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Studies on the outer membrane of *Pseudomonas aeruginosa* and associated resistance properties

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Doctor of Philosophy

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February 2001

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THE UNIVERSITY OF ASTON IN BIRMINGHAM

STUDIES ON THE OUTER MEMBRANE OF *PSEUDOMONAS AERUGINOSA* AND ASSOCIATED RESISTANCE PROPERTIES

A thesis submitted by Michael Loughlin BSc
for the degree of Doctor of Philosophy
2001

Summary

Pseudomonas aeruginosa are ubiquitous Gram-negative bacteria responsible for a number of life-threatening infections including colonisation of the Cystic Fibrosis lung. The high degree of resistance of this organism to a range of antibiotics and disinfectants is widely known and its eradication from some tissues can never be fully achieved.

The aim of this thesis was to investigate antibacterial agents for use in disinfectant formulation in conjunction with benzalkonium chloride (BKC), and if possible, to synthesise novel agents based upon successful structures. Development of resistance to antibacterial agents following long-term exposure of *P. aeruginosa* to BKC was also investigated, examining cross-resistance to clinically relevant antibiotics and determining mechanisms of resistance.

In this study over 50 compounds were examined for antibacterial action against *P. aeruginosa*, both alone and in conjunction with BKC. Successful compounds were used to design novel agents, based upon the acridine ring structure, some of which showed synergy with BKC.

In 15 of the 16 strains exposed to increasing concentrations of BKC, resistance to the disinfectant arose. Strains PAO1 and OO14 were examined further, each showing stable BKC resistance and a slightly varying profile of cross-resistance. In strain PAO1 alterations in the fatty acids of the cytoplasmic membrane, increase in expression of OprG, decrease in susceptibility to EDTA as an outer membrane permeabilising agent and an increase in negativity of the cell surface charge were observed as cells became more resistant to BKC. In strain OO14 a decrease in whole cell phosphatidylcholine content, a decrease in binding/uptake of BKC and an increase in cell surface hydrophobicity were observed as cells became more resistant to BKC.
Resistance to tobramycin in strain OO14 was initially high, but fell as cells were adapted to BKC, this coincided with a quantitative reduction of plasmid DNA in the cells.

Key words: *Pseudomonas aeruginosa*; disinfectant/antibiotic resistance; acridine; outer membrane permeabiliser

For Sarah

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ABBREVIATIONS

APCI-MS	atmospheric pressure chemical ionisation mass spectrometry
API	analytical profile index
BKC	benzalkonium chloride
Bp	base pair(s)
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator protein
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid
FAME	fatty acid methyl esters
GC	gas chromatography
IL	interleukin
kDa	kilodalton
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
MBC/MLC	minimum bactericidal concentration/minimum lethal concentration
MGDA	methylglycinediacetic acid
MIC	minimum inhibitory concentration
min	minute/minimum
MS	mass spectrometry
NMR	nuclear magnetic resonance
NPN	1-N-phenylnaphthylamine
OD	optical density
OPP	orthophenylphenol
Opr	outer membrane protein
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMB	polymyxin B
PMN	polymorphonucleocyte
QAC	quaternary ammonium compound
RAPD	random amplified polymorphic DNA
RND	resistance-nodulation-division
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate protein agarose gel electrophoresis
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
w/v	weight by volume

Chapter One: Introduction

1.1 Pseudomonas aeruginosa: the organism in a clinical setting

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, motile organism found widely in the natural environment. It is a soil dwelling organism and its minimal nutritional needs allow it survive in water for up to 300-days (Bodey *et al.*, 1983). Indeed it is able to utilise a wide range of carbon sources for growth, will survive and reproduce in concentrated salt conditions and tolerate temperatures of between 20 and 42°C (Morrison & Wenzel, 1984). In addition, it is resistant to a range of antimicrobial agents including a number of antibiotics and disinfectants (see section 1.5).

It was first isolated in 1882 by the French pharmacist Gessard, from wounds that exhibited "blue-green" pus. Finklestein documented a case of infant bloodstream infection 4 years later with an adult case reported 3 years after that by Brill and Libman (Morrison & Wenzel, 1984). However, it was not until the widespread use of antibiotics, that the organism became of clinical importance, specifically as a cause of opportunistic, chronic nosocomial infections.

The organism's existence as part of "healthy" human flora has long been recognised (Bodey *et al.*, 1983; Rubenstein & Lev, 1988; Pedersen 1992), but appears to vary with each study and is site specific (skin 0-2%, nasal mucosa 0-3.3%, throat 0-6.6% and faecal samples 2.6-24% of individuals sampled) (Morrison & Wenzel, 1984). It can cause disease in "healthy" individuals, important examples being disease of the eye where it is the most common Gram-negative organism infecting corneal ulcers

and the ear where is causes over 70% of otitis externa cases, "swimmers ear". Indeed the range of illnesses caused by the pathogen is wide and is comprehensively examined in Bodey's review paper (Bodey *et al.*, 1983). However, *P. aeruginosa* has become best known as an important pathogen of debilitated or immunocompromised patients and is a fine example of a pathogen adapted to a man made environment, namely that of the hospital.

1.2 Nosocomial infections

As previously mentioned, *P. aeruginosa* is found as part of the microbial flora of "healthy" individuals. When it has been possible to trace the sources of non-nosocomial infections the results have reflected both the predeliction of the organism for water and its ability to exploit man-made ecological niches. Sources include; whirlpool baths; (Vogt *et al.*, 1982) mascara; (Wilson & Ahearn, 1977) contact lens solutions (Wilson *et al.*, 1981) and contaminated intravenous equipment from heroin users (Rajashekaraiah *et al.*, 1981). However, since the incidence of the organism almost invariably rises once admitted to hospital, especially in those "risk" groups such as burns victims and other forms of immunosuppression, it seems clear that the pathogen is mainly hospital acquired.

The acquisition of *P. aeruginosa* by hospital patients appears to be by three main routes: patient-to-patient, environmental reservoir to patient and re-infection by a previous colonizing strain (Shooter *et al.*, 1969; Morrison & Wenzel, 1984). Of these, the route from an environmental reservoir to a patient is the most prevalent mechanism of transmission. The organism's low nutritional requirements and

adaptability have led to it being isolated from a range of different locations (Morrison & Wenzel, 1984). These include:

equipment - nebulizers, humidifiers, baby baths and hydrotherapy tank.

"sterile solutions" - dialysis fluid, ophthalmic and nasopharyngeal irrigants.

disinfectants - cetrimide, chlorhexidine and benzalkonium chloride.

ocular products - mascara, eye drops, contact lens solutions and ophthalmic ointments.

This seems to reflect both the adaptive nature of the organism and its predilection for moisture.

Patient to patient transmission is rare in the absence of a connecting environmental reservoir, but has been recorded in siblings who were in close contact (Grothues *et al.*, 1988). However, as will be discussed later, patient-to-patient transmission is much more important in immunocompromised patients such as Cystic Fibrosis (CF) patients, where isolation is one of the primary mechanisms of preventing spread. The re-infection of individuals has been examined using serology to differentiate strains and has discovered examples of oncology patients in whom the serotype of the infecting strain was the same as that of the pre-existing colonizing strain (Morrison & Wenzel, 1984). This is just one example of how the status of the host is a key determinant of the likelihood of infection by *P. aeruginosa*.

1.3 Host immunodeficiency

As stated previously, *P. aeruginosa* is predominantly a nosocomial, opportunistic pathogen, and is best known for its infection of immunocompromised individuals. This state of immunodeficiency is achieved in a number of ways, thus a range of patient populations are seen to be at specific risk from infection.

1.3.1 Burns and tissue invasion

Both thermal injury and tissue invasive procedures such as surgery, insertion of a catheter or internal medical device facilitate the establishment of infection where one would not have occurred in a "healthy" individual. *P. aeruginosa* only became important as a cause of serious burn infection after the development of successful anti-staphylococcal therapy. While the organism is not immediately recoverable from the site of injury, by the third week it can be cultured from the wounds of 70% of burns patients (Pruitt, 1974). Prior to successful prophylactic treatment with topical antimicrobial agents, between 60-70% of septicaemia cases associated with burns were as a result of *P. aeruginosa* infection, leading to death in approximately 60% of cases (Bodey *et al.*, 1983). This has been reduced to 10% following such prophylactic measures (Curreri *et al.*, 1970). While the primary result of pseudomonal infection of burns is that of sepsis, the patient is also at risk from eye infection and developing pseudomonal pneumonia.

The illness associated with an invasive medical procedure is dependent upon the location of the implant or surgery. So endocarditis, although rarely associated with *P. aeruginosa*, occurred following heart surgery and insertion of a transvenous pacemaker, umbilical wound infection followed the use of non-sterile umbilical

clamps, pneumonia resulting from a traceostomy and a nasal infection was the result of a retained operational suture (Morrison & Wenzel, 1984; Bodey *et al.*, 1983). In addition, due to *P. aeruginosa*'s resistance to antimicrobials, wound infection also occurs due to contamination of "sterile" preparatory solutions (Morrison & Wenzel, 1984).

1.3.2 Neutropenic patients

Neutropenic patients are at great risk from *P. aeruginosa* infections. This state is often found in patients with cancer or leukaemia, due to either the disease or the related therapy. In addition, pseudomonal infections are now becoming more common in non-neutropenic cancer patients, possibly due to the frequent use or high dosage of broad-spectrum antibiotics in cancer wards (Bodey *et al.*, 1983).

1.4 Cystic fibrosis

Cystic fibrosis (CF) is the most common inherited lethal disorder of Caucasians in the world with a carrier frequency of 1 in 25 (Govan & Deretic, 1996). While more common among Caucasians, it has been reported throughout the races of the world and is thought to have an incidence of 1 in 2,500 live births. The damaged gene responsible for CF is 250kb in length, it is located on the long arm of chromosome 7 and encodes a protein containing 1480 amino acids. While there have been up to 400 point mutations determined as being responsible for the disorder, 70% (86.7% of the Danish CF population) of those with a damaged gene have a singe codon alteration causing a deletion of phenylalanine at position 508 of the protein (Pedersen, 1992; Govan & Deretic, 1996). This protein is known as the Cystic Fibrosis transmembrane conductance regulator protein (CFTR) and is a

chloride ion channel protein of the same family as P glycoprotein which reduces the effect of cancer chemotherapy by excreting chemotherapeutic agents. Those individuals with homozygous mutant alleles of the CFTR gene have defects in chloride ion transport leading to the production of dehydrated "sticky" mucus in such mucosal surfaces as male sex ducts, pancreatic ducts and the airways of the lungs (Govan & Deretic, 1996). It is in the lungs that CF is most often fatal, as the patient is subjected to a huge inflammatory attack, due to chronic respiratory infection. Eventually this leads to irreversible tissue damage due to an excessive release of proteases from neutrophils, eventually leading to respiratory failure and death.

Since it is curious that this often fatal disorder is so commonly carried in its heterozygous form, studies have been carried out to determine whether it carries a selective advantage in resisting another disease. This would be analogous to the way the inherited disorder, sickle cell anaemia, provides heterozygous carriers with an increased resistance to the protozoal disease malaria. Using CF mutant mice it was determined that CF heterozygous mice have an increased resistance to cholera, a potentially fatal diarrheal disease caused by the action of the *Vibrio cholerae* toxin in deregulating the electrolyte transport through the gut mucosa (Govan & Deretic, 1996; Gabriel *et al.*, 1994). Such an advantage, if found in humans, would provide ample explanation for the successful survival of the mutant gene.

While the link between CF and subsequent bacterial colonisation is clear and will be discussed later in this section, it is important to recognise that there is some evidence that the inflammation so typically associated with the disease is not as a direct result of bacterial pathogenicity, more that the bacteria simply exacerbate an underlying

disorder associated with the defective gene. CF patients that had a negative culture result for the normal CF microbial flora, had increased levels of neutrophils and the cytokine interleukin-8 (IL-8) in their bronchoalveolar lavage fluids. In addition airway macrophages found in the lavage fluids showed increased levels of IL-8 mRNA levels (Govan & Deretic, 1996, Khan *et al.*, 1995). Since CFTR is found at low levels in macrophages, it could be that the CFTR defect has wider ranging consequences than originally believed. However, there is ample evidence linking CFTR defects with increased infection by microorganisms, including *P. aeruginosa.* The "sticky mucus" produced by epithelial cells of CF patients may inhibit the mucocilary clearance of bacteria from the lung (Koch & Höiby, 1993) increasing the likelihood of bacterial colonisation. As will be mentioned shortly, CFTR mutations cause undersialylation of receptors providing *P. aeruginosa* with more efficient receptors for its adhesins and so promote attachment (Saiman & Prince, 1993). Finally there is evidence that the airway epithelial layer in CF patients shows inefficient internalisation of *P. aeruginosa* when compared to those cells expressing wild-type CFTR (Pier *et al.*, 1996).

Before the widespread use of antibiotics, most CF patients died in infancy from a staphylococcal infection, whereas now, most succumb to repeated pulmonary disease as a result of persistent *P. aeruginosa* infection after 10-20 years. This would appear to mirror the history of burns infections where, before antibiotics were used, staphylococci were the primary cause of infection whereas now they have been replaced by *P. aeruginosa*. In fact, in modern times, a diagnosed CF individual can expect to survive to early adulthood and the population of adults with the disorder is almost equal that of children. However, by early adulthood most CF patients will

have a chronic infection of mucoid *P. aeruginosa*, the form of the organism most typically associated with CF (Govan & Deretic, 1996).

The overall microbiology of the CF patient is quite specific and seems to be linked to host age. *Staphylococcus aureus* is often the primary coloniser, appearing when the host is in infancy. This is followed swiftly by infection with *Haemophilus influenzae* until *P. aeruginosa* is found as the host goes through adolescence. The presence of *P. aeruginosa* is widely recognised as a very poor prognostic indicator, as despite intensive chemotherapy, it is almost never fully eradicated from the lung. Any infections are localised solely in the lungs where, as stated earlier, it is thought that the dehydrated nature of CF respiratory secretions inhibits the mucocilary clearance of microorganisms (Govan & Deretic, 1996). The link between *P. aeruginosa* and CF is a classic example of microbial adaptation from *in vitro* to *in vivo* growth. The lung is colonised by "typical" non-mucoid forms of *P. aeruginosa*, with the mucoid forms emerging as part of the lung flora during the subsequent chronic pulmonary infection. Indeed, such mucoid forms could be isolated in up to 70% of CF patients colonised with *P. aeruginosa* (Doggett, 1969).

This mucoid form is typified by the production of high levels of an extracellular mucoid exopolysaccharide (MEP), an acetylated form of alginate. This MEP forms a bacterial glycocalyx, leading to a micro-colony or biofilm mode of growth. Such biofilm growth provides altered resistance to host defences and antibiotics as well as that associated with the alginate alone (Demko & Thomassen, 1980; Anwar *et al.*, 1992; Pedersen, 1992; Gander, 1996). This mucoid form of growth is rare, although not unheard of, outside the environment of the CF lung. However, to date, there has

been no natural ecological niche located where the mucoid form of growth could have evolved. This is not to say that mucoid forms are only found in CF patients. At least 40% of non-CF patients with a positive *P. aeruginosa* sputum antibody response contained mucoid forms of the organism (Govan & Deretic, 1996). Analysis has shown that non-CF isolated organisms produce alginate that is chemically very similar to that produced in CF patients (McAvoy *et al.*, 1989; Govan & Deretic, 1996). The advantages of the mucoid form of growth are varied but seem to refer mainly to survival within a mammalian host. Alginate provides protection against the opsonisation action of antibodies and also against any phagocytic attack by immune cells (Pedersen, 1992). Alginate is reported to act as an immunomodulatory molecule including acting to suppress lymphocyte function (Pedersen, 1992). As previously mentioned, the production of this extracellular matrix also contributes to the formation of colony biofilms, structures that themselves are associated with persistent infections and are considered by some to be the "natural" form of bacteria in the environment (Costerton *et al.*, 1999; Singh *et al.*, 2000).

1.4.1 Virulence factors of P. aeruginosa

The intrinsic and acquired resistance to antimicrobial agents possessed by *P. aeruginosa* must be considered as primary virulence factors, that is to say characteristics of the organism that enhance its ability to cause disease. These resistance characteristics are of primary importance when searching for new anti-pseudomonal therapies and are examined later in this introduction. Other virulence factors are primarily linked to invasion, reproduction and survival within a host and a number of them are briefly described in table 1.1.

Table 1.1 Brief summary of virulence factors of *P. aeruginosa* and their interaction with the host where known. (Adapted from Pedersen, 1992)

Virulence factor	Function in promoting bacterial survival	Function in modulating immune response.
Pili	Adherence	Generates antibody response.
Flagella	Motility	Generates antibody response.
Siderophores	Iron acquisition	Generates antibody response.
Pyocyanin and other phenazine pigments.	Inhibits cilia motility in epithelial cells, reducing mucociliary clearance of bacteria.	Inhibits lymphocyte proliferation, has a dose dependant suppression/stimulation action on T and B-lymphocytes, enhances release of TNF and IL-1 and stimulates/inhibits superoxide production.
Elastase	Solubilises lung elastin, degrades collagen, soluble laminin and basement membrane laminin, interrupts tight junctions of respiratory epithelium, inactivates lysozyme.	Cleaves IgG, IgA and sIgA. Inactivates complement but also generates C5a by C5 cleavage, Inhibits PMN chemotaxis and phagocytosis and inactivates TNF, IL-2 and IFN~γ.
Alkaline Protease	Degrades soluble laminin and basement membrane laminin.	Cleaves IgA Inactivates complement, Inhibits PMN chemotaxis and phagocytosis and inactivates TNF, IL-2 and IFN~γ.
Rhamnolipid	Inactivates cilia, reduces active sodium absorption in tracheal epithelium.	Enhances monocyte oxidative burst, releases histamine from mast cells.
Exotoxin A	Inhibits protein synthesis.	Toxic to macrophages.
Phospholipase C	Degrades lecithin.	Releases/induces release of histamine from mast cells.

PMN refers to polymorphonucleocytes, TNF refers to tumour necrosis factor, IL refers to interleukin.

P. aeruginosa has a varied range of virulence factors, some of which have a direct effect on the host immune system. This can be to the benefit or detriment of the microorganism, or as has been observed in some apparently contradictory cases, both. Much of the damage caused to the host in *P. aeruginosa* infections is due to the host's inflammatory response and many of the virulence factors listed in table 1.1 are highly immunogenic. The expression of these virulence factors in *P. aeruginosa* is thought to be co-ordinated by homoserine-lactone mediated quorum sensing (Jones *et al.*, 1993; Singh *et al.*, 2000; Williams *et al.*, 2000). This coordination is based upon the release of diffusible signal molecules to interact with a sensor or transcriptional activator coupling gene expression with concentration of the

molecules and so with population density. This is widely found in Gram-negative species (Parsek & Greenberg, 2000) but has been studied in *P. aeruginosa* usually with regard to biofilm formation (Davies *et al.*, 1998). In addition, recent work has shown that, like many of the virulence factors it regulates, the homoserine lactone that *P. aeruginosa* uses as a quorum sensing signal molecule has its own immunomodulatory activity (Telford *et al.*, 1998).

1.4.2 Disease progression in CF

Colonization by *P. aeruginosa* begins in the upper respiratory tract where it displays chemotaxis toward mucosal surfaces rich in mucin (Nelson *et al.*, 1990; Pedersen, 1992). Initial adhesion is mediated by adhesin factors that have been shown to bind to host laminin, glycolipids, glycosphingolipids and glycoproteins. Certain CFTR mutations cause undersialylation of glycolipids and glycoproteins, providing more efficient receptors for *P. aeruginosa* adhesins (Saiman *et al.*, 1992; Saiman & Prince, 1993; Govan & Deretic, 1996). Trypsin or leukocyte elastase induced damage to epithelial cells of mucus membranes exposes even more receptors for adhesion. Such damage can also occur as a result of the inflammatory response of the host to colonisation by other viruses or bacteria, such as *Staphylococcus aureus*, a common precursor to *P. aeruginosa* in the microbiology of CF as previously described (Govan & Deretic, 1996). From initial colonization there is a period of, on average, 12-months before what is termed persistent colonisation occurs. Circumstantial evidence indicates that this transition from initial to persistent colonisation is mediated by such virulence factors as elastase and alkaline protease. As seen in table 1.1, both of these factors interfere with the immune cells of the host and so may be key in establishing a persistent infection.

As previously described, one characteristic of a persistent infection by *P. aeruginosa* is the presence of mucoid forms of the organism. It has been shown that the change from "normal" non-alginate producing, non-mucoid pseudomonads to the organism commonly found in persistent infections is induced by oxidative stress produced as part of the immune response to the initial infection (Martin *et al.*, 1994; Mathee *et al.*, 1999). Once the organism has entered into this mucoid form, biofilms are produced in the lung accompanied by the development of an increased antibody response against *P. aeruginosa* antigens. While lipopolysaccharide (LPS) is the most common antigen component of immune complexes, as a result of chronic *P. aeruginosa* infection, antibodies are generated against almost all of the bacteria's antigens, including those virulence factors listed earlier (Cash *et al.*, 1983; Shand *et al.*, 1991; Pedersen, 1992). The immune complex mediated inflammation, due to a type-III immunological hypersensitivity reaction, typical of chronic *P. aeruginosa* infection includes increased local concentrations of proinflammatory cytokines such as TNF, IL-1 and IL-8, complement activation and the action of PMNs (Kronborg, 1995). This PMN dominated inflammation results in the release of leucocyte proteases, myeloperoxidase and oxygen radicals in an attempt by the immune system to combat the infection. Due to the biofilm nature of growth of the bacteria at this point in the infection, these immune responses are largely ineffective in dealing with the bacteria and are the cause of the lung tissue damage associated with this chronic infection (Kronborg, 1995). Once a chronic infection is established, the mucoid forms are rarely fully eradicated by even the most aggressive of chemotherapy and the immune mediated tissue damage continues, in many cases with fatal results.

