The production of pigments by the epidemic strain is of some interest. Melanin in black fungi has been proved to provide sufficient antioxidant capacity to negate oxidative killing processes in activated macrophages (Jacobson, Hove & Emery, 1995). The identity of the *B. cepacia* epidemic strain pigment is not clear, but it resembles melanin in appearance. If this pigment is capable of producing a similar effect *in vivo* to that observed with fungal melanin then it may have some role in the pathogenicity of the epidemic strain. Until recently, there was little published evidence to suggest that *B. cepacia* is at any time an intracellular pathogen (Govan & Deretic, 1996) and exposed to an environment where greater resistance to oxidative killing is of great benefit. However, research demonstrating the capability of *B. cepacia* to invade respiratory tract cells has been published within the last few months (Burns *et al.*, 1996).

1.2.5.2 Identification of *Burkholderia cepacia* in Clinical Material.

Identification of this species is problematic and requires specific media for reliable results (Gilligan. 1991). Studies have found that common diagnostic media such as chocolate agar, blood agar and MacConkey agar permit the successful isolation of *B. cepacia* in only 23%, 15% and 11% of cases respectively (Tablan *et al.*, 1987).

1.2.5.2.1 Solid Isolation Media and Biochemical Tests.

The most commonly used medium in diagnostic practice is the commercially available MAST *B. cepacia* medium (MAST Laboratories), which is based on the PC medium of Gilligan *et al.* (Gilligan *et al.*, 1985). This medium contains crystal violet, bile salts, ticarcillin and polymyxin B as selective agents to prevent the overgrowth of *B. cepacia* by other organisms commonly found in the CF respiratory tract such as *S. aureus*, *P. aeruginosa* and *E. coli* (Gilligan *et al.*, 1985). The medium turns a vivid pink colour upon growth of *B. cepacia*. Growth on this medium is not completely diagnostic of *B. cepacia* infection as other pathogens found less commonly in the CF lung such as *S. maltophilia* can also flourish (Burdge *et al.*, 1995). Confirmation of an isolate's identity must be effected using a commercially available diagnostic portfolio such as API 20 NE (Bio-Mérieux) (Burdge *et al.*, 1995).

Another selective medium for the recovery of *B. cepacia* from the respiratory tracts of CF patients is OFPBL (oxidation-fermentation-polymyxin B-bacitracin-lactose) medium (Welch *et al.*, 1987). This medium contains the antibiotics bacitracin and polymyxin B as selective agents in a solid oxidation fermentation (OF) base (Difco OF basal medium). Classically *B. cepacia* can be expected to produce acid from the lactose in the medium and produce a colour change from green to yellow. Paradoxically, this was not always the case in practice (Simpson *et al.*, 1994). This subject is discussed further below (1.2.5.2.2).

Studies have shown OFPBL medium to permit the isolation of *B. cepacia* in 94% of tests as compared to 98% for the selective *B. cepacia* medium of Gilligan *et al.* (Tablan *et al.*, 1987). This study used an artificial isolation base formulated to resemble sputum, and inoculated with *B. cepacia* alone or in combination with other species likely to be present in a typical sputum sample from a CF patient (Tablan *et al.*, 1987). It is not recorded whether the strains used were of clinical origin or not.

Tests of 5 commercially available identification portfolios available in the USA (RapID NF Plus (Innovative Diagnostic Systems); API Rapid NFT (Bio-Mérieux); Vitek Auto Microbic System GNI (Bio-Mérieux); Uni N/F Tek and N/F Screen (Remel) showed a quite varied ability to accurately identify *B. cepacia*, with the API Rapid NFT the worst (43% correct) and the Remel systems the best (86%) (Kiska *et al.*, 1996). A particular problem noted was the occasional inability of all systems to distinguish between different members of the genus *Burkholderia*. This may be expected as most commercial systems will contain one or more key tests to distinguish between closely related species contained within their portfolios (Kiska *et al.*, 1996). When one considers that a species-positive property in many standard texts (such as *Bergey's Manual of Systematic Bacteriology*) refers to a 90% likelihood of that result, there is evidently scope for an atypical result to lead to a misidentification. Illustration of this is provided by *B. cepacia* isolates from the University of Utah Medical Centre, which share an unusual biotype and are lysine decarboxylase (LDC) negative (Welch *et al.*, 1987), *B. cepacia* being typically LDC-positive. This emphasises the value of conventional biochemical tests in the diagnosis of *B. cepacia* (Kiska *et al.*, 1996).

With the API 20 NE system frequently used in the UK, difficulties in the unequivocal identification of Gram-negative nonfermenters such as *B. cepacia* have been noted (Simpson *et al.*, 1994). This illustrates that biochemical identification systems are only suitable for the identification of species within those covered by that system. No inference can be made about the presence or absence of a closely related species that does not fall within the remit of the system in question. This unfortunate pitfall must be borne in mind.

Biochemical tests of use in the isolation of *B. cepacia* include triple sugar iron; citrate; urea; LDC; ornithine decarboxylase; arginine dihydrolase; OF sugar tests with glucose, xylose, lactose, mannitol, sucrose, maltose, fructose, galactose and mannose; *o*-nitrophenyl-β-D-galactoside (OPNG); nitrate reduction and gas, gelatin, esculin, egg yolk agar lecithinase test, litmus milk and cetrimide (Kiska *et al.*, 1996). However, differences in biochemical test results between different studies have been noted in the literature (Urakami *et al.*, 1994). It would appear that selective media such as OFPBL are valuable tools in the accurate identification of *B. cepacia.*

1.2.5.2.2 Fatty Acid Methyl Ester Profiles.

The profiling of cellular fatty acids, extracted and derivatised to the corresponding fatty acid methyl ester (FAME) to permit gas liquid chromatographic (GC) analysis, has proved a useful taxonomic tool in the study of the genus *Pseudomonas* and the species subsequently created via the reclassification of members of that diverse genus. In a study of 340 strains, the relative amounts of 2- and 3- hydroxy fatty acids enabled the grouping of strains into 6 major subdivisions (Stead, 1992). Group 2 in this analysis contained members of the genus *Burkholderia*, which was proposed that same year (Yabuuchi *et al.*, 1992). Group 2 could be further subdivided, but generally the major factor noted was a comparative absence of fatty acids smaller that C14:0. Traces of C10:0 3-OH were occasionally noted (Stead, 1992).

The polar lipids and fatty acids of many members of the genus *Burkholderia* have been closely studied by other groups (Cox & Wilkinson, 1989; Galbraith & Wilkinson, 1991). These produced some differences in the fatty acid profiles compared to those found by Stead (Stead, 1992). This may be due to differences in growth conditions of the strains under study, or due to the time for which cultures were incubated prior to extraction, both of which can influence the fatty acid profile observed.

FAME profiles of 42 clinical isolates of *B. cepacia* from 5 clinical centres revealed a close intra-centre relationship between the profiles produced (Mukawaya & Welch, 1989). This was proposed to be of use in supporting genetic epidemiological techniques (Mukawaya & Welch, 1989). However, FAME profiling has not seen widespread adoption for this purpose.

Microbial identification by GC analysis of FAME profiles is a valuable technique in diagnostic microbiology. The commercial system Sherlock (formerly known as the Microbial Identification System (MIS)) compares the FAME profile of an unknown isolate with a database of profiles and assigns the species in accordance with the best match found (White *et al.*, 1988).

Use of OFPBL medium generally reveals *B. cepacia* by its growth in the presence of the antibiotics present. However, FAME profiling of colonies selected thus revealed the apparent presence of *B. gladioli* (Christenson *et al.*, 1989). The FAME profile of *B. gladioli* is reported as differing from that of *B. cepacia* in the presence of C10:0 3-OH and the presence of much smaller levels of C16:1 and C17:0 Δ (Welch, 1991). DNA hybridisation studies seemed to confirm this finding (Christenson *et al.*, 1989). Amongst the biochemical tests used, these isolates did not produce acid from lactose and thus did not induce a green-to-yellow colour change in the OFPBL medium. Oxidase test results, if positive, were weakly so and LDC tests gave negative results. *B. cepacia* typically produces acid from lactose, and yields positive oxidase and LDC results (Palleroni, 1984).

The clinical course of 3 patients colonised with *B. gladioli* was followed and no indication of a rapid decline in clinical status was recorded. It was tentatively concluded that carriage of *B. gladioli* conferred no greater risk upon the patient (Christenson *et al.*, 1989).

A later study also addresses the issue of the presence of isolates possessing multiple antibiotic resistance and characteristics of both *B. cepacia* and *B. gladioli* from the CF lung (Simpson *et al.*, 1994). Biochemically, these isolates were weakly oxidase-positive. Growth was observed on OFPBL medium, but acid production from lactose was noted as either negative or weakly positive. LDC test results varied. FAME profiles, assessed by MIS, designated 5 of the 6 clinical isolates studied as *B. gladioli*. Distinguishing features in the

FAME profile as reported by this technique were the presence of C10:0 3-OH and the detection of higher levels of C17:0Δ and C19:0Δ in those isolates identified as *B. gladioli* (Simpson *et al.*, 1994). This study concluded that hybrids between the two species *B. cepacia* and *B. gladioli* exist, and that the formation of such hybrids is significant in the emergence from phytopathogens of isolates capable of pulmonary colonisation. Thus it may be too soon to dismiss *B. gladioli* as a harmless commensal. (Simpson *et al.*, 1994). The close relation of these two species is evident (figure 1.3).

These small studies bring to light a facet of CF lung colonisation that requires further investigation. It has been concluded in some studies that the species concept of *B. cepacia* is currently too broad (Yohalem & Lorbeer, 1994a). It has also been widely recorded that lung colonisation with *B. cepacia* leads to a range of outcomes, from asymptomatic carriage to fatality (Gilligan, 1991) and clinical isolates of *B. cepacia* have been noted to display a marked phenotypic variability (Larsen, Stull & Burns, 1993). It may well be that further taxonomic rationalisation of this genus, coupled with practicable methods of recognising the results in the clinical setting, will assist in understanding this diversity.

Interestingly, a recent report has claimed to describe two successfully treated cases of *B. gladioli* infection in CGD (Ross *et al.*, 1995). In this case, FAME profiling and a similar range of biochemical tests to those outlined above were used to confirm the identity of the isolates recovered. The isolates produced a diffusible pigment described as chartreuse in colour. From the limited data in this report, these isolates appear very similar in appearance to the epidemic strain of Govan *et al.* (Govan *et al.*, 1993). Additionally, a pseudomonad similar in some respects to *B. cepacia*, *B. gladioli* and *B. pickettii* has also been described in CGD (Trotter *et al.*, 1990). Possibly, the bacterial hybrids of Simpson *et al.* have wider relevance than CF alone.

In a recent presentation at the 20th European Cystic Fibrosis Conference, a preliminary taxonomic investigation of *B. cepacia* in CF has concluded that Belgian CF isolates identified routinely as *B. cepacia* belong to 3 different *Burkholderia* species. Each of these clusters was deemed to constitute a previously unrecognised species (Vandamme, 1995). How these findings extrapolate to the clinical picture may well prove both significant and exciting.

1.2.5.2.3 Detection of *B. cepacia* Using The Polymerase Chain Reaction.

A recent development has been the application of PCR in bacterial identification. Microbial identification is effected via detection of the DNA amplified by PCR. (Lenstra, 1995).

Detection of *B. cepacia* using PCR has been attempted using 16S rRNA as the amplification target region. This section of the bacterial genome varies in an orderly fashion throughout the phylogenetic tree (see 1.2.5.1), with variable regions highly amenable to the synthesis of species-specific rRNA probes (Campbell *et al.*, 1995). Such probes are not particularly sensitive, but the use of PCR to increase the quantity of the relevant section of DNA present greatly increases the sensitivity of the technique. (O'Callaghan, Tanner & Boulnois, 1994). This procedure has proved effective in clinical practice (O'Callaghan, Tanner & Boulnois, 1994; Campbell *et al.*, 1995), but may prove more expensive than current techniques. However, it has been noted that pulmonary colonisation with *B. cepacia* can remain undetectable for some time by techniques currently in general use and PCR might prove a useful technique for the diagnosis of *B. cepacia* lung infections in CF (LiPuma *et al.*, 1994).

1.2.5.2.4 Radiographic Appearance of *B. cepacia* Lung Infection

The radiographic appearance of *B. cepacia* lung infection has not been reported widely, but infection results in a rapidly progressive mass-like consolidation and reflects an haemorrhagic, necrotising lobular pneumonia (Shah *et al.*, 1995).

1.2.5.3 Treatment of *Burkholderia cepacia* in Cystic Fibrosis.

As outlined above, *B. cepacia* isolates often display a widespread resistance to antibiotics of all types. Frequently, only a minimal clinical response is achieved with antibiotics that appear effective in *in vitro* tests (Gilligan, 1991; Govan & Nelson, 1993). The most effective agents historically were chloramphenicol, co-trimoxazole and tetracycline, although clinical efficacy was limited (Hodson, 1995; Spencer, 1995). Some isolates of *B. cepacia* harbour a dihydrofolate reductase conferring high-level resistance to trimethoprim (and hence co-trimoxazole) (Burns, Lien & Hedin, 1989). Sensitivity tests with 366 strains from CF patients showed widespread resistance to ciprofloxacin, amikacin, gentamicin, tobramycin, carbenicillin, cefuroxime, cefotaxime, imipenem, biapenem, chloramphenicol, tetracycline and co-trimoxazole. 77% of these strains or more were deemed sensitive to meropenem, ceftazidime, and piperacillin alone and in combination with tazobactam (Pitt, 1996). However, no attempt was made to distinguish between strains isolated from the CF lung and those isolated from other sources in this study. A study into the susceptibilities of non-*Pseudomonas aeruginosa* Gram-negative nonfermenters noted apparently greater general antibiotic resistance in *B. cepacia* isolates from the CF lung as opposed to isolates from other clinical sources (Spangler *et al.*, 1996).

Susceptibility tests on 73 isolates from CF patients showed ceftazidime to be the most active antibiotic, with resistance approaching 9% (Bhakta *et al.*, 1992). However, a clinical study of 18 courses of ceftazidime treatment in CF patients showed clinical improvement in only 6 patients (33%), with 4 fatalities (22%) during the study period (Gold, Jin & Levison, 1983). It has been proposed that a ceftazidime-hydrolysing β-lactamase is produced by *B. cepacia*, as resistance seems to correlate with the production of certain β-lactamases (Aronoff & Labrozzi, 1986).

A limited study with temocillin showed some improvement in 9 of 12 courses in 5 patients infected with *B. cepacia* (Taylor, Gaya & Hodson, 1992). Whether this was actually due to an effect against *B. cepacia* or another colonising species is open to debate as no *B. cepacia*-negative patients were included as a control. Of the 5 subjects, only 1 was solely infected with *B. cepacia*, and conclusions drawn from such a small sample must be viewed with circumspection.

Another study comparing intravenous antibiotic treatment in 14 *B. cepacia*-positive and 10 *B. cepacia*-negative CF patients showed improvements in clinical markers of lung function of similar levels for both groups, thus indicating that the use of antibiotics in colonised patients might not be wholly futile (Peckham *et al.*, 1994). This study did not standardise on one particular treatment regimen.

The effects of pH and carbon dioxide (CO_2) on β-lactam susceptibility *in vitro* show a reduction in susceptibility to ceftazidime, cefpirome, piperacillin and piperacillin/ tazobactam in the presence of low pH and higher CO_2 levels (Corkill *et al*, 1994a). The difference between susceptibility test conditions and the environment of the infection may be significant in this case: lung airways can reasonably be expected to contain increased levels of CO_2 compared to ambient air.

The apparent presence of β-lactamase-mediated resistance to the carbapenems is a source of some encouragement in the antimicrobial chemotherapy of *B. cepacia* infections. If a β-lactamase inhibitor capable of inhibiting this enzyme were to be available, it could permit the successful use of such agents in such cases. In this respect, it has been suggested that the carbapenems such as imipenem and meropenem offer the main hope for the future (Simpson *et al.*, 1993).

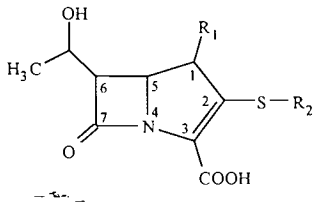
Further treatment for lung infections is of a more general nature: bronchodilators can be administered to try to increase lung function and hypertonic saline can be used to increase expectoration. These procedures are both useful adjuncts to attempts to clear the lungs using physiotherapy (Hodson & Warner, 1992). More recently, deoxyribonuclease (DNase) treatment has been used to break down the DNA debris that collects in the lungs as a result of continued infection and leucocyte actions (Marelich & Cross, 1996).

1.3 The Carbapenems: An Overview.

The carbapenems are currently the most potent and broad spectrum β-lactam antibiotics available. They have been adequately reviewed in the literature (Clissold *et al.*, 1987; Wise, 1990). Figure 1.5 shows the structures of the 9 current members of this group.

Imipenem (Merck, Sharp & Dohme) was the first commercially available carbapenem. It is a semi-synthetic derivative of thienamycin, a naturally produced carbapenem isolated from *Streptomyces cattleya* (Birnbaum *et al.*, 1985). It has recently been joined by meropenem (Zeneca) (Neu, 1994) in the UK and the USA. Biapenem (Lederle) (Malanoski *et al.*, 1993) does not appear to have been the subject of much published research in the past 2 years or so. Currently, four other agents, panipenem (Sankyo) (Shimada & Kawahara, 1994); BO-2727 (Banyu) (Nakagawa *et al.*, 1993); BMS-181139 (Bristol-Myers Squibb) (Kessler *et al.*, 1995); DX-8739 (Daichi) (Giamarellos-Bourboulis, Grecka & Giamarellou, 1995) and DA-1131 (Dong-A) (Kim, Kim & Lee, 1995) are known to be in development. A further carbapenem, L-695,256 (Merck, Sharp & Dohme), has recently been described, but seems to have a greater tendency to anti-Gram-positive activity than anti-Gram-negative (Chambers, 1995).

The carbapenems differ from penems and penicillins in having one atom of sulphur on the side chain at position 2 and a carbon at position 1 of the 5-membered ring attached to the β-lactam. Similar to the penems, that 5-membered ring is unsaturated between positions 2 and 3. Those agents with a methyl group or larger on the 1 position are stable to renal dehydropeptidase-I and do not require co-administration with inhibitors of this degradation. Those agents with a hydrogen at this position are degraded to toxic metabolites as well as failing to achieve suitable levels in the urinary tract. Hence imipenem is co-administered with cilastatin sodium, an inhibitor of renal dehydropeptidase-I (Clissold *et al.*, 1987) and an inhibitor of renal anion transport, betamipron, is under investigation for co-administration with panipenem (Kurihara *et al.*, 1992).



Carbapenem nucleus

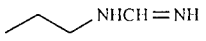
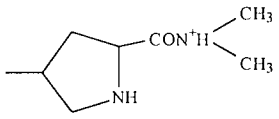
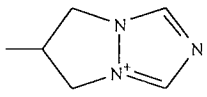
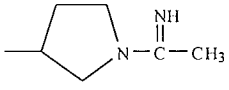
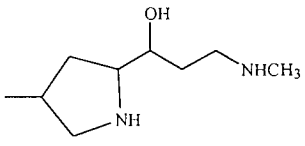
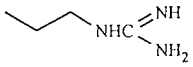
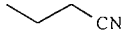
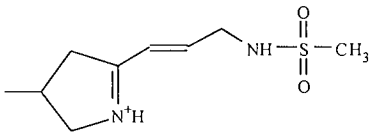
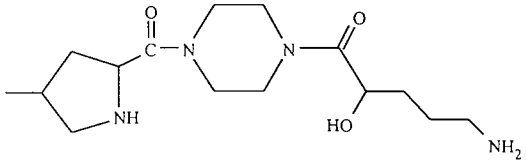
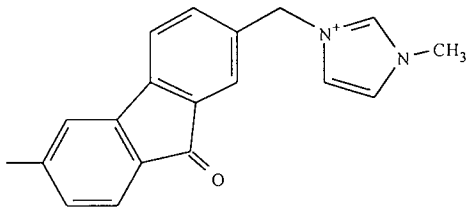
	R_1	R_2
Imipenem	H	
Meropenem	CH_3	
Biapenem	CH_3	
Panipenem	H	
BO-2727	CH_3	
BMS-181139		
DA-1131	CH_3	
DX-8739	CH_3	
L-695,256	H	

Figure 1.5 Structures of the carbapenems.

The carbapenems have particular properties that contribute to their great activity and broad spectrum. Amongst these properties is their outstanding stability to attack by most β-lactamases. This is attributable to a *trans* configuration of the substituent on the 6 position of the β-lactam ring as opposed to the more usual *cis* configuration (Neu, 1985). Additionally, carbapenems appear to have a particular amenability to porin-mediated uptake by the D2 porin of *P. aeruginosa* (Trias & Nikaido, 1990). This property has been attributed to their zwitterionic nature and comparatively low molecular weight (Quinn, 1994). However, some researchers using porin-suppressed mutants have indicated that whilst some carbapenems appear limited to entry via porins, meropenem, BO-2727 and BMS-181139 have other viable routes of entry into a viable bacterial cell (Piddock & Turner, 1992; Sumita & Fukasawa, 1993; Fung-Tomc *et al.*, 1995a; Hazumi *et al.*, 1995). The exact means whereby this is achieved is unclear but it would seem unlikely that the charged carbapenem molecules would be capable of unhindered passage across the hydrophobic Gram-negative outer membrane. There are conflicting reports describing the mode of entry of meropenem into *P. aeruginosa* (Fung-Tomc *et al.*, 1995a).

In *P. aeruginosa*, it has been found that the presence of a basic group at position 2 of the penem moiety is necessary for preferential uptake by the D2 porin and the further addition of a basic group at positions 1 or 6 reduces the reliance of a carbapenem on the presence of a suitable porin for uptake (Fung-Tomc *et al.*, 1995b). It would not be unreasonable to expect a similar situation involving porin-mediated and non-porin-mediated uptake in other species.

These properties give substance to the opinion that carbapenem therapy offers the main hope for the future successful eradication of *B. cepacia* (Simpson *et al.*, 1993). Currently, treatment of *B. cepacia* with a carbapenem is unreliable at best, although meropenem appears more promising than imipenem from a brief comparison of *in vitro* studies (Lewin *et al.*, 1993; Simpson *et al.*, 1993). Elucidation of the resistance mechanisms involved will be vital for the success of carbapenem-oriented treatments for *B. cepacia.*

In vitro antimicrobial sensitivity data against *B. cepacia* in the literature shows biapenem and imipenem to be of comparable potency (Malanoski *et al.*, 1993). Meropenem has greater potency than these two agents and BMS-181139 appears to have slightly greater potency than meropenem (Kessler *et al.*, 1995).

1.4 Bacterial Resistance to β-lactam Antibiotics.

The subject of bacterial resistance to β-lactam treatment is large. The subject of antimicrobial resistance in general has been succinctly reviewed by Neu with an emphasis on the situation with β-lactam agents (Neu, 1992). However, in order to place the following into context, it is necessary to provide a brief précis of the mode of action of β-lactams.

1.4.1 β-Lactams: mode of action.

A detailed discussion of the mode of action of β-lactam antibiotics is beyond the scope of this report. The subject has been well reviewed in the literature (Tipper, 1985). For the purposes of this thesis, it will suffice to mention that the molecular targets of β-lactam antibiotics are the penicillin-binding proteins (PBPs). These are enzymes involved with the end stages of cell wall peptidoglycan manufacture. The inhibition only of certain members of the PBP complement will elicit a fatal outcome. Inhibition of those PBPs with transpeptidase activity (those within groups 1,2 and 3) is fatal. These high molecular weight PBPs are involved in the cross linking of adjacent peptidoglycan strains. The β-lactams act as analogues of the dipeptide substrate of these enzymes, D-alanine-D-alanine, inhibiting this step of metabolism.

