NOVEL AGENTS WITH INHIBITORY ACTIVITY AGAINST THE *BURKHOLDERIA CEPACIA* COMPLEX

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Publications

Langley R, Kenna DT, Vandamme P, Ure R, Govan JR (2003). Lysogeny and bacteriophage host range within the *Burkholderia cepacia* complex. Journal of Medical Microbiology 52, 483-490.

Kenna DT, Barcus VA, Langley RJ, Vandamme P, Govan JR (2003). Lack of correlation between O-serotype, bacteriophage susceptibility and genomovar status in the *Burkholderia cepacia* complex. FEMS Immunology and Medical Microbiology 35, 87-92.

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AHL	Acyl homoserine lactone
AMP	Antimicrobial peptide
Asialo-GM1	Asialo-gangliotetraosylceramide
ASL	Airway surface liquid
ATCC	American type culture collection
ATP	Adenosine 5' triphosphate
BA	Blood agar
Bcc	*Burkholderia cepacia* complex
BCESM	*Burkholderia cepacia* epidemic strain marker
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CAMP	Cationic antimicrobial peptide
Ceftz	Ceftazidime
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
cfu	Colony forming units
CGD	Chronic granulomatous disease
Chlor	Chloramphenicol
CIA	Cepacia isolation agar
Cipro	Ciprofloxacin
CTAB	Cetyltrimethylammonium bromide
DEFA	IIuman alpha (α)- defensin
defb	Murine beta (β)- defensin
DEFB	Human beta (β)- defensin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ENaC	Epithelial sodium channel
ET-12	Electrophoresis type 12
FEV_1	Forced expiratory volume in 1 second
FPLC	Fast protein liquid chromatography
FVC	Forced vital capacity
hBD	Human beta (β)- defensin
HD	Human alpha (α)- defensin

HiB	*Haemophilus influenzae* type B
HIV-1	Human immunodeficiency virus-1
HNP	Human neutrophil α-defensin
HPLC	High performance liquid chromatography
HS	Hypertonic saline
IL-1β	Interleukin 1-β
IL-6	Interleukin 6
IL-8	Interleukin 8
ISA	Iso-sensitest agar
ISB	Iso-sensitest broth
IV	*Intra venous*
Kbp	Kilobase pair
KDO	3-deoxy-d-manno-octulosonic acid
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MBC	Minimum bactericidal concentration
mBD	Murine β defensin
Mbp	Mega base pair
MDR	Multi-drug resistant
Mero	Meropenem
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSD	Membrane spanning domain
MSSA	Methicillin sensitive *Staphylococcus aureus*
MWCO	Molecular weight cut off
NA	Nutrient agar
NADPH	Phosphorylated nicotinamide adenosine dinucleotide
NBD	Nucleotide binding domain
NBYE	Nutrient broth yeast extract
NCTC	National collection of type cultures
NH	Normal (Scottish) honey
NZMh	New Zealand manuka honey
OD	Optical density
ORCC	Outwardly rectified chloride channel
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulsed field gel electrophoresis

PI	Isoelectric point
PIA	*Pseudomonas* isolation agar
PLC	Phospholipase C
Poly	Polymyxin B
psi	Pounds per square inch
QS	Quorum sensing
RBG	Royal botanic gardens
RES	Reticulo-endothelial system
RFLP	Restriction fragment length polymorphism
rhDNase	Recombinant DNase
rRNA	Ribosomal ribonucleic acid
ROI	Reactive oxygen intermediate
SA	Salicyclic acid
SDS-PAGE	Sodium dodedecyl sulphate-polyacrylamide gel electrophoresis
SP-A/B	Surfactant protein A/B
TAP	Tracheal antimicrobial peptide
TLR2	Toll-like receptor 2
Tobra	Tobramycin
TSA	Tryptone soy agar
Trimeth	Trimethoprim
UMF	Unique manuka factor
WGH	Western General Hospital, Edinburgh

ABSTRACT

Pulmonary bacterial infections account for 95% of morbidity and mortality in cystic fibrosis (CF) patients, and include a limited spectrum of bacteria; *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and members of the *Burkholderia cepacia* complex (Bcc). "*B. cepacia*" was first recognised in the late 1970's as a cause of life threatening respiratory infections in CF. Initial clinical observations noted that 20% of colonised CF patients developed "cepacia syndrome", a rapid and fatal necrotising pneumonia. In addition, epidemiological evidence highlighted the potential spread of certain "*B. cepacia*" strains, that most isolates were highly resistant to conventional antibiotics and consequently, Bcc infections are untreatable. Subsequent taxonomic studies have identified the Bcc that contains ten distinct species of bacteria previously termed "*B. cepacia*". Clinical distribution of Bcc species in CF is restricted to mainly *B. cenocepacia* (50%), *B. multivorans* (38%) and *B. vietnamiensis* (7%).

The aim of this study was to investigate novel antimicrobial agents against the Bcc and other problematic and emerging CF associated bacterial pathogens including multi-resistant epidemic *P. aeruginosa* strains, methicillin resistant *S. aureus* (MRSA), and *Stenotrophomonas maltophilia*. The novel antimicrobial strategies examined were based on three main themes: First, the use of natural honey, second, the potential use of bacteriophage and their associated lytic enzymes and third, novel mammalian cationic β-defensins. The project utilised a vast collection of bacterial isolates and included relevant clinical, environmental and epidemic strains. The susceptibility to conventional antibiotics was measured, and resistance was shown to vary across the Bcc. In general, clinical isolates were statistically more resistant to conventional antibiotics than environmental isolates.

Members of the Bcc, and an extended panel of resistant organisms were shown to be sensitive to New Zealand manuka honey (NZMh). The MICs ranged from 9 to 17% (w/v), and the MBCs ranged from 9 to 20% (w/v). The antimicrobial component of NZMh was investigated, and focussed on osmolarity, pH and H_2O_2. All were found to contribute to the antibacterial activity, although none were solely responsible for the activity. Killing-curves suggested that NZMh kills within 24 hours. The NZMh

preparation was applied to a CF patient infected with *B. cenocepacia* J2315, and clinical data highlighted possible benefits to the patient.

Novel Bcc specific bacteriophages were identified from environmental samples and from lysogeny studies. The spectrum of activity of the novel bacteriophages, and previously reported Bcc bacteriophage (NS1 and NS2), was determined using a panel comprising 66 isolates of the Bcc, 55 isolates representing other pseudomonads, and 40 *B. pseudomallei* strains. The novel phages were shown to be very promiscuous and had activity across the Bcc, with some active against *P. aeruginosa*, *B. gladioli*, and *B. pseudomallei*. The wide spectrum of activity was detrimental to therapeutic use, therefore, the phage-encoded lytic enzymes were the focus for further study: bacteriophage therapy with a novel twist. Two enzymes were investigated: the *B. cepacia* bacteriophage Bcep781 endolysin and the *P. aeruginosa* phage D3 endolysin. The Bcep781 phage and phage DNA was not available, therefore the endolysin gene was synthesized using recursive PCR. Briefly, twenty-two overlapping oligonucleotides encoding the entire gene were synthesized and constructed into the endolysin gene using a single PCR reaction. Both genes were cloned into an expression plasmid and the enzymes were recombinantly expressed as 6-His fusion proteins in BL21 *E. coli* cells. Bcep781 endolysin was purified using nickel-affinity chromatography, and the D3 lysin was purified using a Resource S® purification protocol. High-resolution mass spectrometry analysis highlighted discrepancies in both lysins, and neither proved to active against relevant bacteria tested.

The activity of cationic antimicrobial peptides (CAMPs) including: a synthetic novel murine β-defensin (Defr1) with 5-cysteine residues, which forms a covalently bound dimer; its 6-cysteine analog (Def-cys); a chemically reduced Defr1; polymyxin B and colistin; were assessed against the Bcc, as well as several multi-resistant bacterial CF pathogens. Two Bcc isolates, *B. cepacia* type strain ATCC 25416 and *B. cenocepacia* type strain J2315, were found to be inherently resistant to all CAMPs utilised in this study. Epidemic *P. aeruginosa* isolates were found to have a MIC of 6 μg/ml for Defr1 and a MIC of 50-100 μg/ml for Def-cys, suggesting a possible relationship between defensin structure and function. Similarly, the MIC of 6 μg/ml

was also noted for *S. maltophilia* and *Ralstonia* sp. that were found to be resistant to polymyxin B and colistin. The recombinant production of Defr1 was also attempted in a number of expression systems in *E. coli*. However, although Defr1 was successfully expressed, the recombinant proteins were highly insoluble.

This study showed that resistance varies within the Bcc. However, the data show that NZMh exerts a bactericidal effect on members of the Bcc, including *B. cenocepacia* J2315, and that such activity may be utilised clinically. The novel Bcc bacteriophage may prove to be a useful panel for further study, either as vectors for horizontal gene transfer or as therapeutic agents. The data confirm previous observations that the Bcc are inherently resistant to CAMPs, including a novel 5-cysteine defensin. Despite this finding, synthetic Defr1 was shown to be active against a panel of multi-resistant pathogens associated with infections in CF. Further research is required to optimise the recombinant expression of Bcep781 endolysin, D3 lysin, and Defr1, to enable their use in the treatment of multiply resistant infections in CF and the wider hospital environment.

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My friends throughout this thesis have been exceptional; especially Alan, whose support, scientific banter, spare-bed and friendship will never be forgotten! Lastly, but by no means least I must thank my loving and caring family. Mum and Dad- this is your thesis-I dedicate this to the love and faith that you have shown in me throughout my life. Likewise, to my brother and best friend Scott, I will never forget your unrelenting support provided throughout both this PhD and my life.

DECLARATION

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

Ross John Langley 12^{th} of July 2004

1.1 Cystic Fibrosis: Genetics & Pathophysiology

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disorder affecting the Caucasian population with an incidence of approximately 1 in 2500 live births. One in twenty-five Caucasians of European decent are asymptomatic heterozygotes for CF. The hereditary nature of the CF gene is outlined (Fig. 1.1). It is interesting to note that the carrier rate of CF is population dependent and considerably more rare in other ethnic populations (Asians 1 in 32,000 and African Americans 1 in 15, 000). In general, the clinical features of CF are associated with the gastrointestinal and respiratory tract.

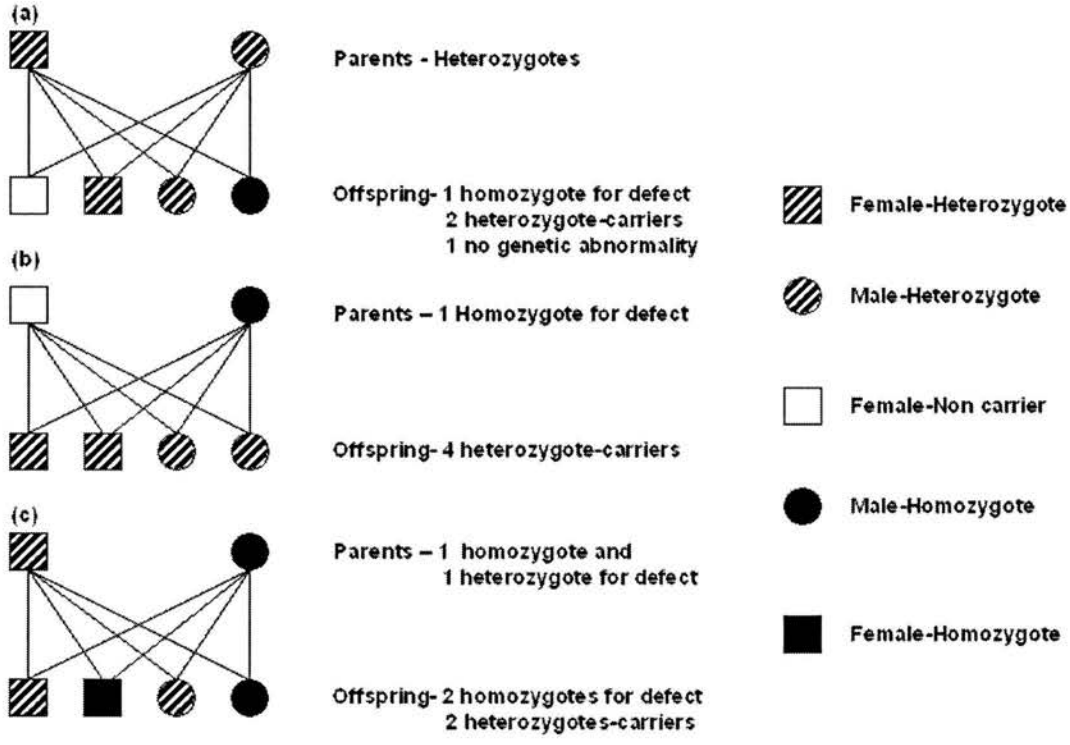


Fig. 1.1 Inheritance of the cystic fibrosis gene. (a) Both parents are heterozygotes; 1 in 4 chance of an affected child, a 2 in 4 chance of a heterozygote, and 1 in 4 chance of non-carrier. (b) Non-carrier plus fertile female homozygote; all offspring will be carriers. (c) Fertile female heterozygote plus homozygote; 1 in 2 chance of affected or carrier offspring.

The CF gene was identified in 1989 (Kerem *et al.*1989, Riordan *et al.*1989, Rommens *et al.*1989) and encodes the CF transmembrane conductance regulator

(CFTR). CFTR, a polypeptide of 1480 amino acids (MW ~168 kDa), is a member of the ATP-binding cassette family of transporter proteins and functions as a cAMP-dependent chloride (Cl^-) channel (Anderson *et al.* 1991, Sheppard and Welsh 1999). CFTR is expressed primarily in epithelial tissues (pancreatic ducts, salivary glands, intestinal crypts, pulmonary submucosal glands, testis and endometrium) and contains five domains: two membrane spanning domains (MSDs), each composed of six transmembrane segments; an R-domain, containing several consensus phosphorylation sequences; and two nucleotide binding domains (NBDs), which interact with ATP (Fig 1.2) (Gibson *et al.* 2003)

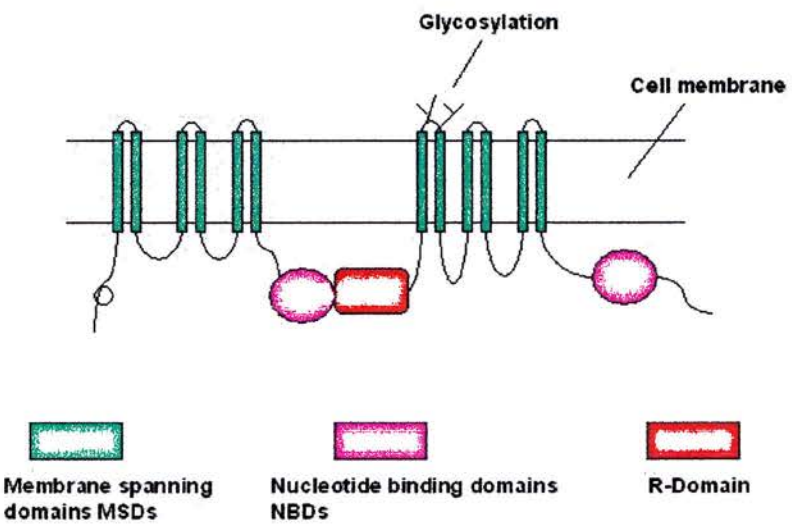


Fig. 1.2 CFTR positioned in the cellular membrane. CFTR contains 2 membrane spanning domains, each composed of 6 transmembrane segments; an R-domain; and 2 nucleotide binding domains.

Mutation in the CF gene, mapped to the long arm of chromosome 7 (7q31.2), result in defective CFTR, and thus disease (Gibson *et al.* 2003, Wainwright *et al.* 1985). To date over 1200 mutations in CFTR have been identified. However, the severity of disease, and indeed the nature of CFTR expression at the molecular level, is

mutation specific. The most common CF mutation is a three base pair deletion at position 508 (ΔF508), accounting for up to 70% of the mutations within the Caucasian population and resulting in loss of a phenylalanine at position 508 in the CFTR protein. Although a large number of mutations can result in a CF phenotype (http://www.genet.sickkids.on.ca/cftr/), some are extremely rare. In this section only the most common will be examined. Each mutation gives rise to a varied CFTR protein defect, which alters the fate of the mature peptide within the cell. To date, six classes of CFTR mutations have been described (Table 1.1).

Table 1.1 Classes of CFTR mutations and outcome on expression

Class	Common CFTR Mutation	Outcome
I	G542X, 3905insT	No synthesis
II	ΔF508, N1303K	No maturation
III	G551D*, G551S	Blocked regulation
IV	R347P, R117H, R334W	Decreased conductance
V	3849 + 10kb C→T, 5T	Decreased abundance
VI	G551D*	Defective regulation

*G551D affects both activation of CFTR and eliminates ORCC regulation

Class I mutations, characterised by G542X, contain premature stop codons, resulting in truncated mRNA and no protein expression. Class II mutations, which are usually missense mutations and include ΔF508, cause misfolding and result in the failure of mature CFTR to traffic to the correct cellular location. Class III mutations, of which G551D is the most common, are localised within the NBDs of CFTR. Since the regulation of CFTR as a chloride channel is dependent on adenosine triphosphate (ATP) stimulation, any mutation occurring in these domains will result in a defective phenotype. Class IV mutations, exemplified by R347P, produce the correct expression and localisation of CFTR, but there is a marked reduction in the chloride conductance of the channel. Such mutations are localised to the MSDs. Class V mutations, for example 3849 + 10kb C→T, result in decreased production of functional CFTR (in contrast to Class I), and are caused by alternative splicing. Class VI mutations (G551D) relate to CFTR's role as a regulator of other channels including outwardly rectified chloride channels (ORCC) (Fulmer *et al.* 1995).

Defective regulation of ORCCs may decrease whole cell chloride conductance, thus, normal function is impaired

Despite increased understanding of CF mutations, in general disease severity does not appear to relate to genotype. Although CFTR genotype, including ΔF508, is closely correlated with pancreatic status, the same cannot be applied to respiratory disease. In general, mutations that are associated with some functional activity of CFTR tend to relate to milder or late onset phenotypes. R117H and R334W are associated with pancreatic sufficiency, although the latter genotype is attributed to late-onset pancreatic insufficiency (Mickle and Cutting.1998). Likewise, 3849+10kb C→T, is associated with "mild" CF, and up to 66% of patients are pancreatic sufficient (Augarten *et al.*1993). In contrast, ΔF508, G542X and G551D are severe mutations and are associated with the classic CF phenotype including pancreatic insufficiency (Kerem *et al.*1990, Kulczycki *et al.*2003, Mickle and Cutting.1998). Interestingly, although G551S is a class III mutation, pancreatic sufficiency has been observed (Strong *et al.*1991). However, for all CF mutations, lung function is variable, even between patients who share genotypes (Gibson *et al.*2003). This suggests that external factors and other genes play an important role in modifying lung disease in CF.

Identification of CFTR led to the hope that gene therapy would provide a "cure" for CF. This expectation is based on the premise that if CFTR function can be restored to an adequate level within the lung epithelia, then chronic infections can be prevented and morbidity and mortality reduced. To date, CFTR DNA has been delivered either by cationic liposomes (Alton *et al.*1999, Caplen *et al.*1995, Porteous *et al.*1997) or via a viral vector such as Adenovirus (Bellon *et al.*1997, Crystal *et al.*1994, Zabner *et al.*1993), adeno-associated virus (Flotte.2002, Flotte *et al.*1993) or Lentivirus (Limbers *et al.*2002). However, despite concerted efforts on increasing both research and funding initiatives, gene therapy remains at the experimental stage. Alternative pharmacologic therapies that focus on restoring CFTR function are also under investigation. These include the use of aminoglycosides in CF patients who are

are homozygous for the ΔF508 mutation. This strategy is based on the interesting observation that gentamicin can overcome the premature stop mutations and restore partial CFTR expression (Howard *et al.* 1996, Wilchanski *et al.* 2000, Wilchanski *et al.* 2003). Since the promise of gene therapy and pharmacologic therapies may be some way off, the need to consider other features of CF is essential.

1.2 Cystic Fibrosis- The Disease

CF was clinically identified in 1938, and initially termed 'cystic fibrosis of the pancreas'. The disease was named according to the characteristic pancreatic lesions observed at autopsy, however several patients in the study had died from pulmonary infections (Anderson 1938). In 1945, it was suggested that CF was an exocrine gland disorder, and termed mucoviscidosis (Farber 1945). Di Sant' Agnese and colleagues observed that in almost all individuals with CF, there was a characteristic increase in sodium and chloride levels (Di Sant' Agnese *et al.* 1953). This observation led to the diagnostic sweat test based on pilocarpine iontophoresis (Gibson and Cooke 1959). Diagnosis of CF can still be based on sweat chloride concentrations although typical gastrointestinal (meconium ileus) and respiratory symptoms are also diagnostic. Recent advances in genetic analysis, however, have allowed clinicians to diagnose the disease according to the patient's genotype. This has led to the introduction of enhanced neonatal screening programmes. Genetic screening, however, does not include all possible CFTR mutations, and thus may be prone to delivering false negatives.

There are three main benefits of newborn screening for CF: 1. To allow increased nutritional management, 2. To allow early management of life-threatening lung disease (this will be discussed later), 3. To provide genetic counselling for the parents and families of affected infants.

1.2.1 Clinical Symptoms of CF

The clinical effect of defective CFTR (Table 1.2), with little or no chloride conductance in epithelial cells, results in thick and viscid secretions (Quinton 1983).

Table 1.2 Main Clinical manifestations of CF (reviewed by Ratjen and Doring 2003)

Newborn	Meconium ileus (accumulation of faecal material)	
	Neonatal jaundice	
Infant	Bronchiolitis (inflammation of the small airways)	
	Chronic cough	
	Staphylococcal infection	**Respiratory**
	Atelectases (failure of part of the lung to expand)	
	Steatorrhoea (passage of increased amounts of fat)	
	Failure to thrive (result of malabsorption of fat)	
	Rectal prolapse	**Intestinal**
	Abdominal distension	
	Hypoproteinaemia	
Childhood	Nasal polyps (benign growth from mucosal membrane)	
	Wheeze	
	Bronchiectasis (widening of the bronchi)	**Respiratory**
	Otitis media	
	Staphylococcal infection	
	Pseudomonal infection	
	Heat exhaustion (hyponatremic dehydration – loss of sodium)	
	Malabsorption	**Other**
	Intestinal obstruction	
	Portal hypertension	
Young Adult	**Pseudomonal infection (Mucoid phenotype)**	
	Wheeze, breathlessness	**Respiratory**
	Pneumothorax (air in the pleural cavity)	
	Haemoptysis (coughing up blood)	
	Delayed puberty	
	Infertility (absence of bilateral Vas Deferens)	
	Cholecystitis (inflammation of the gall bladder)	**Other**
	Pancreatitis (as a result of autodigestion)	
	Diabetes mellitus	
	Obstruction of hepatic ducts (resulting in liver disease)	

Clinical features in neonatal CF predominantly affect the gastrointestinal tract, with the most common complication being meconium ileus. This syndrome affects 10-20% of newborn CF individuals and is almost diagnostic of the disease. The pathological basis of this condition has been attributed to dehydration of intestinal contents caused by epithelial transport dysfunction *in utero*. Complications include abdominal distension, perforation and peritonitis.

Pancreatic enzyme insufficiency is also present at birth, and causes fat and protein maldigestion, producing bulky, greasy and foul-smelling stools. Perhaps the most serious consequence of this disorder is the failure of neonates to thrive. This has implications for respiratory fitness and general well-being of CF patients throughout life. Maintaining nutritional status is also an important consideration for the clinician. Diabetes mellitus may present in older CF patients, as well as pathological changes in the liver. Liver transplantation is not uncommon in CF patients.

Infertility in CF males is approximately 95%, and is due primarily to the absence or blockage of the Vas Deferens. In females, infertility can be as low as 10%. Menstruation can be affected by chronic lung infection and poor nutritional status, and dehydrated cervical mucus may complicate conception.

Despite the considerable number and complexity of clinical symptoms associated with the pathophysiology of CF, between 80 and 95% of the morbidity and mortality in CF patients relates to progressive lung deterioration secondary to chronic endobronchial sepsis and recurrent inflammatory exacerbations.

1.2.2. Lung Disease in CF: Infection vs. Inflammation- Chicken or the Egg?

The lungs of CF patients are usually normal at birth; however, chronic bacterial infections accompanied by recurrent exacerbations result in the extensive morbidity and mortality. Debate continues as to whether inflammation is secondary to microbial infection (Armstrong *et al.* 1995) or is the primary event in the CF lung (Balough *et al.* 1995, Khan *et al.* 1995). An argument favouring the latter hypothesis

was the observation of increased inflammation in the gastro-intestinal tract, an organ not influenced by CF related bacterial infections (Smith *et al.* 2000).

CF lung infections tend to localize in the major and minor airways rather than the alveoli, although the interstitium and alveolar spaces are affected at end-stage disease. Chronic infection and acute, recurring and debilitating inflammatory exacerbations continue throughout the life of the patient (Fig 1.3). Progressive reduction in lung function results in respiratory failure and death. Further pulmonary complications include bronchiolitis, atelectasis, haemoptysis, bronchiectasis, pneumothorax and fibrosis.

The inflammatory process in the CF lung is dominated by a polymorphonuclear leukocyte influx. The predominant immunological cell type is the neutrophil, which accumulates in the airways along with high concentrations of neutrophil-derived mediators, including proinflammatory cytokines such as interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) (Bonfield *et al.* 1995). A consequence of this excessive neutrophil influx and ineffective phagocytic removal of the bacterial pathogen is the release of toxic compounds, including proteases and reactive oxygen species (Birrer *et al.* 1994, Richman-Eisenstat *et al.* 1993, Witko-Sarsat *et al.* 1999). These toxic agents may cause damage to the lower airway and contribute significantly to the pathophysiology of CF lung disease.

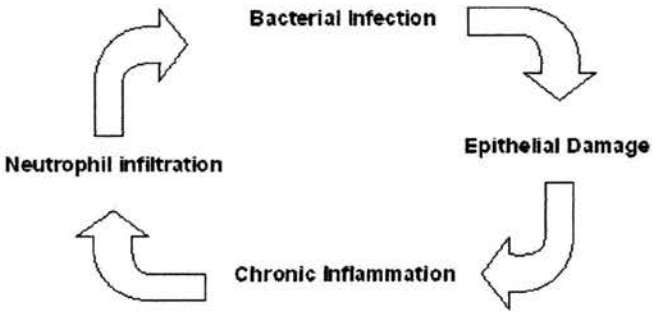


Fig 1.3 The Pathophysiology of Lung Disease in Cystic Fibrosis: The Vicious Cycle

1.2.3 Innate Defences and the Respiratory Tract

To understand the relationship between CF lung disease and bacterial colonisation and infection of the respiratory tract, it is necessary to consider not only the physiological defect that results in CF, but also the innate immune system that is designed to ensure that lungs are protected from microbial pathogens.

Bacterial infections in CF are rarely associated with systemic spread and thus, CF patients can be described as "immunocompetent". This is exemplified by the fact that CF patients exhibit good immune responses to normal vaccination protocols. For example, *P. aeruginosa* vaccines are able to generate an antibody response in most CF patients (reviewed by Holder 2004). Thus, if there is an underlying immunological defect in CF, it is likely to be within the respiratory tract and associated with the innate immune system. Furthermore, there may be a predilection for inflammation and/or infection, as a result of defective CFTR functions.

Innate defences of the respiratory tract are important throughout mammalian species.

The first line of defence in the upper respiratory tract is the nose. This is particularly important in the removal of particulate material (larger than 5 μm and less than 0.01 μm), infectious or otherwise, which is trapped in mucus, propelled to the pharynx and swallowed. Nasal mucus may also contain non-specific antimicrobial substances such as lysozyme, and specific secretory IgA.

The lower respiratory tract is protected by the mucociliary escalator that acts to trap and remove particles which otherwise might cause irritation or infection. Each respiratory epithelial cell has approximately two hundred cilia, which in a normal person beat at approximately 1000 beats per min. Beating cilia provide the propulsion to allow the overlying mucus layer to trap particles and act in mucociliary clearance. The underlying defect in CF, namely the dehydrated and viscid bronchial secretions, impairs this natural process, and leads to microbial infections.

In addition, it is now thought that the normal respiratory tract is protected by a barrier of potent chemical mediators present in airway surface liquid (ASL) (Table 1.3).

Table 1.3 Innate defence molecules in airway surface liquid (ASL) (Devine 2003)

Antimicrobial component	**Produced by cells**
α-Defensins (HNP 1-4)	Neutrophils
α-Defensin (HD5-6)	Epithelial
β-Defensin (HBD 1-4)	Epithelial, macrophages, monocytes, dendritic
LL-37 (cathelicidin)	Neutrophils, epithelial
Lysozyme	Epithelial, neutrophils
Phospholipase A_2	Epithelial, neutrophils
IgA	Epithelial
Lactoferrin	Epithelial, neutrophils
Bactericidal permeability inducing protein	Neutrophils
Serine proteinase inhibitor	Epithelial, macrophages
Surfactant proteins SP-A, SP-D	Epithelial
Anionic peptides	Epithelial
Proline-rich proteins	Epithelial
Trefoil factor family proteins	Epithelial

Each of these substances present in normal ASL may act individually or synergistically to produce an antimicrobial effect. The significance of these

endogenous antimicrobial substances, in particular the antimicrobial peptides, will be discussed later.

1.2.4 High Salt vs. Low Volume Hypotheses

Two opposing hypotheses have been proposed to explain how CFTR malfunction and a defective chloride channel increase susceptibility to bacterial infections in the CF lung.

The first model, the "high salt hypothesis", predicts that an elevated salt concentration in CF-ASL inhibits naturally-occurring salt-sensitive defensins, including human β-defensin 1 & 2 (HBD-1 & 2) (Bals *et al.* 1998, Goldman *et al.* 1997). The model proposes that in normal ASL, salt concentration is low and the antimicrobial activity of defensins prevents potential pathogens from infecting the lower respiratory tract. However, the defective CFTR function brings about reduced Cl^- ion flow into airway epithelial cells. This alteration in ionic potential causes the disruption of Na^+ ion reabsorption, and accounts for the increase in salt content of CF-ASL (Smith *et al.* 1996, Zabner *et al.* 1998) (Fig 1.4). Thus, the increase in salt reduces the killing potential of the salt-sensitive defensins and contributes to bacterial infection.

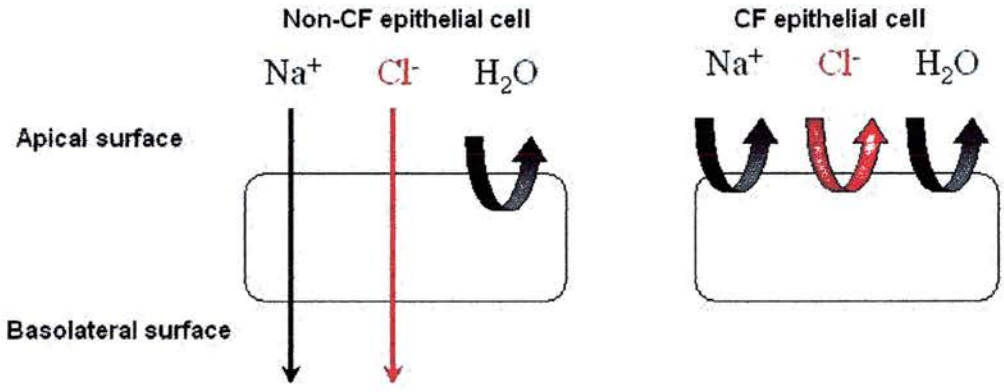


Fig 1.4 High salt hypothesis: In this model, non-CF airway surface liquid has a hypotonic salt concentration. In contrast, CF airways, with defective CFTR, have a hypertonic salt concentration since chloride ions cannot flow back into the epithelial cell. Consequently, endogenous salt-sensitive antimicrobial peptides are inhibited. The ENaC channel is shown in black and CFTR in red.

The second model, "the low volume hypothesis", predicts that fluid absorption is enhanced in CF and conversely the production of fluid, controlled by CFTR, is defective (Guggino 2001, Matsui *et al.* 1998) (Fig 1.5). CFTR is thought to have additional functions, such as a regulator of other membrane proteins, including an epithelial sodium channel (ENaC) (Stutts *et al.* 1997). CFTR appears to regulate the kinetics of ENaC by ensuring that it is open for the minimal amount of time. Thus, in the absence of functional CFTR, ENaC remains open allowing the influx of Na^+ across the airway epithelium. Consequently, Cl^- ions follow, from ASL, into airway epithelial cells via shunt pathways. Furthermore, as the osmotic potential is altered, water follows leading to dehydrated mucus. The reduction in ASL gives rise to a reduction in mucociliary clearance, and thus increased potential for bacterial infection. Tarran and co-workers who examined the nasal epithelium in a CF mouse model have supported this hypothesis (Tarran *et al.* 2001). They found a significant increase in the number and size of goblet cells and a marked reduction in ASL volume in CF mice compared to wild type mice. Furthermore, the authors did not observe a reduction in salt concentration.

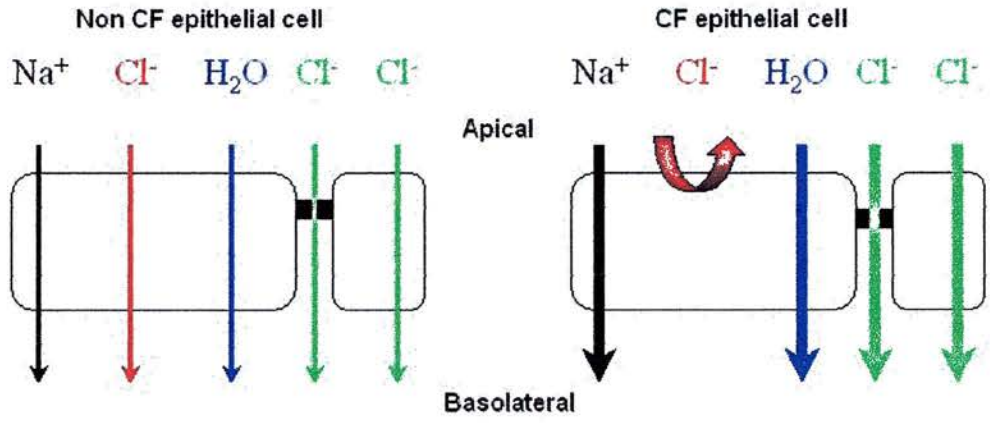


Fig 1.5 Low volume hypothesis: In this model, normal CFTR acts as a regulator of ENaC, thus in its absence in CF, sodium absorption is increased. Chloride ions follow via alternative shunt pathways, as does water via osmosis. The result is a low volume of ASL, and defective mucociliary clearance. ENaC channel is shown in black, CFTR in red, and alternative chloride channels in green.

The technical difficulties of measuring ionic composition of ASL hinder the resolution of the debate surrounding both hypotheses. Recent work, however, has suggested that the ionic composition of ASL in both CF and normal airways is comparable (Caldwell *et al.* 2002, Knowles *et al.* 1997). Nevertheless, controversy over this issue remains (Widdicombe 2001).

1.2.5 CFTR: More Than Just a Chloride Channel?

Adherence is an important aspect in the bacterial colonisation of the lower respiratory tract in CF. Thus, the potential for specific bacterial receptors in the CF host has also been investigated.

It has been suggested that in normal epithelia, CFTR acts as a receptor for *P. aeruginosa*, resulting in the internalisation and removal of potential pathogens by

natural cell desquamation. In CF patients, who lack CFTR on the surface of the airway epithelia, this innate process would be impaired and consequently *P. aeruginosa* remain to infect the respiratory tract (Goldberg and Pier 2000, Pier 2000).

1.2.6 Other Receptors: Asialo-gangliotetraosylceramide (Asialo-GM1)

It has also been suggested that Asialo-GM1 is an apical bacterial receptor, which is increased on the surface of CF epithelial cells, and that *P. aeruginosa* type IV pili bind to this receptor (Imundo *et al.* 1995, Krivan *et al.* 1988, Saiman and Prince 1993). Type IV pili are responsible for twitching motility and are thought to have an important role in establishing adhesion between the pathogen and the epithelial cell in the early stages of infection prior to the formation of biofilms (Hahn 1997). A further study suggests that Asialo-GM1 is increased on the surface of regenerating respiratory epithelial cells (De Bentzmann *et al.* 1996). This is an important observation when one considers the epithelial damage caused by early viral infections in CF, and the possibility of increased *P. aeruginosa* adhesion. Recently, this theory has been disputed (Schroeder *et al.* 2001). These authors highlight several weaknesses in the previous reports including the use of inappropriate commercial antibodies and the failure to use clinical isolates of *P. aeruginosa*. Despite considerable interest and research, the role of "bacterial receptors" in the pathogenesis of CF lung disease remains unproven.

1.3 Microbiology and CF Lung Disease

The microbiology of CF has been described as "a sub-speciality unto itself" (Stutman and Marks 1987). From birth, respiratory complications, due to non-specific viral infections (respiratory syncytial virus, parainfluenza virus and influenza virus), play an important role in the predisposition of the CF child to acute and recurrent respiratory/inflammatory exacerbations and increased hospitalisation (Hiatt *et al.* 1999). It is the latter which may predispose the CF infant to colonisation by the limited spectrum of bacterial pathogens, including *P. aeruginosa* (Armstrong *et al.*

1998), which affect CF patients. The importance of viral infection in CF is unclear, and may be understated (Wat 2003). Despite the influence of viral pathogens on the neonatal CF lung, bacterial pathogens play the most important role in the pathology of chronic CF lung disease.

The spectrum of bacteria capable of infecting the CF lung appears to be limited and includes *Staphylococcus aureus*, non-typeable *Haemophilus influenzae*, *P. aeruginosa, Burkholderia cepacia* complex, *B. gladioli*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* (Burns *et al.* 1998, Gilligan 1991, Govan and Deretic 1996, Krzewinski *et al.* 2001, Mearns *et al.* 1972). Other, and somewhat rarer, bacterial species cultured from CF sputum include, *Ralstonia* sp. (Coenye *et al.* 2002), *Pandoraea* spp. (Jorgensen *et al.* 2003, Segonds *et al.* 2003), atypical *Mycobacteria* (Boxerbaum 1980, Olivier *et al.* 2003), the fungus *Aspergillus fumigatus* (Cimon *et al.* 2001) and the yeast *Candida* (FitzSimmons 1993). The contribution of each individual species to the pathogenesis of lung disease is varied, and dependent on the infecting strain and patient status (the same infecting strain may give rise to different clinical symptoms in different patients). The significance of each species in CF is discussed in two recent reviews (Gibson *et al.* 2003, Saiman and Siegel 2004). Undoubtedly, *P. aeruginosa* is the most common and important pathogen isolated from CF sputum, infecting up to 70% of CF patients (FitzSimmons 1993). The general trend is that the burden of infection, leading to chronic infection, increases with time, suggesting a "window of opportunity" to treat some infections early. This is particularly the case with *P. aeruginosa*, which is intractable if allowed to become chronic.

1.3.1 *S. aureus* and Non-typeable *H. influenzae*: A Role in CF?

S. aureus is a Gram-positive, facultatively anaerobic, catalase-positive bacterium and is usually isolated from the nose in non-CF individuals. *S. aureus* is arguably the only "classic bacterial pathogen" involved in CF, the rest being environmental or commensal organisms. The isolation of *S. aureus* in the respiratory tract of CF infants (Armstrong *et al.* 2002) clearly indicates an infection but the degree of

pathology caused is debatable. Despite extensive debate on whether *S. aureus* is an important CF pathogen (Lyczak *et al.* 2002), it has long been assumed that early treatment with anti-staphylococcal antibiotics provides clinical benefit (Thomassen *et al.* 1987). This dogma, however, has been disputed in a comprehensive review of antistaphylococcal treatment (McCaffery *et al.* 1999). The authors report that although eradication of *S. aureus* from sputum was achieved, there was little or no improvement in lung function. Furthermore, the possibility remains that early and aggressive antistaphylococcal therapy is counter productive and may encourage the acquisition of *P. aeruginosa* (Ratjen *et al.* 2001). Another consequence of antistaphylococcal therapy is the emergence of methicillin resistant *S. aureus* (MRSA). The risk of MRSA in CF is considered a clinical problem for two main reasons: First, unlike methicillin sensitive *S. aureus* (MSSA), MRSA is resistant to many groups of antibiotics and thus is difficult to eradicate. Although vancomycin and linezolid can be used to eradicate MRSA (Ferrin *et al.* 2002), sputum-positive CF patients require significantly more courses of intravenous antibiotics (Miall *et al.* 2001). Second, MRSA is able to spread between patients, and such epidemic transmission of a highly resistant organism, may prove to be an infection control risk in both the hospital and the CF clinic (Jones and Webb 2003, Saiman and Siegel 2004).

H. influenzae is a Gram-negative bacterium that can be considered a commensal of the upper respiratory tract. Its pathological role in CF is not well characterised and is probably underestimated (Hutchison and Govan 1999, Saiman 2004). Historically, *H. influenzae* was included as a "classic CF pathogen" because of its frequent isolation from CF patients in early childhood (Mearns *et al.* 1972). The isolation of non-typeable *H. influenzae* from CF sputum is problematic for the CF clinician for two main reasons: first, the *H. influenzae* type B (HiB) vaccine, provided for all infants, does not provide an effective immunological response against non-typeable *H. influenzae*. Second, non-typeable *H. influenzae* is known to be pathogenic in non-CF pneumonia, bronchitis and bronchiectasis (Jordans and Slack 1995).

The role that *S. aureus* and *H. influenzae* play in the pathophysiology in CF remains to be fully elucidated, but since eradication of both organisms is often attempted by clinicians, antibiotic usage may increase the risk of acquisition of *P. aeruginosa* and members of the *Burkholderia cepacia* complex. Furthermore, early infection of the lower respiratory tract by *S. aureus* and *H. influenzae* may initiate cell damage and predispose to *P. aeruginosa* infection.

1.3.2 *P. aeruginosa*: The Major CF Pathogen

The genus *Pseudomonas* includes diverse species that exhibit striking nutritional and metabolic diversity (Stanier *et al.* 1966). Pseudomonads are also found in a wide variety of environmental niches, ranging from water and soil to plants and mammals. Examples of species within this genus include *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P stutzeri*, *P. solanacearum* and *P. tomato*. *P. aeruginosa* is the most important human pathogen amongst these highly adaptable bacteria.

P. aeruginosa, a Gram-negative bacillus is arguably the most important and prevalent CF pathogen. This ubiquitous saprophyte is non-spore forming, non capsulate and usually motile. It is a strict aerobe, but can grow anaerobically in the presence of nitrate. *P. aeruginosa* is non-glucose fermenting and oxidase positive. A distinguishing feature of *P. aeruginosa* is that most isolates are pigmented. The characteristic green pigment is caused by the combination of a soluble blue phenazine pigment (pyocyanin) and a yellow-green fluorescent pigment (pyoverdin). The latter is a major siderophore important in competitively binding iron (Fe^{3+}) in the human host. These features explain the original name '*Bacillus pyocyanea*' as the causative agent of blue-green pus. Other pigments include pyorubin (red) and melanin (brown). Infections caused by this opportunistic pathogen are varied. They include ventilator-associated pneumonia, burn infections, catheter-associated urinary tract infections, jacuzzi rash (an acute self-limiting folliculitis) and numerous nosocomial infections of immunocompromised patients. It is important to note, that *P. aeruginosa* infections in immunocompromised patients may spread systemically and cause life-threatening septicaemia.

P. aeruginosa infection in CF is thought to arise during infancy. Thus, it is recommended that early and aggressive therapy regimes should be initiated on first isolation, to attempt eradication of this organism from the lung and prevent the emergence of the mucoid form (Rosenfeld *et al.* 2003). The prevalence of *P. aeruginosa* infection increases with age, with 20-30% of infants, 30-40% of young children, up to 60% of adolescents and approximately 80% of CF adults infected with this organism (Cystic 2002).

1.3.3 Virulence Determinants of *P. aeruginosa*

P. aeruginosa has a large genome (~6Mbp) that provides considerable genotypic/phenotypic plasticity including the ability to adapt to multiple environmental niches. A major example of *in vivo* adaptation to a specific human niche, is the biosynthesis of pseudomonal alginate in CF lung disease. This striking adaptation is rarely observed in other infections or indeed within the natural environment. Transition from a non-mucoid to mucoid phenotype is also undoubtedly the most important microbial virulence factor in CF lung infections. The initiation of the mucoid phenotype is genetically complex, and highly regulated (Govan and Deretic 1996). Culture of mucoid *P. aeruginosa* from sputum is almost diagnostic of CF, and associated with chronic infection and increased respiratory exacerbations. Mucoidy, together with other factors controlled by bacterial quorum sensing, contributes to the development of biofilm growth in the CF lung. Biofilms contribute significantly to the pathogenesis of CF lung disease, by enhancing bacteria resistance to antibiotics and phagocytosis. Consequently, infections are difficult to eradicate with antibiotics, and frustrated phagocytes release potent toxic compounds that damage the host epithelium and cause increased pathology.

Other *P. aeruginosa* virulence determinants, both cell surface associated and secreted, contribute to pathogenicity in the CF lung (Table 1.4). Secreted factors which increase potential to utilise nutrients for growth, become invasive and inflict

damage on host tissue include proteases (McIver *et al.* 1995, Rust *et al.* 1996), phospholipases (Vasil *et al.* 1991), siderophores (Meyer *et al.* 1999, Vasil *et al.* 1998) and exotoxins. A particular exotoxin of interest is exotoxin A which acts in a similar manner to diphtheria toxin (Iglewski *et al.* 1977).

Table 1.4 Major virulence factors of *P. aeruginosa*

Alginate
Elastase*
Alkaline protease*
Hydrogen cyanide*
Exotoxin A*
Exo-enzyme S
Secretion proteins*
Catalase*
Rhamnolipid*
Pyocyanin*
Pyoverdin
Lectins*
Phospholipids*
Acylated homoserine lactones*
Superoxide dismutase*
Type III secreted cytotoxins (Exo U, Y, S & T)
Lipopolysaccharide (LPS)

* Quorum-sensing-regulated virulence factors (reviewed by Smith and Iglewski 2003)

1.3.4 Quorum Sensing & *P. aeruginosa*

Quorum sensing (QS) is the mechanism by which bacteria can "sense" population density and coordinate gene expression accordingly (Winson *et al.* 1995). As outlined (Table 1.4), the expression of many *P. aeruginosa* virulence determinants is controlled by two independent but related quorum sensing systems, *las* and *rhl* (Gambello and Iglewski 1991, Ochsner *et al.* 1994 respectively). The first system is the *lasR* & *lasI* system, which was identified due to the high sequence homology to a similar quorum sensing system from the bacterium *Vibrio fischeri* (Dunlap and Greenberg 1988). The transcriptional activator LasR acts with its *N*-acyl homoserine lactone (AHL), *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL). 3-oxo-C12-HSL is synthesized by the LasI autoinducer synthase. LasR-3-oxo-C12-HSL regulates many virulence factors, including elastase and exotoxin A (Pearson *et*

al. 1997, Smith and Iglewski 2003). The second system, *rhl*, is composed of RhlR and the autoinducer RhlI that directs the synthesis of *N*-butyryl-L-HSL (C4-HSL). This system controls the expression of rhamnolipids, and secondary metabolites such as pyocyanin and hydrogen cyanide (Pearson *et al.* 1997, Smith and Iglewski 2003). An important feature of quorum sensing in *P. aeruginosa* is that it appears to contribute to the regulation of biofilm development (Parsek and Greenberg 1999). The cross-talk between both QS-systems is limited within *P. aeruginosa*, and operate almost autonomously. In contrast, *N*-acylhomoserine-lactones expressed by *P. aeruginosa* can exert a unidirectional effect on the *B. cepacia* complex (Lewenza *et al.* 2002, Riedel *et al.* 2001).

1.3.5 Antibiotic Resistance & Hypermutability in *P. aeruginosa*

An important consideration in the pathogenesis of *P. aeruginosa* infection in CF is that many isolates exhibit inherent resistance to antibiotics; this has been attributed to low outer membrane permeability, efflux pumps and other resistance mechanisms (Stover *et al.* 2000). A recent seminal study observed that CF-associated *P. aeruginosa* exhibited a higher degree of hypermutable isolates than expected in non-CF infections (Oliver *et al.* 2000). The significance of such observations is that within a CF lung there might exist a significant percentage of organisms with the potential to evolve rapidly when selection pressure is applied. Such pressure includes antibiotics, and thus their use may rapidly drive an increase in resistance and a highly adaptable bacterial population.

1.3.6 Cross-Infection & *P. aeruginosa*

Despite the high prevalence of *P. aeruginosa* in CF patients, it has long been accepted that each patient acquires, and becomes chronically infected with, a unique strain from the natural environment (Kelly *et al.* 1982). Until recently, cross-infection had never appeared to be a problem, except amongst siblings or patients with close epidemiological connections (Grothues *et al.* 1988). The potential for cross-infection with *P. aeruginosa* infection was first highlighted in a study by

Pedersen and colleagues, in which a multiresistant strain spread throughout a Danish CF centre (Pedersen *et al.* 1986). The observation of multiresistance was significant, and the overuse of antibiotics, in particular third generation cephalosporins, was blamed for selecting resistance. A major caveat in the Danish study was the reliance on epidemiological data based on serology and phage typing, which are important for typing mucoid isolates but inappropriate for LPS-deficient CF isolates. However, in a seminal study in a large CF clinic in Liverpool, Cheng and co-workers observed the extensive spread of a β-lactam resistant *P. aeruginosa* (Cheng *et al.* 1996). In contrast to other studies, Cheng *et al.* based their findings on genomic fingerprinting by pulse-field gel electrophoresis (PFGE) and flagellar polymorphisms, which provided concordant evidence for spread of a single clone. Subsequent studies in the UK, Australia and Brazil, have confirmed that *P. aeruginosa* is capable of epidemic spread within and between CF centres (Anthony *et al.* 2002, Armstrong *et al.* 2003, Armstrong *et al.* 2002, Denton *et al.* 2002, Jones *et al.* 2002, McCallum *et al.* 2001, Pallegrino *et al.* 2002). It would appear that some *P. aeruginosa* strains are more "transmissible" than others, are associated with increased morbidity and can superinfect patients already colonised with *P. aeruginosa* (Al-Aloul *et al.* 2004, McCallum *et al.* 2001).

In summary, the clinical implications of highly transmissible multiresistant *P. aeruginosa* are four-fold: 1. Acquisition of a multiresistant *P. aeruginosa* as the primary pathogen reduces or prevents the opportunity for early therapy to eradicate *P. aeruginosa*. 2. The patient requires prolonged and more frequent periods of hospitalisation. 3. There tends to be a significant decrease in lung function. 4. Prompt and strict isolation of the patient is required to limit spread.

1.4 Emerging CF Pathogens

With aggressive antibiotic therapy increasing life expectancy and hence an increasing population of CF adults, the microbiology of CF is evolving. One development is the identification of novel bacterial species in CF sputum. Emerging and potential CF pathogens include: *S. maltophilia* (Demko *et al.* 1998, Denton and

Kerr 2002, Denton *et al.* 1998), *Pandoraea* spp. (Coenye *et al.* 2001, Jones and Webb 2003), *Ralstonia* spp. (Coenye *et al.* 2002), and *A. xylosoxidans* (Liu *et al.* 2002, Peltroche-Llacsahuanga *et al.* 1998). The pathogenic potential of these species is unclear. However, common features are that they are usually soil organisms, have the potential to infect immunocompromised patients and tend to be antibiotic resistant. With reference to *Pandoraea* spp there may also be cross-infection issues (Coenye *et al.* 2001, Jones and Webb 2003).

1.5. *Burkholderia cepacia*

Arguably the most feared CF pathogen to have emerged in recent decades is *B. cepacia*. First isolated from a CF patient in the 1970s (Ederer and Matsen 1972), the full impact of this organism on the CF community would not be recognised until the 1980s when potential virulence, inherent antimicrobial resistance and transmissibility came to be realised.

1.5.1 *Burkholderia* – A Taxonomic History

Burkholderia cepacia (formerly *Pseudomonas cepacia*) was initially identified as the causative agent of soft rot of onion bulbs (cepa = *Latin* onion) (Burkholder 1950). Identification of other *Pseudomonas* species included isolation of *Pseudomonas multivorans* from soil and water samples (Stainer *et al.* 1966), and *Pseudomonas kingii* from clinical environments (Jonsson 1970, King 1964). However, it was soon recognised that these novel species were in fact synonymous with *P. cepacia* (Ballard *et al.* 1970, Sands *et al.* 1970, Snell *et al.* 1972).

At this time, taxonomic classification of *P. cepacia* was based on rRNA-DNA hybridisation, and *P. cepacia* was grouped in *Pseudomonas* RNA homology group II (Palleroni *et al.* 1973). Subsequently, a new genus, *Burkholderia*, was proposed (Yabuuchi *et al.* 1992) belonging to the β-subdivision of the phylum *Proteobacteria* (Kersters *et al.* 1996). Consequently, the type species was joined by seven other species from homology group II, including *B. gladioli*, *B. pseudomallei*, and *B. mallei*. Of the seven, two were later reclassified into a new genus *Ralstonia*

(Yabuuchi *et al.* 1995). The complex identification and taxonomic classification of these closely-related species continued with the addition of *B. cocovenenans* (Zhao *et al.* 1995), *B. vandii*, *B. glumae* and *B. plantarii* (Urakami *et al.* 1995). A subsequent study has shown that *B. cocovenenans* and *B. vandii* are junior synonyms of *B. gladioli* and *B. plantarii* respectively (Coenye *et al.* 1999). An outline of the phylogenetic relationship between the present 31 species in this genus and closely related genera is shown (Fig 1.6) (Coenye and Vandamme 2003).

Fig 1.6 16S DNA based phylogenetic tree showing the positions of *Burkholderia* species and representatives of related genera. Scale bar indicates 5% sequence dissimilarity (Reproduced by kind permission: Coenye and Vandamme 2003).

Historically, *Burkholderia* species were recognized as phytopathogens and soil bacteria, with two important exceptions; these are the highly virulent human pathogens *B. mallei* and *B. pseudomallei*. Thus, a simplistic approach to *Burkholderia* species is no longer of relevance. Present studies of these highly adaptable microbes must take account of their complex taxonomy and emerging role as human and animal pathogens.

1.5.2 The *Burkholderia cepacia* complex (Bcc): A Complex Complex

In a seminal study published in 1997 isolates previously identified as "*B. cepacia*" were reclassified into five genomovars, termed the *Burkholderia cepacia* complex (Bcc) (Vandamme *et al.* 1997). A genomovar is a group of genomically distinct bacteria, which cannot be separated on phenotypic characteristics (Ursing *et al.* 1995). The Bcc has now been extended to at least nine genomovars (Coenye *et al.* 2001, Coenye *et al.* 2001, Vandamme *et al.* 2002, Vandamme *et al.* 2000). A tenth genomovar, *B. ubonensis*, has been proposed but has yet to be confirmed within the Bcc (Yabuuchi *et al.* 2000). Genomovar status is based on polyphasic taxonomic studies, including: DNA-DNA, DNA-rRNA hybridisations, whole cell protein and fatty acid analysis (Vandamme *et al.* 1997). At this time, all of the original genomovar designations have been superseded by species names (Table 1.5).

Table 1.5 *The B. cepacia* complex

Genomovar	Species	Reference
I+	*B. cepacia*	(Vandamme *et al.* 1997)).
II	*B. multivorans*	(Vandamme *et al.* 1997)).
III	*B. cenocepacia*	(Vandamme *et al.* 2003)).
IV	*B. stabilis*	(Vandamme *et al.* 2000)).
V	*B. vietnamiensis*	(Gillis *et al.* 1995)).
VI	*B. dolosa*	(Coenye *et al.* 2001, Vermis *et al.* 2003)).
VII	*B. ambifaria*	(Coenye *et al.* 2001)).
VIII	*B. anthina*	(Vandamme *et al.* 2002)).
IX	*B. pyrrocinia*	(Imanaka *et al.* 1965, Vandamme *et al.* 2002)).
X*	*B. ubonensis*	(Yabuuchi *et al.* 2000)).

+ Type strain; * Included tentatively, as full classification is yet to be completed (Yabuuchi *et al.* 2000)

By taxonomic convention, genomovar I contains the type species, and thus retains the formal binomial name, *B. cepacia* (Vandamme *et al.* 1997). Genomovar II was named *B. multivorans* on the basis of its unique biochemical characteristics (Vandamme *et al.* 1997). Genomovar IV was named *B. stabilis* due to its low level of genomic variability in comparison to other Bcc genomovars. Further distinctions can be made by the lack of β-galactosidase activity and an inability to utilise sucrose (Vandamme *et al.* 2000). Genomovar V was recognised as a previously-identified species *B. vietnamiensis* (Gillis *et al.* 1995). Genomovar VII was named as the novel species *B. ambifaria* (Coenye *et al.* 2001). The differentiation of *B. ambifaria* from other Bcc members is based on growth at 42°C, β-galactosidase activity, lysine decarboxylase and ornithine decarboxylase activity, acidification of sucrose and β-haemolysis (Coenye *et al.* 2001). Genomovar VIII was identified as a novel species, and termed *B. anthina* (Vandamme *et al.* 2002). Distinction of genomovar VIII from I and III proved difficult, but was unequivocally achieved by *recA*-based restriction fragment length polymorphisms (RFLP) analysis based on rRNA-PCR assays (LiPuma *et al.* 1999). Genomovar IX was recognised as the previously described species *B. pyrrocinia* (Imanaka *et al.* 1965, Vandamme *et al.* 2002). At present, *B. ubonesis* (genomovar X) is included as a member of the Bcc due its taxonomic similarity (Yabuuchi *et al.* 2000).

The differentiation of genomovars III and VI from *B. cepacia* (genomovar I) and *B. multivorans*, respectively, proved to be problematic. However, genomovar III has recently been named as the novel species *B. cenocepacia* ("new-cepacia") (Vandamme *et al.* 2003). Within *B. cenocepacia*, four *recA* lineages have been identified (IIIA-D). Genomovars I and III can also be distinguished using AFLP analysis (Coenye *et al.* 1999) and *recA*-based restriction RFLP (Mahenthiralingam *et al.* 2000). Most recently, genomovar VI has been classified as the novel species *B. dolosa* on the basis of its inability to assimilate tryptamine, azelaic acid and salicin (Vermis *et al.* 2004). Amongst present members of the Bcc, only *B. dolosa* fails to grow on Bcc selective (PCAT) media.

Currently, the most accurate means to identify Bcc species in general, and *B. cenocepacia* and *B. multivorans* specifically, is by species-specific *recA*-based PCR assays (Vermis *et al.* 2002).

For the purpose of this thesis, the taxonomic nomenclature will be as follows: pre-1996: "*B. cepacia*"; post-1996: Bcc and individual species names.

Members of the Bcc are Gram-negative, non-sporing aerobic bacilli. Typically, they are motile and both catalase and oxidase positive. Pigmentation is not uncommon, but may require specific growth media, for example tyrosine agar, to enhance melanin production (Ogunnariwo and Hamilton-Miller 1975). This dark brown pigment is associated with some Bcc species, primarily, although not exclusively *B. cenocepacia*. The nutritional and metabolic diversity of the Bcc is impressive, with some strains able to utilise penicillin G as a sole carbon source (Beckman and Lessie 1979, Vermis *et al.* 2003). This high metabolic activity of the Bcc makes some strains attractive candidates for biological control of plant fungal disease (Homma *et al.* 1989) and bioremediation of contaminated soils (reviewed by Gerhardson 2002, Parke and Gurian-Sherman 2001). The demand for such agents has led to a fierce debate to determine if the use of Bcc species as biopesticides (King and Parke 1993) poses a risk to human health (Govan *et al.* 2000, Holmes *et al.* 1998, Jones *et al.* 2001). Although the jury is still out, there is unequivocal evidence that clinical and environmental Bcc species are indistinguishable and share common habitats (Balandreau *et al.* 2001, LiPuma *et al.* 2002, Vandamme *et al.* 1997). The present regulatory position is that all potential candidates for use as biopesticides must be notified to the United States Environmental Protection Agency (USEPA) for assessment under "New Use Rule" legislation. This system of regulation would allow the USEPA to evaluate, prohibit or limit the use of such candidate strains.