1.4.3 Therapy of P. aeruginosa in CF

While it is clear that *P. aeruginosa* is responsible for a wide range of infections most clinical research has examined the best therapy for dealing with CF infections. However, all of the antibiotics used in an attempt to eradicate *P. aeruginosa* from the lungs of CF patients, have also been used at some point in the treatment of other *P. aeruginosa* infections.

1.4.3.1 Polymyxins: colistin E and polymyxin B

These cyclic polypeptide antibiotics were the first compounds found to be effective in dealing with *P. aeruginosa* in CF. While their mode of action will be examined more closely later in the introduction, they are essentially membrane active agents acting in a detergent-like manner in disrupting the cell membrane of bacteria. This mode of action may well be responsible for the lack of resistance to polymyxin antibiotics reported in clinical settings. Although there is some evidence that this class of antibiotics can cause kidney damage, colomycin (colistin E) is successfully used as part of management of *P. aeruginosa* infections in conjunction with ciprofloxacin, or alone (Littlewood *et al.*, 1985; Valerius *et al.*, 1991; Littlewood *et al.*, 2000).

1.4.3.2 β-lactam antibiotics

β-lactam antibiotics "sabotage" cell wall synthesis in dividing cells by inhibiting the cross-linking of peptidoglycan that provides rigidity and strength to the cell wall. Anti-pseudomonal antibiotics in this category include penicillins (carbenicillin, ticarcillin, piperacillin), cephalosporins (ceftazidime, cefepime), monobactams (aztreonam), and carbapenems (imipenem, meropenem). Recent

research (Christenson *et al.*, 2000) has shown that carbapenems are more effective *in vitro* against *P. aeruginosa* isolates from CF patients than cephalosporins, which are as effective as penicillins.

The lack of penetration of β-lactams into bone and spinal fluid limits their use in treating such infections as meningitis (Coppens & Klastersky, 1974; Rubinstein & Lev, 1988) and many β-lactams are more effective when used in conjunction with an aminoglycoside antibiotic. In addition resistance to β-lactams is common, especially with imepenem (Carmeli *et al.* 1999) although the incidence was reduced when used with an aminoglycoside (Wu *et al.*, 1999). Monobactams such as aztreonam show synergy with aminoglycosides and can be used to replace penicillins when the patient shows an allergy to that group (Rubinstein & Lev, 1988).

1.4.3.3 Aminoglycosides

The use of an aminoglycoside (gentamicin, tobramycin) in conjunction with a β-lactam antibiotic has proven successful both in treating the infection and reducing the incidence of resistance (Farrell *et al.*, 1979; Wu *et al.*, 2000). In the case of piperacillin used alone, 47% of CF patients developed *P. aeruginosa* infections resistant to the antibiotic. If treated with an aminoglycoside and piperacillin, only 17% developed resistant infections (Gribble *et al.*, 1983; Rubinstein & Lev, 1988). In addition, recent treatment of CF has included a nebulised dosage of tobramycin reducing hospitalisation and improving lung function, (Banerjee & Stableworth, 2000; Doring *et al.*, 2000).

1.4.3.4 Quinolones

These antibiotics are generally used as alternatives to the "classical" anti-pseudomonal compounds previously mentioned. They are able to be taken parentally and orally and have good absorption into sputum and bone (Scully *et al.*, 1986). Ciprofloxacin is the most active of this class against *P. aeruginosa* (Hoogkamp-Korstanje, 1997) and few side effects have been reported although resistance has (Jalal *et al.*, 2000).

1.4.3.5 Anti-inflammatories

While some tissue damage in *P. aeruginosa* infections of CF patients is due to bacterial enzymes, much is due to the inflammatory response of the host. One of the key therapies in CF is not to eradicate the infection, but to limit or at least reduce the ongoing lung tissue damage resulting from the immune response. Drugs such as ibuprofen and peroxicam have been used to maintain lung function (Sordelli *et al.*, 1994; Konstan *et al.*, 1995) and the role of suppressors of the neutrophil elastase that is responsible for much of the damage caused to the lung is under investigation (McElvaney *et al.*, 1992; Roum *et al.*, 1999). In addition there is thought to be a role for the immune system in triggering the change from "normal" *P. aeruginosa* to the mucoid form. It is thought that, if the infection is diagnosed before entering the chronic stage, the change to mucoid forms of the bacteria might be prevented by the use of anti-inflammatory drugs. This would keep the bacteria in a "normal" state that, as described, is far more susceptible to antibiotics.

1.4.3.6 Disinfectants and isolation

The most clinically important means of transmission of noscomial infections of *P. aeruginosa* is that of environment to patient from the numerous reservoirs from which the organism has been isolated. Therefore preventing transmission to uninfected CF patients relies upon the eradication of *P. aeruginosa* from the hospital environment and the isolation of infected patients to prevent transmission of the pathogen. Indeed in the case of the latest pathogen to be associated with CF, *Burkholderia cepacia*, isolation of patients is the only effective means of controlling the infection where patient-to-patient transmission is an important epidemiological factor (Whiteford *et al.*, 1995; Govan & Deretic, 1996).

The control of *P. aeruginosa* in the hospital environment relies upon the use of disinfectants. The range of antimicrobial agents used on the floors of aseptic preparation areas in hospitals include quaternary ammonium compounds such as benzalkonium chloride (BKC), bis-biguanides such as chlorhexidine, halogen based groups such as sodium hypochlorite and phenolic disinfectants such as trichlorophenol (Murtough *et al.*, 2000). *P. aeruginosa* has a natural resistance to many of these agents, certainly more so than most Gram-negative and non-mycobacterial Gram-positive organisms, (McDonnell & Russell, 1999) and the organism's acquired resistance to disinfectants is well known (Jones *et al.*, 1989; Guérin-Méchin *et al.*, 1999; Méchin *et al.*, 1999).

1.4.3.7 General therapy

The preferred therapy currently used for any life threatening *P. aeruginosa* infection is a combination of an aminoglycoside and a β-lactam that together show synergy *in vitro* (Banerjee & Stableworth, 2000). In the case of patients with allergies to β-lactam antibiotics, a combination of an aminoglycoside and either a monobactam, such as aztreonam, or ceftazidime is appropriate. In the case of a chronic infection such as CF, long-term use of a single agent is to be avoided to prevent resistance developing. In addition any long-term use of an aminoglycoside, coupled with a monobactam or β-lactam, is likely to result in host tissue damage associated with the aminoglycoside. Therefore a regime of alternating treatment with such agents as ceftazidime, ciprofloxacin and imipenem is most appropriate. Successful therapies often rely on treating the infection in the very early stages, as once *P. aeruginosa* becomes established in the lung it can never be fully eradicated. Recent trials with nebulised tobramycin and colomycin indicate that altering the delivery system of the antibiotic may be as important as the choice of therapeutic agent (Doring *et al.*, 2000). However, even with such advances, it is still true that in the majority of CF cases any therapy used fails to remove the *P. aeruginosa* infection completely and increased lung tissue damage leading to respiratory failure is the most likely final outcome.

1.5 Resistance properties of P. aeruginosa

In choosing one of the many antibiotics mentioned as therapeutic agents against *P. aeruginosa*, an important consideration is the incidence of resistance development to the agent. As previously mentioned, one of the most important virulence factors associated with any *P. aeruginosa* infection is the antimicrobial

resistances associated with the organism. The type of resistance can be separated into three groups: that present in all strains (intrinsic resistance), the type that can develop when genes are activated by mutation, possibly removing the action of a repressor molecule, or acquired by genetic transfer (acquired resistance). Most examples of resistance result from a combination of mechanisms with all strains having a degree of resistance, but some acquiring a higher level that is often of clinical importance.

1.5.1 Permeability to antimicrobial agents

As with all Gram-negative organisms the cell envelope of *P. aeruginosa* comprises two bacterial membranes. The cytoplasmic membrane is made up of a phospholipid bilayer studded with proteins. The main phospholipid constituent is phosphatidylethanolamine (PE) with smaller amounts of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Wilkinson, 1988). This membrane surrounds the bacterial cytoplasm containing the genetic material of the organism and much of the biochemical apparatus of the cell. The membrane provides no real barrier to penetration by hydrophobic antimicrobial agents. Indeed many of the proteins embedded in the membrane are important targets for a range of antimicrobial agents. Outside of this membrane is an area known as the periplasm that contains enzymes and other biochemicals. Surrounding this is a layer of peptidoglycan, the main structural component of the wall, made up of substituted sugars and amino acids. While supplying strength and structure to the cell, the peptidoglycan layer does not contribute its permeability. Superimposed upon this layer, and linked to it by a number of lipoproteins is the outer membrane. It resembles the cytoplasmic membrane in so much as it is a lipid bilayer, and the predominant phospholipid is PE, in the same proportion as in the cytoplasmic membrane (Wilkinson, 1988). However

unlike the cytoplasmic membrane it does not consist of two monolayers of phospholipid but of an asymmetric bilayer, the inner leaflet consisting of phospholipids, the outer leaflet consisting of a glycolipid (Figure 1.1). This glycolipid is lipopolysaccharide (LPS) a highly antigenic molecule, contributing to the immune response of the host to *P. aeruginosa* infection (Goldberg & Pier, 1996).

Illustration removed for copyright restrictions

Figure 1.1 Structure of Gram-negative cell wall including outer and cytoplasmic membrane.

The LPS of all Gram-negative organisms is made up of three sections. The section making up the outer leaflet of the hydrophobic region of the bilayer is lipid A (Figure 1.2). Lipid A is made up of a biphosphorylated 1-6 linked dimer of glucosamine with fatty acids attached via ester and amide bonds to the glucosamine molecules. The fatty acids present are typically dodecanoic and hexadecanoic acid, the hydroxy fatty acids are 2-hydroxydecanoic, 2-hydroxydodecanoic and the amide linked 3-hydroxydodecanoic acid (Kropinski *et al.*, 1985). This 3-hydroxydodecanoic acid is also esterified by other fatty acids leading to either two or three fatty acids attached to each saccharide molecule. The phosphate groups at the 1 or 4 carbon of each

saccharide can be coupled to a range of chemical groups including 4-amino-4-deoxyarabinose (Bhat *et al.*, 1990).

Figure 1.2 Structure of two variants of lipid A of *P. aeruginosa*. Alterations determined by Ernst *et al.* (1999). Diagram taken from Pier (2000).

The second section is that of a core region attached to the lipid A by three residues of ketodeoxyoctonate (KDO). In addition to KDO the core region consists of an oligosaccharide made up of heptose, hexosamine, rhamnose, galactosamine, hexoses and high concentrations of phosphate (Drewry *et al.*, 1975; Wilkinson & Galbraith, 1975; Wilkinson, 1981; Wilkinson, 1983). This region is more variable than the lipid A, which remains much the same within bacterial species. The final section of LPS is one of two structures. Better known is the O antigen (also known as the B band in *P. aeruginosa* LPS), a polysaccharide that is the immunodominant antigen of the organism but is found attached to only about 10% of LPS molecules (Wilkinson, 1983). The second structure is known as the A band, and is a far shorter molecule, primarily a polymer of rhamnose monosaccharides (Drewry *et al.*, 1975; Rivera *et al.*, 1988; Rivera & McGroarty, 1989; Hatano *et al.*, 1993). It is unknown whether the lipid A and core regions of A and B band LPS are the same (Hatano *et al.*, 1993), or whether synthesis of the molecules is distinct (Rivera & McGroarty, 1989). As with the cytoplasmic membrane the outer membrane has proteins associated with it,

in some cases fixed through the membrane itself. A brief summary of the proteins associated with the outer membrane is found in table 1.2.

Table 1.2. Outer membrane proteins of *P. aeruginosa*. Adapted from http://www.cmdr.ubc.ca/bobh/ompknown.htm and Hancock *et al.*, (1990).

Protein	Putative function	Apparent molecular weight Daltons
OprC	Copper transport	74,000
OprJ	Efflux	54,000
OprN	Efflux	50,000
OprM	Efflux	50,000
OprP	Phosphate uptake	48,000
OprD	Imipenem and basic amino acid uptake	45,500
OprE	Possible Porin	43,500
OprF	Porin/structural	38,000
OprG	Ciprofloxacin resistance and changes in LPS structure	25,000
OprH	EDTA/polycation resistance	21,000
OprI	Structural/lipoprotein	8,000

The purpose of the outer membrane is primarily to reduce permeability of chemicals in and out of the cell (Hancock, 1997b). The LPS acts as a barrier to the passage of hydrophobic chemicals through the outer membrane. It is thought that the outer membrane reduces the passage of hydrophobic chemicals by 100 fold when compared to the cytoplasmic membrane (Hancock, 1987). In addition, the LPS is tightly held together by cross bridges of Mg^{2+} between the negatively charged phosphate residues common to LPS (Hancock *et al.*, 1994). In fact the reduced permeability of *P. aeruginosa* when compared to other Gram-negative organisms is primarily due to the enhanced levels of phosphate in the LPS bridged in this way, the LPS of other bacterial species contains 3 phosphate residues while *P. aeruginosa* contains 7 such residues (Wilkinson, 1981).

The transfer of hydrophilic compounds through the outer membrane depends upon the size and number of appropriate channels available. When compared to other

Gram-negative organisms, *P. aeruginosa* has very few porins (proteins that allow hydrophilic compounds to traverse the outer membrane). The hydrophobic nature of the lipid bilayer of the outer membrane limits hydrophilic compounds to the use of the water filled porins to traverse the outer membrane (Hancock, 1987). The primary porin present is the outer membrane protein F (OprF) and is present in two forms. One produces a large channel, with a 6kD exclusion limit, that would allow a range of hydrophilic compounds through, including all antibiotics. However, it is only present in 0.2% of the total number of OprF porins, the huge majority only allowing relative small, solutes to pass through (600D) (Nicas & Hancock, 1983a). This is thought to be because OprF exists primarily in a monomeric form with the larger exclusion limit porins being due to the less common oligomeric form (Woodruff *et al.*, 1986). Recent work has shown that a protein containing the 162-amino-acid N-terminal domain of the protein can only form the smaller channels in a lipid bilayer (Rawling *et al.*, 1998) and both terminals have roles in peptidoglycan association and maintenance of cell shape (Brinkman *et al.*, 2000). Therefore *P. aeruginosa*'s lack of permeability applies to both hydrophobic and hydrophilic compounds. This goes some way to explain the organism's intrinsic low permeability to a range of antimicrobial agents.

Acquired reduced permeability is common among *P. aeruginosa* strains. Mutants deficient in OprF have a significant decrease in permeability to β-lactam antibiotics (Nicas & Hancock, 1983a) whereas mutants with an increase in outer membrane protein H1 (OprH) have reduced susceptibility to membrane permeabilising agents such as EDTA. OprH is thought to bind phosphate residues together in the same manner as Mg^{2+} and has been observed to replace the divalent cation when the

organism is grown in magnesium deficient media (Nicas & Hancock, 1980) and be connected to polymyxin B and gentamicin resistance (Bell *et al.*, 1991) in some strains of *P. aeruginosa*. Other outer membrane proteins that act as porins are used by antimicrobial agents to gain access to the cell, and so their level of expression can affect the organism's resistance properties. The reduction or absence of expression of the protein OprD has been associated with resistance to the β-lactam, imipenem (Quinn *et al.*, 1986). This is thought to be due to the role OprD has in acting as a porin for this antibiotic and for basic amino acids.

There is little evidence that either A or B band oligosaccharides have any effect on the permeability of the outer membrane, although there is evidence that some portions of the LPS may act as binding sites for aminoglycoside antibiotics to promote their own uptake through the outer membrane (Kadurugamuwa *et al.*, 1993). Resistance of this organism to disinfectants tends to occur by reduction in the agent's access to its target. This target is usually the cytoplasmic membrane, although the exact action of many disinfectants is unknown. Alterations in the fatty acids of the outer membrane and the specific phospholipids making up both membranes has been observed to alter the susceptibility of *P. aeruginosa* to disinfectants and membrane active antibiotics such as polymyxin B (Anderes *et al.*, 1971; Conrad & Gilleland, 1981; Gilleland & Farley, 1982; Champlin *et al.*, 1983; Gilleland *et al.*, 1984; Moore *et al.*, 1984; Conrad & Galanos, 1989; Jones *et al.*, 1989; Ernst *et al.*, 1999; Guérin-Méchin *et al.*, 1999; Méchin *et al.*, 1999).

1.5.2 Efflux of Antibiotics.

It is clear that restricted outer membrane permeability contributes to the overall resistance of *P. aeruginosa* to a range of antimicrobial agents. However, the outer membrane merely slows the passage of antimicrobial agents, it does not prevent their access to the periplasm and cytoplasmic cell membrane. In many cases an equilibrium of antibiotic concentration across the outer membrane is achieved in only a very few minutes, even in the case of the "impermeable" *P. aeruginosa*. Therefore such a small alteration in antibiotic passage across the membrane is unlikely to affect the action of an antibiotic in any significant way unless the agent is either inactivated or degraded during, or directly following, its influx (section 1.5.3). The first evidence for another mechanism of resistance was discovered when examining the significant resistance to tetracyclines that is present in *P. aeruginosa* even without the presence of any form of enzymatic inactivation or degradation of the antibiotic (Levy, 1992). It has since been determined that all strains of *P. aeruginosa* contain efflux pumps capable of removing a range of solutes from the cell. Even those strains considered susceptible to agents such as tetracycline are able to remove the antibiotic from the cell interior. Many Gram-positive and Gram-negative organisms have the ability to efflux a wide range of chemicals not all of which are antimicrobial in nature (Nikaido, 1998) including homoserine lactone molecules associated with quorum sensing (Evans *et al.*, 1998). Indeed these pumps are thought to have developed to remove toxic metabolites from the organism rather than to protect against antimicrobial agents. While there are a number of classes of efflux systems, based upon similarity in structure and function, all those associated with *P. aeruginosa* are of the resistance-nodulation-division family (RND). Structurally these are made up of three proteins: an efflux transporter situated in the

cytoplasmic membrane, a membrane fusion protein that traverses the periplasm, and an outer membrane channel protein. In *P. aeruginosa* four homologous RND efflux systems are known; each acting upon a different range of solutes and each system encoded for by a separate operon (Table 1.3).

Table 1.3 RND efflux systems associated with *P. aeruginosa* (adapted from Li *et al.*, 1994; Li *et al.*, 1998; Nikaido, 1998; Aires *et al.*, 1999; Köhler *et al.*, 1999; Li & Poole, 1999; Mine *et al.*, 1999 & Chuanchuen *et al.*, 2001).

Regulator protein	Cytoplasmic membrane transporter protein	Membrane fusion protein	Outer membrane protein	Antimicrobial agents effluxed
Mex R	Mex B	Mex A	OprM	β-lactams, Novobiocin, Erythromicin, Tetracyclines, Chloramphenicol, Fluoroquinlones, organic solvents
Unknown	Mex D	Mex C	OprJ	Tetracyclines, Chloramphenicol, Fluoroquinolones, 4th generation cephems Triclosan, organic solvents
Mex T	Mex F	Mex E	OprN	Chloramphenicol, Fluoroquinolones, organic solvents.
Unknown	Mex Z	Mex Y	OprM	Aminoglycosides

Of these systems only MexAB:OprM and MexZY:OprM are expressed constituitively and therefore contribute to the intrinsic resistance properties of the organism. It also appears that the presence of OprM alone conveys a certain level of resistance. Mutant strains deficient in MexA, MexB or both were 2-4 times more susceptible to quinolones, chloramphenicol and gentamicin whereas OprM deficient mutants were 4-16 times more susceptible to the same antibiotics (Yoneyama, 1997). This may indicate a role for OprM in efflux separate from the other efflux proteins, A, B, Z and Y.

The expression of the other efflux operons is not detectable in wild-type *P. aeruginosa* and only occurs if the operon is de-repressed by a mutation in the

regulator gene. Such a mutation in the MexR gene of the MexAB:OprM system, leads to enhanced acquired resistance to all the antibiotics indicated as effluxed by the system. Such mutants are commonly generated in the laboratory by selection with quinolones, tetracyclines or β-lactam antibiotics and have recently been observed in patients treated with β-lactam antibiotics (Ziha-Zarifi *et al.*, 1999). While resistance to disinfectants has been determined to be at least partially due to efflux in organisms such as *Staphylococcus aureus* (Nikaido, 1994,1998), currently there have been just two reports suggesting that the resistance that *P. aeruginosa* shows to disinfectants may be due to efflux (Schweizer *et al.*, 1998; Chuanchuen *et al.*, 2001).

1.5.3 Enzymatic degradation of antibiotics.

In addition to impermeability or efflux mechanisms there is a final component to the high intrinsic resistance profile present in wild type *P. aeruginosa* strains and the enhanced acquired resistance seen in a growing number of strains. Enzymes that deactivate antibiotics have been observed in a range of organisms including *P. aeruginosa*. The two most common antibiotic classes inactivated in this way are aminoglycosides and β-lactam antibiotics. Aminoglycosides act by binding tightly to a structural component of the 30S ribosomal subunit and interfere with protein synthesis. This binding is much stronger than that of other protein synthesis inhibitors, possibly accounting for the fact that these are the only bactericidal protein synthesis inhibitors. Enzymatic resistance to these antibiotics occurs by modifying the agent in one of three ways: by phosphorylation, acetylation or by adenylation (Wright, 1999). Each of these three classes of enzymes can either be present on acquired plasmids or the bacterial chromosome and in the case of kanamycin, phosphotransferase appears to be constitutively expressed. In most cases however the

enzyme appears to be plasmid carried and it is the presence or absence of such a plasmid that determines resistance.