Of the carbapenems, thienamycin has been shown to bind to all the PBPs, but with a particular affinity to PBPs 1 and 2 (Spratt *et al.*, 1977). Imipenem appears to bind most avidly to PBP 2 (Neu, 1985). Meropenem also binds avidly to PBP 2, but additionally shows a higher affinity than imipenem for the other PBPs with transpeptidase activity (Kitzis *et al.*, 1989). BMS-181139 appears to have a lower affinity for PBP 1A than imipenem and meropenem but a heightened affinity for PBP 2 (Fung-Tomc *et al.*, 1995a) Biapenem shows increased affinity for PBP 1b over imipenem and meropenem and a largely intermediate affinity for the other transpeptidase PBPs (Yang, Bhachech & Bush, 1995). Comparative data does not yet appear to be available for the other carbapenems.

1.4.2 β-Lactams: Microbial Resistance Strategies.

β-lactam antibiotics including the carbapenems may be subject to one or more of four strategies adopted by resistant strains:

a) An alteration of the PBPs in a manner that retains physiological activity whilst rendering them insensitive to β-lactams (Malouin & Bryan, 1986).

b) A reduction in wall permeability resulting in reduced β-lactam penetration. This is the preserve of Gram-negative bacteria, where a reduced expression of porins in the outer membrane impedes β-lactam penetration (Nikaido, 1989).

c) The production of an efflux pump to eliminate the antibiotic from the cell and hence from its site of action. The presence of such elimination systems has only recently been elucidated and is the subject of some surprise: the PBPs are a fairly exposed target located on the outer face of the cytoplasmic membrane and were not previously considered amenable to this strategy (Li *et al.*, 1994). Similar structures have been found in *B. cepacia* but have not yet been discussed in terms of their impact on β-lactam resistance (Burns *et al.*, 1996).

d) The production of a β-lactamase enzyme capable of inactivating (or trapping) the β-lactam antibiotic employed in therapy. This fourth strategy is the most common and clinically relevant mediator of β-lactam resistance (Wiedmann *et al.*, 1989).

Additionally, mechanisms can combine to yield resistance. For example, a combination of altered outer membrane permeability and the production of a β-lactamase together have been found responsible for imipenem resistance in some strains of *P. aeruginosa* (Klare *et al.*, 1994).

1.4.3 β-Lactamases: Diversity and Classification.

β-lactamases (EC 3.5.2.6) were recognised before the use of benzylpenicillin, the first therapeutically useful β-lactam, became commonplace (Bush, 1989a). They are a diverse spectrum of enzymes capable of β-lactam hydrolysis, spanning both Gram-positive and Gram-negative bacteria. Each has a different substrate profile when challenged with the range of available β-lactam antibiotics, although these differences may sometimes be very subtle. It seems that the introduction of a new and apparently β-lactamase-stable β-lactam antibiotic will precede selection of a novel enzyme capable of its hydrolysis by only a few years.

These enzymes function via the cleavage of the β-lactam ring (figure 1.6) (Coleman *et al*, 1994). They can be either constitutive or inducible. The initiation of the induction process appears to arise from the metabolic disturbance of cell wall peptidoglycan caused by the β-lactam (Tölg *et al.*, 1993; Normark, 1995). Stably derepressed mutants, where the β-lactamase is hyperproduced in response to no recognisable induction cue, have also been reported and can be of clinical importance (Tzouvelekis *et al.*, 1992). Genetically, these enzymes can be plasmid- or chromosomally- based. The ease of transmission of the plasmid-based enzymes generally causes them to be of the greatest clinical concern. All β-lactamases so far discovered have either a serine moiety at the active site, or a molecule of water, tightly held by a histidine-chelated atom of zinc (Bush, 1989a).

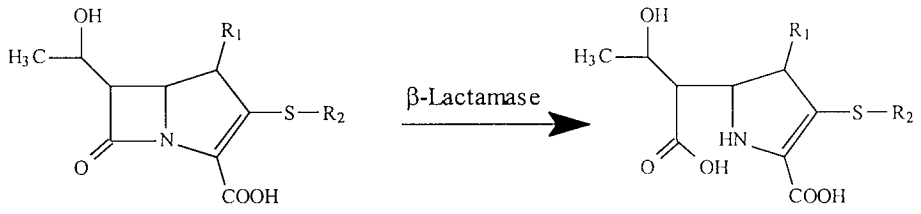


Figure 1.6. Hydrolysis of a carbapenem by a β-lactamase.

The first attempted classification scheme for the β-lactamases from Gram-negative bacteria was that of Richmond and Sykes (Richmond & Sykes, 1973), updated three years later (Sykes & Matthew, 1976). This nomenclature is no longer in general use as the availability of new antibiotic substrates and inhibitors has rendered it obsolete.

1.4.3.1 Ambler's Molecular Classification.

The scheme of Ambler attempts to classify all β-lactamases according to amino acid sequence homology. Two classes were initially proposed: class A (serine penicillinases) and class B (zinc-containing metallo-β-lactamases) (Ambler, 1980). Two further classes were subsequently added; class C (the serine cephalosporinases of Jaurin and Grundström (Jaurin & Grundström, 1981)) and class D (the oxacillin-hydrolysing serine-based enzymes of Huovinen *et al* (Huovinen *et al.*, 1988).

An obvious drawback of this nomenclature is that a tremendous amount of effort must be expended in order to classify an enzyme. Also, the addition of further classes will render this scheme increasingly illogical as allocation of the classes will occur for chronological reasons alone. It will also be appreciated from a brief study of the metalloenzymes that a molecular diversity far beyond one Ambler class is highly likely. The remaining 22 alphabetical classes may be filled very quickly indeed.

1.4.3.2 Bush's Biochemical Classification.

A more recent attempt at classification is that of Bush (Bush, 1989a,b,c). The enzymes are classified according to their substrate profiles and their susceptibility to inhibition by the mechanism-based inhibitor clavulanic acid, and the metal ion chelator ethylene diamine tetra-acetic acid (EDTA). Class 1 comprises the Gram-negative chromosomal enzymes. Class 2 includes most of the clinically important (and clavulanic acid-inhibitable) enzymes, including the Gram-positive penicillinases and the broad and extended spectrum enzymes most commonly found on plasmids in Gram-negatives. This class is subdivided six times. Class 3 comprises the metalloenzymes; all the examples given by Bush are resistant to inhibition by clavulanic acid and susceptible to inhibition by EDTA (Bush, 1989c). Class 4 contains a miscellany of inhibitor-resistant serine-based enzymes. Interestingly, one of these serine-β-lactamases is inhibited by EDTA (Fujii *et al.*, 1985) and another partially inhibited by zinc sulphate (Prince *et al.*, 1988).

This classification scheme appears very complicated at first sight but this is by necessity due to the diversity of the content. It overcomes the need for sequence data prior to classification and seems to be slowly gaining in popularity. However, the scheme of Ambler remains in use for discussions concerning sequence data.

1.4.3.3 The Bush-Jacoby-Medeiros Functional Classification Scheme.

This most recent scheme is largely an update on the scheme of Bush outlined above (Bush, Jacoby & Medeiros, 1995). It attempts to address the discovery of novel β-lactamases over the last 6 years and expand on that functional classification scheme. The differences between molecular and biochemical classification schemes are alluded to and the future prospects for classification by the production of a phylogenetic tree from sequence data is discussed (Bush *et al.*, 1995). This is a laudable aim, but computer-based statistical analysis should allow a polyphasic approach similar to that used in bacterial systematics to produce an encompassing scheme. β-lactamase activity encompasses a spectrum of behaviour. The Bush-Jacoby-Medeiros scheme is too recent to show widespread adoption in the literature at present. Metallo-β-lactamases fall into group 3 of this scheme.

1.5 Bacterial Resistance to Carbapenems.

As mentioned above, carbapenems are extremely adept at gaining cellular entry and largely avoid the overtures of any β-lactamase present due to the *trans*- configured 6-substituent on the β-lactam ring. Imipenem, the only agent in general use, was effective against 95% of strains in 1990, with several notable exceptions (Quinn, 1994). Currently, a review of the literature will reveal a burgeoning resistance problem (Tzouvelekis *et al.*, 1992; Livermore, 1992; Sumita & Fukasawa, 1993; Minami *et al.*, 1993). However, carbapenem (imipenem) resistance is still uncommon and fairly difficult to achieve (Livermore, 1992). The mechanisms of resistance to carbapenems has been discussed recently in the literature (Livingstone, Gill & Wise, 1995).

1.5.1 Mediation of Carbapenem Resistance.

Little evidence exists to suggest that altered PBPs currently play a significant role in carbapenem resistance (Thomson *et al.*, 1993). The role of impaired permeability in carbapenem resistance is significant in the case of imipenem and is well documented in *P. aeruginosa*, where the lack of a specific porin, D2, appears crucial to the development of resistance (Quinn, 1994). A deficiency in the D2 porin appears to give rise to resistance to biapenem and panipenem also (Sumita & Fukasawa, 1993), but not to meropenem (Sumita & Fukasawa, 1993; Piddock & Turner, 1992). No research appears to have been carried out into the possibility of imipenem-extruding efflux pumps.

The involvement of high levels of the chromosomal β-lactamase has been implicated in the development of imipenem resistance in *Enterobacter cloacae* (Thomson *et al.*, 1993) and *Enterobacter aerogenes* (Tzouvelekis *et al.*, 1992).This was definitely in association with impaired permeability in the latter case. From the relative hydrolysis tables compiled by Bush, it is evident that many chromosomal β-lactamases possess a minor imipenem-hydrolysing capability (Bush, 1989b,c). This, coupled with a slight permeability impairment, may be sufficient to confer resistance.

1.5.2 Carbapenemases.

Enzymes competent in the hydrolysis of carbapenems have been reported. These true carbapenemases are usually metallo-β-lactamases (Livermore, 1992). Some serine β-lactamases more commonly considered as cephalosporinases can slowly hydrolyse imipenem and thus contribute to resistance (Zhou, Kitzis & Gutmann, 1993). These are not considered "true" carbapenemases, but their significance should not be underrated. Serine β-lactamases proficient in the hydrolysis of carbapenems are currently a rarity although a notable exception is that reported by Yang *et al.* in *Serratia marcescens* (Yang *et al.*,1990). This enzyme appeared from the initial report to be a metallo-β-lactamase as it was susceptible to inhibition by the metal chelators ethylene diamine tetra acetic acid (EDTA) and ethylene glycol-*bis*-(β-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA). However, sequence data revealed it to be a novel class A β-lactamase (Naas *et al.*, 1994).

Metallo-carbapenemases, although themselves uncommon generally, are more prevalent than their serine counterparts (Livermore, 1992). All those reported to date require zinc as the metal ion, although partial activity can occasionally be restored using other divalent cations including cobalt, calcium and ferrous iron (Payne, 1993). Most are chromosomally based and are widespread amongst some species including *S. maltophilia* and *Bacillus cereus* (Livermore, 1992). A plasmid-borne metallo-β-lactamase has been reported in *P. aeruginosa* (Watanabe *et al.*, 1991) and this may prove significant in the future (Livermore, 1992). Likewise, plasmid-mediated dissemination of a metallo-β-lactamase from *S. marcescens* has been observed in Japan (Ito *et al.*, 1995; Arakawa *et al.*, 1995). At present, novel metallo-β-lactamases are being observed with considerable frequency.

1.5.2.1 Diversity Amongst Metallo-β-lactamases.

From the available data, it would appear that these enzymes are of diverse origins: their molecular weights and isoelectric points differ over a great range. Ambler's classification scheme groups all metallo-β-lactamases in class B, as little supporting data was available at the time (Ambler, 1980). Few sequences are available (Bush *et al.*, 1995) and only one crystal structure, that of *B cereus* β-lactamase II, has been studied at any resolution (Sutton *et al.*, 1987). At least two subclasses of metallo-β-lactamase have been proposed on the basis of molecular data (Massidda *et al.*, 1991). The sequence of the L1 metallo-β-lactamase from *S. maltophilia* has recently been deduced and shows great variation from other sequences known. Some conservation was noted at the purported histidine-rich region proposed as the zinc-binding motif (Walsh *et al.*, 1994). This study proposes the presence of three molecular subclasses within this group (Walsh *et al.*, 1994). Sequence homology is such that a universal genetic probe for the detection of metallo-β-lactamase genes seems unlikely. This does not however discount the use of a mixture of such probes, but more sequence data will be required for a genetic detection approach to become tenable. This genetic detection principle, involving PCR, has been exploited in the molecular characterisation of TEM-type serine β-lactamases (Arlet *et al.*, 1995).

Kinetic studies of substrate hydrolysis reveals a similar diversity in the capabilities of various metallo-β-lactamases (Felici *et al.*, 1993; Felici & Amicosante, 1995), indicating a similar degree of diversity on a functional level. It would appear that a single class in either a molecular or a functional classification scheme does not sufficiently highlight the diversity present in this group of enzymes.

Carbapenemases termed "hidden" and "silent" by their researchers have been observed. In the former case, it was found that a carbapenemase from *Aeromonas hydrophila* was unable to hydrolyse nitrocefin (Hayes, Thomson & Amyes, 1996). Nitrocefin is a chromogenic, clinically useless cephalosporin employed as a β-lactamase indicator on account of the colour change produced by its hydrolysis (O'Callaghan *et al.*, 1972). In the latter case, it was found that minor mutations in the genome of *Bacteroides fragilis* can bring about the production of high levels of a carbapenemase (Podglajen *et al.*, 1992). Both these phenomena may have significant consequences: the former may lead to the non-detection of a carbapenemase and the latter may yield clinically relevant carbapenem resistance.

1.5.2.2 Inhibition of Metallo-β-lactamases.

Metallo-β-lactamases are generally resistant to inhibition by clavulanic acid and other classical β-lactamase inhibitors (Payne, 1993). An exception is a metallo-β-lactamase from the *B. fragilis* group, but the peptide sequence of this enzyme is not known (Nord *et al.*, 1991). All metallo-β-lactamases reported to date are inhibitable by various metal ion chelators such as EDTA.

A detailed discussion of β-lacatamase inhibition is beyond the scope of this thesis as the majority of the research in the literature concerns the serine enzymes. There are currently no clinically usable metallo-β-lactamase inhibitors. The subject of β-lactamase inhibition has been reviewed in the literature (Cartwright & Waley, 1983; Knowles, 1983; Rolinson, 1991).

Kinetic analyses of the interactions between serine β-lactamases and classic mechanism-based inhibitors such as clavulanic acid, sulbactam and tazobactam are indicative of an irreversible, "suicide" mechanism subsequent to an initial competitive binding phase (Bush *et al.*, 1993). Despite conclusions to the contrary involving a single variety of metallo-β-lactamase (Bush *et al.*, 1993), it is unlikely that this is true of all metallo-β-lactamases, which may prove to experience competitive effects only, and not necessarily with preferential binding of the candidate inhibitor over the antibiotic.

Recently, the subject of non-β-lactam phosphonoamidate inhibitors of β-lactamases has seen some consideration in the literature (Payne *et al.*, 1996). These compounds could have promise in the inhibition of metallo-β-lactamases if one considers the success in phosphate containing angiotensin-converting-enzyme (ACE) inhibitors such as fosinopril in inhibiting the zinc-containing peptidase ACE. However, there is no documented evidence of this theory yielding success. Other potential non-β-lactam β-lactamase inhibitors described in the literature include a naturally occurring anacardic acid derivative from the plant *Spondias mombin*, for which no evidence of metallo-β-lactamase inhibitory activity is available (Coates *et al.*, 1994). The 17.5kDa β-lactamase-inhibitor protein (BLIP) isolated from the exocellular products of the fungus *Streptomyces clavuligerus* is a most potent inhibitor of serine β-lactamases, but again there is not particularly effective against metallo-β-lactamases (Strynadka *et al.*, 1994). Interestingly cilastatin, the renal dehydropeptidase I inhibitor co-administered with imipenem, has recently been shown to have inhibitory activity against the metallo-β-lactamase *cphA* from *Aeromonas hydrophila* (Kenyan *et al.*, 1995). However, this seems not to be the case with to some other metallo-β-lactamases (Kenyan *et al.*, 1995).

Additionally, the synthesis of metallo-β-lactamase inhibitors with a β-lactam structure have been described. However, these have only been shown to inhibit the *B. cereus* II enzyme (Vanhove *et al.*, 1995).

1.5.3 Carbapenem Resistance in *Burkholderia cepacia.*

Until the last few years, very little work had been conducted with the aim of elucidating the resistance strategies that are so effective in protecting *B. cepacia* from all antibiotics, including β-lactams. As mentioned above, a recent short report involving clinical isolates of *B. cepacia* has suggested that cell sonicates from this organism can hydrolyse imipenem (Simpson *et al.*, 1993), in a manner inhibitable by the novel penem β-lactamase inhibitor BRL 42715 (Coleman *et al.*, 1989). Sadly, this compound is no longer under development (Coleman, 1994). As also described above, inducible penicillinases have been isolated from *B. cepacia* (Hirai *et al.*, 1980; Prince *et al.*, 1988), but carbapenemase activity has not been unequivocally demonstrated with these. Therefore the source of carbapenemase activity in *B. cepacia* is without confirmation.

1.6 Aims of this study.

This study aims to investigate the biochemical basis for carbapenem resistance in *B. cepacia*. It is intended to conduct this investigation with due attention to the nature of *B. cepacia* isolates from CF and their relationship to *B. cepacia* at large. It would appear from the above that CF isolates of *B. cepacia* may well bear significant similarity to environmental strains, in addition to the display of certain salient divergences. This study is intended to be neither epidemiological nor diagnostic and intends to compare CF strains to those from other, mainly environmental sources. The use of strains from other, related species will at times be necessary for the purposes of comparison.

2 Procurement of Bacterial Strains, Reagents and Apparatus.

2.1 Bacterial Strains.

Strain Storage Code	Source
5BI; 5JIV; 5NVI; 5PII; 5PIV; 5PVI; 5QIV; 5QV; 5RIV; 5SI.	Cystic fibrosis lung isolates. From Dr. E.G. Smith & Dr. D.E. Lacy. Heartlands Hospital, Birmingham, UK.
5OIV.	Burn isolate. From Dr. J. S. Soothill. Accident Hospital, Birmingham, UK.
5OV; 5PI.	Chronic granulomatous disease isolates. From Dr.D.E.Lacy. Heartlands Hospital, Birmingham, UK.
5RI; 5RII; 5RIII.	Cystic fibrosis lung isolates. From Dr D.G. Allison. Manchester University, UK.
J1948; J2315.	Cystic fibrosis lung isolates. From Dr. J.R.W. Govan. Edinburgh University, UK.
J2552.	Botanical Isolate. From Dr. J.R.W. Govan, Edinburgh University, UK.
5SV; 5SVI; 5TI; 5TII; 5TIII; 5TIV; 5TV.	Cystic fibrosis lung isolates. From Dr. D.E. Lacy. Alder Hey Hospital, Liverpool, UK.
3843; 3925; 4325; 4326; 4949; 9275; 12794; 15856.	Cystic fibrosis lung isolates. From Dr.B.Giwercman. Rigshospitalet, Copenhagen, Denmark.

Table 2.1 Origins of clinical strains used in this study.

Clinical strains were requested or donated from the sources indicated (table 2.1). Laboratory strains of the species shown in table 2.2 were obtained directly from the respective culture collections or indirectly via reputable sources (SmithKline Beecham, Brockham Park, Surrey, UK) or the Microbiology Department, City Hospital NHS Trust, Birmingham, UK). Strains were stored in nutrient broth supplemented with 10% glycerol at –70°C until required.

Strain Identificaton Code	Source of Isolation
B. cepacia ATCC 17616	Soil.
B. cepacia NCTC 10744	Blood culture.
B. cepacia NCTC 10661	Trinidad forest soil.
B. gladioli ATCC 10248	Gladiolus leaf.
B. gladioli ATCC 10854	Onion.
B. gladioli ATCC 25417	Gladiolus leaf.
B. vietnamiensis LMG 6998*	Blood culture
S. maltophilia NCTC 10257	Buccal cavity.
P. aeruginosa NCTC 6750	Urine.
P. aeruginosa ATCC 15692	Wound infection.
E. coli NCTC 10418	Unknown

*LMG=University of Gent Culture Collection, Belgium.

Table 2.2 Origins of laboratory strains used in this study.

2.2 Reagents.

Standard laboratory reagents of Analytical Reagent grade of equivalent were obtained from reputable wholesalers via their UK addresses. Reagents specific for the work were obtained from Sigma (with those either less commonplace or obtained at a specific grade included in the list below) unless stated otherwise (UK address unless stated otherwise):

Aldrich:

m-hydroxybenzoate.

Tryptamine.

Bibby Sterilin:

40mm diameter Petri dishes.

BDH:

Electran 4.7-10.6 prestained

Isoelectric point markers.

Proprietary detergent (Extran).

Boehringer Mannheim:

*Xba*I restriction enzyme and buffer concentrate.

Bristol-Myers Squibb:

Aztreonam.

Bio Mérieux:

API 20 NE identification strips.

ATB PSE antibiotic sensitivity test strips.

BioRad:

Bis(N,N[1]-methylene) bisacrylamide (electrophoresis grade).

Cibacron Blue F3GA linked agarose (Affi Gel Blue).

Agarose (chromosomal grade).

Agarose (molecular biology grade).

Lambda Ladder DNA size standards for PFGE.

Econo-Pac S column.

Nitrocellulose membrane, pore size 0.45μm (Trans Blot Transfer Medium).

Polyvinylidene difluoride membrane pore size 0.2 μm (Trans Blot Transfer Medium).

Glaxo:

Nitrocefin.

Hewlett Packard:

Bacterial FAME standard.

Lederle (USA):

Biapenem.

Tazobactam.

Merck, Sharp and Dohme:

Imipenem.

Pharmacia:

Ampholine PAGplate isoelectric focusing gels (range pI 3.5-9.5).

Sigma:

Acrylamide (electrophoresis grade).

Dalton Mark VII-L molecular weight markers.

*Dra*I restriction enzyme and buffer concentrate.

Glycine (biochemical grade).

L-lysine (chromatographically homogeneous).

Lysozyme (molecular biology grade).

Phenylmethylsulphonyl fluoride (PMSF).

Protein A-horseradish peroxidase conjugate

Proteinase K from *Tritrachium album*.

Sodium dodecyl sulphate (electrophoresis grade).

Sodium N-lauroylsarcosinate (Sarkosyl) (molecular biology grade).

L(+)-tartaric acid (cat. no. T6521) for bicinchoninic acid protein assay.

L-tyrosine (chromatographically homogeneous).

SmithKline Beecham:

Clavulanic acid.

Thornton and Ross:

Extra virgin olive oil.

Wellcome:

Polymyxin B sulphate (Aerosporin).

Whatman:

DEAE-cellulose for ion exchange chromatography.

Zeneca:

Meropenem.

2.3 Media.

Constituents for preparing specialist media were obtained from the UK addresses of reputable wholesalers at the appropriate grade. Solid media were solidified with agar; Technical Agar No. 3 (Oxoid) was used unless stated otherwise. The following media and constituents were obtained from the sources indicated:

Mueller-Hinton broth (MHB)	Oxoid.
Mueller-Hinton agar (MHA)	Oxoid.
Nutrient agar (NA)	Oxoid.
Nutrient broth (NB)	Oxoid.
Noble Agar	Difco.
Trypticase soya broth (TSB)	Oxoid.
Basal OF medium	Difco.
Bacto-Peptone	Difco.
Yeast extract	Oxoid.

2.4 Apparatus.

The following specialist pieces of apparatus are available from the sources indicated:

J2 centrifuge, rotors and accessories	Beckman.
CHEF DR-III and accessories	BioRad.
Mini-Protean and Mini Trans Blot	BioRad.
HP-1 capillary column for GC	Hewlett Packard.
Multiphor 2117	LKB (Pharmacia).
Soniprep 150 and accessories	MSE Instruments.
C 16/40 column	Pharmacia.
FPLC System	Pharmacia.
Mono-Q column	Pharmacia.
PU 8750 spectrophotometer	Philips.
GC apparatus and PC-compatible control software	Unicam.

3 Biochemical Analyses and Antibiotic Sensitivities.

3.1 Introduction.

Biochemical tests provide data useful in the definition of an infecting strain (see section 1.2.5.2.1). Here, a commercially available identification portfolio (API 20 NE) was supplemented with additional biochemical and phenotypic observations in an attempt to further distinguish the strains from each other. Emphasis was placed on the differential identification of the closely related species *B. cepacia* and *B. gladioli*: the presence of hybrids between these species is of particular interest in CF, as discussed in section 1.2.5.2.2.