1.5.3. The Genome

It is not the strongest of the species that survives nor the most intelligent, but the one that is the most responsive to change.

- Darwin

All Bcc species have large genomes (mean size approximately 8 Mbp) and multiple replicons that may contribute to genomic plasticity (Mahenthiralingam *et al.* 2002, Parke and Gurian-Sherman 2001, Wigley and Burton 2000).
The genome of the *B. cenocepacia* isolate J2315 (electrophoresis type (ET)-12 lineage) (Govan *et al.* 1996, Mahenthiralingam *et al.* 2000b); has recently been fully sequenced (www.sanger.ac.uk/projects/B-cepacia). The choice of J2315 as the candidate strain was based on five key factors: 1. It is the dominant CF pathogen in the Bcc, 2. It is transmissible, 3. It is associated with poor clinical outcome, 4. The original isolate from the index patient was stored in two separate and established collections (Govan *et al.* 1993), and 5. It is amenable to genetic manipulation. The multireplicon genome of J2315 comprises three "chromosomes" (3.870, 3.212 & 0.876 Mbp respectively) and one large plasmid (92.7 Kbs). Thus, the total genome size is 8.056 Mbp. This compares with a genome size of 6.8 Mbp for *B. multivorans* (ATCC 17616) and 8.0 Mbp for *B. cepacia* (ATCC 25416). With an estimated 7,300 genes and the genomic mass of some protozoa, Darwin himself may have been surprised to note both the size and the potential genomic plasticity of this bacterial genome.

1.5.4 Bcc: The Pathogen

"*B. cepacia*" infections in humans tend to be severe, yet thankfully rare. Historically, "*B. cepacia*" was first associated with endocarditis in the 1950s (Schiff *et al.* 1961, Sorrel and White 1953, Speller 1972). Most infections at this time were nosocomial and/or associated with immunocompromised patients (Jarvis *et al.* 1987, Martone *et al.* 1987). Patients who require intensive care are now known to be

particularly at risk of Bcc infection (Holmes *et al.*1999, Phillips *et al.*1971). Other Bcc nosocomial infections include: renal transplant-associated urinary tract infections (Li *et al.*2003), iatrogenic-urinary tract infections (Keizur *et al.*1993), haemodialysis-associated bacteraemia (Kaitwatcharachai *et al.*2000, Magalhaes *et al.*2003), bacteraemia (Huang *et al.*2001), lung abscesses (Poe *et al.*1977), ventilator-associated respiratory infections (Loukil *et al.*2003) and respiratory infections (Bureau-Chalot *et al.*2003). "*B. cepacia*" has also been implicated as the causative organism in infections (wound, endocarditis and septicaemia) thought to have arisen from contaminated medicines such as antibiotic/disinfectant solutions (Bassett *et al.*1970, Speller *et al.*1971), distilled water (Rapkin.1976) and liquid/aerosol preparations (Noriega *et al.*1975, Ramsey *et al.*2001, Steere *et al.*1977, Van Laer *et al.*1998).

"*B. cepacia*" infections in immunocompetent individuals are rare, but significant. It has been identified as the causative agent of "foot rot" in soldiers serving in swamp environments (Taplin *et al.*1971) and to be the cause of community acquired pneumonia in an immunocompetent host (Pujol *et al.*1992). Contamination of an ear drop preparation, by "*B. cepacia*", was responsible for multiple brain abscesses secondary to otitis media, in an otherwise healthy individual (Hobson *et al.*1995). One group of individuals who are particularly susceptible to life-threatening respiratory infections with "*B. cepacia*" are patients with the inherited condition known as chronic granulomatous disease (CGD) (Clegg *et al.*1986, Govan *et al.*1996, Lacy *et al.*1993, LiPuma.1998, O'Neil *et al.*1986). The susceptibility of CGD patients to "*B. cepacia*" infection is thought to arise due to the underlying genetic defect in neutrophil-associated oxidative killing in conjunction with the inherent resistance of "*B. cepacia*" to neutrophil-derived non-oxidative killing mechanisms: antimicrobial peptides (Speert *et al.*1994).

A recent study indicating the potential of the Bcc to cause animal infections was an outbreak of sub-clinical mastitis in sheep (Berriatua *et al.*2001). This is the first report of a natural Bcc infection in animals. Although Bcc species are not thought to pose the same risk to animals as other zoonotic agents, difficulties in identification

may lead to underreporting. Undoubtedly, the most important group of mammalian hosts affected by Bcc infections are CF patients.

1.5.5. The Bcc and CF

"*B. cepacia*" was initially identified in a CF patient in the early 1970s (Ederer and Matsen 1972). Subsequently, the role of "*B. cepacia*" in the pathogenesis of CF lung disease was recognised with the onset of pneumonia and septicaemia in an adolescent CF patient (Rosenstein and Hall 1980). However, it was not until a seminal retrospective study highlighted the increased prevalence of "*B. cepacia*" colonisation in CF patients in Western Canada, that the variable clinical outcomes were documented (Isles *et al.* 1984). "*B. cepacia*" infection in CF has three main clinical outcomes: 1. The patient may be an asymptomatic chronic carrier, 2. There may be a gradual deterioration in lung function resulting in increased morbidity, similar to *P. aeruginosa* infection, and 3. The most severe outcome is a rapid and fatal necrotising pneumonia accompanied by septicaemia (this is rarely observed in other CF infections). This unexpected and life-threatening outcome, termed "cepacia-syndrome", occurs in 20-30% of "*B. cepacia*" positive patients (Isles *et al.* 1984, LiPuma 1998). Importantly, clinical outcome cannot be predicted even when patients within a single CF centre are infected by the same strain (Govan *et al.* 1993). Host factors, including disease (CF) severity and immunological status, as well as bacterial factors, including Bcc individual species and strains, appear to play important roles in the end result of Bcc infection. Despite the variable outcomes, significant clinical decline is expected in all "*B. cepacia*" positive patients, with an approximate 50% reduction in life expectancy.

The distribution of Bcc species in CF is disproportionate. In general, *B. cenocepacia*, *B. multivorans* and *B. vietnamiensis* account for 95% of isolates (LiPuma *et al.* 2001, Mahenthiralingam *et al.* 2002). Moreover, patient to patient spread and acute pulmonary deterioration are primarily associated with *B. multivorans* and *B. cenocepacia* (Govan *et al.* 1996, LiPuma 1998, Mahenthiralingam *et al.* 2002).

1.5.6 Bcc: Epidemiology, Transmission and Segregation Issues

In addition to the potential virulence and detrimental pulmonary effect of Bcc infection, transmission of this organism between CF patients is a major issue in patient management, in particular cross-infection control. Historically, transmission of "*B. cepacia*" was thought to be restricted to spread amongst siblings or hospitalised patients (Tablan *et al.* 1985, Tablan *et al.* 1987). Clustering of "*B. cepacia*" infections in some CF centres highlighted a possible risk of patient to patient transmission (Goldman and Klinger 1986). In one study, Thomassen and colleagues noted that the increasing prevalence of "*B. cepacia*" could be reduced if stringent infection control and segregation procedures were introduced (Thomassen *et al.* 1986). The risk of direct person to person spread within the clinic (Fisher *et al.* 1993, LiPuma *et al.* 1990, LiPuma *et al.* 1988) or indirect nosocomial transmission from contact with contaminated equipment (Nelson *et al.* 1991) was only fully elucidated with the development of genomic fingerprinting. The use of ribotyping, PFGE and other techniques provided compelling evidence that "*B. cepacia*" was transmissible both within the CF clinic (Doring *et al.* 1996, Smith *et al.* 1993, Taylor *et al.* 1992) and via social contact (Govan *et al.* 1993, Pegues *et al.* 1994).
Despite initial evidence that epidemic strains of "*B. cepacia*" were unique to individual clinics (LiPuma *et al.* 1988), it soon became apparent that within the "*B. cepacia*" population, unique strains and lineages existed which were more capable of inter-regional and inter-continental spread (Govan *et al.* 1996).
A seminal paper by Govan and co-workers identified a highly transmissible strain that was present in both Edinburgh and Manchester CF clinics from 1989 (Govan *et al.* 1993). Genotypic and phenotypic data was used in conjunction with epidemiological evidence examining social contact between patients. Transmission was linked to attendance at weekend CF camps and a weekly fitness class. Although the authors found that 50% of patients that had died during the study were infected with this epidemic strain, clinical outcome was unpredictable and not linked to initial lung function. Likewise, poor respiratory function did not increase the risk of initial infection.

The importance of surveillance, with respect to the epidemiology of CF infections, was fully recognised by the finding that the "Edinburgh epidemic strain" had spread across continents. Later termed the Edinburgh/Toronto ET-12 lineage (Govan *et al.* 1996), it had been isolated in an ongoing epidemic in Toronto, Canada with transmission linked to attendance at summer camps (Johnson *et al.* 1994).

Increased knowledge of Bcc epidemiology and taxonomy indicated that most transmissible strains belong to *B. cenocepacia* (originally genomovar III), including the notorious ET-12 lineage. However, other Bcc species have been responsible for outbreaks including *B. multivorans* (Mahenthiralingam *et al.* 2001, Segonds *et al.* 1999) and others (Agodi *et al.* 2001, Biddick *et al.* 2003, Petrucca *et al.* 2003). Several studies have shown the ET-12 lineage possesses both the so-called *B. cepacia* epidemic strain marker (BCESM) (Mahenthiralingam *et al.* 1997), and a novel cable pilus gene (Sajjan *et al.* 1995, Sun *et al.* 1995). BCESM has recently been shown to be part of a novel genomic island encoding genes associated with virulence and metabolism (Baldwin *et al.* 2004). Important genes identified encode and AHL synthase, a porin (OpcI), and an amidase (AmiI). Mutagenesis studies in a chronic rat lung model demonstrated that AmiI is important in persistence and the AHL synthase and OpcI are important in virulence. The authors conclude that the correlation between BCESM and virulent CF strains is validated by their study, although others argue that neither BCESM or the cable pilus are considered to be frequent enough within the Bcc to be sufficient indicators of virulence or transmissibility (LiPuma *et al.* 2001).

The impact of epidemiological studies on the Bcc have resulted in the publication of national and frequently updated guidelines that focus on Bcc cross infection issues (www.cftrust.org.uk & Saiman and Siegel 2004). As well as describing many high risk factors associated with transmission, the document outlines current cross-infection procedures and states that "*B. cepacia*" positive CF patients should be segregated at both inpatient and outpatient clinics, and encouraged not to mix socially. Not surprisingly, such Draconian measures have had a significant social impact on the CF community (Duff 2002). The implications of such measures were

also highlighted in an emotive article describing how two brothers with CF decided to live apart when one became "*B. cepacia*" positive (Webb 2000). Although cross-infection control measures have reduced the incidence of transmission significantly, outstanding issues remain. The potential for transmission to non-CF individuals (parents, non-CF siblings and partners) is slight, but has been highlighted as a risk (Ledson *et al.* 1998). Environmental acquisition of "epidemic strains" is also considered to be a risk factor (LiPuma *et al.* 2002). Furthermore, cohorting of Bcc positive individuals may encourage "superinfection". Acquisition of a second, more virulent, Bcc (ET-12) strain resulted in increased morbidity and mortality (Ledson *et al.* 1998, Mahenthiralingam *et al.* 2001). At present, all Bcc species are considered equal for the purpose of cross-infection control. Segregation based on species identification within the Bcc has not been recommended. However, species identification is important in patients being considered for lung transplantation (De Soyza and Corris 2003). In these patients, infections with *B. cenocepacia* has been found to carry a high risk and benefit from alternatives to peri-transplant procedures (Aris *et al.* 2001, De Soyza and Corris 2003, De Soyza *et al.* 2001).

1.5.7 Bcc and Virulence

The fact that clinical outcome cannot be predicted in patients infected with the same strain suggests that both bacterial and host factors are important. In contrast to *P. aeruginosa* where alginate biosynthesis and biofilm production are key virulence determinants, no such factors have been demonstrated within the Bcc, including *B. cenocepacia*. The specific virulence determinants required for Bcc infection in CF individuals remain largely unknown. Bacterial factors probably work in combination and include adherence, siderophores, invasion, intracellular survival, exopolysaccharide, type III (Tomich *et al.* 2003) & type IV secretion systems, quorum sensing (and QS regulated genes (Aguilar *et al.* 2003)) and LPS (Hutchison and Govan 1999), as well as several extracellular virulence factors (Table 1.6). The importance of each factor in the ability to colonise and cause tissue damage is unknown, and is probably dependent on the Bcc species, and indeed the strain investigated. Furthermore, the inherent multiple resistance of the Bcc to

antimicrobial agents, including natural defensins, provides another potential factor in the pathogenicity of Bcc lung infections in CF.

Table 1.6 Bcc Virulence Factors (Reviewed by Gessner and Mortensen.1990, Hutchison and Govan.1999, Mohr *et al.*2001, unless otherwise stated, Nelson *et al.*1994)

Extracellular Virulence Factors
Haemolysin (both Phospholipase C and sphingomyelinase activities)
Catalase
Ornithine Decarboxylase
Melanin (Zughaier *et al.*1999)
Valine aminopeptidase
C14 lipase
Alginase
Trypsin
Lipase
36 kDa proteinase (zinc metalloprotease)(Corbett *et al.*2003)
Reduction of nitrate to nitrite
Hydrolysis of urea
Hydrolysis of xanthine

1.5.8 Adherence

Adherence is considered to be the first stage of bacterial colonisation and infection. Pili or fimbriae are surface appendages that allow bacteria to interact with both each other and the host. Type III cable pili and an associated adhesin have been described in the ET-12 lineage and provide convincing candidates for adhesion to epithelial cells and respiratory mucin (Sajjan *et al.*1992, Sajjan and Forstner.1992, Sajjan and Forstner.1993, Sajjan *et al.*1995). Possession of the cable pilus gene, *cblA*, has been suggested as an epidemiological marker for transmissibility in Bcc isolates (Sun *et al.*1995). However, the cable pilus has not been observed in other epidemic strains (Mahenthiralingam *et al.*1997). Furthermore, the *cblA* gene has been identified in a non-CF isolate and a phytopathogenic strain, indicating that the potential expression of the cable pilus is not restricted to CF epidemic strains (Richardson *et al.*2001). As a caveat, however, presence of the *cblA* in a "*B. cepacia*" isolate does not always correlate with the expression of the cable pili (Sajjan *et al.*2002).

1.5.9 Quorum Sensing

A quorum-sensing system in the ET-12 *B. cenocepacia* isolate K56-2, has been identified and consists of *cepI* and *cepR* genes. The expression of *N*-acyl-HSL appears to be conserved throughout the Bcc (Gotschlich *et al.* 2001). K56-2 synthesizes an *N*-octanoyl HSL (C_8-HSL) and an *N*-hexanoyl-HSL (Lewenza *et al.* 1999, Lutter *et al.* 2001). The CepI/R system has been implicated in the negative regulation of siderophores and in the positive regulation of a secreted protease. Likewise, the CepI/R QS-system of *B. cenocepacia* H111 has been shown to be involved in swarming motility and biofilm formation (Huber *et al.* 2001). Similarly, the QS-system in *B. cepacia* ATCC 25416 utilises C6- and C8-HSL molecules. Mutant studies indicated that protease activity was significantly lower in the QS-deficient mutants, and attenuated virulence was observed in an onion maceration model (Aguilar *et al.* 2003).

As with *P. aeruginosa*, the regulation of virulence by QS-systems in the Bcc is unlikely to provide a complete picture. Furthermore, the likelihood of a homologous QS-system within the Bcc is low (Aguilar *et al.* 2003).

1.5.10 Siderophores

The ability to sequester iron from the mammalian host is fundamental to the success of bacteria as pathogens. Microbes acquire iron by the secreting ferric iron chelators, known as siderophores. "*B. cepacia*" is known to produce at least four different siderophores: ornibactins (Meyer *et al.* 1995), pyochelin (Sokol 1986), salicyclic acid (SA) (Visca *et al.* 1993) and cepabactin (Meyer *et al.* 1989). The pathogenic implications of each are unclear, although ornibactins and SA are the predominant siderophores produced by 87 and 92% of clinical Bcc isolates (Darling *et al.* 1998).

1.5.11 Lipopolysaccharide (LPS)

LPS (endotoxin), a membrane glycolipid of Gram-negative bacteria, is an important bacterial virulence factor that has potent immuno-stimulatory activity on mononuclear cells, granulocytes and B-lymphocytes with the induction of pro-inflammatory cytokines (Reitschel *et al.* 1993). The induction of pro-inflammatory cytokines and LPS-mediated macrophage stimulation is thought to play an important role in bacterial pathogenesis in CF (Greally *et al.* 1993). Prior to the current taxonomic revision, *in vitro* studies showed that "*B. cepacia*" LPS exhibited a higher endotoxic activity and induced a greater cytokine response than *P. aeruginosa* and *S. maltophilia* LPS (Shaw *et al.* 1995, Zughaier *et al.* 1999). In a subsequent study by Hughes and co-workers, it was suggested that the inflammatory activity of "*B. cepacia*" LPS may contribute to lung disease in CF by promoting increased neutrophil recruitment and by priming neutrophil respiratory burst responses to microbes and other stimuli (Hughes *et al.* 1997). Although LPS was not found to trigger the respiratory burst directly, it was suggested that the increased potential for release of tissue-damaging proteolytic enzymes and reactive oxygen species might contribute to pathogenic sequelae seen in "*B. cepacia*" lung infections. Differences in stimulatory activities of LPS, together with host factors, could also explain the variable pathogenic sequelae seen in CF lung infections.

It has been suggested that differences in host inflammatory response to Bcc LPS may be related to genomovar/species status of the patient (De Soyza *et al.* 2000). However, a study by Kenna and colleagues investigating the relationship between O-serotype and genomovar status, suggested the biological activity of LPS is likely to be strain- rather than genomovar-specific (Kenna *et al.* 2003). Variation in the O-antigen, the immunodominant component of LPS, involves modification of sugar moieties or changes in the length of the O-antigen sidechain, and is known to be a significant virulence mechanism (Lerouge and Vanderleyden 2001). Individual strain populations within the Bcc can exist as a mixture of smooth and rough LPS chemotypes (Evans *et al.* 1999) and both can be highly endotoxic (Govan 2003, Shaw *et al.* 1995, Zughaier *et al.* 1999). In contrast to environmental strains, clinical

isolates tend to exhibit a rough chemotype (Evans *et al.* 1999). However, both rough and smooth variants are capable of inducing the production of tumour necrosis factor-alpha (TNF-α) to the same extent (Gronow *et al.* 2003). A recent report examined LPS structures of *B. multivorans* (LMG 14273) and *B. cenocepacia* (LMG 12614) (De Soyza *et al.* 2004). Interestingly, the authors suggest that effective induction of pro-inflammatory cytokines by *B. cenocepacia* LPS was due to the presence of a β-hydroxymyristate (3-OH C14:0), which was absent from the less effective *B. multivorans* LPS.

1.5.12 Intracellular Survival and Growth

Several studies have suggested the potential intracellular survival of "*B. cepacia*". Butler *et al.* demonstrated that epidemic CF strains exhibit a rough colonial morphotype and are serum sensitive (Butler *et al.* 1994). In general bacterial strains with rough LPS are more sensitive to serum, less virulent and rarely cause bacteraemia (Crokaert *et al.* 1992). This is not the case with CF isolates of "*B. cepacia*" which frequently produce chronic lung infections and bacteraemia. This apparent contradiction poses an interesting question regarding the mechanism of Bcc survival *in vivo*. In 1996, a seminal study by Burns and co-workers described the invasion of respiratory epithelial (A549) cells by "*B. cepacia*" (Burns *et al.* 1996). Of particular importance, was the observation of intra-epithelial cell replication. Further studies indicated that Bcc isolates were capable of: 1. Surviving in free living amoebae (Landers *et al.* 2000, Marolda *et al.* 1999); 2. Resisting intracellular killing by phagocytic cells and epithelial cells (Saini *et al.* 1999, Tipper *et al.* 1998), and 3. Strain specific intracellular survival (Martin and Mohr 2000). The latter study confirmed that the *B. cenocepacia* ET-12 strain J2315 was able to enter, survive and replicate intracellularly in U937-derived macrophages and A549 pulmonary epithelial cells. Moreover, other epidemic strains, irrespective of cable piliation, were shown to be capable of attaching to and invading murine respiratory tract cells (Chiu *et al.* 2001).

Attachment is an important step in both the colonisation and the invasion of host tissues. Recent work has focused on the role of Bcc flagella in host cell invasion (Tomich *et al.*2002). The authors noted that flagellar defective mutants exhibited reduced invasion of A549 cells and attributed this to impaired flagellum-mediated motility rather than defective adherence.

Additional virulence determinants that may aid intracellular survival of Bcc include the production of catalases which are found in 100% of "*B. cepacia*" isolates (Gessner and Mortensen.1990) and production of melanin-like pigments by some epidemic strains (Zughaier *et al.*1999). Catalase, and superoxide dismutase, are important enzymes which neutralise the potential bactericidal effects of the host defence molecules hydrogen peroxide and reactive oxygen species (Lefebre and Valvano.2001). The melanin-like pigment, produced by ET-12 isolates, has been shown to attenuate the respiratory burst activity by scavenging superoxide anions (Zughaier *et al.*1999).

Another potential argument in favour of the intracellularity of the Bcc relates to antibiotic therapy. Even when *in vitro* antibiotic susceptibility can be demonstrated (Nzula *et al.*2002), this rarely translates into eradication or even reduction of bacteria within sputum during antibiotic therapy (Gold *et al.*1983).

1.5.13 Antibiotic Resistance and The Bcc

The term "multiresistant" is open to interpretation but usually describes organisms that are resistant to at least three major classes of antibiotics; for example the β-lactams, aminoglycosides, quinolones and tetracyclines. Most "*B. cepacia*" isolates exhibit intrinsic resistance to many antimicrobial agents (Lewin *et al.*1993, Nzula *et al.*2002, Pitt *et al.*1996). In addition, resistance can develop during therapy. Not unexpectedly, multiple resistance mechanisms have been observed in the Bcc. These include: 1. Reduced outer membrane permeability; 2. Alteration of intracellular drug targets or variation in extracellular drug binding sites; 3. Enzymatic degradation of antimicrobials and, 4. Active removal of antibiotics by bacterial efflux pumps.

Resistance can also result through several mechanisms acting simultaneously, exemplified by the resistance to β-lactam based antibiotics.

1.5.14 β-Lactam Resistance

Bacterial resistance to the β-lactams can be due to the combination of inducible chromosomal β-lactamases and low outer membrane permeability. The observation that some "*B. cepacia*" isolates can utilise penicillin G as a carbon source exemplifies both resistance and a striking metabolic potential to both degrade and utilise an antibiotic (Beckman and Lessie 1979). Isolates capable of penicillin utilisation were found to possess a highly-inducible chromosomal penicillinase/β-lactamase. One such strain, "*B. cepacia*" 249, has two separate β-lactamases that vary in their activity (Beckman and Lessie 1979, Harai *et al.* 1980, Prince *et al.* 1988). The original penicillinase exhibits around 80% of the activity, and was later found to be a chromosomally-encoded class A β-lactamase (Trepanier *et al.* 1997). The second, is a cephalosporinase, and accounts for the remaining β-lactamase activity found in "*B. cepacia*" 249 (Harai *et al.* 1980, Prince *et al.* 1988).
The β-lactamases of "*B. cepacia*" are also thought to exhibit carbapenemase activity (Iaconis *et al.* 1994, Simpson *et al.* 1993). Interestingly, although the β-lactamase activity extends to imipenem (Trepanier *et al.* 1997) it does not include meropenem (Baxter and Lambert 1994, Iaconis *et al.* 1994). This observation could be clinically important, as Bcc isolates tend to demonstrate *in vitro* susceptibility to meropenem, and this agent is often the drug of choice in the treatment of Bcc infections (Lewin *et al.* 1993, Nzula *et al.* 2002, Pitt *et al.* 1996).

Reduced outer membrane permeability is also a factor in resistance to β-lactam antibiotics. The outer membrane of "*B. cepacia*" is characterised by unusually small porins with low single channel conductance. Permeability studies with the β-lactam nitrocefin, showed that the outer membrane permeability of "*B. cepacia*" was comparable to *P. aeruginosa*, but ten times lower than *Escherichia coli* (Parr *et al.* 1987). Further reductions in permeability, and thus increased resistance to β-

lactams, are achieved by decreased expression of outer membrane proteins or alterations in LPS. Aronoff (1988) described the reduced expression of two outer membrane proteins, 36 kDa and 27 kDa respectively, which resulted in increased resistance to β-lactams. The latter protein may be a major porin or an important component of a major porin complex (Aronoff 1988, Gotoh *et al.* 1994).

1.5.15 Aminoglycoside & Polypeptide Antibiotic Resistance

Binding of aminoglycosides and cationic polypeptide antibiotics, such as polymyxin B and colistin (polymyxin E), to LPS is considered to be the first stage in antimicrobial action of these agents. Both compounds displace divalent cations that stabilise and cross-link LPS in many Gram-negative bacteria. The effect of destabilising the bacterial outer membrane is loss of integrity and stability. Once the outer membrane is disrupted, positively charged polypeptide antibiotics can destroy the cell by acting on the negatively-charged cytoplasmic membrane (Moore and Hancock 1986). Aminoglycosides, on the other hand, act through an intracellular target and inhibit protein synthesis. Most Bcc isolates are inherently resistant to both aminoglycosides and the polymyxins (Nzula *et al.* 2002) and alteration in the initial antibiotic binding site or cross-linking may account for such resistance.

LPS structure in "*B cepacia*" differs from many other Gram-negative bacteria. Essentially, outer membrane stability is achieved by attaching 4-amino-4-deoxyarabinose (Ara4N) to phosphate residues. Further substitution of Ara4N has been observed on mainly the outer sugar (1→8)-*D-glycero-D-talo*-oct-2-ulsonic acid (KO), and to a lesser extent, 3-deoxy-*D-manno*-oct-2-ulsonic acid (KDO) (Gronow *et al.* 2003). Attachment of Ara4N residues to the phosphate groups of lipid A is known to lead to polymyxin resistance (Helander *et al.* 1994). Ara4N contains a positively-charged ammonium group, and thus reduces the negative charge of the lipid A backbone induced by the phosphate groups (Vinion-Dubiel and Goldberg 2003). LPS stability is attained by the interaction of positively-charged arabinose molecules with negatively-charged phosphate residues present in LPS (Cox and

(Cox and Wilkinson.1994, Vaara.1992), therefore, polymyxin resistance is achieved, as divalent cross-linking is no longer required. The ability of "*B. cepacia*" LPS to bind cationic molecules is low, due to low levels of phosphate and KDO (Cox and Wilkinson.1994). Interestingly, "*B. cepacia*" LPS has been shown to bind, with high affinity, to polymyxin B, despite the presence of Ara4N (Shimomura *et al.*2003). Despite the fact that polymyxin B is a potent antagonist of LPS-induced cytokine release (Coyne and Fenwick.1993, Stokes *et al.*1989), it was found to have an unusual interaction with "*B. cepacia*" LPS, in that, polymyxin B was unable to antagonise the induction of TNF-α and IL-6, and interaction was associated with increased IL-1β inducing activity of "*B. cepacia*" LPS (Shimomura *et al.*2003). With respect to aminoglycoside resistance, it is now assumed that because "*B. cepacia*" LPS is not cross-linked by divalent cations, then the "self promoted" uptake mechanism cannot function.

1.5.16 Resistance to Other Antimicrobial Agents

Plasmid-encoded resistance appears to be rare in Bcc isolates, although one study has reported tetracycline and ampicillin resistance determinants in a "*B. cepacia*" strain. The presence of efflux systems in "*B. cepacia*" ensures that drugs entering the cell could be actively exported. An important feature of many efflux systems is the ability to act as "multi-drug resistant (MDR) efflux pumps" (Nikaido.1998). The activity of a Bcc MDR efflux pump, homologous to the *mexA-mexB-oprM* efflux operon in *P. aeruginosa* (Li *et al.*1995), appears to be responsible for cross-resistance to chloramphenicol, trimethoprim and ciprofloxacin (Burns *et al.*1996). Interestingly, the newer fluoroquinolones, such as gemifloxacin and moxifloxacin, are also substrates for this MDR efflux system (Zhang *et al.*2001). Furthermore, the recent cloning and characterisation of the *norM* gene, from *B. vietnamiensis*, which encodes a multi-drug efflux protein, appears to confer some resistance to polymyxin B (Fehlner-Gardiner and Valvano.2002). Wigfield *et al.* have described a Bcc immunodominant efflux pump responsible for both nalidixic acid and tetracycline resistance (Wigfield *et al.*2002). Cross-resistance in the Bcc, to

trimethoprim/sulphamethoxazole, chloramphenicol and the quinolones, has also be attributed to LPS structure and alteration in porin expression (Rajyaguru and Muszynski 1997).

A novel mechanism of resistance observed in "*B. cepacia*" is the *dsbA-dsbB* disulfide bond formation system. Hayashi and co-workers demonstrated that a "*B. cepacia*" DsbA-DsbB mutant exhibits increased sensitivity to β-lactams, kanamycin, erythromycin, novobiocin and ciprofloxacin (Hayashi *et al.* 2000).

Resistance of the Bcc to antibiotics *in vitro* and *in vivo* may be influenced by the environment and growth conditions. Interestingly, the antibiotic resistance observed in *P. aeruginosa* biofilms is related to slow growth and anaerobiosis, not a barrier effect. Planktonic and biofilm cultures were found to be more resistant to ciprofloxacin and ceftazidime (Desai *et al.* 1998). Growth in an atmosphere of 5% carbon dioxide (CO_2) reduces the susceptibility of "*B. cepacia*" to β-lactam antibiotics (Corkhill *et al.* 1994). Likewise, ciprofloxacin and tobramycin resistance is increased if oxygen (O_2) is depleted during growth (McKenney and Allison 1997). Interestingly, the role of "*B. cepacia*" as a plant pathogen may partly explain the inducible nature of certain resistance mechanisms. Salicylic acid is an important signalling molecule in plant defence responses after pathogen attack (Klessig *et al.* 2000). Signalling cascades result in an acute disease response that acts systemically throughout the plant. Early responses include an increase in reactive oxygen species, cross-linking of cell wall proteins and increased expression of plant defence genes (Baker and Orlandi 1995, Hammond-Kosack and Jones 1996, Lamb and Dixon 1997, Yang *et al.* 1997). Some of these defence genes encode biosynthetic enzymes, such as phenylalanine ammonia lyase (PAL). PAL is the first enzyme in the phenylpropanoid pathway, which is involved in the synthesis of antimicrobial compounds: the phytoalexins (Klessig *et al.* 2000).
In "*B. cepacia*" salicylate-inducible antibiotic resistance mechanisms have been observed (Burns and Clark 1992). The authors reported that, in the presence of a weak acid, salicylate, an outer membrane pore-forming protein was absent. This lack

of expression conferred resistance. Thus, in its role as a phytopathogen, an adaptive response of the Bcc may be to reduce outer membrane permeability in the presence of a weak acid, such as salicylic acid, since low pH-mediated signalling results in the production of antimicrobial compounds by the plant. Interestingly, a recent study has shown that salicylate induces an efflux pump in *B. cenocepacia* (Nair *et al.* 2004). The authors state that salicylate is an important siderophore and a natural substrate for the *B. cenocepacia* efflux system; therefore efflux-mediated resistance may be upregulated in low iron concentrations, in the absence of antibiotic selective pressure.

In conclusion, although there are a number of resistance mechanisms utilised within the Bcc, in particular, reduced outer membrane permeability and MDR efflux pumps, resistance varies significantly between isolates and within species (Nzula *et al.* 2002). This variability highlights the need for antibiotic susceptibility testing prior to the administration of antibiotic therapy. However, susceptibility testing in relation to CF lung infections is notoriously unreliable even with more susceptible pathogens such as *P. aeruginosa*.

1.6 Novel Antimicrobial Therapies Against Bcc infections

With the immunological hurdles and physiological barriers facing gene therapy becoming ever more complicated (Ferrari *et al.* 2003), present concerns of recurrent microbial infection and inflammation have to be dealt with in the meantime. Novel antimicrobial therapies do not offer a "cure" for CF, rather they offer a means of disease management. As the major CF pathogens (*S. aureus*, *P. aeruginosa* and the Bcc) are known to have increased virulence, be capable of epidemic spread and multiply antibiotic resistant, novel antimicrobial agents are urgently required to stem this considerable threat. Throughout this thesis, the focus will be on novel agents with activity against the Bcc, since conventional antibiotic therapies are ineffective. Such agents may also act against other CF and non-CF pathogens.

1.7 Medicinal Use of Honey

From within the bee's bodies
A drink of varying colours,
Wherein is healing for men.

Holy Qu'ran

Honey has been used as part of "folk medicine" for centuries but is now gaining a renewed reputation as a possible answer to the growing problem of multiresistant bacterial infections. As with other natural plant-derived compounds, such as allicin, thyme oil, tea-tree extracts and St John's wort, much of the medicinal benefits of honey are hidden in non-scientific literature or simply ignored. Since the antimicrobial activity of honey was first described by Dold *et al.* in 1937, several investigators have attempted to elucidate its antimicrobial components (Dold *et al.* 1937, cited by Wahden 1998.).

1.7.1 Antibacterial Components of Honey

The obvious explanation for the antimicrobial properties of honey is high osmolarity. Honey is a supersaturated solution of sugars with a very low volume of free-water. Free-water is measured as the *water activity* (a_w). Many bacterial species are inhibited by an a_w value in the range 0.94-0.99: mean values for honey range from 0.5-0.6. However, the significance of inhibition by osmotic effects must take in account the dilution of honeys prior to use (reviewed by Molan 1992a). The main component sugars are the monosaccharides, fructose and glucose, and to a lesser degree sucrose and maltose. The sugar/water composition of different types of honey is variable and directly affected by both the season they are produced (Varis *et al.* 1983), and whether the bees are wild or "domesticated" (Chou *et al.* 1994). The high osmotic effect of these saturated sugar solutions on the bacterial cell, results in the diffusion of water across the membrane and a lethal loss of osmotic pressure. Interestingly, the osmotic effect appears to be only partially responsible for the antibacterial activity, as studies using "artificial" honey solutions have concluded that high osmolarity alone does not account for the antimicrobial activity (reviewed by Molan 1992a).

Much of the early work on the antibacterial properties of honey described an antibacterial factor, termed inhibine. Activity extended to both Gram-positive and Gram-negative bacteria, was heat-labile and somewhat sensitive to light (Dold *et al.* 1937). The first attempt to clarify the chemical nature of inhibine described hydrogen peroxide as the likely source of the antibacterial activity in a fall-flower honey (White *et al.* 1963). Hydrogen peroxide was produced by glucose oxidase, an acid-producing enzyme that catalyses production of gluconic acid and hydrogen peroxide from glucose. The oxidase originates from the hypopharyngeal glands of honey bees (Gauhe 1941, in French cited by Weston 2000). Hydrogen peroxide levels can also be influenced by the amount of flower pollen-derived catalase present in honey and by dilution of the honey during sample preparation (Bang *et al.* 2003). A recent comprehensive review by Weston (2000) discounted the contribution of other antibacterial factors present in honey, and indicated that antibacterial action is due solely to pollen-derived catalase and its direct causative effect on the level of hydrogen peroxide (Weston 2000, White 1966). However, an earlier study by Wahden (1998) identified the presence of two antimicrobial phenolic acids (caffeic acid and ferulic acid) as well as flavanoids in trefoil honey (Wahdan 1998). Many of the phenolic compounds present in honey are derived from propolis (Amiot *et al.* 1989, Ferreres *et al.* 1992), a resinous material collected by bees and reputedly used as an antibacterial agent within the hive (Marcucci 1995). A recent study has demonstrated the antimicrobial activity of propolis *in vitro*, against *Streptococcus pneumoniae*, *H. influenza* and *S. aureus* (Drago *et al.* 2000, Miorin *et al.* 2003). Collected from the gum exudates of trees (mainly *Poplar*), propolis contains several potential antimicrobial components including benzoic and cinnamic acids and flavanoids. Although the concentration of these molecules is considerably less in honey than in propolis (Bogdanov 1989), it is conceivable that they may act synergistically and contribute to overall antibacterial activity. Furthermore, the reaction of hydrogen peroxide with benzoic acid can create peroxyacids; these are more stable than hydrogen peroxide and unaffected by catalase (Weston 2000). The presence of peroxyacids in honey has yet to be established.

The pH of honey may also affect its antibacterial activity. Honey is generally acidic, with a pH between 3.2 and 4.5 (Molan.1992a). This acidity is primarily due to the content of gluconic acid. Despite the fact that neutralised honey still has significant antibacterial activity, pH may play a role in unbuffered or undiluted honey.

Other antimicrobial factors may include insect-derived peptides such as lysozyme (Molan.1992a), apidaecins (Casteels *et al.*1989), abaecin (Casteels *et al.*1990), hymenoptaecin (Casteels *et al.*1993) and royalisin (Fujiwara *et al.*1990). These enzymes are strongly induced when an insect becomes infected. The role of these peptides in the antibacterial activity of honey has yet to be fully established, although a study by Weston *et al.* (2000) indicated that their antibacterial role is likely to be minimal (Weston *et al.*2000).

1.7.2 New Zealand Manuka (*Leptospermum scoparium*) Honey (NZMh)

New Zealand manuka honey (NZMh), which has received particular interest for its antimicrobial activity, is derived from the nectar of the manuka tree (*Leptospermum scoparium*: Myrtaceae), a species endemic to New Zealand. Several types of manuka honey exist, but the level of non-peroxide killing is dependent on the region in which the honey is produced (Molan.1995). This variation in bioactivity was explained by the presence of a plant-derived product present in manuka honeys, which is yet to be identified (Molan *et al.*1988). NZMh is presently marketed as a natural antimicrobial product containing a "unique manuka factor" (UMF). The nature of this "UMF" is unlikely to be a unique phenolic compound, as levels do not vary between active and non-active manuka honeys (Weston *et al.*1999). Nor is UMF likely to be two abundant extractable organic components, syringic acid and phenyllactic acid, found in many European honeys (Wilkins *et al.*1993). Furthermore, there is little variation in the oligosaccharide composition of active and non-active manuka honeys (Weston and Brocklebank.1999).

Many potential antimicrobial compounds that have been identified in honey are unique to the preparation used, and dependent on flower source. Furthermore,

because of the natural synthesis of honey, the level of the antimicrobial components may vary between "batches".

1.7.3 Antimicrobial Spectrum of NZMh and Other Honeys

The antimicrobial spectrum of honey is impressive and extends to both Gram-positive and Gram-negative bacteria, as well as to fungi and yeasts. A report by Willix *et al.* (1992), which described the sensitivity of wound-infecting bacterial species to manuka and an alternative monofloral honey, indicated that the activity of both honeys extended to *E. coli*, *Proteus mirabilis*, *Salmonella typhimurium*, *P. aeruginosa*, *Serratia marcecens*, *S. aureus* and *S. pneumoniae* (Willix *et al.*1992). Two further studies described potent antibacterial activity of manuka and pasture honey against clinical isolates of *S. aureus* (Cooper *et al.*1999a) and *P. aeruginosa* (Cooper and Molan.1999b). *P. aeruginosa* burn infections pose an important clinical problem both in their management and treatment. Cooper *et al.* recently described the sensitivity of *P. aeruginosa* isolated from burns, to both manuka and pasture honey (Cooper *et al.*2003).

Honey has been used traditionally to treat dyspepsia in many cultures. Somal and co-workers examined the susceptibility of *Helicobacter pylori* to manuka honey (Somal *et al.*1994). *H. pylori* is known to be an important cause of gastritis and duodenal ulceration, thus the authors hypothesised that manuka honey may be an alternative therapy in eradicating this organism from the stomach. *In vitro* sensitivity studies indicated that *H. pylori* is inhibited by manuka honey, but *in vivo* efficacy was not assessed.

Interestingly, the antibacterial activity of manuka honey extends to antibiotic resistant pathogens including; MRSA, vancomycin-resistant *Enterococcus faecium* (VRE) (Cooper *et al.*2002) and multiresistant CF isolates of "*B. cepacia*" (Cooper *et al.*2000).

1.7.4 Practical Applications of Honey and its Clinical Effect

The main therapeutic use of honey is in the treatment of wounds that fail to heal with conventional therapies. Bergam *et al.* reported that honey accelerated wound healing in mice (Bergam *et al.* 1983). Human studies, which followed, have focused on the effectiveness of topically applied honey-impregnated dressings (Wood *et al.* 1997). An initial study reported the efficacy of topically applied honey onto wounds and ulcers in 58 of 59 patients following one week of honey therapy (Efem 1988). The clinical effects reported were: 1. Gradual separation of necrotic and gangrenous tissue from wall of ulcer; 2. Reduction in surrounding oedema; 3. Dehydration of weeping ulcers; 4. Deodorizing of foul-smelling wounds, and 5. Sterilisation of the infected lesion. In addition, wound healing and epithelialisation were also described. Further studies have highlighted the wound-healing potential of honey with the successful treatment of burns (Subrahmanyam 1978), recalcitrant surgical wounds (Cooper *et al.* 2001) and in the management of pressure ulcers (Van der Weyden 2003). The latter study used a honey/alginate combination, and noted that as well as the well-documented antibacterial activity, there appeared to be significant anti-inflammatory effects associated with the therapy.

A recent report examined the effect of manuka, pasture and artificial honeys on macrophage function (Tonks *et al.* 2001). Macrophages play an important role in regulating wound repair and are primed and activated by the pro-inflammatory cytokine, TNF-α. The authors observed that both pasture and manuka honey caused spontaneous release of TNF-α from a human monocytic (MM6) cell line. Furthermore, pasture, and to a lesser extent manuka honey, reduced production of reactive oxygen intermediates (ROIs) signifying a potential antioxidant effect. Additionally, both manuka and pasture honey have been shown to increase production of other inflammatory cytokines, IL-1β and IL-6, from MM6 cells (Tonks *et al.* 2003), and in some cases to act as a mitogen for B- and T-lymphocytes (Abuharfeil *et al.* 1999). Thus, in therapeutic terms, honey may play an important immunological role *in vivo*.

One problem connected with the therapeutic use of honey, is that it may contain spores of *Clostridium* and *Bacillus* spp. increasing the risk of wound botulism or gangrene (Midura *et al.* 1979). However, sterilisation can be achieved by gamma irradiation without compromising antibacterial activity (Molan and Allen 1996).

Aerosolisation may be an effective method of administering honey for treatment of respiratory infections. Aerosolisation of 50% (vol/vol) acacia honey has been described in the treatment of chronic and exudative bronchitis (Bejan *et al.* 1978 in French) and Kirienko *et al.* advised the inhalation of honey and propolis to supplement conventional therapy in treatment of chronic bronchitis (Kirienko *et al.* 1989, cited by Cooper et al. 2000). Other potential therapeutic applications include the synergistic use of honey with conventional antibiotics (Karayil 1998).

In summary, the full implications of the medicinal use of honey are yet to be realised, and this most natural and useful product may yet provide cheap and novel alternatives to conventional therapies.

1.8 Bacteriophage (Phage)

So, naturalists observe, a flea
Hath smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed ad infinitum.

- Johnathan Swift

The independent discovery of bacteriophage by Fredrick W. Twort and Felix d'Herelle in 1915 and 1917 respectively, was the first indication that these bacterial viruses might have a role in the treatment of bacterial infections (d'Herelle 1917, Twort 1915). Described initially as "filterable particles", bacteriophages were used therapeutically until the antibiotic era began in the 1950s. At this time, the role of

bacteriophage as therapeutic agents was no longer of major concern and instead, they became important tools of the molecular biology revolution. Ironically, it is primarily as a result of increasing bacterial resistance to conventional antimicrobial agents, that the therapeutic use of bacteriophage has once again attracted attention: on this occasion, supported by the molecular technology that these agents helped to create.

1.8.1 Bacteriophage Life Cycle

The global population of phage (or bacterial viruses) is estimated to be more than 10^{30} (Brussow and Hendrix 2002). Bacteriophages infect bacteria by attachment to specific receptors and injection of phage nucleic acid into the cell (reviewed by Stent 1963). Phage particles or virions, exist as a protein capsid surrounding nucleic acid, DNA or RNA. The nucleic acid can be double- or single-stranded, in a linear or circular conformation. The specificity of phage for specific strains within species has resulted in their use as a bacterial typing system.

Two types of phage have been defined: virulent/lytic and lysogenic/temperate phage. Virulent phages propagate through a lytic cycle. Upon infection, lytic phage DNA enters a replicative cycle resulting in the production of new phage particles. The production of a large number of daughter phage particles, and an associated lytic enzyme, leads to lysis of the host. In contrast, temperate bacteriophages are able to form lysogens; in this form phage exist in a latent or quiescent state known as a prophage. In lysogenic bacteria, DNA is integrated into the bacterial chromosome and exists as a prophage. Prophage DNA remains integrated into the bacterial chromosome until induced, whereby the host cell expresses phage genes and lytic growth is initiated.

Some temperate phages can act as vectors for horizontal gene transfer (transduction, and are termed transducing phage. Two types of transduction have been described; generalised and specialised. Generalised transduction is the process by which the

transducing phage, through packaging of bacterial rather than phage DNA during phage replication, can transfer a variety of loci of the bacterial genome from donor to recipient cell, exemplified by the *Salmonella* phage P22. In contrast, specialised transduction is the process by which the transducing phage, having integrated at specific genomic loci, can transfer loci on either side of the integrated site, as is the case with the *E. coli* phage λ. Of particular interest is the ability of phage to transfer antibiotic resistance genes within and between different host species. In *P. aeruginosa*, transduction of imipenem resistance (Blahova *et al.* 1998), as well as ceftazidime (Seginkova *et al.* 1986) and amikacin, gentamicin and tobramycin (Knothe *et al.* 1981) has been well documented. The latter study demonstrated the antibiotic resistance transducing potential of the well-characterised *P. aeruginosa* phage F116.

Lysogenic phage may also play an important role in the evolution of bacteria as pathogens including an intrinsic role in pathogen-host interactions (Miao and Miller 1999). Since the seminal study that demonstrated the essential role played by phage in regulation and biosynthesis of diphtheria toxin (Freeman 1951), many phage-encoded virulence genes have been found (Table 1.7).

Table 1.7 Bacteriophage encoded virulence factors (Adapted from Boyd *et al.* 2001, Miao and Miller 1999)

Virulence Determinant	Bacteria	Bacteriophage
SopE	*S. typhimurium*	SopEΦ
SodC	*S. typhimurium*	Gifsy-2
Cholera toxin	*V. cholerae*	CTXΦ
Toxin-coregulated pilus & CTX Φ receptor	*V. cholerae*	VPIΦ
Diphtheria toxin	*Corynebacterium diphtheriae*	Converting β-phage
Shiga-like toxin-I and -II	Enterotoxigenic *E. coli*	Shiga-like toxin converting phage
Serum resistance	*E. coli*	Lambda (λ)
Enterotoxin A & Staphylokinase	*S. aureus*	Φ 13
Toxic shock syndrome toxin-1 (TSST-1)	*S. aureus*	TSST-1
Streptococcal pyrogenic exotoxins A & C	Group A *Streptococci*	T12 & 3GL16
Neurotoxin	*Clostridium botulinum*	Phage C1
O-antigen acetylase	*Shigella flexneri*	Sf6

The expansion in sequencing whole bacterial genomes has ensured that the importance of phage and their role in bacterial virulence will remain the focus of investigation.

1.8.2 Bacteriophage of the *B. cepacia* complex

Little is known of the phages of the Bcc. Early reports on the "*B. cepacia*" phages CP1 (Cihlar *et al.* 1978) and CP75 (Matsumoto *et al.* 1986) predate revision of Bcc taxonomy. Briefly, Cihlar et al. (1978) characterised an organic solvent-sensitive phage, CP1, present in the lysogenic "*B. cepacia*" strain 249. Phage morphology appeared to be T-even-like comprising a hexagonal head and contractile tail. The authors were unable to demonstrate transduction of auxotrophic "*B. cepacia*" by CP1. Matsumoto and co-workers described a generalised transducing phage, CP75, derived from the lysogenic "*B. cepacia*" strain PCT1. CP75 was insensitive to organic solvents, and had similar morphology to CP1. The CP75 genome was estimated to be approximately 52 Kbp, and generalised transduction was demonstrated by the use of three auxotrophic mutants requiring histidine, leucine or methionine.

More recently, a study characterised two generalised transducing phages (NS1 and NS2) from Bcc strains ATCC 29424 (*B. vietnamiensis*) and ATCC 17616 (*B. multivorans*) respectively (Nzula *et al.* 2000). Evidence suggested that the bacterial receptor was LPS, and both phages were capable of transducing ceftazidime resistance. The broad host range of NS1 and NS2 within the Bcc was surprising, and even extended to *P. aeruginosa* and *B. pseudomallei* (Langley *et al.* 2003). Likewise, some Bcc isolates were found to be sensitive to well-characterised *P. aeruginosa* transducing phages, B3, F116 and G101. These studies highlighted the potential for exchange of genomic loci, both within the Bcc, and between the Bcc, *P. aeruginosa* and other *Burkholderia* species. The broad host range of Bcc phage is unusual and interesting. Lytic phages with interspecies host range within the Bcc have also been reported in association with soil-borne strains of *B. cenocepacia* (LiPuma *et al.* 2000). A novel phage (ϕE125) that can propagate in *B. mallei* has been isolated from *B. thailandensis* strain E125 (Woods *et al.* 2002). *B.*

thailandensis, a non-pathogenic soil saprophyte is closely related to *B. mallei* and *B. pseudomallei*. Prior to this study, eight lysogenic *B. pseudomallei* strains produced bacteriophages that were more active on *B. mallei* than on *B. pseudomallei* (Smith and Cherry 1957). *B. mallei* and *B. pseudomallei* are the causative agents of glanders and melioidosis respectively, and are considered potential agents for bioterrorism. Additionally, *B. pseudomallei* shares insertion sequences with "*B. cepacia*"(Mack and Titball 1998).

1.8.3 *B. cepacia* phage 781 (Bcep781: Accession number- NC_004333)

The first full genome sequence of a *B. cepacia* (genomovar I) phage (Bcep781) has recently been published online (www.ncbi.nlm.nih.gov). Information regarding Bcep781 is scant, however, it was recovered from soil, and was initially propagated on a *B. cepacia* soil isolate. The nucleic acid content is double stranded (ds)DNA and is approximately 48.5 Kbp in size. Bcep781 is classified into the Myoviridae family due to its contractile tail morphology. Two other Bcc phages have been described: Bcep1 (genomovar III soil isolate, NC_005263) and Bcep43 (genomovar I soil isolate, NC_005342). Both have similar genome sizes and are morphologically similar to Bcep781. All three phages are believed to be representatives of a novel family of Bcc bacteriophages.

Further work on the lysogeny and phage host range within the Bcc will be discussed in subsequent sections.

1.8.4 Bacteriophage Therapy

The first clinical studies of the efficacy of bacteriophages as therapeutic agents were performed at the beginning of last century. In 1918, d'Hérelle published his first report describing the involvement of phage in "immunity" to bacillary dysentery (d'Herelle 1918, cited by Stent 1963). d'Hérelle concluded that "the pathogenesis and pathology of dysentery are dominated by two opposing factors: the dysentery bacillus as pathogenic agent and the filterable bacteriophage as agent of immunity".

As a result of this observation, many clinicians began to examine the potential of phage in the treatment of other bacterial infections. Phage therapy was found to be particularly effective in the treatment and control of cholera (Morrison 1932). Following the advent of the antibiotic era in the 1950s, interest in phage therapy declined with the exception of Eastern Europe. More recently, interest in phage therapy has re-emerged as the threat of widespread antibiotic resistance has become a reality (Pirisi 2000).

Bacteriophage therapy has five main advantages over conventional antibiotics: 1. Phages tend to be species specific, and therefore the host commensal flora remains unaffected, 2. Phage replication is localised to the site of infection, 3. No serious side effects have been described, 4. Phage-resistant bacteria remain sensitive to other species-specific bacteriophage and, 5. Novel phages are easily identified. However, there are also disadvantages to phage therapy: 1. The limited host range may impede the treatment of the infection, 2. Phage preparations may contain toxins and bacterial debris that cause severe symptoms in patients and, 3. Phage are rapidly removed from the circulation by the reticuloendothelial system (RES) (Geier *et al.* 1973). In a seminal study, Merril et al. (1996), the authors address each of these disadvantages, and show how phage therapy can be improved (Merril *et al.* 1996). First, the authors examined a range of virulent phages for both *E. coli* and *S. typhimurium* in a murine infection model. Use of virulent phage was important, to prevent phage- infected bacteria surviving as lysogens. Second, they purified the phage preparations on a caesium chloride density-gradient to reduce the levels of endotoxin, and thus adverse effects. Third, they reported a novel technique to increase the capacity of the phage to avoid the RES. After injecting the phage preparation into mice, blood samples were obtained and surviving phage propagated onto a mutator strain of *E. coli* to increase the chance of random mutations in the daughter phage. Interestingly, after the process was repeated several times, a 16,000-fold increase in phage survival was noted in the circulation. Although there are several caveats to this approach, (including the possible presence of phage genes encoding toxins, antibiotic resistance, and lysogeny), the authors concluded that the use of long-circulating phages may have important therapeutic applications.

Two reports by Alisky *et al.* (1998) and Sulakvelidze *et al.* (2001) have systematically reviewed numerous clinical studies carried out over the last 50 years, with particular emphasis on papers published in Russian, Georgian and Polish journals (Alisky *et al.* 1998, Sulakvelidze *et al.* 2001). Of particular interest is the six-part study examining the effectiveness of phages against infections caused by various bacterial pathogens, including multiresistant mutants (Summarised in Slopek *et al.* 1987). In total, 550 cases of suppurative bacterial infections were treated by phage therapy. The bacterial pathogens included *Staphylococci*, *Klebsiella*, *Escherichia*, *Proteus* and *Pseudomonas*, and 94.2% of the cases were resistant to antibiotics. Phage were administered orally, or applied directly to the pleural and peritoneal cavities, or as eye, nose or ear drops. Moreover, phage resistance was monitored and where appropriate the phage pool adjusted. The spectrum of infections studied was widespread, and included the respiratory, gastrointestinal, genitourinary and lymphatic systems. Overall, the authors reported a positive therapeutic result in 92.4% of the cases examined. This extensive analysis of phage therapy in human infections is one of many which recommend the use of virulent phage against multiresistant infections. Further examples include the treatment of urinary tract infections (Perepanova *et al.* 1995), *Staphylococcal* lung infections (Reviewed by Sulakvelidze *et al.* 2001), postoperative skin infections (Cislo *et al.* 1987), neonatal sepsis (Pavelenish and Tsertsvadze 1993) and *P. aeruginosa* infections in CF patients (Shabalova *et al.* 1995). It should be noted that a major caveat in all of these studies is the lack of sufficient control groups.

The use of phage therapy in animal infection models has also been extensively studied. Smith and Huggins compared the efficacy of phage therapy with conventional antibiotics in a mouse model *E. coli* O18:K1 infection (Smith and Huggins 1982). The authors reported that a single intramuscular dose of a single strain of anti-K1 phage was more effective then multiple doses of tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulphamethoxazole (co-trimoxazole). Furthermore, dissemination of bacteria to brain tissue was prevented in phage-treated mice. The authors also attempted to treat diarrhoea caused by an

enteropathogenic strain of *E. coli* O9:K30,99 (B44) in calves, piglets and lambs (Smith and Huggins 1983). In contrast to the previous study, a combination of two phages were used, and were only successful if administered prior to the onset of diarrhoea. However, although post-onset administration of phage did not affect lethality, inoculation of healthy calves with a faecal sample from phage-treated calves did not result in pathology, suggesting that virulence was attenuated in the passaged strain. Smith *et al.* carried out further studies into the control of diarrhoea in calves caused by virulent enteropathogenic *E. coli* and concluded that symptoms could be prevented with a single dose of 10,000 phage particles or by prior phage-treatment of feed (Smith *et al.* 1987a, Smith *et al.* 1987b). In view of the apparent success of these studies, further work was undertaken into the efficacy of phage in the treatment of other animal infections. The successful treatment of *P. aeruginosa* and *Acinetobacter baumannii* was achieved when bacteria and phage were administered simultaneously (Soothill 1992). Later, Soothill described the prevention of skin graft destruction by *P. aeruginosa* in a guinea pig model (Soothill 1994). The author concluded, that the prophylactic topical application of phage in burns patients might prevent *P. aeruginosa* infection.

Phages can also be used as biocontrol agents. Bacterial infection caused by the fish pathogen *Pseudomonas plecoglossicida* in *Plecoglossus altivelis* (Nishimori *et al.* 2000), a fresh water fish, was controlled by the oral administration of phage-impregnated feed (Park *et al.* 2000). The bacterial population in fresh water was also lower in the presence of phage. Studies have also highlighted the potential of phages in the reduction of *E. coli* growing as biofilms polyvinylchloride (Doolittle *et al.* 1995). The authors showed that phage could penetrate a biofilm through a phage-encoded polysaccharide depolymerase specific for the exopolysaccharide produced by the host bacterium (Hughes *et al.* 1998b, Hughes *et al.* 1998a). Hanlon *et al.* also demonstrated that the molecular weight and viscosity of phage-treated *P. aeruginosa* alginate was reduced up to 40% as a result of phage-mediated enzymatic degradation (Hanlon *et al.* 2001).

1.8.5 Bacteriophage Lytic Enzymes as Antibacterial Agents

A new twist to phage therapy removes the problems of using intact phage particles by exploiting the phage-encoded enzymes used to lyse and exit the bacterial cell. These lytic enzymes, produced during the phage lytic cycle, enable phage progeny to escape from the host cytoplasm. In general, large lytic phages (DNA genome greater than 20 Kbp) encode at least two proteins involved in host cell lysis: a holin and an endolysin. This tightly regulated two-component lysis system involves holin accumulation and oligomerization in the bacterial cell membrane and endolysin accumulation in the cytoplasm. At a genetically programmed point in the phage life cycle, the holin oligomers form lesions in the inner cell membrane allowing the endolysin to access and disrupt the peptidoglycan cell wall (Bernhardt *et al.* 2002, Young *et al.* 2000). The substrate for phage endolysin is the bacterial murein layer. In general, phage endolysins have a two-domain structure (Diaz *et al.* 1990, Garcia *et al.* 1990), and exist as soluble proteins with the N-terminal domain containing the catalytic activity of the enzyme. This catalytic activity is directed against the three types of covalent bonds (glycosidic, amide and peptide) of the peptidoglycan polymer of bacterial cell walls (Loessner *et al.* 1999, Navarre *et al.* 1999, Young 1992). The activity may be an endo-β-*N*-acetylglucosaminidase or an *N*-acetylmuramidase (lysozymes), acting on the sugar moiety; an endopeptidase that acts on the peptide cross-linking bridge; or an *N*-acetylmuramyl-L-alanine amidase (amidase) which hydrolyses the amide bond between the sugar and peptide moieties (Reviewed by Fischetti 2003). The C-terminal domain is specific for the cell wall (Garcia *et al.* 1988, Lopez *et al.* 1997, Lopez *et al.* 1992). The endolysins of *Streptococcal* phages have been examined and characterised in some detail (Garcia *et al.* 1987, Garcia *et al.* 1984). The specificity of the lytic enzyme to its substrate is such that without the cell wall binding domain the catalytic domain will not cleave. Resistance to phage lysins has rarely been observed. The specific binding site of the CP-1 endolysin, from the pneumococcal phage CP-1, is choline, a molecule essential for bacterial viability (Garcia *et al.* 1988, Garcia *et al.* 1983, Hermoso *et al.* 2003). This may explain why endolysin resistance is rare since the intact endolysin binding sites are essential for bacterial viability.

The two-domain structure appears to be essential for endolysin activity. Morita *et al.* used mutant peptides to demonstrate that the loss of antibacterial activity corresponded to the loss of the C-terminus (Morita *et al.* 2001). Interestingly, this study also demonstrated the decrease in viability of *P. aeruginosa* on the addition of the endolysin. In a subsequent study, other mutant peptides based on a phage endolysin from *Bacillus amyloliquefaciens* were found to increase the permeability of the outer membrane of *P. aeruginosa* (Orito *et al.* 2004).