β-lactamases act by hydrolysing the β-lactam ring present in all such β-lactam antibiotics. They are present in a wide range of bacteria and classified according to functional characteristics (Bush *et al.*, 1995). They can be produced constitutively, induced by the presence of the target antibiotic, de-repressed by mutation or acquired from plasmids by genetic exchange (Watanabe *et al.*, 1991; Campbell *et al.*, 1997). Both constitutive and inducible enzymes are responsible for the high intrinsic resistance that *P. aeruginosa* has to β-lactam antibiotics, although this is often found in conjunction with efflux and the impermeability of the cell to the antibiotic (Pai *et al.*, 2001).

1.5.4 Alteration of antibiotic target site.

This final mechanism of resistance is, by definition, acquired, not intrinsic and occurs in one of two ways. Either a portion of the bacterial population exposed to the antimicrobial agent has a mutation in the target site and so by Darwinian evolution the population becomes resistant, or a plasmid carried enzyme acquired by the cell acts to alter the target site. While this is a common form of resistance among many bacteria (Spratt, 1994) in *P. aeruginosa* there is evidence for only two antibiotics for which this is a problem. The target site for β-lactam, antibiotics are transpeptidase molecules known as penicillin binding proteins (PBP) and alteration in the structure of these has been observed as a cause of resistance in *P. aeruginosa* (Godfrey *et al.*, 1981). Quinolone antibiotics act upon the DNA gyrase and

alterations in this enzyme have been observed to confer resistance to such antibiotics (Zhanel *et al.*, 1995).

1.5.5 Addressing resistance mechanisms.

It is clear that *P. aeruginosa* has a wide range of mechanisms contributing to its intrinsic and acquired resistance to antibiotics and disinfectants. In addressing the problem of resistance, research has been carried out on each of the 4 mechanisms (Chopra *et al.*, 1997).

The challenge of antibiotic degrading enzymes is met on two fronts. New β-lactam antibiotics such the tribactam sanfetrinem (currently in Phase II clinical trials) are stable to most commonly encountered β-lactamases as well as active against penicillin resistant Streptococci that do not produce β-lactamases (Di Modugno *et al.*, 1994; Sifaoui *et al.*, 1998). Similar β-lactamase reistance is seen in carbapenems, such as imipenem, and monobactams. Also β-lactams are now used in combination with β-lactamase inhibitors such as sulbactam, tazobactam and clavulanic acid (Giwercman *et al.*, 1990). However recent emergence of carbapenemases, extended spectrum β-lactamases and inhibitor resistance β-lactamases may indicate that resistance to such antibiotics is developing (Livermore & Woodford, 2000).

Alteration of target sites can be avoided by synthesis of agents which have the same effect but target a slightly different site due to a small change in the chemical structure. Certain of the macrolides have poor activity due to resistance arising from changes in ribosomal proteins or ribosomal RNA. Antimicrobial activity against previously resistant bacteria has been restored by a simple alteration in one side chain of erythromycin, leading to a new class on antibiotics, the ketolides (Chopra, 1998)

Efflux of antibiotics remains a considerable problem. Much research has been done on the contribution this makes to antibiotic resistance in clinically important pathogens, how often enhanced efflux is caused as a result of selection by antibiotics, and whether efflux itself is connected to selection of resistance to antibiotics unaffected by efflux pumps.

Restricted permeability of bacteria to antibiotics is the classic example of intrinsic resistance. In the case of β-lactam antibiotics, catecholic analogues have been seen to cross more readily the outer membrane in *E.coli* by utilising the siderophore uptake system of that organism (Curtis *et al.*, 1988) and have been developed for use against *P. aeruginosa* (Fung-Tomc *et al.*, 1997). However, in addition to adapting antibiotics to take advantage of bacteria solute uptake systems (as well as avoiding efflux systems) most work involved in the problem of impermeability investigates compounds that act as outer membrane permeabilisers.

1.6 Outer membrane permeabilisers

Brief mention has been made of the outer membrane providing a barrier to the antimicrobial action of aminoglycosides and polymyxin B. Both these agents have sites of action within the cell or cell membrane and facilitate their own uptake by disorganising the outer membrane.

Polymyxin B binds to LPS in the outer membrane via its cationic head group and, once attached, its hydrophobic fatty acid tail inserts into the hydrophobic region of the outer membrane perturbing its structure (Bhattacharjya *et al.*, 1997). This allows increased permeability of the membrane allowing other polymyxin molecules to enter without need for binding. Once within the bacteria, the antibiotic binds to the phospholipids of the cytoplasmic membrane causing disruption of the membrane that is lethal to the organism. A derivation of polymyxin B lacks the acyl chain and terminal amino acid to which this is attached and is known as polymyxin B nonapeptide (PBN). This PBN has all the outer membrane permeabilising properties of polymyxin B but with no antimicrobial action (Vaara, 1992).

This self-promoted uptake is common among some agents that have membrane disorganising properties. Agents with outer membrane disorganising properties that do not go on to have a bactericidal effect on cells have, in general, an action based chelating divalent cations such as the Mg^{2+} that hold adjacent LPS molecules together. The second group, members of which often do have a lethal actions upon cells, possibly due to a disruption of the cytoplasmic membrane, comprises organic cations which can be broadly classified as cationic peptides and organic amines.

1.6.1 Cationic peptides

A wide range of plants and animals employ antimicrobial peptides as part of their host defence mechanism. This indicates a degree of evolutionary conservation which may be for a number of reasons. Firstly the production of small peptides in host cells requires less time and energy than many of the other humoral aspects of an immune response such as antibody production and T-cell activation. Secondly, such small peptides will diffuse at a faster rate than the larger immunoglobulins involved in humoral host defence and so may reach the target tissue faster. Finally, many organisms do not have a lymphocyte-based immune system, so must rely on the production of such peptides as the only means of clearing invading microorganisms. However, many organisms (humans included), use antibacterial peptides as just one of many defences against invading microorganisms (Hancock, 1997a).

Such cationic peptides tend to be in one of four structures. A β-pleated sheet stabilised by disulphide bonds, an α-helix, a loop structure or an extended helix. All of these structures are folded in three dimensions so that they have both a hydrophobic face, comprising non-polar amino acid side chains and a hydrophilic face comprising polar and positively charged amino acid side chains; i.e. the peptides are amphipathic in nature as are many permeabilising compounds (Hancock, 1997a). Polymyxin B has the same structure: a cationic nature coupled with a structure split into hydrophilic and hydrophobic regions.

As well as providing defence for their host, many of these peptides have been isolated and purified for use against micro organisms that pose a threat to man, (table 1.5).

Table 1.4. Antibacterial peptides investigated for use against human pathogens.

Name	Source in nature	Active structure where known.	Anti-microbial activity	Reference
Cecropins	Moths and Flies	α helix	Gram-positive and negative bacteria	Vaara & Vaara, 1994
Magainins	Frog skin	α helix	Gram-positive and negative bacteria and fungi.	Maloy & Kari, 1995
Defensins	Humans, Insects	β pleated sheet	Gram-positive and negative bacteria, fungi and HIV-1	Ganz & Lehrer, 1995
Bactericidal and Permeability increasing Protein (BPIP).	Humans, Rabbits	α helix and β pleated sheet motifs	Gram-negative bacteria	Weiss *et al.*, 1978
Melittin	Bee venom toxin	α helix and β pleated sheet motifs	Gram-positive and negative bacteria	Cornut *et al.*, 1994
Lentivirus Lytic peptide 1 (LLP1)	Human immunodeficiency virus type 1 (HIV-1)	α helix	Gram-negative and Gram-positive bacteria	Tencza *et al.*, 1997
Protegrin	Porcine leukocytes	β pleated sheet	Gram-positive and negative bacteria and HIV-1	Steinberg *et al.*, 1997
Bactenecins	Bovine neutrophils		Gram-negative bacteria	Skerlavaj *et al.*, 1990
Protamine	Vertebrate sperm	β pleated sheet	Gram-negative bacteria	Boussard & Dony, 1988, Boussard *et al.*, 1991
Indolicidin	Bovine neutrophils	extended helix	Gram-negative and positive bacteria, fungi and protozoa.	Falla & Hancock, 1997

The minimum number of cationic amino acids present in peptides of this sort appears to be five based upon the work of Vaara (1990) which examined the properties of oligomers of the cationic amino acid, lysine. However, subsequent work (Vaara, 1992) indicated that while this an appropriate minimum, the number required is dependant upon the final tertiary structure of the peptide. Tossi *et al.* (1997) examined the common structures of many such antimicrobial peptides in order to predict what amino acid sequences would form the peptides with the greatest action against the outer membrane. This work determined that it is the secondary, tertiary

and quaternary structures, which are the more important rather than the primary amino acid sequence.

1.6.2 Organic Amines

Organic amines can acquire a positive charge due to protonation of the nitrogen atom. This positive charge attracts them to the negative charge of the LPS in the outer membrane. Large amines such as tris hydroxymethylaminmethanol (Tris) have been observed to have some permeabilising activity against Gram-negative organisms (Voss, 1967). Other amines that have activity against Gram-negative bacteria include those that have other well-defined clinical properties. For instance, certain neuroleptic drugs including local anaesthetics (Labedan, 1988; Rajyaguru & Muszynnski, 1998) and phenothiazine derivatives (Bourlioux *et al.*, 1992; Kristiansen, 1992; Rajyaguru & Muszynnski, 1997) have activity against both Gram-positive and Gram-negative organisms and many of these compounds have structures that are quite similar. They comprise a hydrophobic triple ring structure with a short akyl chain attached, which contains a secondary, tertiary, or quaternary amino group. Kristiansen has produced two comprehensive reviews on the subject, one looking at the possible use of "non-antibiotics" to treat infections resistant to current therapies (Kristiansen & Amaral, 1997), the second providing a history and present view of the antimicrobial properties of psychotherapeutic drugs (Kristiansen, 1990).

Some work has been completed investigating structure activity relationship in the compounds in relation to their antipsychotic, antibacterial, antiplasmid and anti-cancer multidrug resistance activities (Bourlioux *et al.*, 1992; Molnár *et al.*, 1992; Pajeva & Wiese, 1998). It would appear that each of the four properties might

depend upon a different structure on the molecule, so it may be possible to alter one aspect of the compound's action without significantly altering other properties.

1.6.3 Polymeric organic amines

Two compounds which are made up of repeating units of monomeric amines, compound 48/80 and polyethyleneimine, both have a permeabilising action upon the outer membrane of Gram-negative bacteria.

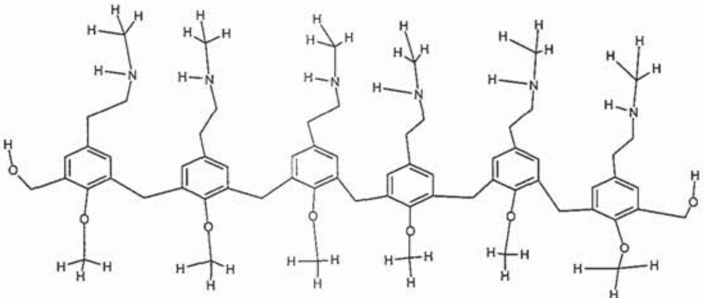


Figure 1.3 Structure of the hexamer of compound 48/80.

Compound 48/80 is a mixture of various length polymers based on the repeating monomeric unit (Lenney *et al.*, 1977) (Figure 1.3). It has been used both as an antibacterial agent and as a potent releaser of histamine from mast cells and other substances responsible for anaphylactic symptoms (Lenney *et al.*, 1977). Studies examining the action of this compound upon mast cells have led to the fractionation of the mixture into 14 components of different chain lengths by repeated thin layer chromatography (Koibuchi *et al.*, 1985). These components were examined for their action upon mast cells in the presence and absence of Ca^{2+}. This enabled the 14 components to be placed in 3 groups. Those of 6 monomeric unit polymers or below had weak releasing properties with or without the presence of Ca^{2+}. Those containing 9 or 10 monomeric units had action upon mast cells that was independent of the

presence of Ca^{2+}. Those components of 13 or 14 units had a strong, but Ca^{2+}-dependant action upon mast cells (Koibuchi *et al.*, 1985).

The relationship between antimicrobial action and chain length has been briefly examined in the action of compound 48/80 as an antibacterial agent. By synthesising monomers, dimers and trimers of this compound each was tested in respect of its antibacterial action (Katsu *et al.*, 1985). It was observed that the monomer was inactive as an antibacterial agent, while the dimer and trimer both permeabilised the outer membrane of *Escherichia coli* and at higher concentrations had lethal action against the organism (Katsu *et al.*, 1985). Similar results have been observed when comparing the degree of polymerisation of lysine polymers with their effect on outer membrane permeability of *P. aeruginosa* (Vaara, 1990). The link between ability to activate histamine release from mast cells and ability to permeabilise the outer membranes of *P. aeruginosa* is also seen in the compound polyethlyeneimine (Suzuki-Nishimura *et al.*, 1995). This polymeric compound releases histamine in a similar manner to compound 48/80, was inhibited by BKC in this action as is compound 48/80, and has been observed to be a potent permeabiliser of the outer membrane of *P. aeruginosa* (Helander *et al.*, 1997).

1.6.4 Divalent metal chelators

The action of chelating agents in binding metal cations has been shown to have a permeabilising action upon the outer membrane. It is thought that this is due to the chelators removing Mg^{2+} or Ca^{2+} ions from between the LPS molecules, causing the destabilisation of the outer membrane and increasing its permeability (Vaara, 1992). Other than the removal of cations, chelators have no other action upon

the outer membrane, so alone they do not produce a killing action and are often coupled with another antibacterial agent. The most widely used chelator is ethylenediamine-tetra-acetate (EDTA) which has action against many Gram-negative organisms. It should be noted that, like many permeabilising agents, the activity of these chelators is severely inhibited by the presence of divalent cations in the bacterial media.

1.7 Aims and objectives of this study:

The quaternary ammonium disinfectant benzalkonium chloride (BKC) is widely used as the active antimicrobial component of a number of common disinfectant solutions. Although it has activity against many Gram-positive and Gram-negative organisms, when tested in the European Standard Disinfectant test (EN1276) BKC fails to kill *P. aeruginosa* effectively. Design and development of agents which enhance the activity of BKC against *P. aeruginosa* would be of great value. This thesis seeks to discover whether the design of such agents is feasible. By examining those compounds previously shown to have activity as outer membrane permeabilising agents against *P. aeruginosa* novel compounds would be designed, synthesised and tested in conjunction with BKC against this test organism. In addition the mechanisms of resistance to BKC intrinsic to and acquired by *P. aeruginosa* would be examined and any co resistance to clinically relevant antibiotics determined.

Chapter 1: A review of the role of *P. aeruginosa* in a clinical setting and a brief review of agents that act to permeabilise the organism's outer membrane.

Chapter 2: Following literature based research, compounds are tested to determine if they have antibacterial action against *P. aeruginosa* both alone, and in conjunction with BKC. This is carried out in both the European disinfectant test and a test of the author's own design. Permeability properties of the agents are also to be determined.

Chapter 3: Using the results of chapter 2 novel agents are synthesised and tested using the EN1276 disinfectant test described in section 3.2.5.1

Chapter 4: Environmental and laboratory isolates of *P. aeruginosa* are adapted to BKC over a period of weeks. Resistance alterations to 12 other agents are examined, as is the strain purity of adapted cells.

Chapter 5: Alterations in properties of resistant bacteria that may be associated with resistance to BKC are examined. These include protein content of the outer membrane, fatty acid content of outer and cytoplasmic membranes, cells surface charge and hydrophobicity and susceptibility of the outer membrane to permeabilisation.

Chapter 2. Search for active compounds

2.1 Introduction

2.1.1 Aims

Compounds were selected by examining appropriate literature and tested for their ability in promoting the antibacterial action of BKC on *Pseudomonas aeruginosa* and their membrane permeabilising properties. Common chemical structures were identified and used to select related compounds.

2.1.2 Agents with permeabilising properties

The concept of chemicals with the ability to permeabilise the outer membrane of *P. aeruginosa* was dealt with in section 1.6. Compounds with a known history of either anti-pseudomonal or membrane permeabilising action were chosen first. Later compounds were chosen based upon any common chemical structures found among those active compounds. Most compounds used were those which were not antibiotic in nature, but agents that have been termed non-antibiotics. These have tended to be compounds with neuroleptic properties, such as members of the phenothiazine group (Bourlioux *et al.*, 1992) and structurally related chemicals that have shown antibacterial action, with or without synergy with antibiotics (Kristiansen, 1992; Kristiansen & Amaral, 1997).

2.1.3 Hydrophobic probes

The resistance properties of many bacteria can be attributed to the permeability of the outer and cytoplasmic membrane to antimicrobial agents. This

was dealt with in detail in section 1.5.1. The most common means for determining the degree of permeability are either by examining the uptake of the agent in question, often by tagging the agent with a fluorescent or radioactive moiety; or by using a probe with uptake properties that mimic those of the antimicrobial agent. Radiolabelled disinfectants and antibiotics have been used to good effect in examining the progress of antimicrobial agents through cell membranes and to their targets of action. However, the cost and availability of radiolabelled compounds often prohibits their use.

There has been successful use of dansyl labelled polymyxin B to measure the incorporation of the antibiotic into the outer membrane of *P. aeruginosa* and *B. cepacia* (Moore *et al.*, 1984, 1986). However a great deal of research on both disinfectants and antibiotics has been achieved by the use of the hydrophobic probe 1-N-phenylnaphthylamine (NPN) (Loh *et al.*, 1984). This compound only fluoresces when in a hydrophobic environment such as that found within a lipid bilayer. Therefore the permeability of a membrane to hydrophobic compounds can be determined by the use of NPN. Another classical method of determining outer membrane permeability in *P. aeruginosa* and *E.coli* is measuring the amount of periplasmic β-lactamase enzyme released when the cell is challenged with an outer membrane permeabilising agent (Zimmerman & Rosselet, 1977; Lambert, 1991). These enzymes are mentioned in section 1.5.3 and are well known as a significant mechanism of β-lactam antibiotic resistance in *P. aeruginosa*.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

P. aeruginosa PAO1 ATCC 15692 and *P. aeruginosa* ATCC 15442 were used for examining enhanced action of disinfectants and the uptake of the hydrophobic probe NPN and PAO1 ATCC 15692 used for action of agents on the release of β-lactamase from the periplasm. The methicillin resistant *Staphylococcus aureus* (MRSA) strain used in EN1276 tests was a clinical isolate from the Queen Elizabeth Hospital, Birmingham and designated 1407461.

All strains were stored on nutrient agar plates for up to two weeks at 4°C. Unless stated otherwise in the specific section, all experiments used bacteria grown at 37°C for 18-22 hrs in aerated nutrient broth (Table 2.1).

Table 2.1. Constituents of complex media.

Nutrient Agar (Oxoid)	Grams/litre	Nutrient Broth (Oxoid)	Grams/litre
'Lab-Lemco' Beef extract	1.0	'Lab-Lemco' Beef extract	1.0
Yeast extract	1.0	Yeast extract	1.0
Peptone	5.0	Peptone	5.0
Sodium Chloride	5.0	Sodium Chloride	5.0
Agar	15.0		

2.2.2 Antibacterial assays

2.2.2.1 Choice of agents

Agents were chosen for a number of reasons. Some had a recorded history of antimicrobial action against Gram-negative organisms, often linked with membrane action; some were metal chelators which were thought to act in the same manner as EDTA in weakening the outer membrane of *P. aeruginosa* and others were chosen due to a similarity in chemical structure to those agents that had proven successful. The structures of those compounds that were chosen due to structural similarity to successful compounds are listed in Appendix 1, as are the structures of the compounds that led to their selection. The sodium silicate and MGDA were gifts from Unilever plc, while all other compounds were purchased from Sigma Aldrich.

2.2.2.2 Enumeration of organisms by total cell counts

When bacterial cultures were required to be diluted to a certain concentration of cells/ml, a total cell count was performed. Cells from an overnight culture were diluted 100-fold in 0.1M phosphate buffer (pH 7.6) and counts made using a 0.1mm haemocytometer slide. For each sample 10 counts were made, 5 from each side of the chamber.

2.2.2.3 Enumeration of organisms by colony counting

When viable counts were required the method used was as follows. Bacteria were serially diluted 10-fold, 100-fold, 1000-fold and in some cases 10,000-fold in 0.1M phosphate buffer, to give about 200 colony forming units per 100μl. 100μl volumes of these dilutions were plated out on nutrient agar plates that were

subsequently incubated inverted for 18-24 hours at 37°C. The resulting colonies were counted and viable cell numbers calculated.

2.2.2.4 *Identification of P. aeruginosa*

When colonies of *P. aeruginosa* were unusual in appearance, or if there was suspected contamination on the agar plate surface, the colony would be subjected to an oxidase test. Upon the addition of 5-10μl of a 0.1% w/v solution of N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride, the colony would turn purple, indicating oxidase activity and suggesting it was *P. aeruginosa* but remain yellow-white if it was a contaminant. A more detailed API test was considered too costly for contamination determination.