3.2 Biochemical tests: methods.

3.2.1 The API 20 NE system.

The API 20 NE system can only discriminate between *B. cepacia*, *B. mallei*, *B. pickettii* and *B. pseudomallei* from amongst *Burkholderia* spp. These are the common human pathogens from this genus. This system forms a convenient basis for the biochemical identification of members of the genus *Burkholderia*.

The API 20 NE system was used in accordance with the manufacturer's instructions. A culture of each strain on MHA, incubated at 37°C for 48 hours, was used as a source for these tests. The test strips were incubated at 30°C and treated or read in the prescribed manner at 24 and 48 hours. Oxidase, which forms the 21st test in this system, was performed by the Kovacs method (Barrow & Feltham, 1993). A negative result was recorded if no visible colour change occurred within 30 seconds. The results were used for species designation via the manufacturer's scoring system and handbook for interpretation. Also, the result of each biochemical test was recorded for separate comparison between strains.

3.2.2 Carbon Source Utilisation Tests.

The utilisation of seven single carbon sources (α-amylamine, 2,3-butylene glycol, *m*-hydroxybenzoate, levulinate, mesaconate, tryptamine and D(-)-tartrate) indicated in the literature as distinguishing between *B. cepacia* and *B. gladioli* (Palleroni, 1984) was investigated using a solid minimal medium as described by Sands (Sands, 1990).

The tests were conducted on a solid medium containing a mineral base with the single carbon source of interest at a concentration of 0.1% w/v. The medium used was medium C of Dye (Dye, 1968), omitting the yeast extract and bromocresol purple and solidified with 1.2% w/v of a purified agar such as Noble agar (Noble, 1950). Tests included both negative and positive controls. The negative control contained no carbon source to prove dependence upon the carbon source added to the test plate for growth. The positive control contained 0.1% w/v D-glucose (a carbon source known to be utilised by both *B. cepacia* and *B. gladioli*). Additionally, carbon sources (adonitol and citraconate) which both *B. cepacia* and *B. gladioli* should be capable of utilising alone were included (Palleroni, 1984). The inclusion of growth factors was not considered necessary: none are described for *B. cepacia* and *B. gladioli* (Stanier, Palleroni & Doudoroff, 1966).

Constituent	Quantity
$NH_4H_2PO_4$	0.5g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	5.0g
Carbon source	1.0g
carbon sources:	
levulinate; mesaconate; D(-)-tartrate; 2,3-butylene glycol; adonitol; tryptamine; α-amylamine; citraconate;. m-hydroxybenzoate; D-glucose.	
Noble Agar (Difco)	12.0g
Distilled Water	1000ml

Table 3.1 Medium for carbon source utilisation tests.

Constituents (table 3.1) excepting the carbon source were mixed in an appropriate vessel, adjusted to pH 7.2 and autoclaved at 121°C/15psi for 15 minutes to sterilise. The medium was cooled to 50°C and the carbon source added in 0.2µm filter-sterilised aqueous solution. The prepared medium was mixed, poured into 40mm diameter Petri dishes in 10ml aliquots, solidified and stored at 4°C until use.

Strains were inoculated onto this medium from a 48-hour culture on MHA. Single-use, sterile disposable plastic inoculating loops were used for this and subsequent inoculations involving the carbon source media to avoid the transfer of any carbon matter that may occur with a flame-sterilised platinum loop.

Each strain was serially transferred three times on the medium to verify growth on the carbon source included. This procedure was repeated for each carbon source tested and ensured the absence of false positive results due to nutrient carryover from the MHA to the carbon source medium (Stanier *et al.*, 1966). Carbon source test plates were incubated at 30 °C and observed daily from the third day of incubation of the first plate. This longer initial incubation ensured the complete utilisation of any nutrients transferred with the inoculum from the primary culture. Serial transfer was effected on the first observation of growth thereafter. A positive result was only declared when serial transfer to a third plate of carbon source medium resulted in growth. If no growth was observed, the test procedure was repeated to confirm the result.

3.2.3 OFPBL Medium and Production of Acid from Sugars.

The production of acid from D-lactose and D-maltose was investigated using OFPBL medium (table 3.2) (Welch *et al.*, 1987). Acid production from these sugars has been noted to yield opposing results for *B. cepacia* and *B. gladioli*. Acid production from the sugars adonitol, D(+)-cellobiose, melibiose, salicin, sucrose and D(+)-trehalose, reported in the literature as yielding opposing results for *B. cepacia* and *B. gladioli* (Yabuuchi *et al.*, 1992) was also investigated using OFPBL medium as a convenient solid oxidation/fermentation test medium, but omitting the antibiotics.

Constituent	Quantity
Basal OF medium (Difco)	9.4g
containing:	
Tryptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Bromothymol Blue	0.08g
Bacto Agar	2.0g
Sugar (see text)	10.0g
Polymyxin B sulphate	300,000 U
Bacitracin	200U
Technical Agar No. 3	15.0g
Distilled Water	1000ml

Table 3.2 Composition of OFPBL medium.

Constituents (table 3.2), excepting antimicrobials, were mixed in an appropriate vessel, adjusted to pH 6.8 (yielding a green-coloured medium) and autoclaved at 121°C/ 15psi for 15 minutes to sterilise. The sterilised medium was cooled to about 50°C before addition of the 0.2μm filter-sterilised antimicrobials. The medium was then mixed, poured into 85mm diameter Petri dishes in 20ml aliquots solidified and stored refrigerated at 4°C. The antibiotic containing variants of this media were used within 60 days of preparation.

Strains were inoculated onto this medium from a 24-hour culture on MHA and incubated at 37°C for 24 hours. After incubation, the plates were examined. A positive result was indicated by the development of a yellow colour in the medium. The development of a blue colour in the medium was indicative of a negative result. A yellow colour in the colony is not necessarily indicative of a positive result in the test under question: it may be due to the pH of the cells themselves.

3.2.4 Lysine Decarboxylase Test.

It has been reported that the LDC test has the potential to differentiate between *B. cepacia* and *B. gladioli* (Simpson *et al.*, 1994). It has also been reported that various centres' epidemic CF isolates identified as *B. cepacia* give atypical results in this test (Welch *et al.*, 1987) as discussed above (section 1.2.5.2.1).

The obligately aerobic nature of some pseudomonads and former pseudomonads precludes the use of many media for the testing of amino acid decarboxylase activity. Commonly used media such as that of Moeller require an anaerobic environment which would be unsuitable for *Burkholderia* spp. (Moeller, 1955). Maccani reported the use of an aerobically incubated agar based medium for amino acid decarboxylase testing (Maccani, 1979). A similar medium was used here to investigate the production of cadaverine from lysine (the product of LDC activity).

Constituent	Quantity
D-Glucose	4.0g
Yeast Extract	3.0g
Bacto-Peptone	1.0g
L-Lysine Hydrochloride	7.0g
Bromocresol Purple	0.02g
Agar	24.0g
Distilled Water	1000ml

Table 3.3 Composition of the LDC test medium.

Constituents (table 3.3) were mixed in an appropriate vessel, adjusted to pH 6.8 and autoclaved at 121°C/15psi for 15 minutes to sterilise. The sterilised medium was mixed, poured into 85mm diameter Petri dishes, allowed to solidify and stored at 4°C until use.

Each strain was inoculated onto a plate of MHA and incubated for 24 hours prior to this test. A quantity of cells was then taken from the plate and suspended in 2.5ml of 0.9% saline to create a visibly turbid suspension. Five 20µl aliquots of a suspension of each strain were used to create 5 well-separated sites of inoculation on a single LDC test plate. Each plate was allowed to dry before it was inverted and incubated at 37°C for 24 hours.

After incubation, the plates were examined. A positive result was indicated by the development of a purple colour in the medium around the sites of inoculation. The development of a yellow colour in the medium around the sites of inoculation was indicative of a negative result.

3.2.5 Lipase Production.

The production of lipases by CF lung isolates of *B. cepacia* is a well documented event. *B. cepacia* is known to produce a variety of lipases (Lonon *et al.*, 1988). A simple plate assay was used to investigate the presence of lipases from all the strains (Kouker & Erich-Jaeger, 1987). The formation of a fluorescent product via the hydrolysis of the trioleoylglycerol component of olive oil in the presence of rhodamine B was used to investigate the presence of lipases from all the strains.

Constituent	Quantity
Tryptone	10.0g
Yeast Extract	5.0g
NaCl	5.0g
Rhodamine B solution 1mg/ml	10.0ml
Extra Virgin Olive Oil	25.0ml
Agar	13.0g
Distilled Water	1000ml

Table 3.4 Composition of the lipase test medium.

Constituents (table 3.4) excepting rhodamine B and olive oil were combined in a bottle twice the capacity of the volume of medium prepared and containing a magnetic stirrer bar. This was autoclaved at 121°C/15psi for 15 minutes to sterilise. Concentrated rhodamine B solution was prepared with distilled water and filter sterilised. The olive oil was sterilised by dry heat. The sterilised base medium was allowed to cool to 60°C with constant stirring, at which point the requisite quantities of rhodamine B solution and olive oil were added. Vigorous shaking ensured the dispersal of the olive oil into an emulsion. The medium was immediately poured into 85mm diameter Petri dishes in aliquots of approximately 20ml and the surfaces gently flamed with an inverted Bunsen burner to remove air bubbles. The bottle of medium was put to stir vigorously whenever not used for pouring.

Strains were inoculated onto this medium from a 24-hour culture on MHA. The plates were then incubated for 48 hours. After incubation, each plate was observed for the presence of a fluorescent product under ultraviolet illumination at 254nm.

3.2.6 Pigment Production.

The production of a brown, melanin-like pigment has been reported as an unusual characteristic of the *B. cepacia* strain identified as the epidemic strain in Edinburgh (Govan *et al.*, 1993). The presence of this pigment was demonstrated utilising a minimal medium (Davis and Mingioli, 1950), fortified with 1% tyrosine. The same technique was previously utilised in the study of pigments produced by *P. aeruginosa* (Ogunnario and Hamilton-Miller, 1975).

Constituent	Quantity
K_2HPO_4	7.0g
KH_2PO_4	3.0g
Trisodium Citrate·$3H_20$	0.5g
$MgSO_4 \cdot 7H_2O$	0.1g
$(NH_4)SO_4$	1.0g
Glucose	2.0g
Agar	15.0g
Distilled Water	1000ml

Table 3.5 Composition of the tyrosine-fortified medium for investigation of melanin-like pigment production by the strains.

Constituents were combined, adjusted to pH 7.0 and autoclaved at 121°C/15psi for 15 minutes to sterilise. The medium was then mixed and poured into 85mm diameter Petri dishes in 20ml aliquots, solidified and stored at 4°C until use.

Strains were inoculated onto this medium from a 24-hour culture on MHA. Additionally, the results obtained were compared with those gained from a similar observation of each strain grown on MHA, although this observation was not made with the plate used for the inoculum mentioned above. Inoculated plates were in all cases incubated at 37°C for 24 hours prior to observation for pigment production.

3.3 Antibiotic Sensitivity Tests and Minimum Inhibitory Concentrations.

Antibiotic sensitivities were investigated using the ATB PSE system, semi-automated using a Vitek inoculator and strip reader. This system assesses susceptibility in accordance with Société Française de Microbiologie (SFM) guidelines. This procedure was conducted by the author of this thesis at the laboratories of SmithKline Beecham (SKB), Brockham Park, Betchworth, Surrey and thanks are extended to Dr. I. N. Simpson for the use of their facilities.

Additionally, the minimum inhibitory concentration (MIC) of selected strains against some common clinically used β-lactam antibiotics was determined (table 3.9). The strains subjected to this procedure were the ones chosen for further investigation in the respect of carbapenemase activity.

The subject of sensitivity testing has been reviewed recently (Brown, 1994), and current guidelines are laid down in the British Society for Antimicrobial Chemotherapy (BSAC) Working Party report (Anonymous, 1990). The MIC of each strain was determined against a number of β-lactam antibiotics in 5ml MHB using 2-fold antibiotic dilutions (the highest concentration used was 64μg/ml in each case) and an inoculum of approximately 10^5 cells/ml. An incubation period of 24 hours at 37°C was used for all tests. This procedure was repeated in the presence of 0.1mM clavulanic acid and again in the presence of 0.1mM tazobactam for isolates 5JIV and NCTC 10661.

Appendix 2 contains a summary of the SFM and BSAC sensitivity test breakpoints for comparison.

3.4 Results.

3.4.1 API 20 NE Identifications.

Use of the API 20 NE system gave a species identification of *B. cepacia* for most (27/32) of the strains (Table 3.6). Some strains (5/32) did not yield an identification from API 20 NE, 3 of which failed to give a positive oxidase test result but would have been identified as *B. cepacia* but for this. Section 3.4.2 considers the results of individual tests apart from this result.

Strain	API no. (& Identification)
NCTC 10661	0467577 (*B. cepacia* 99.9%)
NCTC 17616	1447577 (*B. cepacia* 99.9%)
NCTC 10744	1477577 (*B. cepacia* 99.9%)
ATCC 25417	0067573 (*B. cepacia* 99.9%)
ATCC 10248	0067573 (*B. cepacia* 99.9%)
ATCC 10854	1067573 (*B. cepacia* 99.9%)
LMG 6998	1447577 (*B. cepacia* 99.9%)
5BI	1047577 (*B. cepacia* 94.8%)
5JIV	0067577 (*B. cepacia* 99.9%)
5NVI	5467577 (*B. cepacia* 99.9%)
5OIV	0467537 (No ID in API manual)
5OV	1067577 (*B. cepacia* 99.9%)
5PI	0067577 (*B. cepacia* 99.9%)
5PII	1067577 (*B. cepacia* 99.9%)
5PIV	1004573 (No ID in API manual)
5PVI	1067573 (No ID in API manual)
5QIV	1004576 (No ID in API manual)
5QV	0046577 (*B. cepacia* 94.1%)
5RIV	0046573 (No ID in API manual)
5SI	1047577 (*B. cepacia* 94.8%)
5RI	1477477 (*B. cepacia* 99.9%)
5RII	5467577 (*B. cepacia* 99.9%)
5RIII	0477477 (*B. cepacia* 99.9%)
3843	1047577 (*B. cepacia* 94.8%)
3925	1047577 (*B. cepacia* 94.8%)
4325	1047577 (*B. cepacia* 94.8%)
4326	1047577 (*B. cepacia* 94.8%)
4949	1047577 (*B. cepacia* 94.8%)
9275	1067577 (*B. cepacia* 99.9%)
12794	1047577 (*B. cepacia* 94.8%)
15856	1047577 (*B. cepacia* 94.8%)
5SV	0477777 (*B. cepacia* 99.9%)
5SVI	0047577 (*B. cepacia* 92.2%)
5TI	0046577 (*B. cepacia* 94.1%)
5TII	0047577 (*B. cepacia* 92.2%)
5TIII	0447577 (*B. cepacia* 99.9%)
5TIV	0447577 (*B. cepacia* 99.9%)
5TV	0447577 (*B. cepacia* 99.9%)
J1948	0447577 (*B. cepacia* 99.9%)
J2315	1047577 (*B. cepacia* 94.8%)
J2552	1767777 (No ID in API manual)

Table 3.6 API 20 NE identification of the strains.

3.4.2 Biochemical Tests.

The biochemical test results are best considered with reference to the strain groupings elicited from identification by FAME profile (MIS), FAME profile cluster analysis, chromosomal DNA analysis by PFGE and OMP profile (chapters 4, 5 and 6 respectively). Therefore, results have been displayed as the percentage of positive results gained for the strains in each of these groups (table 3.7). Full results for individual strains can be found in appendix 1.

3.4.2.1 API 20 NE Biochemical Tests.

The results of individual tests from the API 20 NE portfolio have been included in table 3.7 with the results of other tests. The results of the assimilation tests are worthy of further note. Of the strains in this study, four (5PIV, 5PVI, 5RIV and 5TI) could not utilise a single nutrient of those tested using the minimal medium described in section 3.2.2. Within this group, all can utilise at least some of the carbon sources used in the assimilation tests of the API 20 NE system.

The API 20 NE assimilation tests are conducted in AUX medium. This contains 2.0g/l $(NH_4)_2SO_4$ 1.5g/l agar, 82.8mg/l mineral base, 250.0mg/l amino acids and 35.9mg/l trace nutrients, in 0.04M pH 7.1 phosphate buffer. Therefore, if a group of *B. cepacia* isolates contains any auxotrophic strains, as would appear possible in a sample containing clinical isolates (Barth & Pitt, 1995), this characteristic may not be detected. For the purposes of this research, the PFGE cluster (cluster 4b; figure 5.2, page 100) containing these and several other strains with apparent metabolic deficiencies does seem to show some narrowing of nutritional diversity.

3.4.2.2 Carbon Source Test Results.

Carbon source tests using the solid minimal medium described in section 3.2.2 revealed the nutritional diversity inherent to this taxonomic niche. The exception to this lies in PFGE group 4b, where there appears to be a tendency towards a lesser ability to utilise such a wide array of carbon sources.

3.4.2.3 Acid Production from Sugars.

Acid production from the sugars tested amongst these isolates was most widely prevalent with the sugars D(+)-cellobiose, D-lactose and D-maltose. Acid production from all sugars (with the marginal exception of sucrose) was less in FAME cluster 3, PFGE cluster 4, OMP group 2, and when MIS identified the isolate as *B. gladioli*. Figure 3.1 shows some typical results with this medium.

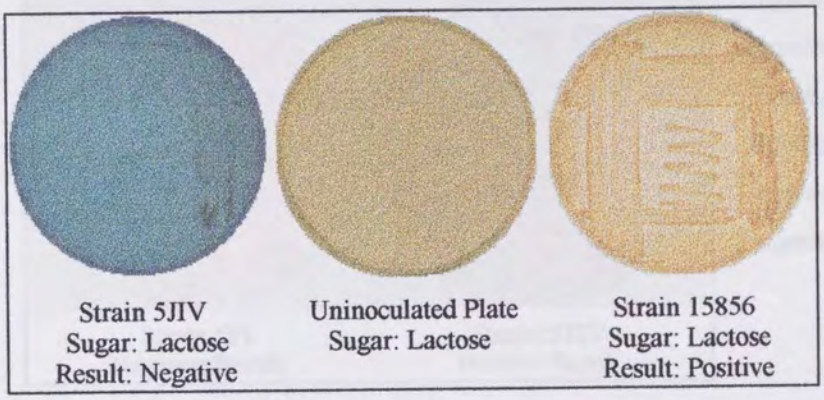


Figure 3.1 Some typical results with the OF test medium. An uninoculated plate (middle) has been included for comparison.

Growth on OFPBL medium revealed a group of strains comprising both some of those identified by MIS (chapter 4) as *B. gladioli* and some of those identified by the same technique as *B. cepacia* (strains 5PIV, 5QIV, 5QV, 5RIV, 5TI, 5TII, 5TIII, and 5PVI) that did not produce acid from D-lactose or D-maltose and adopted a very pale colonial appearance when grown in this manner. These strains were subsequently found to be quite closely related by PFGE. Furthermore, all but strains 5QV and 5TIII fall into subgroup b of PFGE cluster 4.

3.4.2.4 Pigmentation.

Growth on the tyrosine-fortified medium revealed a group amongst these isolates that produced the red-brown pigment described in section 1.2.5.1.5. These strains (appendix 1) were typically associated with FAME cluster 3, PFGE cluster 4, OMP group 2, and an identification by MIS of *B. gladioli*. Figure 3.2 shows some typical results for this medium.

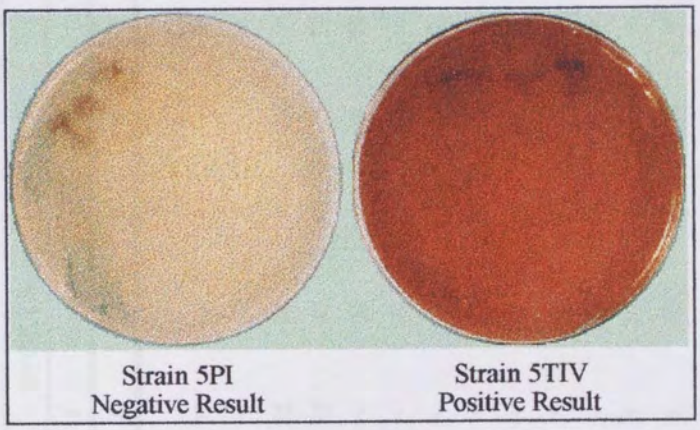


Figure 3.2 Typical results with the tyrosine-fortified medium for the investigation of pigment production by the strains. An uninoculated plate is very similar in appearance to the negative result (left hand plate).

Pages 78 and 79: **Table 3.7** Biochemical test results for the strains in the groupings observed by subsequent investigation by the techniques outlined in chapters 4, 5 and 6. Full results for individual isolates are shown in appendix 1.

	FAME Cluster				PFGE Cluster							Species by MIS		OMP Group	
	1	2	3	4	1	2	3	4 (total)	4 (a)	4 (b)	4 (c)	*B. cepacia*	*B. gladioli*	1	2
Number of isolates:	12	4	12	6	3	7	4	17	5	8	4	22	12	25	15
Utilisation of:															
N-acetyl-glucosamine	83	+	+	+	+	+	75	+	+	+	+	+	+	+	+
Adipate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adonitol	83	+	67	+	+	+	75	76	+	50	+	77	+	+	67
α-amylamine	58	25	42	17	33	29	25	47	60	25	75	36	50	36	40
Arabinose	+	+	67	83	+	+	+	71	+	50	75	77	+	+	75
2,3-butylene glycol	+	75	67	83	+	+	+	71	+	38	+	82	+	+	73
Caprate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citraconate	+	+	67	50	67	86	+	71	+	38	+	77	83	88	73
Citrate	+	+	+	83	+	+	+	+	+	88	+	+	+	+	+
Gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose (media)	+	+	67	+	+	+	+	76	+	50	+	86	+	+	73
D-glucose(API20NE)	+	+	+	83	+	+	+	88	+	75	+	+	+	+	+
Levulinate	83	+	58	83	+	86	+	65	80	38	+	77	83	80	67
m-hydroxybenzoate	+	+	42	67	+	+	+	41	80	38	–	68	75	80	53
Malate	+	+	+	+	+	+	+	+	+	88	+	+	+	+	+
D-maltose	–	–	–	–	–	–	–	–	20	–	–	–	–	–	–
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	83	+	+	+	88	+	75	+	+	+	+	+
Mesaconate	50	50	–	17	33	29	75	–	–	–	25	36	–	44	–
Phenyl-acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(-)-tartrate	75	+	17	67	+	86	75	18	20	–	50	77	–	80	27
Tryptamine	+	50	25	67	67	86	+	29	20	–	+	77	83	80	67

+ = ⩾90% of results positive; – = ⩽10% of results positive; percentage shown otherwise.

	FAME Cluster				PFGE Cluster							Species by MIS		OMP Group	
	1	2	3	4	1	2	3	4 (total)	4 (a)	4 (b)	4 (c)	*B. cepacia*	*B. gladioli*	1	2
Number of isolates:	12	4	12	6	3	7	4	17	5	8	4	22	12	25	15
Acid production from:															
Adonitol	–	25	–	–	–	29	–	18	40	13	–	14	–	20	–
D(+)-cellobiose	83	75	–	67	+	+	+	–	20	–	–	68	15	72	13
Glucose	–	–	–	–	–	–	–	–	–	–	25	–	–	–	–
D-lactose	+	75	17	67	+	+	+	24	60	13	–	73	38	72	38
D-maltose	+	75	17	67	+	+	+	24	60	13	–	73	38	72	38
Melibiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	–	–	–	–	20	–	–	–	–	–	–
Sucrose	25	25	33	17	–	14	25	35	60	13	50	47	–	16	44
D(+)-trehalose	58	50	25	67	+	71	75	24	60	13	–	55	31	44	25
Other tests:															
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Esculin hydrolysis	42	–	42	17	–	–	25	41	60	25	50	18	58	28	50
β-galactosidase	75	25	25	–	–	14	+	29	20	25	50	36	42	52	31
Gelatin hydrolysis	17	–	–	–	–	–	25	–	20	–	–	–	–	–	13
Indole production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lysine decarboxylase	67	75	50	83	+	72	+	47	20	63	50	+	17	68	50
Nitrate reduction	75	75	25	83	+	+	75	29	20	38	25	68	42	80	25
Oxidase	+	75	83	+	+	+	+	88	+	75	+	+	+	88	88
Urease	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pigment production	17	–	67	33	–	14	–	71	+	38	+	14	83	12	56

+ = ⩾90% of results positive; – = ⩽10% of results positive; percentage shown otherwise.