The antimicrobial activity of phage lysins resembles a process termed "lysis from without". Historically, this term relates to the phenomenon of immediate lysis of the host bacteria at high phage multiplicity, but even then the presence of lytic enzymes was thought to play a vital role (Stent 1963).

Recently, a seminal study by Nelson and co-workers described the prophylactic use of the C_1 phage lysin, from a *Streptococcal* phage, to control and prevent Group A *Streptococcal* pharyngitis (Nelson *et al.* 2001). C_1 phage lysin is a murein hydrolase (*N*-acetylmuramoyl-L-amidase). The authors also reported that the lysin activity was specific to Group A, C and E *Streptococci*, whilst other commensal *Streptococci* were unaffected. The efficacy of the lysin was tested *in vitro* and an *in vivo* mouse infection model. *In vitro*, the authors demonstrate that as little as 10 nanograms (ng) of lysin can sterilise a culture containing approximately 10^7 Group A *Streptococci* within five seconds. The *in vivo* study highlighted the potential use of the lysin as both a prophylactic treatment to prevent *Streptococcal* colonisation and as a treatment for an active infection.

Other studies have focussed on other *Streptococcal* phage lytic enzymes. Loeffler and colleagues described a pneumococcal phage-encoded 34 kDa amidase, termed Pal, that can specifically digest the pneumococcal cell wall within seconds, resulting in rapid cell death (Loeffler *et al.* 2001). In a follow up study, Loeffler and Fischetti successfully used a synergistic combination of lytic enzymes (Cpl-1 & Pal) to kill penicillin-sensitive and -resistant pneumococci (Loeffler and Fischetti 2003). A recent report has confirmed the *in vivo* antimicrobial activity of Cpl-1 against pneumococci. Cpl-1, a muramidase (Garcia *et al.* 1987), was found to be an

effective agent in controlling bacteraemia in a mouse model (Loeffler *et al.* 2003). This result was confirmed by a subsequent study that also found that a combination of phage enzymes (Cpl-1 and Pal) could successfully control antibiotic-resistant pneumococcal bacteremia in a murine sepsis model (Jado *et al.* 2003). The crystal structure of Cp-1 has recently been published and should aid future studies on the interaction of this enzyme with its substrate (Monterroso *et al.* 2002).

In addition to the growing concerns of multiresistant pathogens, the urgency to develop novel antimicrobial agents was intensified after the terrorist attacks on the USA on September the 11th 2001. In a remarkable and timely report, Schuch and co-workers identified a lysin, PlyG, isolated from the γ phage of *Bacillus anthracis* (Schuch *et al.* 2002). Protein analysis suggested that this enzyme was homologous to *N*-acetylmuramoyl-L-alanine amidases. Homology was noted in the N-terminal, or catalytic domain, but absent in the cell wall specific C-terminal binding domain. Interestingly, the lysin was active against *B. anthracis* isolates and other members of the *B. anthracis* "cluster" of bacilli. The bactericidal activity of PlyG was demonstrated *in vivo* and *in vitro*, and extended to both vegetative cells and germinating spores. Furthermore, the ability of PlyG to kill germinating spores was exploited to develop a rapid and specific identification system for *B. anthracis*, or as it is euphemistically called "the white powder". The specificity of phage lysins for their bacterial host was further emphasised by Zimmer et al (2002) who described a murein hydrolase, from bacteriophage ϕ3626, which specifically killed *Clostridium perfringens* isolates (Zimmer *et al.* 2002).

Finally, a novel study investigated the potential use of a bacteriolytic protein from phage P4282, against wilt disease in tobacco, caused by *Ralstonia solanacearum* (Ozawa *et al.* 2001). The authors cloned and overexpressed the lytic gene in *E. coli*, and observed specific killing of *R. solanacearum*. Interestingly, the authors concluded that the production of transgenic plants containing the bacteriolytic gene of phage P4282 might enhance resistance to bacterial wilt in nature.

1.9 Antimicrobial Peptides (AMPs)

Despite continuous exposure to potential pathogenic organisms, humans remain relatively free from infection. This apparent paradox can, in part, be explained by a highly efficient acquired immune system. Mediated by cellular responses, acquired immunity requires highly specific antigen recognition to initiate both humoral and cellular arms. However, the initial acquired response may take from several days to a few weeks to reach maximum efficiency. The outcome may be immunological memory, but the infection must be contained to ensure survival of the host.
The first line of defence against potential pathogens is the innate immune system, which is inducible, and whose role is to recognise and eliminate microbial challenges via effector molecules. The innate system is not autonomous and signalling molecules allow communication with the acquired immune system. Effector molecules in innate immunity include: reactive oxygen species, nutrient limiting molecules, proteases and antimicrobial peptides (AMPs).

AMPs are part of an ancient group of highly conserved peptides that are found across a diverse range of organisms including unicellular prokaryotes, plants, insects, amphibians and mammals. These defence molecules form part of a multifaceted defensive system that is under intensive selective evolutionary pressure.

AMPs are gene-encoded and are initially expressed as a pre-propeptide, containing a N-terminal endoplasmic reticulum targeting sequence (signal pre-sequence), the adjacent precursor sequence (propeptide sequence) and the mature peptide at the C-terminus. The mature peptide is proteolytically cleaved and generally has broad-spectrum antimicrobial activity. Structurally, AMPs are generally amphipathic and cationic, but anionic peptides have been described. AMP expression can be both constitutive and inducible.
The classification of AMPs can be based on chemical structure, anatomical site of expression or gene family. Mammalian AMPs are generally characterised by the presence of intramolecular disulfide bonds. Protegrins, identified from porcine neutrophils, contain two intramolecular disulfide bonds (Harwig *et al.* 1995). Bovine dodecapeptide, purified from bovine neutrophils, has a single disulfide bond (Romeo

et al. 1988). Defensins, the most studied mammalian AMP, generally contain three disulfide bonds. At present, three groups of mammalian defensins have been observed: alpha (α), beta (β), and theta (θ). The classification of defensins is generally based on the position of the cysteine residues and the connectivity of the disulfide pairings (Fig 1.7). In contrast, classification can also be based on the amino acid composition, such as: proline-rich bactenecins, from bovine leukocytes (Frank *et al.* 1990); histidine-rich histatins, from human parotid secretions (Oppenheim *et al.* 1988); proline-rich prophenin, from porcine leukocytes (Harwig *et al.* 1995); proline-rich AMPs, from ovine and caprine leukocytes (Shamova *et al.* 1999); and tryptophan-rich indolicidin, from bovine neutrophils (Selsted *et al.* 1992). Other examples of AMPs include: the linear cathelicidin LL-37, characterised by an alpha (α)-helical structure, isolated from human neutrophils and epithelial cells (Gudmundsson *et al.* 1996), and a number of anionic peptides (Brogden *et al.* 1997, Brogden *et al.* 1996).

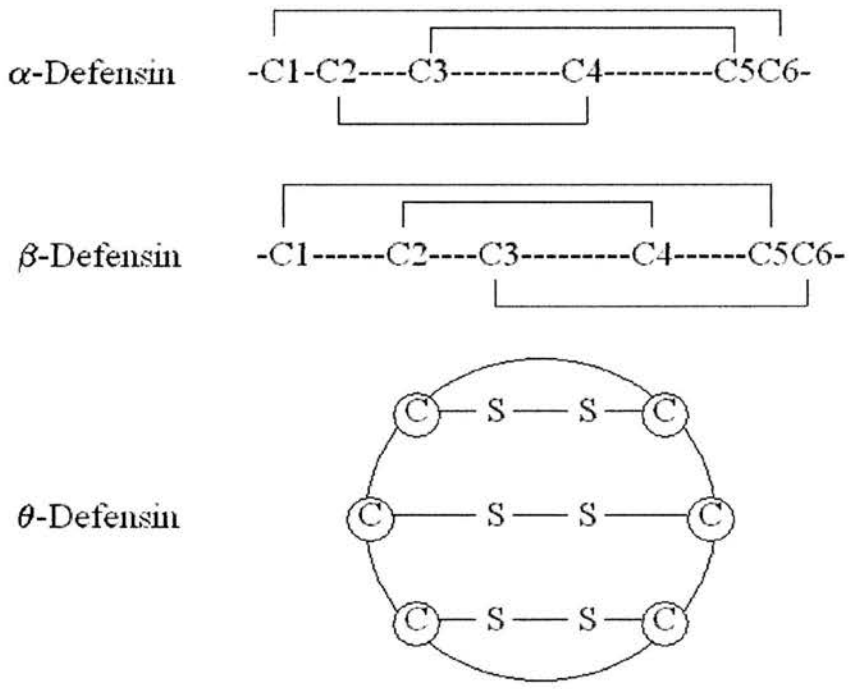


Fig 1.7 The disulfide bridging connectivity in α, β, and θ-defensins: α-defensins are characterised by C1-C6, C2-C4 and C3-C5 disulfide bridging (Selsted and Harwig 1989). β-defensins are characterised by C1-C5, C2-C4 and C3-C6 disulfide connectivity (Tang and Selsted 1993). θ-defensins are characterised by an amide-linked backbone that forms a cyclic structure with three disulfide bonds (Tang *et al.* 1999).

Grouping AMPs according to gene family provides a more simplistic approach than classification based on structural relationship or disulfide bond connectivity. In this respect, two families of AMPs are recognised, the defensins and the cathelicidins. For the purpose of this thesis, attention will be focussed on defensins.

1.9.1 Defensins

Defensins are a family of small, cationic antimicrobial peptides identified in both mammals and plants. To date, the defensin family has been subdivided into three main classes (α, β, and θ) on the basis of structural characteristics. The role of θ-defensins will not be discussed. In humans, genes encoding α- and β-defensins are clustered on chromosome 8p23 (Linzmeier *et al.* 1999, Sparkes *et al.* 1989). The taxonomic nomenclature of defensins is complex: α-defensins can be referred to with the prefix DEFA or HNP or HD; β-defensins can be referred to with the prefix DEFB or hBD, and murine β-defensins are designated by the prefix defb. For recent and extensive reviews of defensins see Ganz (2003) & Schutte and McCray (2002).

1.9.2 Alpha (α)-Defensins

α-Defensins are 29-35 amino acids in length, and are characterised by six highly-conserved cysteine residues that form three disulfide bonds. In humans, α-defensins are expressed in neutrophils (HNP-1-4: DEFA1, DEFA2, DEFA3, DEFA4) (Lehrer *et al.* 1993), Paneth cells (HD-5 and –6: DEFA5/6) (Jones and Bevins 1993, Jones and Bevins 1992) and other epithelia including the epithelium of the vagina, endocervix and fallopian tubes (DEFA5, DEFA6) (Quayle *et al.* 1998, Schutte and McCray 2002). In neutrophils, α-defensins make up to 50% of the protein content of the azurophilic granules. During phagocytosis, α-defensins are released into the phagolysosome where they exert antimicrobial effects. In Paneth cells, DEFA5 and DEFA6 are present in cytoplasmic granules (Mallow *et al.* 1996).
A recent seminal study by Salzman *et al.* has described the potent *in vivo* activity of HD-5 (DEFA5) in protecting mice from oral administration of virulent *Salmonella typhimurium* (Salzman *et al.* 2003). Using transgenic mice expressing HD-5, the

authors demonstrated that this defensin protected animals from an infection that was fatal for wild-type mice. Protection was achieved quickly upon administration of bacteria, and lower counts of *S. typhimurium* were observed in the intestinal lumen, with less dissemination to other organs. Interestingly, protection was only observed if the dose of *S. typhimurium* was given orally, not intraperitoneally, supporting the hypothesis that defensins act as "locally secreted antibiotics".

1.9.3 Beta (β) Defensins

The first β-defensin to be identified was tracheal antimicrobial peptide (TAP) isolated from bovine trachea (Diamond *et al.* 1991). A subsequent study characterised the primary structure and the antimicrobial activities of thirteen novel bovine antimicrobial peptides that were also classified as β-defensins (Selsted *et al.* 1993). These peptides shared high sequence homology with TAP, and were structurally different from α-defensins. β-defensins are of similar size to α-defensins, and contain six highly conserved cysteine residues. However, β-defensins differ from α-defensins in the pattern of pairing of their three disulfide bonds. To date, four human β-defensins have been characterised. The first human β-defensin was identified in human plasma (Bensch *et al.* 1995). Human β-defensin-1 (hBD-1) is a cationic antimicrobial peptide, which is expressed constitutively in: salivary glands and epithelial cells (Sahasrebudhe *et al.* 2000, Zhao *et al.* 1996), gingiva (Krishnaprakornkit *et al.* 1998), buccal mucosal (Mathews *et al.* 1999), respiratory epithelia (Goldman *et al.* 1997, McCray and Bentley 1997) and urogenital tissues (Valore *et al.* 1998). Human β-defensin-2 (hBD-2) was isolated from psoriatic scales of human skin and in the lung (Bals *et al.* 1998, Harder *et al.* 1997a, Harder *et al.* 1997b). Human β-defensin-3 (hBD-3) was initially isolated from human lesional psoriatic scales, but also shown to be expressed in keratinocytes and respiratory epithelial cells (Harder *et al.* 2001). Unlike the other studies, identification of human β-defensin-4 (hBD-4) was based on genomic sequence alignments (Garcia *et al.* 2001). In contrast to the constitutive expression of hBD-1, hBD2 and hBD3 are inducible in response to pro-inflammatory stimuli, such as LPS, TNF-α and IL-1β (Bals *et al.* 1998, Harder *et al.* 2001, Harder *et al.* 1997a, Singh *et al.* 1998). Within

the promoter regions of both hBD-2 and hBD–3 there are binding sites for NF-κB, an important inflammatory mediator (Tsutsumi-Ishii and Nagaoka 2002). Conversely, hBD-4 does not contain such binding sites, and is unaffected by inflammatory stimuli (King *et al.* 2003). It is, however, inducible upon stimulation by bacterial factors, including heat inactivated *P. aeruginosa* (Garcia *et al.* 2001). In a recent report, activation of Toll-like receptor 2 (TLR2) was shown to induce the expression of hBD-2 (Hertz *et al.* 2003).

To date, the antimicrobial activity of all human β-defensins extends to both Gram-positive and Gram–negative bacteria. Human β-defensin-1 has been shown to kill *E. coli*, *P. aeruginosa* and *Listeria monocytogenes* (Goldman *et al.* 1997, Singh *et al.* 1998, Valore *et al.* 1998). Interestingly, hBD-1 can be secreted in five different forms (Valore *et al.* 1998), with three forms existing in the respiratory tract (Singh *et al.* 1998). Human β-defensin-2 has bactericidal against *P. aeruginosa* (Singh *et al.* 1998), *E. coli* (Bals *et al.* 1998) and *C. albicans* (Harder *et al.* 1997a), but has lower activity against Gram-positive bacteria, such as *S. aureus* (Harder *et al.* 1997a). The bactericidal activity of hBD-3 extends to *S. aureus*, vancomycin-resistant *E. faecium*, *P. aeruginosa*, *C. albicans* and *Streptococcus pyogenes* (Harder *et al.* 2001). An early report claimed that hBD-3 was active against "*B. cepacia*" (Garcia *et al.* 2001); significantly, this unusual killing activity was contradicted in a later study (Sahly *et al.* 2003). The killing ability of hBD-4 appears to be limited to *Staphylococcus carnosus* and *P. aeruginosa* (Garcia *et al.* 2001).
In general, the microbicidal activity of hBD-1 and hBD–2 is directed against Gram-negative bacteria, whereas hBD-3 has a broader spectrum of activity. It has been suggested that this broad spectrum of bactericidal activity may result from a greater density of cationic residues in hBD-3 compared to hBD-1 and hBD–2 (Harder *et al.* 2001).

The antimicrobial action of hBD-1, -2 and -4 is affected by the concentration of salt (NaCl) (Bals *et al.* 1998, Garcia *et al.* 2001, Goldman *et al.* 1997), and in the case of hBD-1 appears to be dependent on the secreted form (Valore *et al.* 1998). hBD-3 is

less salt sensitive in its activity against *S. aureus*, and high salt concentrations are needed to reduce the antimicrobial activity (Harder *et al.* 2001).

In addition to human-β defensins, mouse homologs have also been described with antimicrobial activity against *E. coli*, *P. aeruginosa*, and *S. aureus* (Bals *et al.* 1998, Morrison *et al.* 1999, Morrison *et al.* 1998, Yamaguchi *et al.* 2001). Moreover, a novel five cysteine defensin, termed Defr1, has been identified and characterised (Morrison *et al.* 2002). Antimicrobial activity of Defr1 extends to *S. aureus*, *E. coli*, *P. aeruginosa* and J2315, the type strain of *B. cenocepacia* (Vandamme *et al.* 2003). Activity against this highly-resistant isolate (Nzula *et al.* 2002) is of special interest since previous reports indicate that "*B. cepacia*" are inherently resistant to AMPs (Hancock and Chapple 1999).

1.9.4 Mechanism of Killing by Defensins

Interaction between host membranes and the defensin molecules is thought to be derived from the amphipathic nature of the latter; the positively-charged amino acids of the defensin molecule interact with the negatively-charged phospholipid residues of the host membrane. As a result of electrostatic interactions, the defensin molecules can be drawn towards the membrane and accumulate as a "carpet" on the membrane surface. At a critical point, the defensin carpet aggregates and inserts into the membrane, forming a pore and reducing membrane integrity. α-defensins are known to permeabilise sequentially the outer and inner membranes of *E. coli* (Lehrer *et al.* 1989), although the exact nature of pore formation is still the subject of debate (Ganz and Lehrer 1999, Hoover *et al.* 2000, Sawai *et al.* 2001).

1.9.5 Defensins: Alternative Functions

Human α-defensins play an important role as innate defence molecules but can also interact with the acquired immune system. Evidence indicates that α-defensins stimulate the release of cytokines from airway epithelial cells (Van Wetering *et al.*

1997) and from T cells (Lillard *et al.* 1999), and act as chemoattractants for immature dendritic and naïve T cells (Yang *et al.* 2000), monocytes (Territo *et al.* 1989) and for granulocytes and lymphocytes (Welling *et al.* 1998).

Human β-defensins have also been shown to be chemoattractants for monocytes (Garcia *et al.* 2001), T cells and immature dendritic cells (Yang *et al.* 1999), suggesting a link between innate and adaptive immunity. The latter study postulated that the chemoattractant activity may be due to defensin binding to the chemokine receptor CCR6 (Yang *et al.* 1999). Murine-β-defensin-2 has been shown to activate immature dendritic cells via Toll-like receptor 4 (Biragyn *et al.* 2002). The authors found that such binding results in the upregulation of costimulatory molecules and dendritic cell maturation.

Defensins have also been shown to be active against some enveloped viruses (Daher *et al.* 1986, Lehrer *et al.* 1985). In addition, two recent studies have highlighted the importance of defensins during HIV-1 infection. Zhang *et al.* observed that human α-defensins 1, 2 and 3 are amongst the molecules involved in the antiviral activity secreted by $CD8^+$ T cells of HIV-non-progressors (Zhang *et al.* 2002). Moreover, a recent report has described the induction of β-defensin expression in human oral epithelial cells by HIV-1 (Quinones-Mateu *et al.* 2003). In addition, the authors observed that HIV-1 replication was inhibited by hBD-2 and –3 through direct interaction with virions and via modulation of the chemokine receptor CXCR4. Some defensins can also bind glycoproteins, which may explain their antiviral activity (Wang *et al.* 2003).

1.10 Aims

The aims of this study were to identify and investigate novel antimicrobial agents whose activity extended to the *Burkholderia cepacia* complex and other multiresistant CF pathogens. The project focused on three major themes: 1. The antimicrobial activity of New Zealand manuka honey, 2. Novel Bcc bacteriophages and their lytic enzymes and, 3. Novel antimicrobial peptides, in particular, antimicrobial activity of a novel synthetic defensin, Defr1.

2.1 Materials

2.1.1 Bacterial Isolates

All bacterial isolates were provided by Prof. John Govan from the Edinburgh Cystic Fibrosis Microbiology Laboratory and Strain Repository (ECFML), and by Prof. Peter Vandamme of the Belgium National Repository, Gent. Bcc strains used are listed (Table 2.1). Other bacterial strains used are listed (Table 2.2). Where appropriate and possible, further information regarding each isolate is provided.

Table 2.1 *B. cepacia* complex isolates used.

Species	Strain	Gent Lab No.	Description
B. cepacia	ATCC 25416	LMG 1222	Onion isolate; type strain
	C2970	LMG 17997	Urine; Gent
	C3159	LMG 18821	CEP 509; CF; Australia
	ATCC 17759	LMG 2161	Soil; Trinidad
B. multivorans	C2775		Sputum (CF); Aberdeen
	C3161	LMG 13010	CF; Belgium
	C3162	LMG 18825	CF-A1-1; CF-e UK
	C3163	LMG 18824	CGD; USA
	C3164	LMG 18823	249-2, LAB USA
	ATCC 17616	LMG 17588	Soil; Canada
	C1962	LMG 16665	Cerebral abscess; Aberdeen
	C1576	LMG 16660	Sputum (CF); Glasgow; CF-e
	C3160	LMG 18822	C5393; CF; Canada
B. cenocepacia	J415	LMG 16654	Sputum (CF); Edinburgh;
	J2315	LMG 16656	Sputum (CF); Edin; ET12; index case
	C1394	LMG 16659	Sputum (CF-e); Manchester
	J2956		Sheep Mastitis; Spain
	C2836		BC 226 Miss (CF-e); Mississippi
	C517		CF, Edinburgh, UK
	C3165	LMG 18826	BC7; CF-e; Canada
	C3166	LMG 18863	K56-2; CF-e; Canada; ET12
	C3167	LMG 18827	C5424; CF-e; Canada
	C3168	LMG 18828	C6433; CF-e; Canada
	C3169	LMG 18829	PC184; CF-e; USA
	C3170	LMG 18830	CEP511; CF-e; Australia
	ATCC 17765	LMG 18832	Urinary-tract infection
	C1632		Sputum (CF); Newcastle, CF-e
	C1773		Blood Culture; Papworth
	C1511		Sputum (CF); Manchester
	R2817	J2967	(Balandreau *et al.* 2001)
	R2827	J2970	"

B. cenocepacia	R8351		"
	R8571	J2969	"
	R8574	J2964	"
	R8580	J2971	"
	R8605	J2972	"
	R9235	J2968	"
	R9239	J2965	"
	R9243	J2966	"
	R9342		"
B. stabilis	C3171	LMG 14294	CF; Belgium
	C3172	LMG 18870	C7322; CF; Canada
	C3173	LMG 18888	Clinical; Belgium
	C3174	LMG 14086	Respirator; UK
B. vietnamiensis	C2978	LMG 16232	CF isolate; sputum
	C3175	LMG 18835	PC259; CF USA
	C3176	LMG 18836	FC441; CGD Canada
	C3177	LMG 10929	Rice; Vietnam
	J2962		Sheep Mastitis, Spain
B. dolosa	E12		
B. ambifaria	J2742	LMG 19182	Biological control; AMMD; pea rhizosphere
B anthina	J2552	LMG 16670	Soil; Royal botanic gardens; Edin
	J2553		RBG; Samseveira leaf; Edin
	J2863	LMG 20980	Soil; Rhizosphere; Nashville
	J2927		Soil; Dundee
	J2928		Soil; Dundee
	J2941		Rhizosphere; Dundee
	J2943		Rhizosphere; Dundee
	J2944		Rhizosphere; Dundee
	J2945		Rhizosphere; Dundee
	J2946		Rhizosphere; Edin
	J2949		Rhizosphere; Dundee
	J2950		Rhizosphere; Dundee
	J2951		Rhizosphere; Edin
	J2862		Soil; Rhizosphere; Vine plant
	C1658	LMG 20982	Environmental; Manchester hospital
	C1765	LMG 20983	Sputum (CF); Blackpool
B. pyrrocinnia	J2536		Soil; Royal botanic gardens; Edin
	J2542		Soil; Royal botanic gardens; Edin
	C1469		Sputum (CF); Manchester
	C3909		
B. pyrrocinnia	C3918		
	C3928		

B. pyrrocinnia	C3930		
	C3993		
	C3995		
	C3997		
B. ubonensis	E26		
	E27		
	E571		
	E551		
Gnvr Unclassified	J2540	LMG 16672	Soil; Royal botanic gardens; Edin

CF-e: Strains involved in epidemic outbreaks. Edin: Edinburgh.

Table 2.2 Other bacterial strains used

Species	Strain	Description
Pseudomonas aeruginosa	NCTC 10662	Antibiotic Control
	PAO1	Lab Strain
	PAO579/P55	Mucoid: muc-28
	C3425	Manchester; Epidemic
	H183	Liverpool; Epidemic
	C4269	Brisbane; Epidemic
	C3781	Melbourne; Epidemic
	P235	Rough LPS (PAC 605)
	J1385	Jacuzzi Strain
	C3567	
	C3487	
	C3542	
	C1659	Sputum; Manchester
	C1665	Environment; WGH; Edin
	C2903	
	C3063	
	C3781	
	C3801	
	J2673	Environment; R.I; Glas
	J2467	Wound; WGH; Edin
	J2758	Rowett Institute, Aberdeen
	J2795	Blood; R.I; Glas
	J2432	Washer; R.I; Glas
	J2826	Delhi; India
	J2845	Delhi; India
	C3659	
	C3667	
	C3712	
	C3547	
	C3551	
	C3703	
	C3522	
	C3523	
	C3461	
	C3468	
	C3064	

P. aeruginosa	C3067	
	C3037	
	C3038	
Stenotrophomonas maltophilia	C1980	Clinical Isolate
	C3625	Clinical Isolate; WGH; Edin
	C3626	Clinical Isolate
	C3627	Clinical Isolate
	C3642	Clinical Isolate
	J2796	
	J2801	
	J2802	
	JN79	
	J2807	
	J2809	
	C3497	Clinical Isolate
Burkholderia caledonica	J3059	
B. gladioli	J2512	
	C3654	
Comamonas acidovorans	C3641	
P. fluorescens	C3049	
	J2729	
	JN72	
P. mendocina	JN71	
P. stutzeri	JN78	
	C3643	
P. testosteroni	JN76	
P. syringae pv. tabaci	J2391	
Achromobacter xylosoxidans	J2907	
	C3184	
	C3209	
P. putida	JN77	
Ralstonia pickettii	C3079	R4050 CF Isolate
	J2513	
R. eutropha	C3081	LMG 1199 (Type Strain)
Escherichia coli	ATCC 25922	
Enterococcus faecalis	ATCC 29212	
Staphylococcus aureus	ATCC 25923	
MRSA	J2918	S113

Candida albicans	J2922
Bordetella bronchoseptica	J3083
Acinetobacter baumannii	U45

Edin: Edinburgh. Glas: Glasgow, WGH: Western General Hospital, Edinburgh

2.1.2 Media

Unless otherwise stated all media were provided by Oxoid Ltd., prepared according to the manufacturer's instructions using distilled water, and sterilised at 121°C at 15 psi for 15 minutes.

Nutrient Agar (NA): Columbia agar base, 39 g/L.

"*B. cepacia*" isolation agar (CIA): 32.5 g/L (MAST Diagnostics) plus one selectab (MAST) per 100 ml of agar. The selectab is added post-autoclaving once the media has cooled to approximately 50°C. The final antibiotic concentration is 100 μg/ml ticarcillin and 300 units/ml of polymyxin B.

Blood Agar (BA): Columbia agar base plus 5% defibrinated horse blood added post autoclaving, once the media has cooled to approximately 50°C.

Pseudomonas Isolation Agar (PIA): 45 g/L (Difco Laboratories, U.S.A) plus 2% (v/v) glycerol (Sigma-Aldrich, UK) added prior to autoclaving.

Iso-sensitest Agar (ISA): 31.4 g/L.

Iso-sensitest Broth (ISB): 23.4 g/L.

Nutrient Broth Yeast Extract (NBYE): Nutrient broth (No. 2) (25g/L) plus 5% yeast extract (Difco).

Luria-Bertani (LB) Broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl.

LB Agar: 1 x LB broth, 1.5% bacto-agar.

2YT Broth: 1.6% Bacto-tryptone, 1.0% Yeast Extract, 0.5% NaCl, pH 7.5.

SOC Medium: 2% Tryptone, 0.5% Yeast Extract, 0.05% NaCl, pH 7.0.

Soft Overlay Agar: 2.5% (w/v) Nutrient broth (No.2), 0.38% Bacteriological agar.

Tryptone Soy Agar (TSA): 40 g/L.

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

Phosphate Buffered Saline (PBS): One PBS tablet per 100 ml of distilled water.

Saline: 0.85% sodium chloride (w/v) (Sigma).

Bacteriophage Buffer: 10mM Tris/HCl, pH 8.0; 10mM $MgCl_2$.

2.1.3 Chemicals

All chemicals used in the following sections were provided by Sigma-Aldrich, unless otherwise stated. The details of all the companies used are provided in Appendix 1.

2.2 Bacteriological Methods

2.2.1 Storage and recovery of isolates

Bacteria were stored at –70°C in 1 ml of 10% w/v skimmed milk. Post-thawing, Bcc isolates were inoculated onto NA and incubated at 37°C. Isolates were subcultured onto CIA to ensure purity of stored cultures. Similarly, *P. aeruginosa* isolated were initially inoculated onto NA and incubated at 37°C. For identification, *P. aeruginosa* isolates were also subcultured onto PIA to enhance pigmentation production. *S. aureus*, *E. coli* and *Candida* isolates were recovered and subcultured weekly onto BA, grown at 37°C, and stored at 4°C for up to a fortnight. All other isolates used were inoculated onto NA and grown at 37°C.

2.2.2 Standardisation of Bacterial Cultures

Bacteria were inoculated in 10 ml NBYE and incubated for 18 hours at 37°C in an orbital incubator. 1 ml of the overnight bacterial suspension was added to 9 ml of fresh NBYE, incubated at 37°C for a further 4-5 hours, and centrifuged (4,500 x g for 15 minutes). The pellet was resuspended in an appropriate buffer; the optical density was measured (λ=590 nm) and adjusted to 1. The approximate number of bacteria in the culture was 1 x 10^8 colony-forming units (cfu)/ ml.

2.2.3 Antibiotic Sensitivity Testing: Agar Dilution Method

Stock solutions of antibiotics were prepared and stored at concentrations of 1 mg/ml, 100 μg/ml, 10 μg/ml and 1 μg/ml, according to the manufacturer's instructions. The stock solutions were then added to 20 ml of ISA (cooled to 60°C) to give final concentrations of doubling dilutions from 0.06 μg/ml to 512 μg/ml. The antibiotics were mixed gently, and plates were flamed to ensure sterility. A control plate containing no antibiotic was also prepared.

Bacteria to be investigated were inoculated into 10 ml of ISB and incubated overnight. The bacteria were then standardised as described (section 2.2.2) to give approximately 10^8 cfu/ml. After diluting the bacteria to approximately 10^7 cfu/ml in saline, 1 μl of the suspension was inoculated onto the pre-dried antibiotic agar plates by a multipoint inoculator (Denley, Billingshurst, Sussex), to give a final inoculum of approximately 10^4 cfu. The plates were incubated overnight at 37°C before the MIC was determined. Some species required an additional 18 hours at room temperature before the MIC could be verified. The MIC was defined as the lowest concentration at which there was no visible growth. Resistance was defined as an MIC greater than the BSAC breakpoints for *Pseudomonas* species (MacGowan and Wise 2001, Nzula *et al.* 2002).

2.2.4 Antibiotic Sensitivity Testing: Broth Dilution Method

Bacteria and antibiotic stock solution were prepared as described for the agar dilution method. Antibiotic stocks were added to 10 ml of ISB to a final concentration of doubling dilutions between 512 μg/ml and 0.06 μg/ml. 100 μl of the bacterial suspension was inoculated into broth/antibiotic solution to give a final inoculum of 10^5 cfu/ml. Tubes were incubated overnight at 37°C in an orbital incubator, prior to the determination of the MIC. The definition of the MIC was as described previously.

2.2.5 Production of Catalase

Bacteria were grown overnight at 37°C on NA. A drop of 3% H_2O_2 was applied to a colony and catalase production was assessed semi-quantitatively by production of bubbles (+/+++).

2.3 Molecular Cloning

Cloning vectors are outlined in Appendix 3.

2.3.1 DNA Digestion with Restriction Endonucleases

Restriction endonucleases (Invitrogen or New England BioLabs) were used in conjunction with the manufacturers buffers. Digests were normally prepared by 3 hours treatment in a 25-50µl reaction at a temperature suggested by the manufacturer (usually 37°C).

2.3.2 Polymerase Chain Reaction (PCR)

Standard PCR reactions were typically performed in a 50 µl final volume using 2 x Ready To Go PCR™ beads (Amersham Biosciences, UK), 1 µl of template DNA, 5 µl of each primer, and 39 µl of dH_2O. The final concentration of $MgCl_2$ in a 50 µl reaction was 3.0 mM. All PCR primers were purchased from Sigma-Genosys (Appendix 2), and kept at –20°C in 10 µM stocks. Reactions were overlayed with mineral oil, and unless otherwise stated all PCR and sequencing reactions were performed in a Perkin Elmer 480 thermal cycler.

2.3.3 Agarose Gel Electrophoresis

DNA was electrophoresed on gels consisting of 1-1.5% agarose. Gels were cast with 1 x TAE buffer containing 0.5 µg/ml (w/v) ethidium bromide. DNA samples were mixed with 10 x loading dye, vortexed and loaded onto the gel. Electrophoresis was

performed at 70 volts in horizontal electrophoresis apparatus containing TAE buffer. DNA fragments sizes were estimated by comparison to DNA size markers (HyperMarkers I & IV, Bioline). Following electrophoresis agarose gels were visualised with a short wave UV transilluminator.

2.3.4 Agarose Gel Extraction of DNA Fragments with a QIAquick Gel Extraction Kit (QIAGEN)

DNA fragments were extracted according to the manufacturer's instructions. In brief, DNA fragments were marked in the gel using a sharp scalpel blade under UV-light, and fully excised under normal room lighting. Depending on the weight of the fragment (three volumes of buffer QG to one volume of gel fragment), approximately 250 µl of buffer QG was added and, ensuring that the fragment was fully immersed, incubated at 50°C for 10 minutes. During the incubation period the sample was frequently vortexed to aid dissolving. Once the fragment had fully dissolved, one gel volume of isopropanol was added and mixed thoroughly by inverting. The mixture was then added to a QIAquick spin column, and centrifuged at 15,000 x g for 1 minute. The flow through was discarded and the column-bound DNA was washed (and centrifuged at 15, 000 x g for 1 minute) once with 500 µl of buffer QG and once with 750 µl of buffer PE. Prior to the final elution step, the column was centrifuged at 15,000 x g to ensure complete removal of buffer PE. 25 µl of sterile distilled water was added to the column, and incubated at room temperature for 1 minute, before centrifugation at 15,000 x g for 1 minute to elute the DNA. DNA samples were stored at –20°C.

2.3.5 DNA Fragment Ligation

Ligations were performed such that the insert to vector ratio was typically 7:1. Reactions contained 10% ligase buffer (New England Biolabs, UK), 5% T4 DNA Ligase (New England Biolabs, UK), and dH_2O to a final volume of 20 µl. Ligation reactions were incubated overnight at room temperature (16-23°C). Ligation

reactions always incorporated negative controls, including a vector minus insert control, to determine the contribution of plasmid re-ligation to transformation efficiency.

2.3.6 Purification of Plasmid DNA with a QIAprep Spin Miniprep Kit (QIAGEN)

Plasmid DNA was purified according to the manufacturer's instructions. In brief, a single transformant was inoculated into 10 ml of 2YT broth containing the appropriate antibiotic (100 μg/ml of ampicillin or 50 μg/ml of kanamycin), and incubated overnight at 37°C in an orbital incubator. The bacterial suspension was centrifuged at 1,500 x g for 10 minutes, the pellet resuspended in 250 μl of buffer P1 and transferred to a microfuge tube. 250 μl of buffer P2 was added and mixed thoroughly by inverting several times. 350 μl of buffer P3 was added and mixed carefully, before the mixture was centrifuged at 15,000 xg for 15 minutes. The supernatant was applied to a QIAprep spin column, and plasmid DNA was bound by centrifugation at 15,000 x g for 1 minute. The DNA was washed by addition of 500 μl of buffer PB (15,000 x g for 1 minute) and 750 μl of buffer PE. After an additional centrifugation step (15,000 x g for 1 minute), plasmid DNA was eluted by the addition of 50 μl of sterile distilled water. Plasmid DNA was stored at –20°C.

2.3.7 Preparation of Glycerol Stocks of Transformants

Glycerol stocks were prepared by the addition of 200 μl of sterile 60% glycerol to 800 μl of an overnight bacterial culture. After vortexing, the aliquot was stored at -70°C. Recovery of the culture was achieved by spreading a loopful of the suspension onto LB-agar containing the appropriate antibiotic and incubating overnight at 37°C.

2.3.8 Automated DNA Sequencing

Automated DNA sequencing was carried out on an ABI prism 377 DNA sequencer, using the Sanger dideoxy chain termination method. Sequencing reactions were performed in 0.5 ml PCR tubes with 2 µl of template DNA, 1 µl of primer, 13 µl of dH_2O, and 4 µl of BigDye™ V. 3.0 (PE Biosystems, UK). Reactions were overlaid with mineral oil and transferred to a thermal cycler; 95°C for 30 seconds, 54°C for 15 seconds and 72°C for 4 minutes, repeated for 30 cycles.

2.3.9 Cloning of PCR Products

PCR products containing restriction endonuclease sites at their 5' end were digested with the appropriate restriction endonuclease prior to cloning into the desired vector. However, if no such sites were present, PCR products were cloned using a PCR 2.1-TOPO TA cloning kit, (Invitrogen) (Appendix 3). Cloning reactions contained a 1µl aliquot of the PCR 2.1 vector, 1µl of 200 mM NaCl and 3µl of PCR product. The mixture was mixed and incubated at room temperature for 30 minutes prior to transformation of One Shot TOP10 chemically competent *E. coli* (Invitrogen, UK).

2.3.10 Transformation of One Shot TOP10 Chemically Competent *E. coli*

One Shot chemically competent cells (Invitrogen, UK) were transformed according to the manufacturer's instructions. Briefly, a 25 µl aliquot of competent cells was thawed on ice, prior to the addition of 2 µl of the TOPO cloning reaction. The sample was incubated on ice for 30 minutes, before heat shocking for 30 seconds at 42°C. The sample was then immediately incubated on ice for 2 minutes, after which 250 µl of SOC medium was added. The sample was incubated in an orbital incubator for 1 hour at 37°C, spread onto an LB-ampicillin agar plate, and incubated overnight at 37°C. Colonies were picked and analysed further by miniprep and restriction endonuclease digestion.

2.4 Protein Expression and Purification

2.4.1 Transformation of Chemically Competent *E. coli* (DH5α,HMS174, BL21 (DE3) & BL21 (PlysS) Novagen) with Vector DNA

The chemically competent cells were transformed according to the manufacturer's instructions. Briefly, a 25 µl aliquot of competent cells was thawed on ice, prior to the addition of 2 µl of expression plasmid containing the gene of interest. After incubation on ice for 5 minutes, the sample was placed at 42°C for 30 seconds, and immediately re-incubated on ice for 2 minutes prior to the addition of 250 µl of SOC medium. The sample was incubated in an orbital incubator for 1 hour at 37°C. The mix was spread onto an LB-agar plate containing the appropriate antibiotic and incubated overnight at 37°C. Single colonies were used to inoculate fresh broth to achieve overexpression of the gene.

2.4.2 Protein Over Expression

Overexpression was achieved by transforming chemically competent *E. coli* with the desired expression plasmid containing the gene of choice. A single colony was used to inoculate 500 ml of 2YT broth supplemented with the appropriate antibiotic (ampicillin 100 µg/ml; kanamycin 50 µg/ml) and grown overnight at 37°C, in an orbital incubator (140 revs/min). This seed culture was then used to inoculate 5 litres of 2YT supplemented with the appropriate antibiotic, and grown at 37°C to OD = 1 (λ=600nm) before induction with 10 mM isopropyl thio-β-D-galactoside (IPTG). After a further 3 hours the cells were harvested by centrifugation (4000 x g for 15 minutes at 4°C) and washed in binding buffer (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 5 mM imidazole). The cells were resuspended in binding buffer (5 ml per gram of wet cell paste) and disrupted by sonication (15 pulses of 30 seconds at 30-second intervals) at 4°C. The cell debris was removed by centrifugation at 27, 000 x g for 20 minutes at 4°C, after which the supernatant was filtered through a 0.45 µm membrane (Millipore, USA) prior to chromatography.

2.4.3 Protein Purification

The filtered cell lysate was applied to a Hitrap® chelating affinity column (Amersham Biosciences, UK) previously washed with 5 column volumes of dH_2O, loaded with 3 column volumes of charge buffer (100 mM $NiSO_4$) and equilibrated with binding buffer at room temperature. The column was then washed with 5 column volumes of binding buffer before bound material was eluted using a linear gradient of 0-100% elution buffer (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 1 M imidazole). Fractions were analysed by SDS/PAGE.

2.4.4 Purification of Periplasmic Proteins

Periplasmic proteins were purified by a modification of the method by Berish *et al.* (1992). Briefly, the cell pellet was resuspended in 50 ml of 1 M Tris (pH 8.0). 50 ml of 2% CTAB was prepared and stirred at 37°C until dissolved. After mixing, the solution was slowly stirred overnight at 37°C. The white insoluble material was removed by centrifugation (10,000 x g for 15 minutes at 4°C). The supernatant (cell-free extract) was incubated overnight at 4°C to ensure that all the CTAB had precipitated. After diluting to a final voume of 1 litre, the cell-free extract was filtered using Whatman number 4 paper (Fischer). The cell free extract was applied to a RESOURCE®S cation exchange column (Amersham Biosciences, UK) and connected to a Fast Protein Liquid Chromatography (FPLC) system, washed to the manufacturer's instructions, and equilibrated with 10 mM Tris buffer (pH 8.0). The cell free extract was loaded onto the column at a flow rate of 4 ml/min. Unbound protein was removed by extensive washing with low salt buffer. Proteins were eluted with a linear NaCl gradient of low-to-high salt (0-1 M NaCl over 20 column volumes in 10 mM Tris buffer). Fractions were collected and analysed by SDS-PAGE.

2.4.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

25 µl of each fraction was mixed with 25 µl of SDS Loading buffer, vortexed, and boiled for 10 minutes in a 70°C water bath. 20 µl of each sample was then loaded onto a 12% Nu-PAGE Bis/Tris gel (Invitrogen, UK). A low molecular weight standard was also loaded. The gel was run in vertical electrophoresis apparatus containing Nu-PAGE MES running buffer for 35 minutes at 200 volts, and stained with comassie blue.

2.4.6 Mass Spectrometry

Mass spectrometry was performed on a MicroMass Platform II quadruple mass spectrometer equipped with an electrospray ion source, under the supervision of Mr Nick Tomczyk, School of Chemistry, University of Edinburgh. In brief, the spectrometer cone voltage was ramped from 40 to 70 volts and the source temperature was set to 140°C. Protein samples were separated with a Waters High Performance Liquid Chromatography (HPLC) 2690 with a Phenomenex C5 reverse phase column directly connected to the spectrometer. The proteins were eluted from the column with a 5-95% acetonitrile (containing 0.01% trifluoroacetic acid) gradient at a flow rate of 0.4 ml/min. The total ion count in the range of 500-2000 *m/z* was scanned at 0.1 second intervals. The scans were accumulated and spectra combined and the molecular mass determined by the Maxent and Transform algorithms of the Mass Lynx software (MicroMass).

2.4.7 Sequence Analysis and Bioinformatics

DNA sequence analysis was performed using Vector NTI 6 software (Informax), and BLASTn (http://www.ncbi.nlm.nih.gov:80/BLAST). In addition, protein sequence analysis was performed using Vector NTI 6 software (Informax), BLASTp (http://www.ncbi.nlm.nih.gov:80/BLAST) and www.expasy.com .

2.5 Antimicrobial Activity of Honey

2.5.1 Preparation of Honey Samples

New Zealand manuka honey (NZMh) (Comvita, New Zealand) with an antibacterial equivalent of 4% carbolic acid, an alternative NZMh (with a UMF level of 10), and a traditional Scottish honey (Tweedside) were diluted to a final concentration range of 5-50% (v/v) with dH_2O. Samples were autoclaved and centrifuged at 3200 x g for 20 mins to remove debris. The supernatant was then passed through a 0.2 μm membrane (Millipore). A sample of each solution was examined for potential anaerobic organisms by inoculating an egg yolk agar plate (Oxoid), which had been pre-reduced for 24 hours, and incubated anaerobically for 24-48 hours at 37°C. An artificial honey solution (40.5% fructose, 33.5% glucose, 7.5% maltose, 1.5% sucrose) was prepared as described previously (Wahdan.1998).

2.5.2 Antimicrobial Activity of Honey

Test bacteria were grown to mid-logarithmic phase in ISB (Oxoid, UK) and then diluted to 2×10^6 cfu/ml. 100 μl of bacteria was added to 1 ml of test honey and the minimum inhibitory concentration (MIC), based on turbidity, was assessed after overnight incubation at 37°C. The minimum bactericidal concentration (MBC) was determined by inoculating 100 μl of each non-turbid honey dilution onto ISA. The MIC and MBC were deemed to be the first dilutions in which there was no turbidity and no growth.

2.5.3 Killing Rate of Honey

To determine the rate of bacterial killing by honey, the experiment described previously was modified. Briefly, 10 ml of honey (33% v/v) was inoculated with 1 ml of 10^6 cfu/ml bacteria and incubated at 37°C for 24 hours. At 30-minute time points, 100 μl was removed, serially diluted onto NA and bacterial viability examined after incubation at 37°C for 18 hours.

2.5.4 Effects of Catalase, Light, and pH on the Antimicrobial Effect of Honey

The assay described in section 2.5.2 was repeated with the following modification. To determine the effect of H_2O_2 on bacterial killing, catalase was added to the reaction at a final concentration equivalent to ten times the estimated concentration of H_2O_2 (NZMh contains approximately 45 mM of H_2O_2). To assess the effect of light on antibacterial activity, honey samples were exposed to 24 hours of direct sunlight prior to use. The pH of honey samples ranged from 3.6 to 4.5, this was neutralised with 1 M NaOH prior to use.

2.5.5 Time-Lapse Light Microscopy

Test bacteria (10^6 log phase) were inoculated into 10 ml of a 33% (v/v) honey solution and viewed under an inverted microscope (Olympus: IX70) and recorded using time-lapse video equipment. The morphological changes were monitored for 24 hours at room temperature.

2.5.6 *In vivo* Use of NZMh

Patient Information

Name:	CA	
Gender:	Male	
DOB:	22/01/77	
Diagnosis:	At birth (Sister also had CF)	
Mutation:	508/508 (ΔF508 homozygote)	
1st Bcc Isolation:	28/9/1990; *B. cenocepacia* ET-12	
Liver Transplant:	08/1999	
Admitted	11/05/2001	FEV1/FVC 1.5/3.0
	06/06/2001	FEV1/FVC 1.4/2.9
24 Hour Sputum:	96 gm	
Microbiology:	19/04/2001	+ *P. aeruginosa* (fully sensitive)
		+++ *B. cenocepacia*

25/05/2001 +++ *B. cenocepacia*

Nebuliser: Honey was nebulised using both a Ventstream nebuliser with mouthpiece at a flow rate 71/min (air), and a sidestream nebuliser.

Dose: Honey was administered at 33% (v/v) and 25% (v/v) with 35% (O_2).

2.6 Bacteriophage Assays

Control bacteriophages used are listed (Table 2.3).

Table 2.3 Control Bacteriophage (Holloway and Krishnapillai 1975, Nzula *et al.* 2000)

Bacteriophage	Host	Propagating Strain
E79	*P. aeruginosa*	PAO1
D3	*P. aeruginosa*	PAO1
F116L	*P. aeruginosa*	PAO1
NS1	*B. vietnamiensis*	ATCC 29424
NS2	*B. multivorans*	ATCC 17616

2.6.1 Isolation of New Lysogenic Bacteriophages for the Bcc

Temperate phage were identified and maintained as described (Nzula *et al.* 2000). Lysogenised bacteria were investigated using the following method: 20 strains of the Bcc were prepared as saline suspensions (approximately 10^6 cfu ml^{-1}), inoculated onto TSA (Oxoid, UK) using a multipoint inoculator and incubated at 30°C for 6 hours. Bacterial growth was inverted over chloroform vapour for 15 minutes and allowed to air-dry for 15 minutes. Soft agar overlays (2.5 ml), inoculated with 100 µl log-phase culture of each of the 20 strains, were layered over the original bacterial growth and the plates incubated overnight at 37°C. Phage plaques, identified in the overlay in the proximity of the original inoculum were used to prepare single-plaque preparations as follows: an agar plug containing a single phage plaque was removed using a sterile glass pipette, transferred to 10 ml phage buffer, vortexed for 30

seconds, centrifuged at 3000 x g for 30 minutes and filtered (pore size 0.2 μm; Millipore)

2.6.2 Isolation of Environmental Bacteriophages

Twenty samples of soil, river sediment and rhizosphere (soil plus root material) were collected and the presence of phage investigated using a modification of the phage enrichment technique described previously (Weiss *et al.* 1994). Briefly, approximately 10 g sample was suspended in 15 ml LB broth and dispersed by shaking in an orbital incubator (140 rev/min) for 30 minutes at 30°C. After removal of soil particles by centrifugation (4000 x g for 20 minutes), the supernatant was filter-sterilized (pore size 0.2 μm; Millipore) and 1 ml aliquots added to 15 sterile tubes. To each tube, was added 25 μl of exponential-phase cultures from one of 15 potential propagating strains chosen to represent genomovars I-V of the Bcc strain panel (Mahenthiralingam *et al.* 2000b), and the contents incubated at 37°C overnight. Bacterial cells were removed by centrifugation (4000 x g for 30 minutes), the supernatant was membrane-filtered as before and 10 μl filtrate was spotted onto lawns of the propagating strain. Phage plaques were identified after overnight incubation at 37°C, and single-plaque stocks were prepared as described in section 2.6.1.

2.6.3 High-Titre Bacteriophage Preparations

High-titre phage preparations were prepared as follows: 100 μl single-plaque preparation, containing approximately 10^5 pfu ml^{-1}, was added to 2.5 ml soft nutrient agar, previously seeded with 100 μl exponential-phase culture of the propagating strain. The mixture was then overlaid on NA and allowed to set. After 18 hours at 37°C, overlays showing semi-confluent lysis were harvested into 10 ml phage buffer. The lysate was vortexed and centrifuged at 3200 x g for 30 minutes and the supernatant membrane-filtered. Phage titres were determined, as pfu ml^{-1}, by incorporating 100 μl host bacteria (exponential-phase NBYE culture) and 100 μl

phage stock in 2.5 ml soft agar overlay, and counting lytic plaques after an 18 hour incubation at 37°C. Stock preparations were maintained at 4°C.

2.6.4 Host Range of Bacteriophages

Stock phage preparations were diluted in phage buffer to approximately 10^8 pfu/ml against the propagating strain, and 10 µl was spotted onto lawns (prepared from exponential-phase NBYE cultures) of potential host bacteria. Lytic activity was recorded after 24 hours at 37°C on a scale ranging from <10 plaques (+) to confluent lysis (+++). In addition to a panel of 66 Bcc strains, 55 other strains were investigated: *P. aeruginosa* (n=30); *S. maltophilia* (n=12); *B. caledonica* (n=1); *B. gladioli* (n=2); *Comamonas acidovorans* (n=1); *P. fluorescens* (n=3); *P. mendocina* (n=1); *P. stutzeri* (n=2); *P. putida* (n=1); *P. testosteroni* (n=1); *P. syringae* pv. *tabaci* (n=1); *R. pickettii* (n=2).

2.6.5 Electron microscopy

Stock phage preparations (approximately 10^8 pfu ml^{-1}) were centrifuged at 3,200 x g to remove cell debris, and then at 100, 000 x g for 1 hour. Phage pellets were resuspended in 1 M ammonium acetate, negatively stained with 2% (w/v) potassium phosphotungstate solution (pH 7.0), and phage morphology examined with a Hitachi model HU-12A transmission electron microscope.

2.6.6 Bacteriophage DNA Extraction and RFLP Profiling

In preparation for DNA extraction, high-titre phage stocks (containing at least 10^{10} pfu ml^{-1}) were prepared using soft agar overlays, as described previously. DNA was extracted from 10 ml phage stock using the Wizard Lambda preparation DNA purification system in conjunction with the Vac-Man laboratory vacuum manifold (Promega, UK). Briefly, 40 µl of nuclease mix was added to 10 ml of phage lysate and after incubation at 37°C for 15 minutes, 4 ml of phage precipitant was added and the reaction was placed on ice for 30 minutes. The reaction was centrifuged (10,000

x g for 10 minutes) and the pellet resuspended in 500 µl of phage buffer. Insoluble particles were removed by centrifugation (10, 000 x g for 10 seconds), the supernatant transferred to a clean microcentrifuge tube, 1 ml of purification resin was added and thoroughly mixed. A Wizard® minicolumn was prepared and attached to the vacuum manifold. The phage resin/lysate mix was applied to the syringe barrel and a vacuum applied to draw the mix into the minicolumn. The column was washed by adding 80% isopropanol and applying a vacuum to draw the solution through the minicolumn. The vacuum was applied for a further 30 seconds after the solution was removed to dry the resin. The syringe barrel was removed and the minicolumn was transferred to a 1.5 ml microcentrifuge tube. Residual isopropanol was removed by centrifugation (10, 000 x g for 2 minutes). The minicolumn was transferred to a clean microcentrifuge tube, 100 µl of preheated (80°C) water was applied, and the column centrifuged (10, 000 x g for 20 seconds). Extracted DNA was eluted in sterile distilled water and stored at –20°C. DNA quality was assessed on a pre-cast 0.8% agarose gel "E-gel" (Invitrogen, UK). In cases where DNA was not pure enough for DNA restriction, purification was performed using the PCR protocol from the QIAquick gel extraction kit (Qiagen, UK). In brief, 5 volumes of buffer PB were added to 1 volume of DNA. The mix was transferred to a QIAquick spin column and DNA was bound by centrifugation (15,000 x g for 1 minute). 750 µl of buffer PE was added to the spin column. After centrifugation (15,000 x g for 1 minute), the flow through was discarded and the column was centrifuged as before. The spin filter was transferred to a clean microcentrifuge tube and purified DNA was eluted in 30 µl of distilled water by centrifugation (15,000 x g for 1 minute). Purified DNA was stored at –20°C. To determine genome size and to confirm that the phages were different from one another, approximately 1 µg DNA was restricted using 10 U *Hind*III (Promega, UK), incubated for 3 hours at 37°C and visualized on 0.3% TBE agarose gel alongside 1 µl Ready-Load Lambda DNA/*Hind*III fragments (Invitrogen, UK).

2.6.7 Alternative Method for Bacteriophage DNA Extraction

The alternative method is based on a protocol described by Sambrook *et al.* (1989). Phages were prepared by infecting the host bacteria at high multiplicity. Briefly, 50 ml of bacteria were grown in NBYE overnight at 37°C in an orbital incubator (140 rev/min), prior to inoculating 4 x 500 ml of NYBE. The bacterial cultures were incubated for 3-4 hours before stock phages with 10^{10} plaque forming units (pfu) were added (1:100). The culture was incubated for a further 3-5 hours, with vigorous shaking, until lysis was visible. 10 ml of chloroform was added to each flask, and incubated for a further 10 minutes at 37°C. Flasks were cooled to room temperature, prior to the addition of Dnase I and Rnase, each to a final concentration of 1 μg/ml. The reaction was incubated at room temperature for 30 minutes. NaCl was added and dissolved to give a final concentration of 1 M, and incubated on ice for 1 hour. Solid polyethylene glycol (PEG 8000) was added to a final concentration of 10% (w/v). PEG was dissolved by stirring at room temperature then transferred to ice water for approximately 1 hour to allow the precipitation of phage particles. Phage particles were recovered by centrifugation at 11,000 x g for 10 minutes at 4°C. Supernatants were discarded, and the pellet was resuspended in bacteriophage buffer (8 ml for each 500 ml flask). PEG and bacterial cell debris was removed by adding an equal volume of chloroform and vortexing for 30 seconds. The organic and aqueous phases were separated by centrifugation at 3000 x g for 15 minutes at 4°C, and latter retained. The aqueous phase was subjected to centrifugation (25, 000 rpm for 2 hours at 4°C), and the supernatant discarded. The pellet was resuspended in 1-2 ml of phage buffer and incubated at 4°C overnight. Proteinase K (final concentration of 50 μg/ml) and SDS (final concentration 0.5% w/v) were added, mixed and incubated at 56°C for 1 hour. The digestion mix was cooled to room temperature, prior to the addition of phenol equilibrated with 50 mM Tris (pH 8.0). An emulsion was formed by invert mixing, and separated into separate phases by centrifugation (3000 x g for 5 minutes at room temperature). The aqueous phase was removed and extracted with 50:50 mix of equilibrated phenol and chloroform. The aqueous phase was recovered as before, and extracted with an equal volume of chloroform. Sodium

acetate (to a final concentration of 0.3 M: pH 7.0) was added to the aqueous phase and mixed thoroughly prior to the addition of 2 volumes of ethanol. The solution was mixed and incubated at room temperature for 30 minutes. Thread-like DNA precipitate was transferred, using a Pasteur pipette, to a DNA microfuge tube containing 1 ml of 70% ethanol. DNA was recovered by centrifugation (12, 000 x g for 2 minutes, at 4°C). The supernatant was removed and the DNA pellet allowed to dry at room temperature. The DNA was dissolved in 250 µl of dH_2O and stored at -20°C. DNA was quantified on a 0.6% agarose gel.

2.6.8 Bcep 781 Endolysin: Recursive (Gene Assembly) PCR

The Bcep 781 bacteriophage genome was analysed and the hypothetical endolysin gene was identified (Accession Number/Locus: NP_705653 [255 amino acids]; gene: ORF25). The gene assembly technique was based on the Recursive PCR protocol as described (Prodromou and Pearl 1992). Twenty-two oligonucleotides representing a synthetic Bcep 781 endolysin were synthesised (Sigma-Genosys) (Appendix 2). The oligonucleotides were 37-46 bp long with overlapping regions of 7-15 bp in length. Subsequent insertion of the synthesized gene into an expression vector was facilitated by the incorporation, in the outermost oligonucleotides, of an *Nco*I site at the 5' end and a *BamH*I site at the 3' end of the final gene. The recursive PCR (30 cycles of 2 minutes at 95°C, 2 minutes at 56°C and 1 minute at 72°C, with a final 10 minutes at 72°C) was performed in a Techgene (Techne) thermal cycler. The reaction contained 20-30 pmol of the outermost 5' oligonucleotides of each strand, 2-3 pmol of the internal primers, 10 µl of 10 x Herculase buffer, 5 units of Herculase® polymerase (Stratagene), 4 µl of Dimethyl-sulfoxide (DMSO), 4 µl of 100 µM of each dinucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), and dH_2O added to a final volume of 100 µl. The product was subjected to agarose gel electrophoresis and DNA extracted from the gel as described previously. The purified PCR product (10 µl) was then subjected to another round of recursive PCR, using only the outermost oligonucleotides. After agarose gel electrophoresis, extracted DNA was cloned into PCR 2.1 as described previously, and the integrity of

the sequence was determined by automated sequencing. Mutations and mis-base pairing was corrected using the standard PCR protocol, and a mega primer PCR reaction using primer-pair specific temperatures (lysin fix primers outlined in Appendix 2). In brief, an internal primer with the fix sequence was coupled with the reverse outermost primer in a PCR reaction using the standard protocol and the original template DNA. The PCR product was purified using gel electrophoresis and the product used as a reverse primer coupled with the forward outermost primer and the initial template DNA. After purification, the product was cloned into PCR 2.1 and the integrity of the sequence assessed. Once the template sequence was correct, the PCR product was cloned into an expression vector.