2.2.2.5 *Zone assays*

In an attempt to assay the antibacterial activity of a large number of compounds quickly, a disk diffusion assay was developed. An overnight culture of bacteria was diluted to approximately $1x10^4$ cells/ml in 0.1M phosphate buffer (pH 7.5). Approximately 2-4ml of the bacterial suspension was added to each nutrient agar plate that had been dried at 37°C for 30-minutes. The suspension was distributed evenly over the surface of the agar by rocking gently. Excess fluid was removed by pipette and the surface of the agar allowed to dry for 5-minutes before replacing the lid. Whatman antibiotic assay discs of 6mm diameter were placed firmly onto the agar plates, using sterile forceps, at points equidistant from each other. A 15μl sample of each compound at a chosen concentration was added to a disk. The lids were replaced and the plates inverted and incubated for 24-hours at 37°C. After 24-hours the diameter of any zones of growth inhibition were measured and recorded.

The compounds were also tested for their ability to affect the zone produced by the disinfectant benzalkonium chloride (BKC). In these experiments, the disks containing the compounds to be tested were placed on the agar plate in such a way that they were all equidistant from a central disk that contained a fixed amount of BKC. Again the plates were incubated for 24-hours at 37°C and any zones of inhibition recorded.

2.2.2.6 Log cycle reduction; non-disinfectant test of enhancement of BKC

Cells from a *P. aeruginosa* stationary phase liquid culture at a concentration of $1x10^8$ cells/ml were added to a permeabiliser at a range of concentrations and incubated for 30-minutes at 37°C. A viable count of these cells was performed using 0.1M phosphate buffer (pH 7.6-7.8) as a diluent followed by plating out on nutrient agar that was subsequently incubated for 18-hours at 37°C. A sample of the pre-treated cells was then added to phosphate buffer containing a non-lethal concentration of the disinfectant, BKC (0.625μg/ml). These cells were then incubated for 60-minutes at 37°C and a viable count performed. Due to the nature of BKC as a membrane active agent, the first dilution of each viable count was performed in letheen broth to neutralise this action and prevent carry-over bacteriostasis. Subsequent dilutions were performed in phosphate buffer as previously described. This procedure was repeated with each compound at a range of concentrations. The concentration of BKC used was shown to have no effect on permeabilising the outer membrane of *P. aeruginosa* to NPN and so was considered not to be acting as a permeabiliser to the agents investigated.

2.2.2.7 European standard disinfectant test (EN1276)

A range of compounds was examined using the modified EN1276 disinfectant test that is detailed in section 3.2.3.1 but without the use of BKC. Compounds were tested at the two concentrations 0.05% and 0.1% w/v dissolved in DMSO (final concentration 1% v/v), at both pH 7 and pH 10, against two organisms, *P. aeruginosa* ATCC 15442 and *Staphylococcus aureus*. DMSO at 1% had no antibacterial action against either organism when tested alone.

2.2.3 Permeability assays

2.2.3.1 Permeability assay using NPN

NPN was dissolved in acetone to a concentration of 500μM. It was used at a final concentration of 10μM when added to stationary phase bacteria ($5x10^7$ cells/ml) in phosphate buffer. The final concentration of acetone was 2% v/v. These cells had been pre-treated for 30-minutes with permeabilisers at the range of concentrations used in the log cycle reduction assay (section 2.2.3.6). Control experiments showed no change in viability or permeability of the outer membrane due to the added acetone. After a 15-minute incubation at 22-25°C with NPN, the fluorescence of each sample was measured using a Perkin Elmer spectrophotometer. Excitation and emission wavelengths were set at 350 and 420nm respectively with slit widths of 5nm (Lambert, 1991). All compounds tested were examined for fluorescence in the absence of cells.

2.2.3.2 Permeability assay using nitrocefin

Preliminary experiments were carried out by mixing 50µl of stationary phase bacteria grown in nutrient broth containing 50µg/ml 6-aminopenicillanic acid ($1x10^9$cells/ml), 50µl nitrocefin (1mg/ml) and 50µl of 0.1M phosphate buffer (pH 7.0) containing permeabiliser compound at a sub-lethal concentration. The change in colour, yellow to red upon hydrolysis of the antibiotic, was then recorded over time by measuring absorbance at 492nm using an Anthos Labtech plate reader (Lambert 1991). In subsequent experiments bacterial cells, $1x10^7$ cells/ml, were centrifuged at 7840 x g for 15-min and resuspended in the same volume of phosphate buffer (pH 7.0) to remove any extracellular β-lactamase. They were then pre-treated with permeabilisers or sonicated for 50-seconds with an MSE Soniprep sonicator equipped with a 3mm diameter tip at an amplitude of 20 microns. In the case of pre-treatment with permeabilisers, treated cells were centrifuged for 1-minute at 7840 x g and the supernatant frozen at -20°C. This was repeated at regular intervals to develop a time course of the permeabiliser action. The frozen supernatant containing any β-lactamase released by permeabiliser action was assayed with nitrocefin as previously described.

2.3 Results

2.3.1 Zone assays

The diameter of the inhibition zones were measured and compared to determine the relative antibacterial activity of each chemical tested, an example of this is shown in figure 2.1

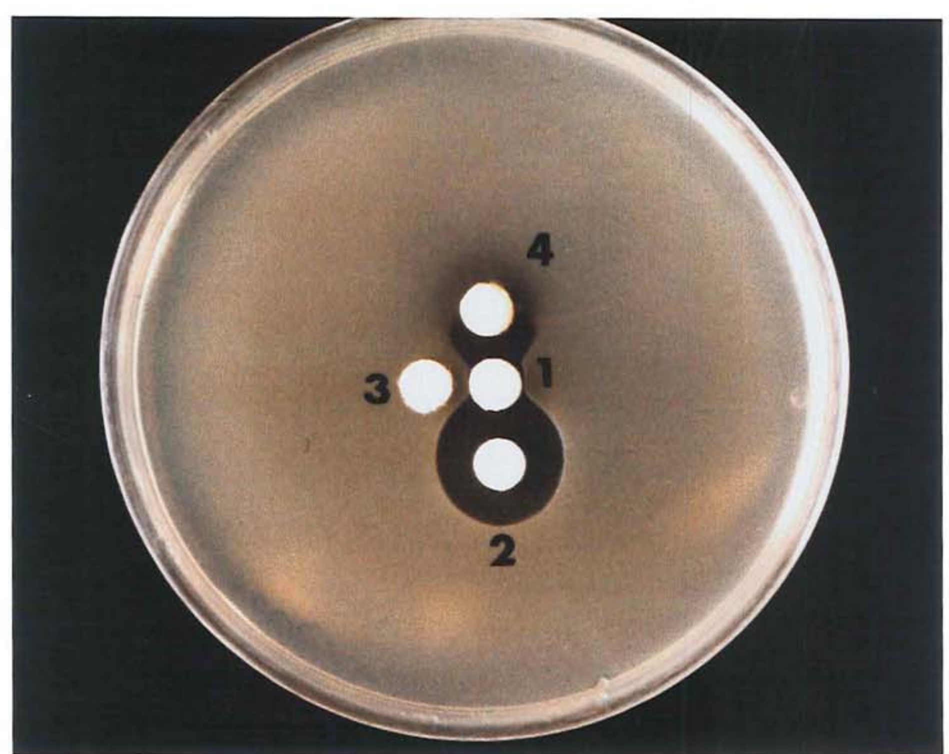


Fig. 2.1 Example of zone assay. The disk in the centre (1) contained 100µg BKC, the disk below (2) contained 10µg of Polymyxin B, the disk to the left (3) contained double distilled H_2O and the disk above (4) contained 400µg of EDTA. These were all placed on a confluent lawn of *P. aeruginosa*, strain PAO1.

The obvious inhibition zone seen around the polymyxin B disk(2) was enhanced slightly by BKC. The disk containing water(3) showed neither inhibition nor evidence of synergy with BKC. However the disk containing EDTA(4), while not inhibiting alone, did exhibit classic synergy with BKC.

The concentration of bacteria used in fig 2.1 (1×10^6 cells /ml), was too high to show the action of BKC alone, so in further experiments lower concentrations of 1×10^4 cells/ml were used. This gave clearer results where slight changes in inhibitory activity could be measured accurately as shown in figure 2.2.

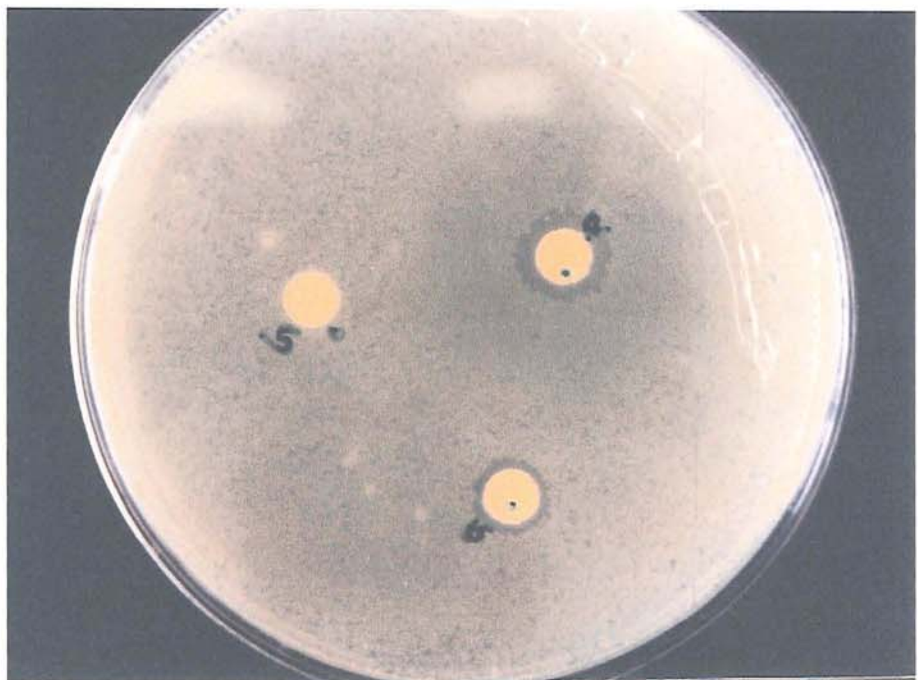


Fig 2.2. Example of inhibition assay alone. Cell concentration of 1×10^4 cells/ml, with no synergy test. This shows the killing action of three compounds. Disk number 4 contained amitryptiline, 5 fluorenone and 6 dioxolone. All disks contained 1200μg of the compound. Only fluorenone showed no inhibitory effect, with amitryptilline having shown a greater activity than dioxolone.

This method was repeated for a range of new compounds being examined and those that had shown activity previously. Details of the inhibition zones produced and the action of the compound when combined with BKC are reported in table 2.2.

Table 2.2 Growth inhibition zones indicating inhibitory action of compounds against *P. aeruginosa* PAO1 and, where applicable, action in conjunction with BKC

Compound name	Zone diameter (mm) at 1200ug of compound	Action with BKC
Ethylenediaminetetraacetic acid (EDTA)	6	Synergy
Quinacrine	10.5	None
Amitryptiline	10	Synergy
[3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl]trimethyl-ammonium chloride	8	None
9-methyl amino methyl anthracene	6.5	None
4(ethylaminomethyl) pyridine	8	None
2-dimethyl amino fluorine	6	None
2-dimethyl amino-9-fluorenone	6	None
2 (2-methylaminoethyl) pyridine	11.5	None
2-methyl amino methyl 1,3, dioxolane	9	None
6-(dimethyl amino fulvene)	7.5	None
Chlorpromazine	9.5	Synergy
3-[-(cholamidopropyl) dimethyl ammonio)2-hydroxy-1-propane sulfonate	7	None
BKC	9.5	N/A
Double distilled H_2O	6	None

These results indicate that 10 of the compounds tested showed some antibacterial activity against *P. aeruginosa* PAO1 in this assay. Only EDTA, amitryptiline and chlorpromazine showed any synergy with BKC, while no compounds antagonised the action of BKC in this test.

2.3.2 Log cycle reduction; non-disinfectant test

Data from the log cycle experiments were converted into graphical form indicating the level of killing action each compound had alone and in conjunction with BKC. These graphs showed the sub-lethal concentration of the compound, that is the highest concentration at which the compound has no significant effect on the viability of *P. aeruginosa* (the sub-lethal concentration) and the potentiating action on BKC at this sub-lethal concentration. In figure 2.3 the sub-lethal concentration of Polymyxin B would be considered to be 0.25μg/ml and the log cycle increase in the action of BKC determined as approximately 2 log cycles as indicated by the arrow shown.

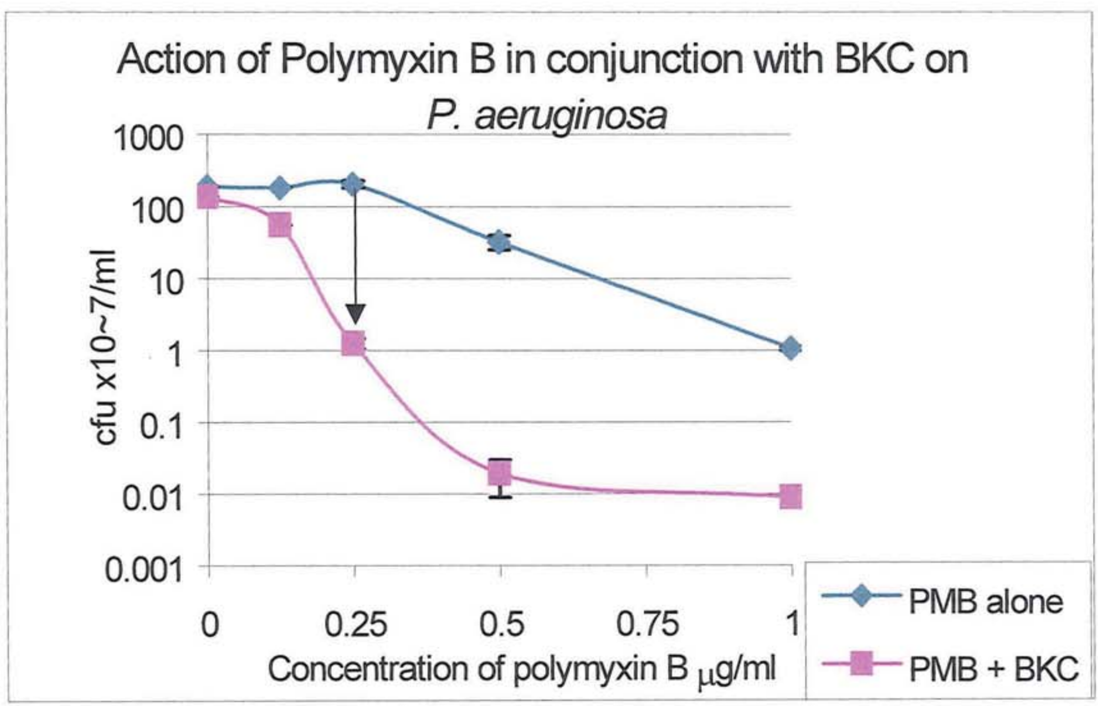


Fig. 2.3 Graph showing log cycle reduction and disinfectant enhancement properties of polymyxin B. against *P. aeruginosa* PAO1.

The activity of compounds as both antimicrobial agents and enhancers of the action of BKC is listed in table 2.3.

Table 2.3 Antimicrobial and synergistic properties of compounds against *P. aeruginosa* PAO1

Compound	Classification	Max log cycle kill (lck) alone and concentration	Synergy with BKC	Highest concentration used with no effect or at which synergy was maximum.
EDTA	Chelator	0	4 log cycles	400μg/ml
MGDA*	Chelator	0	0.25 log cycles	400μg/ml
Sodium Citrate	Chelator	0	0	400μg/ml
Sodium pyrophosphate	Chelator	0	0	400μg/ml
Sodium tripolyphosphate	Chelator	0	0.4 log cycles	400μg/ml
Sodium Silicate	Chelator	0	0	400μg/ml
EGTA*	Chelator	0	0	400μg/ml
Zeolite A4	Chelator	0	0	400μg/ml
Lidocaine	Neuroleptic	0	0	2mM
Dibucaine	Neuroleptic	0	1.7 log cycles	2mM
Tetracaine	Neuroleptic	0	0	2mM
Prochlorperazine	Neuroleptic	2.25 lck /2mM	0	2mM
Imipramine	Neuroleptic	1.2 lck / 1mM	1.05 log cycles	1mM
Chlorpromazine	Neuroleptic	2.5 lck/ 0.125mM	0.99 log cycles	0.06mM
Theophyline	Cationic drug	0	0	1mM
Theobromine	Cationic dug	0	0	1mM
Famotidine	Cationic drug	0	0	1mM
Coumarin 152	Anti coagulant	0	0	1mM
Amitryptiline	Neuroleptic	2.8 lck /0.6mM	1.66 log cycles	0.6mM
Compound 48/80	Polymeric amine	2.77 lck/ 5μg/ml	3.09 log cycles	2.5 μg/ml
Triethylamine	Linear amine	0	0	50 mM
Ethylamine	Liner amine	0	0	50 mM
3-aminopropanol	Linear amine	0	0	50 mM
n-butylamine	Linear amine	0	0	50 mM
Sec-butylamine	Linear amine	0	0	50 mM
Diethanolamine	Linear amine	0	0	50 mM
duethylamine	Linear amine	0	0	50 mM
Iministolibene	Related structure	0	0	2mM
Quinacrine	Related structure	1.1 lck / 3.4mM	3.09 log cycles	1.69mM
Chlorprothixene	Neuroleptic	3.9 lck / 0.56mM	0	4.5mM
2(2-methylamino ethyl) pyridine	Related structure	0	0	11.7mM
9-methylamino methyl anthracene	Related structure	2.25 lck / 3.6mM	1.6 log cycles	1.8mM
2-methylamino methyl1,3dioxolone	Related structure	0	0	13.6mM
4 (ethylamino) pyridine	Related structure	0	0	11.6mM
6-(dimethylamino) fulvene	Related structure	1.6 lck / 6.6mM	0.7 log cycles	6.6mM
2-dimethylamino 9 fluorenone	Related structure	0	0	7.17mM
2-dimethylamino 9 fluorene	Related structure	0	0	7.65mM
Ammon*	Related structure	3.5 lck / 0.49mM	2.6 log cycles	0.1225mM
Cholamido*	Related structure	0	0	2.54mM
Promazine	Related structure	4.2 lck / 1.56mM	3.1 log cycles	0.78mM
Benzox*	Related structure	0	0	5.93mM

Coumarin*	Related structure	0	0	5.13mM
Triflupromazine	Related structure	1.1 lck / 5.14mM	0	5.14mM
Polymyxin b	Peptide	2.5 lck / 1μg/ml	1.6 log cycles	0.25 μg/ml
Protamine	Peptide	1.76 lck /5μg/ml	0.8 log cycles	2.5 μg/ml
Polylysine	Peptide	4.2 lck/ 193μg/ml	2.8 log cycles	12 μg/ml

Ammon* refers to 3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride, Cholamido* refers to 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate, Coumarin* refers to 3-[2-(Diethylamino)ethyl]-7-hydroxy-4-methylcoumarin, Benzox* refers to 2-chloro-3-ethyl benzoxulum fluoroborate, EGTA* refers to ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, MGDA refers to methylglycinediacetic acid, Related structure refers to compounds chosen due to their structural similarity to membrane active agents.

These results show that the compounds can be separated into four groups according to their action against *P. aeruginosa*. A number of agents that include the linear amines, tetracaine and iministolibene, showed neither antimicrobial action alone, nor synergy with BKC. Some agents, typified by chelators such as EDTA and the anaesthetic dibucaine showed no antibacterial action at any concentration used but did increase the activity of BKC. A small group (triflupromazine, chlorprothixene and prochlorperazine) showed antimicrobial action but no effect upon the action of BKC. However a dozen agents showed both antimicrobial activity alone and the ability to enhance the disinfectant BKC.

2.3.3 EN1276 disinfectant test

2.3.3.1 Results against Staphylococcus aureus

A range of compounds, including examples from each of the four groups described in section 2.3.2, were tested against a methicillin resistant strain of *Staphylococcus aureus*. Data are shown in table 2.4.

Table 2.4 Log cycle kill of compounds tested with EN1276 disinfectant test against *S. aureus* at two concentrations and at two pHs.

Compounds	Log cycle kill at pH7		Log cycle kill at pH10	
	0.1% w/v	0.05% w/v	0.1% w/v	0.05% w/v
N,N Dimethyl dodecylamine	0	0	0	0
Quinacrine	0	1	2	1
Amitryptiline	5	3	3	5
Dibucaine	1	0	1	0
Coumarine 152	1	1	1	1
9-methyl amino methyl anthracene	2	1	0	0
Ammon*	5	4	5	5
Prochloperazine	5	4	3	1
Cholamido*	1	1	0	0
2-dimethyl amino-9-fluorenone	0	0	2	1
Promazine	0	0	4	2
Chlorprothixene	2	1	3	2
Lidocaine	0	0	0	2
Imipramine	2	0	5	1
Iminostilbene	0	0	0	1
Triflupromazine	4	2	4	3
Chlorpromazine	2	2	3	2
MGDA	0	0	0	0
n-hexylamine	1	0	2	2
EDTA	0	0	0	0
Sodium silicate	1	0	1	2

Ammon* refers to 3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride, Cholamido* refers to 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate.

All compounds except for the chelators EDTA and MGDA showed some antimicrobial action against *S. aureus*.

2.3.3.2 Results against *P. aeruginosa ATCC 15442.*

The action of each compound against *P. aeruginosa* ATCC 15442 in the EN1276 disinfectant test is shown in table 2.5.

Table 2.5 Log cycle kill of compounds tested with EN1276 disinfectant test against *P. aeruginosa* ATCC 15442 at two concentrations and at two pHs.