3.4.2.6 Lipase Production.

Lipase detection via the production of fluorescent products with rhodamine B upon the hydrolysis of olive oil showed all the strains to possess lipase activity. This was more marked in some than in others, apart from the strains' differing potential for growth on this medium (figure 3.3). Generally, the strains that produced a red/brown exocellular pigment appeared to be associated with less fluorescence when viewed by eye. Such strains are frequently proven by genetic typing to be closely related to the CF epidemic strain which generally lacks C14 lipase in its exocellular products, whilst producing a red/brown pigment. (Govan *et al.*, 1993). Whether this reduction in fluorescence is due to a lack in exocellular C14 lipase or a straightforward obstruction of fluorescence by the pigment is open to question.

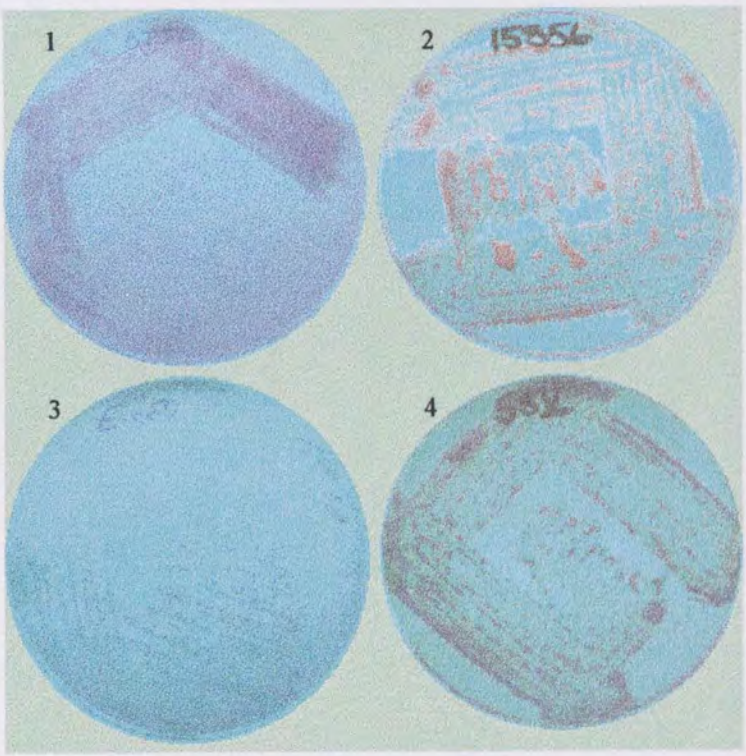


Figure 3.3 Examples of the lipase test medium, viewed under ultraviolet light. Plates 1 and 3 are cultures of *P. aeruginosa* NCTC 6750 and *E. coli* NCTC 10418, as positive and negative controls respectively. Plate 2 contains a culture of 15856, showing a marked positive response, and plate 4 contains a culture of 5SV, a pigmented strain, showing a markedly less prominent positive result.

3.4.3 Antibiotic Sensitivity Tests.

Antibiotic sensitivity tests revealed widespread resistance to ticarcillin (with or without clavulanic acid), cefsulodin, imipenem, the aminoglycosides, colistin and fosfomycin (Table 3.8). More prevalent susceptibility was observed with piperacillin (marginally enhanced by the addition of tazobactam), ceftazidime and co-trimoxazole. Resistance to imipenem was widespread, with only 1 isolate displaying full susceptibility. Strains identified as *B. gladioli* by MIS were highly resistant, more so than those identified as *B. cepacia*, and included several that were not sensitive to any antibiotic included in this study. The difference in resistance to co-trimoxazole between the group identified as *B. cepacia* and that identified as *B. gladioli* provided the most striking illustration of this. These strains fell mostly within FAME cluster 3, PFGE cluster 4 and OMP group 2.

Table 3.8 Antibiotic sensitivities of the strains (continued on following page).

Strain	Antibiotic														FAME	PFGE	MIS	OMP
	TIC	TCC	PIP	CFS	TZP	IMI	CAZ	ATM	AMG	COL	FOS	PEF	CIP	COT	cluster	cluster	species	group
5RIII	R	R	R	R	R	R	I	R	R	R	R	I	I	I	1	–	*B. cepacia*	1
5RII	R	R	S	R	S	R	S	I	R	R	R	I	S	S	1	–	*B. cepacia*	1
J2552	S	S	S	R	S	S	S	S	*	R	R	S	S	S	1	–	*B. cepacia*	1
4325	R	R	S	R	S	R	S	I	R	R	R	R	R	S	2	1	*B. cepacia*	1
4949	R	R	S	R	S	R	S	S	R	R	R	S	S	S	1	1	*B. cepacia*	1
3843	R	R	R	R	I	R	S	I	R	R	R	R	R	S	4	1	*B. cepacia*	1
4326	R	R	S	R	S	R	S	S	R	R	R	R	R	S	4	2	*B. gladioli*	1
3925	R	R	R	R	S	R	S	I	R	R	R	I	I	S	2	2	*B. cepacia*	1
5OV	R	R	S	R	S	R	S	I	R	R	R	I	S	S	1	2	*B. cepacia*	1
12794	R	R	R	R	I	R	I	R	R	R	R	R	R	S	4	2	*B. gladioli*	1
15856	R	R	R	R	I	R	S	R	R	R	R	R	R	S	4	2	*B. cepacia*	1
5SI	R	R	S	R	S	R	S	S	R	R	R	S	S	S	1	2	*B. cepacia*	1
5PII	S	R	S	R	S	R	S	S	R	R	R	I	S	S	1	3	*B. cepacia*	1
5PI	S	S	S	R	S	S	S	S	R	R	R	S	S	S	–	3	*B. cepacia*	1
9275	R	R	S	R	S	R	S	S	R	R	R	R	R	S	1	3	*B. cepacia*	1
5RI	R	R	R	R	I	I	I	R	R	R	R	R	R	S	1	3	*B. cepacia*	2

* Variable results for the different aminoglycosides tested: tobramycin=I; amikacin=S; gentamicin=R; netilmicin=I.
TIC=ticarcillin; TCC=ticarcillin+clavulanic acid; PIP=piperacillin; CFS=cefsulodin; TZP=piperacillin+tazobactam; IMI=imipenem; CAZ=ceftazidime; ATM=aztreonam; AMG=aminoglycosides (tobramycin, amikacin, gentamicin & netilmicin); COL=colistin; FOS=fosfomycin; PEF=perfloxacin & other 4-quinolones; CIP=ciprofloxacin; COT=co-trimoxazole.

Strain	Antibiotic														FAME	PFGE	MIS	OMP
	TIC	TCC	PIP	CFS	TZP	IMI	CAZ	ATM	AMG	COL	FOS	PEF	CIP	COT	cluster	cluster	species	group
5SVI	R	R	S	R	S	R	S	I	R	R	R	R	R	I	3	4 (a)	*B. cepacia*	2
5TV	R	R	R	R	I	R	S	I	R	R	R	R	R	R	3	4 (a)	*B. gladioli*	2
5SV	R	R	R	R	R	R	I	R	R	R	R	R	R	I	3	4 (a)	*B. gladioli*	2
J1948	R	R	R	R	I	R	S	R	R	R	R	R	R	I	3	4 (a)	*B. gladioli*	2
J2315	R	R	R	R	R	R	I	R	R	R	R	R	R	R	1	4 (a)	*B. gladioli*	2
5OIV	R	R	R	R	R	R	I	R	R	R	R	R	R	I	–	4 (b)	*B. gladioli*	2
5TIV	R	R	R	R	R	R	I	R	R	R	R	R	R	I	3	4 (b)	*B. gladioli*	2
5TII	R	R	R	R	I	R	S	I	R	R	R	R	R	I	3	4 (b)	*B. cepacia*	2
5PIV	R	R	S	R	S	I	S	R	R	R	R	R	R	S	3	4 (b)	*B. cepacia*	2
5RIV	R	R	S	R	S	I	S	R	R	R	R	R	R	S	3	4 (b)	*B. cepacia*	2
5PVI	R	R	S	R	S	I	S	R	R	R	R	R	R	S	3	4 (b)	*B. gladioli*	2
5TI	R	R	R	R	R	R	R	R	R	R	R	R	R	I	3	4 (b)	*B. cepacia*	2
5NVI	R	R	R	R	S	R	S	I	R	R	R	R	R	I	3	4 (c)	*B. gladioli*	1
5JIV	R	R	S	R	S	R	S	I	R	R	R	R	R	I	1	4 (c)	*B. gladioli*	1
5TIII	R	R	S	R	S	R	S	I	R	R	R	R	R	I	4	4 (c)	*B. gladioli*	2
5QV	R	R	R	R	I	R	I	1	R	R	R	R	R	R	3	4 (c)	*B. cepacia*	2
5BI	Strain died in storage at SmithKline Beecham														2	2	*B. cepacia*	1
5QIV	Strain died in storage at SmithKline Beecham														4	4 (b)	*B. cepacia*	1

Notes: TIC=ticarcillin; TCC=ticarcillin+clavulanic acid; PIP=piperacillin; CFS=cefsulodin; TZP=piperacillin+tazobactam; IMI=imipenem; CAZ=ceftazidime; ATM=aztreonam; AMG=aminoglycosides (tobramycin, amikacin, gentamicin & netilmicin); COL=colistin; FOS=fosfomycin; PEF=perfloxacin & other 4-quinolones; CIP=ciprofloxacin; COT=co-trimoxazole

Those strains for which MICs were measured showed widespread resistance to the antimicrobials tested, consistent with the results of the ATB PSE system (table 3.9). MIC values indicative of resistance were taken as those results not less than the breakpoint value quoted in the BSAC guide to sensitivity testing. These were: ceftazidime: >2μg/ml; imipenem, meropenem and biapenem: >4μg/ml; aztreonam: >8μg/ml; piperacillin and azlocillin: >16μg/ml.

Carbapenem resistance was observed in all but one isolate, 5QIV, for all 3 carbapenems tested. This group of strains also showed considerable resistance to every antibiotic tested, including ceftazidime.

Antibiotic	Strain: MIC(μg/ml)								
	5JIV	5NVI	5QIV	5QV	5RI	5RII	5RIII	5RIV	NCTC 10661
Imipenem	>64	>64	2	>64	16	32	32	32	8
Meropenem	32	32	16	32	16	>64	32	4	8
Biapenem	32	>64	8	>64	16	16	16	4	4
Ceftazidime	>64	64	8	64	>64	>64	64	32	>64
Aztreonam	>64	>64	32	>64	>64	>64	>64	>64	>64
Piperacillin	>64	>64	8	>64	>64	>64	>64	>64	>64
Azlocillin	>64	>64	16	>64	>64	>64	>64	64	>64

Table 3.9 MIC values for the strains shown against a variety of clinically utilised β-lactams.

The addition of a fixed 0.1M concentration of one of the β-lactamase inhibitors clavulanic acid or tazobactam did not render either isolate 5JIV or the laboratory strain NCTC 10661 sensitive to imipenem. The addition of clavulanic acid as indicated did reduce the MIC of imipenem against NCTC 10661 from 8μg/ml to 4μg/ml, but the MIC of imipenem against isolate 5JIV was not reduced to less than 64μg/ml by either β-lactamase inhibitor (table 3.10).

Antibiotic	Strain: MIC (μg/ml)	
	5JIV	NCTC 10661
Imipenem + 0.1M Clavulanic acid	>64	4
Imipenem + 0.1M Tazobactam	>64	>64

Table 3.10 The effect of adding one of two β-lactamase inhibitors to the clinical strain 5JIV and *B. cepacia* NCTC 10661 at a fixed concentration.

4. Analysis of Cellular Fatty Acids.

4.1 Introduction.

As described in 1.2.5.2.2, fatty acid analysis in bacteria (via derivatisation to the corresponding FAME) has uses in both identification and taxonomy. Both approaches were utilised in this study. The former permitted the further confirmation of strain identity and the latter allowed a more detailed classification of the strains.

4.2 Methods.

4.2.1 Strain Identification via Fatty Acid Analysis and the Microbial Identification System.

FAME profiles were analysed using the commercially available MIS (currently marketed as Sherlock). This system couples standard conditions for cultivation and harvesting of cells to a similar procedure for fatty acid extraction and derivatisation to that outlined below in section 4.3. Automated GC analysis of the resulting extracts (with reference to a standard FAME mix) permits comparison of the profiles gained with those of a prepared database. Microbial identification is made by matching the profile to entries in the database (White *et al.*, 1988; Sasser, 1990).

This procedure was generously undertaken by staff at SKB, Worthing, UK. Grateful thanks are extended to Dr. I. N. Simpson of SKB, Brockham Park, Betchworth, Surrey, UK for arranging this. NA slopes of the strains were submitted to SKB and the printed results returned. This permitted exploitation of the MIS database for microbial identification, which would not otherwise have been possible.

4.2.2 Fatty Acid Extraction and Analysis.

An alkaline hydrolysis method was utilised to extract fatty acids from whole cells. This method excludes those fatty acids that are amide-linked to the diglucosamine component of lipid A: these require harsher acidic conditions for liberation. Free fatty acids liberated by alkaline hydrolysis were derivatised to the methyl esters and analysed by (GC).

4.2.2.1 Fatty Acid Extraction and Derivatisation.

All glassware and caps including adaptors for the rotary evaporator were prepared by immersion in a proprietary detergent overnight, followed by rinsing alternately with hot deionised water, acid and cold deionised water. Contact between plastic and all reagents used in the following procedure was not permitted. This prevented contamination of the extracts with plasticisers.

Cells of each *B. cepacia* strain were cultivated for 24 hours on agar plates of trypticase soya broth agar (TSBA, produced by preparation of a commercially available TSB according to the manufacturer's instructions and the addition of 1.5%w/v agar). About 50mg wet weight of cells from each strain was taken from the plate using a flamed wire inoculating loop and transferred to separate 10ml pyrex glass hydrolysis tubes, sealable with teflon-lined caps. Two samples for treatment were created thus for each strain.

Cells were then mixed by vortexing the tightly sealed hydrolysis tubes with 1ml of 3.8M NaOH in 50% aqueous methanol, sealed and incubated at 100°C for 5 minutes using a covered bath of boiling water. Each sample was removed from the water bath and carefully vortexed once more to ensure complete homogenisation of cells and reagent. Then, the homogenised sample was incubated in the same manner at 100°C for a further 25 minutes. After this period had elapsed, the samples were removed and left to cool to room temperature.

Methyl esters of the free fatty acids liberated in the preceding step were formed by the addition of 6ml of a solution of 6M HCl and methanol (1:1) and heating at 80°C for exactly 10 minutes in a covered water bath. After heating, samples were removed and allowed to cool to room temperature once more.

FAMEs were extracted from these preparations with 1ml hexane/diethyl ether (1:1). This solvent mixture was combined with the aqueous product from the preceding methylation step by repeated inversion of the sealed glass tubes for 10 minutes to allow partitioning of the FAMEs from the aqueous phase to the organic phase. Samples thus combined were allowed to stand for 5 minutes to permit the aqueous and organic phases to form 2 separate layers.

The upper solvent layers from the two samples prepared from each strain were carefully removed and combined in a similar sealable glass tube containing 3ml of 0.3M NaOH. These were mixed for 10 minutes by repeated inversion. The organic phase was recovered after centrifugation (5000×g for 10 minutes at 10°C) and transferred to a 2ml glass sample tube with a foil lined cap. This procedure extracts any unwanted residues present back from the organic phase into the aqueous phase.

The organic solvent was evaporated to a volume of about 50µl by the passage of nitrogen gas into the tube at room temperature. This procedure removes the diethyl ether from the solvent system, leaving the extracted FAMEs in hexane alone. The completed preparation was sealed in these tubes under nitrogen and stored at –20°C until analysis. This procedure was repeated on 3 separate cultures of *B. cepacia* NCTC 10661 and *B. gladioli* ATCC 25417 to confirm the reproducibility of this technique.

4.2.2.2 Chromatographic Analysis of Fatty Acid Methyl Ester Profiles.

1µl of each sample was loaded in turn onto a Hewlett Packard HP-1 capillary column (crosslinked methyl silicone gum, ID 0.32mm, film thickness 0.17µm, length 25m) on a Unicam 610 series GC operating with 1:50 sample splitting and flame ionisation detector. The gas phase comprised helium at 6.5psi with nitrogen as the makeup gas. The flame ionisation detector used hydrogen and air, the injector temperature was set at 200°C and the detector temperature was set at 280°C. The GC was controlled by Microsoft Windows-compatible software (Unicam) that permitted integration of the peaks and storage of both the integration table and the detector trace. Fatty acids were identified by comparison of retention times with those of a standard bacterial FAME mix containing 25 bacterial FAMEs prepared according to manufacturer's instructions (Hewlett-Packard). Integration readings were calculated for each peak and expressed as a percentage of the total peak integration.

4.2.2.3 Numerial Analysis of Fatty Acid Methyl Ester profiles.

The areas of the 9 peaks comprising the FAME profiles of the strains (figure 4.1) were expressed in terms of a percentage of total peak area. Statistical analysis was carried out using Unistat (Unistat Ltd.), running in unison with Microsoft Excel 5.0. Levels of similarity between FAME profiles was calculated by coefficients based on Euclidean distance and clustering of isolates was then achieved with the unweighted pair-group mean of arithmetic averages (UPGMA) method (Rainey, 1994). The result of this process was displayed as a dendrogram.

4.3 Results.

4.3.1 Microbial Identification System Identification of the Strains.

Fatty acid analysis by MIS gave an identification of *B. cepacia* for 20 of the strains; the remaining 12 strains were identified as *B. gladioli* (Table 4.1). Those strains identified as *B. gladioli* typically possessed a fatty acid profile containing a higher percentage of fatty acids C17:0Δ and C19:0Δ than those identified as *B. cepacia*. Additionally, 11/12 of those strains identified as *B. gladioli* produced trace quantities of C10:0 3-OH whereas all of those identified as *B. cepacia* did not. Previous studies have confirmed the ability of MIS to distinguish between ATCC/NCTC strains of *B. cepacia* and *B. gladioli* (Simpson *et al.*, 1994).

Strain	Fatty acid identification (probability index)	Presence of C10:0 3-OH	Amount of C17:0 Δ (%)	Amount of C19:0 Δ (%)
5RI	*B. cepacia* (0.079)	ND	2.01	0.52
5RII	*B. cepacia* (0.775)	ND	4.37	2.32
5RIII	*B. cepacia* (0.889)	ND	4.08	1.79
5OV	*B. cepacia* (0.210)	ND	2.62	0.00
5PI	*B. cepacia* (0.449)	ND	2.20	0.99
5PII	*B. cepacia* (0.656)	ND	2.31	0.86
5SI	*B. cepacia* (0.504)	ND	3.62	1.12
J2552	*B. cepacia* (0.305)	ND	1.19	0.23
5SVI	*B. cepacia* (0.571)	ND	10.96	4.59
5PIV	*B. cepacia* (0.486)	ND	9.64	5.08
5QIV	*B. cepacia* (0.361)	ND	13.14	3.96
5QV	*B. cepacia* (0.268)	ND	11.50	4.18
5RIV	*B. cepacia* (0.187)	ND	12.51	3.30
5TI	*B. cepacia* (0.555)	ND	6.82	2.42
5TII	*B. cepacia* (0.299)	ND	14.94	4.60
5BI	*B. cepacia* (0.327)	ND	4.40	0.97
3843	*B. cepacia* (0.392)	ND	4.03	0.81
3925	*B. cepacia* (0.762)	ND	2.46	0.00
4325	*B. cepacia* (0.467)	ND	6.84	2.76
4949	*B. cepacia* (0.863)	ND	1.43	0.61
9275	*B. cepacia* (0.652)	ND	2.33	1.12
15856	*B. cepacia* (0.175)	ND	13.03	2.86
5JIV	*B. gladioli* (0.758)	ND	9.44	5.22
5NVI	*B. gladioli* (0.577)	D	12.03	5.60
5SV	*B. gladioli* (0.656)	D	16.14	6.54
J1948	*B. gladioli* (0.397)	D	15.78	8.95
J2315	*B. gladioli* (0.533)	D	17.48	11.10
5OIV	*B. gladioli* (0.535)	D	18.58	8.28
5PVI	*B. gladioli* (0.396)	D	19.33	8.89
5TIII	*B. gladioli* (0.652)	D	11.99	6.52
5TIV	*B. gladioli* (0.555)	D	16.98	7.42
5TV	*B. gladioli* (0.741)	D	15.56	6.81
4326	*B. gladioli* (0.147)	D	26.50	11.55
12794	*B. gladioli* (0.500)	ND	7.99	2.22

Table 4.1 Identification of the strains via FAME analysis by MIS and the detection of C10:0 3-OH, C17:0 Δ and C19:0 Δ by this technique. (D=detected; ND=not detected. C17:0Δ and C19:0Δ are expressed as a percentage of total FAMEs)

4.3.2 Fatty Acid Methyl Ester Profile Analysis.

Those strains included in this study possessed similar FAME profiles of 9 peaks on GC analysis (figure 4.1). The major FAMEs present in these strains are C16:0, C17:0Δ, C18:1 *cis*-9 & *trans*-11, and C19:0Δ, with the other types shown in table 4.2 present in much smaller amounts. The unidentified FAME peak (retention time 14.7 minutes) shown in figure 4.1 as present in the sample but not in the standard is possibly C16:0 3-OH. Such a FAME would be more polar than either C16:0 or C16:0 2-OH, both of which are present in the sample. A peak corresponding to C15:0 3-OH would therefore have a correspondingly greater retention time compared to these. A similar situation is illustrated by the peaks corresponding to C14:0, C14:0 2-OH and C14:0 3-OH FAMEs present in the standard trace in figure 4.1.

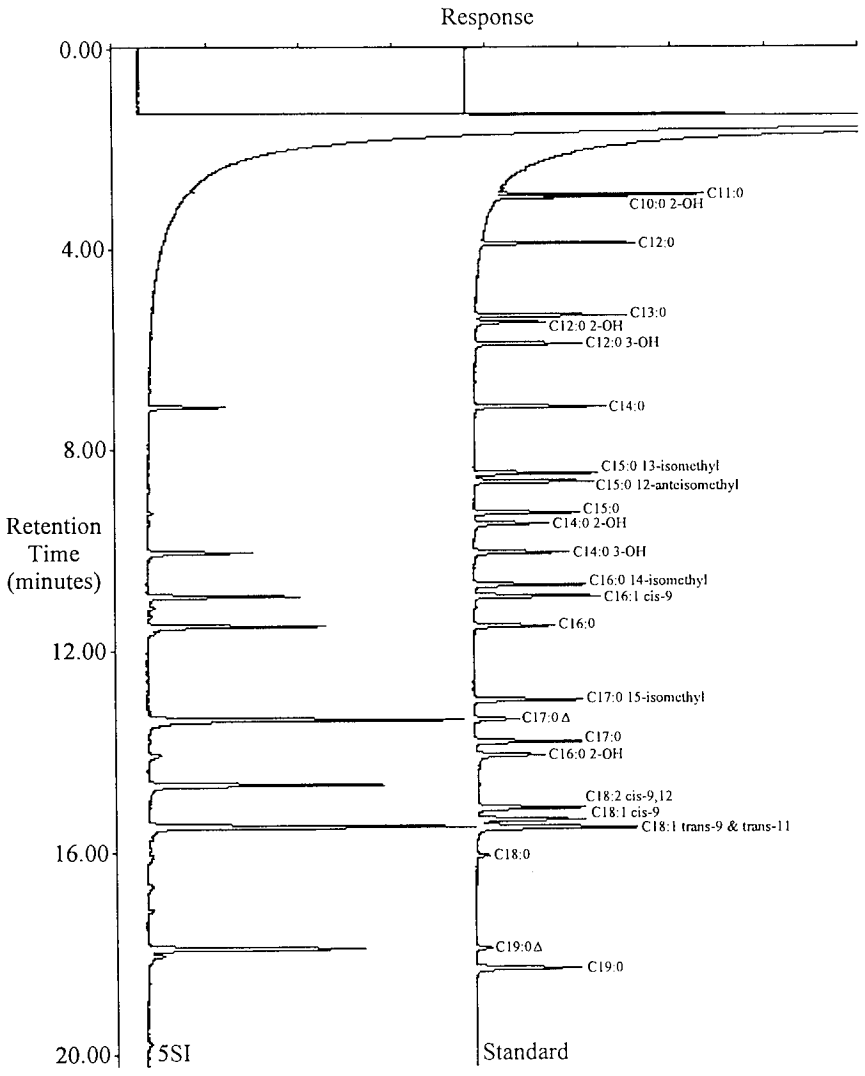


Figure 4.1 GC trace of a typical FAME profile from the group of strains studied, that from strain 5SI, together with the trace produced by GC analysis of the FAME standard mixture.