2.6.9 Bacteriophage D3 Endolysin Cloning

Bacteriophage DNA was extracted as described previously. Endolysin specific primers were identified from the sequenced genome (ORF 31: 160 amino acids) (Kropinski 2000), and synthesized with a 5' *NcoI* site and a 3' *BamHI* site to aid latter cloning steps (Sigma-Genosys) (Appendix 2). The PCR reaction combined; 5 µl of both forward and reverse D3 lysin primers (Appendix 2), 5 µl of D3 template DNA, 5 µl of 10 x Herculase buffer, 5 units of Herculase® polymerase (Stratagene), 2 µl of Dimethyl-sulfoxide (DMSO), 2 µl of 100 µM of each dinucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), and dH_2O added to a final volume of 50 µl for 30 cycles (95°C for 1 minute, 56°C for 1 minutes and 72°C for 1 minute). The PCR product was extracted from an agarose gel, and used as the template for a second round of PCR (95°C for 1 minute, 56°C for 1 minutes and 72°C for 45 seconds). The PCR product was then cloned into PCR 2.1 and subjected to automated sequencing. Mutations were corrected using the Mega-primer PCR protocol outlined previously. Once the template sequence was correct, the PCR product was cloned into an expression vector.

2.6.10 Antibacterial Activity of Bacteriophage Endolysins

The *in vitro* activity of the phage lysins was determined as outlined by (Schuch *et al.* 2002). 1 ml of log-phase bacteria were treated with lysin and plated after 3 hours onto ISA, incubated overnight at 37°C at which time bacteria were enumerated.

2.6.11 Expression of a Bacteriophage Endolysin in a Bacterial Overlay

E. coli transformants containing an expression plasmid/bacteriophage lysin gene were spread onto ISA containing the appropriate antibiotic and IPTG (final concentration 1 mM) and grown overnight at 37°C. Control plates with *E. coli* transformants containing the vector minus the lysin insert were also prepared. Bacterial growth was inverted over chloroform vapour for 15 minutes and then allowed to air-dry for 15 minutes. Soft agar overlays (2.5 ml), inoculated with 100 µl exponential-phase culture of the propagating bacteria were layered over the original bacterial growth and allowed to set. Evidence of lysis was assessed after overnight incubation at 37°C.

2.7 Cationic Antimicrobial Peptide (CAMP) Assays

Experiments involving CAMPs were carried out in collaboration with Mr David Clarke, School of Chemistry, University of Edinburgh.

2.7.1 Preparation of peptide

The synthetic peptides (Albachem, Edinburgh) were lyophilised (in 250 µg aliquots) and kept at –70°C. Prior to use, the peptide was dissolved in 250 µl of 0.01% acetic acid to give a stock solution with a final concentration of 1 mg/ml. The peptide preparations were held at 4°C during use and after which were stored at –70°C.

2.7.2 Antimicrobial activity of CAMPs

Test bacteria were grown to mid-log phase in ISB (Oxoid) and then diluted to 2×10^6 cfu/ml in 10 mM sodium phosphate containing 1% (v/v) ISB, pH 7.4. The bacterial samples were washed three times in buffer to ensure removal of salt. Varying concentrations of test peptide (peptide range 1.5 μg/ml to 100 μg/ml) were incubated in 100 μl of the bacterial suspension at 37°C for 1 or 3 hours. 10-fold serial dilutions of the incubation mixture were plated on ISA plates, incubated at 37°C, and the number of cfu determined the following day. The MIC of the peptide was the concentration required to fully inhibit bacterial growth. All assays were repeated at least three times and experimental errors found generally to be within one doubling dilution. Dose-response curves were determined from surviving cfu.

2.7.3 Salt Sensitivity of CAMP

The salt sensitivity of the CAMP was determined as follows. The assay was conducted as described previously, but after the final washing step the bacteria were resuspended in 10 mM sodium phosphate (1% v/v ISB, pH 7.4) containing varying concentrations of NaCl (range 25 mM to 300 mM).

2.7.4 Inhibition of Antimicrobial Activity with LPS

Briefly, peptide (final concentration equivalent to 4 x the MIC) was incubated with varying concentrations of LPS (range 3.15 μg/ml to 200 μg/ml) from *E. coli* (Sigma-Aldrich, UK), *B. cenocepacia* J2315 and *P. aeruginosa* PAO1 for 1 hour at 37°C. Bacterial suspensions were added as before and inhibition ascertained after overnight incubation at 37°C.

2.7.5 Inhibition of Antimicrobial Activity with Lipoteichoic Acid

The inhibition of antimicrobial activity by lipoteichoic acid (Sigma-Aldrich, UK) from *S. aureus* was determined as described (section 2.7.4). In this case, LPS was replaced with varying concentrations of lipoteichoic acid (range 1.5 μg/ml to 100 μg/ml).

2.7.6 Phase-Contrast Microscopy

At 30 minute intervals during the bacterial inhibition assay, a sample was observed under phase contrast microscopy (Olympus, UK) and morphological changes in bacteria observed.

2.7.7 Radial Diffusion Assay

This antimicrobial assay was performed using a well-established radial diffusion method described by (Cole and Ganz 2000, Lehrer *et al.* 1991, Yount and Yeaman 2004). Test bacteria were grown to log-phase and diluted to a final concentration of 10^7 cfu/ml. 1 ml of bacterial suspension was added to 9 ml of ISA, vortex mixed and poured into a Petri-dish. The agar was allowed to set prior to the formation of 5 mm holes by an Ouchterlony punch, to which 5 μl of test peptide was added (peptide concentration range 0.5 μg/ml- 25 μg/ml). The plates were incubated at 37°C for 3 hours, before 10 ml of soft agar was overlayed, dried and incubated overnight at 37°C after which time the zone of killing was determined.

2.7.8 Time-Kill Assay

The assay described in section 2.7.2 was used but the reaction was carried out in a 200 μl volume with a final peptide concentration of 25 μg/ml. At 30 minute intervals 10 μl of suspension was removed and serially diluted onto ISA. This procedure was repeated for 3 hours. After the plates were incubated overnight at 37°C, bacterial viability was assessed and the cfu/ml determined.

2.8 Statistical Analysis

Statistical analysis was carried out using Graphpad Prism software (Graphpad). A *P* value of <0.05 was taken to indicate significance.

Appendix 1. Commercial suppliers of reagents and equipment

Albachem UK, Ltd., Elvinston Science Centre, East Lothian, EH33 1EH, UK. www.albachem.co.uk

Amersham Biosciences UK, Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK. www.amershambiosciences.com

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, UK. www.bio-rad.com

Comvita, Bay of Plenty, New Zealand. www.comvita.com

Difco Laboratories, Detroit, Michigan, U.S.A. www.bd.com

Graphpad, San Diego, CA. www.graphpad.com

Invitrogen, Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK. www.invitrogen.com

MAST Diagnostics, Bootle, Merseyside, UK. www.mastgrp.com

Millipore, 80 Ashby Road, Bedford, Massachusetts, USA. www.millipore.com

New England Biolabs (UK) Ltd., 73 Knowl Piece, Wilbury Way, Hitchin, Hertordshire, SG4 0TY, UK. www.neb.com

Olympus UK Ltd., 2-8 Honduras Street, London EC1Y OTX, UK. www.olympus.co.uk

Oxoid Ltd., Basingstoke, Hampshire, UK. www.oxoid.com

PE Biosystems, Warrington, England, UK. www.appliedbiosystems.com

Promega UK, Ltd., Delta House, Chilworth Research Centre, Southampton, SO16 7NS, UK. www.promega.com

Roche Diagnostics Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, UK. www.roche.com

QIAGEN Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 9AX, UK. www.qiagen.com

Sigma-Aldrich Ltd., Dorset, UK. www.sigmaaldrich.com

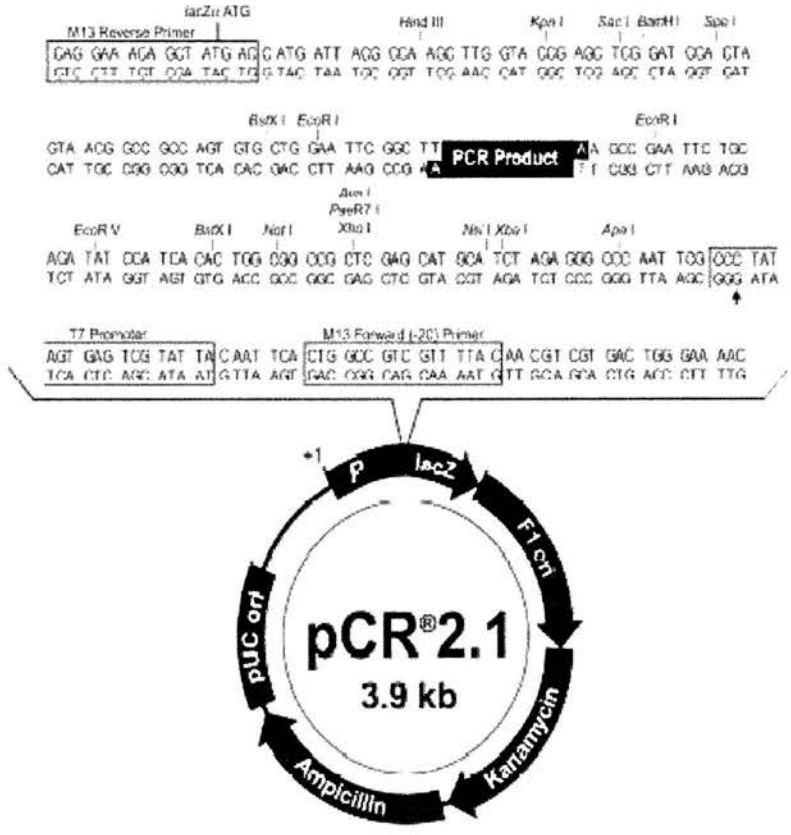
Sigma-Genosys, www.sigma-genosys.eu.com

Stratagene Europe, Gebouw California, Hogehilweg 15, 1101 CB Amsterdam, Zuidoost, The Netherlands. www.stratagene.com

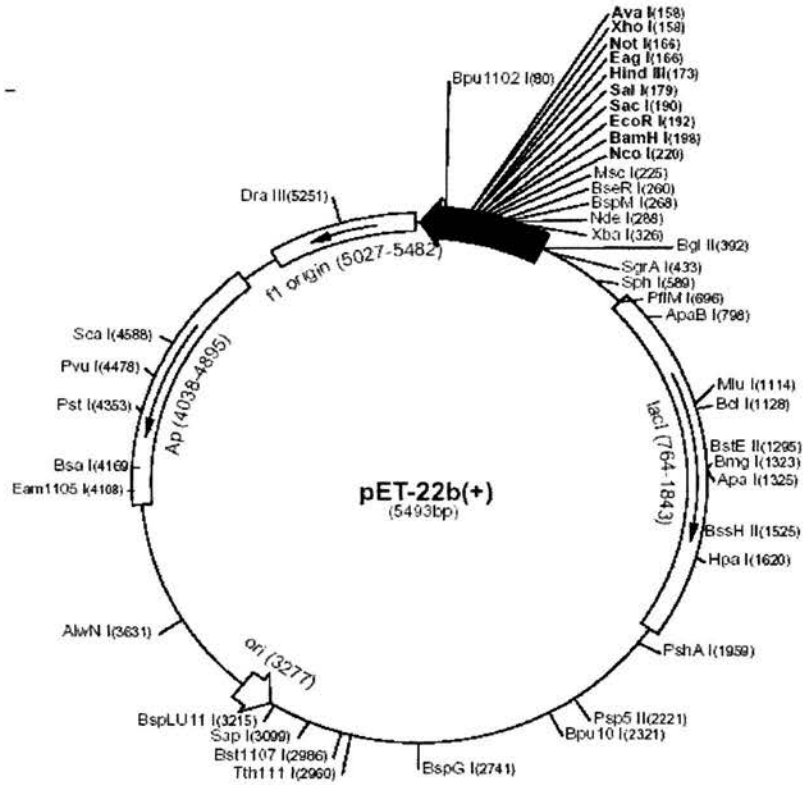
Appendix 2. Oligonucleotides utilised during this study

Bcep781 Primer No.	5' - 3'	Tm (°C)
1	CCATGGCCGCGCCGCTGATCGTCGGCGCGTCGGGTCGCGCCGTGGT	80.2
2	TGGCCCGACTGCGCGAGGCCGAGCCGCGCCTGCAGAAACACCACGG	81.1
3	CGCGCAGTCGGGCCAATTCGACGCGAGCGTCGCAACGGCGCTGCGC	79.4
4	CACGCCATCCGGCGTCATCCCGTGCGCCTCCTGCCACTGGCGCAGC	81.1
5	ACGCCGGATGGCGTGTACGGATCGCAGACGAATGCGGTGATGACGG	98.1
6	GGCGCGCAGCCGCGTCGGCGATGTCCGGCAGCGCGCGCGCCGTCAT	82.7
7	ACGCGGCTGCGCGCCTGAACGTCGACACGCCTGCGCTCCAAGCCAT	97.0
8	TCGGGCAGGAAGCCGCTACCCGTTGTCTCGACCTGGATAATGGCTT	91.6
9	CGGCTTCCTGCCCGACGGGCGGCCGCGCATCCTGCTCGAACGCCAC	80.2
10	TAGCACGCGCTGCGCCGGGCTCGTCGCCGCCCATACCTTGTGGCGT	99.2
11	GCGCAGCGCGTGCTACTTGGCGCGCAGGATTGCAACCCGACGCCCG	80.2
12	CGCCGCGCGCGTTTGCGTCCGGCCCCGTGGCGTAGCCGCCGGGCGT	84.3
13	CAAACGCGCGCGGCGCGGGCGAGTGGGTGCGTTTCGAGCGCGTGGC	81.1
14	GAGCAGCACTGCGCGGCCACTTCGTCGCCCGTGACGGCTGCCACGC	80.2
15	GCGCAGTGCTGCTCCTGGGGACTCGGGCAGGTTATGGGCGCCAACT	94.6
16	ACATCAGACCGACGGCGTTCGTGAAGCCGCACGTCGCATAGTTGGC	95.9
17	CCGTCGGTCTGATGTTCGCGAGCGCCCTGAACGAGCGCGCGCAACT	97.1
18	AGGCCGGCCTGCGGCAGCGCGAAGCGCACCATCACGTCAAGTTGCG	79.4
19	GCCGCAGGCCGGCCTGCTCGGGGCATTACGTGCGCATCAATGGGCG	80.2
20	GATCGCGAAGTTAGGCCCGTTCCAGATGCGCGCGACGGCCGCCCAT	96.1
21	CCTAACTTCGCGATCAACCAGTACGACACGAAGCTGTCCGATGCGTA	87.8
22	GGATCCTTATCGCGACGTCAATGTGGTGTACGCATCGGAC	85.7
Bcep781Lysin Fix		
1 (For)	AACTTGACGTGATGGTGCGC	68.5
2 (Rev)	GCGCACCATCACGTCAAGTT	68.5
3 (For)	CGAGCGCGCGCAACTTGACGTG	80.3
4 (For)	CGAGCGCGCGCAACTTGACGTT	78.6
5 (Rev)	TGCGGCAGCGCGAAGCGCACCATC	86.1
6 (For)	CAACTTGACGTTATGGTTCGTTTCGCTCTG	74.8
D3 Lysin		
1 (For)	CCCATGGCAGTTGTTTCCGA	70.2
2 (Rev)	GGATCCTCACGACAGCACCCCGCCCGCTGCAAG	89.7
D3 Lysin Fix		
1 (Rev)	GGATCCGTGGGGCGGGCGTCGATCA	86.0

Appendix 3. Cloning vectors utilised in this study



Vector pCR 2.1. Vector pCR 2.1, 3929bp in size, was obtained from Invitrogen.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Eag I(166)
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Sal I(179)
Sac I(190)
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BamH I(198)
Nco I(220)
Msc I(225)
BseR I(260)
BspM I(268)
Nde I(288)
Xba I(326)
Bgl II(392)
SgrA I(433)
Sph I(589)
PflM I(696)
ApaB I(798)
Mlu I(1114)
Bcl I(1128)
BstE II(1295)
Bmg I(1323)
Apa I(1325)
BssH II(1525)
Hpa I(1620)
PshA I(1959)
Psp5 II(2221)
Bpu10 I(2321)
BspG I(2741)
Bpu1102 I(80)
Dra III(5251)
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Pvu I(4478)
Pst I(4353)
Bsa I(4169)
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AlwN I(3631)
ori (3277)
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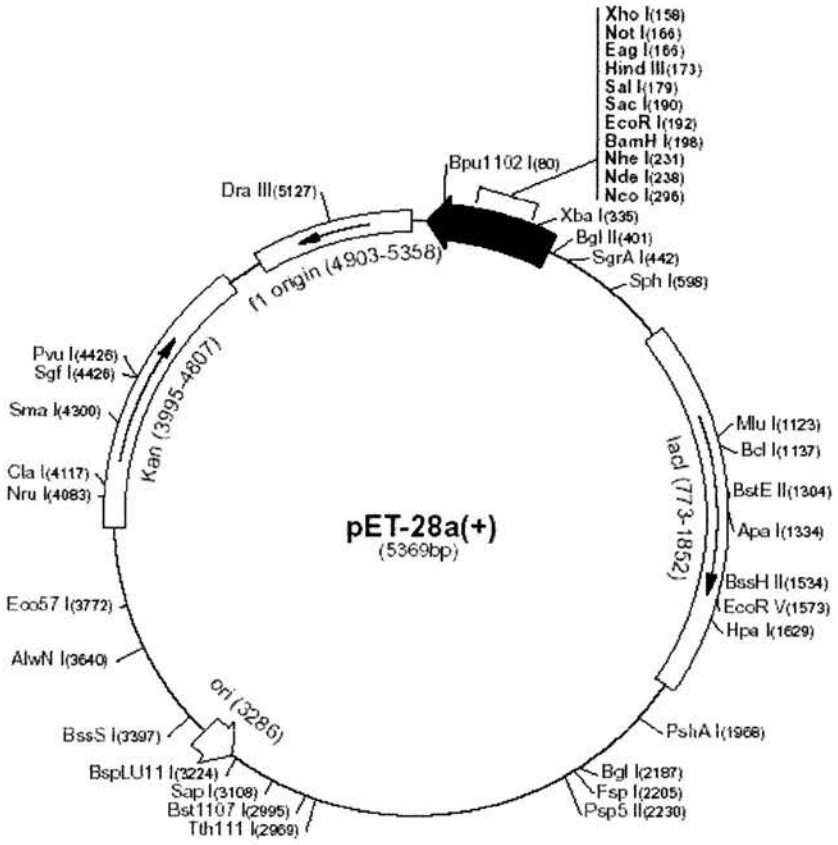
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Bgl II(401)
SgrA I(442)
Sph I(598)
Mlu I(1123)
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Sma I(4300)
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Nru I(4083)
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AlwN I(3640)
ori (3286)
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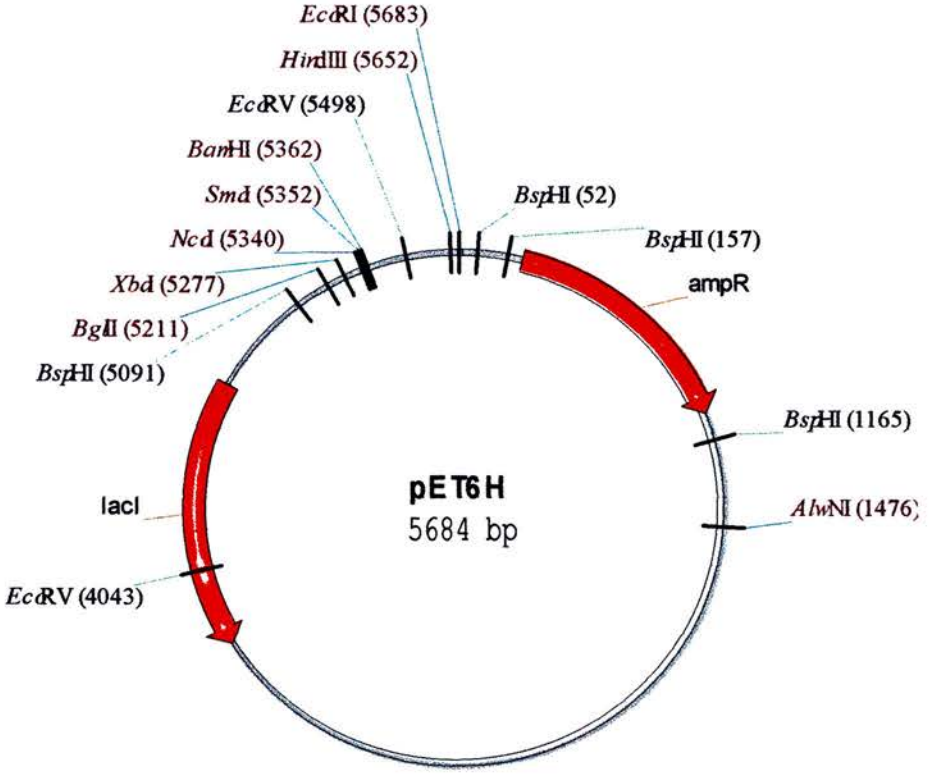
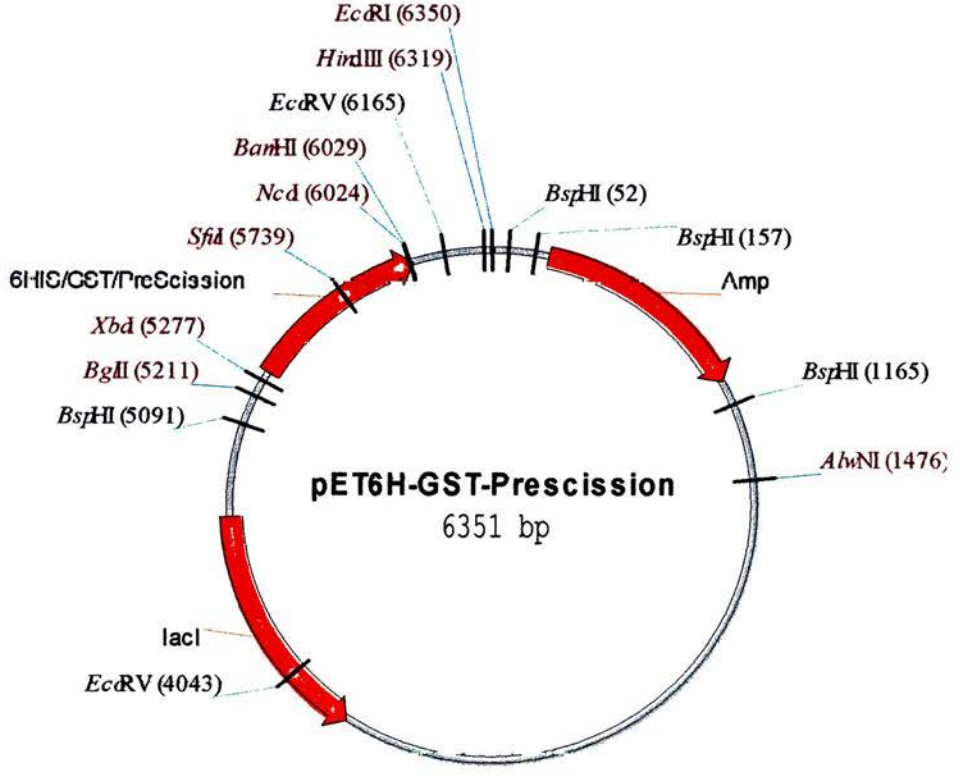




pET Plasmids were obtained from Novagen.

3.1 Aims

The aim of this section was to determine or confirm the antibiotic susceptibilities of a panel of Bcc isolates derived from environmental and clinical sources. The extent of resistance within some Bcc species has been recently investigated (Nzula *et al.* 2002), and concordance of this study was sought prior to examination of novel therapies.

3.2 Results: Sensitivity Testing

The antibiotic susceptibility profiles of Bcc species have been previously investigated to some degree (Lewin *et al.* 1993, Nzula *et al.* 2002, Pitt *et al.* 1996). In the present study, susceptibility of a representative panel of Bcc strains was determined by the agar dilution method. MIC values are presented in Table 3.1.

Sensitivity of the Bcc panel to the antibiotics investigated varied within and between species. Strikingly, all Bcc species examined were resistant to polymyxin B (MICs $\geq$ 512 μg/ml). No strains examined were sensitive to tobramycin; MICs ranged from 4 to 256 μg/ml. MICs of ceftazidime ranged from 0.12 to 256 μg/ml, with 81% (n= 39) of the isolates examined sensitive. Similarly, 85.4% (n= 41) of the strains investigated were sensitive to meropenem, although the MIC range varied from <0.06 to 8 μg/ml. Sensitivity to ciprofloxacin was moderate, with 64.6% (n= 31) of the strains sensitive, and a MIC range of 0.12 to 128 μg/ml. Only a few Bcc strains were sensitive to chloramphenicol (12.5%, n= 6) and trimethoprim (6.3%, n= 3), with MICs between 4 to 128 μg/ml and 0.25 to 64 μg/ml respectively. These findings indicate that meropenem, ceftazidime and ciprofloxacin exhibit the most activity against members of the Bcc.

Clinical Bcc isolates (shown in black) were significantly more resistant to the antibiotics studied than environmental Bcc isolates (shown in red) (P= 0.0001). Resistance between Bcc species varied (Fig 3.1), but all Bcc strains examined were resistant to at least 40% of the 7 antibiotics tested.

Of particular interest are the epidemic strains C1576 (*B. multivorans*; Glasgow epidemic) and J2315 (*B. cenocepacia*; Edinburgh epidemic, ET-12). Both exhibit high-level (100%) resistance to all of the conventional antibiotics examined, in contrast to non-epidemic clinical isolates that show variable resistance.

3.3 Results: Multi-Resistance of *B. cenocepacia*

When attention was focussed on *B. cenocepacia*, resistance was less in environmental isolates than in clinical isolates (Table 3.2). The mean percentage resistance is 67.83 (Standard Deviation, SD± 14.3) and 51.9 (SD± 7.215) for clinical and environmental strains respectively. Clinical isolates of *B. cenocepacia* are also significantly more resistant to the antibiotics examined than environmental isolates ($P = 0.0023$). However, the data also indicate that resistance is strain dependant, irrespective of origin. Interestingly, 100% of environmental *B. cenocepacia* isolates (n= 11) were sensitive to the ciprofloxacin and meropenem. In contrast, clinical isolates show 50% (n=8) sensitivity to ciprofloxacin, with 81.3% sensitive to meropenem.

Table 3.1 MICs of conventional antibiotics against members of the Bcc

Species	Strain	Description	Ceftaz	Chlor	Cipro	Trimeth	Tobra	Poly	Mero
B. cepacia	ATCC 25416	Onion isolate	4	16	4	2	64	>512	1
	C2970	Urine; Gent	2	8	2	2	16	>512	2
	C3159	CF; Australia	8	16	4	4	128	>512	1
	ATCC 17759	Soil, Trinidad	4	8	0.12	4	32	>512	1
B. multivorans	C3161	CF; Belgium	2	32	2	2	16	>512	4
	C3162	CF-e UK	4	16	4	4	64	>512	1
	C3163	CGD; USA	4	16	2	2	64	>512	0.5
	C3164	LAB USA	2	8	0.12	0.5	16	>512	4
	ATCC 17616	Soil; Canada	4	8	0.5	2	32	>512	4
	C1962	Cerebral abscess	1	8	8	4	32	>512	0.5
	C1576	CF; Glasgow	256	32	16	4	64	>512	8
	C3160	CF; Canada	8	128	4	32	32	>512	1
B. cenocepacia	J415	CF; Edinburgh	4	64	2	4	64	>512	0.5
	J2315	CF; ET12	16	32	8	32	256	>512	8
	C1394	CF; Manchester	1	16	2	64	128	>512	0.12
	C3165	CF; Canada	2	16	0.12	0.25	8	>512	8
	C3166	CF; Canada	4	16	4	32	128	>512	1
	C3167	CF; Canada	2	8	0.12	0.25	8	>512	16
	C3168	CF; Canada	2	16	32	32	32	>512	1
	C3169	CF; USA	0.12	4	16	0.5	8	>512	0.5
	C3170	CF; Australia	2	16	32	32	128	>512	2
	ATCC 17765	Urinary-tract	4	16	2	4	32	>512	1
	C1632	CF; Newcastle	8	32	16	8	64	>512	0.5
	C1773	Blood Culture	32	8	64	1	32	512	0.5
	C1511	CF; Manchester	4	32	8	32	32	>512	2
	C2374	CF; Edinburgh	8	16	128	32	64	>512	0.5
	R2817	Env; Philippines	2	4	0.25	1	4	>512	0.25
	R2827	Env; Philippines	4	16	0.25	16	16	>512	0.5
	R8351	Env; Philippines	1	8	0.25	1	8	>512	0.25
	R8571	Env; Philippines	4	4	0.25	4	8	>512	0.25
	R8574	Env; Philippines	4	8	0.25	1	16	>512	0.5
	R8580	Env; Philippines	4	8	0.25	1	16	>512	0.5
	R8605	Env; Philippines	4	16	0.25	1	16	>512	0.5
	R9239	Env; Philippines	2	8	0.25	1	8	>512	0.5
	R9243	Env; Philippines	2	8	0.25	4	8	>512	0.5
	R9235	Env; Philippines	2	8	0.25	1	16	>512	0.5
	R9342	Env; Philippines	2	8	0.25	2	16	>512	0.5
B. stabilis	C3171	CF; Belgium	8	4	64	32	8	>512	<0.06
	C3172	CF; Canada	2	16	4	32	64	>512	0.5
	C3173	Clinical; Belgium	2	16	0.12	0.25	8	>512	<0.06
	C3174	Respirator; UK	8	16	0.12	2	8	>512	1

B. vietnamiensis	C2978	CF isolate	2	8	1	4	16	>512	0.25
Species	Strain	Description	Ceftaz	Chlor	Cipro	Trimeth	Tobra	Poly	Mero
B. vietnamiensis	C3175	CF USA	2	8	0.12	32	16	>512	0.5
	C3176	CGD Canada	2	8	0.12	2	16	>512	0.12
	C3177	Rice; Vietnam	2	4	0.12	32	16	>512	0.25
B. ambifaria	J2742	AMMD	0.5	8	0.25	0.5	4	>512	1
B anthina	J2552	Soil; RBG; Edin	1	8	1	1	8	512	1
Gnvr Unclass	J2540	Soil; RBG; Edin	0.12	4	0.25	0.5	8	>512	<0.06
P. aeruginosa	NCTC 10662	Antibio Contol	2	128	0.5	32	0.12	1	1
		Break Point Values	8	8	4	0.5	4	4	4

Ceftz, ceftazidime; Chlor, chloramphenicol; Cipro, ciprofloxacin; Trimeth, Trimethoprim; Tobra, tobramycin ; Poly, polymyxin; Mero, meropenem. Gnvr unclass, genomovar/species unclassified. Antibio control, antibiotic control *P. aeruginosa* NCTC 10662. RBG Edin, Royal botanical gardens, Edinburgh. Environmental (Env) isolates are highlighted in red. Clinical isolates are in black.

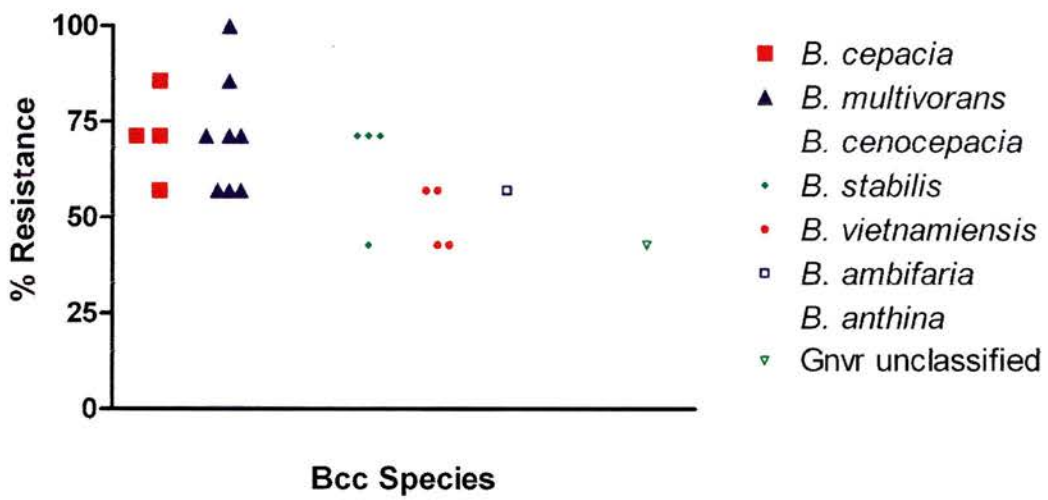


Fig 3.1 The resistance of the *B. cepacia* complex to antibiotics. The figure shows the percentage antibiotic resistance of each isolate, of each species, within the Bcc.

Table 3.2 Antibiotic Resistance in *B. cenocepacia*

CF/Non CF/ ENV	Ceftaz	Chlor	Cipro	Trimeth	Tobra	Poly	Mero	% Resistance
C3165	S	R	S	S	R	R	R	57.1
C3170	S	R	R	R	R	R	S	71.4
C1394	S	R	S	R	R	R	S	57.1
C3167	S	R	S	S	R	R	R	57.1
C3168	S	R	R	R	R	R	S	71.4
C3166	S	R	R	R	R	R	S	57.1
C3169	S	S	R	R	R	R	S	57.1
J415	S	R	S	R	R	R	S	57.1
C517	S	R	S	R	R	R	S	57.1
C1335	S	R	S	R	R	R	S	57.1
J2315	R	R	R	R	R	R	R	100
C2374	R	R	R	R	R	R	S	85.7
C1632	R	R	R	R	R	R	S	85.7
C1511	S	R	R	R	R	R	S	71.4
C1773	R	R	R	R	R	R	S	85.7
ATCC17765	S	R	S	R	R	R	S	57.1
R2817	S	S	S	R	R	R	S	42.8
R2827	S	R	S	R	R	R	S	57.1
R8351	S	R	S	R	R	R	S	57.1
R8571	S	S	S	R	R	R	S	42.8
R8574	S	R	S	R	R	R	S	42.8
R8580	S	R	S	R	R	R	S	57.1
R8605	S	R	S	R	R	R	S	57.1
R9239	S	R	S	R	R	R	S	42.8
R9243	S	R	S	R	R	R	S	57.1
R9235	S	R	S	R	R	R	S	57.1
R9342	S	R	S	R	R	R	S	57.1
Break Point	8	8	4	0.5	4	4	4	

CF isolates are shown in black. Non-CF clinical isolates are shown in blue. Environmental isolates are shown in red. Ceftz, ceftazidime; Chlor, chloramphenicol; Cipro, ciprofloxacin; Trimeth, Trimethoprim; Tobra, tobramycin ; Poly, polymyxin B; Mero, *meropenem*. % Resistance is defined as the number of antibiotics each strain is resistant to (based on the MIC breakpoint) in relation to the number of antibiotics used (n=7). R; Resistance, S; Sensitive.

3.4 Discussion:

"*B. cepacia*" is often described as a pan-resistant organism. This is not supported by these data, which demonstrate considerable variation in resistance between and within Bcc species. As with some other bacterial pathogens, the concept of describing the antibiotic sensitivity of an individual species in general terms is of little use in the case of the Bcc.

With few exceptions, the data obtained with this extended Bcc panel correlated with the findings of Nzula *et al.* (2002). The data support the hypothesis that the grouping of species into antibiograms does not provide relevant information on individual-strain resistance profiles. Thus, clinical relevance of conventional antibiotic therapy can only be established if individual strains are examined to determine the full extent of their resistance. The variation within species may result in less effective therapy, and antibiotics that are successful against one strain may be ineffective against another isolate of the same species and from the same environment.

B. cenocepacia is arguably the most important Bcc species in CF lung disease. The observation that clinical isolates of *B. cenocepacia* tended to be more resistant than environmental isolates could be explained by prior exposure to antibiotics. Investigation of a greater number of environmental isolates from *B. cenocepacia* and other Bcc species is necessary to confirm this trend. Unfortunately, environmental examples of Bcc species are not readily available. In particular, despite the significant prevalence of *B. multivorans* amongst CF isolates, this species is seldom identified in natural environments.

Despite the well-established clonality of three *B. cenocepacia* isolates (ET-12 isolates; C3165, C3166 and J2315), the MIC data and extent of resistance against individual antibiotics varied. The data highlight two important facts recently confirmed in outbreaks of *P. aeruginosa* infections (Jones *et al.*2002). First, when cross-infection is suspected or investigated routinely, antibiograms are an unreliable marker of clonality. Antibiograms are useful, however, in monitoring resistance patterns within clinics. Second, variability in MIC data for individual strains and

antibiotics emphasises the need to base therapy on individual isolates (Hobson *et al.* 1995, Pitt *et al.* 1996). This is strikingly evident for the most active agent meropenem (C3165 and J2315: MIC 8 μg/ml; C3166: MIC 1 μg/ml).

Only two Bcc strains investigated were resistant to all antibiotics; C1576, a Glasgow epidemic strain, and J2315, the Edinburgh representative of the ET-12 lineage. Both epidemic strains highlight the characteristics most feared of the Bcc: high virulence, transmissibility and multi-resistance. Interestingly, the high resistance of the Edinburgh isolate was noted and commented on in one of the earliest antibiotic studies (Lewin *et al.* 1993) which predated its epidemic spread and the clinical use of meropenem.

A major caveat in current definitions of antibiotic resistance is the reliance on MIC breakpoints. At present, breakpoints are based on a concentration of antibiotic that can be achieved by *intra venous* (IV) administration, and that has been shown to inhibit bacterial growth. Thus, breakpoints may not be relevant to infections at specific sites, such as the lung. In CF, antibiotics may be delivered to the lung by aerosol, a process that may achieve a concentration of the drug approximately 1000-fold higher than IV. In such cases as CF, present definitions of resistance are probably unreliable and need redefined. To compound these difficulties, clinical improvement and bacterial reduction in sputum is rarely observed in the treatment of Bcc infections (Isles *et al.* 1984). Furthermore, this study has demonstrated that all the Bcc isolates are resistant to at least 40% of the 7 most relevant antibiotics investigated emphasising the need for novel and effective therapeutic agents against the Bcc.

4.1 Aims

As the results from Chapter three indicate, many Bcc isolates exhibit extensive resistance to multiple groups of antibiotics fulfilling the criteria to be termed multiresistant bacteria. These data also emphasize that novel antimicrobial agents are required to provide therapeutic options against life-threatening Bcc infections.

Plant products, including honey, are traditional remedies which have created renewed interest as broad spectrum antimicrobial agents (Cooper *et al.* 1999a, Somal *et al.* 1994) and as a wound dressings (Van der Weyden 2003). Significantly, this antimicrobial activity has been shown to extend to multiresistant pathogens (Karayil 1998). The aim of this section was to investigate the antimicrobial activity of NZMh against Bcc and other problematic CF pathogens.

4.2 Results: Antibacterial Activity of Honey

The potential antibacterial activity of NZMh was investigated against a panel of Bcc isolates chosen to represent a variety of clinical isolates, plus epidemic and biopesticide strains. Two strains were included from a recent outbreak of untreatable ovine mastitis. NZMh was sterilised as described and the absence of anaerobic organisms confirmed. The MIC and MBC were determined for each strain (Table 4.1). MICs ranged from 9 to 17% (v/v), and MBCs from 9 to 20% (v/v). Catalase production was determined for each strain used. All Bcc strains produced catalase with the exception of ATCC 25416, C3159 and C3175. To investigate the spectrum of activity against multiresistant strains, a selection of such strains were selected and tested for susceptibility to 33% (v/v) NZMh. The panel of resistant bacteria included *S. aureus* (n= 3), *Acinetobacter baumannii* (n= 5), *E. coli* (n= 3), *Klebsiella pneumoniae* (n= 3), *Enterococcus faecium* (n= 3), *E. faecalis* (n= 3) and *S. maltophilia* (n =1). Interestingly, all strains were completely inhibited by the concentration of NZMh used, with the exception of one strain of *E. faecium*, which produced a small number of CFUs post-exposure to NZMh. Inoculation of these

surviving colonies in 33 % (v/v) NZMh resulted in confluent growth, consistent with resistance.

4.3 Results: Killing Curves

Log phase bacteria were challenged with 33% (v/v) NZMh (approximately 3 x MIC) and monitored for growth for 24 hours. Kill curves for NZMh against: *P. aeruginosa* PAO1, *E. coli* ATCC 25922, *B. cenocepacia* J2315 and *B. cepacia* ATCC 25416, are shown in Fig 4.1. Experiments were carried out in triplicate and repeated twice. The kill curves show that bacterial killing becomes obvious at 5 hours, with 100% killing achieved by 24 hours. After 24 hours exposure all strains showed 100% killing with the exception of *E. coli*. No *E. coli* was recovered after 36 hours.

4.4 Results: Antibacterial Components of Honey

The antibacterial components of honey have been discussed in detail and include: osmolarity, H_2O_2, pH and phytochemicals (Molan 1992a, 1992b, 1995). For the purpose of the present study each of these putative killing agents was assessed and its importance determined.

Log-phase cultures of bacterial strains outlined in Table 4.1 were inoculated into 100% artificial honey, which comprised high concentrations of sugars with low water content. All strains showed growth in 100% artificial honey suggesting that osmolarity is not solely responsible for the antibacterial activity of honey.

The importance of H_2O_2 in the antibacterial activity of both NZMh and Scottish honey (NH) was determined by attempting to remove all traces of both H_2O_2 and the H_2O_2 generating enzyme glucose oxidase. Diluting honey, to a concentration of 33% (v/v) has been shown to increase H_2O_2. Thus it was important to ensure that this phenomenon did not affect the antibacterial activity (Bang *et al.* 2003). Prior to the assay, all honey preparations were autoclaved to destroy glucose oxidase, and

catalase was added to neutralise any H_2O_2 present. The data suggest that H_2O_2 is important in the antibacterial activity of NH. All strains were killed by a 33% solution, however, killing was inhibited on the addition of catalase (Table 4.2). In contrast, catalase did not affect the antibacterial activity of NZMh against J2315, PAO1 and NCTC 10662. Interestingly, however, ATCC 25416 was able to grow in 33% NZMh in the presence of catalase. Catalase production did not seem to correlate with resistance to NZMh; C1576 and ATCC 25416 exhibit different levels of catalase production but shared identical NZMh MICs (Table 4.1). Exposure to 24 hours of sunlight did not alter killing potential of either honey sample. Light has been previously reported to reduce the antibacterial effect of honey; this was not observed in this study (Table 4.3). Likewise, neutralisation of pH did not alter antibacterial activity (Table 4.3)

Various components of honey were extracted using organic solvents and high performance liquid chromatography (HPLC). Disappointingly, however, individual fractions did not exert antibacterial activity (data not shown) and thus further investigation was discontinued.

4.5 Results: Time-Lapse Phase Contrast Microscopy

Bacteria were observed for 24 hours under phase contrast microscopy in the presence of NZMh at room temperature and in NBYE. Initially, no obvious morphological changes, such as blebbing or filamentation, were observed. However, motility was disrupted and ceased within 6 hours. At 24 hours few intact cells remained and the preparation had been reduced to bacterial debris. In contrast, control cells cultured in NBYE remained intact and motile at 24 hours incubation.

Table 4.1 Catalase production and sensitivity of the Bcc and *P. aeruginosa* isolates to NZMh (% v/v)

Strain	Catalase	MIC for NZMh (% v/v)	MBC for NZMh (% v/v)
J 2315†	+++	10	11
J 2962†	+	11	12.5
J 2742†	++	11	12.5
J 2956†	+/-	12.5	14
C 3159†	-	12.5	14
C 3173†	+/-	11	12.5
C 3175†	-	10	12.5
C 1576†	+++	17	20
ATCC 25416†	-	17	17
PAO1*	+++	9	10
NCTC 10662*	+++	12.5	14

Catalase production was scored according to severity of reaction with H_2O_2 with +++; high, ++; intermediate, +; low, +/-; very low & -; no production. †Bcc Strains; **P. aeruginosa* strains.

Table 4.2 The effects of catalase on the antibacterial activity of honey

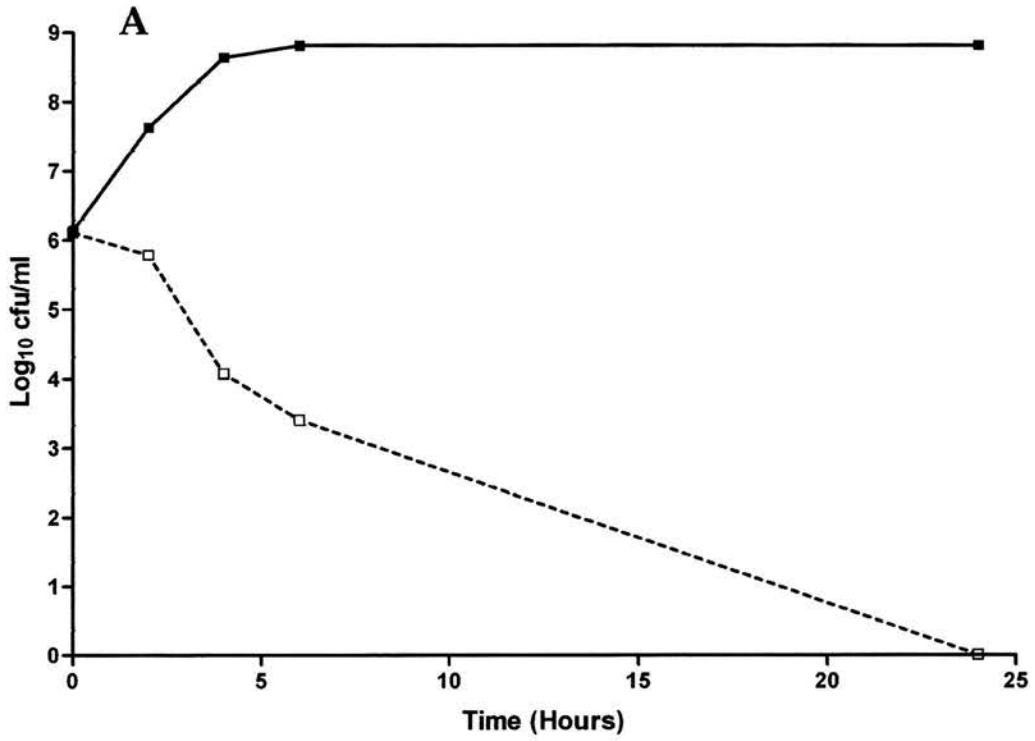
Strain	NZMh	NH	NBYE	NZMh + C	NH + C	NBYE + C
ATCC 25416†	-	-	+++	+++	+++	+++
J 2315†	-	-	+++	-	++	+++
PAO1*	-	-	+++	-	+	+++
NCTC 10662*	-	-	+++	-	++	+++

NZMh, 33% (v/v) New Zealand manuka honey; NH, 33% (v/v) Scottish honey; NBYE, nutrient broth-yeast extract; C, catalase. +++, 500-1000 colonies; ++, 200-500 colonies; + 5-200 colonies; -, no growth. †Bcc Strains; **P. aeruginosa* strains.

Table 4.3 The effects of light and neutral pH on the antibacterial activity of honey

Strain	NZMh + L	NH + L	NZMh (pH 7)	NH (pH 7)	NBYE (control)
ATCC 25416†	-	-	-	-	+++
J 2315†	-	-	-	-	+++
PAO1*	-	-	-	-	+++
NCTC 10662*	-	-	-	-	+++

NZMh, 33% (v/v) New Zealand manuka honey; NH, 33% (v/v) Scottish honey; NBYE, nutrient broth-yeast extract. L, exposure to 24 hours of sunlight prior to use. +++, 500-1000 colonies; ++, 200-500 colonies; + 5-200 colonies; -, no growth. †Bcc Strains; **P. aeruginosa* strains.


A
Log10 cfu/ml
Time (Hours)
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6
7
8
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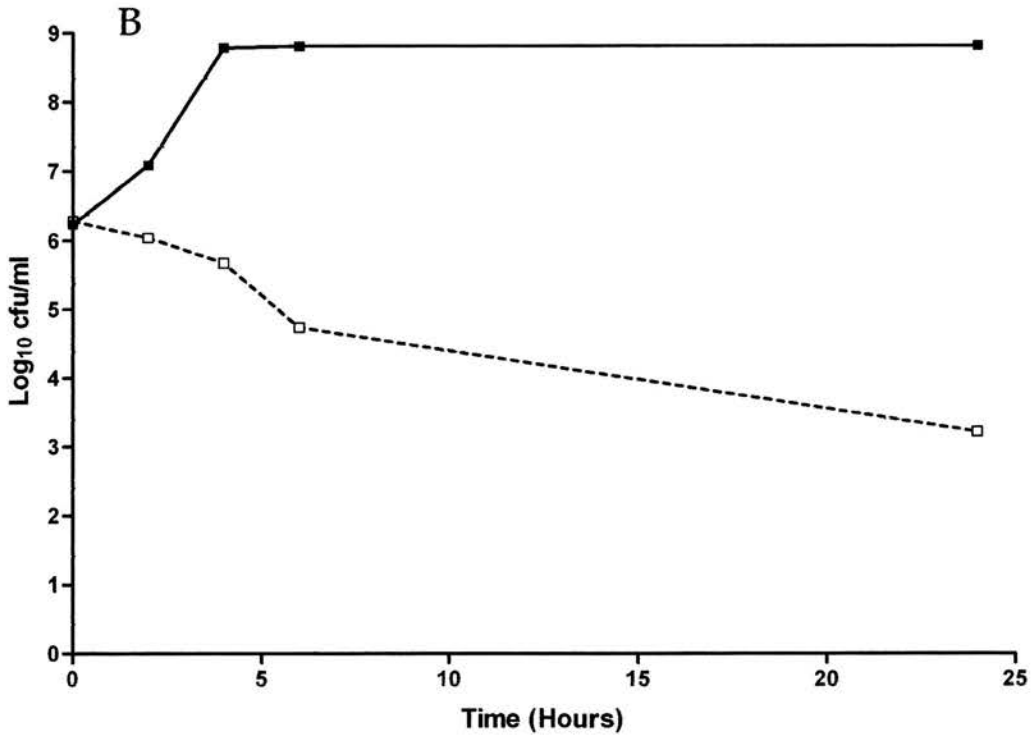
25


B
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Time (Hours)
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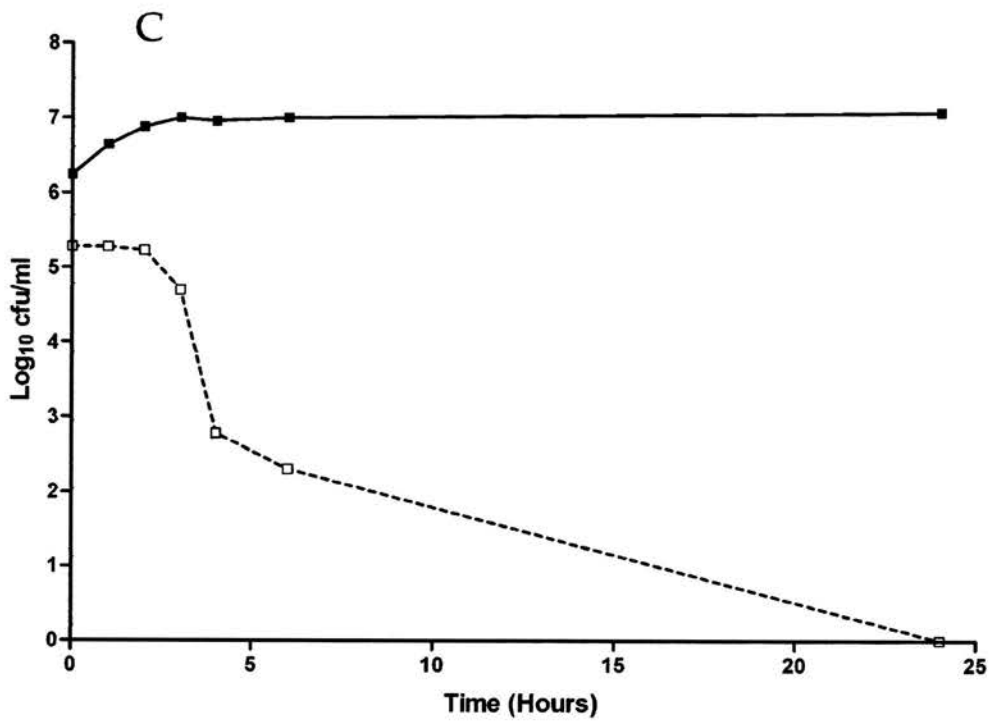
C
Log10 cfu/ml
Time (Hours)
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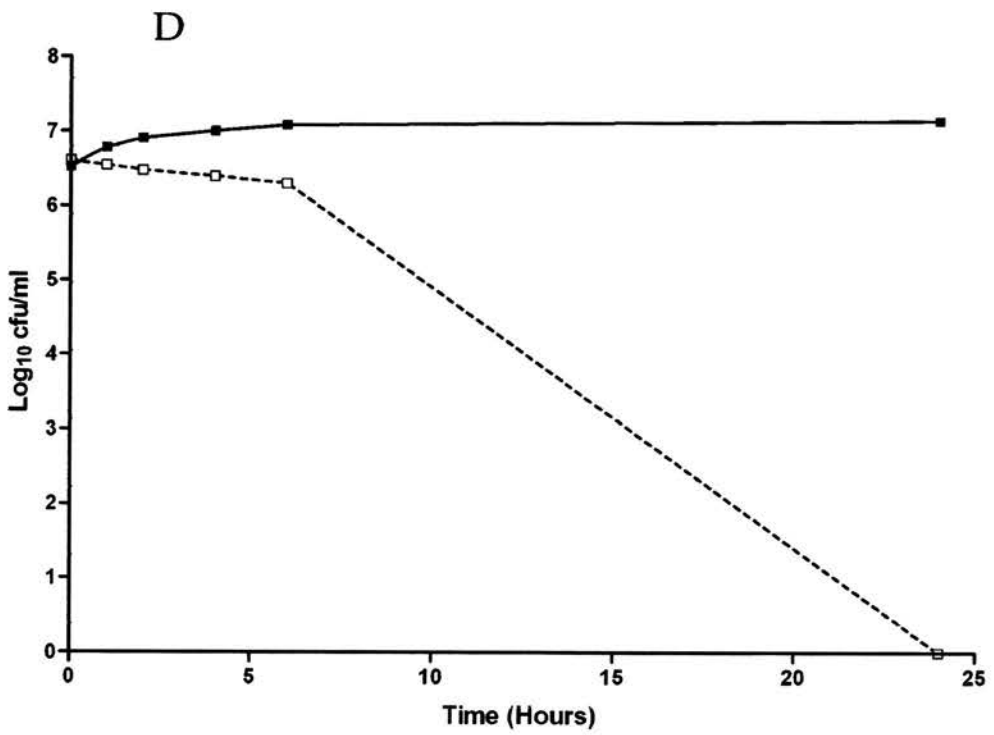
D
Log10 cfu/ml
Time (Hours)
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25

Fig 4.1 Killing Curves. Bactericidal activity of NZMh against: **A**, *P. aeruginosa* PAO1; **B**, *E. coli* ATCC 25922; **C**, *B. cenocepacia* J2315; **D**, *B. cepacia* ATCC 25416. Bacterial cultures were grown to log-phase and inoculated into honey preparations (33% v/v). At time points; designated samples were removed and CFUs determined. Whole lines and closed symbols represent control growth in NBYE. Dotted lines and open symbols represent viability in 33% NZMh.

4.6 Results: *In vivo* Use of NZMh in a CF Patient

A patient infected with the multiresistant, virulent and transmissible *B. cenocepacia* ET-12 lineage was admitted with a respiratory exacerbation. Since the patient had shown little response to conventional antibiotic therapy it was decided to administer NZMh as a last resort.

The initial dose was 4 ml of 33% (v/v) NZMh, nebulised using a ventstream nebuliser and mouthpiece. This is a breath-enhanced nebuliser with a one-valve system and is commonly used for nebulised antibiotics. Despite concomitant administration of bronchodilators, the patient's spirometry fell and he felt short of breath. In addition, the NZMh appeared to be crystallising on the respiratory filter of the nebuliser (Fig 4.3). The second dose of NZMh was at a reduced concentration (25% v/v) and was administered using a sidestream nebuliser (4 ml, once daily). This valveless system reduces the labour of breathing for the patient. On this occasion, the patient reported feeling fine, and no oxygen desaturation was noted. At this point, it was decided to continue with a dose of 25% (v/v) although the mechanism of nebulisation was modified. NZMh was delivered via a mouthpiece as before, but the mouthpiece was inserted into a mask to allow the simultaneous administration of 35% oxygen. After three doses it became apparent that the patient had to work particularly hard to inhale the NZMh, in contrast to the relative ease at which antibiotics, Dnase and bronchodilators were tolerated. However, the latter mechanism of administration appeared to be the most comfortable and the patient was willing to double the daily dose. The patient's temperature appeared to stabilise from day 9 (Fig 4.2), and thus NZMh therapy was continued for a further five days. NZMh appeared to have little effect on sputum production and oxygen saturation (Table 4.4). After several days significant oxygen desaturation was observed (even with the administration of oxygen) and all therapies were stopped. The patient died comfortably three days later.

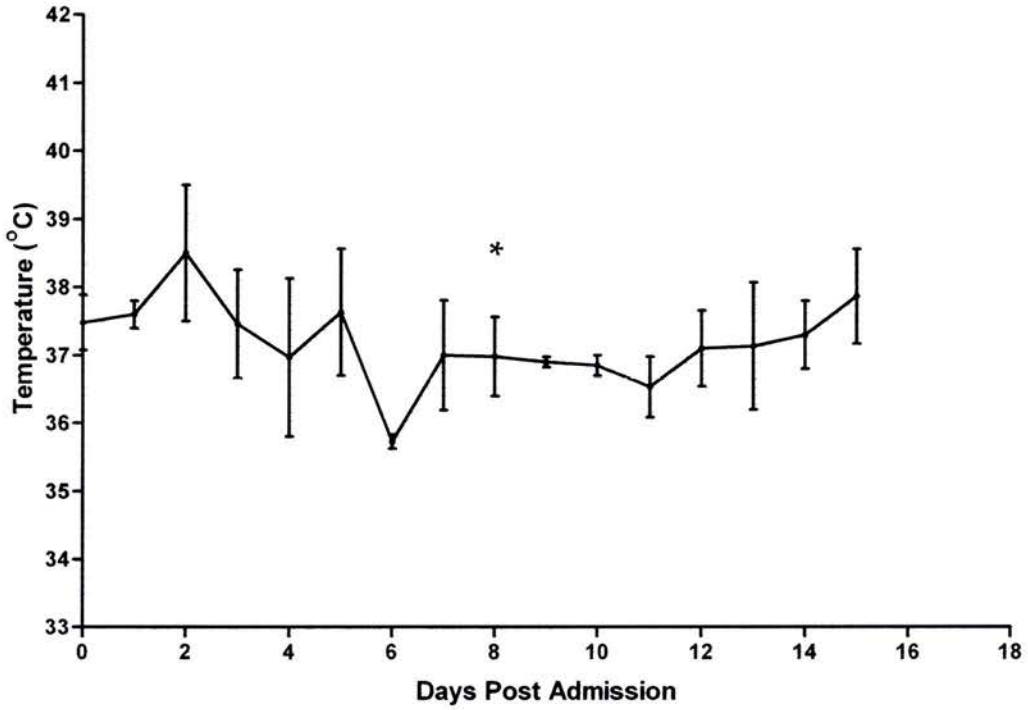


Fig 4.2 Temperature variation in CF patient being treated with NZMh. The error bars denote the range of temperatures noted at daily time points. * denotes the start of NZMh nebulisation.

Table 4.4 Patient details post admission to hospital

Days Post Admission	Honey Concentration (% w/v)	Sputum (g/d)	Oxygen Saturation (%)
6	0	175	95
7	0	265	93
8	33	100	93
9	25	194	91
10	25	200	93
11	25	199	93
12	25	87	90
13	25	138	96
14	25	175	NM
15	25	109	NM

Oxygen saturation highlighted in red denotes use of air, blue denoted use of oxygen. g/d; grams/day. NM: not measured.