Compounds	Log cycle kill at pH7		Log cycle kill at pH10	
	0.1% w/v	0.05% w/v	0.1% w/v	0.05% w/v
N,N Dimethyl dodecylamine	1	1	2	2
Quinacrine	0,0,0	1,0,1	1	1
Amitryptiline	1,1,0	1,0,0	1	0
Dibucaine	1	0	1	1
Coumarine 152	0,0,2	0,0,0	1	0
9-methyl amino methyl anthracene	1,0,1	0,1,0	0	0
Ammon*	0	0	1	1
Prochloperazine	1,	1,	1	1
Cholamido*	0	0	0	0
Fluorenone	0	0	0	0
Promazine	0	0	4	2
Chlorprothixene	2	1	1	1
Lidocaine	1	0	2	0
Imipramine	2	1	1	0
Iminostilbene	0	0	1	1
Triflupromazine	1	0	1	0
Chlorpromazine	0	1	3	2
MGDA	1	2	1	0
EDTA	0	0	0	0
Sodium silicate	1	1	0	1

Ammon* refers to 3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride, Cholamido* refers to 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate

While no compounds showed the requisite 5 log cycle kill required for a compound to pass the EN1276 test, a number of chemicals showed some action against *P. aeruginosa*. From the data collected it was possible to examine whether the compound tested had actions upon BKC that could be termed synergistic, additive or antagonistic, these data are shown in table 2.6 and 2.7.

Table 2.6 Log cycle of antagonism/synergy on the action of BKC (0.25% w/v) by compounds at min (0.05%w/v) and max (0.1%w/v) concentration against *P. aeruginosa* ATCC 15442 in the EN1276 test.

Compounds	Log cycle kill at pH7		Log cycle kill at pH10	
	0.1% w/v	0.05% w/v	0.1% w/v	0.05% w/v
N,N Dimethyl dodecylamine	1	0	**2s**	**2s**
Quinacrine	0,0,**1s**	1, 2, 0	1	3
Amitryptiline	A,0,0	**1s,1s,1s**	1	1
Dibucaine	A	**1s**	0	A
Coumarine 152	A,**1s,1s**	**2s**, 1,**1s**	A	0
9-methyl amino methyl anthracene	**3s**,,0	**2s,1s,1s**	2	2
Ammon*	**2s**,0	**1s**,0	0,0	0,0
Prochloperazine	0,0	0,0	**1s**,0	A,0
Cholamido*	2,0	0,0	2,0	1,0
Fluorenone	**1s**,0	**1s**,0	0,**1s**	0
Promazine	**1s**	0	A	A
Chlorprothixene	0	1	A	0
Lidocaine	**1s**	0	2	0
Imipramine	A	A	0	0
Iminostilbene	0	0	0	0
Triflupromazine	0	2	0	1
Chlorpromazine	1	0	0	1
MGDA	**1s**,0	A,**1s**	0	0
EDTA	**3s,1s**	**3s**,A	**1s**,0	**1s**, 0
Sodium silicate	0, 1	**2s**, 1	0	0

Numbers in plain type indicate a log cycle inhibition of BKC by the compound, while numbers in bold followed by an s indicate the log cycle increase that the compound enhances the action of BKC in a synergistic manner. The letter A indicates that the compound acted in an additive manner. Ammon* refers to 3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride, Cholamido* refers to 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate.

MGDA, EDTA, Fluorenone, Promazine, Ammon*, Imipramine, Iminostilbene and Dibucaine all showed no antagonism action when combined with 0.25% w/v BKC, and all but Iminostilbene showed either synergy or additive action with BKC. Only Triflupromazine, Chlorpromazine and Cholamido* showed either no action or antagonism with BKC. The remaining compounds showed a mixture of synergy, antagonism and additive action with BKC.

Table 2.7 Log cycle of antagonism/synergy of the action of BKC (0.125% w/v) by compounds at min (0.05%w/v) and max (0.1%w/v) concentration against *P. aeruginosa* ATCC 15442 in the EN1276 test.

Compounds	Log cycle kill at pH7		Log cycle kill at pH10	
	0.1% w/v	0.05% w/v	0.1% w/v	0.05% w/v
N,N Dimethyl dodecylamine	2	1	**1s**	**1s**
Quinacrine	0, 1,1	**1s,1s**,0	1	0
Amitryptiline	0,0,0	**1s**,A,1	2	1
Dibucaine	A	1	0	1
Coumarine 152	0,**1s**,0	A,**1s**,0	0	0
9-methyl amino methyl anthracene	**2s**, A, **1s**	**2s,1s**,0	**1s**	1
Ammon*	**1s**	**1s**	A	A
Prochloperazine	**1s**	0	A	0
Cholamido*	1	1	0	0
Fluorenone	**1s**	0	**1s**	0
Promazine	**1s**	0	A	A
Chlorprothixene	0	1	A	A
Lidocaine	1	0	2	1
Imipramine	A	0	0	1
Iminostilbene	0	0	0	A
Triflupromazine	0	**1s**	1	0
Chlorpromazine	**1s**	**1s**	0	2
MGDA	**2s**, 0	**1s, 1s**	A	**1s**
EDTA	**3s,2s**	**3s,1s**	**2s,1s**	**1s,1s**
Sodium silicate	0,1	A, 1	0	0

Numbers in plain type indicate a log cycle inhibition of BKC by the compound, while numbers in bold followed by an s indicate the log cycle increase that the compound enhances the action of BKC in a synergistic manner. The letter A indicates that the compound acted in an additive manner. Ammon* refers to 3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride, Cholamido* refers to 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate.

EDTA, MGDA, Fluorenone, Promazine, Prochloperazine, Ammon*, Coumarine 152, and Iminostilbene all showed no antagonistic action when combined with BKC 0.125%w/v and also some level of either synergy or additive behaviour. Only Cholamid* and Lidocaine showed either antagonism alone or no action at all with BKC. The remaining compounds showed a mixture of synergy, antagonism and additive action with BKC.

2.3.4 Permeability assays

2.3.4.1 Permeability assay using NPN

The increase in fluorescence of NPN due to incorporation of the compound into the outer membrane of *P. aeruginosa* was measured, as was the control value. This led to three sets of data being produced. A gross level of fluorescence, a control level of fluorescence (produced by the compound in the absence of bacterial cells) and a net level of fluorescence which is found by subtracting the control level from the gross level. Results are shown in Table 2.8.

Table 2.8 Effect of potential permeabilising agents upon fluorescence of NPN in presence of *P. aeruginosa* PAO1 whole cells

Compound	Classification	Net fluorescence increase over untreated cells	Concentration at which flourescence increase is maximum or maximun concentration used
EDTA	Chelator	3.09 fold	5 µg/ml
MGDA	Chelator	0*	400µg/ml
Sodium Citrate	Chelator	0	400µg/ml
Sodium pyrophosphate	Chelator	0	400µg/ml
Sodium Silicate	Chelator	0	1mM
EGTA	Chelator	1.15 fold	1mM
Zeolite A4	Chelator	0*	400µg/ml
Lidocaine	Neuroleptic	0	2mM
Dibucaine	Neuroleptic	0*	2mM
Tetracaine	Neuroleptic	0	2mM
Prochlorperazine	Neuroleptic	0*	2mM
Imipramine	Neuroleptic	2.99 fold	1mM
Chlorpromazine	Neuroleptic	0*	2mM
Theophyline	Cationic drug	0	2mM
Theobromine	Cationic dug	0	1mM
Famotidine	Cationic drug	0	1mM
Coumarin 152	Anti coagulant	1.221 fold	0.5mM
Amitryptiline	Neuroleptic	0*	200µg/ml
Compound 48/80	Polymeric amine	2.4 fold	5µg/ml
NN dodecylamine	Linear amine	0	50mM
Triethylamine	Linear amine	0	50mM
Ethylamine	Liner amine	0	50mM
3-aminopropanol	Linear amine	0	50mM
n-butylamine	Linear amine	0	50mM
Sec-butylamine	Linear amine	0	50mM
Diethanolamine	Linear amine	0	50mM
duethylamine	Linear amine	0	50mM
Iministolibene	Related structure	0*	2mM
Quinacrine	Related structure	Coloured compound	200µg/ml

Chlorprothixene	Neuroleptic	0*	1600µg/ml
2(2-methylamino ethyl) pyridine	Related structure	0	1600µg/ml
9-methylamino methyl anthracene	Related structure	0*	1600µg/ml
2-methylamino methyl1,3dioxolone	Related structure	0*	1600µg/ml
4 (ethylamino) pyridine	Related structure	0	1600µg/ml
6-(dimethylamino) fulvene	Related structure	0	1600µg/ml
2-dimethylamino 9 fluorenone	Related structure	0	200µg/ml
2-dimethylamino 9 fluorene	Related structure	0	200µg/ml
Ammon*	Related structure	0*	1600µg/ml
Cholamido*	Related structure	0*	200µg/ml
Promazine	Related structure	0*	250µg/ml
Benzox*	Related structure	0	1600µg/ml
Coumarin*	Related structure	0	1600µg/ml
Triflupromazine	Related structure	0*	250µg/ml
Polymyxin b	Peptide	2.08 fold	0.5 µg/ml
Protamine	Peptide	1.38 fold	5µg/ml
Polylysine	Peptide	1.49 fold	25µg/ml

Ammon* refers to 3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride, Cholamido* refers to 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate, Coumarin* refers to 3-[2-(Diethylamino)ethyl]-7-hydroxy-4-methylcoumarin, Benzox* refers to 2-chloro-3-ethyl benzoxulum fluoroborate. Those compounds whose fluorescence is marked 0* produced a higher degree of fluorescence in the absence of bacteria than when cells were present.

A number of the compounds examined showed synergistic activity with BKC, but any permeabilising action was masked by the fluorescence recorded in the absence of bacteria. A number of these compounds were used in a different assay to determine if they had any outer membrane permeabilising action.

2.3.4.2 Permeability assay using nitrocefin

There was no noticeable increase in the rate of hydrolysis of nitrocefin following the addition of BKC, EDTA, Polymyxin B or compound 48/80 to cells of *P. aeruginosa* PAO1. However in the experiments where at time points after the addition of a permeabiliser cells were removed by centrifugation and the supernatant harvested, it was possible to determine an increase in the release of β-lactamase by measuring the hydrolysis of nitrocefin after 10-minutes incubation with the supernatants harvested. Each compound was also added to nitrocefin alone (no supernatant) at appropriate concentrations to ensure that colour changes were due to β-lactamase release alone. The final results are shown graphically in figures 2.4, 2.5 (Promazine), 2.6 (Amitryptiline), 2.7, 2.8 (PMB) and 2.9 (EDTA).

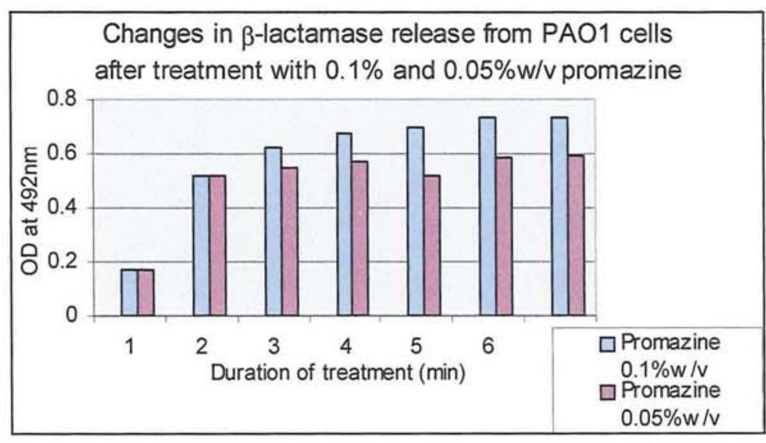


Figure 2.4 Action of two concentrations of promazine on the time course of release of β-lactamase from PAO1 cells.

OD at 492nm indicates the release of β-lactamase by the conversion of nitrocefin (yellow) to its hydrolysis product (red) through the action of enzyme released from the cells by promazine.

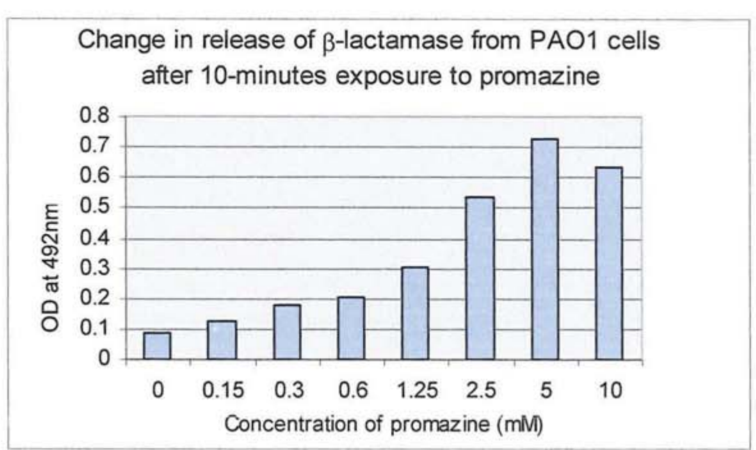


Figure 2.5 Action of increasing concentrations of promazine in releasing β-lactamase from PAO1 cells

Overall these results indicate that promazine exerts a permeabilising action upon the outer membrane of *P. aeruginosa,* with a maximum effect occurring at approximately 5mM, (0.16%w/v).

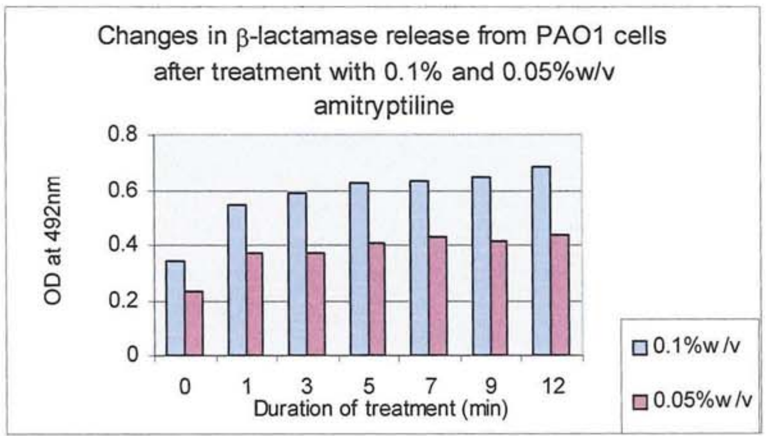


Figure 2.6 Action of two concentrations of amitryptiline in increasing the release of β-lactamase from PAO1 cells over time.

From the results shown it appears that amitryptiline has an outer membrane permeabilising action upon PAO1 cells, which is dependant upon the concentration of the agent and the duration of exposure.

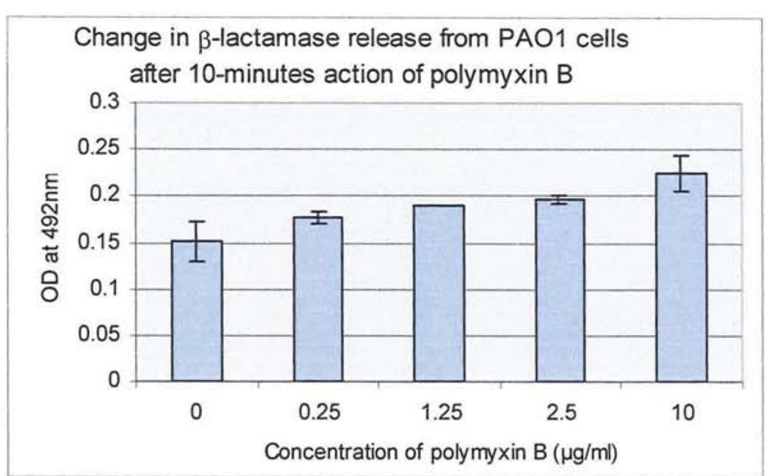


Figure 2.7 Action of a range of polymyxin B concentrations in increasing the release of β-lactamase from PAO1 cells after 10-minutes.

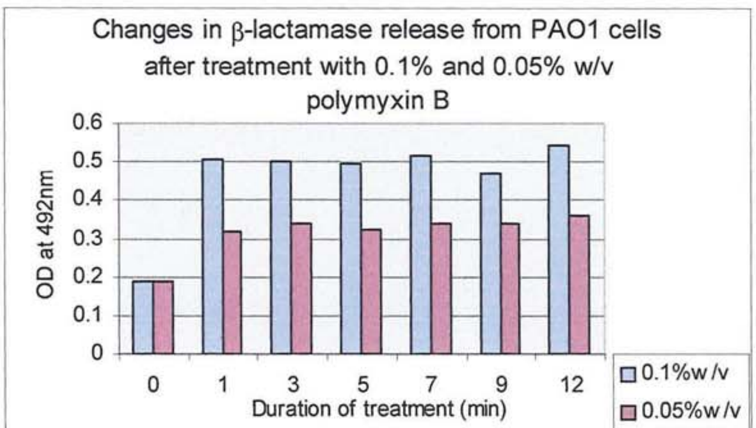


Figure 2.8 Action of two concentrations of PMB on the release of β-lactamase from PAO1 cells over time.

These results support those in table 2.6, that polymyxin B does indeed have an outer membrane permeabilising action, and so can validate the use of this nitrocefin assay to examine those compounds unsuited to the previously used NPN uptake assay.

These results show how the action of polymxin B over time is similar to that of amitryptiline.

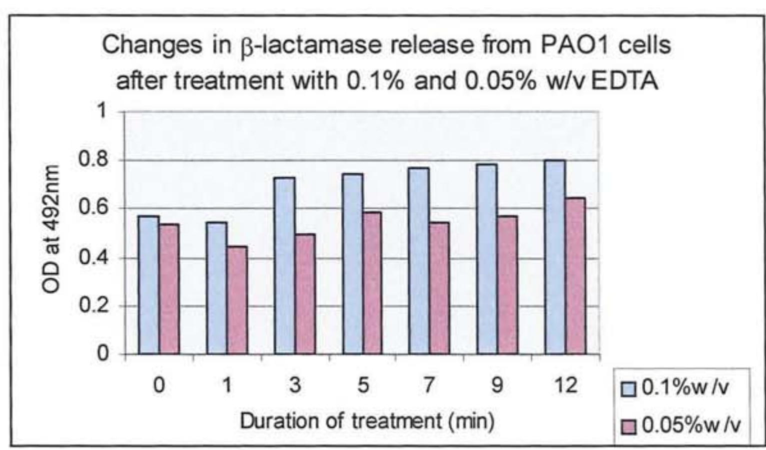


Figure 2.9 Action of two concentrations of EDTA in increasing the release of β-lactamase from PAO1 cells over time.

These results further validate this assay as EDTA has been long established to have outer membrane permeabilising properties.

2.4 Discussion

Previous work has examined the properties of a range of compounds, (Voss, 1967; Vaara & Vaara, 1983a,b; Hancock & Wong, 1984; Labedan, 1988; Vaara, 1992; Ayres *et al.*, 1999) in both reducing the barrier posed by the outer membrane to hydrophobic probes and in enhancing the action of antibacterial agents. In the results for this chapter each agent was classified due to its structure, proposed mechanism of action or its best-known biological property. It is proposed to continue with this classification when discussing the action of the agents.

2.4.1 Chelating agents

EDTA has shown the most consistent permeabilising action against *P. aeruginosa* of all compounds tested, while showing no antibacterial action itself. It has been used in the past both as an example of a permeabilising agent when comparing other putative permeabilisers (Ayres *et al.*, 1999) and as a means of defining the outer membrane permeability of bacteria resistant to other agents. EDTA showed up to 3 log cycles of synergy with BKC in the EN1276 disinfectant test, and showed no antagonistic action whatsoever. MGDA showed a lower level of synergy and no antagonism, while sodium silicate showed a mixture of antagonism and synergy. Both MGDA and sodium silicate showed antimicrobial activity alone against *P. aeruginosa* and *S. aureus*.

In the non-disinfectant enhancement of BKC study only sodium polyphosphate, MGDA and EDTA showed any enhancement of BKC log cycle kill, all at the same concentration but with EDTA giving an enhancement 10-fold larger than any of the other chelators.

EDTA increased the incorporation of NPN into the outer membrane by a greater amount and at a lower concentration that any other metal chelators. Indeed of all the other chelators studied, only EGTA and EDTA showed any enhancement of NPN incorporation, an unusual result when considering that MGDA showed synergy with BKC in both previous tests.

The conclusion of these tests show that EDTA is the most active of the chelators studied. Its use however in disinfectant solutions to be tested by the EN1276 test is still limited by the presence of divalent cations in the media used for this test as these cations are known to reduce EDTA's activity. In addition, previous work has stated that the action of EDTA is reduced in phosphate buffers and in the absence of Tris or other organic monovalent cations. It is thought that these cations replace the chelated divalent cations holding the adjacent LPS molecules together and so destabilise the outer membrane (Voss, 1967). This would appear to contradict the results for enhancement of NPN incorporation that took place in a 0.1M phosphate buffer.

2.4.2 Peptides and Polymyxin B

Polymxin B, poly-lysine and protamine all have a history of antimicrobial and permeabilising activity and show both enhancement of BKC action in the non – disinfectant test and enhancement of NPN incorporation in the outer membrane. None of the three were used in the EN1276 test due to both the high concentrations required in the test (0.025% w/v would be 250μg/ml a 10-fold increase in concentration over that of other assays) and communication from Unilever that protein based enhancers of BKC would not be appropriate in disinfectant formulations. However the action of polymyxin B as an outer membrane

permeabiliser is well known and was used in addition to EDTA to validate the nitrocefin work. The synergy of polymyxin B and the disinfectant BKC is not without precedent, as work showing synergy between polymyxin antibiotics and the disinfectant chlorhexidine against *P. aeruginosa* (Al-Najjar & Quensel, 1979) and the antibiotic is often described as having a detergent like mode of action.