Numerical analysis of percentage peak areas grouped the strains into four clusters at a distance of 12 Euclids (figure 4.2). From the top to bottom in figure 4.2, these comprise 5SI to 5RI; 5BI to ATCC 25417 (c); 5SV to 5TIV and 5QIV to 3843. The mean percentage peak areas are shown in table 4.2 and seem to correspond to a decrease in levels of C14:0 3-OH and C18:1 *cis*-9 and *trans*-11 FAMEs. Beneath the 12 Euclid level, as one moves to smaller distances, the strains become progressively more divided. The only samples linked below 4 Euclids are cases where more than one sample was prepared from the same strain. The close linkage of such samples demonstrates the reproducibility of the technique.

The 2 laboratory strains, *B. cepacia* NCTC 10661 and *B. gladioli* ATCC 25417, which are members of clusters 1 and 2 respectively, link at a distance of about 13.5 Euclids. Clusters 1 and 2 do not link with clusters 3 and 4 until a distance of 19.5 Euclids. Therefore, the former and latter pair of clusters are linked only at more distant levels, a feature worthy of further investigation. Strains 5OIV and 5PI were considered too distant to cluster 1 for inclusion therein.

Table 4.2 FAME profiles of the bacterial isolates grown on trypticase soya broth agar. The strains can be divided into 4 major clusters at a distance of 12 Euclids, as described in the text.

Peak Identity:	C14:0	C14:0 3-OH	C16:1	C16:0	C17:0Δ	C16:0 2-OH	C16:0 3-OH ?	C18:1 *cis*-9 & *trans*-11	C19:0Δ
Retention Time (minutes):	7.158	10.046	10.925	11.575	13.413	14.067	14.671	15.483	17.904
Cluster	Mean % Peak Area (sample standard deviation)								
1	5.094	3.163	5.392	29.409	17.464	1.318	4.115	17.420	16.693
	(1.551)	(1.125)	(1.981)	(3.709)	(2.091)	(0.698)	(1.635)	(2.402)	(3.343)
2	5.474	3.448	6.893	37.032	20.280	0.949	4.287	12.368	9.273
	(0.625)	(0.948)	(2.764)	(1.284)	(2.059)	(0.395)	(0.856)	(1.917)	(3.314)
3	4.321	2.911	2.023	31.980	28.998	1.014	4.280	6.856	17.621
	(0.937)	(0.834)	(0.893)	(3.177)	(2.856)	(0.527)	(1.838)	(2.054)	(3.606)
4	7.184	1.772	4.354	40.868	28.985	0.837	3.097	4.154	8.745
	(1.821)	(1.020)	(2.245)	(3.593)	(3.960)	(0.536)	(1.785)	(1.772)	(1.118)

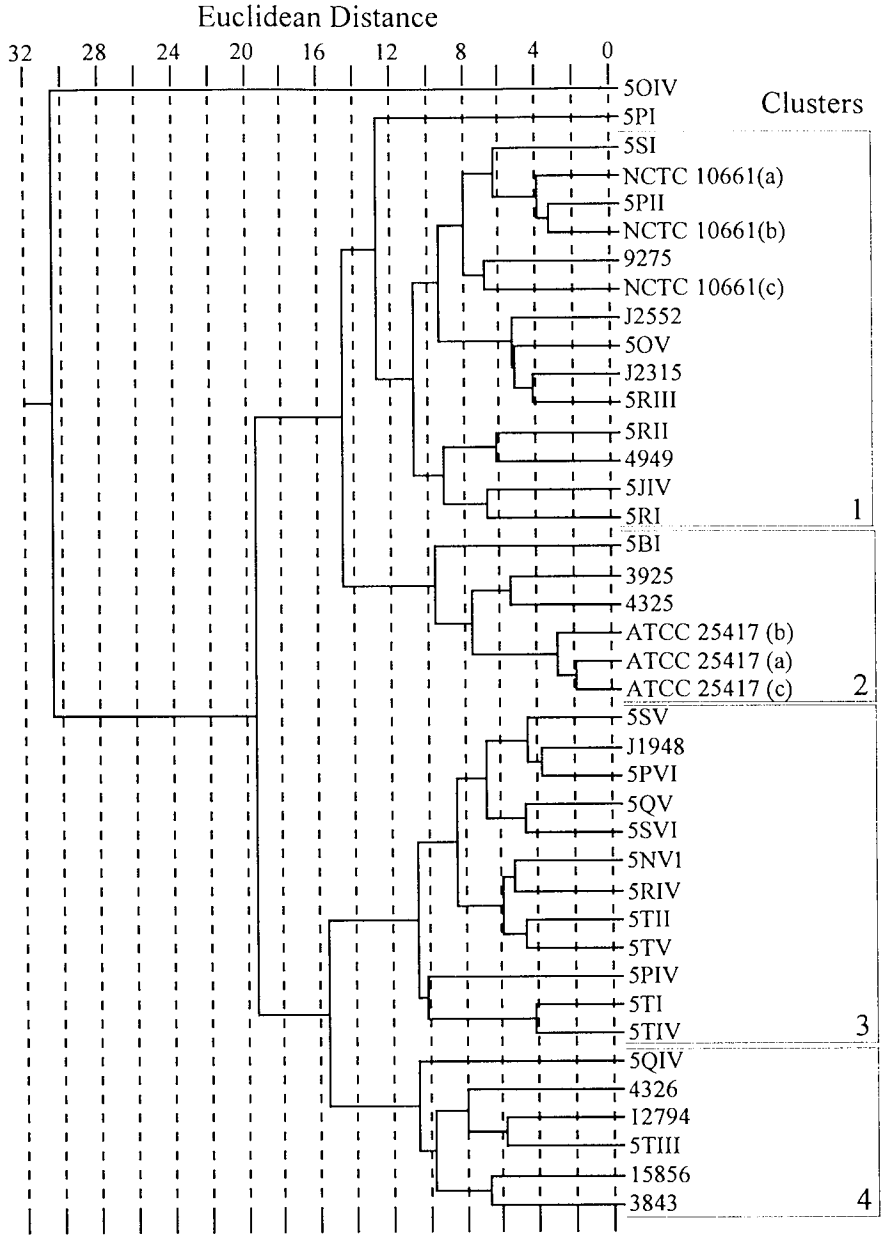


Figure 4.2 Dendrogram of the strains created via cluster analysis of FAME profiles using the UPGMA method.

5. Pulsed-Field Gel Electrophoresis.

5.1 Introduction.

As mentioned in section 1.2.5.1.1, PFGE has both epidemiologic and taxonomic applications. The epidemiologic relationship between infecting strains of *B. cepacia* has been studied at length. In this investigation, data from PFGE studies with one rare cutting restriction enzyme is combined with that from studies with a second such enzyme in order to gain a more detailed picture of inter-strain relationships. Strains from other species are included to clarify this investigation with respect to a broader taxonomic picture.

5.2 Method.

The contour-clamped homogeneous electric field (CHEF) method of PFGE was used here. This technique, based on the BioRad CHEF DR-III system, permits versatility of operating parameters with relative ease of use.

5.2.1 Preparation of master blocks and DNA isolation for PFGE.

50ml MHB was inoculated from a 36-hour culture of a single isolate incubated at 37°C on MHA. The liquid culture was then incubated for 48 hours at 37°C with continuous shaking before harvesting by centrifugation (10,000×g, 10 minutes, 25°C). The cells were washed twice by resuspension in sterile 0.9%w/v saline solution and recovered by centrifugation as before after each washing. The desired wet weight of cells (typically 5mg) was then transferred to a sterile microcentrifuge tube and resuspended in 1ml NET-100 (0.1M NaCl, 0.1M EDTA pH 8.0, 0.01M Tris(Tris-(hydroxymethyl) aminomethane)-HCl pH 8.0) with thorough vortexing to ensure an even suspension.

A stock of 0.9%w/v chromosomal grade agarose in NET-100 was prepared and autoclaved at 15psi for 5 minutes to ensure complete melting of the agarose powder. This was kept molten, at 60°C. 0.6ml of cell suspension was combined with an equal volume of molten agarose, mixed, and dispensed into a 9mm perspex block mould. The filled mould was placed on ice and left for 20 minutes to permit complete solidification of the agarose. This resulted in cells embedded evenly within a matrix of agarose.

Solidified agarose blocks were transferred to 5ml plastic bijou bottles and 5ml of lysis solution (6mM Tris-HCl pH 7.6, 1M NaCl, 0.1M EDTA pH 8.0, 0.5%w/v Sarkosyl, 1mg/ml molecular biology grade lysozyme) was added. The bottles were tightly sealed with screw caps and incubated at 37°C for 24 hours. This lysed the cells in situ within the agarose matrix. The lysis solution was then removed, and 5ml of ESP solution (0.5M EDTA pH 9.0, 1%w/v Sarkosyl, 1.5mg/ml proteinase K) added in its place. The bottles were sealed as before and incubated at 50°C for a further 48 hours. This enabled degradation of cellular debris resulting from lysis, leaving the DNA intact and unsheared in the agarose matrix. Blocks were subsequently stored in ESP at 4°C for further treatment.

Prior to digestion of the DNA with a restriction enzyme, the ESP was removed and 3ml of TE-PMSF (10mM Tris pH 8.0, 1mM EDTA pH 8.0, containing 30µl of a 0.1M stock of phenylmethyl sulphonyl fluoride (PMSF) in isopropyl alcohol) was added. The bottles were resealed once more and the blocks left to bathe in this solution for 2 hours, with constant gentle agitation on a rotating platform. This process was then repeated once with fresh TE-PMSF. After the second treatment, the TE-PMSF was removed and the blocks were washed 3 times for 20 minutes with 5ml fresh TE (10mM Tris pH 8.0, 1mM EDTA pH 8.0), and constant gentle agitation as before. Washed blocks were stored at 4°C in tightly sealed bijou bottles, in a further 5ml of fresh TE until required.

5.2.2 DNA digestion for pulsed-field gel electrophoresis.

Two restriction enzymes were used separately here: *Xba*I (cutting at 5'-TCTAGA) and *Dra*I (cutting at 5'-TTTAAA). The procedure for both was similar. A single block containing DNA from the desired strain was removed from storage in TE, placed on a piece of Parafilm and gently blotted to remove excess fluid. A uniformly proportioned 9mm×1mm×1mm sliver was cut from the block and transferred to a 1.5ml sterile microcentrifuge tube. The remainder of the block was stored in 5ml of fresh TE as before.

Slivers were bathed in 200µl of an appropriate buffer for the restriction enzyme used. Buffer was prepared according to the manufacturer's instructions by addition of the recommended sterile diluents to the manufacturer's commercially available concentrate. Slivers were equilibrated with buffer for 15 minutes with constant cooling on ice. Then, buffer was removed, with care taken not to mechanically damage the sliver. 196µl of fresh buffer and 4µl of the relevant restriction enzyme stock was added in its place. These were mixed thoroughly, left for 15 minutes on ice to equilibrate and then incubated at 37°C for 20 hours to permit digestion of the DNA present.

5.2.3 Electrophoresis: gel preparation, loading and running.

A stock of 10×TBE (1.0M Tris, 0.89M boric acid, 0.02M EDTA pH 8.0) was prepared by dissolution of the constituents in distilled water and diluted with distilled water for use. A 100ml, 1.2%w/v gel of molecular biology grade agarose was prepared by completely melting the agarose in 100ml 0.5×TBE using a 650W domestic microwave oven operating at full power with frequent pauses for stirring. On cooling to about 50°C, a gel suitable for use with the BioRad CHEF DR-III apparatus was cast onto a supporting plate using the same manufacturer's gel casting mould. This was placed on a levelled platform to ensure a uniform gel.

The digest liquid was removed from the microcentrifuge tube, taking care not to damage the agarose sliver. 200µl of ES (0.5M EDTA pH 9.0, 0.1% w/v Sarkosyl) was added in its place and the sliver was incubated in this medium for an accurately timed 15 minutes at 50°C to quench and remove any remaining restriction enzyme, without threatening the mechanical properties of the sliver too greatly. When 15 minutes had elapsed, the ES was removed immediately and completely. ES-treated slivers were washed with gentle mixing by hand in 1.5ml of TE and left to stand in this solution for 15 minutes.

The CHEF DR-III electrophoresis cell was prepared by filling with 2,000ml of fresh 0.5xTBE. The circulating pump was set to maximum and the buffer cooled to 10°C using the apparatus' cooling unit. The apparatus was left to equilibrate to 10°C.

TE-washed slivers were removed from the microcentrifuge tubes one at a time and transferred to a strip of Parafilm. The solidified agarose gel was loaded with these slivers, one into each lane, and the slivers were sealed into place with a coating of molten 0.5%w/v molecular biology grade agarose in 0.5×TBE. The final lane on each gel was filled with a 2mm sliver of a commercially available DNA size standard for PFGE (BioRad Lambda Ladder; concatamers of λ cl857 Sam7 providing DNA bands in multiples of 48.5kb up to approximately 1,000kb). This was sealed in place as before.

When the sealing agarose had solidified, the electrophoresis cell flow rate was cut to 70% of maximum, a flow rate of approximately 1,000ml/minute. The gel was removed from the mould on the supporting plate and transferred to the locating bracket within the cell, in which position it was totally submersed in 0.5×TBE running buffer. The lid to the cell was then placed into position and the gel left for 5 minutes to equilibrate to 10°C with the running buffer. The DNA fragments were then electrophoretically separated using the running conditions for each enzyme shown in table 5.1.

	Restriction enzyme *Xba*I	Restriction enzyme *Dra*I
Program	Block 1	Block 1
Initial pulse time	7 seconds	2 seconds
Final pulse time	60 seconds	25 seconds
Total run time	22 hours	22 hours
Temperature	10°C	10°C
Electric field	6 V/cm	6 V/cm
Electrode angle	120°	120°

Table 5.1 Parameters for CHEF-PFGE with the 2 separate restriction enzymes used.

Upon completion of the separation procedure, the gel was stained by immersion in 500ml of ethidium bromide solution (0.5μg/ml in distilled water) for 30 minutes and destained by immersion in 1,000ml of distilled water for 2 hours to reveal the DNA bands. Banding patterns were visualised by placing the gel on an ultraviolet transilluminator and recorded by photography with a black and white Polaroid film, keeping the same distance between the camera and the gel to ensure a consistent size reproduction in the photographs produced.

5.2.4 Analysis of restriction patterns.

The numerical assessment described by Grothues and Tümmler was used to assess the relatedness of the strains (Grothues and Tümmler, 1991) and its statistical significance. A computer-based spreadsheet (Microsoft Excel 4.0) was used to assist in this process.

Relatedness was determined by conducting a comprehensive, exhaustive pair-wise comparison of restriction fragment sizes within the sample group. This permitted the calculation of the Dice coefficient for each pair using equation (1):

$$S_D = \frac{2n_c}{(n_A + m_B)} \qquad (1)$$

where S_D is the Dice coefficient, n_c is the number of common bands and n_A and m_B are the total number of bands in patterns A and B respectively.

The gel photographs were divided into intervals with respect to their powers of resolution. *Xba*I-digested samples and *Dra*I-digested samples were deemed to possess 28 and 30 intervals respectively. Statistical significance was then evaluated using equation (2):

$$P_{(S_{D\,AB})} = \frac{2}{\sum(n_K + m_K)} \times \left[\sum \left(\frac{n_K \times m_K}{I_K} \right) \pm \left(\frac{t}{2} \times \sqrt{\left(\sum n_K \right)} \right) \right] \qquad (2)$$

where $P(S_{D\,AB})$ is the predicted upper or lower boundary of the confidence limit, n_K and m_K are the total number of bands in both the *Xba*I and *Dra*I patterns, I_K is the number of intervals into which the range of restriction fragments is divided and t is the value of Student's t-distribution (1.96 for the 95% confidence interval and 2.57 for the 99% confidence interval).

The hierarchical UPGMA method of analysis was used to group the strains into clusters based on the S_D values calculated. The computer-based statistical analysis software Unistat, working in unison with Microsoft Excel 5.0, was used for this step and to generate a dendrogram of the data.

5.3 Results.

Numerical analysis of the strains as outlined in section 5.2.4 gave rise to the dendrogram in figure 5.2. A single cluster is present above a distance of about 0.58 Euclids, with subsequent subdivisions in evidence.

The laboratory strains *P. aeruginosa* NCTC 6750 and ATCC 15692, *S. maltophilia* NCTC 10247 and *B. gladioli* ATCC 10248, ATCC 10854 and ATCC 25417 depart from the majority of the strains at distances of 0.58 Euclids, 0.53 Euclids and 0.46 Euclids respectively. Isolates 5RII, 5RIII and J2552 do not group with the rest of the environmental strains. The former two are both clinical isolates from Manchester (table 2.1) and not closely linked to J2552, which is a botanical isolate from Edinburgh (table 2.1). They appear somewhat surprisingly to be most closely associated with *S. maltophilia* NCTC 10257, but diverge from this laboratory strain at a level of 0.45 Euclids. This is a higher level than that at which some separately designated species diverge from each other; e.g. *B. cepacia* and *B. vietnamiensis* (see below). A third clinical isolate from Manchester, 5RI, is more closely associated with the remainder of the clinical strains.

The majority of the clinical strains fall into 4 main clusters at a level of 0.4 Euclids (figure 5.1). Of these, cluster 4 contains isolates previously identified as the CF epidemic strain, such as the Edinburgh strains J1948 and J2315. This cluster appears to divide into 3 subgroups at a level of 0.12 Euclids, although this level of distance is quite small. Typically, these subdivisions each possessed distinctive banding patterns when analysed by digestion with *Xba*I or *Dra*I. These patterns were not found when other isolates were analysed. A variety of such patterns are shown in figure 5.2.

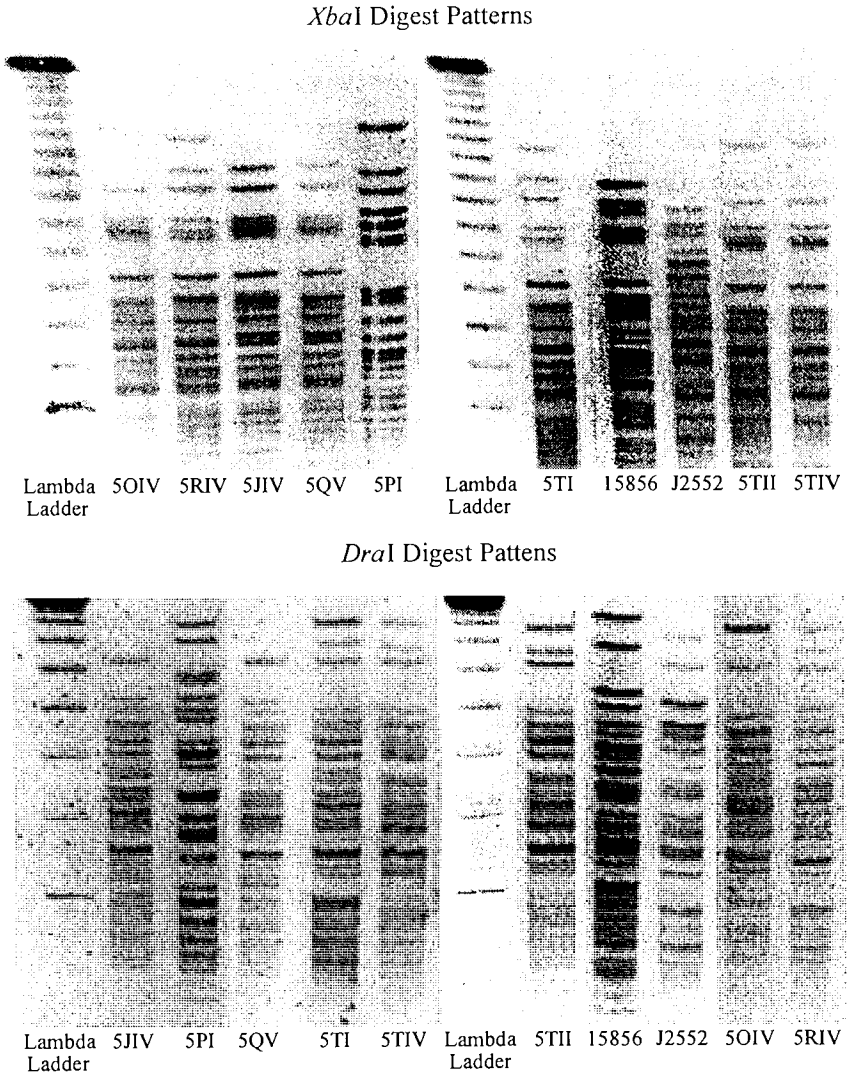


Figure 5.1 PFGE gels showing characteristic patterns produced by members of cluster 4 (figure 5.2) and others among the strains analysed. Lambda Ladder (BioRad) provides marker bands in multiples of 48.5kb. Negative images are shown for clarity.

B. cepacia ATCC 10744 ATCC 17616 and NCTC 10661, and *B. vietnamiensis* LMG 6998 remain associated with the 4 clusters discribed above until a distance of 0.41 Euclids and diverge from each other at a distance of 0.31 Euclids. Therefore, these two distinct species are genetically more related than many clinical isolates considered to be *B. cepacia*. This subject will be continued in chapter 8.

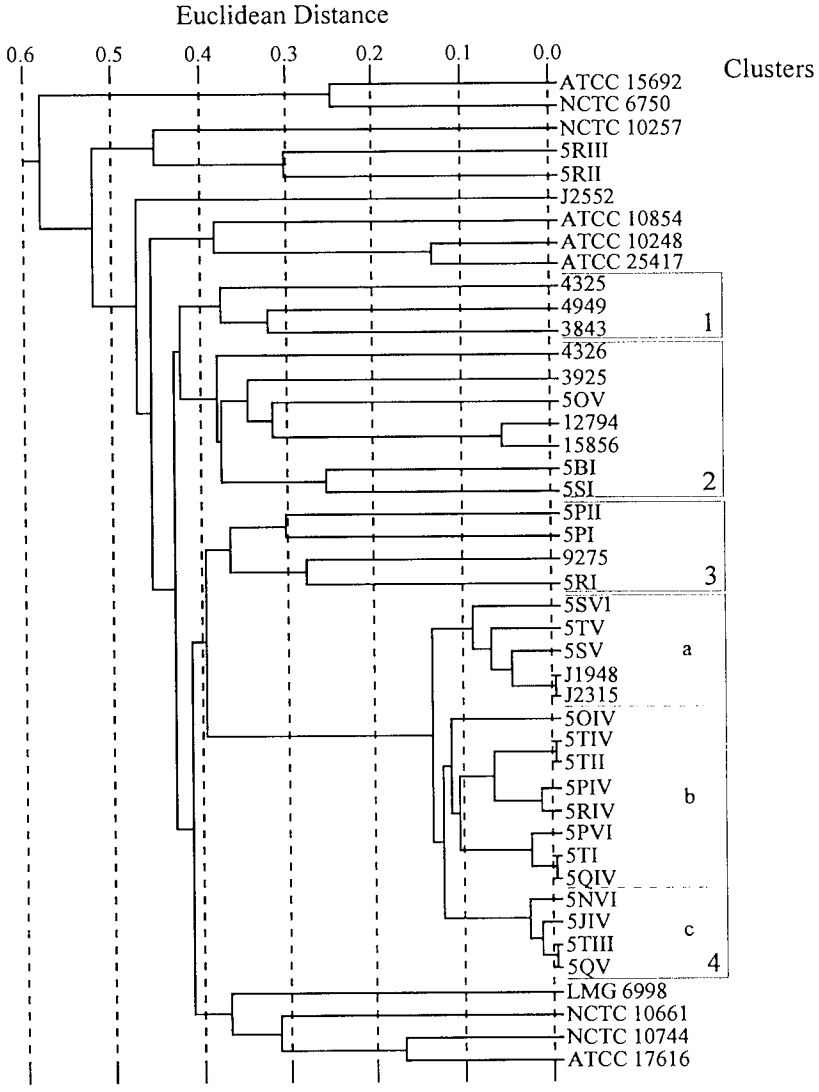


Figure 5.2 Dendrogram of the strains created via cluster analysis of Dice coefficients using the UPGMA method.