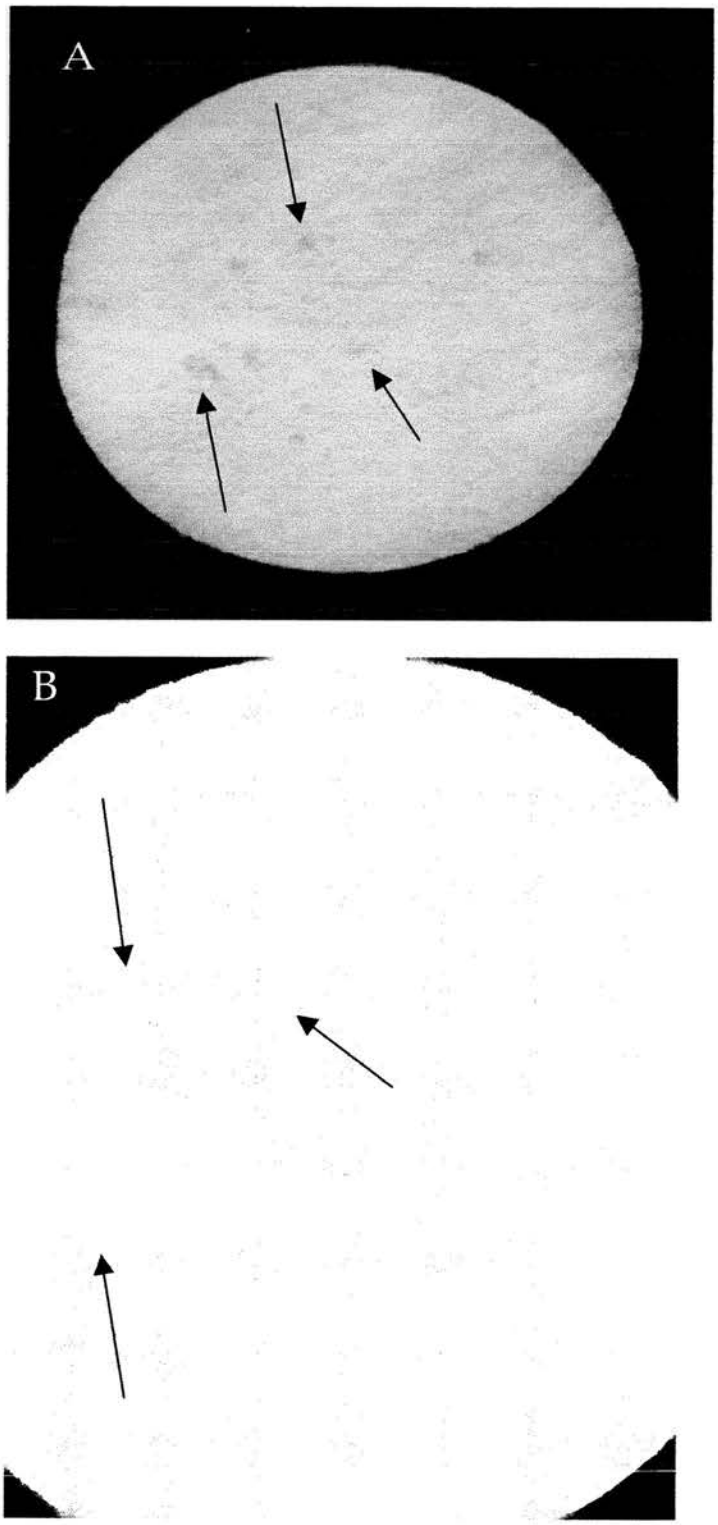


Fig 4.3 Honey crystal formation on respiratory filter during nebulisation.
The arrows indicate the honey crystals. **A**, respiratory filter with crystallised honey residue; **B**, respiratory filter x 10.

4.7 Discussion

These data demonstrate the broad antimicrobial spectrum of NZMh, which covers Gram-positive and Gram-negative bacteria and individual isolates exhibiting multiresistance to conventional antibiotics. This activity has considerable implications for therapy as multiresistant infections become more widespread. It is interesting to note that resistance to conventional antibiotics does not confer resistance to NZMh. Indeed, *B. cenocepacia* J2315, arguably the most resistant member of the Bcc to conventional antibiotics, was amongst the most sensitive strains to NZMh. In contrast, the *B. multivorans* epidemic strain C1576 and *B. cepacia* type strain ATCC 25416 were the most resistant to NZMh. These data suggest that conventional resistance mechanisms, including MDR efflux systems, do not play a significant role in resistance to NZMh. This hypothesis is supported by the fact that although resistance was observed, it is a rare event. Resistance of *E. faecium* strain was observed and appeared to be stable. Inherent resistance was not identified in other *Enterococci* spp., and so does not appear to be widespread even in this species.

The antibacterial components of honey have been well studied, but no definitive antibacterial factor has been identified. Weston *et al.* (2000) discounted the effects of osmolarity, pH and phytochemicals, and stated that activity was due solely to H_2O_2. In some respect, the data presented in this thesis support these findings. The effect of osmolarity was reduced by autoclaving, and removing much of the caramelised sugars from honey samples. A control artificial honey had little effect on bacterial growth. Likewise, neutralising the pH did not affect the killing potential of honey. However, the role of H_2O_2 is more complex. For example, the data show that resistance does not correlate with high level of bacterial catalase production. Furthermore, glucose oxidase is sensitive to heat, thus autoclaving will destroy the enzyme. Cumulatively, addition of catalase and the destruction of glucose oxidase did not inhibit the antibacterial activity of NZMh against *B. cenocepacia* J2315, *P. aeruginosa* PAO1 and NCTC 10662. In contrast, NH was unable to exert bactericidal activity. This finding suggests that either H_2O_2 levels are higher in NZMh than in NH and therefore the treatments did not reduce it significantly, or that

important factor in NH samples. Furthermore, the retention of antimicrobial activity after autoclaving offers a safe alternative to the use of gamma irradiation in sterilisation of honey preparations for therapeutic purposes (Molan and Allen 1996).

HPLC was unable to provide a suitable candidate for the "UMF" as antibacterial activity was not observed for individual fractions. Since no antibacterial activity was noted in any of the 20 fractions attempts to isolate an antimicrobial factor in this way were discontinued. Other extensive investigations described in the literature have attempted to elucidate the unique antibacterial factor in honey by a number of chemical techniques. To date, no study has provided a definitive antibacterial factor (Mato *et al.* 2003, Suarez-Luque *et al.* 2002, Suarez-Luque *et al.* 2002).

From the data presented in this thesis, it appears that the antibacterial activity of NZMh involves a complex synergism between H_2O_2 and plant derived chemicals. This is supported to some extent by batch variation- a fundamental problem in studies of the antibacterial activity of NZMh is batch-to-batch variation. Despite attempts to over come this problem, clinical efficacy may be difficult to predict. Explanations for variation in potency include flower source, season of harvest and the position of the hive in the farm (Molan 1992b). Certain NZMhs are marketed as having standardised potency: this was not supported by the data in this thesis.

The use of NZMh in an *in vivo* model was a unique opportunity to contribute to the treatment of a CF patient. The patient presented with a respiratory exacerbation and late stage "cepacia-syndrome" was suspected. Response to conventional treatments was also minimal. Although the patient eventually succumbed to *B. cenocepacia* infection, valuable lessons can be learned from this compassionate use of NZMh, including optimal dose and drug delivery
Interestingly, the patient showed an improvement during treatment with honey in combination with antibiotics and bronchodilators, including reduction in pyrexia. Honey has been shown to exert effects on the immune system and influence cytokine levels (Abuharfeil *et al.* 1999, Tonks *et al.* 2001). Therefore, it has been speculated

that NZMh may have a positive clinical effect by reducing the inflammatory response, and hence fever.

The patient was at end stage disease, pre-organisation of treatment was minimal, and valuable data including sputum microbiology and inflammatory markers were not observable. Earlier intervention may have prompted a more significant improvement and allowed more in-depth pre-planning and analysis before initiation of therapy. Nevertheless, the study was challenging and provided a unique insight into the application of a novel therapy in a clinical setting.

During this study, a paper was published reporting the susceptibility of multiresistant strains of "*B. cepacia*" to honey (Cooper *et al.* 2000). Despite reporting significant sensitivity among 20 "*B. cepacia*" strains isolated from CF sputa, the authors failed to take account of the diversity and strain variation within the Bcc. No distinction was made between individual Bcc genomovars or strains. The authors also failed to mention possible clonality between the clinical isolates used. Thus, although the study was encouraging, it is difficult to draw any firm conclusions. Cooper *et al.* (2000) also compared data from a previous publication (Cooper and Molan 1999b) and concluded that "*B. cepacia*" was more sensitive to honey than *P. aeruginosa*. Unfortunately, the authors fail to take in account batch variation of honey samples.

The potential use of a honey as a therapeutic agent in CF can be considered to be two-fold. Not only are CF-associated bacteria, including multiresistant epidemic strains, sensitive to the antibacterial activity of honey but also high osmotic solutions may alter local physiological conditions within the upper airway. It is hypothesised that in CF airways, isotonic hyperabsorption of ASL, in the absence of CFTR, results in diminished ASL volume, low mucus transport and mucus plug accumulation to which bacteria can adhere (Boucher 1999, Matsui *et al.* 1998). An alternative hypothesis suggests that an increase in salt concentration in ASL inhibits antimicrobial defensins resulting in defective bacterial killing (Goldman *et al.* 1997, Smith *et al.* 1996). In either case, osmolytes which would draw water into the airway lumen may offer an alternative therapy (Tarran *et al.* 2001). The use of hyperosmotic agents has recently been reviewed (Hirsh 2002). Several studies have

highlighted the potential use of non-ionic osmolytes. The carbohydrate, mannitol, is an osmotic agent that has been used to increase mucociliary clearance in several respiratory conditions including: asthma (Daviskas *et al.* 1997), bronchiectasis (Daviskas *et al.* 1999, Daviskas *et al.* 2001), mucociliary dysfunction (Daviskas *et al.* 2002) and CF (Robinson *et al.* 1999). Administered as a dry powder, mannitol is well tolerated and appears to be as effective as hypertonic saline (HS) (Robinson *et al.* 1997). Mannitol may also be retained in the lung for longer as it is less permeable than Na^+ and Cl^- (Yankaskas *et al.* 1987). The therapeutic use of HS is disputed and appears to be less effective than recombinant human deoxyribonuclease (rhDNase) (Suri *et al.* 2001). Furthermore, HS is ionic and may inhibit the action of salt-sensitive defensins. Interestingly, although use of mannitol in CF improved mucociliary clearance, the authors observed a fall in forced expiratory volume in one second (FEV_1) immediately after administration of both HS and mannitol despite premedication with a bronchodilator. Data presented in this thesis also highlighted a fall in FEV_1 during and after administration of NZMh. Mucociliary clearance may be assisted by the fact that mannitol has been shown to stimulate mucus secretion in an *in vivo* animal model (Kishioka *et al.* 2003). Alternatively, amiloride, which blocks uptake of salt and water across the airway epithelium, has been used as a strategy to enhance the degree of hydration and dilute the composition of CF ASL (Tomkiewicz *et al.* 1993). A five-carbon sugar, xylitol, has been shown to have low transepithelial permeability, be poorly metabolised by bacteria, lower the concentration of salt in CF ASL, and increase the volume of ASL (Zabner *et al.* 2000). The main problem of aerosolised osmolyte therapy concerns the difficult in achieving a uniform distribution of aerosol, particularly in the lung periphery (Daviskas *et al.* 1999).

Irrespective of the outcome of the pathophysiological debate that surrounds CFTR and the CF lung, the use of honey and osmolytes may have therapeutic benefits (Zabner *et al.* 2000). A potential osmolyte would require five important characteristics. First, it must be non-ionic. Second, the compound must have low transepithelial permeability. Third, the osmolyte must not be readily metabolised by bacteria. Fourth, the compound must well-tolerated in humans. Fifth, because

defensins kill rapidly, the osmolyte does not have to be retained in the lung for long periods as transient exposure may reduce salt significantly. Interesting, honey in general and NZMh in particular, fulfils these criteria providing a high osmolar solution capable of rehydrating the airway with broad-spectrum antibacterial activity. In future, NZMh may prove to be an important therapeutic agent either individually or in combination with conventional therapies.

5.1 Aims

The aim of this study was to investigate the bacteriophages of the Bcc, in terms of lysogeny and the presence of lytic phages in natural environments. Bcc phages have received little attention, although lysogeny had been reported previously (Cihlar *et al.* 1978, Matsumoto *et al.* 1986, Nzula *et al.* 2000). Lytic Bcc phages, from soil and the plant rhizosphere, had not been described previously. The aim was to isolate a panel of Bcc phages and determine their host range against the Bcc, *Burkholderia* and *Pseudomonas* species.

A further aim was to clone, recombinantly express, purify, characterise and determine the antibacterial activity of an endolysin from the Bcc bacteriophage (Bcep781), and from the *P. aeruginosa* serotype converting phage D3, using DNA sequence data from the published genomes.

The work in the next three sections was carried out in collaboration with Dr Dervla Kenna (Medical Microbiology, University of Edinburgh).

5.2 Results: Lysogeny Within the *B. cepacia* Complex

Of the 21 Bcc strains that were investigated, 10 strains (*B. cepacia*: ATCC 25416, ATCC 17759; *B. multivorans*: C3161, C1576, C1962 and C3163; *B. cenocepacia*: J2315, C3166, and C3170; *B. stabilis*: C3174) were found to be lysogenized. These provided 14 temperate phages (DK1-4 and MM1-10) (Table 5.1). The *P. aeruginosa* temperate bacteriophages D3 and F116L were used as turbid plaque positive controls. Polylysogeny was noted for four strains J2315, J673, J675 and C3170.

5.3 Results: Isolation of Environmental Bacteriophage

Samples were collected from a variety of natural environments, and retained at room temperature until they could be investigated further. Five virulent phages (JB1, JB3, JB5, RL1c and RL2) were isolated from soils and from the rhizosphere of various plants. Most positive samples included decayed plant material collected from moist environments, but phages were also isolated from dry soils.

5.4 Results: Host Range of *B. cepacia* Complex Bacteriophage

The host range of the newly-isolated phages identified in both the lysogeny experiments and the environmental screen was determined. Individual phages were tested against a preliminary host panel comprising the 21 Bcc isolates used in the lysogeny screen. This information was then used in conjunction with *Hind*III RFLP profiles of each phage to confirm that distinct phages were accumulated. The RFLP profiles also provided approximate genome sizes (Table 5.2). If two or more shared the same host range, or RFLP pattern, only one was used for further study. An exception was made for phages RL1c and RL1t, which shared the same host range and RFLP pattern but produced different plaque morphologies: clear plaques in the case of the virulent phage (RL1c) and turbid plaques in the case of the RL1t (Fig 5.1 & Table 5.2). Interestingly, a similar host range and RFLP profile was observed for both temperate phages DK2 and DK3, which had been isolated from *B. cenocepacia* C3166 and *B. stabilis* C3174 respectively. As previously observed for other "*B. cepacia*" phage, none of the newly identified phages were inactivated by treatment with the organic solvent chloroform.

The host range of phages NS1, NS2 (Nzula *et al.* 2000) and eight novel phages (JB1, JB3, JB5, DK1, DK3, RL1c, RL1t and RL2) was determined against an enlarged bacterial host panel comprising 66 Bcc isolates and 55 isolates representing other pseudomonads. The Bcc host panel included examples of each Bcc species, however, only 1 strain of *B. dolosa* and *B. ambifaria* was available for study. Additionally, in collaboration with Dr Ty Pitt (Health Protection Agency, Colindale, London, UK), the Bcc phages were tested against 40 *B. pseudomallei* strains.

The host range of individual phages included multiple Bcc species (Table 5.3). Interestingly, no phage activity was detected against the single representatives of *B. dolosa* and *B. ambifaria*. However, within each Bcc species, there was wide variation in susceptibility to an individual phage. The following paragraph describes the species and number of strains within a species that acted as hosts for each phage.

Phage **JB1**: *B. cepacia* (n=1); *B. cenocepacia* (n=3); *B. stabilis* (n=1); *B. vietnamiensis* (n=1); *B. anthina* (n=3); *B. pyrrocinia* (n=6); *B. ubonensis* (n=1). Phage **JB3**: *B. multivorans* (n=1); *B. cenocepacia* (n=2); *B. anthina* (n=2); *B. pyrrocinia* (n=1); *B. ubonensis* (n=1). Phage **JB5**: *B. cepacia* (n=1); *B. cenocepacia* (n=4); *B. vietnamiensis* (n=2); *B. anthina* (n=3); *B. pyrrocinia* (n=5). Phage **DK1**: *B. multivorans* (n=1); *B. cenocepacia* (n=3); *B. vietnamiensis* (n=1); *B. pyrrocinia* (n=1). Phage **DK3**: *B. cepacia* (n=1); *B. multivorans* (n=1); *B. cenocepacia* (n=3); *B. stabilis* (n=1); *B. vietnamiensis* (n=1). Phage **NS1**: *B. cepacia* (n=1); *B. multivorans* (n=1); *B. cenocepacia* (n=4); *B. stabilis* (n=1); *B. vietnamiensis* (n=1); *B. anthina* (n=2); *B. pyrrocinia* (n=2); Genomovar unclassified (n=1). Phage **NS2**: *B. cepacia* (n=1); *B. multivorans* (n=1); *B. cenocepacia* (n=9); *B. vietnamiensis* (n=2); Genomovar unclassified (n=1). Phages **RL1c/RL1t**: *B. cepacia* (n=1); *B. multivorans* (n=1); *B. cenocepacia* (n=2); *B. stabilis* (n=1); *B. vietnamiensis* (n=2); *B. anthina* (n=1). Phage **RL2**: *B. cenocepacia* (n=6); *B. vietnamiensis* (n=1); *B. anthina* (n=2); *B. pyrrocinia* (n=4); *B. ubonensis* (n=1).

Amongst the Bcc species used as potential hosts *B. multivorans* appeared to be least susceptible to the phages investigated: of the nine *B. multivorans* strains examined, only strain C2775 showed susceptibility. As has been previously observed for phages NS1 and NS2, the host range of some phages was not restricted to the Bcc. *P. aeruginosa* strains C1546 and J2852 were susceptible to phage JB3, and *B. gladioli* strain C3654 was susceptible to phages NS2, DK1, RL1c/RL1t and JB5. Interestingly, NS2 was found to be the only phage active against *B. cenocepacia* J2315. NS2 was also active against 13 of the 40 *B. pseudomallei* strains tested. All other pseudomonads investigated were not able to act as hosts for Bcc phages.

Although there may be species bias with respect to environmental and clinical isolates investigated, there does not appear to be a significant correlation between the environment of isolation and bacteriophage sensitivity.

Table 5.1 Lysogenic Bcc and their associated bacteriophage

Species	Lysogenic Bcc Strain	Temperate Phage
B. cepacia	**ATCC 17759/J675**	**MM1**
B. multivorans	C3161	MM2
B. cenocepacia	C3170	MM3
B. cepacia	**ATCC 25416/J673**	**MM4**
B. multivorans	C3163	MM5
B. cenocepacia	**C3170**	**MM6**
B. cepacia	**ATCC 17759/J675**	**MM7**
B. cenocepacia	**J2315**	**MM8**
B. multivorans	C1576	MM9
B. multivorans	C1962	MM10
B. cepacia	**ATCC 25416/J673**	**DK 1**
B. cenocepacia	C3166	DK 2
B. stabilis	C3174	DK 3
B. cenocepacia	**J2315**	**DK4**

Polylysogenic strains are indicated in red

Table 5.2 Bacteriophage genome size, plaque morphology and source

Phage	Phage genome size (bp)	Source	Plaque Morphology
JB1	40,000	Soil, The Caribbean.	Turbid Plaques, variable size (0.5-1 mm in diameter)
JB3	34, 000	Plant rhizosphere, U.K.	Clear Plaques (1 mm in diameter)
JB5	40,000	Plant rhizosphere, U.K.	Turbid Plaques, variable size (0.5-1 mm in diameter)
DK1	45,900	Lysogeny	Turbid Plaques, variable size (0.5-1 mm in diameter)
DK2/DK3	25,800	Lysogeny	Turbid Plaques (1 mm in diameter)
NS1	48,000*	Lysogeny	Turbid Plaques, variable size (0.5-1 mm in diameter)
NS2	48,000*	Lysogeny	Semi-clear plaques, variable size (0.5-1 mm in diameter)
RL1c/RL1t	44,300	Plant rhizosphere, U.K.	RL1c, clear, lytic plaques (1 mm in diameter) RL1t, turbid plaques (1 mm in diameter)
RL2	40,000	Pond sediment, U.K.	Semi-clear plaques, variable size (0.5-1 mm in diameter)

Table 5.3 Host range of bacteriophages within the *B. cepacia* complex

Species name	JB1	JB3	JB5	DK1	DK3	NS1 ¥	NS2 ¥	RL1t/ RL1c	RL2
B. cepacia									
ATCC 25416*§	-	-	-	-	-	-	-	-	-
C2970	+	-	+	-	-	+	+	+	-
C3159§	-	-	-	-	-	-	-	-	-
ATCC 17759*§	-	-	-	-	+	-	-	-	-
B. multivorans									
C2775	-	+	-	+	+	+	+	+	-
C3161*§	-	-	-	-	-	-	-	-	-
C3162§	-	-	-	-	-	-	-	-	-
C3163*§	-	-	-	-	-	-	-	-	-
C3164§	-	-	-	-	-	-	-	-	-
ATCC 17616	-	-	-	-	-	-	-	-	-
C1962*§	-	-	-	-	-	-	-	-	-
C1576*§	-	-	-	-	-	-	-	-	-
C3160§	-	-	-	-	-	-	-	-	-
B. cenocepacia									
J415	-	-	-	+	-	-	+	-	+
J2315*§	-	-	-	-	-	-	+	-	-
C1394	-	-	-	-	+	-	+	-	+
J2956	+	+	+	-	+	-	-	+	+
C2836	-	-	+	-	-	+	+	-	+
C3165	-	-	-	-	-	+	+	-	-
C3166*§	-	-	-	-	-	-	+	-	-
C3167§	-	-	-	-	-	-	-	-	-
C3168§	-	-	-	-	-	-	-	-	-
C3169	+	+	+	+	+	+	+	-	+
C3170*§	-	-	-	-	-	+	+	-	+
ATCC 17765	+	-	+	+	-	-	+	+	-
B. stabilis									
C3171	-	-	-	-	-	+	-	+	-
C3172	+	-	-	-	-	-	-	-	-
C3173§	-	-	-	-	+	-	-	-	-
C3174*§	-	-	-	-	-	-	-	-	-
B. vietnamiensis									
C2978§	-	-	-	-	-	+	+	+	-
C3175§	-	-	+	-	-	-	-	-	-
C3176§	-	-	-	-	-	-	+	-	-
C3177§	+	-	+	+	+	-	-	+	+
B. dolosa									
E12	-	-	-	-	-	-	-	-	-

B. ambifaria									
J2742	-	-	-	-	-	-	-	-	-
B. anthina									
J2552	-	-	-	-	-	-	-	-	-
J2553	-	-	-	-	-	-	-	-	-
J2863	-	-	-	-	-	-	-	-	-
J2927	-	-	-	-	-	-	-	-	-
J2928	-	-	-	-	-	-	-	-	-
J2941	-	-	-	-	-	-	-	-	-
J2943	-	-	-	-	-	-	-	-	-
J2944	-	-	-	-	-	-	-	-	-
J2945	-	-	-	-	-	-	-	-	-
J2946	-	-	-	-	-	-	-	-	-
J2949	-	-	-	-	-	-	-	-	-
J2950	-	-	-	-	-	-	-	-	-
J2951	+	-	+	-	-	+	-	-	+
J2862	-	-	-	-	-	-	-	-	+
C1658	+	+	+	-	-	-	-	-	-
C1765	+	+	+	-	-	+	-	+	-
B. pyrrocinia									
J2536	-	-	-	-	-	-	-	-	-
J2542	-	-	-	-	-	-	-	-	-
C1469	-	-	-	-	-	-	-	-	-
C3909	+	-	+	-	-	-	-	-	-
C3918	+	-	+	+	-	+	-	-	+
C3928	-	-	-	-	-	-	-	-	-
C3930	+	-	+	-	-	+	-	-	+
C3993	+	-	+	-	-	-	-	-	+
C3995	+	-	+	-	-	-	-	-	+
C3997	+	+	-	-	-	-	-	-	-
B. ubonensis									
E26	-	-	-	-	-	-	-	-	+
E27	-	-	-	-	-	-	-	-	-
E571	-	-	-	-	-	-	-	-	-
E551	+	+	-	-	-	-	-	-	-
Genomovar unclassifiable									
J2540	-	-	-	-	-	+	+	-	-

-, lack of sensitivity to phage; +, < 10 plaques at phage inoculum site; ++, >10 plaques at phage inoculum site; +++, confluent lysis at phage inoculum site

§ Strains used in lysogeny experiments. * Lysogenic strains

¥ (Nzula *et al.* 2000)

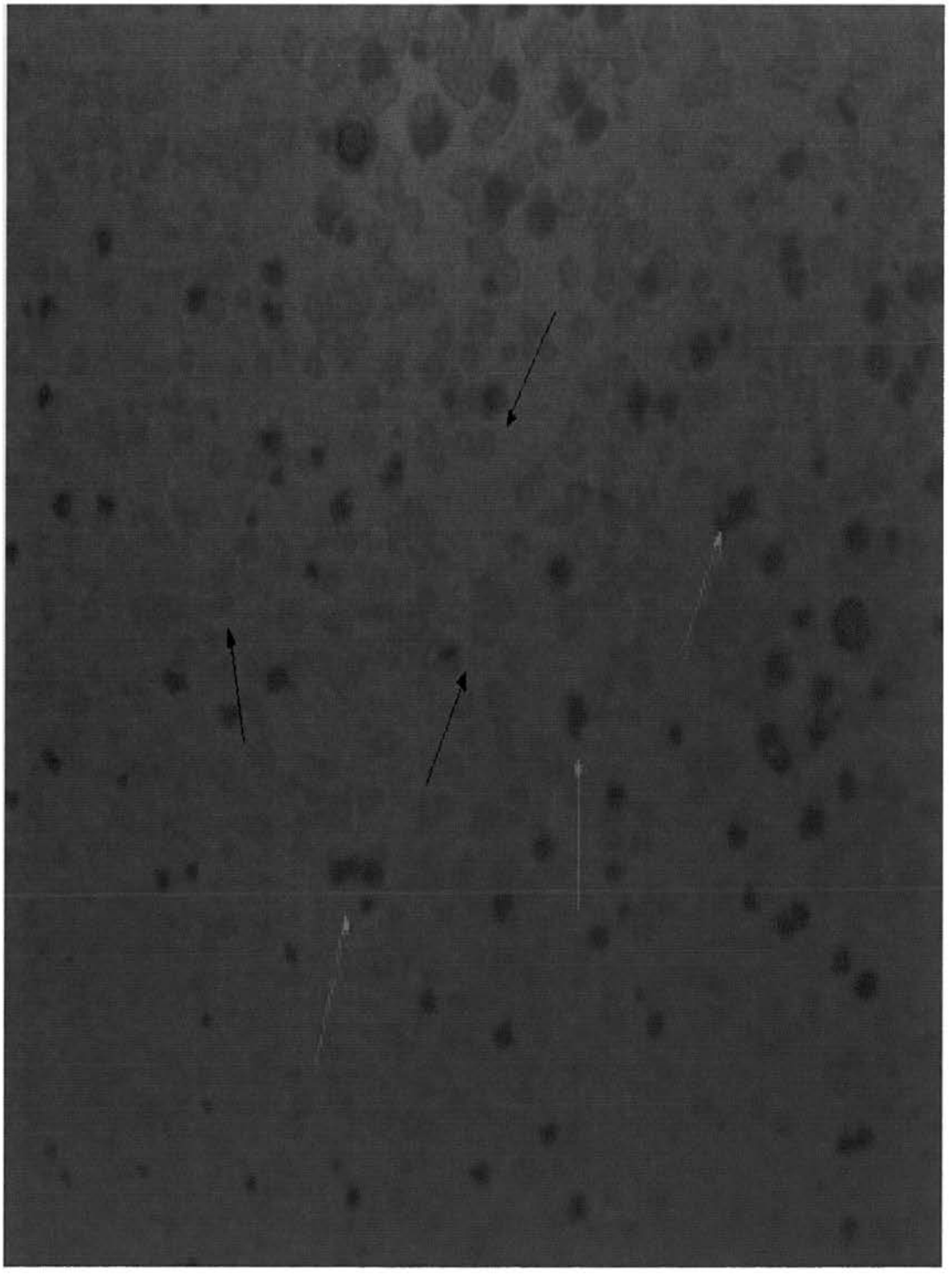


Fig 5.1 Plaque morphology of RL1c and RL1t. RL1c (wild-type phage) clear plaques indicated by red arrows. RL1t (mutant phage) turbid plaques indicated by black arrows.

5.5 Results: Electron Microscopy

Electron microscopy was used to identify the structural morphology of the Bcc phages. In accordance with phage taxonomy (Ackermann 2001, Ackermann and Krisch 1997); phages JB1, JB5, DK2/DK3, DK4, RL1c/RL1t and RL2 could be described as possessing hexagonal heads and contractile tails of variable length, characteristic of T-even-like phages (Fig 5.2). Such phages remain unclassified but can be placed in the type genus of the *Myoviridae* family. In contrast, phages JB3 and DK1 were revealed to have the hexagonal heads and flexuous, non- contractile tails characteristic of lambda-like phages (Fig 5.2). Such phages remain unclassified but can be placed in the type genus of the *Siphoviridae* family.

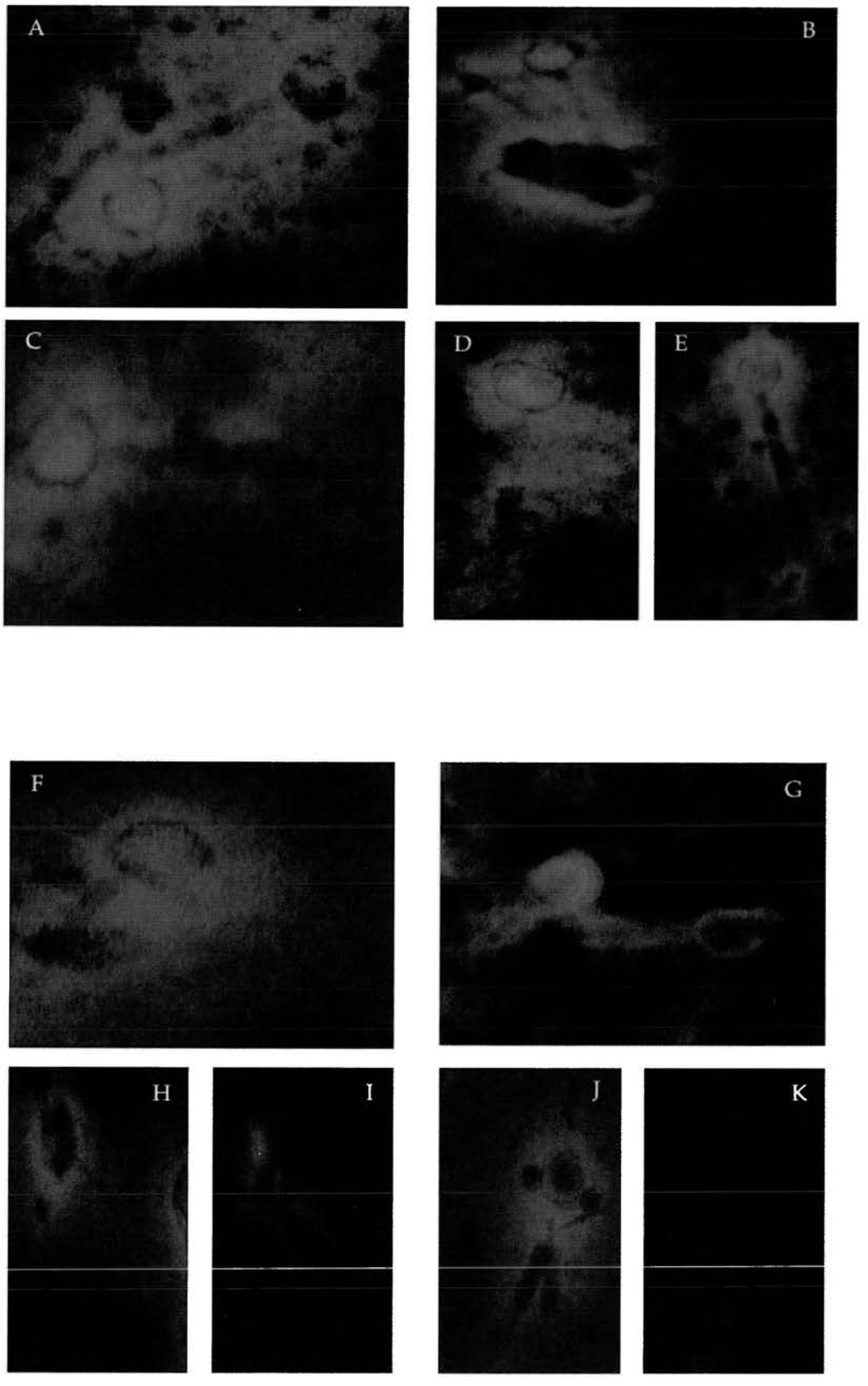

A
B
C
D
E
F
G
H
I
J
K

Fig 5.2 Electron-micrographs of Bcc bacteriophages: **A.** JB1, **B.** JB3, **C.** JB4, **D.** JB5, **E.** RL1c, **F.** RL1t, **G.** RL2, **H.** DK1, **I.** DK2, **J.** DK3, **K.** DK4 (Magnification x 200,000).

5.6 Discussion

The data presented show that lysogeny is relatively common in Bcc isolates and demonstrate that virulent Bcc bacteriophages are present in the natural habitats of these bacteria. Interestingly, the host range of the Bcc phages was not species-specific and, in some cases (for example phage JB1), included the majority of Bcc species presently known. These data are in agreement with the earlier study by Nzula *et al.* (2000) on the Bcc phages NS1 and NS2. This broad host range, which in some cases extended to the related pseudomonads *P. aeruginosa* and *B. gladioli*, is unusual. With the exception of unusual phages such as the plasmid-like 'phasmid' P4 (Gutmann *et al.*1990), the host range of most phages is species specific. In terms of biodiversity of the Bcc and scope for horizontal gene transfer it is also potentially important.

The method for detecting lysogeny can also be utilised to identify bacteriocin production. There was however, no apparent bacteriocin production and activity in the panel of Bcc investigated. This finding is in agreement with previous studies which highlight the low prevalence of bacteriocinogeny in "*B. cepacia*" (Govan and Harris.1985).

A broad host range for Bcc bacteriophages could contribute to genomic plasticity and biodiversity of these bacteria, and their evolution from highly metabolically active soil saprophytes to plant, human and animal pathogens. The data show that there is little correlation between phage sensitivity and whether isolates were isolated clinically or from the natural environment suggesting that prophages did not contribute significantly to virulence. Of particular interest was the finding that the biopesticide strain *B. ambifaria* (J2742: AMMD) was not susceptible to any of the Bcc bacteriophages investigated, a factor which could support its commercial use (Parke and Gurian-Sherman.2001).

Lysogenic conversion and transduction are important processes by which chromosomal host genes can be acquired and exchanged between bacteria (Canchaya

et al. 2004). *In vitro* studies have shown that antibiotic resistance genes can be transferred between *B. vietnamiensis* strains by phages NS1 and NS2 (Nzula *et al.* 2000). In relation to the therapeutic use of Bcc phages, their broad host range poses a potential problem. The MIC data presented previously highlight the variation in antibiotic resistance within the Bcc. Bcc phages appear to be promiscuous and thus the potential for horizontal gene transfer within and between Bcc species needs to be considered. This applies in particular to the data showing that Bcc phages can use *P. aeruginosa* and *B. pseudomallei* as bacterial hosts. The fact that Bcc phages can use the highly virulent *B. pseudomallei* as hosts is perhaps not surprising given their close taxonomic relationship within the Palleroni 'RNA II' group. The role of *P. aeruginosa* as hosts is more surprising given the taxonomic 'distance' between Bcc and *P. aeruginosa*. Any therapeutic benefit for the Bcc phages may be lost by the potential spread of resistance genes between pseudomonad species. Furthermore, as reported for *Shigella flexneri* (Allison and Verma 2000), *P. aeruginosa* (Kropinski 2000) and the *B. cepacia* complex (Kenna *et al.* 2003), phages can modulate bacterial virulence by contributing to O-antigen modification pointing to a potential role for Bcc LPS as a potent virulence determinant. This possibility is even more significant following recent evidence for the important role of Bcc LPS in cytokine induction and its unique biological and structural properties (Hughes *et al.* 1997, Shaw *et al.* 1995, Shimomura *et al.* 2003, Zughaier *et al.* 1999). The potential for Bcc phages to encode pathogenic factors has recently been confirmed (Summer *et al.* 2004). The authors describe a *B. cenocepacia* phage BcepMu, which carries amongst other factors, a LPS modification acyltransferase.

Opportunities for transduction and lysogenic conversion within the Bcc would not only exist in natural environments shared by various Bcc species and related bacteria, but also in CF airway secretions, where mixed infections of *P. aeruginosa* and Bcc are frequent and bacterial populations can reach densities in excess of 10^9 cfu/ml. Additionally, the presence of bacteriophages in sputum has been reported (Ojeniyi *et al.* 1991, Tejedor *et al.* 1982).

Based on the close taxonomic relationship and shared insertion sequences and environmental habitats, the observation that NS2 can utilise *B. pseudomallei*, the causative agent of melioidosis and a potential agent for bioterrorism (Mack and Titball 1998), as a propagating host is particularly significant. The potential importance of broad-host-range phages such as NS2 is also highlighted by recent reports of *B. pseudomallei* infection in CF patients (including coinfection with Bcc species) following travel to Thailand where melioidosis is endemic (Schulin and Steinmetz 2001, Visca *et al.* 2001).

The shared host range of DK2 and DK3 isolated from *B. cenocepacia* and *B. stabilis* respectively, supports the host range studies and suggests that integration of the same phage can occur in different species within the Bcc. While the presence of multiple prophages within a single strain is common in *P. aeruginosa* (Holloway *et al.* 1960), polylysogeny appeared relatively rare for Bcc species.

The turbid and lytic plaque morphology exhibited by the otherwise similar (host range and restriction profile), environmental phages RL1c and RL1t was not observed during the initial sample screen, and has not been reported previously for Bcc phages. This variable plaque morphology was observed in early phage studies and explained by the development of host range mutants (Luria 1947). These mutant phages are able to overcome phage resistance because of changes in the structure of the adsorption 'adhesin' on the phage. This structural alteration allows the phage to re-adsorb to bacteria resistant to the wild type phage. Normally, such mutant phages demonstrate an extended or curtailed host range, but this was not observed for bacteriophage RL1. The co-existence of RL1c (wild-type) and RL1t (mutant) bacteriophage in the same population illustrates the ongoing mutational equilibrium between Bcc phage and host, a phenomenon first recognised for the classic *E. coli* phage T2 (Delbruck 1945, Hershey 1946).

All "*B. cepacia*" bacteriophages identified to date [Bcep 1, 43, 781, Mu; CP1 (Cihlar *et al.* 1978) and CP75 (Matsumoto *et al.* 1986); NS1 and NS2 (Nzula *et al.* 2000); phage-42 (Ackermann and Krisch 1997)] have been shown to be morphologically

similar to T-even like bacteriophages. Likewise, the majority of Bcc phages identified in this study were morphologically similar to previously identified Bcc phages. However, lambda-like Bcc phages were also observed in this study.

The relative lack of susceptibility shown by *B. multivorans* to any of the phage panel is interesting. Together, *B. multivorans* and *B. cenocepacia* account for over 90% of clinical isolates of the Bcc (LiPuma *et al.* 2001); however, in contrast to *B. cenocepacia*, *B. multivorans* is rarely isolated from natural environments (Bevivino *et al.* 2002). The data presented here show that of the nine *B. multivorans* strains tested (including the Glasgow epidemic strain C1576), only one strain (C2775) exhibited phage susceptibility. However, further studies have tentatively identified a novel Bcc phage from soil (RU2) which plates on *B. multivorans* C3164, as well as three other strains (J2552 and J2553, both *B. anthina* & E571, *B. ubonensis*). Each of these strains was resistant to the phage panel tested in this thesis.

Whole-genome sequencing of bacteria provides increasing evidence of widespread exchange of chromosomal genes and other extrachromosomal elements mediated by phages. The genome of *B. cenocepacia* isolate J2315 has recently been fully sequenced (http://www.sanger.ac.uk/Projects/B_cenocepacia/) and an initial BLAST search of the provisional genome sequence identified a single prophage, supporting the scarcity of polylysogeny in the studies for this thesis. However, these studies tentatively identified two temperate bacteriophages, DK4 and MM8, from J2315. Interestingly, four phage-related islands have been identified on chromosome one, with another identified on chromosome three (Personal communication; Mahenthiralingam, 2003). Further genomic analysis is necessary to elucidate the importance of prophage in the evolution and genomic plasticity of this well characterised epidemic lineage.

A characteristic feature of the Bcc phages identified in this study was that they were not the easiest of phages to work with. Despite rigorous attention to protocols including storage buffers and bacterial growth conditions, in some cases results were difficult to repeat. These difficulties were not encountered with the classic *P.*

aeruginosa phages E79, D3 and F116L suggesting that, like their bacterial hosts, Bcc phages have idiosyncrasies. This applied in particular to an apparent variability in host range. Attempts to explain these discrepancies by attention to the depth of agar overlays and the influence of anaerobiosis were unsuccessful. A possible explanation is that variability in the host range may be, in part, due to a propensity for Bcc phages to undergo mutations affecting host range.

In recent years, the major disadvantage of phage therapy, namely bacterial and/or mammalian host resistance, has been addressed by identifying novel phages or by selecting long circulating phage mutants resistant to clearance by the human reticulo-endothelial system (RES) (Merril *et al.* 1996). In the case of Bcc, the risk of gene transfer between and within species of the Bcc and related pathogens, as a consequence of phage therapy, adds an additional risk to their widespread clinical use in CF. A novel twist to these problems would be to utilise the phage enzyme that is required to exit, lyse and destroy the bacterial host cell: namely the phage endolysin.

5.7 Bacteriophage Lytic Enzymes as Antibacterial Agents

Aims

The aim of this study was to investigate the antimicrobial potential of two phage-derived endolysins. The first endolysin was derived from the Bcc phage Bcep781, which has been fully sequenced. The second investigation focused on the endolysin derived from the *P. aeruginosa* phage D3. Unlike the latter, the Bcep781 genomic DNA was unavailable.

5.8 Results: Proteomic Analyses of the Bacteriophage Endolysins Bcep781 & D3

The protein sequences of the phage endolysins were obtained from a protein database (www.pubmed.com) and, using a BLASTn search, were compared with other proteins in the database. Sequences of high similarity were aligned using AlignX software (Informax). Bcep781 endolysin was almost identical to two other

endolysins in the database identified in Bcc phages Bcep1 and Bcep43 (Fig 5.3). The data highlight similarities and note amino acid differences, although all sequences used were based on bioinformatics, and thus protein functionality has yet to be determined. Furthermore, the endolysin identified in the Bcc phage BcepNazgul (127 aa in length) shared no similarity with the Bcep lysins. Further protein analysis of the Bcep781 endolysin is provided (Table 5.4). The protein sequence of phage D3 lysin was subjected to a similar study described above. Interestingly, it appeared to show protein sequence homology to an endolysin described in *P. putida* KT22 440, although to a lesser extent than the Bcep phage lysins (Fig 5.4). Further protein analysis of the D3 endolysin is provided (Table 5.5).

```
                          1                                                  50
Bcep781 Lysin       (1)   MAAPLIVGASGRAVVFLQARLGLAQSGQFDASVATALRQWQEAHGMTPDG
Endolysin Bcep1     (1)   MAAPLIVGASGRAVVFLQARLGLAQSGQFDASVATALRQWQEAHGMTPDG
Endolysin Bcep43    (1)   MAAPLIVGASGRAVVFLQARLGLAQSGQFDASVATALRQWQEAHGMTPDG
Consensus           (1)   MAAPLIVGASGRAVVFLQARLGLAQSGQFDASVATALRQWQEAHGMTPDG
                          51                                                 100
Bcep781 Lysin       (51)  VYGSQTNAVMTARALPDIADAAARLNVDTPALQAIIQVETTGSGFLPDGR
Endolysin Bcep1     (51)  VYGSQTNAVMTARALSDIADAAARLRVDVPAFQAIIQVETMGSGFLPDGR
Endolysin Bcep43    (51)  VYGSQTNAVMTARALPDIADAAARLNVDTPALQAIIQVETTGSGFLPDGR
Consensus           (51)  VYGSQTNAVMTARALPDIADAAARLNVDTPALQAIIQVETTGSGFLPDGR
                          101                                                150
Bcep781 Lysin       (101) PRILLERHKVWAATSPAQRVLLGAQDCNPTPGGYATGPDANARGAGEWVR
Endolysin Bcep1     (101) PRILLERHKVWAATSPAQRVLLGAQDCSPTPGGYATGPDADARGAGEWVR
Endolysin Bcep43    (101) PRILLEPHKVWAATSPAQRVLLGAQDCNPTPGGYATGPDANARGAGEWVR
Consensus           (101) PRILLERHKVWAATSPAQRVLLGAQDCNPTPGGYATGPDANARGAGEWVR
                          151                                                200
Bcep781 Lysin       (151) FERVAAVTGDEVAAQCCSWGLGQVMGANYATCGFTNAVGLMFASALNERA
Endolysin Bcep1     (151) FERVAAVTGDEVAAQCCSWGLGQVMGANYATCGFTNAVGLMFASALNERA
Endolysin Bcep43    (151) FERVAAVTGDEVAAQCCSWGLGQVMGANYATCGFTNAVGLMFASALNERA
Consensus           (151) FERVAAVTGDEVAAQCCSWGLGQVMGANYATCGFTNAVGLMFASALNERA
                          201                                                250
Bcep781 Lysin       (201) QLDVMVRFALPQAGLLGALRAHQWAAVARIWNGPNFAINQYDTKLSDAYT
Endolysin Bcep1     (201) QLDVMVRFALPQAGLLGALRAHQWAAVARIWNGPNFAINQYDTKLDKAYI
Endolysin Bcep43    (201) QLDVMVRFALPQAGLLGALRAHQWAAVARIGNGPNFAINQYDTKLSDAYT
Consensus           (201) QLDVMVRFALPQAGLLGALRAHQWAAVARIWNGPNFAINQYDTKLSDAYT
                          251
Bcep781 Lysin       (251) TLTSR
Endolysin Bcep1     (251) TLTSQ
Endolysin Bcep43    (251) TLTSR
Consensus           (251) TLTSR
```

Fig 5.3 Proteomic alignment of Bcc bacteriophage endolysins. Three protein sequences of Bcep endolysins (Bcep 1, 43 and 781) were aligned using AlignX software (Informax). Sequence homology is shown in red, partial homology is shown in blue, no homology is shown in green. The consensus sequence is also provided.

Table 5.4 Bacteriophage Bcep781 endolysin analysis

Analysis	Entire Protein
Length	255 aa
Molecular Weight	26854.78
Isoelectric Point	7.01
Charge at pH 7	0.01

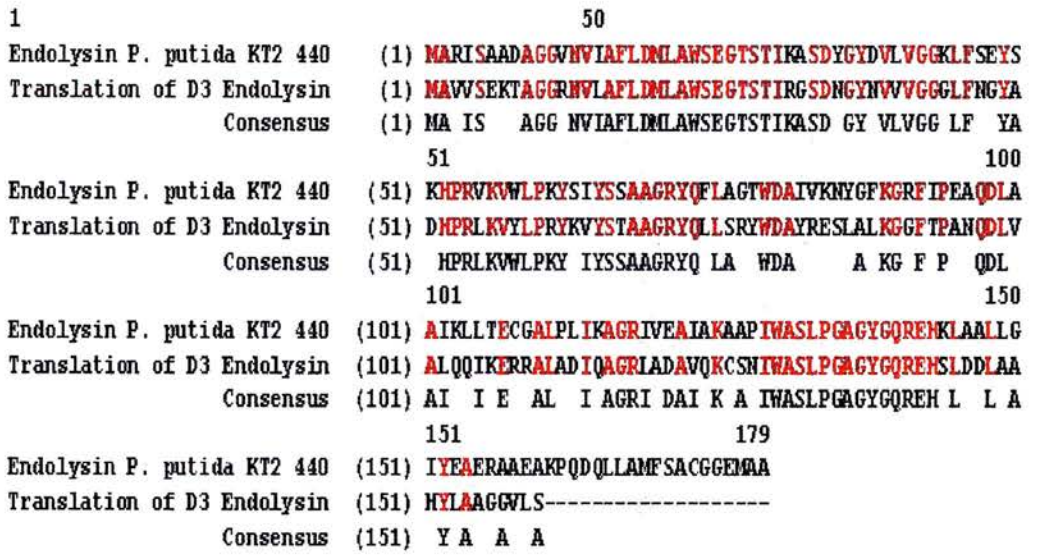


Fig 5.4 Proteomic alignment of the bacteriophage D3 phage endolysin. The protein sequences of the D3 endolysin were aligned with an endolysin from *P. putida* KT22 440 using AlignX software (Informax). Sequence homology is shown in red, unique sequence is shown in black. The consensus sequence is also provided.

Table 5.5 Bacteriophage D3 endolysin analysis

Analysis	Entire Protein
Length	160 aa
Molecular Weight	17310.84 m.w.
Isoelectric Point	9.02
Charge at pH 7	3.02

5.9 Results: Recombinant Synthesis of the Bacteriophage Bcep781 Endolysin Gene by PCR

Since neither intact Bcep781 phage particles nor genomic DNA were available, we had to rely on the phage genomic sequence for gene synthesis. Twenty-two primers were designed and synthesized, with restriction endonuclease sites engineered to the 5' and 3' ends to aid later cloning steps. Each primer was designed to overlap at the 3' end, as represented in Fig 5.5. The one-step recursive PCR method was used to synthesize the 776 bp Bcep781 endolysin gene (*Nco*I/*BamH*I) (Fig 5.6). Herculase® Hotstart DNA polymerase was chosen in preference to *Taq* polymerase because of its high fidelity amplification. This was required to reduce the occurrence of point mutations. The most critical aspects of this technique are the relative concentrations of the internal and outer (amplifying) oligonucleotides and the design of overlapping ends. The initial round of PCR did not appear to be successful and the extracted DNA fragment was subjected to another round of PCR with the outermost oligonucleotides, prior to being cloned into PCR 2.1 (Fig 5.7). Automated DNA sequencing highlighted sequence error at two specific sites. The 5' *Nco*I site contained errors and a deletion of a guanine base at position 614 was also observed. The 5' *Nco*I site was fixed by repeating the PCR protocol, using the outermost primers (1 and 22) and the synthesised gene as the template. The base pair deletion at position 614 proved to be problematic, and required several "fix" primers to be tried before the integrity of the gene was assured. The fix method was based on a mega-primer PCR and used a *Taq* polymerase with a lower fidelity than the proof-reading polymerase used in the gene synthesis step. The Bcep781 endolysin gene was finally completed by a mega-primer PCR and site-directed mutagenesis to reduce the GC content of the region in which the base pair deletion was present. Four base pair changes (G→T) were made, although each mutation was silent to retain the amino-acid sequence integrity.

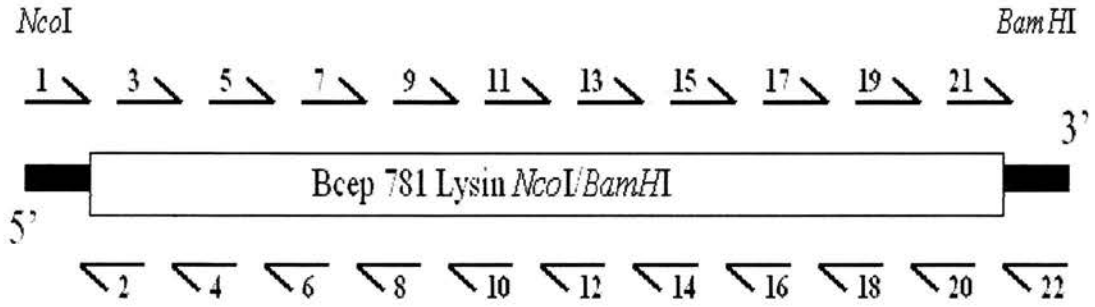


Fig 5.5 The design of primers for the synthesis of the bacteriophage Bcep781 endolysin gene. Twenty-two primers were designed to encode the entire genomic sequence of the hypothetical endolysin gene. The even numbered primers overlapped the odd number primers to ensure integrity of the synthesized fragment.

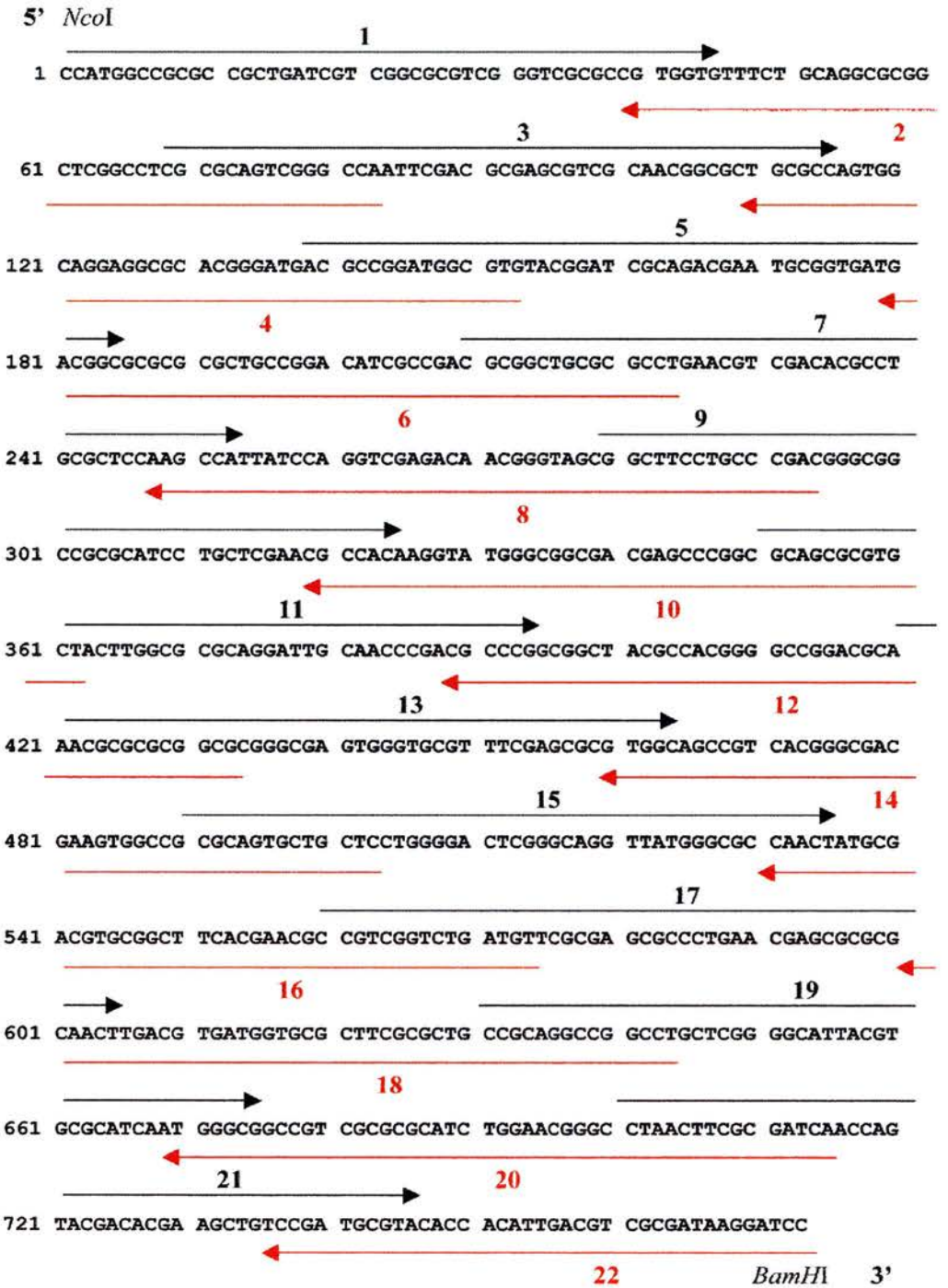


Fig 5.6 Gene synthesis of bacteriophage Bcep781 endolysin gene using recursive PCR. The figure shows the 22 primers used in the synthesis of the Bcep 781 endolysin gene (Appendix 2), their positions across the gene, and the location of the engineered *NcoI* and *BamHI* sites. Black arrows represent forward primers (5' – 3'). Red arrows represent reverse primers (3'-5').

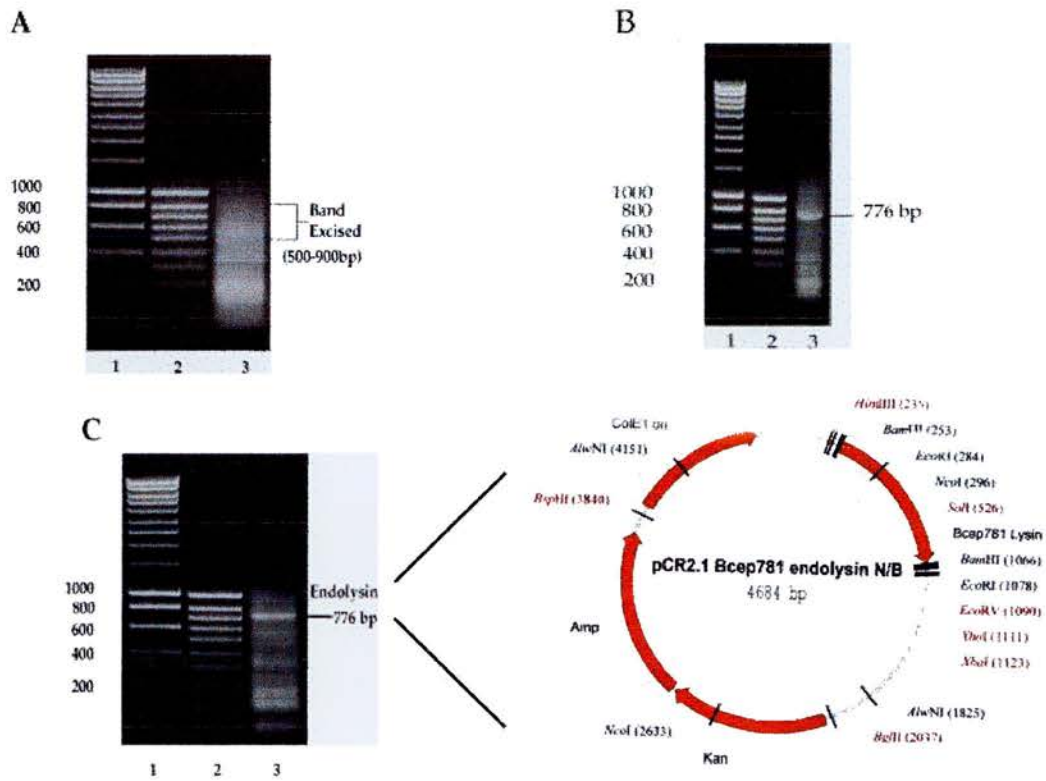


Fig 5.7 Bacteriophage gene synthesis and subsequent cloning steps. The 22 Bcep781 lysin primers were added together with dNTPs and Herculase polymerase. The external primers (1 & 22) were added at 10 x the concentration of the internal primers (2-21). **A.** The initial round of PCR produced a smear of DNA and a band between 500 and 900 bp was extracted and the DNA content was purified. **B.** The second round of PCR used the extracted band from **A** in conjunction with the external primers (1 & 2). This round of PCR resulted in a DNA band of 776 bp, consistent with the expected number of base pairs for the Bcep781 endolysin gene. The band was excised, and the DNA was extracted and purified. **C.** The excised band from **B** was subjected to another round of PCR using the external primers (1 & 22). The last round of PCR was utilised to increase the integrity of the synthesized gene, prior to cloning into PCR 2.1. Lane 1: DNA Hyperladder I; Lane 2: DNA Hyperladder IV; Lane 3: Gene synthesis product.