2.4.3 Amines: Polymeric and Linear

The inclusion of linear amines as possible permeabilisers is based upon the work of Voss (1967) examining the action of these and related compounds upon *E. coli* in conjunction with EDTA. The simple linear amines had no antibacterial, synergistic or outer membrane permeabilising activities whatsoever, although N,N, dimethyl dodecylamine did show synergy with BKC in the EN1276 test when used at pH10, but showed no action against *S. aureus* in the same test. Its antibacterial action alone was examined in the EN1276 test and again was higher at pH10 then at pH7. The agent showed no permeabilising properties when examined with NPN but may be of use in formulations due to its synergy with BKC. The polymeric amine compound 48/80 showed both antibacterial and synergistic action in the non-disinfectant test and increased the incorporation of NPN into the outer membrane indicating it has outer membrane permeabilising properties. Further work with this compound is dealt with in chapter 3.

2.4.4 Neuroleptic agents, anti-coagulants, cationic drugs and compounds with similar structures

In the 1990s a number of papers were published examining the antibacterial action of non-antibiotic agents more commonly used in the treatment of psychiatric disorders (Kristiansen, 1992; Kristiansen & Amaral, 1997). In addition, local anaesthetics were observed to have permeabilising properties on the outer membranes of *E. coli* cells (Labedan, 1988). Later that same decade the action of neuroleptic agents, local anaesthetics and other cationic drugs was examined in enhancing the action of antibiotics against the cystic fibrosis associated organism *Burkholderia cepacia* (Rajyaguru & Muszynski, 1997, 1998).

In this work a number of these compounds were tested at concentrations that were used successfully against *B. cepacia* in synergising antibiotic action.

From the results of the preliminary non-disinfectant BKC enhancement experiments it was clear that a number of these compounds could synergise the action of BKC and had anti-pseudomonal properties. The compounds imipramine, chlorpromazine and amitryptiline all showed both these properties, and shared structural similarities, having a tricyclic ring structure with a carbon chain attached at C'9 via a variety of links, the chain terminating with an nitrogen atom attached to 2 methyl (-CH_3) groups. The compounds prochloperazine and chlorprothixene had antibacterial action but showed no synergy, and have similar structures to those already described. The only non-chelating agent that showed no antibacterial action, but synergised BKC was dibucaine, a local anaesthetic with a dicyclic ring structure with a short carbon chain terminating in a nitrogen atom with 2 ethyl (-C_2H_5) groups attached. New compounds were selected due to their structural relation with active agents;

namely easily available chemicals with structures with a mono-, di- or tricyclic ring base with or without a side chain containing a secondary, tertiary or quaternary amine.

Of these structures quinacrine, 9-methyl amino anthracene, 6-(dimethylamino) fulvene, ammon* and promazine all showed both antibacterial and synergistic properties in the non-disinfectant synergy test and triflupromazine showed antibacterial action alone. All but 6-(dimethylamino) fulvene had structures comprised of tricyclic rings with a side chain terminating with a nitrogen atom and either methyl, ethyl or diethyl group(s) attached.

The results in the EN1276 test against *S. aureus* appear to confirm that many of the compounds have antibacterial action; no synergy with BKC was examined at this point as BKC already has an adequate action against Gram-positive organisms. All compounds showed at least 1-log cycle of kill at 1 of the 2 pHs the compounds were tested at and some such as amitryptilline and ammon*, showed up to 5-log cycles of kill against the organism. The results for the same compounds tested against *P. aeruginosa* ATCC 15442 were similarly mixed. All such compounds showed action against the organism at either pH or concentration, but no compound showed higher than 4-log cycle kill alone.

Synergy with BKC was observed with amitryptiline, dibucaine, coumarin 152, 9-methyl amino methyl anthracene, ammon*, quinacrine, prochloperazine, fluorenone, promazine and lidocaine, although only promazine, flurenone, prochlorperazine, ammon* and dibucaine showed no evidence of antagonism with the disinfectant.

This antagonism may indicate a common site of action for the agents with BKC since it seems unlikely that charge considerations would preclude the action of BKC and these compounds.

One property for certain of these compounds is the high level of fluorescence produced in experiments with NPN in the absence of bacterial cells. This prevented accurate use of NPN as a hydrophobic probe of the outer membrane of the cell and has been observed in other work, where the addition of compounds to cells reduced the fluorescence of NPN (Rajyaguru & Muszynski, 1997). This was assumed to be due to a lack of permeabilising activity although there is no evidence in the literature that the compounds were tested with NPN in the absence of cells. The action of both promazine and amitryptilline in increasing the release of the chromosomal β-lactamase from the periplasm by disruption of the outer membrane permeability barrier indicates that both these compounds act as permeabilisers, regardless of their lack of activity in the NPN test. In addition, drug efflux in *E.coli* has been shown to be inhibited by such agents (Molnár *et al.*, 1997), although no work has examined the action of agents in *P. aeruginosa*. As described in the introduction to this thesis, there is increasing evidence that efflux plays a role in the resistance of *P. aeruginosa* and other organisms to antibiotics. Any sensitisation to antibiotics as described in Rajyaguru and Muszynski (1997, 1998) may be due to reduction in efflux action in addition to membrane permeabilisation.

2.4.5 Proposals for novel agents.

Due to time constraints the choice of novel agents for synthesis was based upon the results from the BKC enhancement work, not from the EN1276 work. The

common structure linking successful agents would appear to be a tricyclic ring base with side chain attached as previously described. Much work has been completed on structure activity relationships for these tricyclic compounds with reference to their antiplasmid, antibacterial and use in cancer therapy. Bourlioux *et al.* (1992) when examing phenothiazine molecules (those with a tricyclic ring substituted with an S atom at point 2 and an N atom at point 9), suggested that antibacterial activity is linked to the presence on the C'2 position of the tricyclic base of a halogen or methyl thio group and make no comment on the nature of the side chain attached to C'9. There is evidence in this chapter of antibacterial activity in compounds such as promazine, imipramine, amitryptilline and 9-methylaminomethyl anthracene which have no side chain or halogen attached save that at position C'9 ending in an amine. This evidence would seem to contradict the need for such other side groups or atoms in promoting antibacterial action, although there is much evidence that such groups are essential for other of the properties of the compounds. Molnár *et al.* (1993) suggest that while the substitution at the C2' position of either Cl or CF_3 is important in the anti-plasmid activity of such compounds, the side chain length at position C9' is also important. Previous work (Motohashi *et al.*, 1992a, b) agreed with this and suggested that the properties of antiplasmid, and antibacterial action are controlled by similar moities in the molecule, separate from those associated with other activities. Other work has examined the action of these agents in preventing the efflux of anti-cancer drugs from human cells as part of therapy. It would appear that this action may be associated with the length of the side chain previously mentioned, or more specifically the distance between the N atom at beginning the side chain and the last N atom at the end of the side chain (Motohasi *et al.*, 1997).

While there is much evidence suggesting that compounds such as chlorpromazine may be useful as the basis of generating novel compounds to add to disinfectants, there are a number of disadvantages to this work. They are, as their primary use, neuroleptic agents, with action on mammalian nervous systems that would be inappropriate for use in a disinfectant. Many of the active agents contain halogen groups, moities that are difficult to biodegrade and would make disposal of disinfectants a problem in an industry closely regulated by ecological directives. Finally, while the chemistry involved in creating novel agents is possible, it is also beyond the practical limits of a project of this kind.

Agents based upon the acridine molecule (quinacrine) have a history of anti-bacterial, and predominantly anti-plasmodial action as treatments for malaria. A very recent paper reviewed the use of such acridine agents in history describing acridine as "a neglected antibacterial chromophore" (Wainwright, 2001).
Their safety has already been evaluated for use as anti-malarial agents, and the example used in this work, quinacrine, showed good activity in the non-disinfectant BKC enhancement test. In addition, the chemistry involved in creating compounds with varying side chains, and tri-cyclic bases with or without halogen or methyl groups is relatively straightforward (Atwell *et al.*, 1998; Dibyendu *et al.*, 1998). While quinacrine's performance in the EN1276 test was not as successful in either antibacterial action alone or synergising BKC it provided the most realistic basis for novel compound synthesis for agents to act against *P. aeruginosa*.

2.5 Conclusions

There is ample evidence that certain of the non-antibiotic compounds examined have a measure of antimicrobial activity and that this activity appears to be linked to the common triple ring and side chain structure as described in figure 3.1. By examining alterations in this structure based upon a known antiplasmodial agent, quinacrine, it is hoped to develop novel agents for safe use in disinfectants.

Chapter 3 Synthesis and testing of novel compounds

3.1 Introduction

3.1.1 Aims

Having determined that one of the common structures found in compounds that synergise BKC action against *P. aeruginosa* comprises three fused aromatic rings with an aliphatic side chain attached to the central ring terminating in an amine, the next step was to synthesise novel compounds based upon this element. Once synthesised, compounds could be tested using the EN1276 European standard disinfectant test. In addition, compound 48/80 was subjected to gel permeation chromatography to isolate the different sized oligomers that make up this membrane active agent. Two peptides based upon the structure of a viral peptide known to have antipseudomonal properties were also synthesised and examined.

3.1.2 Synthesis of novel compounds.

Compounds based upon the phenothiazine fused aromatic ring structure were thought to have too strong a neuroleptic activity to be safe as disinfectants. However the quinacrine molecule is based upon the acridine ring structure, a structure found in a range of antimicrobial agents including antibacterials, for example proflavine, and antiprotozoals such as quinacrine, used in treating malaria. In addition, the chemistry required to produce phenothiazine derivatives is far more complex that that for novel compounds based upon an acridine ring (see fig 3.1).

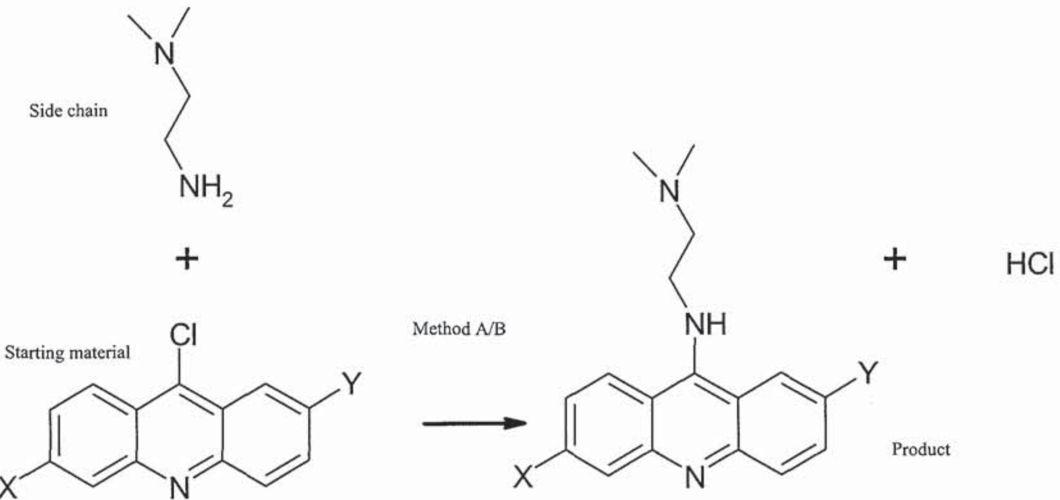


Figure 3.1 Route for synthesis of substituted acridine compounds.Triple ring starting materials are 6,9 dichloro-2-ethoxyacridine in the case of α compounds and 9-chloroacridine for β compounds. All triple ring starting materials and side chains are shown in figure 3.2

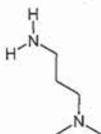
Method A was used to synthesise α compounds, described in section 3.2.1.1 and based upon the method of Dibyendu *et al.*, (1998). Method B was used in the synthesis of β compounds, described in section 3.2.1.2 and based upon the methods of Atwell *et al.*, (1998).

Starting material 1 (SM1)

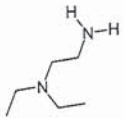
Starting material 2 (SM2)

Side chain 1

Side chain 2



Side chain 3

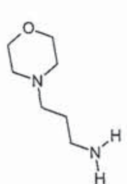


Side chain 4

Side chain 5

Side chain 6

Side chain7



Side chain 8

Side chain 9

Side chain 10

Side chain 11

Figure 3.2 Structure of compounds used in synthesis of novel antimicrobials.

3.1.3 Thin Layer Chromatography (TLC)

When producing novel compounds for screening it is important to have evidence to confirm that the synthesis process has worked. Thin layer chromatography is often used in this manner as it is quick, uses little of the compound and is inexpensive. It should be noted that this process will distinguish "product" from starting materials if a sample of each is run in the same solvent and their relative frontal mobility (R_F value) compared. However it gives little information about the nature of the "product", so to distinguish between the desired product and unwanted contamination or byproducts other techniques are required.

3.1.4 Mass Spectrophotometry (MS)

This technique for identification of compounds involves the disintegration of organic compounds into fragment ions that are accelerated in an electric field and separated on the basis of their different masses. The first ions produced are parent ions that rapidly disintegrate to give smaller fragments. It is possible to determine the molecular weight of the fragments and parent ion, but this gives no information as to the purity of the compound.

3.1.5 Nuclear Magnetic Resonance (NMR)

Electromagnetic radiation can be used to identify and determine purity of compounds. This is possible due to the atomic nuclei of many elements being magnetic due to their being charged and possessing spin. Typical examples include ^{1}H, ^{13}C, ^{14}N, ^{15}N, ^{19}F and ^{31}P. When these nuclei interact with a uniform magnetic field they align themselves in a direction either parallel or antiparallel to the field. These two positions have different energy levels; the difference between them

corresponds to a particular electromagnetic frequency. When an isotope containing a magnetic nucleus is placed in a magnetic field and exposed to an appropriate radiofrequency, transitions between the energy levels will occur when the energy gap and the applied frequency are in resonance. However the immediate chemical environment surrounding the magnetic nucleus will effect the resonant frequency observed. So, in the simple chemical acetic acid (CH_3COOH) there would be two different ^1H resonances, one for the protons in $-CH_3$ one for the signal from $-COOH$. Furthermore the relative intensities of the signals are proportional to the number of contributing nuclei. Therefore, in the example of acetic acid the relative intensities of the signals due to the protons in CH_3 and COOH would be 3:1 respectively. This can be used to determine the purity of a product by comparing signal intensities of ^1H known to be in the product with that of a starting material or other impurity.

3.1.6 European Standard Disnfectant Test (EN1276)

The European standard disinfectant test, EN1276, is now the standard by which all chemicals to be defined as the active ingredient in a disinfectant solution are evaluated. In addition, it has been used for the testing of disinfectant products for use in food, industrial and domestic markets (Taylor *et al.*, 1999). The standard test is performed at a range of temperatures, in both clean and dirty conditions. The method used in this work is adapted for a high throughput method and is currently used in comparing different disinfectant formulations in the Unilever laboratories in The Wirral, Merseyside.

3.1.7 Novel peptide synthesis.

The history of antibacterial peptides was dealt with in section 1.6.1. To create a novel peptide there are two main routes; by examining primary amino acid sequence and by making substitutions, deletions or additions, different versions of a peptide known to have antimicrobial activity can be produced. In this way the key areas of the original peptide can be determined. The second route is to examine the secondary structure of antimicrobial peptides, including hydrophobicity, proportion of alpha helical and beta pleated sheet structures and charge. Then, by substitution of amino acids that confer such secondary structures that have been shown to confer antimicrobial activity, truly novel peptides with potential activity can be produced. A recent review by Tossi (1997) examines this process in great detail. In particular work has been completed on the effects of such modifications upon the antibacterial properties of peptides isolated from lentivirus envelopes against a range of organisms including *P. aeruginosa* (Tencza *et al.*, 1997; Tencza *et al.*, 1999).

3.1.8 Separation of different chain length components of compound 48/80

As stated in the introduction to this work compound 48/80 is made up of a number of varied length polymers of a single unit (Lenney *et al.*, 1977), (figure 3.3).

Figure 3.3 Structure of the hexamer of compound 48/80

Each length of polymer has associated with it certain biochemical properties including, but not solely, antimicrobial actions on organisms such as *P. aeruginosa*. While synthesis of polymers with up to 3 units has been achieved experimentally, larger length polymers have not been synthesised alone and so their antimicrobial properties are unknown. Results shown in chapter 2 confirmed this agent's antimicrobial properties against *P. aeruginosa* when tested as a mixture of different length polymers. Therefore separating compound 48/80 by gel permeation chromatography into different sized oligomers would allow examination of the antimicrobial properties of each oligomer in turn.

3.2 Materials and Methods

3.2.1 Synthesis of acridine based compounds

3.2.1.1 General procedure for the synthesis of compounds 3α to 13α from starting material 6,9 dichloro-2-methoxyacridine, (SM1)

A mixture of 100mg (0.357 mmol) SM1 and 73mg 0.72mmol triethylamine* and 0.54mmol (1.5mol equivalent) of the appropriate primary amine (figure 3.2 side chains 1-11) in 2ml butanol was heated in a sealed vial at 120°C for 48-hrs. The butanol was then removed by evaporation, the remaining product triturated in diethyl ether and the washings stored. The product was then desiccated under high vacuum overnight and weighed.

*The triethylamine was used as a scavenging agent to remove excess Cl ions.

3.2.1.2 General procedure for the synthesis of compounds 3β to 13β from starting material 9-chloroacridine, (SM2)

A mixture of 100mg (0.468mmol) SM2 and 0.5258mmol (1.68mol equivalent) of the appropriate primary amine in 2ml anhydrous methanol was heated in a sealed vial at 55°C for 24-hrs. The methanol was then removed by evaporation, the remaining product triturated in diethyl ether and the washings stored. The product was then desiccated under high vacuum overnight and weighed.

3.2.1.3 Nomenclature

Compounds based upon the tricyclic compound 6,9 dichloro-2-ethoxyacridine (starting material 1, SM1) were designated as alpha (α) compounds while those based upon 9-chloroacridine (starting material 2, SM2) were designated as beta (β) compounds. Side chain 1 is used to produce 3α and 3β compounds, side chain 2 to produced 4α and 4β compounds. This nomenclature continues until side chain 10 is used in the synthesis of 13α and 13β compounds.

3.2.1.4 Calculation of yield

The approximate percentage yield of each product was calculated by weighing the dry mass of triturated product and comparing with the combined mass of triple ring starting material and side chain.

3.2.2 Identification of products

3.2.2.1 Thin layer chromatography

SM1, SM2 and samples (3-13 α+β) were dissolved in chloroform and applied to aluminium-backed silica gel chromatography plates (20 x 20cm, layer thickness 200μm, particle size 2-25μm, pore size 60Å, Sigma) using a microcapillary tube and allowed to dry at room temperature. The mobile phase (10%v/v methanol in dichloromethane) was allowed to equilibrate in a glass tank with sealed lid for at least half an hour. The dry plate was placed within the glass tank and developed until the solvent front had progressed nearly to the top of the plate. The plate was then removed and left to dry. The product and starting material were visualised by UV illumination and R_F values calculated.

3.2.2.2 Mass spectrometry

Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) of samples in methanol was carried out on a Hewlett-Packard 5989B quadrapole instrument connected to an electrospray 59987A unit with an APCI accessory and automatic injection using a Hewlett-Packard 1100 series autosampler.

3.2.2.3 NMR

Proton NMR (^{1}H) spectra were obtained on a Bruker AC250 instrument operating at 250 MH_z at a temperature of 300 Kelvin where samples were solutions in d6-DMSO and referenced from δDMSO = 2.50ppm. Where the DMSO solvent peak masked peaks from the samples the solvent d6-CDCl3 was used and referenced from δCDCl3 = 7.27ppm. By comparing the magnitude of signal from starting material and product a value for compound purity was estimated.

3.2.3 Testing of compounds

3.2.3.1 Disinfectant test EN1276

3.2.3.1.1 Preparation of bacteria.

Streaking from frozen stock culture onto tryptone soya agar slopes and incubating at 37°C for 16-20-hours produced the first subculture of bacteria. A second subculture was produced by streaking from the first subculture to a fresh slope, and from this second subculture a third was also produced. The second and third subcultures were used in the experiments.

3.2.3.1.2 Preparation of novel compounds

Hard water was produced as follows. Solution A: 1.984g $MgCl_2$ and 4.624g $CaCl_2$ in 100ml ddH_2O. Solution B: 3.502g $NaHCO_3$ in 100ml ddH_2O. 6.0ml of solution A added to 8.0ml of solution B then made up to 1L with ddH_2O filter sterilised and stored at 4°C for up to 1-month.Compounds were prepared in DMSO at 10x the concentration to be tested. Brief sonication was used to fully dissolve the compounds. Compounds were diluted in filter sterilised hard water to the appropriate concentration, 0.1%w/v or 0.05%w/v. BKC was added to the hard water to a final concentration of either 0.025%w/v or 0.0125%w/v. In experiments using only β-compounds a phenolic disinfectant, orthophenylphenol, was used in the place of BKC but at the same concentrations (w/v).

3.2.3.1.3 Method of testing

A loopful of subculture was transferred from a slope to a sterile flask containing 10ml tryptone soya broth (TSB) and 5g of sterile glass beads. The flask was shaken for 5-minutes at 37°C at 250 rpm. The bacterial suspension was then transferred to an empty sterile petri dish. A 96-well microtitre plate had been prepared as described in annotated figure 3.3, taking care that no more than 2-hours passed between compounds being dissolved in hard water and being used to challenge the organism.