6. Investigation of Outer-Membrane Proteins.

6.1 Introduction.

OMP profiles have previously proved useful in the epidemiologic and taxonomic rationalisation of bacterial strains in some species, e.g. *Haemophilus influenzae* (Van Alphen *et al.*, 1983). *B. cepacia* was subjected to a similar approach, in order to observe any differences between the OMPs of the strains. Outer membranes (OMs) were isolated using an anionic detergent to differentially solubilise and remove cytoplasmic membrane proteins from cell envelopes prepared by sonic disruption of the cells (Filip *et al.*, 1973). Specific OMP constituents separated by SDS-PAGE were then subjected to immunological and N-terminal amino acid sequencing investigation in an attempt to differentiate further between the groups of strains identified by FAME analysis and PFGE. All the environmental strains were included in this study. Laboratory strains *B. cepacia* NCTC 10661 and ATCC 17616; *B. gladioli* ATCC 10248, ATCC 10854 and ATCC 25417; and *B. vietnamiensis* LMG 6998 were included for comparison.

6.2 Method for the isolation and purification of outer membrane proteins.

1000ml MHB was inoculated with a 50ml, 24-hour culture of a single isolate incubated at 37°C with continuous shaking in the same medium. The larger volume culture was then incubated for 16 hours with continuous shaking at 37°C before harvesting by centrifugation (10,000×g, 10 minutes, 25°C). The cells were washed once by resuspension in double distilled water and recovered by centrifugation as before, then resuspended in 20ml double distilled water containing 250μg/ml deoxyribonuclease II to reduce viscosity on sonication.

Cells were ruptured by sonication using an MSE Soniprep 150 with a logarithmic probe of tip 4mm operating at maximum power in 30 cycles of 30-second bursts of sonication, with intervening pauses of 30 seconds and constant cooling on ice. Any whole cells remaining after sonication were excluded by further centrifugation as before. The resulting supernate was made up to a volume of 35ml by the addition of double distilled water. A suitable volume of 20% w/v Sarkosyl was then added, yielding a concentration of 2% w/v in the diluted supernate. This mixture was left to stand for 5 minutes to dissolve and remove the cytoplasmic membrane components.

Centrifugation (35000×g, 60 minutes, 4°C) deposited a pellet containing partially purified outer membranes. This pellet was resuspended in 35ml 2% w/v Sarkosyl, left to stand for 5 minutes and centrifuged again as before to yield a pellet containing purified outer membranes. This was resuspended in 2ml double-distilled water and stored at –20°C for analysis by SDS-PAGE.

6.2 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis.

SDS-PAGE separates proteins with respect to their size and hence molecular weight (MW) by denaturation and subsequent normalisation of the charge-to-MW ratio. The latter is achieved by the saturation of each protein present with SDS. The method of Lugtenberg *et al.* was used to separate the OMPs for observation (Lugtenberg *et al.*, 1975).

	Separating gel (12%)	Stacking gel	Sample denaturing buffer	Electrode buffer
Stock I	5.0ml	-	-	-
Stock II	-	2.5ml	-	-
10% w/v SDS	0.5ml	0.15ml	5.0ml	20ml
1.5M Tris-HCl buffer, pH 8.8	6.2ml	-	-	-
0.5M Tris-HCl buffer, pH 6.8	-	3.8ml	2.5ml	-
Double-distilled water	8.0ml	8.0ml	5.0ml	to 2,000ml
TEMED	50µl	40µl	-	-
10% w/v AMPS, freshly prepared	70µl	50µl	-	-
Glycerol	-	-	2.5ml	-
2-mercaptoethanol	-	-	0.25ml	-
5% w/v Bromophenol blue	-	-	0.2ml	-
Tris	-	-	-	6.0g
Glycine	-	-	-	28.8g

Notes: Stock I: solution of 44% w/v acrylamide and 0.8% w/v bis(N,N'-methylene) bisacrylamide; Stock II: solution of 30% w/v acrylamide and 0.8% w/v bis(N,N'-methylene) bisacrylamide; Glycine=biochemical grade.

Table 6.1 Reagents used to prepare the SDS-PAGE system.

Reagents were prepared as outlined (table 6.1) and stocks of acrylamide were filtered before use. The BioRad Mini Protean system was used. The tank was filled with an electrode buffer (table 6.1). Two 12 % acrylamide separating gels were cast. A stacking gel was cast on top of these for loading the system. Gels are prepared by the combination with gentle mixing of the constituents, with the final steps being the addition of the correct volumes of AMPS and TEMED and pouring the liquid solution into the mould. This procedure is carried out first for the separating gel and, when this has solidified in position, the stacking gel.

Samples for SDS-PAGE were each mixed with equal volumes of denaturing buffer (table 6.1) and heated for 30 minutes at 100°C. Molecular weight standards (Dalton Mark VII-L) were prepared by dissolution in 1ml of double distilled water and addition of an equal volume of denaturing buffer, followed by heating for 30 minutes at 100°C as before. A suitable volume of each denatured sample (typically between 5µl and 10 µl) was loaded into separate lanes of a prepared SDS-PAGE gel. A constant voltage of 200V was applied until the bromophenol blue marker dye in the denaturing buffer reached the bottom of the gel.

Gels were stained permanently by immersing in 40ml 0.1% Coomassie Brilliant Blue R-250 in 80%v/v methanol-20%v/v glacial acetic acid solution for 1 hour. Destaining in 2×40ml 10%v/v methanol-20%v/v glacial acetic acid solution (once for 90 minutes and a second time overnight) revealed the protein bands. Destained gels were rinsed with tap water for 4 hours, mounted on thick chromatography paper and dried for 6 hours using a vacuum gel drier.

6.4 Immunological Examination of Outer Membrane Proteins.

6 strains were subjected to this procedure as a preliminary investigation; *B. cepacia* ATCC 17616 and *B. gladioli* ATCC 10248, J1948, 5PIV, 5TI and 5PVI.

6.4.1 Western Blotting.

Proteins were first separated on a 12% SDS-PAGE gel (section 6.2) and then subjected to western blotting (transferring the protein to a nitrocellulose membrane of pore size 0.45μm) following a procedure modified from that of Towbin *et al.* (Towbin *et al.* 1979), using a BioRad Mini Trans Blot system.

The SDS-PAGE gel and nitrocellulose membrane were soaked in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol, pH 8.3) for 5 minutes and then placed in uninterrupted contact with one another between two appropriately sized pieces of chromatography paper soaked in the same buffer. Two similarly sized pieces of Scotch Brite pad (also soaked in the transfer buffer) were placed either side of this and the resulting stack clamped into a plastic support frame.

This assembly was placed into the Trans Blot cell such that the nitrocellulose membrane was positioned between the gel and the anode (the proteins, having been negatively charged by denaturation with SDS, will move towards this electrode). Blotting was carried out in transfer buffer at 100V for 1 hour under conditions of constant cooling to 4°C with ice. After blotting, unoccupied sites on the nitrocellulose membrane were blocked by soaking in TBS-Tween (0.01M Tris-HCl pH 7.4, NaCl 0.9% w/v, 0.3% v/v Tween-20) for 2 hours.

6.4.2 Preparation of Antiserum.

Antiserum raised against the 80kDa Sarkosyl-purified outer membrane protein from strain 5NVI was kindly donated by Dr D.E. Lacy and Dr P.A. Lambert (Aston University). This had been prepared by electroelution of OMP samples according to the following method (Lacy, 1996).

750µl of an OM preparation (prepared from 2,000ml of cells as described in section 6.2) was electrophoresed on a preparative 12% SDS-PAGE gel. This process utilised the BioRad Protean II Vertical Electrophoresis cell with a single small sample well for the molecular weight standards and a large well spanning the maximum possible width of the rest of the gel. Electrophoresis was performed at 50V until the marker dye front had reached the bottom of the gel. The 80kDa band was located by comparison with molecular markers separated on the same gel and by removal and Coomassie Blue-staining of a small sliver from the edge of the broad preparative lane. Staining was conducted as described above (section 6.2), but reducing the staining and destaining periods to a total of 2 hours. The relevant unstained portion of the SDS-PAGE gel was then located and excised for further processing.

The 80kDa protein was electroeluted from the excised segments of gel using the BioRad Electroeluter Model 422 in accordance with the manufacturer's instructions, at a constant current of 8mA per tube for 4 hours (Harrington, 1990). Protein recovered thus was stored in solution at -20°C until use.

The presence of the electroeluted protein was confirmed by SDS-PAGE using the Mini-Protean system. The 80kDa structure is known to be an aggregate of 36kDa and 27kDa subunits, which dissociate only after electroelution (Parr *et al.*, 1987). Electroelution resulted in a product mainly comprised of 36kDa protein, with little detectable 27kDa protein and some undissociated 80kDa protein. Of these two products, only the 36kDa protein proved immunologically active on subsequent western blotting (Lacy, 1996).

The electroeluted material was emulsified in Freund's complete adjuvant for the initial dose and Freund's incomplete adjuvant for eleven subsequent weekly doses. Two adult female New Zealand White rabbits were injected with 1ml of these emulsions subcutaneously at multiple sites in the neck. Blood was recovered by cardiac puncture one week after the final administration date and allowed to clot. The serum was stored at –20°C until use.

6.4.3 Immunological Detection of Proteins.

The nitrocellulose membrane containing OMP profiles from section 6.3.1 was immunologically investigated with the prepared antiserum outlined in section 6.3.2. After soaking with TBS-Tween to block unoccupied sites (section 6.3.1), the membrane was reacted for 3 hours at 4°C with a 1 in 50 dilution of the antiserum. The antiserum-treated membrane was washed for 3×15 minutes in further TBS-Tween.

The washed membrane was then soaked with gentle agitation in protein A-horseradish peroxidase conjugate (50μg/ml in TBS-Tween) for 2 hours at 25°C. This was then removed and the membrane washed for a further 3×15 minutes in fresh TBS-Tween and once for 15 minutes in 0.01M Tris-HCl pH 7.4.

The IgG-conjugate interaction was visualised by addition of a freshly prepared solution of a chromogenic substrate (4-chloronaphthol 25μg/ml in 0.01M Tris-HCl, pH 7.4 and 10μl of H_2O_2) for 10 minutes. Colour development was stopped by washing with distilled water.

6.5 N-Terminal Sequencing.

This procedure involves two steps: electroblotting to a polyvinylidene difluoride (PVDF) membrane and subsequent Coomassie Blue staining, followed by solid phase microsequencing via the technique of Edman (Edman, 1950). Two strains were subjected to this procedure as a preliminary investigation; *B. cepacia* ATCC 17616 and *B. gladioli* ATCC 10248.

6.5.1 Electroblotting.

A suitably sized section of PVDF membrane with pore size 0.2 μm was cut and soaked in 100% methanol and distilled water for 5 seconds each, then soaked in CAPS buffer (0.01M 3[cyclohexylamino]-propane sulphonic acid, 10% methanol, pH 11.0) for 15 minutes. An SDS-PAGE gel of OM samples produced as described above (section 6.2) was also equilibrated in CAPS buffer for 15 minutes to remove tris and glycine from the previous SDS-PAGE separation process. This membrane and gel were assembled in a Mini Trans Blot cell in the manner described for western blotting, with the PVDF membrane taking the place of the nitrocellulose membrane used therein (section 6.3.1).

Electroblotting was conducted at a constant 0.3A for 30 minutes in CAPS buffer, with constant cooling to 4°C using ice. Following this, the membrane was rinsed in distilled water (3×5-minute washes). The washed membrane was stained for 5 minutes in Coomassie Brilliant Blue 0.1% in 50% methanol, and destained for 15 minutes with 50% methanol, 10% acetic acid. The membrane was then dried in air and stored at -20°C prior to microsequencing.

6.5.2 Microsequencing.

Microsequencing was conducted on the author's behalf by Alta Bioscience, Birmingham University School of Biochemistry, UK, utilising an Applied Biosystems 473A automatic protein sequencer. This uses the Edman process to remove successive single amino acids from the N-terminus of the protein. Subsequent identification of the moeties thus liberated by high-performance liquid chromatography permits the identification of up to 50 amino acids from the N-terminus of the protein under investigation. However, the presence of reactive species at many stages of the process prior to microsequencing may lead to the chemical blockage of the N-terminus.

6.6 Results.

6.6.1 Outer Membrane Protein Profiles.

SDS-PAGE-separated OMP profiles revealed 2 major profile groups amongst the environmental strains on the basis of the presence and absence of 2 prominent protein bands at about 80kDa in the OMP profile (table 6.2).These were denoted groups 1 and 2 (table 6.2). Figure 6.1 shows examples of these 2 OMP profiles, with the profiles obtained similarly from one laboratory strain of each species included for comparison.

Group	Strains
Group 1	LMG 6998; ATCC 17616 NCTC 10744; ATCC 10248; ATCC 10854; ATCC 25417; 3843; 3925; 4325; 4326; 4949; 9275; 12794; 15856; J2552; 5BI; 5JIV; 5NVI; 5OV; 5PI; 5PII; 5QIV; 5RII; 5RIII; 5SI.
Group 2	NCTC 10661; J1948; J2315; 5OIV; 5PIV; 5PVI; 5QV; 5RI; 5RIV; 5SV; 5SVI; 5TI; 5TII; 5TIII; 5TIV; 5TV.

Table 6.2 Strains comprising OMP groups I and 2.

Comparing these groups with table 3.7, it can be seen that group 2 contains the 77% of those strains producing the red/brown pigment (10 out of 13 strains). Also, OMP group 2 accounts for 92% of FAME cluster 3 (11 out of 12 strains) and 82% of PFGE cluster 4 (14 out of 17 strains). This correlation is discussed further in chapter 8.

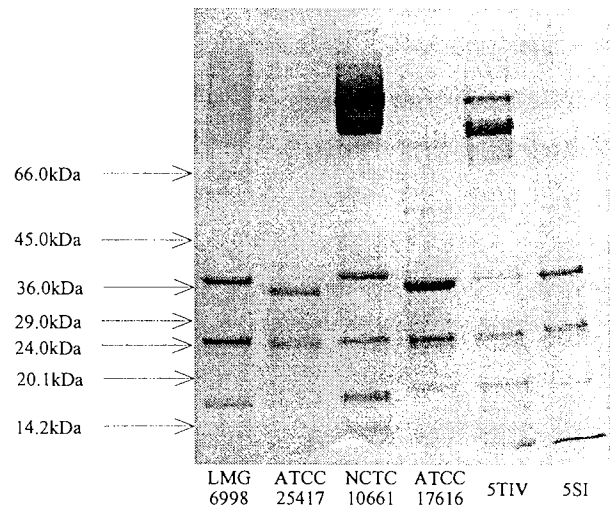


Figure 6.1 SDS-PAGE gel of typical OMP profiles obtained in this investigation. The profile from strains 5SI and 5TIV illustrate typical examples of OMP groups 1 and 2 respectively.

This method revealed *B. vietnamiensis* and *B. gladioli* laboratory strains to have similar OMP profiles to that of *B. cepacia* (figure 6.1). Interestingly, the major protein band at approximately 17kDa appears to be of diminished intensity in *B. gladioli* (figure 6.1).

Lesser heating when denaturing samples (70°C as opposed to 100°C for 30 minutes) led to the production of patterns resembling group 2 from strains classified as group 1 above (figure 6.2). Furthermore, an extended period of heating at 100°C (50 minutes as opposed to 30 minutes) led to the absence of the high-MW band on SDS-PAGE analysis (figure 6.2). Therefore, is is assumed that the manner in which the constituents of this high-MW band are held together differs between groups 1 and 2 in some manner.

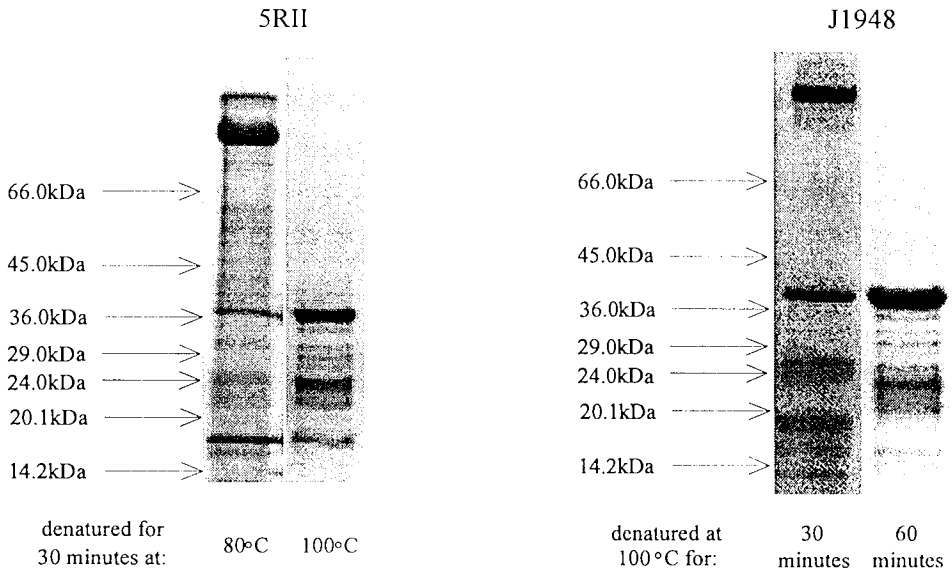


Figure 6.2 SDS-PAGE gel lanes showing examples of the effects of different denaturing conditions on two strains, 5RII and J1948, from groups 1 and 2 (table 6.2) respectively.

6.6.2 Immunological Investigation.

Immunoblotting as described in section 6.3 revealed a significant reaction with most of the protein material present in the OMP profiles. This may have been due to contamination of the material used to create the antiserum used with LPS. Therefore, this investigation was not extended to include further strains and no additional data was gained from this investigation.

6.6.3 N-Terminal Sequencing.

Unfortunately, both samples on which N-terminal sequencing was attempted proved not to be amenable to this procedure. Alta Bioscience reported both to be blocked at the N-terminus. Therefore, no further strains were subjected to this analysis and no additional data was gained from this investigation.

7. Investigation of Carbapenemase Activity in *Burkholderia cepacia*.

7.1 Introduction.

Previous chapters have shown that there is a closely related cluster of strains within the genus *Burkholderia* responsible for pulmonary infection in CF patients. Finally, the biochemical basis for carbapenem resistance in *B. cepacia* will be considered in this light.

Purification of the β-lactamase was attempted on the cell free extract from one isolate, 5JIV, as this organism was not troublesome to culture and produced an isoelectric focusing (IEF) pattern of β-lactamase activity typical of the strains investigated (section 7.2.2). Also, a spectrophotometric assay (section 7.4) of cell free extracts prepared from this strain showed it to possess a good imipenem-hydrolysing capability. A further 8 strains were briefly studied by way of comparison.

7.2 β-lactamase Isolation and Purification.

7.2.1 Cell Free Extract Preparation.

Cells were grown for 18 hours at 37°C with vigorous shaking in 50ml MHB. These cultures were then used to inoculate 5-litre flasks containing 2 litres of the same medium and incubated for a further 12 hours under the same conditions. Each strain was grown in the presence and absence of 4μg/ml imipenem, to act as an inducer of β-lactamases. Cells were harvested by centrifugation (10,000×g, 10 minutes, 5°C), washed in 5mM phosphate buffer (pH8) and resuspended in the same buffer (4ml for every 2 litres of original culture) containing 250μg/ml of deoxyribonuclease II to reduce viscosity on sonication. Cells were disrupted by sonication with a Soniprep 150 with a 2cm diameter probe operating at maximum power in 40 cycles of 30-second periods of sonication with intervening 30 second pauses and constant cooling on ice. Sonicates were clarified by centrifugation at 35,000×g for 1 hour at 0°C. The supernatants (3ml from every 2 litres of culture) were stored at –20°C until use.

Features of this method worthy of further comment are the lengthy period of sonication used to rupture the cells and the relatively high speed of centrifugation needed to clarify the resulting suspension. Fung *et al.*, investigating *Moraxella catarrhalis* β-lactamases, found that higher centrifugation speeds and longer periods of sonication resulted in a better quality of cell free extract (Fung *et al.*, 1994). Therefore justification can be made for the use of such extreme conditions. Additionally, *B. cepacia* isolates proved exceptionally difficult to rupture and lengthy sonication proved necessary to achieve this.

7.2.2 Analytical Isoelectric Focusing (IEF).

IEF separates proteins with respect to their isoelectric points (pI) to a high degree of resolution in an electrophoretically produced pH gradient (Matthew *et al*, 1975). The procedure is well described in established texts (Arbuthnott & Beeley, 1975).

The IEF of β-lactamases is usually conducted on thin layers of polyacrylamide gel in accordance with a method based on that of Matthew *et al.* (Matthew *et al.*, 1975). Commercially available kits containing pre-cast gels make this procedure relatively simple to perform.

β-lactamases present in a heterogeneous protein mixture can be differentially visualised using the chromogenic cephalosporin nitrocefin (O'Callaghan *et al*, 1972). Nitrocefin is highly susceptible to β-lactamase-mediated hydrolysis and undergoes a colour change from yellow to red when thus challenged. This is not a permanent stain: the result of nitrocefin addition must be photographically recorded. The amount of nitrocefin required to obtain a sufficient colour intensity for photographic purposes is very expensive when purchased.

IEF was firstly used to confirm the presence of β-lactamases in the cell free extracts prepared. Later, it was used to prove the presence of a single band of β-lactamase activity in purified samples showing carbapenemase activity. Samples (6μl of a purified sample or 6μl of a 1 in 8 dilution of a cell free extract) were each applied to thin glass fibre application strips 10mm from the anode of a pH 3.5-9.5 polyacrylamide IEF gel and focused at 30W constant power for 90 minutes with cooling to below 5°C using a Multiphor LKB 2117 apparatus, coupled to a suitable power supply. The electrodes at either edge of the gel were prepared in accordance with the gel manufacturer's instructions (thick filter paper strips soaked in 0.1M H_3PO_4 for the anode and 0.1M NaOH for the cathode). The development of

the pH gradient was followed using pre-stained pI markers for the pI range 4.7 to 10.6. β-lactamase activity was visualised by overlaying the gel surface with filter paper soaked in nitrocefin solution (0.1mM) and in each case was recorded by photocopying the gel through the plastic backing sheet. Less nitrocefin was required to record the gel by photocopying than by photography, but the former procedure produced a result of lower (although still acceptable) quality.

7.2.3 β-lactamase Purification.

7.2.3.1 Purification: Methodological Choices.

Several means of purification were attempted before a successful method was found. Ammonium sulphate precipitation, preparative isoelectric focusing anion exchange chromatography (using a DEAE-cellulose filled column and a Mono-Q column) and cation exchange chromatography (using an Econo-Pac S column) all proved unsuccessful. These are established techniques dealt with at length in the literature (Scopes, 1982). Dye affinity chromatography, also an established technique, enabled successful isolation of the β-lactamase and is therefore discussed in detail here.