5.10 Results: Cloning, Expression and Purification of the Bacteriophage Bcep781 Endolysin

The phage Bcep781 endolysin gene was cloned into pCR 2.1. DNA sequencing confirmed the presence of four point mutations at positions 614, 620, 623 and 629. Subsequently, the endolysin gene was cloned into a pET expression vector with a N-terminal His_6-tag (pET 6-His), for expression in various *E. coli* strains (DE3 lysogens); optimum recovery of protein was achieved with *E. coli* strain BL21 (DE3) strain. The Bcep781 endolysin gene was also cloned into pET 28a to enable purification of the native protein; unfortunately protein expression was not successful. Cells were grown in shake flasks at 37°C and expression induced with 1 mM IPTG. The endolysin cell lysate was first purified by nickel-affinity chromatography (FPLC). The protein was eluted with 200 mM imidazole and twenty-five 5 ml fractions were collected. Absorbance was measured at 280 nm for each (Fig 5.8). All fractions were subjected to SDS-PAGE on a 4-12% gel and Coomassie staining highlighted a protein band with a molecular weight of approximately 27-28 kDa (Fig 5.9). The molecular weight of Bcep781 lysin was based on protein analysis software, Vector NTI (Table 5.6). Sample fractions were pooled and concentrated using a spin filter of a molecular weight cut off (MWCO) of 10 kDa. The concentrated sample was then dialysed against 50 mM phosphate buffer (pH 8.0) with 100 mM NaCl and concentrated as before. The dialysed sample was then subjected to platform mass spectrometry and its antimicrobial activity investigated.

5.11 Results: Mass Spectrometry of the Bacteriophage Bcep781 Endolysin

The mass spectrum of the endolysin sample revealed the presence of a distinct species of mass 27917.0 Da (Fig 5.10). This figure did not correspond to the primary structure analysis of the 6-His-Bcep781 endolysin, and differed by +37.12 mass units.

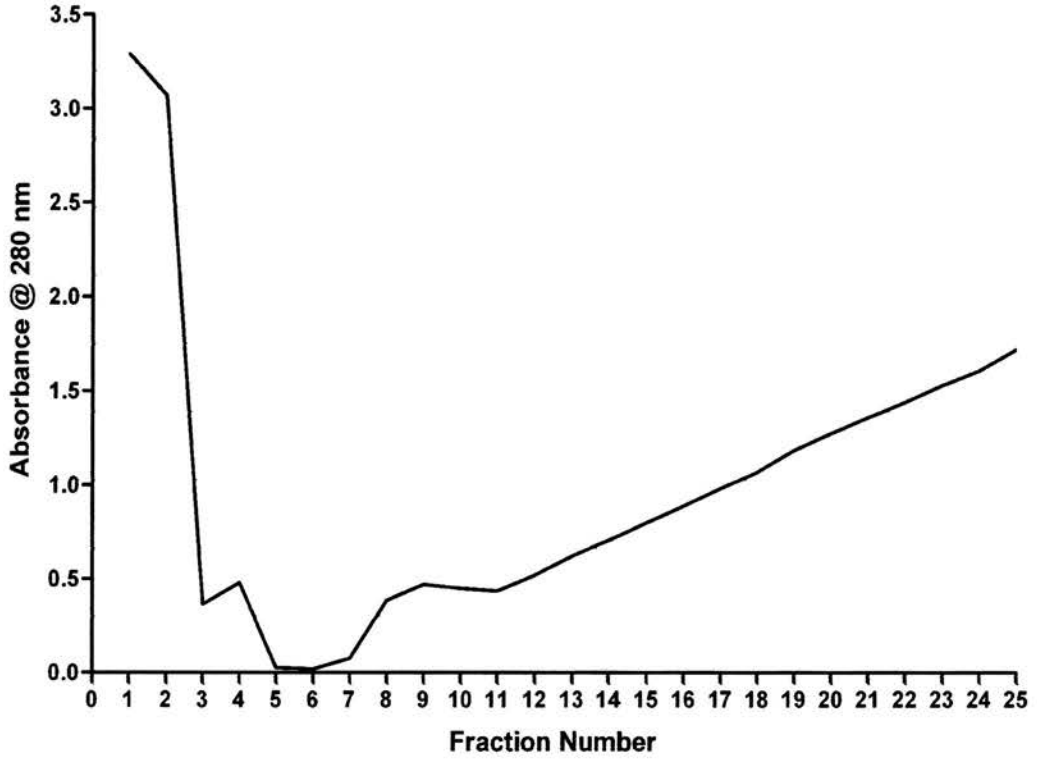


Fig 5.8 The absorbance values of FPLC fractions following Nickel affinity purification. The fractions that demonstrated a significant absorbance value were subjected to SDS-PAGE analysis.

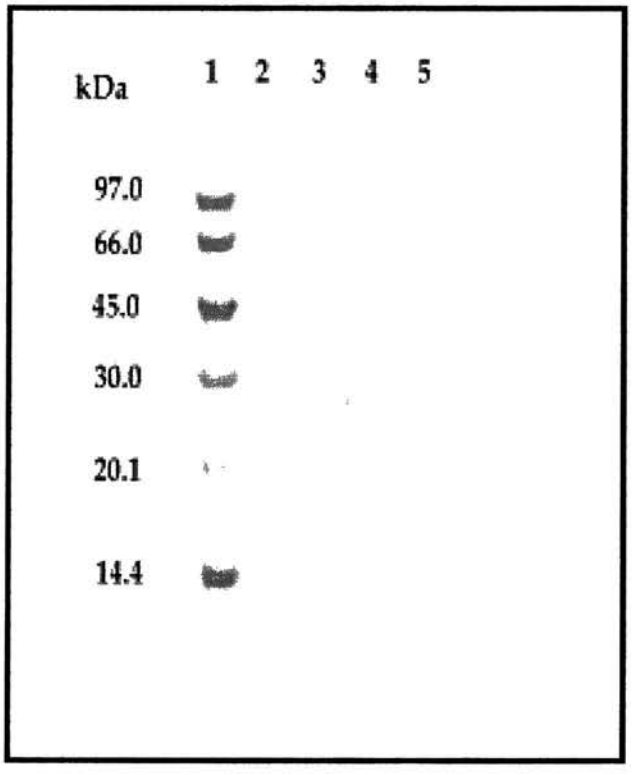


Fig 5.9 Purification of 6-His Bcep781 endolysin. The extract was purified using Nickel affinity chromatography. The sample fractions were run on a 4-12% Bis/Tris SDS-PAGE gel and stained with Commassie blue. Lane 1: Low molecular weight marker. Lane 2: fraction 8. Lane 3: fraction 9. Lane 4: fraction 10. Lane 5: fraction 11. Lanes 2,3,4 show a band at approximately 27 kDa, which corresponds to the predicted molecular weight of the 6-His Bcep781 endolysin.

Table 5.6 6-His Bcep781 endolysin protein analysis

Analysis	**Entire Protein**
Length	263 aa
Molecular Weight	27879.88 m.w
1 microgram =	35.868 pMoles
1 A[280] corr. to	0.68 mg/ml
Isoelectric Point	7.27
Charge at pH 7	0.53

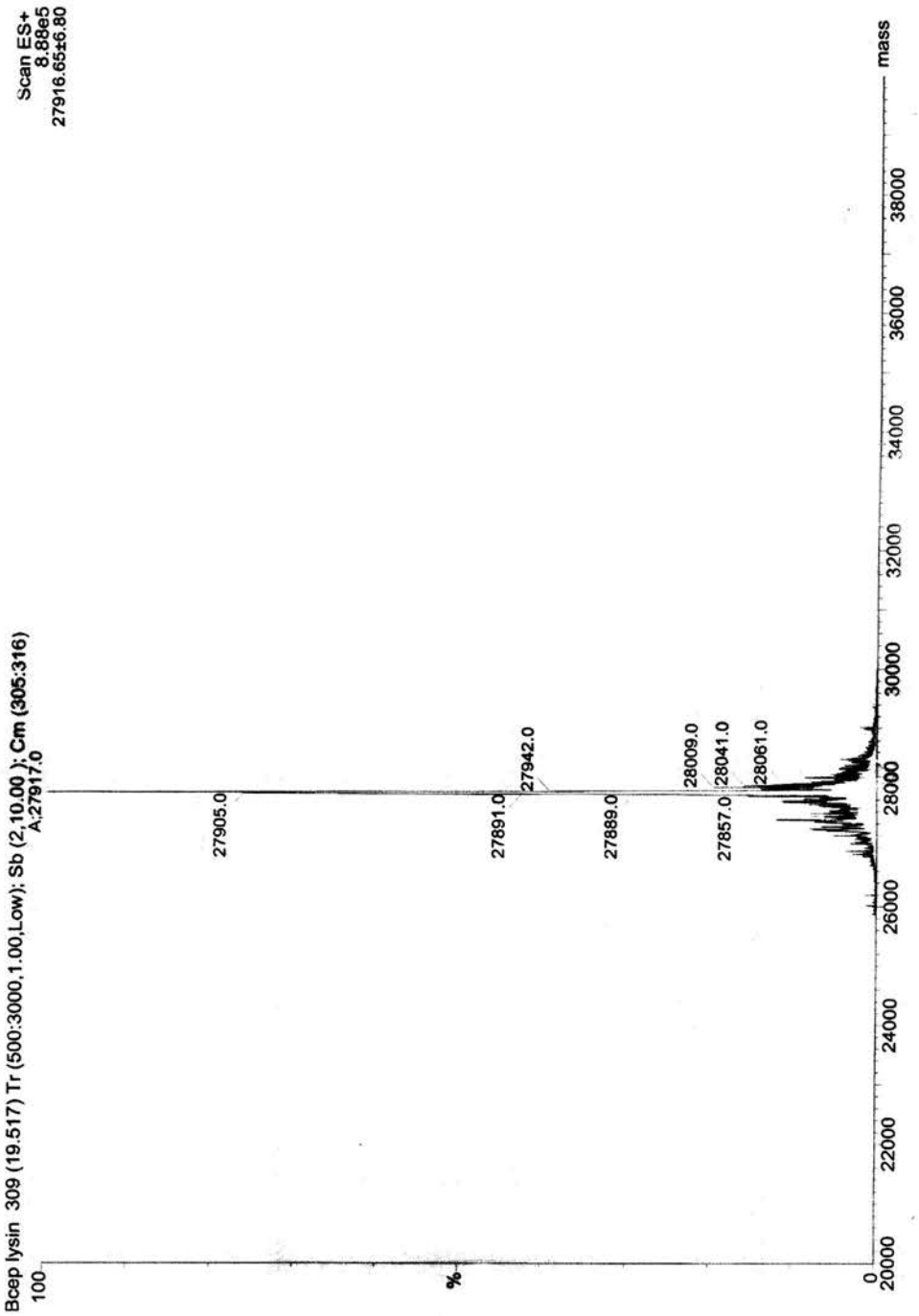


Fig 5.10 Mass spectrum of 6-His-Bcep781 endolysin. The data show that the predominant mass (indicated by A) was 27916.65 ± 6.80.

5.12 Results: Antibacterial Activity of the Bcep781 Endolysin

The antimicrobial potential of the recombinant- purified 6-His-Bcep781 endolysin was assessed using ten Bcc isolates: two *B. cepacia* isolates (ATCC 25416 & ATCC 17759), three *B. multivorans* isolates (C1576, C1962 & ATCC 17616), four *B. cenocepacia* isolates (C517, J2315, R9239, R2817), and one *B. ambifaria* isolate (J2742-AMMD). The recombinant protein did not appear to have any activity against the panel of Bcc isolates examined. Unfortunately, the propagating host strain, now known to be a *B. cepacia* (genomovar I) isolate, was unavailable as a positive control.

5.13 Results: PCR of the Bacteriophage D3 Lysin Gene

The D3 endolysin gene was identified and specific primers were designed to amplify the lysin (492 bp) gene from D3 genomic DNA (Fig 5.11). A 5' *Nco*I restriction endonuclease site, and a 3' *BamH*I site had been engineered into the gene. The PCR product was then cloned into pCR 2.1. DNA sequencing showed that the 3' *BamH*I site had not been correctly inserted, although the consensus gene sequence was correct. The absence of the restriction endonuclease made subsequent cloning steps difficult. Therefore, a fix primer was designed and, by using a mega primer PCR the restriction endonuclease site was corrected.

5.14 Results: Cloning, Expression and Purification of the Bacteriophage D3 Endolysin

The endolysin gene was cloned into a pET expression vector with a N-terminal His_6-tag (pET 6-His), for expression in various *E. coli* strains (DE3 lysogens); the optimum recovery of protein was achieved using *E. coli* strain BL21(DE3). Cells were grown in shake flasks at 37°C and expression induced with 1 mM IPTG. The endolysin cell lysate was first purified by nickel-affinity chromatography (FPLC), the protein was eluted with 200 mM imidazole and twenty-five 5 ml fractions were collected. Each fraction was subjected to SDS-PAGE and run on a 4-12% gel. Commassie staining highlighted a protein band with a molecular weight of approximately 19-21 kDa (Fig 5.12). The molecular weight of D3 endolysin was

based on protein analysis software, Vector NTI (Table 5.7). However, the D3 endolysin appeared to be in the initial fractions (fractions 1 & 2) indicative of non-binding to the Nickel affinity column. Sample fractions 1 and 2 were pooled and dialysed (MWCO 18, 000) for 18 hours against 25 mM Tris buffer (pH 7.5). The pH of the buffer was important, as this would enable latter purification steps. Due to its PI, the D3 lysin would have an overall positive net charge, and thus could be purified on a Resource S® (Sulfonic acid) column. It is very unlikely that *E. coli* proteins would bind during Resource S® purification steps as they tend to have PIs of 3-7, and be negatively charged at pH 7.5.

The dialysed (and desalted) sample was subjected to Resource S® purification and the protein was eluted with 1 M NaCl and twenty-five 5 ml fractions were collected. The absorbance at 280 nm was measured for each fraction (Fig 5.13). All fractions were subjected to SDS-PAGE on a 4-12% gel and Coomassie staining highlighted a protein band with a molecular weight of approximately 18-19 kDa (Fig 5.14). The dialysed sample was then subjected to MALDI-TOF mass spectrometry and its antimicrobial activity investigated.

5.15 Results: Mass Spectrometry of the Bacteriophage D3 Endolysin

The sample was subjected to MALDI-TOF mass spectrometry (Fig 5.15). The data showed that the predominant mass in the sample was 16927.72 ± 6.49. The mass of 6His-D3 endolysin was predicted to be 18335.94, thus a mass discrepancy of 1408.22 ± 6.49 was noted. Further analysis did not provide an exact mass for the expressed protein in the initial sample.

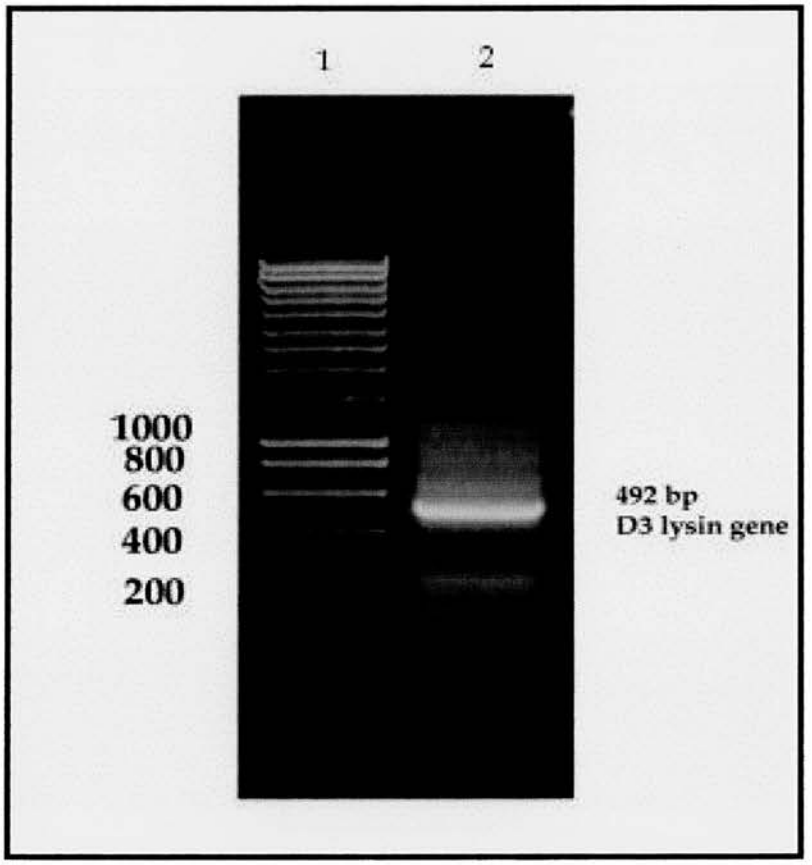


Figure 5.11 The Bacteriophage D3 lysin gene amplified by PCR. Lane 1: DNA hyper ladder 1. Lane 2: D3 lysin gene. The data show the amplification of the D3 lysin gene (492 bp) from extracted and purified D3 genomic DNA, using lysin specific primers.

Table 5.7 Analysis of 6-His D3 endolysin

Analysis	**Entire Protein**
Length	163 aa
Molecular Weight	18335.94 m.w
1 microgram =	54.538 pMoles
1 A[280] corr. to	0.61 mg/ml
Isoelectric Point	9.02
Charge at pH 7	3.54

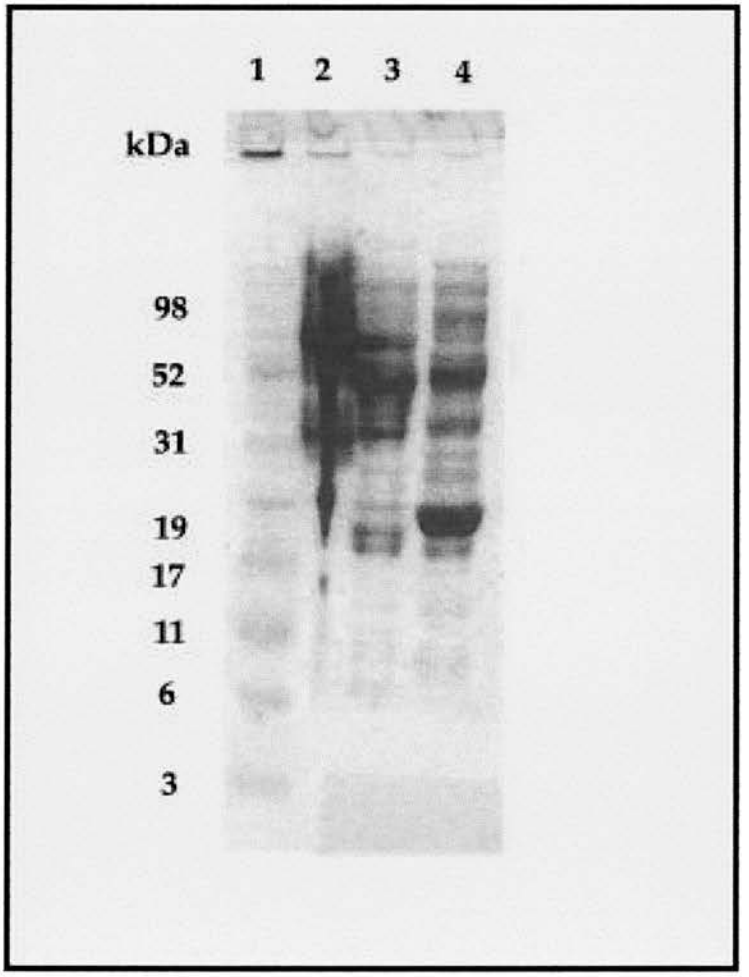


Figure 5.12 SDS-PAGE analysis of Nickel affinity purification 6-His D3 lysin. The cell free extract was purified on a Nickel affinity column and collected fractions were subjected to SDS-PAGE (4-12% gel). Lane 1: multiplex marker. Lane 2: cell free extract. Lane 3: flow through. Lane 4: fraction 1 & 2 from Ni-affinity purification step. The data show that an over-expressed protein is present in fractions 1 and 2, which is indicative of no binding to the affinity column.

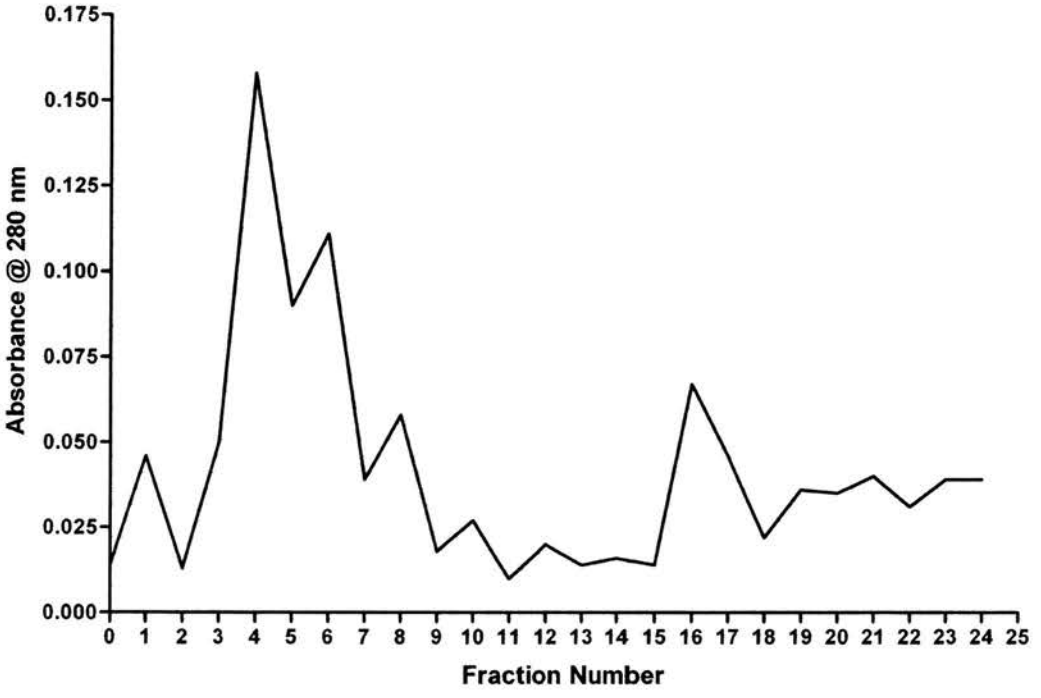


Fig 5.13 Resource-S purification. The D3 lysin recombinant protein was dialysed into 25 mM Tris buffer (pH 7.5) to ensure that the protein had an overall positive charge. The extract was then purified using a Resource S purification step, and 25 fractions were collected. The absorbance of each fraction at 280 nm was measured and this analysis provided a number of peaks. The corresponding fraction was then subjected to SDS-PAGE analyses.

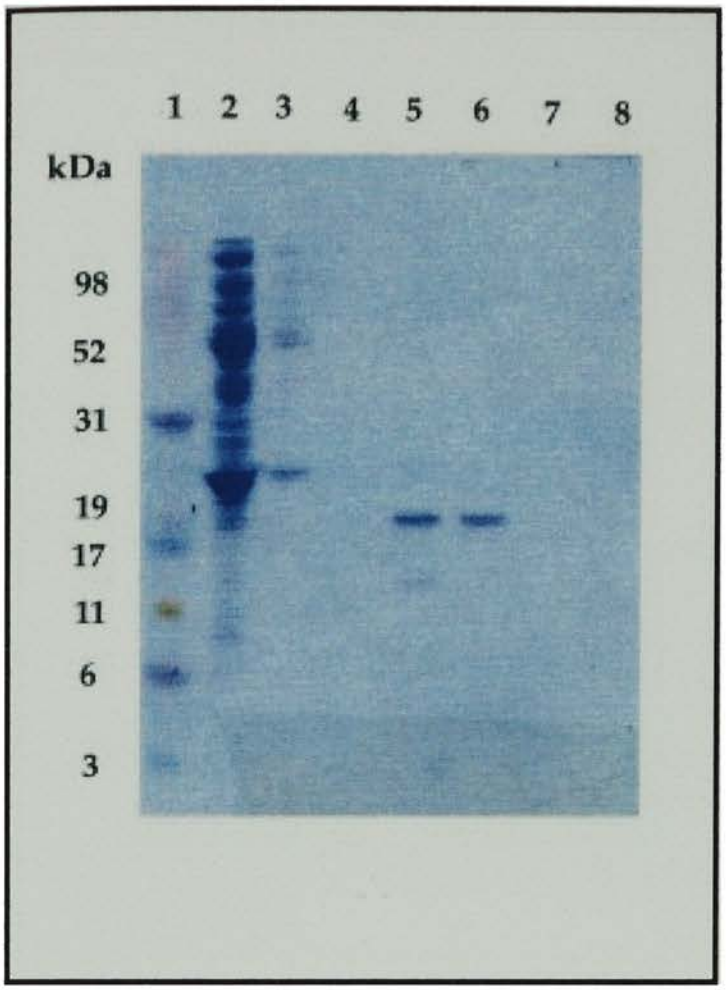


Fig 5.14 SDS-PAGE results after Resource S purification of 6-His D3 lysin. The purified fractions from the Resource S purification that had previously been shown to have a significant absorbance at 280 nm were subjected to SDS-PAGE (4-12% gel). Lane 1: Multiplex marker. Lane 2: flow through loading. Lane 3: flow through wash. Lane 4: fraction 1. Lane 5: fraction 4. Lane 6: fraction 6. Lane 7: fraction 8. Lane 8: fraction 10. The results indicate that fraction 4 and fraction 6 contained a protein with a molecular weight consistent with the 6-His D3 lysin protein.

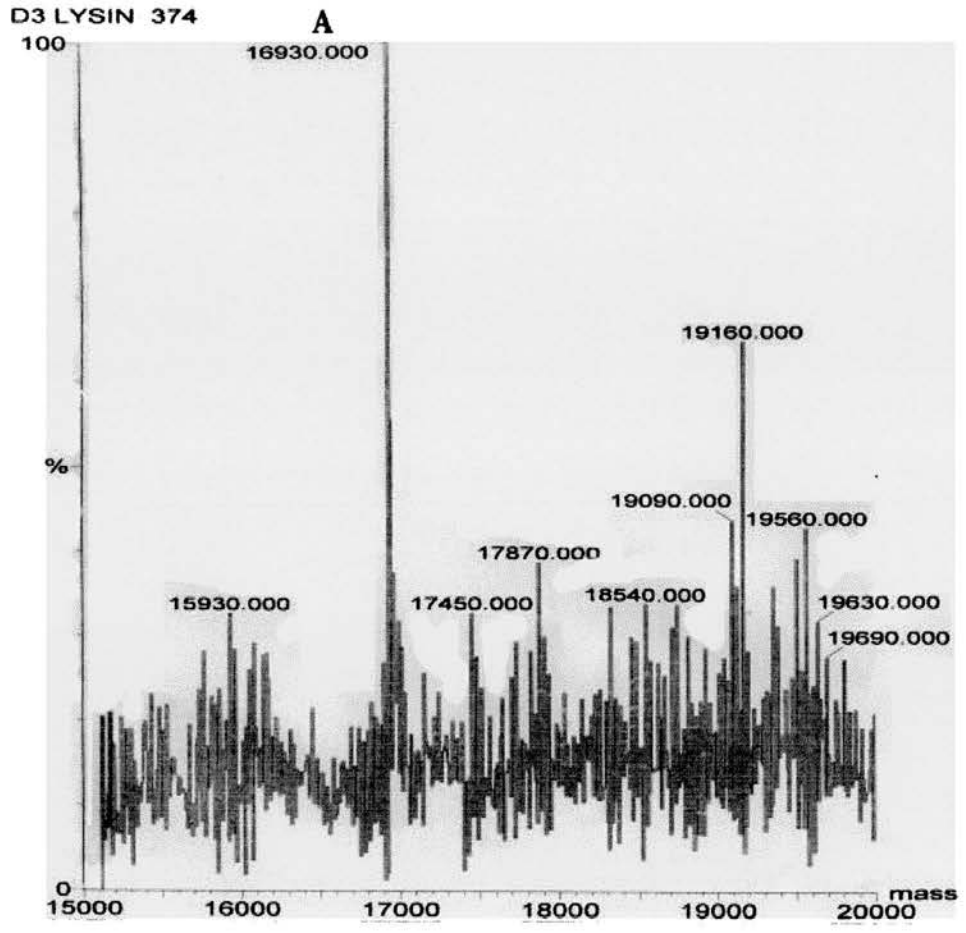


Figure 5.15 Mass spectrometry of recombinant 6-His D3 lysin. The data show that the predominant mass (indicated by A) was 16927.72 ± 6.49.

5.16 Results: Antibacterial Activity of the Bacteriophage D3 Endolysin

The antimicrobial potential of the recombinant- purified 6-His-D3 endolysin was assessed using three *P. aeruginosa* isolates: PAO1 (positive control), H183 (Liverpool epidemic strain) & C3425 (Manchester epidemic strain). The recombinant protein did not show any antimicrobial activity against the small panel of *P. aeruginosa* isolates examined, including the propagating strain used as a positive control.

5.17 Results: Expression of Bacteriophage D3-6-His Lysin Fusion Protein in *E. coli* HMS-174

The endolysin gene cloned into a pET expression vector with a N-terminal His_6-tag (pET 6-His) was expressed in *E. coli* cells HMS 174. Cells were grown in shake flasks at 37°C and expression induced with 1 mM IPTG. During the mini-induction, expression appeared to be consistent with expression in BL21 (DE3) cells (Fig 5.16). Subsequently, large-scale induction of a 2-litre culture was prepared and purified using nickel-affinity chromatography (FPLC). The protein was eluted with 200 mM imidazole and twenty-five 5 ml fractions were collected. The absorbance at 280 nm was determined for each fraction prior to SDS-PAGE. It was noted that the recombinant protein had not bound to the affinity column (Fig 5.17). In addition, the protein appeared to be completely insoluble, as highlighted by the large amount of expressed protein in the cell pellet fraction (Fig 5.18).

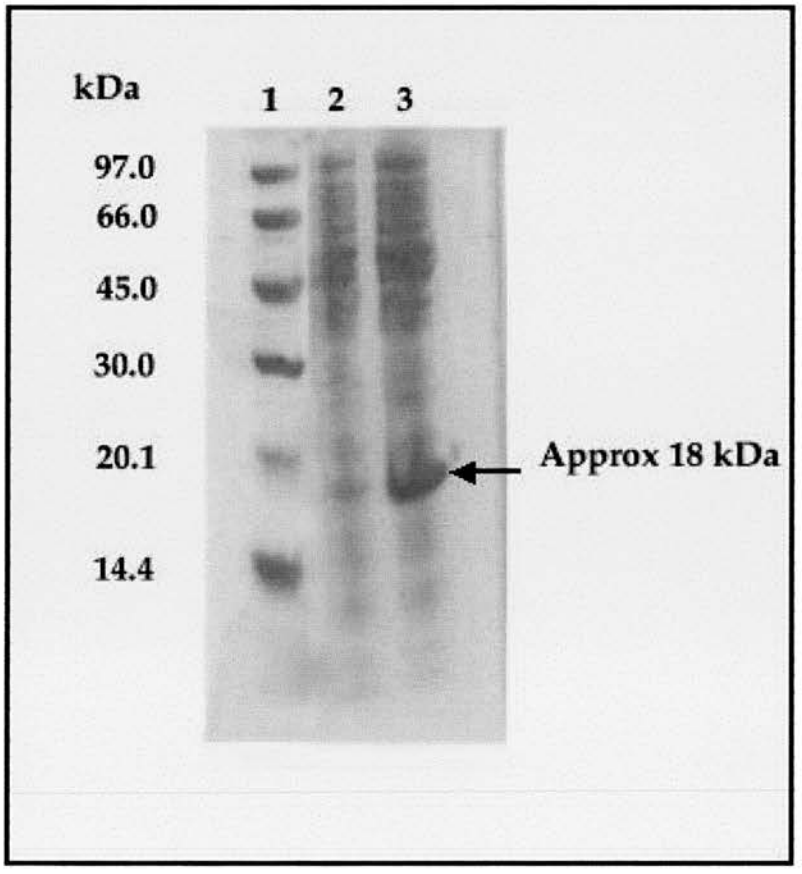


Fig 5.16 Bacteriophage D3 lysin expressed in *E. coli* HMS 174 mini induction. The HMS-174 *E. coli* strain, transformed with the 6His-D3 lysin expression plasmid, was grown in 1 ml of 2YT broth and induced with 1 mM for 3 hours. Lane 1: LMW marker. Lane 2: cell free extract with no IPTG induction. Lane 3: cell free extract from cells induced with 1 mM IPTG. The band in Lane 3 is approximately 18-19 kDa, as was the predicted molecular weight of the 6His-D3 protein.

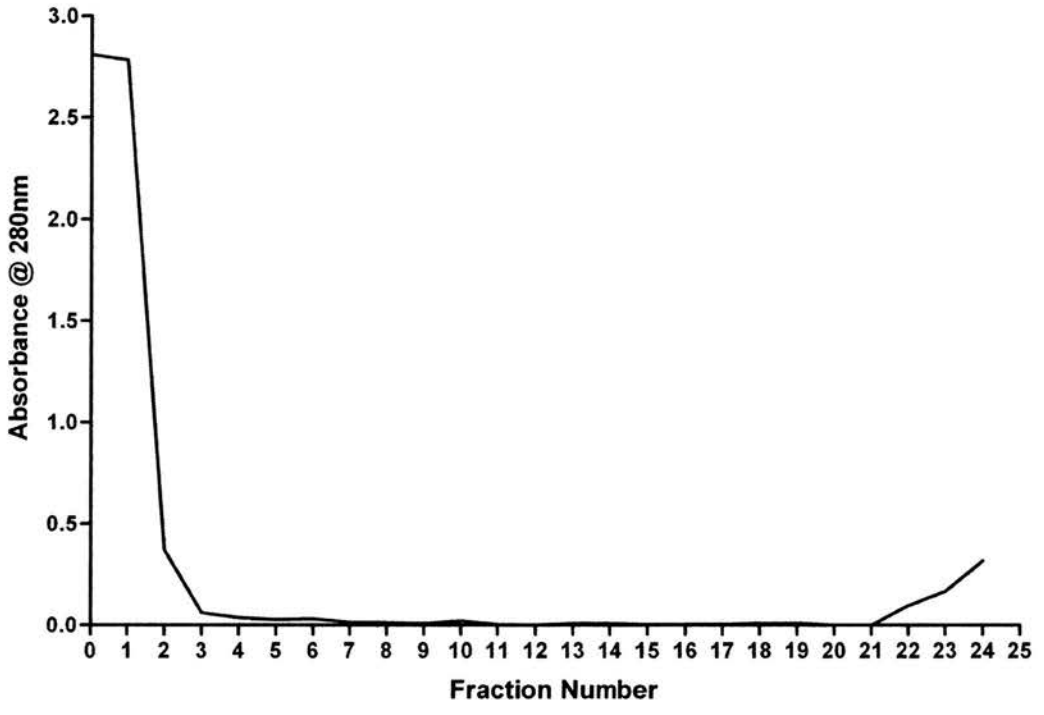


Fig 5.17 Bacteriophage D3 lysin HMS-174 nickel affinity purification. The cell free extract from the large scale induction of HMS-174 *E. coli* transformed with the 6His-D3 expression plasmid was subjected to purification on a nickel affinity column, and 25 protein fractions were collected. The data show that no significant level of protein was eluted by the imidazole concentration gradient, and thus the over-expression did not result in soluble recombinant D3 lysin.

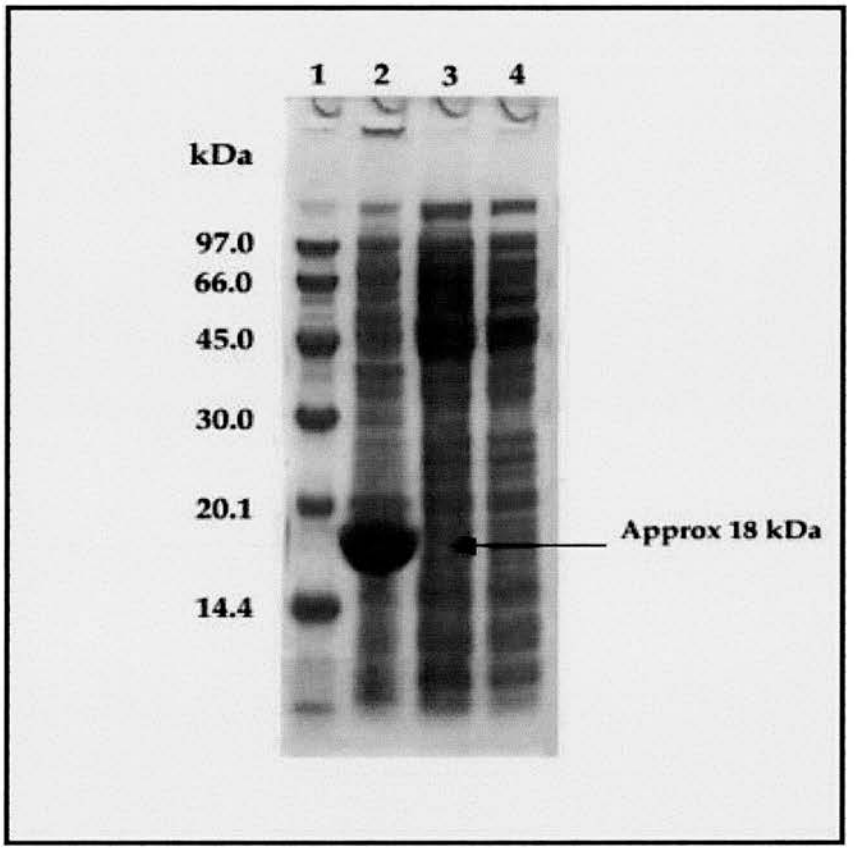


Fig 5.18 Insoluble D3 lysin expressed in HMS 174. The fractions collected from the Nickel purification step were subjected to SDS-PAGE and run on a 4-12% gel. Lane 1: LMW marker. Lane 2: Cell pellet. Lane 3: Cell free extract. Lane 4: Flow through. The results show that the over-expression of the recombinant D3 lysin was successful. However, the resultant protein was insoluble as indicated by its presence in the cell pellet.

5.18 Discussion

Unlike other microbial-derived antibiotics (with the exception of bacteriocins), the activity of phage endolysins typically remains specific to the bacterial species acting as the phage host. With the exception of one study by Ozawa *et al.* that investigated the phage encoded bacteriolytic protein of phage P4282 and its lytic activity against *R. solanacearum* (Ozawa *et al.* 2001), the available literature on phage endolysins has focused on Gram-positive organisms. Thus, to the author's knowledge, this is the first study to investigate the potential use of bacteriophage enzymes against *P. aeruginosa* and the Bcc.

The 776 bp Bcep781 endolysin gene was synthesized using 22 overlapping oligonucleotides in a single reaction. Importantly, the success of this process was dependant on the relative concentration of the internal and outer (amplifying) oligonucleotides. Likewise, the choice of DNA polymerase was fundamental to successful gene synthesis. The synthesis was not without its problems, and several attempts were required to correct a one base pair deletion. However, the technique allows both optimisation of codon usage for *E. coli*, and the easy creation of mutants as novel primers can be introduced at any stage during synthesis to alter genomic integrity. This makes the technique useful in situations where template DNA is unavailable, mutant genes are desired, or the optimisation of codons is required to increase protein yield.

Disappointingly, neither recombinant endolysin created and purified in this study exhibited antibacterial activity. Explanations to account for this result include: First, the function of both genes identified as endolysins, is based solely on bioinformatics and full proteomic characterisation is required to identify their role in the phage life cycle. Second, both endolysins were expressed with a N-terminal His_6-tag that may inhibit activity by altering the tertiary structure of the endolysin, thus stearically altering its ability to bind to its target. The difficulty in the purification of the 6-His D3 lysin supports this hypothesis, as the recombinant protein did not bind to a Nickel affinity column, suggesting that the His-tag may have been buried within the tertiary

structure. To avoid problems associated with His-tagging, attempts were made to express the native endolysins in another pET system (pET 28a). However, although both genes were successfully cloned into the pET 28a expression system, gene expression was not achieved. The use of different *E. coli* strains was also investigated. Interestingly, over-expression in *E. coli* HMS-174 may have been too high and may explain why the resultant protein was insoluble. High-level over-expression can reduce the fidelity of protein folding and therefore affect solubility. Third, mass spectrometry indicated that neither endolysin possessed the expected molecular weight despite the evidence of SDS-PAGE analysis. Thus, both endolysins may have been subjected to modification or have been affected by *E. coli* proteases and their structure altered during the purification steps. Fourth, to be fully functional, phage lytic enzymes may require the presence of a holin, an enzyme which is important during phage release from the host cell. Holins are small peptides that are required for endolysins to gain access to bacterial peptidoglycan (Young and Blasi 1995) and to lyse the host cell during "timed" lysis (Grundling *et al.* 2001). The membrane lesions formed by holin/endolysin activity, are stable and non-specific and may be essential for the activity of endolysins in Gram-negative organisms. Further studies are required to explore the roles of both holins and endolysins as potential, and possibly synergistic, antibacterial agents. Fifth, the ability of endolysins to kill Gram-negative cells from the outside-in remains to be clarified. Furthermore, in this study, no positive host control strain for phage Bcep781 was available and thus potential antibacterial activity may have been missed.

The use of phages as therapeutic agents has been the topic of extensive research for over a century; however, only recently has the novel use of phage derived-enzymes as antibacterial agents received attention in the microbiological community. Although recent focus has been on those phage-encoded enzymes that are responsible for the exit of phage progeny from the bacterial host, other essential cellular functions, such as host DNA replication and transcription machineries, can also be disrupted by phage-encoded peptides (Liu *et al.* 2004). Liu and co-workers utilised a phage genomics approach to obtain information from the whole genome

sequencing of 26 *S. aureus* phages, and identified several polypeptides with antibacterial activity. Such studies will become more commonplace as data from genomic sequencing becomes more accessible.

6.1 Aims

The aim of this study was to investigate the antimicrobial activity of a newly characterised five-cysteine murine β-defensin (Defr1) against multiresistant pathogens, including members of the Bcc, epidemic *P. aeruginosa* and *S. maltophilia*. Both Defr1 and the 6-cysteine analog (Def-cys) were produced synthetically (Albachem: Edinburgh) to enable further investigations. The 6-cysteine analog, in conjunction with two conventional cationic antimicrobial peptides polymyxin B sulfate and colistin sulfate (polymyxin E), were included in the study to enable structural and functional comparisons, as well as to determine possible mechanisms of action. Chemical synthesis of peptides with intra-molecular disulfide bridging was incredibly expensive; therefore an alternative means of production, namely recombinant expression, was also an aim of this project.

6.2 Results: Peptide Characterisation of Synthetic Defr1

The isolation and functional characterisation of the novel murine β-defensin, Defr1 - a polymorphism of Defb8, which codes for only 5 cysteines was previously described (Morrison *et al.* 2002). The structural characteristics of Defb8 resemble a normal β-defensin in disulfide connectivity and amphipathicity (Fig 6.1). The synthetic Defr1 peptide displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria, demonstrating that the 3-disulfide bonds that characterise β-defensins are not a prerequisite for activity. Furthermore, Defr1 was reported as being active against the ET-12 isolate *Burkholderia cenocepacia* J2315. The activity against *B. cenocepacia* strain J2315 was worthy of further investigation, as no previously described CAMP has been shown to be active against the Bcc. In addition, synthetic Defr1 appeared to be structurally novel in that it exists as covalently bound dimer (Fig 6.2 & Fig 6.3), suggesting a possible structure/function relationship. Protein analyses of Defr1 and Def-cys are shown in Table 6.1 and 6.2.

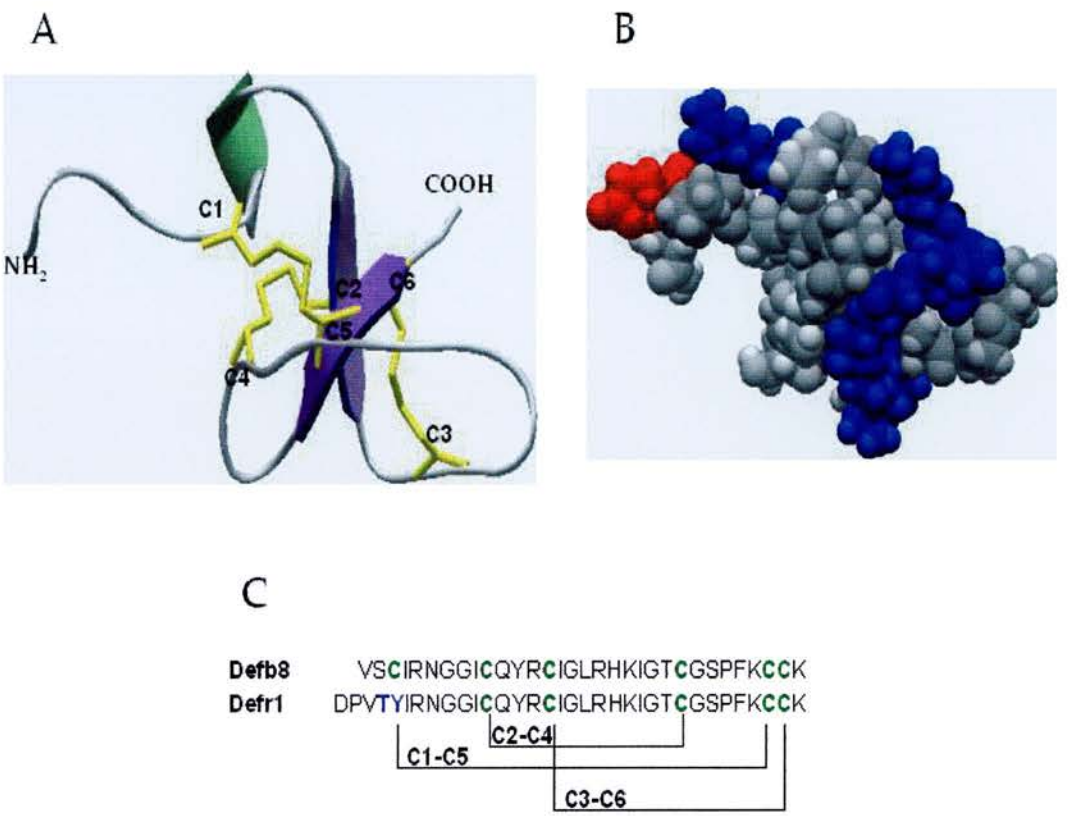
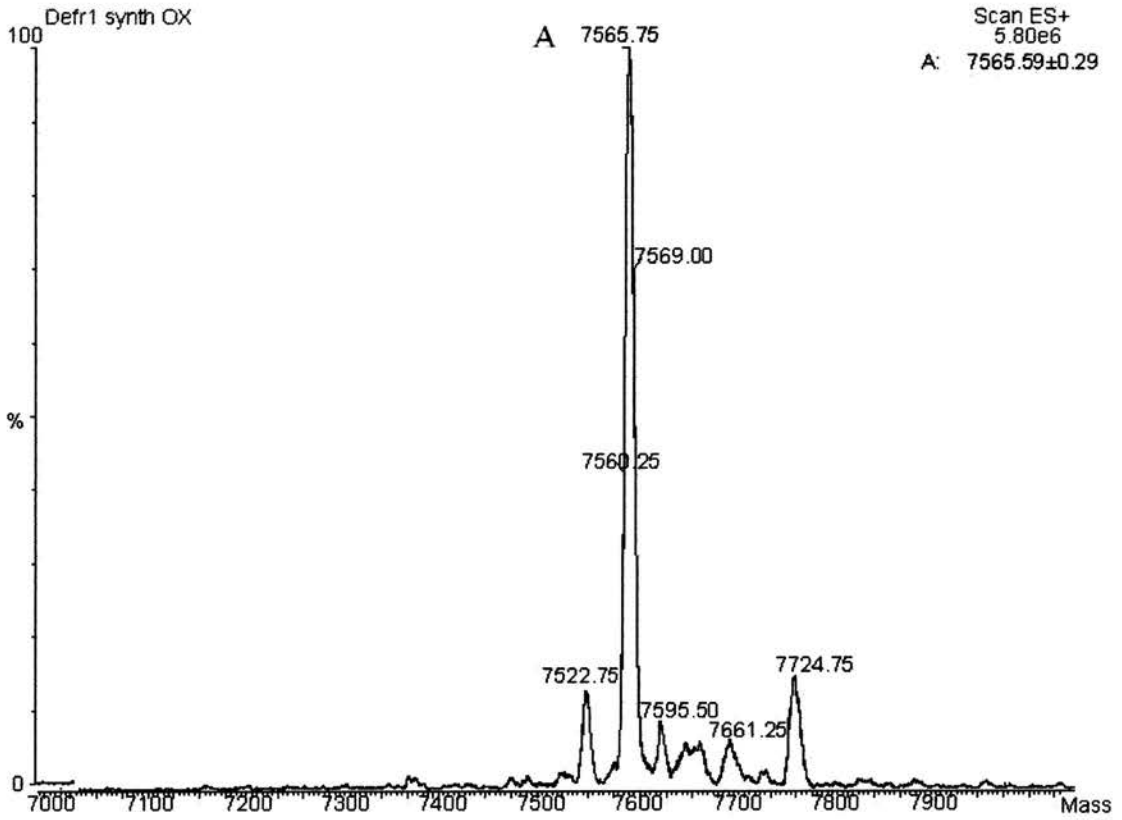


Fig 6.1 Peptide characterisation of Defb8 and its polymorphism Defr1. **A:** The 3D NMR structure of Defb8 demonstrates the characteristic disulfide bond connectivity in a β-defensin (Bauer *et al.* 2001). **B:** The electrostatic surface plot of murine Defb8 demonstrates the cationic nature of the peptide. Acidic and basic residues are represented in red and blue respectively. **C:** A primary sequence alignment of the β-defensin Defb8 and the polymorphism Defr1. The characteristic cysteines are highlighted in green and the intramolecular disulfide bridge connectivity is shown below. Notice the loss of C1 in Defr1 (C1 is replaced by a tyrosine residue). Mass spectrometry analysis of synthetic Defr1 indicates it dimerises via an intermolecular disulfide bridge through two free C5 cysteines.


Defr1 synth OX
Scan ES+
5.80e6
A: 7565.59±0.29
A
7565.75
7569.00
7560.25
7522.75
7595.50
7661.25
7724.75
100
%
0
7000
7100
7200
7300
7400
7500
7600
7700
7800
7900
Mass

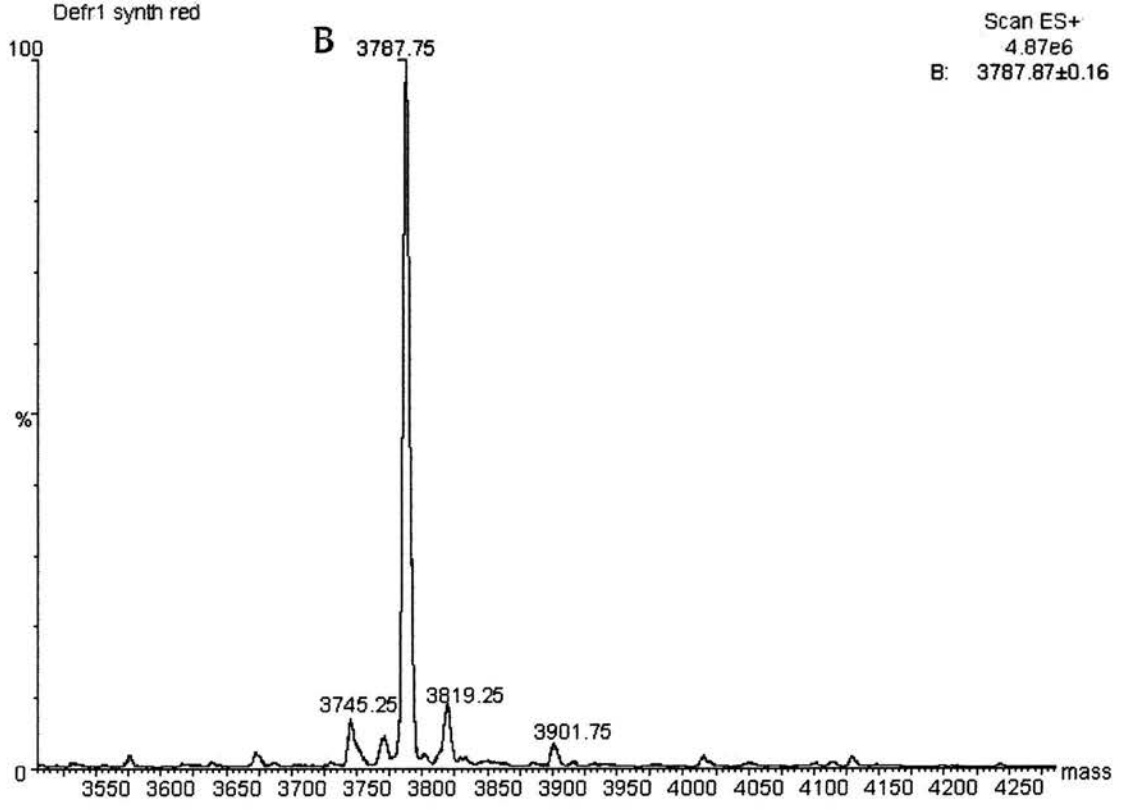

Defr1 synth red
Scan ES+
4.87e6
B: 3787.87±0.16
B
3787.75
3745.25
3819.25
3901.75
100
%
0
3550
3600
3650
3700
3750
3800
3850
3900
3950
4000
4050
4100
4150
4200
4250
mass

Fig 6.2 Electrospray Mass Spectrum (ESI-MS) of synthetic Defr1 (A: Oxidised & B: Reduced). The deconvoluted data represent the mass of the peptide. For Defr1 (A) the mass is given as 7565.59±0.29. This is consistent with a dimer containing intermolecular disulfide bridging when one examines Defr1 reduced. For Defr1 reduced (B) the mass is given as 3787.87±0.16, which is consistent with the predicted mass. (Masses are in the top right hand corner of each spectrum). For each disulfide formed there is a loss of 2 Hydrogen atoms (therefore a mass loss of 2 x 1.008 Da). So mass is consistent with a dimer containing 5 disulfide bonds, ie 2 x (Mass of red peptide) - 5 (2 x 1.008 Da): 2 x 3787.87 - 10.08 = 7565.66 Da. NB: This data does not show how many disulfides are intermolecular or intramolecular. This data was compiled with the help of David Clarke (School of Chemistry, University of Edinburgh).

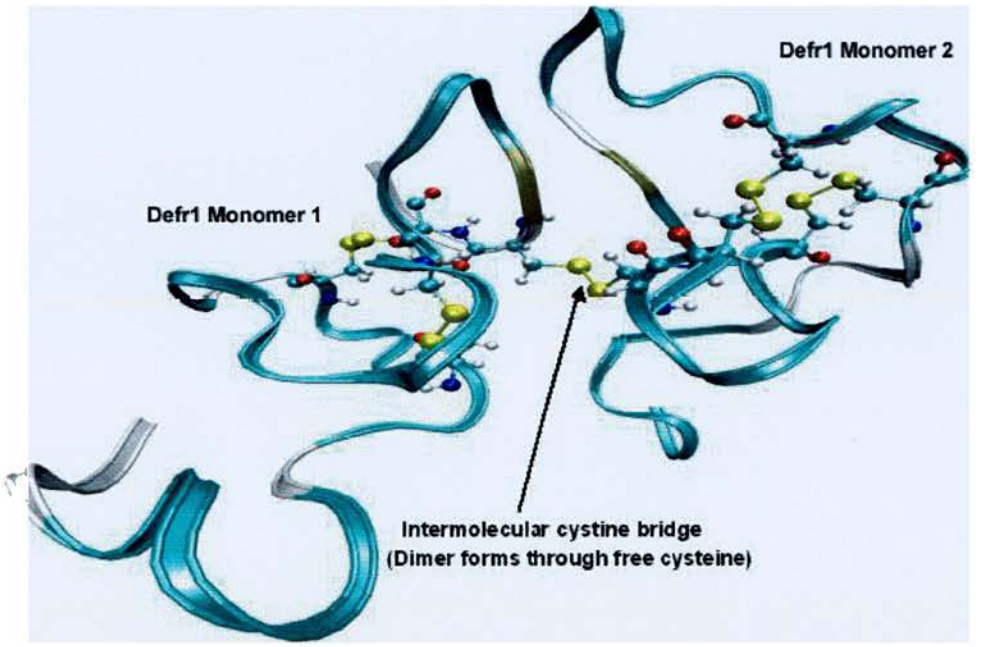


Fig 6.3 Model-ribbon structure of synthetic Defr1. The model illustrates two Defr1 monomers, with two intramolecular disulfide bonds, linked by an intermolecular disulfide bridge between the free cysteines. Disulfide bonds are shown in yellow. Reprinted by kind permission of Dr Perdita Barran (School of Chemistry, University of Edinburgh).

Table 6.1 Defr1 monomeric protein analysis

Analysis	Entire Protein
Length	34 aa
Molecular Weight	3788.20
Isoelectric Point	9.26
Charge at pH 7	4.79

Table 6.2 Def-cys protein analysis

Analysis	Entire Protein
Length	34 aa
Molecular Weight	3728.27
Isoelectric Point	9.15
Charge at pH 7	4.78

6.3 Results: Antimicrobial Activity of Defr1 & Other CAMPs

The initial MIC and dose response assays of the CAMPs focussed on two Bcc isolates, *B. cepacia* ATCC 25416 and *B. cenocepacia* J2315. It became apparent that both were highly resistant to Defr1 (MIC > 100 μg/ml) and other CAMPs investigated, including Def-cys (MIC > 100 μg/ml), polymyxin B (MIC >512 μg/ml) and colistin (MIC >512 μg/ml) (Table 6.3). The dose response data confirmed that both Bcc isolates are resistant to Defr1 and Def-cys (Fig 6.4 & Fig 6.5). The study was therefore expanded to include other resistant and problematic CF organisms including: four multiresistant epidemic *P. aeruginosa* isolates from Manchester (C3425), Liverpool (H183), Brisbane (C4269) and Melbourne (C3781) as well as a PAO1 mutant (PAO579) that secretes copious amounts of a positively charged polysaccharide (alginate) and a rough LPS CF isolate (P235); five CF *S. maltophilia* isolates (C1980, C3625, C3626, C3627, C3642); two strains from the genus *Ralstonia* sp. (C3019 and C3081); one *S. aureus* strain (ATCC 25923), and an MRSA isolate (S113); one *C. albicans* strain (J2922). Other bacterial pathogens investigated included *E. coli* (ATCC 25922), *E. faecalis* (ATCC 29212), *Bordetella bronchoseptica* (J3083) and *Acinetobacter baumannii* (U45). The MICs for each peptide were determined by aliquoting the peptide at concentrations ranging from 0.75-100 μg/ml in phosphate buffer containing log-phase bacteria. The combination was incubated for 3 hours; the incubation time was predetermined by the result of the time-kill assay using PAO1 and Defr1 at five times the MIC (25 μg/ml) (Fig 6.6). Under normal circumstances, lyophilised Defr1 and Def-cys were dissolved in 0.01% acetic acid as this helps to stabilise cysteine formation and inter-bridging (acetic acid provides a source of H^+); however, when investigating the activity of reduced Defr1, the peptide was re-suspended in phosphate buffer and the assay was incubated for 1 hour to limit the level of oxidation which may lead to intermolecular disulfide bridge formation (Fig 6.7). Excluding the Bcc isolates examined, the MIC ranges were: polymyxin B; 0.12- >512 μg/ml, colistin; 0.5- >512 μg/ml, Defr1; 3-25 μg/ml, Def-cys; <12.5-100 μg/ml, Defl reduced; 25-100 μg/ml (Table 6.3). Interestingly, resistance to conventional cationic antimicrobial peptides did not confer resistance to Defr1. All *S. maltophilia*, *Ralstonia* sp., *E. faecalis*, *S. aureus*, MRSA and *C.*

albicans strains examined were highly resistant to both polymyxin B (MIC range 4- >512 μg/ml) and colistin (MIC range 8- >512 μg/ml), but remained as sensitive to Defr1 (3-25 μg) as *P. aeruginosa* strains which were all sensitive to polymyxin B (MIC range 0.5-1 μg/ml) and colistin (MIC range 1- 2 μg/ml). The converse was also true. Sensitivity to both polymyxin and colistin did not confer sensitivity to Defr1. Two strains demonstrated this phenomenon: *P. aeruginosa* PAO579 and *S. maltophilia* C3642. The data suggest that different CAMPs may have different modes of action. Furthermore, in contrast to polymyxin B and colistin, Defr1 is active against both Gram-negative and -positive bacteria.