A 25μl sample of the culture was transferred aseptically to wells A1-H1 and A12, of the 96-well microtitre plate, using a multichannel pipette. After 5-minutes, 25μl of the culture in wells A1-H1 and A12 was transferred to wells A2-H2 and B12 respectively. Drawing the suspension up and down in the pipette tips mixed the culture in the wells and allowed the letheen broth to neutralise the action of BKC. After a further 5-minutes, 25μl of culture was transferred from wells A2-H2 and B12 to wells A3-H3 and C12 respectively. Serial transfer of 25μl of culture from wells A2-H2 to each column then serially diluted the culture until reaching column 11 and from C12 to H12. The microtitre plate was then sealed and incubated for 24-hours at 37°C. For each compound the experiment was repeated 3 times at 3 different pHs, (4.5,7.0, 10.5). The contents of each well of the microtitre plate are shown in figure 3.4.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Columns 3-10 contained 225µl of TSB and Column 2 contained 225µl of letheen broth
A1 contained compound at 0.1% w/v in hard water. Total volume 225µl.
B1 contained compound at 0.1% w/v and BKC at 0.25% w/v in hard water. Total volume 225µl.
C1 contained compound at 0.1%w/v and BKC at 0.0125% w/v in hard water. Total volume 225µl.
D1 contained compound at 0.05% w/v in hard water. Total volume 225µl.
E1 contained compound at 0.05% w/v and BKC at 0.25% w/v in hard water. Total volume 225µl.
F1 contained compound at 0.05% w/v and BKC at 0.125% w/v in hard water. Total volume 225µl.
G1 contained BKC at 0.25% w/v in hard water. Total volume 225µl.
H1 contained BKC at 0.125% w/v in hard water. Total volume 225µl.
A12 contained 225µl of hard water, B12 contained 225µl of letheen broth
C12-H12 contained 225µl of TSB.

Figure 3.4. Diagram of microtitre plate showing contents of the wells prior to commencing the EN1276 test.

3.2.4 Production of novel peptides

Solid-phase peptide synthesis was carried out using an Applied Biosystems model 432A Synergy personal peptide synthesizer according to the method developed by Merrifield *et al.* (1966). The C-terminus amino acid of the sequence was covalently bound to a resin support and subsequent amino acids were added until the N-terminal residue of the sequence was reached. Each addition involved 3 reactions: de-protection of the solid phase amino acid, activation and coupling. Due to the nature of the support there was a significant reduction in yield when peptides of more than 20 amino acids were produced due to swelling of the resin. Since this peptide was 28 amino acids in length, synthesis was completed in two sections. The first produced a peptide containing the first 20 amino acids from the C-terminus. The resin was then split into 2 equal portions, only 1 of which was used to couple the remaining 8 amino acids. The process therefore produced a small quantity of the 20 amino acid peptide and a larger quantity of the full length 28 amino acid peptide. After synthesis both peptides were cleaved from the resin support by addition of cleavage mixture cooled to -5°C. This mixture contained 100µl thioanisole, 50µl water, 50µl ethanedithiol and 1.8ml trifluoroacetic acid (TFA). A 500µl sample of the cleavage mixture was added to each peptide and incubated for 2-hrs at 22-25°C. A further 500µl of cleavage mixture was added and incubated at 22-25°C for a final 2-hours. The resin was filtered from the mixture using a Pasteur pipette plugged with glass wool and washed with 500µl of TFA to remove any remaining peptide.

The filtrate produced was then added dropwise to 8mls of methyl t-butyl ether (MTBE), cooled to 4°C. This formed a white precipitate of de-protected peptide. This solution was centrifuged at 314 x g for 10-minutes and the supernatant removed. The pellet was resuspended in fresh MTBE and re-centrifuged. The pellet was washed in

this way a total of 4 times. The final supernatant was removed and the pellet allowed to air dry. The final peptide pellet was resuspended in 2ml of water and lyophilised. This produced approximately 52mg of the 28 amino acid peptide and 12mg of the shorter 20 amino acid peptide. The final freeze dried peptide was weighed and dissolved in a mixture of water, 10% acetic acid and dimethyl sulphoxide in the ratio 26:1:1 respectively to give peptides of concentration 5mg/ml. This concentration was verified by means of ninhydrin protein estimation.

As stated earlier two peptides were produced, one with 20 amino acids and one with 28 amino acids both based upon the lentivirus derived protein LLP1 as shown in figure 3.5

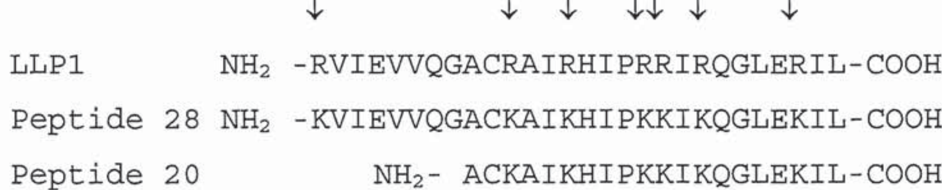


Figure 3.5 Amino acid sequence of lentivirus peptide LLP1 and the two novel peptides based upon its structure. The arrows indicate Arginine amino acids in LLP1 and the Lysine amino acids that replace them in the novel peptides, 28 and 20.

The antibacterial action of the peptides was determined by the log cycle synergy method as detailed in section 2.2.3.5 and examined by the NPN permeability assay as described in section 2.2. The helical wheel structures of the peptides produced were determined by use of the University of Wisconsin Genetics Computer Group (GCG) software (http://gcg.hgmp.mrc.ac.uk). Kyte-Doolittle and Hopp-Woods hydropathy and Chou-Fasman secondary structure data predictions were generated from the peptide sequences using the Protylze Structure Prediction Version 3.0 software (Scientific Educational software).

3.2.5 Fractionation of compound 48/80

A 50mg/ml aqueous solution of compound 48/80 was passed through a Superose 12 HR 10/30 column eluted with water at a flow rate of 0.2ml/minute. 2-minute fractions were taken over a total of 120-minutes, each fraction having a volume of 400µl. A 100µl sample of each fraction was diluted in 2.9ml water and its absorbance at 280nm recorded using a PU 8700 series UV/Vis spectrophotometer. In addition 100µl of each sample was freeze dried, re-dissolved in methanol and 0.5µl subjected to mass spectrometry by atmospheric pressure chemical ionisation (APCI), using a Hewlett Packard 5989B mass spectrometer.

3.3 Results

3.3.1 Thin layer chromatography and approximate yield

The R_f values of both starting materials and products were compared to check that synthesis had occurred (see table 3.1).

Table 3.1 R_f values of starting materials and putative products of chemical synthesis and the approximate yield.

Product	Appearance	Rf Product	Rf triple ring	Approximate yield (%w/w)
3α	yellow/orange solid	0.118	0.967	10.01
4α	orange/brown solid	0.135	0.967	76.1
5α	orange/brown solid	0.096	0903	54.9
6α	yellow/orange solid	0.07	0.898	39.7
7α	red/orange solid	0.065	0.918	16.8
8α	pale brown solid	0.098	0.918	72.8
9α	red/brown solid	0.186	0.847	80.5
10α	dark red solid	0.189	0.847	90.5
11α	brown solid	0.189	0.847	70.2
12α	brown/black solid	0.37	0.833	86.2
13α	brown/black solid	0.315	0.833	85.4
3β	yellow solid	0.415	0.84	89
4β	yellow solid	0.087	0.877	27.9
5β	orange/brown solid	0.212	0.78	82.6
6β	yellow solid	0.277	0.836	88.7
7β	brown solid	0.4	0.836	67.25
8β	dark brown solid	0.15	0.867	87.0
9β	yellow/orange solid	0.148	0.907	86.2
10β	pale brown solid	0.164	0.89	99
11β	yellow/orange solid	0.13	0.83	89.8
12β	pale brown/orange solid	0.35	0.85	99
13β	yellow solid	0.28	0.90	90.4

In general it would appear that putative β compounds were produced at a higher yield than α compounds, although no conclusions can be made of the nature of the products.

3.3.2 Mass spectrometry

Each sample was dissolved in HPLC grade methanol and examined by APCI-MS as described in section 3.2.3. The mass:charge ratio of each peak was compared with the calculated molecular weight of the product and starting materials (Table 3.2). Due to the nature of the mass spectrometry ionisation method used the peaks present will contain one additional hydrogen atom, giving a mass:charge ratio (m/z) one unit higher than the molecular weight of that expected.

Table 3.2 Molecular weight (MW) of products of synthesis compared to data generated by APCI-MS. Only data peaks with above 20% of the abundance of the main peak are noted here.

Product	MW of product	MS peaks(m/z) (peak of product is in bold type)	MW triple ring structure
3α	413	260, **414**	260
4α	343	149, 259	260
5α	357	149, 260, 274, **358**, 438	260
6α	427	114, 149, 187, 260, **428**	260
7α	371	149, 260, 338, **372**	260
8α	329	149, 260, 274, **330**, 338	260
9α	385	113, 149, 167, 260, **386**	260
10α	355	149, 260, **356**, 391, 434	260
11α	397	113, 149, 167, 259, **398**	260
12α	371	259, **372**, 423	260
13α	369	260, **370**, 419	260
3β	349	210, **350**	210
4β	279	196, 210, 294	210
5β	293	196, 210, **294**	210
6β	363	**364**	210
7β	307	196, 210, 249, 338, 364	210
8β	265	196, 210, **266**	210
9β	321	210, **322**	210
10β	291	196, 210, **292**	210
11β	333	196, 210, **334**	210
12β	307	196, 210, **308**	210
13β	305	196, 210, **306**	210

The results indicate that of the samples produced, all but 4α, 4β and 7β contained at least some compound of the predicted m/z for the proposed novel antimicrobial.

3.3.3 Nuclear magnetic resonance

NMR was used to determine purity of each product as described in sections 3.1.5 and 3.2.4.

Table 3.3 Purity of products as determined by NMR ^1H.

Product	Solvent	Estimated Abundance
3α	DMSO	≤ 10%
4α	$CDCL_3$	≤ 10%
5α	DMSO	≤ 10%
6α	DMSO	≤ 10%
7α	DMSO	≤ 10%
8α	DMSO	≤ 10%
9α	DMSO	≤ 10%
10α	DMSO	≤ 10%
11α	DMSO	≤ 10%
12α	DMSO	≤ 10%
13α	DMSO	≤ 10%
3β	DMSO	88%
4β	$CDCL_3$	88%
5β	DMSO	88%
6β	DMSO	50%
7β	DMSO	≤ 10%
8β	DMSO	95%
9β	DMSO	89%
10β	DMSO	30%
11β	DMSO	95%
12β	DMSO	95%+
13β	DMSO	31%

In chemical synthesis such as this, an estimated purity of below 10% is considered unacceptable for further experimentation, whereas purity of 80% and above is considered an acceptable result for a compound to be used in further tests.

The NMR results would seem to indicate that the synthesis of α compounds was unsuccessful, while mass spectrometry detected the presence of the correct size MW product, further NMR examination showed this to be of low abundance.

3.3.4 Disinfectant test EN1276

3.3.4.1 Killing by novel compound alone

Log cycle survival from *P. aeruginosa* challenged with 2 concentrations of the novel compounds was compared to the log cycle survival of untreated cells. The difference gives a log cycle kill. The results for alpha compounds are presented in Table 3.4, beta compounds in Table 3.5. Neither starting ring (SM1 and SM2), nor any side chain used showed bactericidal activity when tested against *P. aeruginosa* at the same concentrations as the synthesised compounds

Table 3.4 Log cycle kills of compounds 3-13α alone against *P. aeruginosa* strain ATCC 15442 using the modified EN1276 protocol.

	Log cycle kill alone (0.1% w/v)			Log cycle kill alone (0.05% w/v)		
Compound	pH 4.5	pH 7.0	pH 10.5	pH 4.5	pH 7.0	pH 10.5
3α		0/2/0	0/2/0		0/2/1	0/1/1
4α		0/2/1	1/0/0		0/1/1	0/0/0
5α		0/1/1	0/0/0		0/1/2	0/0/0
6α		1/1/1	0/1/0		1/1/1	0/1/0
7α		0/0/0	1/0/1		0/1/2	0/0/0
8α		1/1/1	0/1/0		1/1/0	0/0/0
9α		0/0/0	1/1/1		0/0/0	0/0/0
10α		0/1/0	1/1/0		0/0/0	0/0/1
11α		1/0/1	0/1/0		0/0/0	0/0/0
12α		0/0/0	0/0/1		0/0/0	0/0/0
13α		2/0/0	1/0/0		1/0/0	1/0/0

Tests were completed at the two pHs shown before NMR data showed the low level of purity of alpha compounds. Once this was discovered no attempt was made to repeat the experiment at pH 4.5. Data on synergy however could already be extracted from the experiments completed and is included in table 3.11 and 3.12 for completion.

Table 3.5 Log cycle kills of compounds 3-13β alone against *P. aeruginosa* strain ATCC 15442 using the modified EN1276 protocol.

	Log cycle kill alone (0.1% w/v)			Log cycle kill alone (0.05% w/v)		
Compound	pH 4.5	pH 7.0	pH 10.5	pH 4.5	pH 7.0	pH 10.5
3β	0/0/0	0/0/0	1/0/1	0/1/0	0/0/0	0/0/1
4β	1/0/0	1/1/1	0/0/0	0/0/0	1/1/1	0/0/0
5β	0/0/0	0/0/0	0/0/0	0/0/0	0/0/1	1/0/1
6β	1/0/0	1/0/0	0/1/0	1/0/0	0/0/1	0/0/0
7β	0/0/1	0/1/0	1/0/0	0/0/1	0/0/0	1/0/0
8β	1/1/0	1/0/0	1/0/1	1/0/0	1/0/0	0/1/0
9β	1/0/1/0	1/1/0	0/0/0	1/0/1/0	1/2/0	0/0/0
10β	0/1/0	1/2/0	1/0/0	0/0/0	1/2/0	0/0/0
11β	1/0/1/0	0/0/0	0/0/0	1/0/1/0	0/0/0	1/0/0
12β	1/1/0	0/1/0	0/0/0	1/0/0	0/0/1	0/0/0
13β	0/0/0	0/0/0	1/0/0	0/0/1	0/1/0	1/0/0

Since most compounds had little or no bactericidal action against the test organism, the alpha and beta compounds were tested against *Escherichia coli*, which is regarded as less resistant to membrane active agents. The results are shown in table 3.6 and 3.7

Table 3.6. Log cycle kills of compounds 3-13α alone against *E. coli* strain DC0 using the modified EN1276 protocol.

	Log cycle kill alone (0.1% w/v)		Log cycle kill alone (0.05%w/v)	
Compound	pH 7.0	pH 10.5	pH 7.0	pH 10.5
3α	0/1	2/1	0/0	2/1
4α	0/0	0	0/1	0
5α	1/1	1	0/1	0
6α	0/1	0	0/1	0
7α	0/1	0	0/0	1
8α	1/0	0	0/2	0
9α	1/0	0	1/0	0
10α	2/1	0	1/1	0
11α	2/0	0	1/1	0
12α	1/0	0	0/1	0
13α	0/1	0	½	0

Table 3.7 Log cycle kills of compounds 3-13β against *E. coli* strain DC0 using the modified EN1276 protocol.

	Log cycle kill alone (0.1% w/v)		Log cycle kill alone (0.05% w/v)	
Compound	pH 7.0	pH 10.5	pH 7.0	pH 10.5
3β	1/0	2/0	1/0	0/1
4β	0/0	1/0	1/0	0/0
5β	0/0	1/0	1/0	0/0
6β	2/1	5/5	3/0	2/3
7β	0/0	1/0	0/0	0/0
8β	0/0	1/0	0/0	1/1
9β	0/0	0/0	0/0	0/1
10β	0/0/3	0/0	0/0/1	1/1
11β	1/0	0/2	1/1	0/3
12β	0/1	0/1	0/0	0/1
13β	1/1/0	0/0	1/2/1	0/3

A selection of compounds showing at least some action against *P. aeruginosa* and *E. coli* were used at twice and four times the previous highest concentration to see if any further action could be seen. Apart from the increase in concentration the EN1276 was repeated in the same manner as before. Results are shown in table 3.8.

Table 3.8 Log cycle kills of compounds 3β, 6β, 7β and 13β alone against *P. aeruginosa* strain ATCC 15442 using the modified EN1276 protocol.

Compound	Log cycle kill alone (0.4% w/v)		Log cycle kill alone (0.2% w/v)	
	pH 7	pH 10.5	pH 7	pH 10.5
3β	4/2/3/4	1/0/3/1	1/1/1/0	0/0/1/0
6β	1/1/1/0/1	2/1/3/2	0/1/1/1/0	3/3/0/2
7β	0/0/1/0	2/1/1/2	0/0/0/1	1/1/0/0
13β	0/0/2/0	1/1/2/1	0/0/0/1	0/0/0/1

Of those compounds examined for antibacterial action alone rather than synergy only a few showed any significant action when compared to those of commercially obtained compounds as shown in table 2.7. Compound 3β showed the highest levels of action primarily at pH 7, while compound 6β, 7β and 13β showed an increase in action at pH10.5.

3.3.4.2 Antagonistic action of compounds

It became evident after analysing the results that while some synthesised compounds had bactericidal activity against *P. aeruginosa* it was also true that these compounds inhibited the action of BKC when used in combination. Therefore the data were reanalysed to measure levels of BKC antagonism. Results are shown in tables 3.9-3.14.

Table 3.9 Log cycle of antagonism of the action of BKC (0.025% w/v) by β compounds at min (0.05%w/v) and max (0.1%w/v) concentrations.

Compound	Antagonism at pH 4.5		Antagonism at pH 7		Antagonism at pH 10.5	
	Max conc	Min conc	Max Conc	Min Conc	Max conc	Min Conc
3β	0,2,0	2,2,0	3,3,0	4,2,1	2,1	2,0
4β	3,3,2	3,3,2	0,5,2	2,0,2	4	2
5β	1,2,2,	1,1,2	5,5,2	4,1,3	2	2
6β	3,1,3,	2,1,2	3,3,1	3,2,1	3	2
7β	3,2,3	3,0,3	3,3	3,3	3,4	1,2
8β	2,2,3,2	1,**1s**,2,2	3,3,2	2,3,4	1,4	0,2
9β	2,3,2,3	2,3,3,2	4,3,1	2,3,0	3,5	0,0
10β	1,0,3,2	1,**1s**,3,2	3,4,2	3,4,0	2,4	2,1
11β	2,3,**1s**,3	2,3,**1s**,3	3,2,2,2	3,2,2,3	3,4,2	0,1,0
12β	3,3,3	2,2,2	4,1,3,3	5,1,2,3	3,4,2	0,2,1
13β	1,2,2,	1,3,1	5,4,3,2	4,4,3,4	3,4,2,3	1,2,0,1
Triple ring SM2	0,0,	0,0	4,3	2,2	0,0	0,0

Numbers relate to the reduction in log cycle kill (antagonism) of BKC by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

Table 3.10 Log cycle of antagonism of the action of BKC (0.0125% w/v) by β compounds at min (0.05%w/v) and max (0.1%w/v) concentrations.

Compound	Antagonism at pH 4.5		Antagonism at pH 7		Antagonism at pH 10.5	
	Max conc	Min conc	Max Conc	Min Conc	Max conc	Min Conc
3β	0,2,1	0,2,2	3,3,0	3,3,2	1,5,2	1,1,1
4β	1,0,1	1,0,1	1,3,1	1,2,1	2,3	2,3
5β	1,0,1	1,0,1	4,3,1	4,2,**1s**	1,3	0,3
6β	1,0,1	0,**1s**,1	1,3,0	2,0,0	1	0
7β	0,1,0	0,1,0	3,3	3,3	3,3	1,2
8β	2,1,0,0	2,0,0,0	2,2,2	2,2,3	1,3	1,1
9β	2,1,1,2	1,1,0,2	2,3,3	2,4,1	1,3	1,1
10β	1,0,0	2,0,0**1s**	2,1,2	2,**2s** ,0	1,2	1,1
11β	1,1,0,0	2,1,0,0	2,1,2,0	2,1,2,0	1,0,1	0,1,0
12β	1,3,0	1,3,0	2,2,2,1	2,1,2,1	1,2,2	0,2,2
13β	1,1,1	1,1,1,**1s**	2,1,3,3	2,1,2,0	0,3,1,2	0,2,0,1
Triple ring SM2	0,0	0,0,	2,3	1,2	**1s**,0	1,**1s**

Numbers relate to the reduction in log cycle kill (antagonism) of BKC by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

Table 3.11 Log cycle of antagonism of the action of BKC (0.025% w/v) by α compounds at min (0.05%w/v) and max (0.1%w/v) concentrations.

Compound	Antagonism at pH 4.5		Antagonism at pH 7		Antagonism at pH 10.5	
	Max conc	Min conc	Max Conc	Min Conc	Max conc	Min Conc
3α			2,0	0,0	3,2,5	**1s**,1,3
4α			2,0	2,0	4,2,3	0,1,2
5α			2,0	2,0	2,3,2	3,2,1
6α			2,0	2,0	2,3,1	1,4,1
7α			1,0	1,1	2,2,2	1,4,1
8α			1,1	1,0	2,3,4	1,5,4
9α			1,1	2,1	1,4,2	1,3,1
10α			0,2	1,2	3,5	3,5
11α			1,1	2,1	2,3,2	1,4,1
12α			3,2	3,2	3,4,3	1,3,0
13α			2,1	3,1	3,2,3	2,1,2
Triple ring SM1			0	0	0	0

Numbers relate to the reduction in log cycle kill (antagonism) of BKC by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

Table 3.12 Log cycle of antagonism of the action of BKC (0.0125% w/v) by α compounds at min (0.05%w/v) and max (0.1%w/v) concentrations.