Many different chromatographic media exist for the separation of biological matter. Often, these are eluted with a salt gradient using a system such as the Pharmacia FPLC (fast protein liquid chromatography) system, which provides an automated, accurate and conveniently reproducible way of controlling the desired elution parameters (Payne *et al*, 1990). Cibacron Blue was used as the stationary dye affinity phase for dye affinity chromatography in this case. This medium has also proved useful for the separation of the chromosomal serine β-lactamase from *P. aeruginosa* (Giwercman *et al.*, 1994).

Cibacron Blue itself is a vague structural analogue of nicotinamide adenine dinucleotide (Scopes, 1982). However, β-lactamases do not appear to be dependent on any particular cofactor (Sykes & Matthew, 1976). Therefore, the basis for the separation of β-lactamases by Cibacron Blue dye affinity, chromatography remains unclear.

7.2.3.2 Dye Affinity Chromatography Purification: Method.

80ml of Cibacron blue F3GA coupled agarose was washed with a solution of 0.1M acetic acid and 1.4M NaCl in 40% isopropyl alcohol to remove loosely bound dye. The washed gel was suspended in 100ml of 3-[N-morpholino] propanesulphonic acid-NaOH (MOPS) Buffer (pH 6), washed in several changes of the same buffer, packed in a Pharmacia C 16/40 column (dimensions 400mm × 16mm) with column adapter and washed at 1.5ml/min with the buffer for 24 hours. 5ml of cell free extract from strain 5JIV was applied to the column and washed with MOPS buffer for 2 hours at 1ml/min.

Bound protein was then eluted with a NaCl gradient increasing at 4mM/min over the range 0-2M as above. The flow rate was 1ml/min. The eluate was collected in 10ml fractions and each was tested for imipenem-hydrolysing activity using a spectrophotometric assay (below). Active fractions were subjected to analytical IEF. Those fractions containing a single band of β-lactamase activity and hydrolysing imipenem were pooled and stored at −20°C for further analysis.

7.3 Protein Assay.

The protein contents of the original cell free extract and the purified β-lactamase sample used for spectrophotometric analysis were measured using the bicinchoninic acid (BCA) assay of Smith *et al.* (Smith *et al.*, 1985). This is similar in principle to the more widely used protein assay of Lowry *et al.* (Lowry *et al.*, 1951), but the reagent used is more stable and the assay more robust. Protein content was measured to quantify the purification achieved.

7.4 Spectrophotometric Assay.

The ability of the purified enzyme to hydrolyse various β-lactam antibiotics including imipenem was investigated using this assay. Those β-lactams tested are listed in table 7.1. The presence of imipenem-hydrolysing activity in cell free extracts and the fractions resulting from purification processes were also investigated in this way.

Hydrolysis was followed for 3 hours in triplicate at the appropriate wavelength using a Philips PU8730 spectrophotometer with 3ml cuvettes maintained at 37°C. Absorbance readings were taken every 15 minutes. Inhibition of imipenem hydrolysis was investigated by pre-incubation of the enzyme with a fixed concentration of inhibitor prior to the addition of imipenem. Table 7.2 includes the incubation times permitted. In the cases of the metal ion chelators EDTA and *o*-phenanthroline (OP) an attempt was made to restore activity by addition of an equimolar concentration of zinc sulphate after addition of the imipenem substrate. Enzyme and chelator were incubated before addition of imipenem, zinc sulphate was then added after 3 hours.

All assays were conducted in 5mM MOPS buffer (pH7) and all antibiotics and inhibitors were freshly prepared in this buffer. MOPS buffer is known to chemically stabilise solutions of the carbapenems, thus ensuring minimal degradation of these agents by non-β-lactamase means during the assays (N.P. Brenwald, City Hospital NHS Trust, Birmingham, UK, personal communication). The starting concentration of each antibiotic tested (table 7.1) gave an absorbance reading of approximately 2.5. The enzyme sample obtained as described in section 7.2.3.2 was added to achieve a final concentration of 80µl/ml in the cuvette.

7.5 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis.

β-lactamases subjected to SDS-PAGE can recover activity if the gel is thoroughly washed with buffer after electrophoresis has been conducted (P.A. Lambert, Aston University, personal communication). Recovery of activity may be easier if a less rigorous denaturing procedure is adopted, but it is important to ensure that this is not to the detriment of resolution if this technique is to be used for MW determination.

The Mini Protean system (BioRad) was used as described above (section 6.1). 12% polyacrylamide separating gels were utilised once again and samples were prepared by the addition of equal volumes of denaturing buffer (section 6.2). One gel was loaded with this combination after 5 minutes incubation at 37°C (gel 1). The remaining mixture was heated for 20 minutes at 100°C and then used to load the other gel (gel 2). A constant voltage of 200V was applied until the bromophenol blue marker dye in the denaturing buffer reached the bottom of the gel.

When electrophoresis was completed (about 60 minutes), gel 1 was removed and bathed for 1 hour in 40ml 5mM phosphate buffer pH7. This was repeated 3 times to remove SDS from the gel, in an attempt to renature any β-lactamases present. Gel 1 was then overlaid with filter paper soaked in nitrocefin solution (0.1mM). Bands of β-lactamase activity visualised by this were marked by injection of a small volume of Indian ink and the gel stained permanently with Coomassie Blue as described previously (section 6.2) to reveal protein bands.

Gel 2 was stained permanently using Coomassie Blue as above. This gel was used to verify protein band positions using samples denatured under more stringent conditions. The stained and destained gels were rinsed with tap water for 4 hours, mounted on thick chromatography paper and dried for 6 hours using a gel drier.

7.6 Bioassay of Imipenem-Hydrolysing Activity.

7.6.1 Explanation of the Bioassay.

The bioassay used here is similar in execution to the disc approximation test for inducible β-lactamases described in the BSAC working party report (Anonymous, 1990). Assays of this type have been described as more sensitive than a spectrophotometric assay (Paton *et al.*, 1993). An antibiotic assay disc moistened with the antibiotic under investigation is placed onto the surface of an agar-filled Petri dish that has been surface seeded with a susceptible organism shortly before. Another assay disc moistened with an extract of the resistant strain under investigation is placed in close proximity to the first, and the plate is then incubated to allow the surface seeding to grow. If the extract from the resistant strain can protect the sensitive strain from the antibiotic, the normally circular zone of inhibition will be blunted in the region closest to the disc bearing the extract (figure 7.1).

Here, wells cut into the agar replace the antibiotic assay discs. However, the principle remains the same.

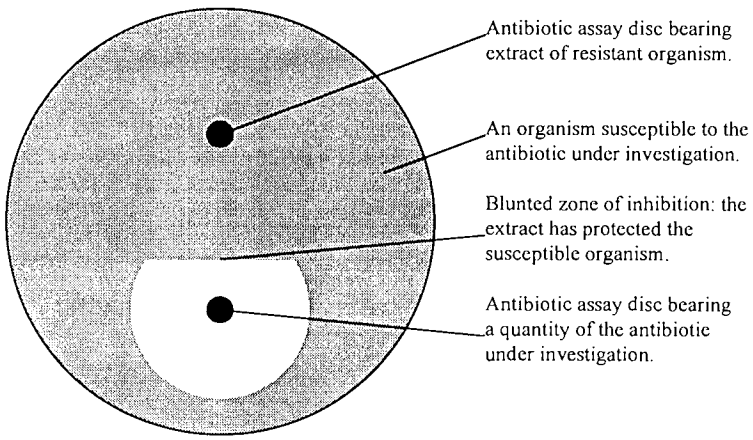


Figure 7.1 Idealised representation of a positive result in the BSAC bioassay: the extract from the resistant organism (on the top disc) has protected the susceptible organism used to surface seed the plate and has produced a blunted edge to the zone of inhibition.

7.6.2 Bioassay Method.

20ml aliquots of MHA were poured into Petri dishes and allowed to set. The surfaces of these plates were seeded with 2ml of a culture of *E. coli* NCTC 10418 grown for 18 hours in MHB. Excess fluid was drained from the surface and the plates were dried for 1 hour at 37°C. Wells (diameter: 8mm) were cut into the agar in the centre of each plate and filled with 50µl of a freshly made 1mg/ml solution of imipenem in 5mM MOPS buffer (pH6). This was surrounded by 6 similar wells 15mm distant, each filled with 50µl of either the cell free extract from strain 5JIV or a fraction collected during dye affinity chromatography. The plates were incubated at 37°C for 24 hours to allow the *E.coli* to grow. The zones of inhibition that developed were then inspected for regions of blunting and the results recorded by photography.

7.7 Results.

7.7.1 Purification of the β-lactamase.

The imipenem-hydrolysing enzyme was eluted from the dye affinity column at a NaCl concentration of 0.80-0.92M (figure 7.2). Of the three 10ml fractions collected over this range (21-23), fraction 23 showed only weak activity and was discarded. Fractions 21 and 22 were pooled and used for investigation. Protein measurements using the technique outlined in section 7.3 showed that 98% of the protein in the cell free extract had been excluded by the purification process. No carbapenemase activity was present in fractions outside the range 21-23.

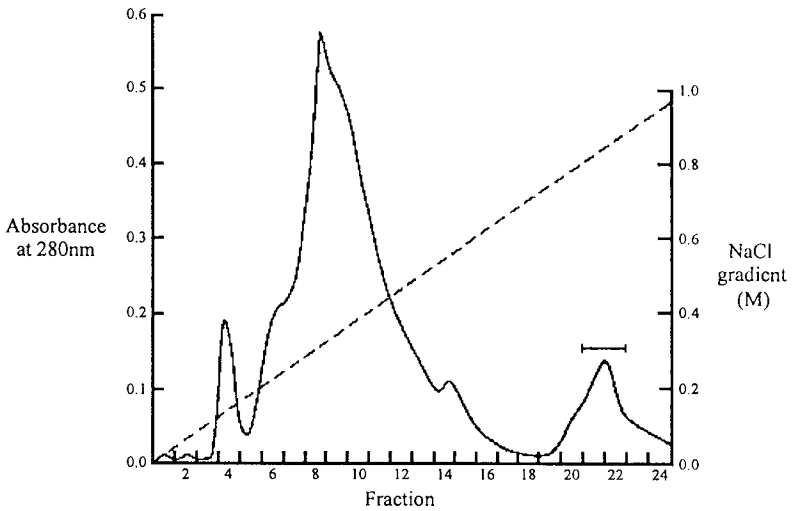


Figure 7.2 Trace of the dye affinity column eluate, monitored by the FPLC detector at 280nm. The carbapenemase was eluted in fractions 21-23. The horizontal bar indicates fractions pooled for further use (21 and 22).

7.7.2 Analytical Isoelectric Focusing of the Strains.

Analytical IEF of column fractions 21 and 22 showed a single band of β-lactamase activity at pI 8.45 (figure 7.3). Analytical IEF of a cell free extract from each of the 10 strains included in this investigation confirmed the presence of the pI 8.45 β-lactamase in all the strains, but only when induced with imipenem (figure 7.4). If this sample is representative of *B. cepacia* as a species, this enzyme would appear to be inducible and ubiquitous.

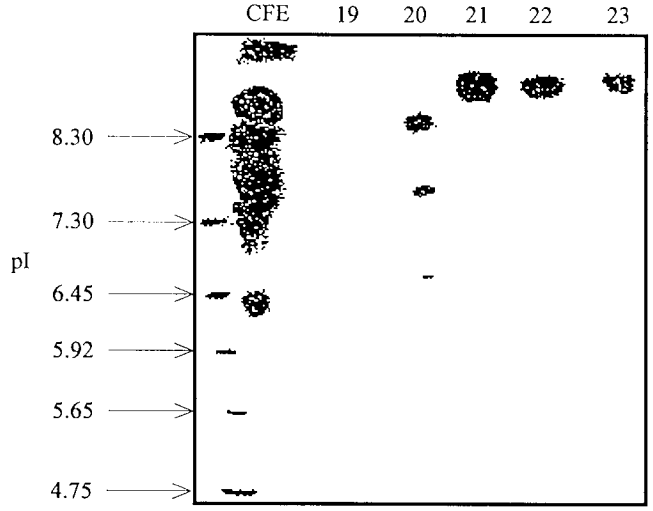


Figure 7.3 IEF of fractions 19-23 from the affinity column; the carbapenemase appears as a single band of activity in fractions 21-23 (pI 8.45). The cell-free extract was included for reference.

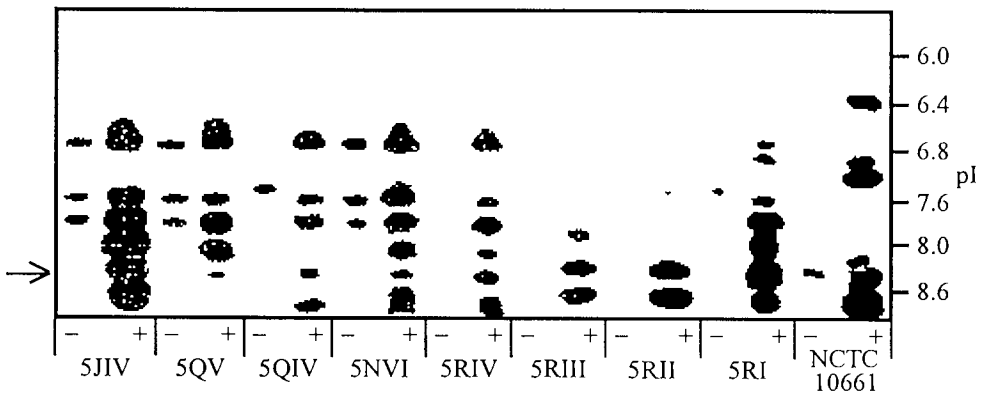


Figure 7.4 IEF of the cell-free extracts prepared from the ten strains used, with and without imipenem (4μg/ml) induction. Note the almost universal appearance of a band of activity in the region pI 8.4-8.5 after induction. + indicates an induced strain; – an uninduced strain. The arrow to the left indicates pI 8.45.

7.7.3 Spectrophotometric Characterisation of the Enzyme.

The enzyme hydrolysed each of the three carbapenems tested, showing greatest activity against imipenem (table 7.1). Some cephalosporins and penicillins were also hydrolysed, but aztreonam and cloxacillin were resistant to hydrolysis.

Substrate	Starting Concentration Employed / μM	Hydrolysis Rate (nmol/ml/min)*	Relative Rate (Cephaloridine=100)
Benzylpenicillin	842	0.525	32
Ampicillin	334	0.258	16
Cloxacillin	180	< 0.213	< 13
Carbenicillin	287	0.505	31
Cephaloridine	193	1.650	100
Ceftazidime	132	0.135	8
Imipenem	378	6.793	412
Meropenem	274	1.220	74
Biapenem	288	0.583	35
Aztreonam	367	< 0.418	< 25

Table 7.1 Hydrolysis of β-lactam antibiotics by the novel carbapenemase from *B. cepacia* at 37°C in 5mM pH7 MOPS buffer.
*Rate=nanomoles of substrate hydrolysed per minute, per millilitre of the reaction mixture

The enzyme was susceptible to inhibition by a range of compounds including aztreonam, cloxacillin, the inhibitors clavulanic acid and tazobactam, and the cysteine-modifying agent, *p*-chloromercuribenzoate (pCMB) (table 7.2). A dependence of the enzyme on zinc for activity was demonstrated by inhibition with two structurally dissimilar metal ion chelators, EDTA and *o*-phenanthroline (OP), and subsequent recovery of activity on addition of an equimolar concentration of zinc.

Inhibitor	Concentration Employed	Preincubation Time / minutes	% Inhibition	% Recovery with Equimolar Zinc
Aztreonam	1.0mM	5	95	–
Cloxacillin	1.0mM	5	98	–
Clavulanic Acid	0.1mM	5	98	–
Tazobactam	0.1mM	5	98	–
EDTA	1.0mM	10	93	14
OP	1.0mM	10	90	100
pCMB	0.5mM	10	100	–

Table 7.2 Effects of the various inhibitors tested against imipenem-hydrolysing activity.

7.7.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

SDS-PAGE analysis of 6 fractions from around the point of elution of the carbapenemase from the dye affinity column showed the presence of 10 well resolved Coomassie blue-stained bands remaining in fractions 21 and 22 (those containing significant carbapenemase activity). Attempts to identify the β-lactamase *in situ* with nitrocefin by the method described in section 7.5 were unsuccessful. However, one band (indicating a MW in the region of 40.5kDa) appears to be associated with carbapenemase activity, as indicated by its presence only in fractions which hydrolyse imipenem (figure 7.5). This observation is tentative and requires further substantiation.

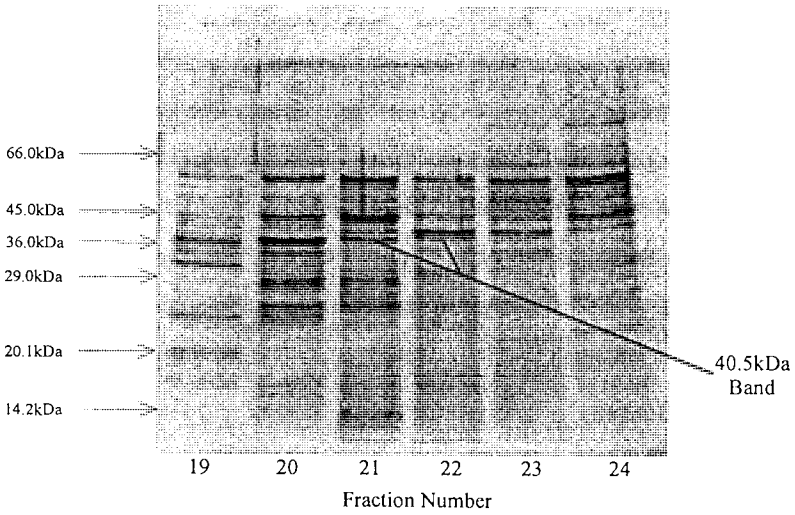


Figure 7.5 SDS-PAGE gel of fractions 19 to 24 from the dye affinity chromatographic partial purification of the β-lactamase. Activity was limited to fractions 21 and 22. These fractions possess a unique protein band at approximately 40.5kDa.

7.7.5 Bioassay of Imipenem-Hydrolysing Activity.

The growth of *E.coli* 10418 within the zone of inhibition around the well filled with imipenem solution was promoted slightly by the carbapenemase-containing cell free extract from *B. cepacia* strain 5JIV and to a much lesser extent by dye affinity-purified fractions of that extract (figure 7.6). However, a reduction in absolute activity (as opposed to activity per weight of protein present) is likely upon purification.

This assay is claimed to be more sensitive than spectrophotometric monitoring of antibiotic hydrolysis (Paton *et al.*, 1993). However, this did not seem to be the case in this instance. This assay is time consuming and tedious to execute and the results given are not quantitative. However, it has the advantage of clearly demonstrating a loss in activity of a given antibiotic due to the actions of the extract under test.

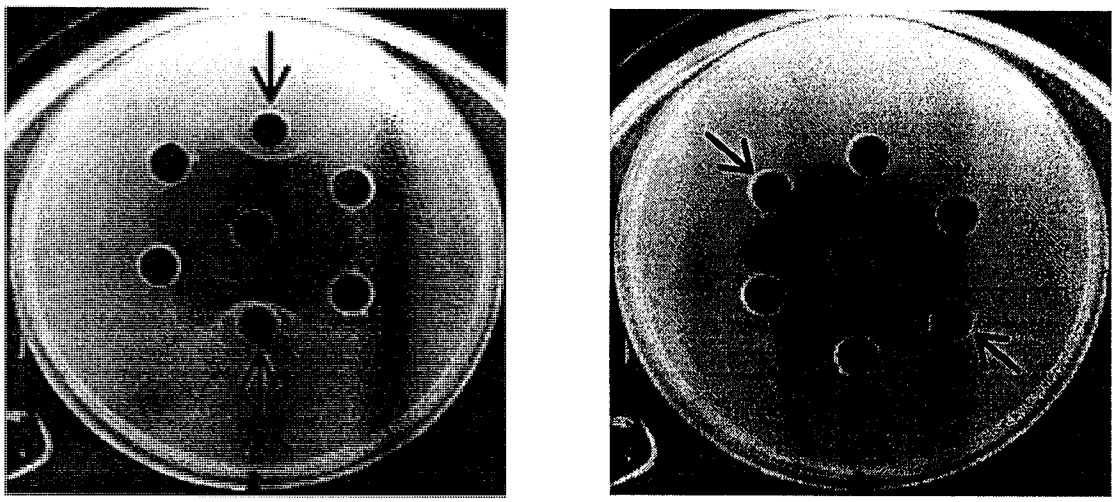


Figure 7.6 Photographs of 2 bioassay plates. The central well contains imipenem. The arrows locate wells filled with carbapenemase-containing cell free extract from strain 5JIV (left hand plate) and carbapenemase-containing pooled fractions 21 and 22 from dye affinity purification (right hand plate). Diminished zones of inhibition are clearly present around the wells containing cell free extract. The same phenomenon is visible, although much less marked, around the wells containing dye affinity eluate. The other wells shown contain fractions from the dye affinity separation distant from fractions 21 and 22 as a control.

7.7.6 Summary.

Metallo-β-lactamases with carbapenemase activity have previously been described in a number of different species (Livermore, 1992; Payne, 1993), but none appears to combine such a marked affinity for imipenem with a susceptibility to inhibition by clavulanic acid. This enzyme was designated PCM-I (*Pseudomonas cepacia* metallo-enzyme I).

8 Discussion and Concluding Comments.

8.1 Clustering of *Burkholderia cepacia* and Supporting Biochemical Data.

This group of isolates has been characterised in detail via investigation of their biochemical profiles, FAME profiles, PFGE restriction pattern profiles and OMP profiles.

8.1.1 Fatty Acid Methyl Ester Profiles.

Many clinical isolates share similar FAME profiles. From figure 4, it is noticeable that strains from clusters 3 and 4 are more distantly related to *B. cepacia* NCTC 10661 than *B. gladioli* ATCC 25417. For this reason, it could be concluded that a considerable proportion of the strains under study (which are identified as *B. cepacia* by API 20 NE and the standard procedures outlined in section 1.2.5.2) fit poorly with that description because they are more distant to a laboratory isolate of that species than an isolate of a closely-related species. This may mean that such strains require nomenclature of their own. On a slightly less detailed level, MIS separates these isolates into two categories under the headings of *B. cepacia* and *B. gladioli*. On this basis, the initial study of Simpson *et al.* has brought to light a difference between isolates similar to these that appears highly significant (Simpson *et al.*, 1994).

It is worth commenting that, as an instrument designed for the purpose of bacterial identification in line with current taxonomic thinking, MIS cannot make any finer distinction and its inability to do so is probably a function of its programming combined with the taxonomic proximity of the species from *Burkholderia* spp. to one another. However, it provides a most useful standardisation of procedure. Fatty acid extraction and FAME derivatisation contains at least one critically time-dependent step and it is difficult for a single operator to remain totally faithful to this whilst achieving a reasonable rate of productivity. Consequently, it was felt that FAME profiles would require confirmation by the use of further techniques. PFGE, with a proven applicability in this sphere, was therefore a suitable technique to adopt.

8.1.2 Pulsed-Field Gel Electrophoresis Restriction Fragment Profiling.

PFGE analysis illustrated the marked prevalence of a narrow genetic lineage amongst those isolates arising from CF lung infections of which CF researchers are greatly aware. However, the combining of data arising from the use of 2 separate restriction enzymes revealed this group to consist of 3 closely related subtypes. One subtype is noted for its relative metabolic deficiency, a feature not commonly associated with *B. cepacia.* Furthermore, clusters of isolates from PFGE analysis, especially that containing the CF epidemic strain (cluster 4) and those containing other clinical isolates (clusters 1, 2 and 3) were less similar to laboratory isolates of *B. cepacia* than isolates of the closely-related species *B. vietnamiensis* and linked with *B. cepacia* at a similar level to *B. gladioli.* If these named species are considered to be worthy of a species distinction, then it could reasonably be suggested that the majority of clinical isolates in this study are also worthy of such. Further taxonomic investigation, employing a large sample of isolates and submission of those isolates to a detailed polyphasic investigation, will be needed to support this ultimately but it can only be a matter of time before this proposed investigation is effected.