The data show that, with respect to *P. aeruginosa* PAO1, reduced Defr1 and Def-cys are ten times less active than oxidised Defr1. This lends support to the hypothesis that dimerised Defr1 is more active as a result of disulfide bonds, and in particular an inter-molecular disulfide. Def-cys may dimerise, but not covalently.

6.4 Results: Killing Curve

The rate of killing of *P. aeruginosa* PAO1 with Defr1 was studied. The period required to reduce bacterial viability (initial inoculum 10^5 cfu/ml) to almost zero was found to be between 0 and 30 mins post Defr1 challenge (Fig 6.6).

6.5 Results: Dose Response Curves

The bactericidal activity of Defr1, Def-cys and reduced Defr1 was measured at a range of concentrations for all strains presented in Table 6.1. A representation of the data is shown for PAO1 (Fig 6.8) and *E. coli* ATCC 25922 (Fig 6.9). Bacterial killing by the CAMPs was shown to be dose dependant. Defr1 was found to be most effective at reducing bacterial viability.

Table 6.3 MIC values of cationic antimicrobial peptides against a panel of pathogens

Organism	Strain	Antimicrobial Peptide MIC μg/ml				
		Pol.	**Col.**	**Defr1**	**DefCys**	**Defr1 Red**
P. aeruginosa	PAO1	0.5	2	6	50	50
PA	PAO579/P55	1	1	25	50	100
PA Manchester	C3425	1	1	6	50	100
PA Liverpool	H183	1	1	6	100	100
PA Brisbane	C4269	1	1	6	>100	100
PA Melbourne	C3781	0.5	2	6	100	100
PA (Rough)	P235	1	2	6	>100	50
B. cepacia	ATCC 25416	>512	>512	>100	>100	ND
B. cenocepacia	J2315	>512	>512	>100	>100	ND
S. maltophilia	C1980	32	64	12.5	>100	>100
S. maltophilia	C3625	32	64	6	>100	50
S. maltophilia	C3626	32	64	3	>100	>100
S. maltophilia	C3627	64	256	6	>100	>100
S. maltophilia	C3642	4	8	25	>100	>100
R. pickettii	C3079	>512	>512	3	>100	>100
R. eutropha	C3081/LMG1199	128	>512	3	>100	>100
E. faecalis	ATCC 29212	256	512	6	100	>100
E. coli	ATCC 25922	0.25	0.5	6	>100	>100
S. aureus	ATCC 25923	256	>512	12.5	>100	>100
MRSA	S113/ J2918	64	256	6	>100	25
C. albicans	J2922	>512	>512	3	25	25
B. bronchoseptica	J3083	0.12	0.5	6	>100	>100
A. baumannii	U45	1	1	3	<12.5	ND

Pol: Polymyxin B sulfate, Col: Colistin sulfate, Defr1: Murine β-defensin with 5-cysteine residues, DefCys: Murine β-defensin analog with 6-cysteine residues, Defr1Reduced: Defr1 chemically reduced with DTT (essentially breaking disulfide bonds). ND: Not done. *P. aeruginosa* PA (rough): rough LPS isolate. Experiments were carried out in duplicate and repeated twice, and the MIC values were based on the average.

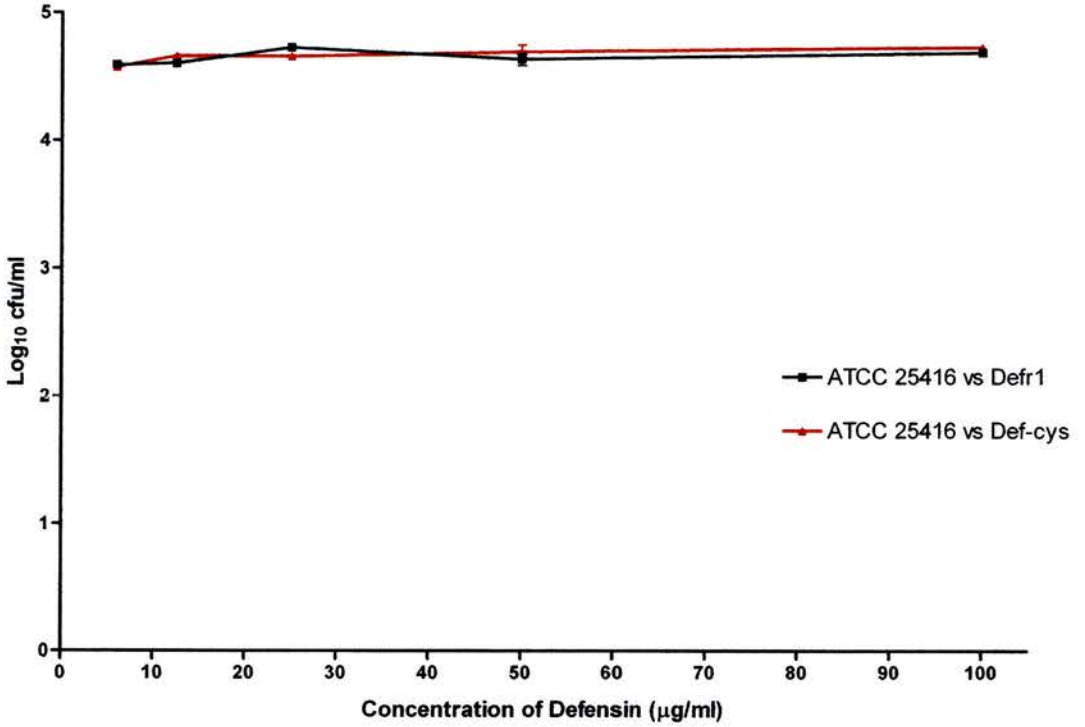


Fig 6.4 Dose response curve. ATCC 25416 vs Defr1 (black line) and Def-cys (red line).

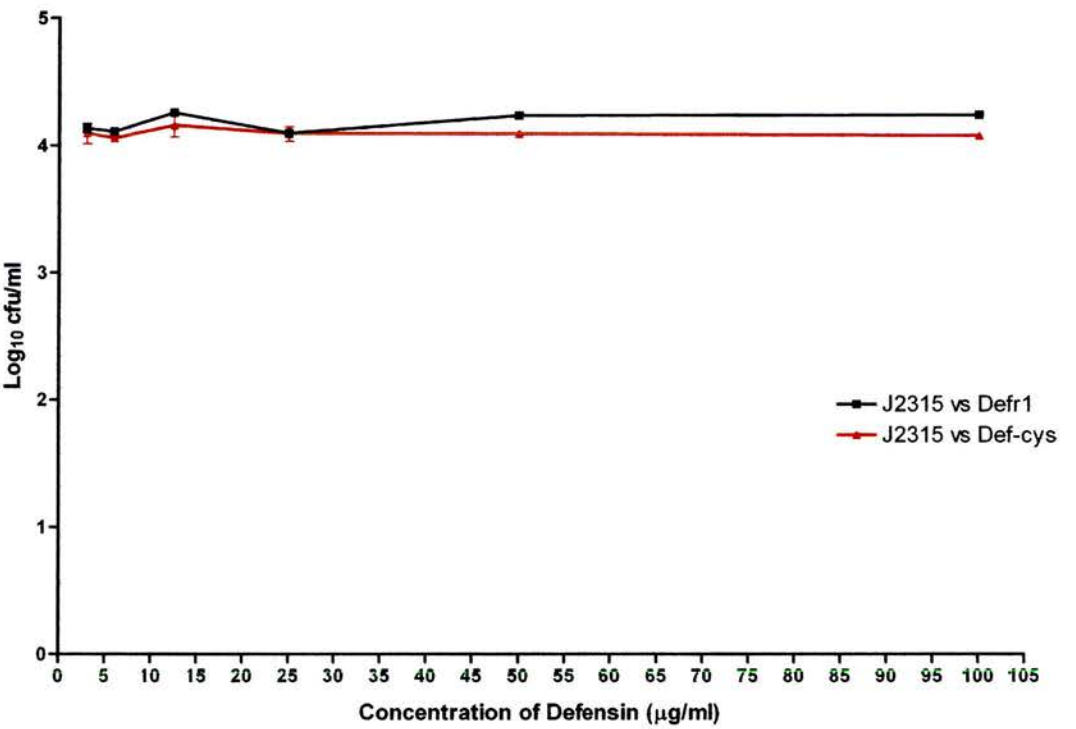


Fig 6.5 Dose response curve. J2315 vs Defr1 (black line) and Def-cys (red line)

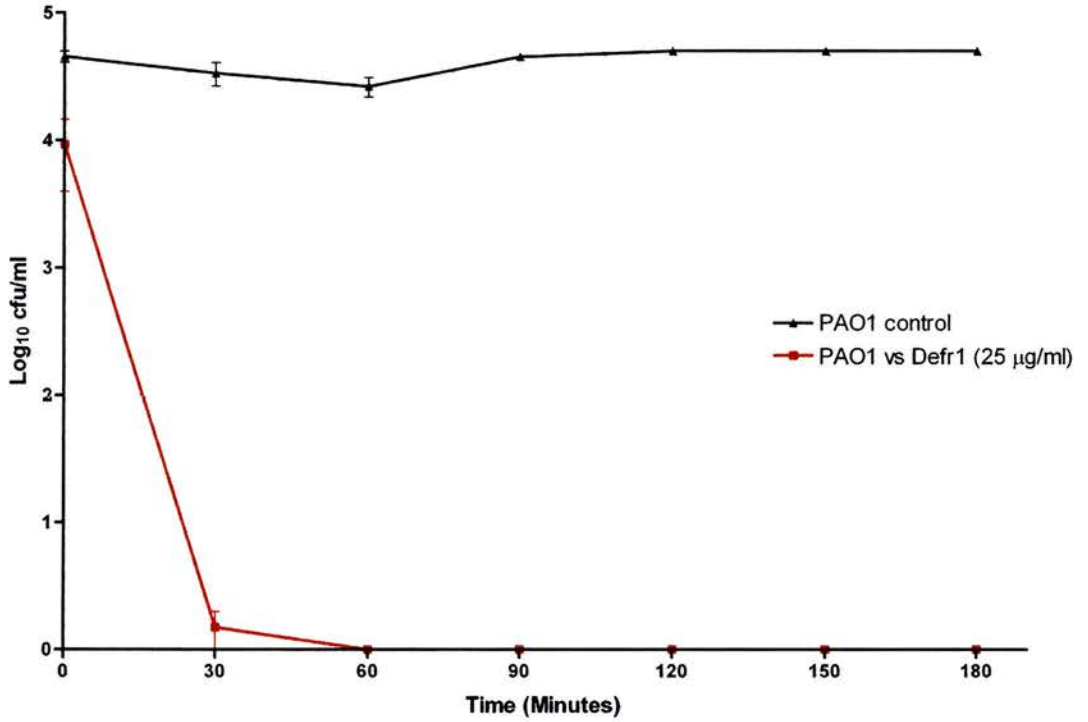


Fig 6.6 Time-Kill Assay. Defensin killing assay (of PAO1 and Defr1 at 5 x MIC: 25 μg/ml). Bacterial samples were removed at 30-minute intervals and plated to determine the viable count (cfu/ml). The data show that bacterial viability was reduced significantly within the first 30 minutes and completely diminished by 60 minutes.

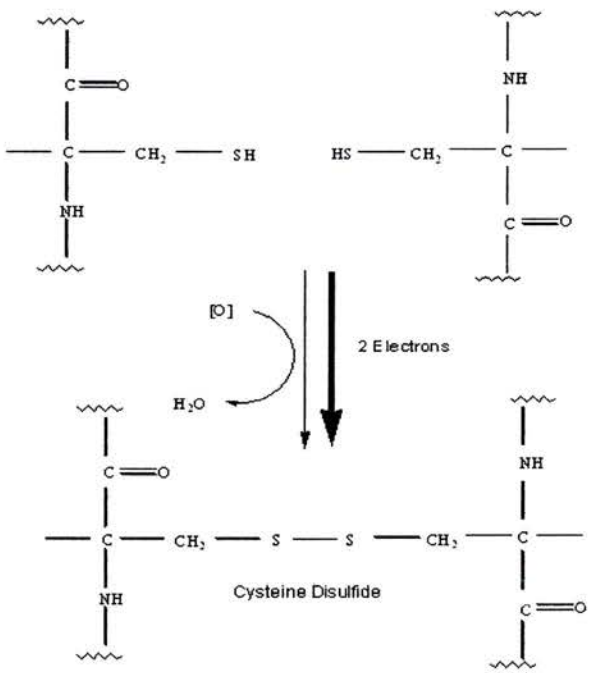
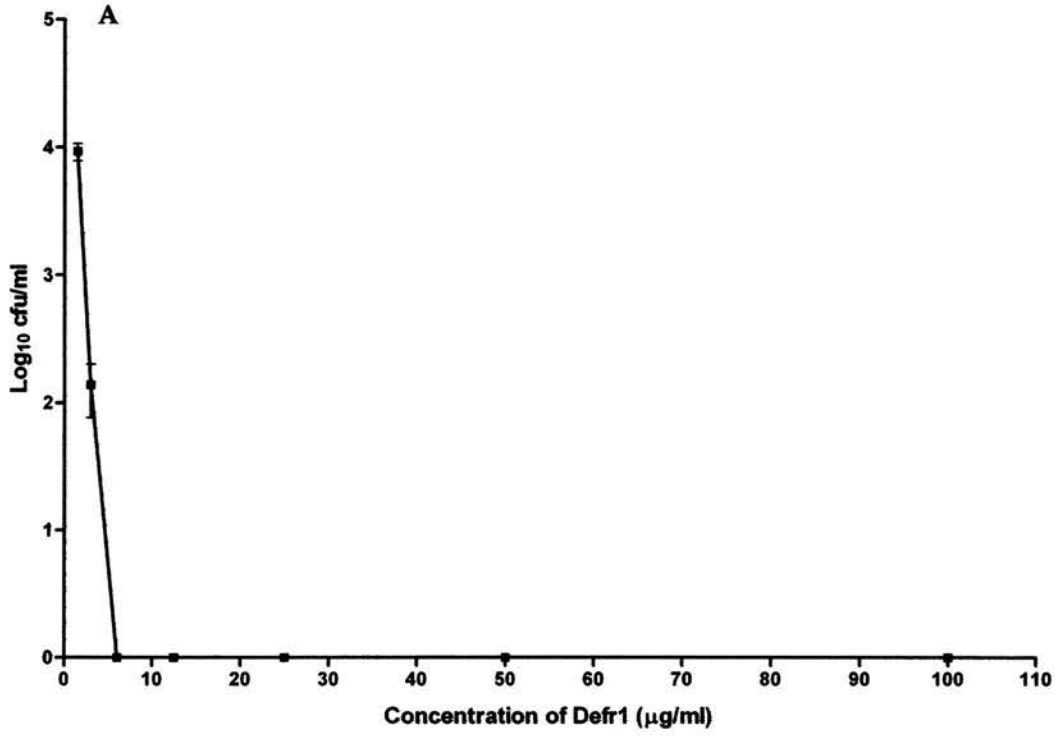
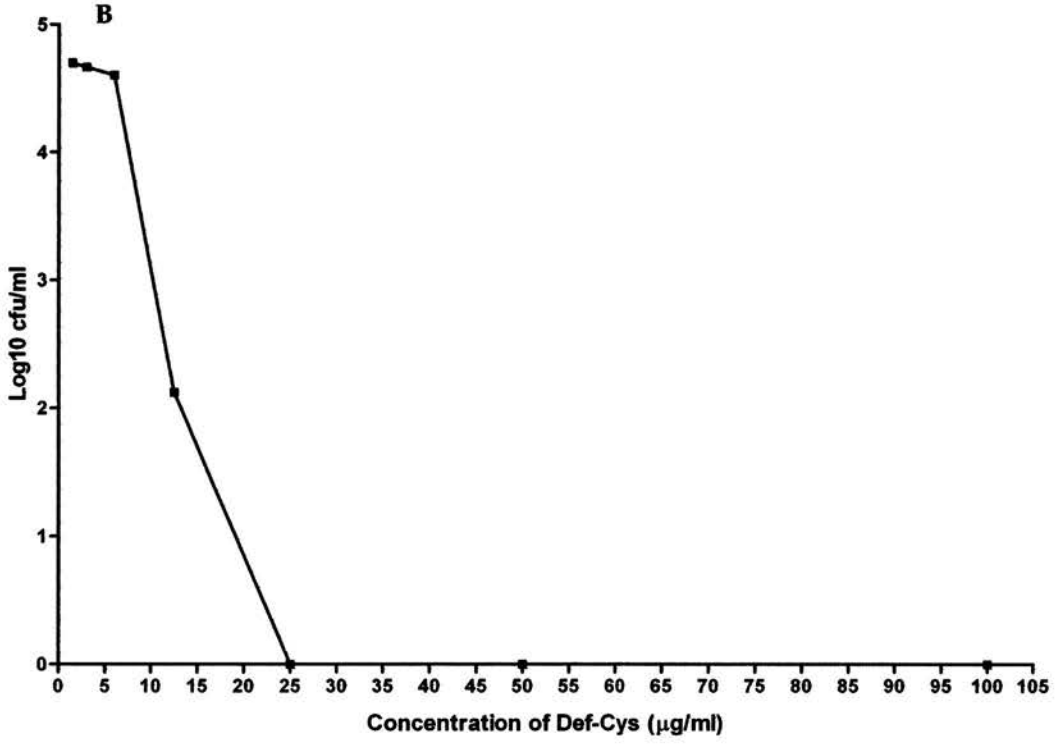


Fig 6.7 Schematic of disulfide bond formation between two cysteine thiol sulfurs


A
Log10 cfu/ml
0
1
2
3
4
5
0 10 20 30 40 50 60 70 80 90 100 110
Concentration of Defr1 (μg/ml)


B
Log10 cfu/ml
0
1
2
3
4
5
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
Concentration of Def-Cys (μg/ml)

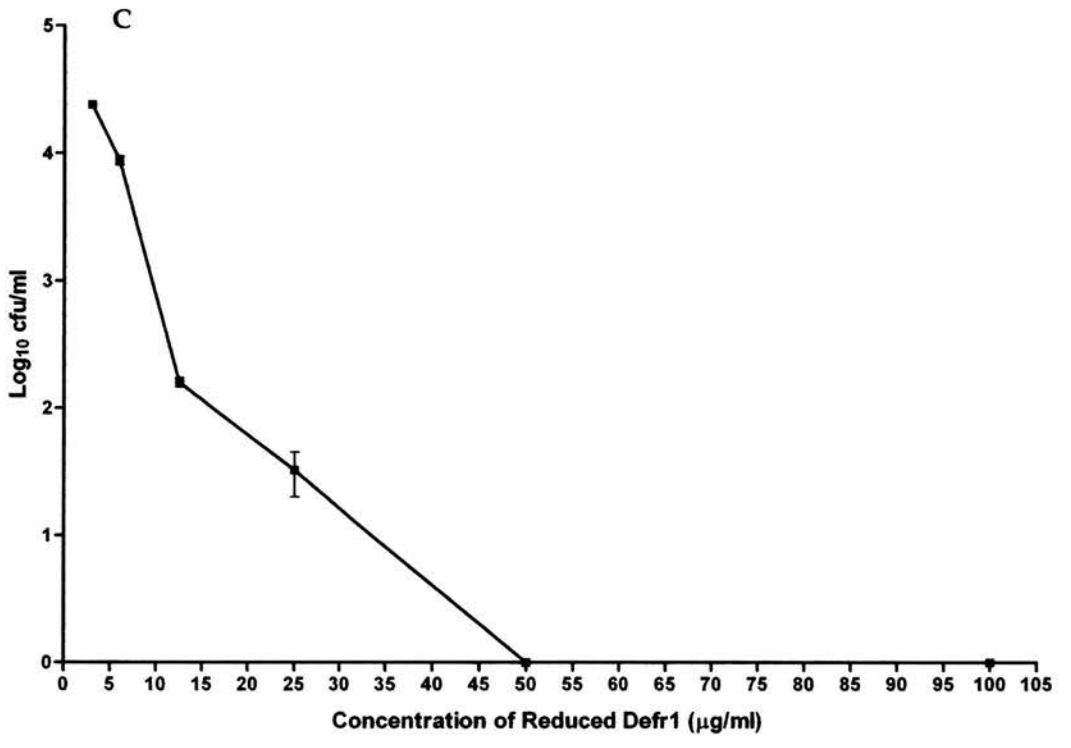
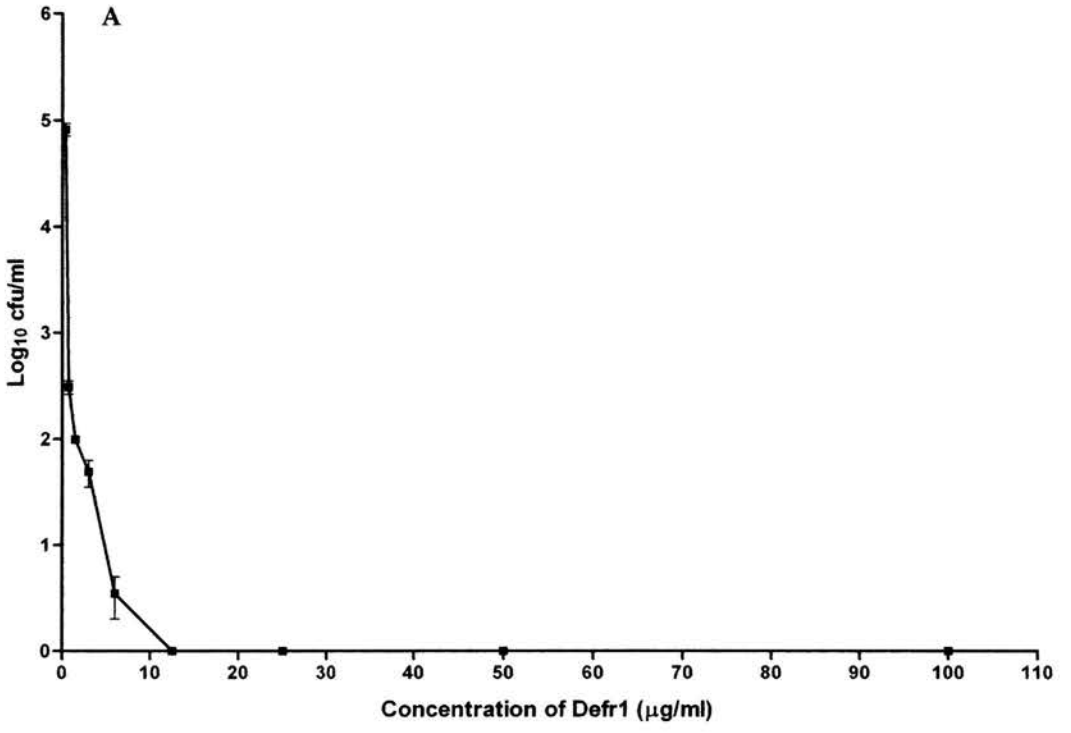


Fig 6.8 Defensin dose response curves: PAO1 vs CAMPs. A: PAO1 vs Defr1 (1.5-100 μg/ml). B: PAO1 vs Def-cys (1.5-100 μg/ml). C: PAO1 vs Reduced Defr1 (1.5-100 μg/ml). Error bars represent the standard error between experimental values.


A
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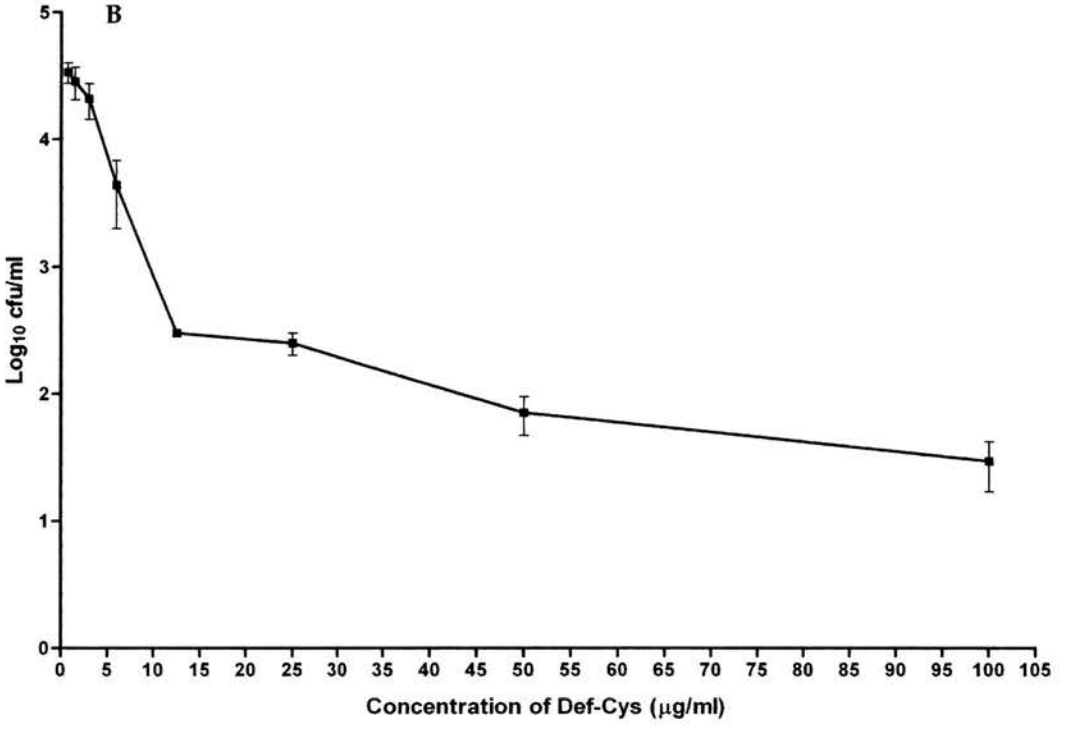
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100 110
Concentration of Defr1 (μg/ml)


B
Log10 cfu/ml
0
1
2
3
4
5
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
Concentration of Def-Cys (μg/ml)

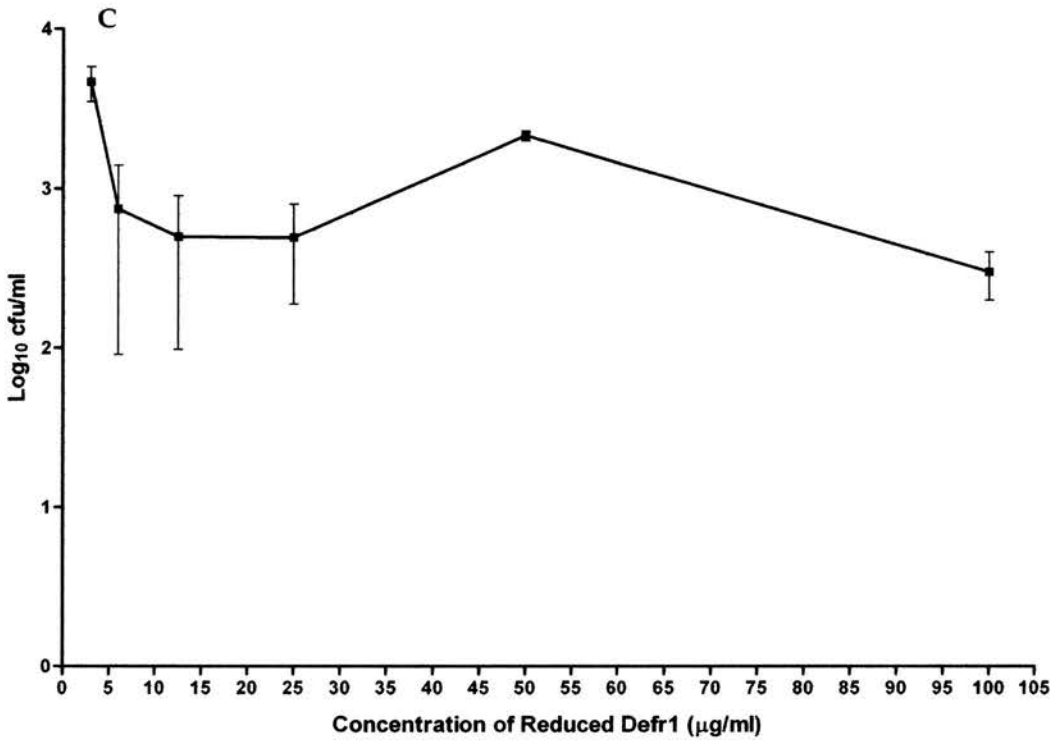


Fig 6.9 Defensin dose response curves: *E. coli* vs CAMPs. A: *E. coli* vs Defr1 (0.375-100 μg/ml). B: *E.coli* vs Def-cys (1.5-100 μg/ml). C: *E.coli* vs Reduced Defr1 (1.5-100 μg/ml). Error bars represent the standard error between experimental values.

6.6 Results: Salt Sensitivity of Defr1

The salt sensitivity of Defr1 was investigated. The standard killing assay was performed with 25 μg/ml of Defr1 in the presence of NaCl (0-300mM). The data show that the inhibitory activity of Defr1 remained uneffected in the presence of 50mM NaCl (Fig 6.10). However, the antimicrobial activity of Defr1 was inhibited as the concentration of NaCl rose from 50-300mM.

6.7 Results: The Inhibition of Defr1 With LPS and LTA

The ability of LPS and LTA to inhibit the action of Defr1 was investigated. The standard killing assay was performed with 25 μg/ml of Defr1, preincubated with either LPS (*P. aeruginosa* PAO1, *E. coli* 0184, *E. coli* 0111:B4 [Sigma], *B. cenocepacia* J2315) or LTA (*S. aureus*: Sigma) for one hour at 37°C, prior to the addition of 10^5 cfu/ml *P. aeruginosa* PAO1. LPS concentrations varied from 0.75-100 μg/ml. LTA concentrations varied from 1.5-100 μg/ml. The data show that LPS inhibits the antimicrobial activity of Defr1 (Fig 6.11). The degree of inhibition varied between the bacterial sources of LPS. Both *E. coli* samples were found to have a similar inhibitory effect on Defr1 activity, but were less effective than PAO1 and J2315. *S. aureus* LTA also inhibited the action of Defr1 (Fig 6.12). Taken together, the data show that both LPS and LTA are capable of inhibiting Defr1, although LTA appears to be more potent.

6.8 Results: Inhibition of Polymyxin B and Colistin With LPS

The ability of *P. aeruginosa* PAO1 LPS to inhibit the action of polymyxin B and colistin was investigated. The standard killing assay was performed with both antibiotics (at 4 μg/ml) preincubated with *P. aeruginosa* PAO1 LPS (0.75-100 μg/ml), as described above. *P. aeruginosa* PAO1 LPS (50 μg/ml) inhibited the bactericidal activity of both conventional cationic peptide antibiotics, but had increased potency at inhibiting colistin with 4.5 times (Log scale) more bacteria surviving (Fig 6.13).

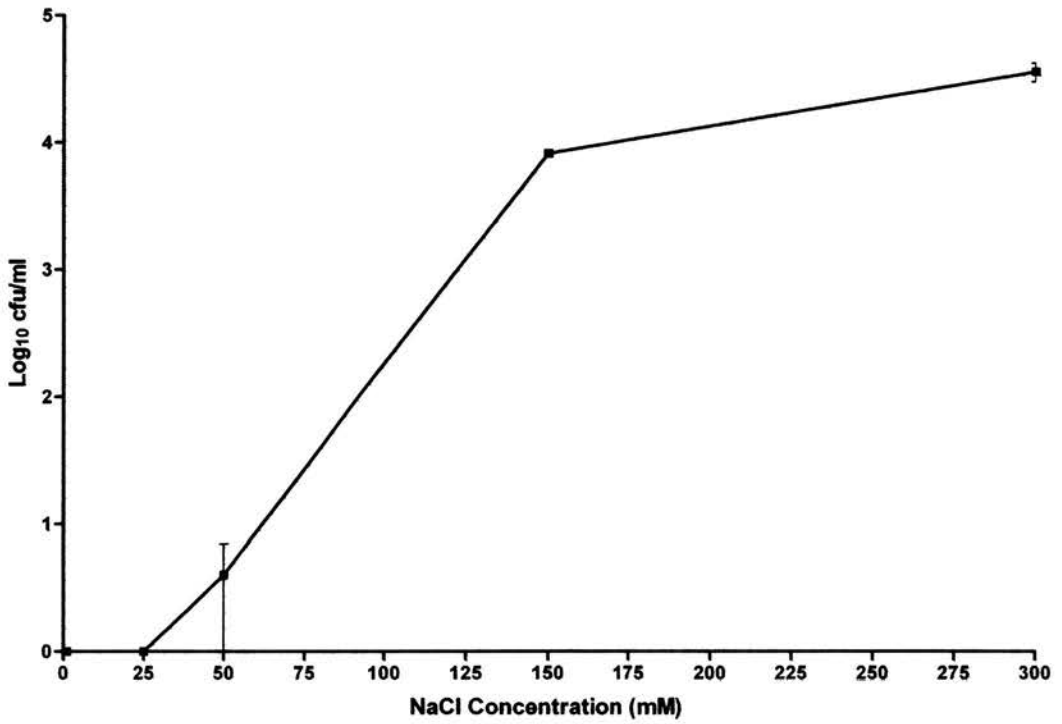


Fig 6.10 Salt sensitivity of Defr1 against PAO1. Defr1 concentration was approximately 5 x the MIC (25 μg/ml) and the salt concentration in the reaction buffer varied from 0-300mM NaCl. Error bars represent the standard error between experimental values.

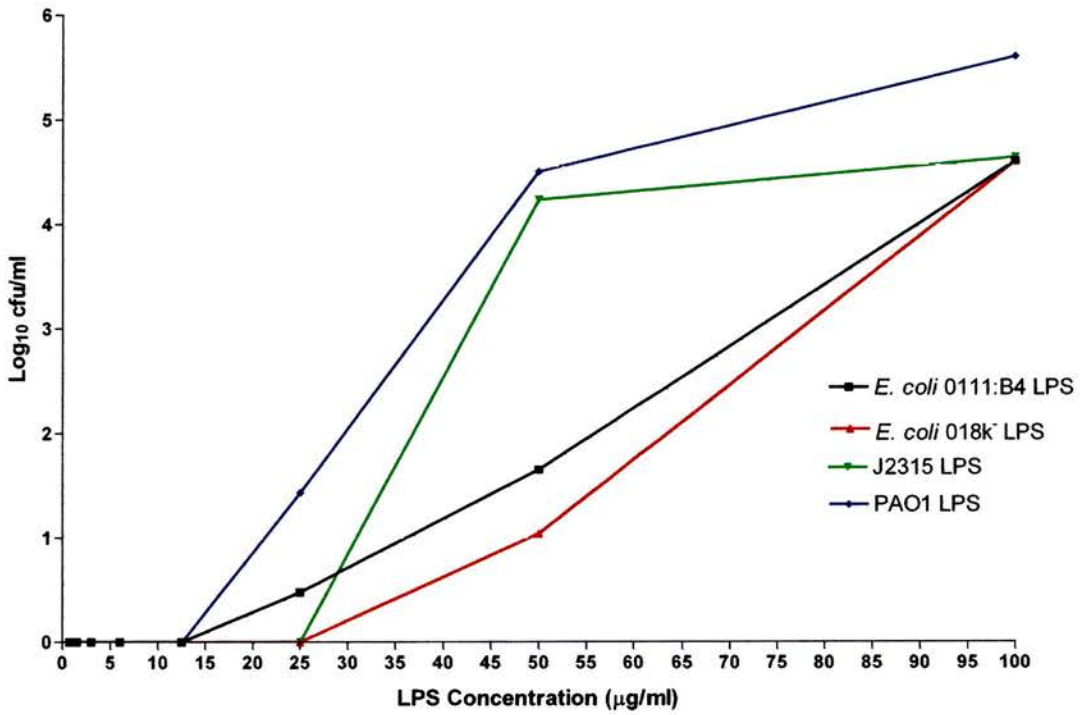


Fig 6.11 LPS inhibition of Defr1 killing against PAO1. Killing assay using PAO1 against Defr1 at a concentration approximately 5 x the MIC (25 μg/ml). Defr1 was preincubated with different LPS at varying concentrations (0.75-100 μg/ml). Bacterial survival is directly proportional to the concentration of LPS added.

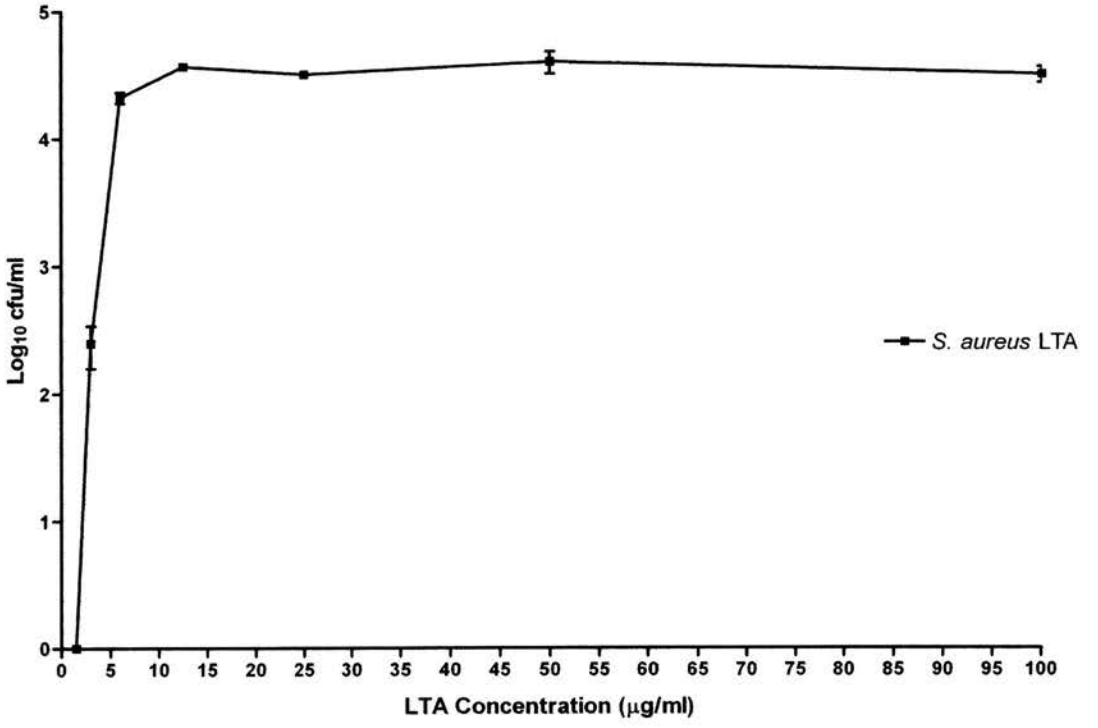


Fig 6.12 LTA inhibition of Defr1 killing against PAO1. Killing assay using PAO1 against Defr1 with a concentration approximately 5 x the MIC (25 μg/ml). LTA was preincubated with Defr1 at varying concentrations (1.5-100 μg/ml). Bacterial survival is directly proportional to the concentration of LTA added. Error bars represent the standard error between experimental values.

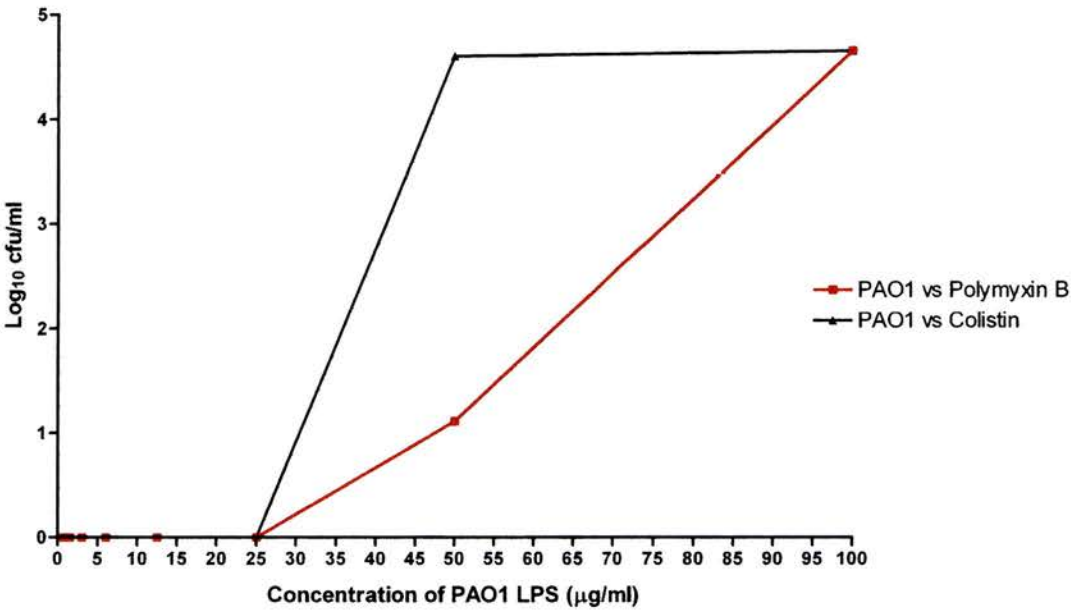


Fig 6.13 Inhibition of polymyxin B and colistin with PAO1 LPS. Killing assay using PAO1 against either polymyxin B and colistin at a concentration of 4 μg/ml. PAO1 LPS was preincubated with either polymyxin B and colistin at varying concentrations (0.75-100 μg/ml). Bacterial survival is proportional to the concentration of LPS added.

6.9 Results: Recombinant Expression of Defr1

The Defr1 gene was amplified from murine cDNA (C57BL/6J) using PCR and the fragment was subsequently cloned into a pET expression vector with a N-terminal His_6-tag (pET 6-His), for expression in various *E. coli* strains (DE3 lysogens). Optimum recovery of protein was achieved by using the BL21 (DE3) strain. Bacteria were grown in shake flasks at 37°C and expression induced with 1 mM IPTG. The Defr1 cell-free lysate was initially subjected to SDS-PAGE and run on a 4-12% gel. Coomassie staining showed no protein band of the expected molecular weight of approximately 5 kDa (Fig 6.14). The molecular weight of 6-His Defr1 was based on protein analysis software, Vector NTI (Table 6.4). However, 6-His Defr1 fusion protein was not expressed successfully. The 6-His tag was also problematic, as it could not be proteolytically cleaved from Defr1. A further expression system, pET-30c (Fig 6.15) was also utilised to help both with expression and to allow cleavage of the tag. Expression was achieved, but insolubility proved to be a problem that was not overcome. Subsequently, Defr1 was cloned into a pET expression system (pET 6His-Glutathione-S-Transferase [GST]), which had a Precission protease site to enable cleavage of the purification tag. This vector was utilised because GST is a large (~29 kDa), highly soluble protein and it was hoped that co-expression with Defr1 would result in high expression and would solve any solubility problem. Expression was induced as before, but the fusion protein was found to be insoluble (Fig 6.16). An alternative route of expression was investigated using the pET-22b expression vector. This system of expression exports induced proteins into the periplasm of *E. coli*. Interestingly, the periplasm is the location of disulfide bond isomerases, which catalyse the formation of disulfide bonds in many *E. coli* proteins (Hayashi *et al.*2000). It was hypothesised, that the complex folding of the two intra-molecular disulfides in Defr1 may be contributing to its high insolubility, thus enzymatic formation may increase the likelihood of soluble protein expression. The *E. coli* competent cells (BL21 DE3) were transformed with the pET-22b plasmid containing the Defr1 gene, as described previously. The cells were grown to OD =1, and then induced with 1 mM IPTG, prior to proteins being extracted from the periplasm using CTAB. The periplasmic extract was then purified

S® protocol with FPLC. Forty fractions were collected and the absorbance for each was measured (Fig 6.17). Fractions were then subjected to SDS-PAGE analysis (Fig 6.18A). A curious band, suspected to be approximately 3 kDa, was identified in fraction 36, which was concentrated at 4°C, using a 3000 MWCO spin filter, and analysed by SDS-PAGE (Fig 6.18B). Unfortunately, mass spectrometry analysis did not confirm the presence of Defr1 in fraction 36, suggesting that Defr1 had not been successfully purified.

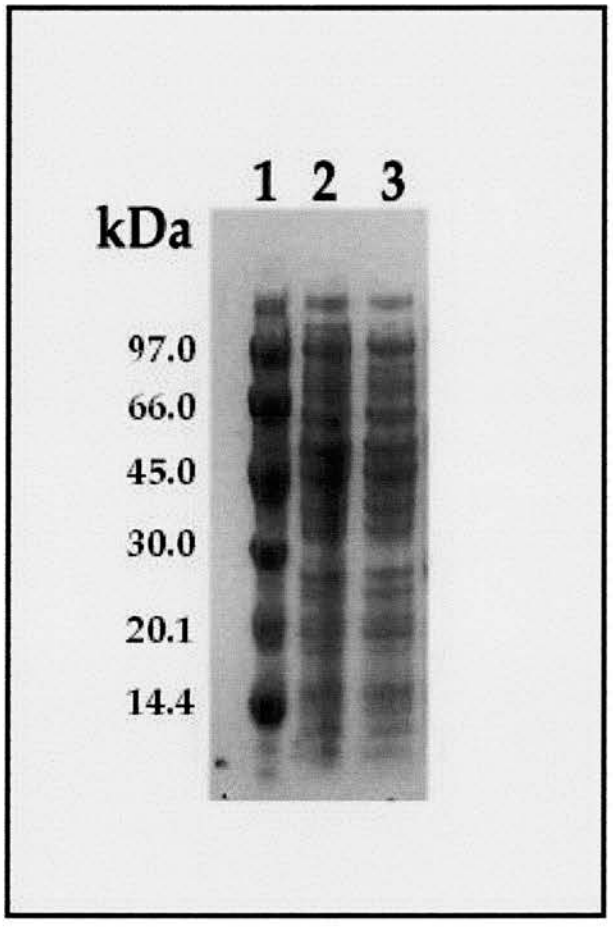


Fig 6.14 Expression of 6His-Defr1 fusion protein. Lane 1: LMW marker; Lane 2: Control (no IPTG); Lane 3: IPTG induced expression.

Table 6.4 pET 6His-Defr1 analysis

Analysis	Entire Protein
Length	50 aa
Molecular Weight	5733.36
Isoelectric Point	9.45
Charge at pH 7	6.32

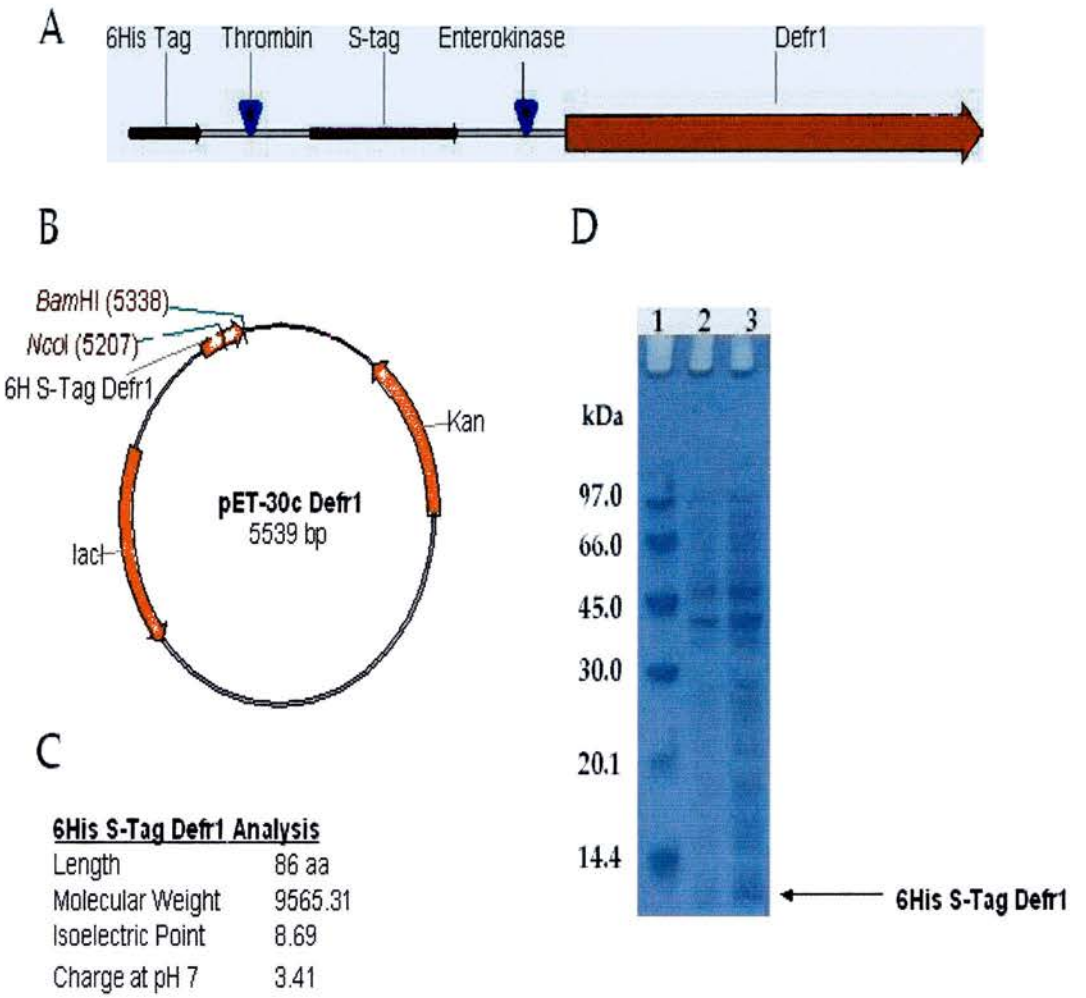


Fig 6.15 Recombinant expression of Defr1 in pET-30c system. **A**. Schematic of 6His S-Tag Defr1 fusion protein. The thrombin and enterokinase protease sites enabled full cleavage of 6His-S tag. **B**. pET-30c expression plasmid. **C**. Protein analysis of 6His S-Tag Defr1 prior to cleavage. **D**. SDS-PAGE analysis of expressed fusion protein (4-12% SDS-PAGE gel stained with commassie blue), Lane 1: LMW marker; Lane 2: Control (no IPTG); Lane 3: IPTG induced expression of fusion protein.

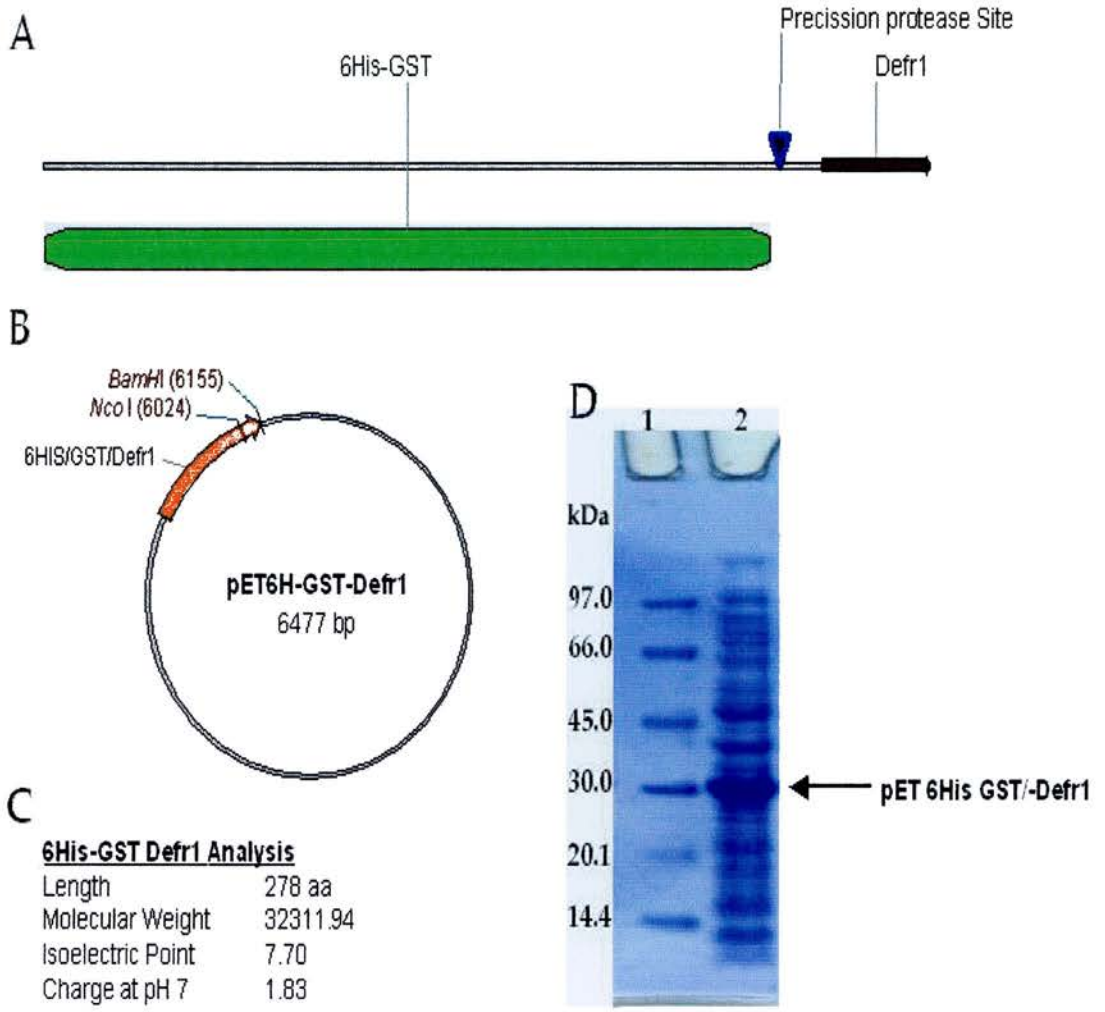


Fig 6.16 Recombinant expression of Defr1 in pET6H-GST system. **A**. Schematic of 6His-GST Defr1 fusion protein. The Precission protease site enabled cleavage of 6His-GST tag. **B**. pET His-GST Defr1 expression plasmid. **C**. Protein analysis of 6His-GST Defr1. **D**. SDS-PAGE analysis of expressed fusion protein (4-12% SDS-PAGE gel stained with commassie blue), Lane 1: LMW marker; Lane 2: Cell pellet showing highly expressed fusion protein.

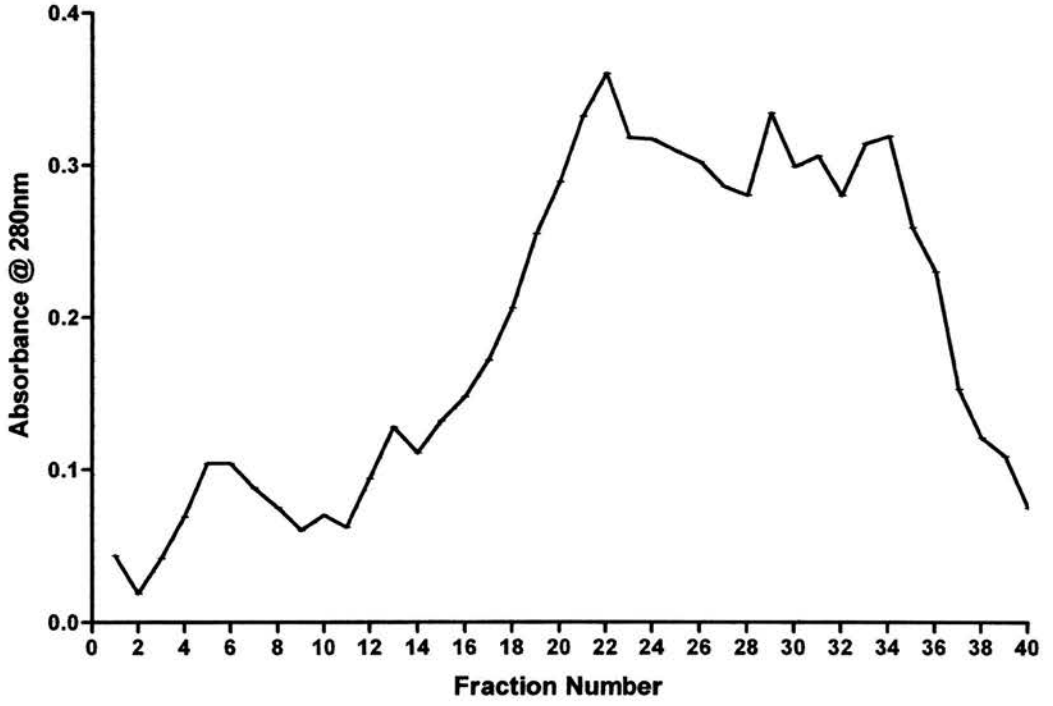


Fig 6.17 Resource S® purification (FPLC) of periplasmic Defr1 extract. OD was measured at 280 nm.

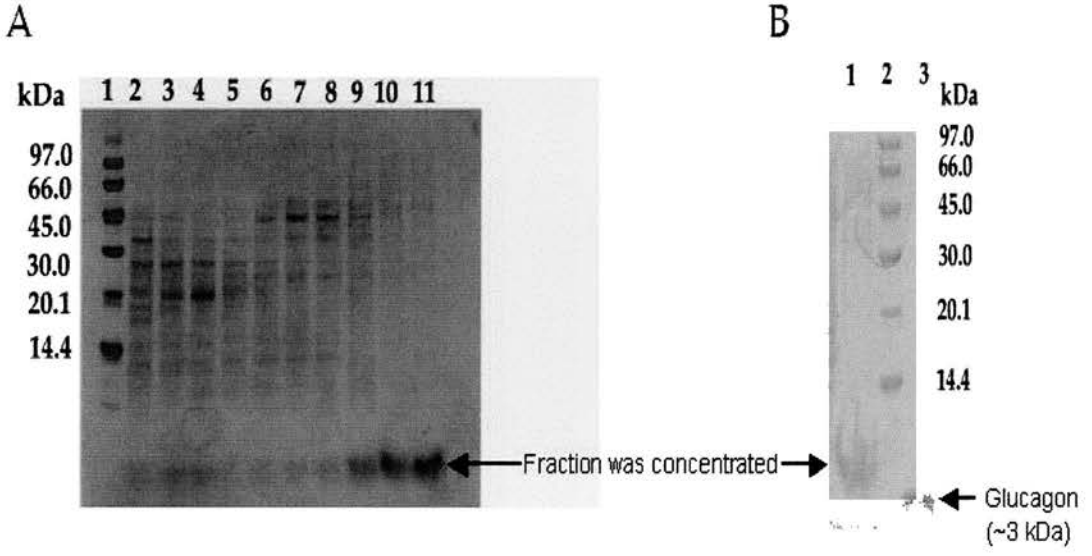


Fig 6.18 SDS-PAGE analysis of periplasmic extract purified by FPLC. **A:** Lane 1: LMW marker; Lane 2: fractions 18/19; Lane 3: fractions 20/21; Lane 4: fractions 22/23; Lane 5: fractions 24/25; Lane 6: fractions 26/27; Lane 7: fractions 28/29; Lane 8: fractions 30/31; Lane 9: fractions 32/33; Lane 10: fractions 35/35; Lane 11: fraction 36. **B:** Lane 1: concentrated (x2) fraction 36; Lane 2: LMW marker; Lane 3: glucagon (~3 kDa).

6.10 Discussion

The sensitivity of bacteria to CAMPs has been described as a potential "Achilles heel" (Zasloff 2002). In this chapter, the antimicrobial activity of a novel murine defensin was investigated in relation to structure/function since Defr1 is unusual in containing five cysteine residues (as opposed to six) and initial mass spectrometry analysis inferred the presence of a covalently linked dimer. The importance of intra- and inter-molecular disulfide bonds was also examined.