The lower end of the 99% confidence limit between certain strains in cluster 4 and laboratory strains is shown in table 8.1, together with the actual Dice coefficient calculated. The strains included were selected for their positioning within figure 5.2. The lower end of the confidence range is of greatest interest here as this thesis is concerned primarily with difference between strains and not with similarity. Similarity could be considered as the essence of an epidemiological investigation. This aspect of *B. cepacia* infections in CF is not the aim of this thesis and has been considered in the literature already (Govan *et al.*, 1993).

	NCTC 10257	ATCC 10248	LMG 6998	NCTC 10744	4949	15856	5PI	5SV	5PIV	5JIV
NCTC 6750	0.44 (0.37)	**0.41 (0.52)**	**0.35 (0.44)**	**0.39 (0.41)**	0.45 (0.43)	**0.41 (0.49)**	**0.44 (0.45)**	**0.42 (0.48)**	**0.39 (0.49)**	**0.39 (0.48)**
NCTC 10257		**0.50 (0.53)**	0.47 (0.44)	**0.36 (0.42)**	0.50 (0.44)	**0.40 (0.52)**	**0.36 (0.46)**	**0.44 (0.47)**	**0.43 (0.48)**	**0.41 (0.48)**
ATCC 10248			**0.56 (0.63)**	**0.49 (0.59)**	**0.50 (0.63)**	**0.44 (0.74)**	**0.53 (0.65)**	**0.57 (0.69)**	**0.60 (0.71)**	**0.54 (0.70)**
LMG 6998				0.69 (0.49)	**0.52 (0.53)**	0.63 (0.60)	**0.47 (0.55)**	0.69 (0.58)	**0.57 (0.60)**	0.68 (0.59)
NCTC 10744					0.72 (0.49)	0.63 (0.55)	0.60 (0.51)	0.62 (0.54)	0.58 (0.55)	0.70 (0.55)
4949						0.61 (0.60)	0.60 (0.55)	**0.56 (0.58)**	**0.54 (0.60)**	0.68 (0.59)
15856							**0.51 (0.64)**	**0.65 (0.68)**	**0.48 (0.70)**	**0.61 (0.69)**
5PI								**0.57 (0.61)**	**0.60 (0.62)**	0.62 (0.60)
5SV									0.86 (0.65)	0.85 (0.66)
5PIV										0.87 (0.68)

Table 8.1 Dice coefficients between selected pairwise comparisons within the sample (upper number in each cell) compared to the lower limit of the 99% confidence level (in parentheses). If the Dice coefficient is lower than the confidence limit, then the two strains are significantly dissimilar as judged by equation 2 in chapter 5, but with the reservations made in the text in section 8.1.2. Figures shown in bold type represent significant dissimilarities within this selected group.

If the calculated Dice coefficients are lower than the confidence value, one can be certain that the strains are significantly different by this analysis. These figures must be viewed with some caution. Observing equation 2 in chapter 5, it can be seen that the confidence equation is heavily depended on I_K, the interval factor, which is the most subjective measurement in the system. Small variations in I_K have a fairly large impact on the confidence value. Therefore, it is probably as valid to consider the isolates in relation to several closely related species included in the study for that purpose, to look at the relationships between previously assigned species and to attempt to lay down a minimum species level on the dendrogram against this backdrop. In figure 5.2, such a minimum level would appear to be between 0.3 and 0.4 Euclids, at which level the laboratory isolates of *B. cepacia* and *B. vietnamiensis* diverge. This approach may not be as useful when considering a taxonomic niche less closely interrelated than *Burkholderia* spp. and thus highlights the care and skill necessary in interpreting such data as that presented in chapter 5 and table 8.1.

The figures in bold type in table 8.1 indicate the positions where statistical analysis returned a result of significant dissimilarity. The strains falling within the cluster containing the CF epidemic strain (PFGE cluster 4) are significantly dissimilar to all isolates except *B. vietnamiensis* LMG 6998 and *B. cepacia* NCTC 10744 and, in the case of isolate 5JIV only, isolate 5PI. It is noticeable that this analysis does not reveal a significant difference between LMG 6998 and NCTC 10744 either, so the powers of a *Dra*I/*Xba*I 2-enzyme PFGE system to differentiate between these groups appear weak. The exploitation of further rare-cutting restriction enzymes such as *Spe*I to investigate this relationship might yield useful data, but the published study of Gillis *et al.* provides compelling evidence for considering this difference to be significant insofar as it draws on polyphasic taxonomy to justify the creation of *B. vietnamiensis* as an entity distinct from the other members of *Burkholderia* spp.(Gillis *et al.*, 1995).

From direct observation of the dendrogram, cluster 4 appears to split into 3 major subgroups, although statistical data is not available to support this as these groups are closely related by this analysis. From table 3.7, it appears that these clusters possess differing metabolic capabilities. Outstanding in this respect is the cluster denoted 4b, which

contains strains of apparently great inferiority in their metabolic diversity. This group contains several strains that appear to be auxotrophs (5PIV, 5PVI, 5RIV and 5TI) and others with limited metabolic capabilities, such as 5QIV. The higher prevalence of auxotrophic *B. cepacia* strains in the CF lung is reasonably explained by Barth and Pitt as due to the abundance of nutrients arising from pulmonary damage and the destruction of lung tissue (Barth & Pitt, 1995). The occurrence of amino acid auxotrophs in the CF lung has also been described for *P. aeruginosa* (Barth & Pitt, 1996). However, this reliance on growth factors and narrow metabolic capability is not a feature of *B. cepacia* sensu stricto and it would appear that the species definition of *B. cepacia* (Burkholder, 1950) has become confused during the intervening 46 years since the original description.

Evidence to support this conclusion is provided by Yohalem & Lorbeer in their detailed studies of this species and its relatives (Yohalem & Lorbeer 1994a,b). The presence of three or more novel species to be created from those isolates currently considered as *B. cepacia* but poorly fitting this species description is also supported by this work and by that of Vandamme (Vandamme, 1995). With respect to the work conducted in this thesis, all 4 clusters in figure 5.2 could be suggested as new species, but would require further biochemical characterisation to support this. The conclusions arising from PFGE analysis of these strains are that a number of species await description in this taxonomic niche. Amongst these is a species defining the CF epidemic strains, which appears from initial biochemical analyses to be comprised of 3 pathovars.

8.1.3 Outer Membrane Profile Assessment.

2 broad OMP types were observed in this sample on the basis of the heat-modifiability of an 81kDa protein previously described as the major porin oligomer of *B. cepacia* (Gotoh *et al.*, 1994). As mentioned in chapter 6, the second of these groups was largely comprised of pigmented strains that fell within FAME cluster 3 and PFGE cluster 4. This corresponds well with both the CF epidemic isolates, the group of strains lacking in metabolic diversity and the group of strains identified by MIS to be *B. gladioli*. The nature of this difference is not known, but it could be due to the way in which the subunits of this oligomer are associated. However, the functional relevance of this has not been assessed.

Electroelution studies show this oligomer to dissociate upon electroelution. This technique is commonly used to remove LPS from a bacterial protein sample (Harrington, 1990). One could therefore speculate that this oligomer is assisted in its association by LPS and that differing LPS type could possibly be involved with this property. However, electroelution could reasonably be expected to generate a great deal of heat and thus heat-modify the proteins anyway. It may also be that this difference in association of the major porin explains at least in part the difference in metabolic capability observed by an altered ability to take up a range of nutrients. Similarly, the presence and absence of cable pili may correlate with this observation. From the literature, this high-MW structure has not been described as a nonfimbrial adhesin in *B. cepacia* but the presence of differentially heat-modifiable proteins of this type has been described in *E. coli* (Ahrens *et al.*, 1993). It should be reiterated that these suggestions are speculations alone, since no attempt was made to isolate fimbrial adhesins from the strains. Therefore, further work must be conducted into this observation.

8.2 Carbapenemase production by *Burkholderia cepacia.*

Evidence is presented in chapter 7 for the presence in *B. cepacia* of an inducible, zinc-dependent carbapenemase that is inhibited by the clinically available agents clavulanic acid, tazobactam, aztreonam and cloxacillin (although at high concentrations in the last 2 cases). Susceptibility tests in the presence of the first two of these agents suggest that this observation will not improve the current clinical situation immediately.

When investigating potential metalloenzymes, the use of chemically diverse chelators should overcome the unpredictable possibility that one structural class of chelator in some way inhibits the enzyme in a manner not related to chelation of a metal ion from the active site. Table 7.2 shows that the degree of recovery from addition of EDTA and OP by supplementation with equimolar Zn^{2+} is dissimilar. EDTA has been reported to inhibit one serine β-lactamase at least partially (Fujii *et al*, 1985). Therefore one must be alert to the possibility that EDTA and analogues are capable of inhibiting β-lactamase activity by a mechanism other than chelation. It must be borne in mind that recovery from inhibition by a chelator is at least as significant as inhibition itself. Although inhibition by any single chelator is not necessarily conclusive evidence of a metalloenzyme, the use of two or more structurally diverse agents such as in this study is firmer proof of a true chelating effect. The use of structurally diverse chelators was proposed by Bush and Sykes (1986) as part of an extensive review of techniques suitable for the investigation of β-lactamases. In this study, the use of two structurally diverse chelators produced vastly different extents of recovery. If that recovery were solely due to an effect of zinc on imipenem, the extent of recovery should be similar in both cases.

Currently, it seems that sequence data remains the ultimate determinant in this field of investigation. However, the construction of a universal gene probe for the detection of metallo-β-lactamases would not immediately appear to be a viable proposition. Those enzymes studied thus far have proved to be an heterogeneous group at the molecular level. It has not yet been possible to purify PCM-I sufficiently to attempt a N-terminal sequence for cloning attempts.

Considering the hydrolysis of the three carbapenems tested, it would appear that the presence of a bulky substituent on the 2-position of the 5-membered ring may cause steric hindrance to their enzymatic degradation. This observation might be useful in the design of a β-lactamase-stable carbapenem, although the availability of more carbapenems would assist the clarification of this.

It is notable that PCM-I appears to be ubiquitous amongst isolates of *B. cepacia* including NCTC 10661. Carbapenemases and metallo-β-lactamases are a rarity in most species, but if these results are representative it would appear that, in common with *S. maltophilia*, most if not all strains of *B. cepacia* are capable of expressing this metallo-carbapenemase. This finding was recently proposed by Simpson *et al.* (Simpson *et al.*,1993). It is evident that, as inferred by these authors, the combination of a carbapenem with a suitable β-lactamase inhibitor (if one could be found) would be clinically advantageous.

The elucidation of clavulanic acid-sensitive metallo-β-lactamases brings to light a point in classification. It is becoming increasingly evident that the inclusion of every metallo-β-lactamase in the same group of any classification scheme is unsatisfactory. Whilst extension of the Ambler classification scheme to class E and beyond must be supported by sequence data, the extension of the biochemical based classification of Bush (Bush, 1989b,c) is feasible. This would be effected by the inclusion of an extra class, which could be subtitled MET-Y and numbered 3a in order to preserve the subgrouping of the metalloenzymes in the scheme. PCM-I and the metalloenzyme described by Nord *et al.* from *Bacteroides distasonis* (Nord *et al.*, 1991) would be suitable members for inclusion in this group. Further subdivision may arise from elucidation of the mechanism of inhibition. This could be suicide-based, whereby the inhibitor becomes irreversibly bound within the active site, or competitive inhibition via preferential occupation of the active site of the enzyme.

The work from this thesis concerning the isolation of this enzyme was published in 1994 (appendix 3). Subsequently, this enzyme has been classified in group 3 of the Bush-Jacoby-Medeiros scheme by Bush *et al.* (Bush *et al.*, 1995). From current research communications, it would appear that the biochemical subdivision of metallo-β-lactamases is under consideration by this group and their collaborators (Yang & Bush, 1996).

8.3 Concluding Remarks.

The relationship of clinical isolates identified as *B. cepacia* to laboratory strains of that species and other, closely related species has been addressed. This leads to the conclusion that further subdivision in this taxonomic niche is required. The species name *B. cepacia* is alone insufficient to describe the great diversity found therein.

B. cepacia is an important cause of infections in the CF lung and it appears that metallo-β-lactamase production is widespread within this species. Considering this, and the clinical implications of metallo-β-lactamases in general, the carbapenemase from *B. cepacia* is evidently worthy of further study. The discovery of novel metallo-β-lactamase inhibitors will be of great future therapeutic benefit, both for of infections with *B. cepacia* and for other organisms capable of expressing metallo-β-lactamases. There is a great diversity apparent amongst the metallo-β-lactamases characterised thus far. This would tend to indicate that any biochemical screen for a potential lead compound for development as a β-lactamase inhibitor should contain a variety of enzymes selected to achieve maximum coverage of the whole field.

Regarding *B. cepacia* as a species, it is evident from the above that the clinical ramifications of a diagnosis of *B. cepacia* will not be fully elucidated until a detailed taxonomic investigation of this genus and the closely related species has been effected. The most accurate assessment of this situation to be published is that of Yohalem and Lorbeer (Yohalem & Lorbeer, 1994a). Put simply, "the species concept of *B. cepacia* is too broad". I conclude that the CF-associated pigmented strains clustering within FAME cluster 3 and PFGE cluster 4 comprise a novel species currently without taxonomic designation.

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Appendix 1. Biochemical Test Results Strain-by-Strain.

Strain	Utilisation of:										
	None	GLU	MHB	LEV	ADO	MES	CIT	TRY	AMY	BUT	TAR
NCTC 10661	–	+	+	–	+	–	+	+	–	+	+
ATCC 17616	–	+	–	–	+	–	+	+	–	+	+
ATCC 10744	–	+	+	–	+	–	+	+	–	+	+
ATCC 25417	–	+	+	+	+	+	+	–	–	–	+
ATCC 10248	–	+	+	+	+	+	+	–	+	+	+
ATCC 10854	–	+	–	+	+	+	+	–	–	+	+
5RI	–	+	+	+	–	–	+	+	–	+	+
5RII	–	+	+	+	+	+	+	+	+	+	+
5RIII	–	+	+	+	+	–	+	+	–	+	+
5OV	–	+	+	+	+	–	+	+	+	+	+
5PI	–	+	+	+	+	+	+	+	–	+	+
5PII	–	+	+	+	+	+	+	+	–	+	+
5SI	–	+	+	–	+	+	+	+	+	+	+
5SIV	–	+	+	+	–	+	+	+	+	+	+
5SVI	–	+	–	+	+	–	+	+	+	+	+
3843	–	+	+	+	+	–	–	+	–	+	+
3925	–	+	+	+	+	+	+	+	–	+	+
4325	–	+	+	+	+	–	+	–	+	+	+
4949	–	+	+	+	+	+	+	+	–	+	+
15856	–	+	+	+	+	–	+	+	–	+	+
5JIV	–	+	–	+	+	–	+	+	+	+	–
5NVI	–	+	–	+	+	–	+	+	+	+	–
5SV	–	+	+	+	+	–	+	–	+	+	–
5SII	–	+	+	–	+	–	+	–	–	+	–
5TIV	–	+	+	+	+	–	+	–	–	+	–
5SIII	–	+	+	+	+	–	+	–	+	+	–
5TV	–	+	+	+	+	–	+	–	–	+	–
5OIV	–	+	+	+	+	–	+	–	+	+	–
4326	–	+	+	+	+	–	–	–	–	+	–
9275	–	+	+	+	+	+	+	+	+	+	–
12794	–	+	+	+	+	–	+	+	–	+	+
5PIV	–	–	–	–	–	–	–	–	–	–	–
5QV	–	+	–	+	+	–	+	+	+	+	+
5RIV	–	–	–	–	–	–	–	–	–	–	–
5TI	–	–	–	–	–	–	–	–	–	–	–
5TII	–	+	+	+	+	–	+	–	+	+	–
5TIII	–	+	–	+	+	+	+	+	–	+	+
5PVI	–	–	–	–	–	–	–	–	–	–	–
5BI	–	+	+	+	+	–	+	+	–	+	+
5QIV	–	+	–	–	+	–	–	–	–	–	–

None = no carbon source in medium; GLU = glucose utilisation in medium C of Dye;
MHB = *m*-hydroxy benzoate; LEV = levulinate; ADO = adonitol; MES = mesaconate; CIT = citraconate;
TRY = tryptamine; AMY = α-amylamine; BUT = 2,3-butylene glycol; TAR = D(-)-tartrate.
NB: *B. vietnamiensis* LMG 6998 excluded from this study due to time of arrival of the isolate.

Strain	Acid Production From:								LDC	PIG
	ADO	CEL	LAC	MAL	MEL	SUC	SAL	TRE		
ATCC 10744	+	+	+	+	+	+	+	+	+	–
NCTC 10661	–	+	+	+	–	+	–	–	+	–
ATCC 17616	–	+	+	+	–	–	–	+	+	–
ATCC 25417	–	–	–	–	–	–	–	–	–	–
ATCC 10248	+	–	–	–	–	–	–	–	–	–
ATCC 10854	+	–	–	–	–	–	–	–	–	–
LMG 6998	–	+	+	+	–	+	–	–	+	–
5RI	–	+	+	+	–	+	–	+	+	–
5RII	–	+	+	+	–	–	–	+	–	–
5RIII	–	+	+	+	–	+	–	+	+	–
5OV	–	+	+	+	–	–	–	+	+	–
5PI	–	+	+	+	–	–	–	–	+	–
5PII	–	+	+	+	–	–	–	+	+	–
5SI	+	+	+	+	–	–	–	–	–	–
5SIV	–	+	+	+	–	–	–	–	+	–
5SVI	–	–	+	+	–	+	–	+	+	+
3843	–	+	+	+	–	–	–	+	+	–
3925	–	+	+	+	–	+	–	+	+	–
4325	–	+	+	+	–	–	–	+	+	–
4949	–	+	+	+	–	–	–	+	+	–
15856	–	+	+	+	–	–	–	+	+	–
5JIV	–	–	–	–	–	–	–	–	–	+
5NVI	–	–	–	–	–	–	–	–	–	+
5SV	–	–	–	–	–	+	–	+	–	+
5SII	–	–	–	–	–	–	–	–	–	+
5TIV	–	–	–	–	–	–	–	+	–	+
5SIII	–	–	+	+	–	–	–	–	–	+
5TV	–	–	+	+	–	–	–	–	–	+
5OIV	–	–	+	+	–	–	–	–	–	+
4326	–	+	+	+	–	–	–	+	–	+
9275	–	+	+	+	–	–	–	+	+	–
12794	–	+	+	+	–	–	–	+	+	–
5PIV	–	–	–	–	–	–	–	–	+	–
5QV	–	–	–	–	–	+	–	–	+	+
5RIV	–	–	–	–	–	–	–	–	+	–
5TI	–	–	–	–	–	–	–	–	+	–
5TII	+	–	–	–	–	+	–	–	+	+
5TIII	–	–	–	–	–	+	–	–	+	+
5PVI	–	–	–	–	–	–	–	–	–	–
5BI	+	+	+	+	–	–	–	–	+	–
5QIV	–	–	–	–	–	–	–	–	+	–

ADO = adonitol; CEL = cellobiose; LAC = lactose; MAL = maltose; MEL = melibiose; SUC = sucrose; SAL = salicin; TRE = trehalose; LDC = lysine decarboxylase; PIG = pigment production.

	NO2	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OXI
NCTC 10744	+	–	–	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
NCTC 10661	–	–	–	–	–	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
ATCC 17616	+	–	–	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
ATCC 25417	–	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–
ATCC 10248	–	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–
ATCC 10854	+	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–
LMG 6998	+	–	–	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5RI	+	–	–	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+
5RII	+	–	+	–	–	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
5RIII	–	–	–	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+
5OV	+	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
5PI	–	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
5PII	+	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
5SI	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
J2552	+	–	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5SVI	–	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
3843	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
3925	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
4325	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
4949	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
15856	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+

NO2 = nitrate reduction; TRP = indole production; GLU = acid production from glucose; ADH = arginine dihydrolase; URE= urease; ESC = esculin hydrolysis; GEL = gelatin hydrolysis; PNG = β-galactosidase; GLU = glucose utilisation in API 20 NE galery; ARA = arabinose utilisation; MNE = mannose utilisation; MAN = mannitol utilisation; NAG = N-acetyl-glucosamine utilisation; MAL = maltose utilisation; GNT = gluconate utilisation; CAP = caprate utilisation; ADI = adipate utilisation; MLT = malate utilisation; CIT = citrate utilisation; PAC = phenyl-acetate utilisation; OXI = oxidase.

	NO2	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OXI
5JIV	–	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
5NVI	+	–	+	–	–	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
5SV	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
J1948	–	–	–	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5TIV	–	–	–	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
J2315	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5TV	–	–	–	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5OIV	–	–	–	–	–	+	–	+	+	+	+	+	+	–	+	+	+	–	+	+	+
4326	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
9275	+	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
12794	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5PIV	+	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	+	+	+	–
5QV	–	–	–	–	–	–	–	–	+	–	+	+	+	–	+	+	+	+	+	+	+
5RIV	–	–	–	–	–	–	–	–	+	–	+	+	+	–	+	+	+	+	+	+	+
5TI	–	–	–	–	–	–	–	–	+	–	+	+	+	–	+	+	+	+	+	+	+
5TII	–	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5TIII	–	–	–	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5PVI	+	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–
5BI	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+
5QIV	+	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	+	–	+	+

NO2 = nitrate reduction; TRP = indole production; GLU = acid production from glucose; ADH = arginine dihydrolase; URE= urease; ESC = esculin hydrolysis; GEL = gelatin hydrolysis; PNG = β-galactosidase; GLU = glucose utilisation in API 20 NE gallery; ARA = arabinose utilisation; MNE = mannose utilisation; MAN = mannitol utilisation; NAG = N-acetyl-glucosamine utilisation; MAL = maltose utilisation; GNT = gluconate utilisation; CAP = caprate utilisation; ADI = adipate utilisation; MLT = malate utilisation; CIT = citrate utilisation; PAC = phenyl-acetate utilisation; OXI = oxidase.

Appendix 2. Comparison of Société Française de Microbiologie (SFM) and British Society for Antimicrobial Chemotherapy (BSAC) Breakpoint Values.

Antimicrobial Agent	BSAC Breakpoint MIC (μg/ml)		SFM Breakpoint MIC (μg/ml)	
	S≤	R≥	S≤	R≥
Imipenem	4	4	4	8
Aztreonam	8	8	4	32
Cefsulodin	8	8	8	8
Ceftazidime	2	2	4	32
Ticarcillin	16	64	64	64
Ticarcillin + Clavulanic Acid	16	64	64	64
Piperacillin	16	64	16	16
Piperacillin + Tazobactam	16	64	16	64
Tobramycin	1	4	4	8
Amikacin	4	16	8	16
Gentamicin	1	4	4	8
Netilimicin	1	4	8	16
Colistin	–	–	4	4
Fosfomycin	-	–	32	32
Perfloxacin and other 4-Quinolones	1	4	1	4
Ciprofloxacin	4	4	1	2
Co-Trimoxazole	0.5	2	2	8

S = Sensitive; R = Resistant. BSAC breakpoint values are based on the recommendations for the treatment of Enterobacteriaceae and *Pseudomonas* spp. No breakpoint value was quoted for either colistin or fosfomycin in the BSAC guidelines (Anonymous, 1991).

Appendix 3. Publications Submitted in the Course of this Research.

Baxter, I.A. & Lambert, P.A. 1994. Isolation and partial purification of a carbapenem-hydrolysing metallo-β-lactamase from *Pseudomonas cepacia*. *FEMS Microbiology Letters* **122:** 251-256.

Baxter, I.A. & Lambert, P.A. 1995. Are all carbapenems created equal? *Journal of Antimicrobial Chemotherapy* **35:** 708-709.

Baxter, I.A., Lambert, P.A. & Simpson, I.N. 1996. Isolation of *Burkholderia cepacia* from clinical sources possessing characteristics of *Burkholderia gladioli*. *Journal of Antimicrobial Chemotherapy*, in press.