To the author's knowledge, this is the first report of a covalently bound defensin dimer with broad-spectrum activity against multiresistant Gram-positive, Gram-negative and fungal pathogens. Recent structural studies have suggested that, in contrast to HBD1 and HBD2, HD3 forms a non-covalently bound dimer that may account for its increased activity by increasing the positive surface charge (Schibli *et al.* 2002). Thus, the increased activity noted for synthetic Defr1 may be explained in a similar manner. The relationship between defensin structure and activity has been investigated in other studies (Wu *et al.* 2003). These authors produced protein homologs lacking cysteine residues, and concluded that intramolecular disulfide bonds in HBD3 did not contribute to antibacterial activity, although such bonding appeared essential for the chemotactic properties of the peptide. In contrast to these findings, the data presented in this thesis suggest that antibacterial activity is intrinsically linked to disulfide bond formation in Defr1. MIC data for covalently dimerised synthetic Defr1, its six-cysteine homolog, and a chemically reduced form of Defr1 support the hypothesis that inter-molecular disulfide bonding increases the antibacterial activity. Thus, dimerised Defr1 is more active. Furthermore, the fact that many of the bacterial pathogens investigated demonstrated resistance to the polymyxins yet were sensitive to Defr1 suggests that not all CAMPs necessarily have the same mechanism of action.

Unfortunately, as has been reported for other defensins (Sahly *et al.* 2003), Bcc species were not sensitive to the action of Defr1. This result contradicts a previous study by Morrison and co-workers who reported 40% killing of *B. cenocepacia*

J2315 by Defr1 (Morrison *et al.* 2002). We were unable to reproduce this activity. A possible explanation may be that the "batch" of Defr1 used in the earlier study was not identical to the one used in this thesis. Unfortunately, the original sample was unavailable for comparison. Other multiresistant CF pathogens, including *S. maltophilia*, epidemic *P. aeruginosa* and MRSA were each found to be sensitive to Defr1. Since conventional treatments for such pathogens remain limited, novel antimicrobial molecules could be therapeutically significant. The decision to include the polymyxins in this study was deliberate and informative. The polymyxins are amphiphilic cyclic polycationic peptides and constitute a separate class of CAMPs consisting of five different molecules, polymyxin A-E (Gales *et al.* 2001). Only two polymyxins are used therapeutically, polymyxin B and E (colistin), and their activity extends mainly to Gram-negative bacteria (Conrad and Glanos 1995). Polymyxin B, a fermentation product from *Bacillus polymyxa*, has an overall net charge of +5 (Hancock and Chapple 1999). Polymyxin E (colistin), a fermentation product from *Bacillus colistinus*, became clinically available in 1959, and exists in two forms: colistin methanesulfonate and colistin sulfate. The former has a superior therapeutic ratio, but cannot neutralise endotoxin to the same extent as colistin sulfate which was used in this thesis (Anonymous 2002, Li *et al.* 2001).

A major caveat in the use of 'defensins' as therapeutic agents is the development of bacterial resistance to mammalian derived CAMPs. A recent review outlined the need for further research prior to the widespread use of mammalian, and in particular human, CAMPs (Bell and Gouyon 2003). Much of the concern has focussed on the possibility that widespread use of human derived CAMPs may result in the development of resistance in bacteria rendering the human host unable to mount an effective innate response against bacterial pathogens. The study of a murine defensin has even greater therapeutic significance therefore, as any resistance generated may not affect human CAMPs, unless the resistance mechanism to all mammalian CAMPs is the same. Conventional opinion appears to be that resistance to CAMPs is both rare and unlikely (Hancock and Chapple 1999, Zasloff 2002). Despite this re-assurance, the presence of CAMP resistance has been recognised and cannot be ignored. The interaction of the CAMP with negatively charged moieties on the

bacterial surface appears to be the site of resistance. In the case of Gram -negative bacteria, alterations in LPS structure affect sensitivity to CAMPs. In *Salmonella* the PhoP/PhoQ two-component system plays an important role in resistance to CAMPs and virulence (Miller *et al.*1990). This system is widespread in Gram -negative bacteria and related to virulence via alterations in the bacterial cell membrane (Oyston *et al.*2000). Interestingly, a popular explanation for the intrinsic resistance of the Bcc to the polymyxins is based on reduced negativity of LPS by the addition of Ara4N to the lipid A moiety of LPS. This alteration in LPS has been shown to confer polymyxin resistance in a number of other bacteria, including *E. coli* (Boll *et al.*1994, Numimila *et al.*1995), *Salmonella enterica* (Vaara *et al.*1981), and *Proteus mirabilis* (Radziejewska-Lebrecht *et al.*1988). Resistance via this mechanism is assumed to be due to a lower binding efficacy of the positively charged polymyxin and the reduced negatively charged LPS. Yet, the data presented in this thesis has shown that Bcc LPS can bind CAMPs (including the polymyxins) and prevent them from killing sensitive bacteria at five times the MIC. It has been suggested that CAMPs block binding of LPS to lipopolysaccharide binding protein (LBP) (Scott *et al.*2000). However, the data presented show that free LPS is capable of "mopping up" CAMPs, suggesting that increased resistance may be achieved by shedding LPS. Further novel and potentially important evidence of an interaction between Bcc LPS and polymyxin B was described recently (Shimomura *et al.*2003). The authors demonstrated that polymyxin B can bind Bcc LPS without reducing its ability to induce TNF-α or IL-6 and, increase its capacity to induce IL-1β at concentrations of polymyxin B that completely removed the cytokine stimulating effect of *Salmonella typhimurium* LPS. Therefore, despite the apparent reduced negativity associated with the addition of Ara4N, Bcc LPS can bind and alter the interaction between CAMPs and bacterial membranes. Thus, it could be hypothesised that inherent resistance in the Bcc to CAMPs is not due to lack of binding, but downstream of this initial interaction.

The recombinant expression of a mammalian defensin was attempted in a prokaryotic expression system as described. Unfortunately, the expression of an active defensin was not achieved. There are several reasons that may account for this

failure. First, this study has shown that synthetic Defr1 has antibacterial activity against *E. coli*. Therefore expression of a toxic molecule in a system based in *E. coli* cells may appear naive. However, recombinant expression was attempted on the basis of the cost of synthetic Defr1 and because successful expression of HBD3 had previously been described in *E. coli* (Harder *et al.* 2001). However, key points were omitted from this study and despite the authors' claims, recombinant expression of HBD3 must remain questionable. Harder and co-workers failed to mention solubility issues, the refolding conditions for the intra-molecular disulfides, the yield of protein obtained from recombinant expression, or whether they observed *E. coli* toxicity associated with HBD3 expression. In this thesis, attempts to resolve these issues were unsuccessful since attempts to contact Harder and co-workers electronically or otherwise failed. The second reason for failure may be related to toxicity. It was noted, for example, that the expression of Defr1 impaired and slowed bacterial growth, suggesting that toxicity may play a role in poor expression. A third explanation is that the *E. coli*-derived protein lacks post-translational modifications that may be necessary for successful expression. Fourth, insolubility may be associated with unchecked over-expression, where protein folding is prone to error. Fifth, mass spectrometry analysis of synthetic Defr1 suggests that it exists as a dimer, linked covalently by an inter-molecular disulfide bond, in addition to two intra-molecular disulfide bonds in each monomer. Disulfide bond formation is a complex process and misfolding may affect expression and antimicrobial activity.

The therapeutic significance of a novel molecule with an extended spectrum of activity against multiresistant CF pathogens cannot be understated. The chronic infections associated with CF lung disease are continuingly being exposed to high concentrations of antibiotics, including aerosolised colistin and tobramycin. The selective pressure that such therapy applies to bacteria is considerable and may contribute to selecting more highly resistant strains (including epidemic and multiresistant *P. aeruginosa*), or increase colonisation by multiresistant bacteria such as *S. maltophilia* and MRSA. In contrast to CF isolates of *P. aeruginosa*, resistance of non-CF isolates has been shown to be relatively low and unchanged over the last five years (Henwood *et al.* 2001). An intriguing explanantion for the high levels of

resistance in CF isolates is a corresponding high level of hypermutable (mutator) strains in the CF patients (Oliver *et al.* 2000). Interestingly, colistin resistance in CF isolates is thought to be low, with approximately 3% of CF isolates resistant based on conventional breakpoints (Catchpole *et al.* 1997). The level of colistin resistance in CF isolates may, however, be under-estimated, with 33% resistance noted during a recent clinical trial of nebulised tobramycin versus colistin (Govan 2002, Hodson *et al.* 2002). Furthermore, bacterial sputum loads were only partially reduced, suggesting that colistin may exert both a bactericidal and anti-inflammatory effect (Hodson *et al.* 2002). In addition, colistin resistant *P. aeruginosa* isolates emerged, and appeared transmissible, after use of IV colistin in a paediatric CF centre in Leeds, UK (Denton *et al.* 2002).

Treatment of a CF individual with synthetic Defr1 may be beneficial, but not without hazards. First, resistance to Defr1 in sensitive organisms was not observed within the laboratory, and although it would be naïve to assume that it could not arise, it does appear to be a rare event. Second, multiresistant bacteria have been shown to be sensitive to the action of Defr1, even if they are highly resistant to the polymyxins, thus selection of such organisms would be reduced. It could be argued that the acquisition of Bcc may be increased, due to their inherent resistance to CAMPs. However, there is no evidence that the increased use of colistin in the 1990s in early therapy against *P. aeruginosa* is associated with the emergence of the Bcc. Nor did Bcc emerge during the recent trial of tobramycin versus colistin (Govan 2002). Bcc acquisition is reduced, but not eliminated, by strict cross infection procedures and thus the therapeutic use of Defr1 should not increase the incidence significantly if suitable cross infection measures are in place. In addition, bacterial killing is not necessarily all that is desired of a novel therapy. Synthetic Defr1 has been shown to bind to *B. cenocepacia* (J2315) LPS, suggesting potential use as an anti-inflammatory agent. However, the recent seminal paper by Shimomura *et al.* (2003) emphasises the caution that is required in attributing LPS-CAMP binding to anti-inflammatory effects.

7.1 Conclusions

The need for novel antimicrobial agents has seldom been so urgent. The widespread use (and mis-use) of conventional antibiotics has resulted in an upsurge in infections caused by multiresistant bacteria. Aggressive and long-term use of antibiotics is particularly typified during the treatment of CF lung infections, with the benefits of reduced morbidity and mortality. However, the cost of such therapeutic regimes is that the bacterial pathogen demonstrates increasing resistance throughout the course of the disease, and more highly resistant species are acquired. Multiresistant species, such as members of the Bcc, epidemic *P. aeruginosa*, *S. maltophilia* and MRSA are problematic, and virtually untreatable. In the case of *P. aeruginosa* and Bcc this situation is exacerbated by potential for epidemic spread and increased virulence (Jones *et al.* 2002).

7.2 General Conclusions

This thesis has focussed on three main areas, New Zealand manuka honey, bacteriophage and their endolysins, and a novel murine β-defensin: Defr1. The significance of both broad-spectrum agents, NZMh and Defr1, is that antimicrobial activity extended to highly resistant Gram-negative and Gram-positive bacteria. NZMh has been shown to be bactericidal for members of the Bcc, as well as other multiresistant bacteria. The active components were not identified, although osmolarity, H_2O_2 and pH were not found to be solely responsible. Interestingly, this agent was used clinically, and appeared to show considerable promise during an episode of "cepacia syndrome".

The bacteriophages of the Bcc have been shown to be promiscuous, their spectrum of activity extending across the complex and to other pseudomonads. This widespread activity is undesirable for therapeutic agents and may contribute to antibiotic resistance via horizontal transfer. The recombinant expression of bacteriophage-derived endolysins was investigated. Although both failed to demonstrate bacterial killing, further work is required to optimise expression.

The activity of a novel five-cysteine containing murine defensin, Defr1, was found to extend to multiresistant organisms that demonstrated considerable resistance to conventional CAMPs, including polymyxin B and colistin. Despite the fact that

members of the Bcc were resistant, Defr1 was capable of binding LPS derived from *B. cenocepacia* J2315. This binding was capable of inhibiting the action of Defr1 against a sensitive strain at five times the MIC suggesting that resistance to Defr1, in the Bcc, is downstream of LPS binding. The relationship between Defr1 structure and its antimicrobial activity was also investigated. It was found that intra- and inter-molecular disulfide bridging are important factors in the potency of this molecule as an antimicrobial agent. Recombinant expression was attempted in a number of *E. coli* based expression systems. The Defr1 protein expressed was invariably insoluble; therefore further work is required to express a denatured form of Defr1 with subsequent refolding steps.

Although some results were not as we would have wished, the use of the novel agents described could have a significant therapeutic impact in the treatment of CF lung infections. Possible synergism with conventional therapies may increase their efficacy, particularly Defr1. Antibiotic resistance in the Bcc can result from reduced OM permeability, thus agents capable of disturbing the membrane may sensitise the bacteria to hydrophilic antibiotics. Such "sensitisation" has been suggested for polymyxin derivatives (Tsubery *et al.* 2000). Therefore, although Defr1 did not demonstrate killing against the Bcc, it may yet prove to be an important Bcc antagonist.

Taken together, these results show that resistance against antimicrobial agents varies within the Bcc. NZMh exerts a promising bactericidal effect on members of the Bcc, including *B. cenocepacia* J2315. The novel Bcc bacteriophages may prove to be a useful panel for further study, either as vectors for horizontal gene transfer or as therapeutic agents. The results confirm previous reports showing that Bcc isolates are inherently resistant to CAMPs, including the novel 5-cysteine defensin, Defr1. However, synthetic Defr1 was shown to be active against a panel of multiresistant pathogens associated with infections in CF and the respiratory tract. Further research is required to optimise the recombinant expression of Bcep781 endolysin, D3 lysin, and Defr1, to allow investigation of their antimicrobial properties and their potential as therapeutic agents against multiresistant CF pathogens.

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Lysogeny and bacteriophage host range within the *Burkholderia cepacia* complex

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The *Burkholderia cepacia* complex comprises a group of nine closely related species that have emerged as life-threatening pulmonary pathogens in immunocompromised patients, particularly individuals with cystic fibrosis or chronic granulomatous disease. Attempts to explain the genomic plasticity, adaptability and virulence of the complex have paid little attention to bacteriophages, particularly the potential contribution of lysogenic conversion and transduction. In this study, lysogeny was observed in 10 of 20 representative strains of the *B. cepacia* complex. Three temperate phages and five lytic phages isolated from soils, river sediments or the plant rhizosphere were chosen for further study. Six phages exhibited T-even morphology and two were lambda-like. The host range of individual phages, when tested against 66 strains of the *B. cepacia* complex and a representative panel of other pseudomonads, was not species-specific within the *B. cepacia* complex and, in some phages, included *Burkholderia gladioli* and *Pseudomonas aeruginosa*. These new data indicate a potential role for phages of the *B. cepacia* complex in the evolution of these soil bacteria as pathogens of plants, humans and animals, and as novel therapeutic agents.

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INTRODUCTION

In the last decade, bacteria previously identified as *Burkholderia cepacia sensu lato* have become recognized as important human pathogens, and particularly as a cause of life-threatening pulmonary infections in individuals with cystic fibrosis (CF) or chronic granulomatous disease (Govan *et al.*, 1996; LiPuma, 1998; Jones *et al.*, 2001; Mahenthiralingam *et al.*, 2002). Concurrently, polyphasic taxonomic approaches revealed that *B. cepacia sensu lato* comprises at least nine phylogenetically related but genomically distinct species (genomovars) (Vandamme *et al.*, 1997, 2003; Coenye *et al.*, 2001). Known as the *B. cepacia* complex, the group currently comprises *B. cepacia* (previously genomovar I), *Burkholderia multivorans* (genomovar II), '*Burkholderia cenocepacia*' (genomovar III), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *B. cepacia* genomovar VI, *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genomovar VIII) and *Burkholderia pyrrocinia* (genomovar IX). All species in the *B. cepacia* complex have been isolated from clinical specimens; however, the clinical significance of individual genomovars in human disease remains unclear. Approximately 90 % of *B. cepacia* complex isolates cultured from CF patients belong to *B. multivorans* and '*B. cenocepacia*' (Agodi *et al.*, 2001; LiPuma *et al.*, 2001; Mahenthiralingam *et al.*, 2002; Speert *et al.*, 2002; Vandamme *et al.*, 2003). These two species account for most episodes of epidemic spread in CF and non-CF patients (Holmes *et al.*, 1999; Mahenthiralingam *et al.*, 2002); '*B. cenocepacia*' is also the species most associated with the rapid pulmonary decline known as cepacia syndrome, and with post-transplant mortality (Aris *et al.*, 2001).

Most isolates of the *B. cepacia* complex exhibit high-level resistance to all major classes of antibiotics (Lewin *et al.*, 1993; Pitt *et al.*, 1996; Nzula *et al.*, 2002). The *B. cepacia* complex is also one of the few groups of bacteria to exhibit intrinsic resistance to cationic antimicrobial peptides (Hancock, 1997). Some strains are susceptible *in vitro* to ceftazidime and meropenem, arguably the most potent 'anti-cepacia' agents; however, the majority of strains, including the highly transmissible '*B. cenocepacia*' lineage ET12, are resistant to these agents (Lewin *et al.*, 1993; Nzula *et al.*, 2002).

All bacteria in the *B. cepacia* complex have large genomes (mean size approx. 8 Mbp), comprising multiple replicons that may contribute to genomic plasticity (Lessie *et al.*, 1996; Wigley & Burton 2000; Parke & Gurian-Sherman, 2001; Mahenthiralingam *et al.*, 2002). Ironically, the *B. cepacia* complex could be considered as both friend and foe, as some strains are highly effective as biopesticides in the control of plant fungal diseases and in bioremediation of contaminated

Abbreviations: CF, cystic fibrosis; NBYE, nutrient broth with yeast extract.

soils (Holmes *et al.*, 1998; Parke & Gurian-Sherman, 2001). These dual roles raise important medical, agricultural and ecological issues (Govan *et al.*, 1996, 2000; Govan & Vandamme, 1998; Environmental Protection Agency, 2002), including the significance of horizontal gene transfer in assessment of the risk to humans of using these bacteria as biopesticides or in bioremediation (Holmes *et al.*, 1998; LiPuma & Mahenthiralingam, 1999; Govan *et al.*, 2000; Parke & Gurian-Sherman, 2001).

Attempts to explain the genomic plasticity, adaptability and virulence of the *B. cepacia* complex have paid little attention to the potential contribution of bacteriophages. In many pathogens, these bacterial viruses are recognized as important contributors to virulence, in the form of bacterial lysogens, or as vectors in horizontal gene transfer. Interest in the use of phage-induced bacterial lysis for therapeutic purposes was widespread in the 1920s, but declined with the arrival of the antibiotic era. However, as antibiotic resistance increasingly threatens standard therapies against bacterial infections, there is renewed interest in the antimicrobial properties of these highly specific agents (Pirisi, 2000; Sulakvelidze *et al.*, 2001).

Little is known of the phages of the *B. cepacia* complex. Early reports on the '*B. cepacia*' phages CP1 (Cihlar *et al.*, 1978) and CP 75 (Matsumoto *et al.*, 1986) predate the revision of *B. cepacia* taxonomy. However, a recent report from our laboratories described two transducing bacteriophages, NS1 and NS2, whose host range included the five genomovars (I–V) that were known at the time, and also extended to *Pseudomonas aeruginosa* (Nzula *et al.*, 2000). Lytic phages with an interspecies host range within the *B. cepacia* complex have also been reported in association with soil-borne strains of '*B. cenocepacia*' (LiPuma *et al.*, 2000). These results suggest that lysogenic conversion and transduction could play a role in the evolution of species of the *B. cepacia* complex as human pathogens, and indicate the need for further studies on the host range and properties of phages associated with the *B. cepacia* complex and related bacteria.

In this study, we investigated the prevalence of lysogeny within the nine current species of the *B. cepacia* complex and isolated lytic phages from natural habitats of these bacteria, including the plant rhizosphere. Our results show that the host range of the phage panel includes seven genomovars and, in the case of individual phages, is not genomovar-specific.

METHODS

Bacterial strains. The 66 strains of the *B. cepacia* complex used in this study are listed in Table 1. The collection comprised environmental and clinical isolates belonging to genomovars I–V that were included in the *B. cepacia* strain panel (Mahenthiralingam *et al.*, 2000a), and isolates representing the recently identified genomovars VI–IX (Coenye *et al.*, 2001). Four isolates identified as *Burkholderia ubonensis*, a putative tenth genomovar of the *B. cepacia* complex (Vermis *et al.*, 2002), were also included. Isolates were identified using *recA* RFLPs, whole-cell protein electrophoresis and DNA–DNA hybridization (Mahenthiralingam *et al.*, 2000b; Coenye *et al.*, 2001). In addition, 55 strains of related pseudomonad species were screened as potential phage hosts: *P. aeruginosa* (30 strains), *Stenotrophomonas maltophilia* ($n = 11$), *Burkholderia caledonica* ($n = 1$), *Burkholderia gladioli* ($n = 2$), *Comamonas acidovorans* ($n = 2$), *Pseudomonas fluorescens* ($n = 2$), *Pseudomonas mendocina* ($n = 1$), *Pseudomonas stutzeri* ($n = 2$), *Pseudomonas putida* ($n = 1$), *Pseudomonas testosteroni* ($n = 1$), *Pseudomonas syringae* pv. *tabaci* ($n = 1$) and *Ralstonia pickettii* ($n = 1$). Clonal relationships were excluded by PFGE fingerprinting using a Bio-Rad CHEF Mapper PFGE system (Butler *et al.*, 1995).

Media. Bacteria were grown in nutrient broth with 0·5 % yeast extract (NBYE) at 37 °C in a shaking incubator. Soft overlay agar for phage experiments comprised Luria–Bertani (LB) broth with 0·3 % bacteriological agar (Difco). The nutrient agar used was Columbia agar base (39 g l^{-1}; Oxoid).

Isolation of lysogenic phages. Temperate phages were assayed and maintained as described previously (Nzula *et al.*, 2000). Lysogeny was investigated using the following method: the 20 strains designated in Table 1 with the symbol † were prepared as saline suspensions (approx. 10^6 c.f.u. ml^{-1}), inoculated onto tryptone soy agar (TSA; Oxoid) using a multipoint inoculator and incubated at 30 °C for 6 h. Bacterial growth was inverted over chloroform vapour for 15 min and then allowed to air-dry for 15 min. Soft agar overlays (2·5 ml), inoculated with 100 µl exponential-phase culture of each of the 20 strains, were layered over the original bacterial growth and allowed to set; the plates were incubated overnight at 37 °C. Phage plaques were identified in the overlay in the proximity of the original inoculum and used to prepare single-plaque preparations as follows: an agar plug containing a single phage plaque was removed using a sterile glass pipette, transferred to 10 ml phage buffer (10 mM Tris/HCl, pH 8·0; 10 mM $MgCl_2$), vortexed for 30 s, centrifuged at 3000 ***g*** for 30 min and filtered (pore size 0·2 µm; Millipore).

Isolation of lytic environmental phage. The natural habitats of the *B. cepacia* complex include soils, river sediments and plants, particularly the plant rhizosphere (Fisher *et al.*, 1993; Butler *et al.*, 1995; Parke & Gurian-Sherman, 2001). Therefore, 20 samples of soil, river sediment and rhizosphere (soil plus root material) were collected. The presence of phage was then investigated using a modification of the phage enrichment technique described by Weiss *et al.* (1994), as follows: approximately 10 g sample was suspended in 15 ml LB broth and dispersed by shaking in an orbital incubator for 30 min at 30 °C. After removal of soil particles by centrifugation (4000 ***g*** for 20 min), the supernatant was filter-sterilized (pore size 0·2 µm; Acrodisc) and 1 ml aliquots were added to 15 sterile tubes. To each extract was added 25 µl exponential-phase culture from one of 15 propagating strains chosen to represent the genomovars of the *B. cepacia* complex strain panel (Mahenthiralingam *et al.*, 2000a), and the contents were incubated at 37 °C overnight. The bacterial cells were removed by centrifugation (4000 ***g*** for 30 min), the supernatant was membrane-filtered as before and 10 µl filtrate was spotted onto single-layer lawns of the propagating strain. Phage plaques were identified after overnight incubation at 37 °C, and single-plaque stocks were prepared as described in the previous section.

High-titre phage preparations. High-titre phage preparations were prepared as follows: 100 µl single-plaque preparation, containing approximately 10^5 p.f.u. ml^{-1}, was added to 2·5 ml soft nutrient agar, previously seeded with 100 µl exponential-phase culture of the propagating strain. The mixture was then overlaid on nutrient agar and allowed to set. After 18 h at 37 °C, overlays showing semi-confluent lysis were transferred into 10 ml phage buffer. The lysate was then vortexed and centrifuged at 3200 ***g*** for 30 min and the supernatant was membrane-filtered. Phage titres were determined, as p.f.u. ml^{-1}, by

incorporating 100 µl host bacteria (exponential-phase NBYE culture) and 100 µl phage stock in 2·5 ml soft agar overlay, and lytic plaques were enumerated after 18 h incubation at 37 °C. Stock preparations were maintained at 4 °C.

Host range of phages. Stock phage preparations were diluted in phage buffer to approximately 10^8 p.f.u. ml^{-1} against the propagating strain, and 10 µl was spotted onto single-layer lawns (prepared from exponential-phase NBYE cultures) of potential host bacteria. Lytic activity was recorded after 24 h at 37 °C on a scale ranging from < 10 plaques (+) to confluent lysis (+++) (Table 1).

Electron microscopy. Stock phage preparations (approx. 10^8 p.f.u. ml^{-1}) were centrifuged at 100 000 *g* for 1 h. Phage pellets were resuspended in 1 M ammonium acetate, negatively stained with 2 % (w/v) potassium phosphotungstate solution (pH 7·0) and examined with a Hitachi model HU-12A transmission electron microscope.

Phage DNA extraction and RFLP profiling. In preparation for DNA extraction, high-titre phage stocks (containing at least 10^{10} p.f.u. ml^{-1}) were prepared using soft agar overlays, as described above. DNA was extracted from 10 ml phage stock using the Wizard Lambda preparation DNA purification system in conjunction with the Vac-Man laboratory vacuum manifold (both from Promega). Extracted DNA was eluted in sterile distilled water and stored at −20 °C. DNA quality was assessed on an E-gel pre-cast 0·8 % agarose gel (Invitrogen Life Technologies). In cases where DNA was not of sufficient quality for DNA restriction, purification was performed using the PCR protocol from the QIAquick gel extraction kit (Qiagen). Purified DNA was eluted in 30 µl elution buffer and stored at 4 °C. To determine genome size and to confirm that the phages were different from one another, approximately 1 µg DNA was restricted using 10 U *Hin*dIII (Promega), incubated for 3 h at 37 °C and visualized on 0·6 % 0·5× TBE agarose gel alongside 1 µl Ready-Load Lambda DNA/*Hin*dIII fragments (Invitrogen Life Technologies).

RESULTS

Lysogeny

Of the 20 strains of the *B. cepacia* complex that were investigated, 10 strains [ATCC 25416^T and ATCC 17759 (genomovar I); C3161^T, C1576, C1962 and C3163 (*B. multivorans*); J2315^T, C3166 and C3170 ('*B. cenocepacia*'); and C3174 (*B. stabilis*)] were found to be lysogenized. These provided 14 temperate phages (DK1–DK4 and MM1–MM10) for further study.

Isolation of environmental phages

Five virulent phages, JB1, JB3, JB5, RL1c and RL2, were isolated from soils and from the rhizosphere of various plants (Table 2). Most positive samples included decayed plant material collected from moist environments, but phages were also isolated from dry soils.

Host range of *B. cepacia* complex phages NS1, NS2 and newly isolated phages

To confirm that distinct phages were being accumulated and investigated, we determined the host range of the 19 phages against a preliminary bacterial panel comprising the 20 isolates of the *B. cepacia* complex that were used in the lysogeny screen, and also the *Hin*dIII RFLP profile (and hence an approximate genome size; Table 2). With the exception of phages JB3, DK2 and DK3, the genomes of the *B. cepacia* complex phages were within the range 40–48 kbp (Table 2). If several phages shared the same host range and RFLP profile, only one phage was used for further study. An exception was made for phages RL1c and RL1t, which shared the same host range and RFLP profile but produced different plaque morphologies: clear plaques associated with virulent phage (RL1c) or turbid, temperate phage plaques (RL1t). Similar host ranges and RFLP profiles were observed with the temperate phages DK2 and DK3, which had respectively been isolated from '*B. cenocepacia*' C3166 and *B. stabilis* C3174. As previously observed for NS1 and NS2, none of the newly identified phages was inactivated by treatment with chloroform. In addition, no evidence of bacteriocin activity was found during the search for *B. cepacia* complex phages.

The host range of phages NS1, NS2 and eight novel phages (JB1, JB3, JB5, DK1, DK3, RL1c, RL1t and RL2) was then determined against an enlarged panel of 66 isolates of the *B. cepacia* complex and 55 isolates representing other pseudomonads. The host range of individual phages included multiple species of the *B. cepacia* complex. Collectively, the host range of the phage panel included seven of the presently recognized *B. cepacia* genomovar species; no phage activity was detected against the single representatives of *B. cepacia* genomovar VI or *B. ambifaria* (Table 1). However, within each *B. cepacia* species, there was wide variation in susceptibility to an individual phage. *B. multivorans* appeared to be least susceptible to phages investigated in this study: of nine *B. multivorans* strains examined as potential phage hosts, only strain C2775 showed susceptibility. As observed previously for NS1 and NS2, the host range of some of the phages was not restricted to the *B. cepacia* complex. *P. aeruginosa* strains C1546 and J2852 were susceptible to phage JB3, and *B. gladioli* strain C3654 was susceptible to phages NS2, DK1, RL1c, RL1t and JB5.

Electron microscopy

In accordance with previous studies on phages NS1 and NS2 (Nzula *et al.*, 2000), electron microscopy revealed the novel phages JB1, JB5, DK2/DK3, RL1c/RL1t and RL2 to be morphologically T-even-like phages, with hexagonal heads and contractile tails of variable length. In contrast, phages JB3 and DK1 were lambda-like, with hexagonal heads and flexuous, non-contractile tails.

DISCUSSION

This study confirmed that lysogeny is relatively common in isolates of the *B. cepacia* complex and demonstrated the presence of virulent *B. cepacia* complex phages in the natural habitats of these bacteria. In agreement with previous observations of phages NS1 and NS2 (Nzula *et al.*, 2000), the host range of the newly isolated phages was not genomovar-specific and, in some phages (for example JB1), it included the majority of *B. cepacia* complex species. This broad host range, which in some phages extended to the

Table 1. Host range of bacteriophages within the *B. cepacia* complex

−, Lack of sensitivity to phage; +, < 10 plaques at phage inoculation site; ++, >10 plaques at phage inoculation site; +++, confluent lysis at phage inoculation site.

Strain	JB1	JB3	JB5	DK1	DK3	NS1	NS2	RL1t/RL1c	RL2
B. cepacia									
ATCC 25416^T (= LMG 1222^T)*†	−	−	−	−	−	−	−	−	−
C2970 (= LMG 17997)	+	−	+	−	−	+	+	+++	−
C3159 (= LMG 18821)†	−	−	−	−	−	−	−	−	−
ATCC 17759 (= LMG 2161)*†	−	−	−	−	+++	−	−	−	−
B. multivorans									
C2775	−	+	−	+	+	+	+	+++	−
C3161^T (= LMG 13010^T)*†	−	−	−	−	−	−	−	−	−
C3162 (= LMG 18825)†	−	−	−	−	−	−	−	−	−
C3163 (= LMG 18824)*†	−	−	−	−	−	−	−	−	−
C3164 (= LMG 18823)†	−	−	−	−	−	−	−	−	−
ATCC 17616 (= LMG 17588)	−	−	−	−	−	−	−	−	−
C1962 (= LMG 16665)*†	−	−	−	−	−	−	−	−	−
C1576 (= LMG 16660)*†	−	−	−	−	−	−	−	−	−
C3160 (= LMG 18822)†	−	−	−	−	−	−	−	−	−
'*B. cenocepacia*'									
J415 (= LMG 16654)	−	−	−	+	−	−	+	−	+++
J2315^T (= LMG 16656^T)*†	−	−	−	−	−	−	+++	−	−
C1394 (= LMG 16659)	−	−	−	−	+	−	+	−	++
J2956	+++	+++	+++	−	+++	−	−	+	+++
C2836	−	−	+	−	−	+	+	−	++
C3165 (= LMG 18826)	−	−	−	−	−	−	+	−	−
C3166 (= LMG 18863)*†	−	−	−	−	−	+	+	−	−
C3167 (= LMG 18827)†	−	−	−	−	−	−	−	−	−
C3168 (= LMG 18828)†	−	−	−	−	−	−	−	−	−
C3169 (= LMG 18829)	+++	+++	++	+++	+++	++	++	−	+++
C3170 (= LMG 18830)*	−	−	−	−	−	+	+	−	+++
ATCC 17765 (= LMG 18832)	++	−	+	+++	−	−	+	+	−
B. stabilis									
C3171^T (= LMG 14294^T)	−	−	−	−	−	+	−	+++	−
C3172 (= LMG 18870)	+	−	−	−	−	−	−	−	−
C3173 (= LMG 18888)†	−	−	−	−	+	−	−	−	−
C3174 (= LMG 14086)*†	−	−	−	−	−	−	−	−	−
B. vietnamiensis									
C2978 (= LMG 16232)†	−	−	−	−	−	+	+	+++	−
C3175 (= LMG 18835)†	−	−	+	−	−	−	−	−	−
C3176 (= LMG 18836)†	−	−	−	−	−	−	+	−	−
C3177^T (= LMG 10929)†	+++	−	+++	+	+++	−	−	+	+++
Genomovar VI									
E12	−	−	−	−	−	−	−	−	−
B. ambifaria									
J2742^T (= LMG 19182^T)	−	−	−	−	−	−	−	−	−
B. anthina									
J2552 (= LMG 16670)	−	−	−	−	−	−	−	−	−
J2553	−	−	−	−	−	−	−	−	−
J2863 (= LMG 20980)	−	−	−	−	−	−	−	−	−
J2927	−	−	−	−	−	−	−	−	−
J2928	−	−	−	−	−	−	−	−	−
J2941	−	−	−	−	−	−	−	−	−
J2943	−	−	−	−	−	−	−	−	−
J2944	−	−	−	−	−	−	−	−	−

(continued overleaf)

Table 1. cont.

Strain	JB1	JB3	JB5	DK1	DK3	NS1	NS2	RL1t/RL1c	RL2
J2945	–	–	–	–	–	–	–	–	–
J2946	–	–	–	–	–	–	–	–	–
J2949	–	–	–	–	–	–	–	–	–
J2950	–	–	–	–	–	–	–	–	–
J2951	+++	–	+++	–	–	+	–	–	+++
J2862	–	–	–	–	–	–	–	–	+++
C1658 (= LMG 20982)	+++	+	+++	–	–	–	–	–	–
C1765 (= LMG 20983)	+++	+	++	–	–	+	–	++	–
B. pyrrocinnia									
J2536	–	–	–	–	–	–	–	–	–
J2542	–	–	–	–	–	–	–	–	–
C1469	–	–	–	–	–	–	–	–	–
C3909	++	–	++	–	–	–	–	–	–
C3918	+	–	+	+	–	+	–	–	+++
C3928	–	–	–	–	–	–	–	–	–
C3930	+++	–	+++	–	–	+++	–	–	++
C3993	+++	–	+++	–	–	–	–	–	++
C3995	+++	–	+++	–	–	–	–	–	++
C3997	+	–	–	–	–	–	–	–	–
B. ubonensis									
E26	–	–	–	–	–	–	–	–	++
E27	–	–	–	–	–	–	–	–	–
E571	–	–	–	–	–	–	–	–	–
E551	+	++	–	–	–	–	–	–	–
Genomovar unclassifiable									
J2540 (= LMG 16672)	–	–	–	–	–	+	+	–	–

*Lysogenic strains.

†Strains used in lysogeny experiments.

Table 2. Phage genome size, plaque morphology and source

Phage	Genome size (bp)	Source	Plaque morphology*
JB1	40 000	Soil, the Caribbean	Turbid plaques, variable size (0·5–1 mm in diameter)
JB3	34 000	Plant rhizosphere, UK	Clear plaques (1 mm in diameter)
JB5	40 000	Plant rhizosphere, UK	Turbid plaques, variable size (0·5–1 mm in diameter)
DK1	45 900	Lysogeny	Turbid plaques, variable size (0·5–1 mm in diameter)
DK2/DK3	29 300	Lysogeny	Turbid plaques (1 mm in diameter)
NS1	48 000†	Lysogeny	Turbid plaques (0·5–1 mm in diameter)
NS2	48 000†	Lysogeny	Semi-clear plaques, variable size (0·5–1 mm in diameter)
RL1c/RL1t	44 300	Plant rhizosphere, UK	RL1c, clear plaques (1 mm in diameter); RL1t, turbid plaques (1 mm in diameter)
RL2	40 000	Pond sediment, UK	Semi-clear plaques, variable size (0·5–1 mm in diameter)

*Plaque morphology on propagating strains (i.e. strain J2956 for phages JB1, JB3 and JB5; strain C3169 for phages DK1, DK3, NS2 and RL2; strain C2978 for phages RL1c/RL1t; strain C3166 for phage NS1).

†Data from Nzula *et al.* (2000).

related pseudomonads *P. aeruginosa* and *B. gladioli*, is interesting. With the exception of unusual phages such as the plasmid-like 'phasmid' P4 (Gutmann *et al.*, 1990), the host range of most phages is species-specific. Lack of bacteriocin activity in our study could be explained by the techniques and conditions used and the low prevalence of bacteriocinogeny in *B. cepacia sensu lato* (Govan & Harris, 1985).

A broad host range for *B. cepacia* complex phages could contribute to the genomic plasticity of these bacteria, and their evolution from highly metabolically active soil saprophytes to plant and human pathogens and, recently, also animal pathogens (Berriatua *et al.*, 2001). Lysogenic conversion and transduction are important processes by which chromosomal host genes can be acquired and exchanged between bacteria. We have previously demonstrated *in vitro* transfer of antibiotic-resistance genes between *B. vietnamensis* strains by phages NS1 and NS2 (Nzula *et al.*, 2000).

As reported for *Shigella flexneri* (Allison & Verma, 2000), prophages may contribute to O-antigen modification in the *B. cepacia* complex (Kenna *et al.*, 2003), and to the role of *B. cepacia* complex LPS as a potent virulence determinant (Shaw *et al.*, 1995; Hughes *et al.*, 1997). Opportunities for transduction and lysogenic conversion would exist not only in natural environments shared by various *B. cepacia* complex species and related bacteria, but also in CF airway secretions, where mixed infections are frequent and bacterial populations can reach densities in excess of 10^9 c.f.u. ml^{-1}. Based on a close taxonomic relationship and shared insertion sequences and environmental habitats, we were particularly keen to test the phage panel against isolates of *Burkholderia pseudomallei*, the causative agent of melioidosis and a potential agent for bioterrorism (Mack & Titball, 1998). In collaboration with Dr Ty Pitt (PHLS, Colindale, London, UK), only phage NS2 was found to be active, lysing 13 of 40 *B. pseudomallei* strains tested (unpublished results). The potential importance of broad-host-range phages such as NS2 is also suggested by recent reports of *B. pseudomallei* infection in CF patients (including coinfection with *B. cepacia*) following travel to Thailand, where melioidosis is endemic (Schulin & Steinmetz, 2001; Visca *et al.*, 2001).

Further studies are required to determine the transducing potential and other biological properties (e.g. nucleic acid content and bacterial receptors) of the *B. cepacia* complex phages that were accumulated in this study. Meanwhile, several preliminary observations merit comment. The shared host range and RFLP profiles of the temperate phages DK2 and DK3, respectively isolated from strains of '*B. cenocepacia*' and *B. stabilis*, suggest that integration of the same phage can occur in different species of the *B. cepacia* complex. While the presence of multiple prophages within a single strain is common in *P. aeruginosa* (Holloway *et al.*, 1960), we found no evidence of polylysogeny during our investigation of *B. cepacia* complex phages. The variable plaque morphology exhibited by the environmental phages RL1c and RL1t is interesting and, to our knowledge, has not been reported previously for *B. cepacia* complex phages.

The relative lack of susceptibility of *B. multivorans* to the phage panel was interesting. Taken together, *B. multivorans* and '*B. cenocepacia*' account for almost 90% of clinical isolates of the *B. cepacia* complex; however, in contrast to '*B. cenocepacia*', *B. multivorans* is rarely isolated from natural environments (Bevivino *et al.*, 2002; authors' unpublished data). In our study, of nine *B. multivorans* strains tested, only one (C2775) exhibited phage susceptibility. However, this resistance may be misleading, as a recent study in our laboratories identified a novel *B. cepacia* complex phage (RU2) from soil, which plates on *B. multivorans* C3164 and also on three other *B. cepacia* complex isolates that are resistant to the primary phage panel (Table 1), namely J2552 and J2553 (both *B. anthina*) and E571 (*B. ubonensis*).

Whole-genome sequencing of bacteria provides increasing evidence for widespread exchange of chromosomal genes and other extrachromosomal elements, mediated by phages. Thus, analyses following the recent sequencing and annotation of '*B. cenocepacia*' J2315^T (= LMG 16656^T = NCTC 13227^T; http://www.sanger.ac.uk/Projects/B_cepacia/) are keenly awaited. In relation to the issue of multiple lysogeny in the *B. cepacia* complex, we performed a BLAST search of the provisional J2315^T genome sequence and found evidence of a single prophage. If confirmed, this would be an interesting result as, in this study and in a more extensive search for lysogeny in J2315^T, we isolated only one temperate phage, DK4 (authors' unpublished data). The availability of broad-host-range phages complements the panel of *B. cepacia* complex strains (Mahenthiralingam *et al.*, 2000a) and should facilitate future research on these highly adaptable and increasingly important bacteria. Furthermore, in addition to the established therapeutic use of lytic phages, phage-encoded lytic enzymes may provide novel therapeutic agents against *B. cepacia* complex infections (Schuch *et al.*, 2002), for which there are few antibiotic options at present (Nzula *et al.*, 2002).

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Lack of correlation between O-serotype, bacteriophage susceptibility and genomovar status in the *Burkholderia cepacia* complex

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Abstract

The *Burkholderia cepacia* complex comprises at least nine phylogenetically related genomic species (genomovars) which cause life-threatening infection in immunocompromised humans, particularly individuals with cystic fibrosis or chronic granulomatous disease. Prior to recognition that '*B. cepacia*' comprise multiple species, in vitro studies revealed that the lipopolysaccharide (LPS) of these Gram-negative bacteria is strongly endotoxic. In this study, we used 117 *B. cepacia* complex isolates to determine if there is a correlation between O-antigen serotype and genomovar status. Isolates were also tested for their ability to act as bacterial hosts for the LPS-binding bacteriophages NS1 and NS2. The absence of genomovar II (*Burkholderia multivorans*) in 'historical *B. cepacia*' isolates was notable. Neither O-serotype nor phage susceptibility correlated with genomovar status. We conclude that variability in LPS may contribute to the success of these highly adaptable bacteria as human pathogens.

Keywords: *Burkholderia cepacia* complex; Cystic fibrosis; Lipopolysaccharide; Serotypes; Bacteriophage

1. Introduction

The *Burkholderia cepacia* complex consists of phylogenetically related Gram-negative bacteria that can be divided into at least nine genomic species (genomovars) [1]. The *B. cepacia* type strain, ATCC 25416, which belongs to genomovar I, was identified originally as the cause of onion rot [2]. In the last decade, however, these versatile bacteria have emerged as the cause of life-threatening human infections, notably pulmonary infections in individuals with cystic fibrosis (CF) or chronic granulomatous disease [3–5], of bacterial ovine mastitis [6] and, ironically as highly effective biopesticides and bioremediators [7]. All nine genomovars have been cultured from clinical specimens, however, the prevalence of individual genomovars is disproportionate with strains belonging to genomovar III and *Burkholderia multivorans* (formerly genomovar II) responsible for almost 90% of isolates from CF patients [5,8]. In individuals with CF, patient-to-patient spread and the acute pulmonary deterioration, referred to as 'cepacia syndrome', are also primarily associated with genomovar III and *B. multivorans* [3–5]. Following lung transplantation, the prognosis for patients infected with genomovar III is less favourable than for those infected by strains belonging to other genomovars [9]. It has been suggested that this phenomenon reflects differences in lipopolysaccharide (LPS)-mediated proinflammatory activity between *B. cepacia* genomovars [10].

LPS (endotoxin) is an important bacterial virulence factor whose biological properties include immunostimulation of mononuclear cells, B cells and granulocytes [11]. In vitro studies, performed before it was known that the *B. cepacia* complex comprises multiple species, showed that '*B. cepacia*' LPS exhibits a higher endotoxic activity and a more pronounced proinflammatory cytokine response compared to *Pseudomonas aeruginosa* LPS [12,13]. Other studies, at this time, indicated that '*B. cepacia*' LPS may contribute to the inflammatory nature of *B. cepacia* infection in CF patients both by promoting in-

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creased neutrophil recruitment and by priming neutrophil respiratory burst responses [14]. The presence of a rough or smooth LPS chemotype was also found to be variable both within and between genomovars, with clinical isolates more likely to possess rough LPS than environmental isolates [15].

The aim of our study was to investigate whether LPS variability in the *B. cepacia* complex extends to the O-antigen, the immunodominant component of LPS, and whether O-serotype might correlate with genomovar status. Genomovar status was identified by *recA*-based PCR [16] in isolates whose LPS endotoxicity had been studied prior to the identification of the *B. cepacia* complex [12], and in a collection of 'historical' isolates whose O-serotype was known [17–19,20]. In addition, isolates were examined for their ability to act as hosts for the *B. cepacia* LPS-binding bacteriophages NS1 and NS2 [21].

2. Materials and methods

2.1. Bacteria

The bacterial strains used in this study comprised the five '*B. cepacia*' strains investigated by Shaw et al. [12], 98 isolates of known O-serotype used in the Rabkin et al. study [20], and 19 additional serotyped isolates kindly supplied by Dr H. Monteil, Strasbourg, France. The majority of isolates had been cultured from human respiratory secretions or from contaminated solutions and hospital equipment. All isolates had previously been identified as *B. cepacia* complex using biochemical tests and the API 20 NE system (bioMérieux, Marcy l'Etoile, France). Serotyping had been performed by the immunisation of rabbits with O antigen, [17–19] and subsequent agglutination of heat-treated bacteria with O antisera. Included in the 117 isolates we examined were representatives of the nine known serotypes (O1–O9) and the polyagglutinating types, O1/O4, and O1/O7.

2.2. Media and DNA extraction

Bacteria were grown overnight at 37°C on nutrient agar (Oxoid Ltd., UK) or on selective agar (Mast cepacia agar, Mast Diagnostics, Ltd., UK). For genomovar identification by *recA* PCR [16], DNA was extracted using a modified version of the technique supplied with the Puregene DNA isolation kit (Gentra systems, Minneapolis, MN, USA). Briefly, 500 μl of an overnight broth culture (nutrient broth +0.5% yeast extract–NBYE) was centrifuged at 13 000 rpm for 2 min, the supernatant discarded, the bacteria resuspended in 300 μl of cell lysis solution and the cells incubated at 80°C for 5 min. Thereafter, 1.5 μl of RNase A solution was added to the cell lysate, the sample inverted several times, and incubated at 37°C for 30 min. The sample was allowed to return to room temperature, 100 μl of protein precipitation solution was added, the mixture vortexed for 20 s, and centrifuged at 13 000 rpm for 5 min. The supernatant containing the DNA was removed, mixed with 300 μl 100% isopropanol, inverted several times and centrifuged at 13 000 rpm for 2 min, before the supernatant was discarded. The DNA pellet was washed with 300 μl of 70% ethanol, the tube inverted several times, and centrifugation repeated. The ethanol was discarded, and the pellet left to air dry for 15 min. 100 μl re-hydration solution was added and the DNA incubated at 65°C for 1 h.

2.3. PCR

DNA amplification was based on the technique of Mahenthiralingam et al. [16]. Briefly, a 25-μl reaction was set up using PCR reagents from Qiagen (Qiagen Ltd., UK): 20 ng of DNA, 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1×PCR buffer, 20 pmol each primer (Cruachem Ltd., UK), 1 U *Taq* DNA polymerase, and sterile distilled water. DNA was amplified in a Progene thermal cycler (Techne Ltd., UK) using the conditions specified by Mahenthiralingam et al. [16] and visualised on a 1% 0.5×TBE agarose gel alongside a 1-kb DNA ladder (Gibco BRL, Life Technologies, Grand Island, NY, USA). Strains of known genomovar status from the *B. cepacia* complex strain panel [22] were used as positive controls; negative controls consisted of reaction mixture without DNA.

2.4. Propagation and host range of bacteriophages NS1 and NS2

Bacteriophages NS1 and NS2 were propagated, maintained, and their host range investigated using the methods described previously by Nzula et al. [21].

3. Results

3.1. Genomovar typing using PCR

The genomovar status of 117 '*B. cepacia*' isolates of known serotype was identified by *recA*-based PCR. Two of the 117 isolates (1.7%) were identified as genomovar I, while the majority of isolates (48/117; 41%) belonged to genomovar IIIA. In addition, 17 of the 117 isolates (14.5%) belonged to genomovar IIIB, 24/117 (20.5%) were *Burkholderia stabilis* (formerly genomovar IV), and 26/117 (22.2%) were *Burkholderia vietnamiensis* (formerly genomovar V). No *B. multivorans* (formerly genomovar II) were identified.

3.2. Serotyping data

Table 1 shows the distribution of *B. cepacia* complex

Table 1
Distribution of *B. cepacia* complex serotypes within genomovars

Genomovar (No.)	Number of isolates with each serotype (%)										
	O1	O2	O3	O4	O5	O6	O7	O8	O9	O1,7	O1,4
I (2)	0	0	0	0	0	0	2 (100)	0	0	0	0
II (0)	0	0	0	0	0	0	0	0	0	0	0
IIIA (48)	6 (13)	9 (19)	12 (25)	5 (10)	8 (17)	1 (2)	0	4 (8)	2 (4)	1 (2)	0
IIIB (17)	5 (29)	3 (18)	6 (35)	0	0	0	0	2 (12)	1 (6)	0	0
IV (24)	5 (21)	3 (13)	4 (16)	1 (4)	3 (13)	0	0	1 (4)	7 (29)	0	0
V (26)	4 (15)	6 (23)	8 (31)	0	2 (8)	0	0	3 (11)	2 (8)	0	1 (4)

serotypes within the genomovars identified. Although 11 serotypes were represented amongst the 117 isolates investigated, the majority comprised serotypes O1, O2, O3, O5, O8 and O9. When the data for serotype and genomovar was combined, there was no correlation between O-serotype and genomovar status. For example, strains belonging to genomovars IIIA, IV, and V were distributed amongst several serotypes; similarly, each of the major serotypes comprised multiple genomovars. Nine serotypes were identified amongst the 48 genomovar IIIA strains, seven serotypes amongst 24 isolates of genomovar IV (*B. stabilis*) and seven serotypes amongst 26 isolates of genomovar V (*B. vietnamiensis*).

3.3. *Sensitivity of* 'B. cepacia' *isolates to bacteriophages NS1 and NS2*

The 117 isolates were investigated for their ability to act as bacterial hosts for the transducing bacteriophages NS1 and NS2. Eleven of the 117 isolates (9.4%) were sensitive to NS1, but not to NS2, while 10/117 (8.5%) were sensitive to NS2, but not to NS1. Forty-two isolates (36%) were sensitive to both bacteriophages, and 54/117 (46%) were sensitive to neither NS1 nor NS2. Isolates belonging to each of the major serotypes showed the ability to act as hosts for NS1 and NS2, but there was no correlation between phage susceptibility and an individual serotype (Table 2) or genomovar (Table 3).

4. Discussion

Knowledge of the phenotypic and genomic diversity of *B. cepacia* complex isolates is essential to clarify the molecular epidemiology and pathogenic potential of this group of highly adaptable bacteria. In our study, we were fortunate to have the opportunity to investigate isolates of '*B. cepacia*' which had originally been cultured, in Europe and North America, more than 20 years ago [17–19,20]. Although the isolates were well characterised on the basis of phenotypic properties, their genomovar status was unknown.

Initial identification of the genomovar status of these 'historical' isolates produced an interesting finding, namely the absence of *B. multivorans*. This absence was unexpected since a recent study of clinical isolates submitted to the United States *B. cepacia* complex referral laboratory reported a disproportionate distribution of genomovars amongst the *B. cepacia* complex, with *B. multivorans* (37.8%) and genomovar IIIA and IIIB (50.0%) accounting for almost 90% of isolates [8]. In our study, the prevalence of genomovar III (65 of 117, 55.6%) is similar to that reported by LiPuma et al., and once more demonstrates the adaptation this genomovar as a human pathogen. In contrast, the absence of *B. multivorans* in our study is intriguing. Recent reports have indicated that *B. multivorans* accounts for 9.3% of *B. cepacia* complex isolates in Canada [23] and 5.2% in Italy [24]. However, similar to

Table 2
B. cepacia complex serotypes and susceptibility to phages NS1 and NS2

Serotype	Number of isolates (%) susceptible to:			
	NS1 only	NS2 only	NS1 and NS2	neither NS1 or NS2
O1	1 (5)	5 (25)	7 (35)	7 (35)
O2	2 (9.5)	2 (9.5)	7 (33)	10 (48)
O3	6 (20)	2 (7)	11 (36.5)	11 (36.5)
O4	1 (17)	0	1 (17)	4 (66)
O5	1 (8)	1 (8)	6 (46)	5 (38)
O6	0	0	0	1 (100)
O7	0	0	1 (50)	1(50)
O8	0	0	4 (40)	6 (60)
O9	0	0	4 (33)	8 (66)
O1,4	0	0	1 (100)	0
O1,7	0	0	0	1 (100)

Table 3
Phage susceptibility patterns in different genomovars of *B. cepacia*

Genomovar	Number of isolates (%) susceptible to:			
	NS1 only	NS2 only	NS1 and NS2	neither NS1 or NS2
I	0	0	1 (50)	1 (50)
II	0	0	0	0
IIIA	2 (4)	7 (14)	17 (35)	22 (46)
IIIB	1 (6)	1 (6)	2 (12)	13 (76)
IV	2 (8)	2 (8)	10 (42)	10 (42)
V	6 (23)	0	12 (46)	8 (31)

the United States data, isolation of *B. multivorans* elsewhere is considerably higher, with prevalence rates in France, United Kingdom, Belgium and Germany ranging from 47 to 65% (authors unpublished). Our results cannot be explained by the apparent scarcity of *B. multivorans* in natural environments [25] since the isolates we examined were predominantly clinical in origin, or associated with contaminated equipment implicated in human infections [17–19,20]. Since epidemic spread features less strongly in the epidemiology of *B. multivorans* compared to that of genomovar III [3–5], it is probable that the reason(s) for the apparent emergence and increased prevalence of *B. multivorans* is (are) species rather than strain specific. Furthermore, the suggestion [26] that *B. multivorans* is of less importance, as a human pathogen, than genomovar III needs to be treated with caution. Since *B. multivorans* is increasingly recognised as potentially virulent and transmissible [22], it is tempting to speculate that this species has emerged more recently than genomovar III as a human pathogen, and that similar adaptation may occur in other species of the *B. cepacia* complex.

In our study, the distribution of O-serotypes amongst the 117 strains did not correlate with genomovar status. In most cases, individual serotypes were represented in several genomovars, and no particular serotype predominated amongst strains of any single genomovar. Of interest, seven serotypes were identified amongst the 24 genomovar IV (*B. stabilis*) isolates. This was another unexpected result. In contrast to the genomic diversity and plasticity of most members of the *B. cepacia* complex, genomic analyses by random amplified polymorphic DNA analysis and pulsed-field gel electrophoresis have suggested that the genome of *B. stabilis* is highly conserved [27]. Our results confirm data from a small study by Vinion-Dubiel et al., which identified serotypes O2, O3, O4 and O9 in four genomovar III isolates [28]. The authors suggested that multiple serotypes may result from transfer of the O antigen locus between strains from different genomovars, or alternatively that different genomovars may arise from strains sharing an identical serotype. Since the latter hypothesis would require considerable genetic shift, this seems an unlikely explanation. Alternatively, antigenic variation within genomovars could occur through mutation and horizontal gene transfer. Strains exhibiting a hypermutator phenotype have been identified in several '*B. cepacia*' genomovars (authors' unpublished results). In addition, evidence of lysogeny and transducing bacteriophages within the *B. cepacia* complex [21,29,30], together with insertion sequences [31–33], and homologues of the *P. aeruginosa wbp* locus [28], provides ample scope for horizontal transfer and alteration of genes involved in O antigen synthesis. It is known that *P. aeruginosa* and *Shigella flexneri* phages can alter the serotype of the strain they lysogenise [34,35]. Furthermore, O-antigen variability, involving modification of sugar moieties or changes in the lengths of the O antigen side chains, is utilised as a virulence mechanism in many bacteria, [36–38]. In the *B. cepacia* complex, the LPS within a particular strain population can exist as a mixture of smooth and rough chemotypes [15] and in either form be highly endotoxic [12,13]. Thus, it is perhaps to be expected that this potent virulence factor is utilised by bacterial pathogens to their advantage resulting in genomovars exhibiting multiple serotypes.

Since the bacterial receptor for phages NS1 and NS2 is LPS [21], we looked for a possible correlation between O-serotype and sensitivity to these phages. For most serotypes, there was little correlation between serotype and sensitivity to either NS1 or NS2 (Table 3). The small numbers of isolates available hampered conclusions on serotypes O6–O9, and the polyagglutinating serotypes. However, our results pose several possibilities. First, phages NS1 and NS2 may not bind solely to the O antigen moiety of LPS, but rather to regions within the LPS core. This would explain why the epidemic ET12 lineage, which exhibits a rough LPS chemotype [15], is capable of acting as a host for NS2 [21]. LPS core receptors have also been found with the *Escherichia coli* phage U3, and the phi CTX phage of *P. aeruginosa* [39,40]. This hypothesis would explain the lack of serotype specificity for NS1 and NS2. Alternatively, the '*B. cepacia*' phages may bind non-specifically or inefficiently to the O antigen, or have the ability to utilise additional non-LPS receptors on the bacterial cell surface. Phages that have multiple bacterial binding sites have been described and include phage P127 which binds reversibly to several cell wall components of *Lactococcus lactis* subspecies *cremoris* [41]. A low adsorption rate due to inefficient binding of NS1 and NS2 might also result in failure to detect phage susceptibility.

Recent results described by Woods and colleagues [42] suggest another possibility. They observed that lack of sensitivity of certain strains of *Burkholderia mallei* to the *Burkholderia thailandensis* phage E125 is due to the presence of an insertion sequence within the O antigen genes *wbiE* and *wbiG*. Since the isolates used in our study had been originally cultured over two decades previously, it is also possible that instability through storage or re-culture has led to alterations in LPS-associated genes. This phenomenon has been noted in other bacterial pathogens [43,44] and may perhaps be more prone to occur in a region with the potential for variability, resulting in host populations with mixed LPS chemotype and variable phage susceptibility.

Combining previous data for LPS activity [12] with genomovar status from this study showed that the degree of endotoxicity and tumor necrosis factor-α induction varies within and between genomovars III and *B. multivorans* (data not shown) indicating that endotoxicity is variable within the *B. cepacia* complex. Variable endotoxicity and inflammatory potential may be particularly relevant to CF patients infected by *B. cepacia* complex bacteria who are being considered for lung transplantation. Patients infected by *B. cepacia* complex pre-operatively, in particular genomovar III, exhibit lower post-operative survival [9,10]. The phenomenon also appears to be genomovar- rather than strain-specific [9], suggesting that it is not due to the impact of highly transmissible strains such as ET12 [3,45,46]. Further studies are required to determine if LPS endotoxicity plays a role in reduced survival following transplantation, and if strain-specific endotoxicity in other genomovars, in particular *B. multivorans*, influences post-transplantation survival.

In conclusion, whilst taxonomic analyses continue to reveal the diversity of genomovars within the *B. cepacia* complex, our data suggest that the biological activities of *B. cepacia* complex LPS, as a major virulence factor, are strain and not genomovar specific. Furthermore and unfortunately, serotyping based on O-antigenicity does not provide a simple and rapid alternative to DNA-based and other molecular approaches to identify genomovar status.

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