Compound	Antagonism at pH 4.5		Antagonism at pH 7		Antagonism at pH 10.5	
	Max conc	Min conc	Max Conc	Min Conc	Max conc	Min Conc
3α			**1s**,2	**1s**,2	1,0	1,1
4α			0,0	0,**1s**	0,2	**1s**,1
5α			0,0	0,0	1,0	1,0
6α			0,1	0,**1s**	0,1	0,0
7α			**1s**,1	**1s**,1	1,**1s**	2,**1s**
8α			0,1	0,2	2,1	2,1
9α			1,0	2,0	2,0	2,0
10α			1,1	1,1	2,2	2,1
11α			0,1	0,1	1,1	0,0
12α			2,1	1,1	1,0	1,0
13α			0,1	1,0	1,**1s**	1,**1s**
Triple ring SM1			0	0	0	0

Numbers relate to the reduction in log cycle kill (antagonism) of BKC by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

Table 3.13 Log cycle of antagonism of the action of BKC (0.025% w/v) by four β compounds at enhanced min (0.2%w/v) and max (0.4%w/v) concentration

Compound	Antagonism at pH 7		Antagonism at pH 10.5	
	Max conc	Min conc	Max conc	Min conc
3β	**2s,1s,2s**	2,3,3	3,3,3,3	2,3,2,2
6β	3,1,1,3	1,0,1,1	2,0,2,1	2,0,2,1
7β	3,2,3	2,2,2	3,1,4,3	0,0,2,0
13β	6,2	3,2	3,3,3	1,2,1

Numbers relate to the reduction in log cycle kill (antagonism) of BKC by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

Table 3.14 Log cycle of antagonism of the action of BKC (0.0125% w/v) by four beta compounds at enhanced min(0.2%w/v) and max (0.4%w/v) concentration.

Compound	Antagonism at pH 7		Antagonism at pH 10.5	
	Max Conc	Min Conc	Max conc	Min Conc
3β	**2s,1s,2s**	0,1,0	5,1,1,4	3,1,1,3
6β	0,0,0,0	2,**1s**,0,0	**2s**,3,0,**2s**	1,3,0,1
7β	2,3,2	2,3,2	1,2,3,1	1,1,3,1
13β	5,2	4,2	3,1,3	2,1,2

Numbers relate to the reduction in log cycle kill (antagonism) of BKC by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

3.3.4.3 Action of β compounds in conjunction with the disinfectant orthophenylphenol (OPP)

BKC was replaced in the disinfectant test by the antimicrobial compound OPP commonly used in disinfectant solutions. β-compounds were tested for synergy with this compound. Results are shown in table 3.15

Table 3.15 Log cycle antagonism of the action of OPP (0.0125% w/v and 0.025% w/v) by β-compounds at min (0.05%w/v) and max (0.1%w/v) concentration at pH 7

Compound	0.025% OOP 0.1% compound	0.0125% OOP 0.1% compound	0.025% OOP 0.05% compound	0.0125% OOP 0.05% compound
3β	6/4/0	1/0/0	6/4/0	1/0/0
4β	4/2/3	0/1/0	4/2/3	0/0/**1s**
5β	5/3/3	0/1/0	5/4/3	1/1/**1s**
6β	4/3/**3s**	**1s**/0/0	2/3/**3s**	**1s**/0/0
7β	4/4/2	0/1/1	4/3/2	0/0/1
8β	3/0/0	0/0/0	3/2/0	0/0/0
9β	3/4/4	0/0/1	3/4/3	0/0/1
10β	4/3/5	0/**1s**/0	3/2/5	0/**1s**/**1s**
11β	3/3/3	0/1/0	3/3/3	1/**1s**/0
12β	3/3/5	0/**1s**/1	3/4/6	1/**1s**/1
13β	3/0/2	0/1/1	0/3/1	1/1/0

Numbers relate to the reduction in log cycle kill (antagonism) of OPP by the compound, numbers in bold followed by **s** indicate the synergistic increase in log cycle kill of BKC as a result of formulation with each compound.

3.3.5 Separation of compound 48/80

All fractions produced by gel permeation chromatography were examined by spectrophotometry to determine which contained any of the compound 48/80. Only fractions 7-13 contained detectable material as shown in figure 3.6.

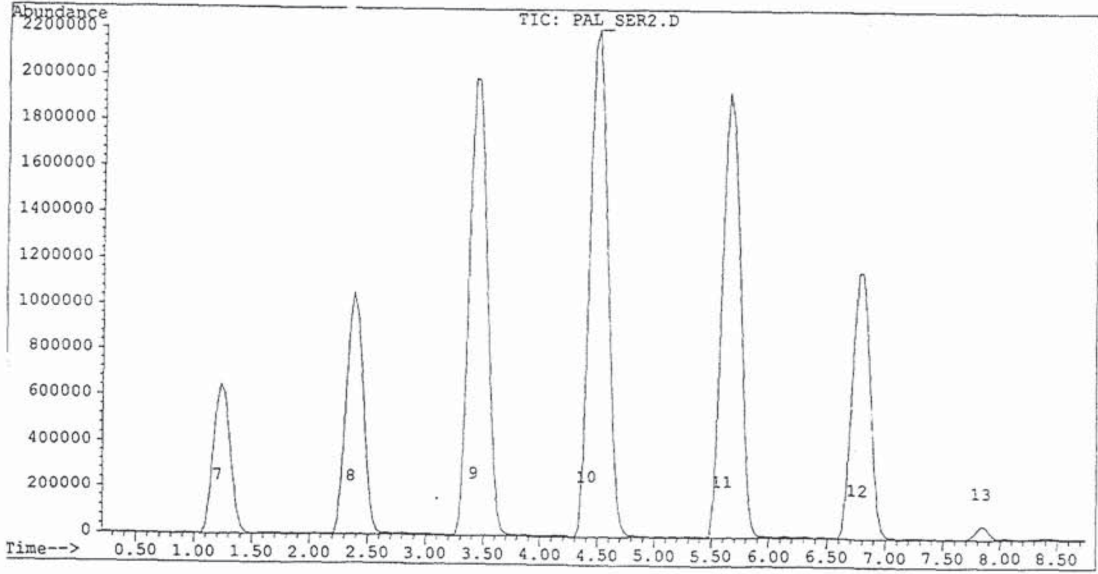


Figure 3.6 Mass spectrometry data showing abundance of material in fractions 7-13.

Fractions 7-13 were examined further by mass spectrometry to determine the molecular weight of the material present and from this data determine the size of each oligomer of 48/80.

Table 3.16 Presence of each oligomer of compound 48/80 found in fractions 7-13. Y refers to presence of oligomer detectable my mass spectrometry, N refers to absence of oligomer.

	Presence (Y/N) of each oligomer of compound 48/80 in each fraction						
Oligomer of compound 48/80	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12	Fraction 13
(1-mer)	Y	N	N	N	N	N	N
(2-mer)	Y	N	N	N	N	N	N
(3-mer)	Y	Y	N	Y	Y	Y	Y
(4-mer	Y	Y	Y	Y	Y	Y	Y
(5-mer)	Y	Y	Y	Y	Y	Y	Y
(6-mer)	Y	Y	Y	Y	Y	Y	Y
(7-mer)	Y	Y	Y	Y	Y	Y	Y
(8-mer)	Y	Y	N	Y	Y	N	N
(9-mer)	Y	N	N	N	N	N	N

No fraction contained a sole oligomeric unit, the nearest was fraction 9 which contained molecules of 4 different oligomeric sizes. No further antimicrobial testing of fractions was attempted.

3.3.6 Synthesis of novel peptides

3.3.6.1 Predicted structures of novel peptides

The two peptides investigated, one with 20 amino acids and one with 28 amino acids, were based upon the lentivirus peptide LLP1.

↓ ↓ ↓ ↓↓ ↓ ↓

LLP1 NH_2 -RVIEVVQGACRAIRHIPRRIRQGLERIL-COOH

Peptide 28 NH_2 -KVIEVVQGACKAIKHIPKKIKQGLEKIL-COOH

Peptide 20 NH_2- ACKAIKHIPKKIKQGLEKIL-COOH

Figure 3.7 Amino acid sequences of novel peptides and viral peptide from which they were derived. Arrows indicate sites where Arginine(R) was replaced by Lysine (L).

To determine how the alterations made in the peptides would affect the alpha helical structures of the peptides, helical wheel diagrams were generated (figures 3.8, 3.9 and 3.10).

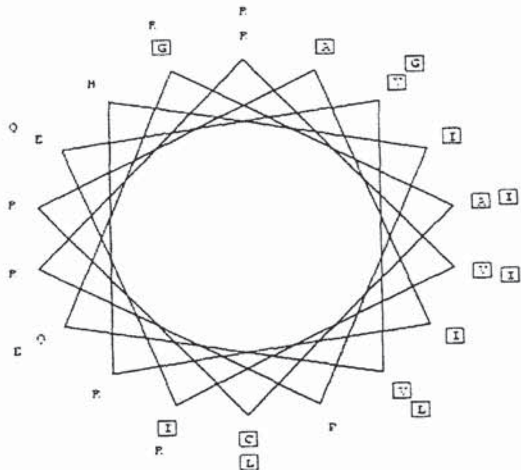


Figure 3.8 Helical wheel of viral peptide LLP1. Amino acids with a hydrophobic nature are boxed.

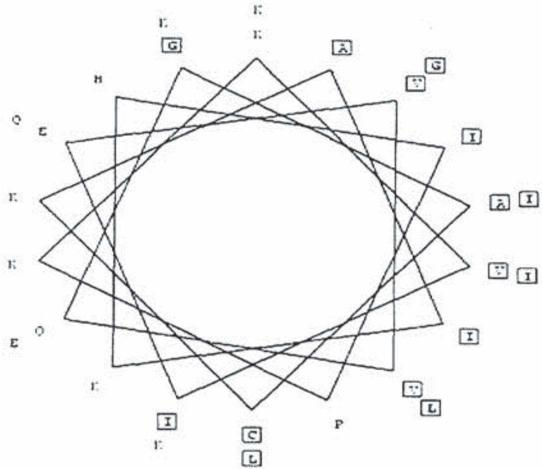


Figure 3.9 Helical wheel of Peptide 28. Amino acids with a hydrophobic nature are boxed.

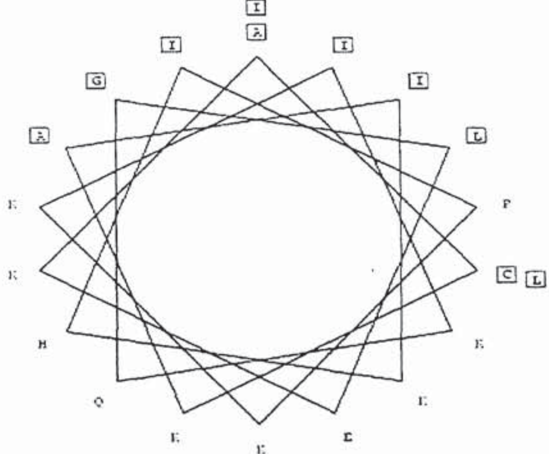


Figure 3.10 Helical wheel of Peptide 20. Amino acids with a hydrophobic nature are boxed.

The viral peptide LLP1, peptide 20 and peptide 28 all conform to an alpha helical structure with a hydrophobic, and a hydrophilic "face".

The Kyte-Doolittle and Hopp-Woods values for each amino acid in peptide 20, peptide 28 and LLP1 were determined and graphically presented in figure 3.11 and 3.12.

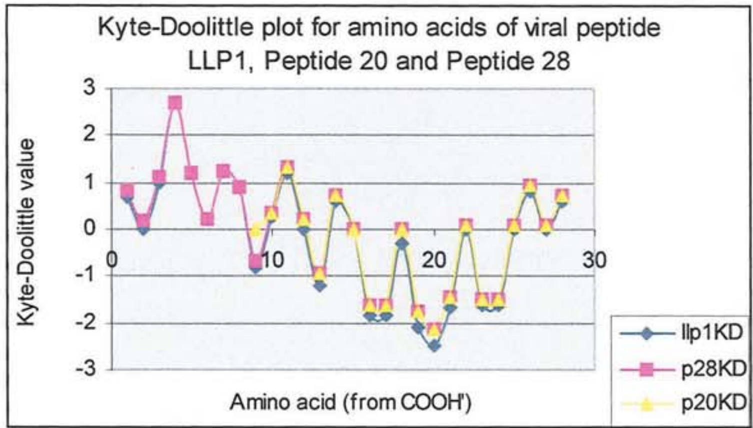


Figure 3.11 Kyte-Doolittle values for LLP1, Peptide 20 and Peptide 28. Positive values indicate hydrophobic areas of the peptide, negative values indicate hydrophilic amino acids.

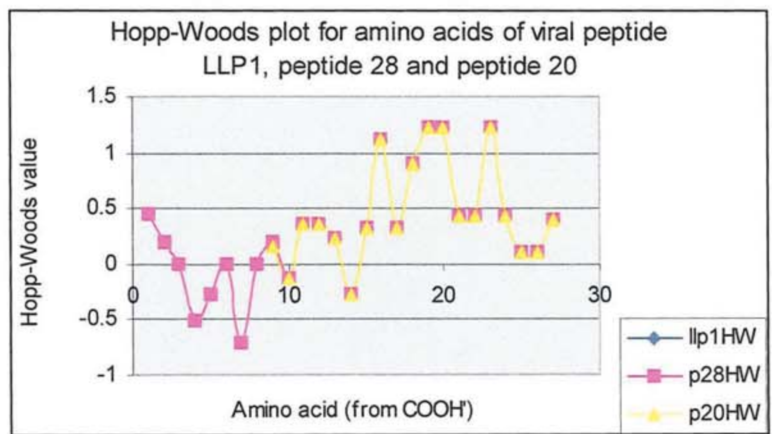


Figure 3.12 Hopp-Woods values for LLP1, Peptide 20 and Peptide 28. Positive values indicate polar (hydrophilic) amino acids, negative values indicate apolar (hydrophobic) amino acids.

Chou-Fasman data for each amino acid was used to predict the secondary structure for each peptide as shown in table 3.17.

Table 3.17 Secondary structure predictions (Chou-Fasman) for viral peptide LLP1 and novel peptides 20 and 28.

Amino acid sequence	Alpha helix	Beta sheet	Turn	Amino acid sequence	Alpha helix	Beta sheet	Amino acid sequence	Alpha helix	Beta sheet	Turn
Peptide 28				Peptide 20			LLP1			
K	-						R		+	
V		+					V		+	
I		+					I		+	
E		+					E		+	
V		+					V		+	
V		+					V		+	
Q		+					Q		+	
G		+	+				G		+	+
A		+	+	A			A		+	+
C		+	+	C			C		+	+
K			+	K			R			+
A				A			A			
I				I			I			
K				K			R			
H				H			H			
I	+		+	I	+		I			
P	+		+	P	+		P			
K	+		+	K	+		R		+	
K	+		+	K	+		R		+	
I	+			I	+		I		+	
K	+			K	+		R		+	
Q	+			Q	+		Q		+	
G	+			G	+		G		+	
L	+			L	+		L		+	
E	+			E	+		E	+		
K	+			K	+		R	+		
I	+			I	+		I	+		
L	+			L	+		L	+		

While both novel peptides are predicted to contain a large degree of alpha helical secondary structure peptide 28 also is predicted to contain a degree of beta pleated sheet structure. The replacement of Arginine with Lysine has increased the length of peptide that is of an alpha helical structure.

3.3.6.2 Antimicrobial action of novel peptides

The antibacterial action of the novel peptides derived from LLP1 was determined by the BKC enhancement test detailed in section 2.2.2.6

Table 3.18 Antimicrobial action and synergy with BKC of novel peptides against *P. aeruginosa* ATCC 15442

Compound	Max log cycle kill (lck) alone and concentration	Synergy with BKC	Maximum concentration used or at which synergy was maximum.
Peptide 20	0 at 50μg/ml	1.67 log cycles	50μg/ml
Peptide 28	0 at 50μg/ml	0	50μg/ml

Neither of the peptides had antibacterial action alone against *P. aeruginosa* ATCC 15442, but peptide 20 showed synergy with BKC.

3.3.6.3 Permeabilising action of novel peptides upon the outer membrane of P. aeruginosa.

Each peptide was examined to determine if it acted as a permeabiliser of the outer membrane of *P. aeruginosa* ATCC 15442 by the measurement of NPN incorporation detailed in chapter 2.

Table 3.19 Action of novel peptides on enhancing incorporation of NPN into the outer membrane of *P. aeruginosa* ATCC 15442.

Compound	Net fluorescence increase over untreated cells	Concentration at which flourescence increase is maximum or maximun concentration used
Peptide 20	2.0	50μg/ml
Peptide 28	1.42	50μg/ml

Both peptides exhibited permeabilising activity of the outer membrane of this strain of *P. aeruginosa* although peptide 20 had a higher activity than peptide 28.

3.4 Discussion

There are three groups of agents examined in this chapter and so this section is divided accordingly.

3.4.1 Compound 48/80

The action of compound 48/80 as an antibacterial agent with potent outer membrane permeabilising properties is well known (Katsu *et al.*, 1984; Varra, 1992) and has been shown in chapter 2 of this work. The activity of each of the oligomeric portions that make up its whole has been less well documented although synthesised dimers and trimers of the compound are seen to be active against *E. coli* (Katsu *et al.*, 1985).

With the sepharose column used, it was impossible to separate compound 48/80 into fractions with sufficiently high proportions of each of the component oligomers, to test the antibacterial action of said oligomers. Separation of the compound has been achieved in earlier work (Lenney *et al.*, 1977) but attempts to separate the histamine releasing and antibacterial properties of the oligomers met with mixed results and no accurate determination of oligomeric size or purity of the fractions produced was ever achieved.

3.4.2 Peptides based upon LLP1

Both peptides synthesised have similar Kyte-Doolitle and Hopp-Woods plots which confirmed by the helical wheel data suggests that the peptides share the "hydrophobic face and hydrophilic face" motif common to many antibacterial peptides. It is believed that the peptides form hydrophilic channels in the outer

membrane of Gram-negative bacteria, the hydrophobic amino acids interacting with the fatty acids of the membrane, the hydrophillic amino acids forming the channel. Certainly the permeabilising action of both peptides would seem to support this. Peptide 28 had no synergising properties with BKC at the concentrations tested, while peptide 20 did. The difference in molecular weights of the two peptides means that when both were tested at 50µg/ml peptide 28 was at a concentration of 16.6µM while peptide 20 was at a concentration of 19.2µM. However, peptide 20 had synergistic action with BKC at concentrations of 9.6µM and above, a concentration where peptide 28 showed no activity. As is shown in the results, apart from the number of amino acids the structures of the peptides are, as expected, very similar. It is possible that the difference in activity between the two is linked to the higher proportion of lysine amino acids in peptide 20 (30% of amino acids) than peptide 28 (25% of amino acids) or to the presence in peptide 28 of a region of beta-sheet conformation absent from peptide 20.

3.4.3 Novel acridine agents.

The synthesis of alpha compounds (those based upon 6,9 dichloro-2-ethoxyacridine as a tricyclic ring base) failed to produce any product of above 10% purity. Of those beta compounds synthesised only 7β showed the low level of purity associated with alpha compounds, although 6β and 13β showed less than the 80% purity generally accepted as a minimum for use in work such as this.

The antibacterial action of the compounds alone against *P. aeruginosa* ATCC 15442 was low, no more than 2 log cycles at either concentration or at any pH. In addition

there was little difference between the action of the "pure" β-compounds and the α- and β-compounds with purities of less that 10%. When tested at higher concentrations than advised by Unilever it was possible to observe log cycle kills of between 2 and 4 for compound 3β at a concentration of 0.4% w/v when used at pH 7. This action was reduced at a higher pH, unlike the action of 6β, 7β and 13β, all of which had increased action at pH 10.5. When compounds were tested against *E. coli* strain DC0 there was an increase in the log cycle kills for many of the β-compounds but not for the α-compounds. This was not unexpected, as *E. coli* is regarded to be more susceptible to antimicrobial agents than *P. aeruginosa*.

Synergy with BKC was rare, more often both α- and β-compounds would inhibit the action of BKC. This antagonistic action seemed more common when BKC was used at a higher concentration (0.025% w/v) than at the lower concentration (0.0125% w/v). The only compound that showed a consistent degree of synergy was 3β. When used at 0.4% w/v at pH7 this compound showed synergy with both 0.025% w/v and 0.0125% w/v BKC with no incidence of antagonistic action. Compound 6β also showed synergy at 0.4% w/v and 0.2% w/v, but antagonism with BKC was also recorded.

When BKC was replaced by a phenolic agent (orthophenylphenol) and tested with β-compounds synergy without antagonism was observed between both concentrations of compound 6β and 10β and the 0.0125% w/v phenolic agent.

Certainly 3β and 6β have similar structures, both having a pair of 4 carbon chains leading off from the nitrogen atom in the side chain, a nitrogen atom attached to the tricyclic base by a 2 or 3 carbon chain respectively.

As mentioned in the previous chapter work in determining the structure activity relationships in non-antibiotics has been achieved. Since it has been stated that the presence of a halogen side group upon such molecules as synthesised here promotes antibacterial activity it is unfortunate that none of the alpha compounds were of sufficient purity to be tested against their halogen free beta counterparts.

One reason for the apparent variability in the results from the EN1276 test for not only the "pure" compounds in this chapter but those purchased compounds in the preceding chapter is the test organism. A recent study has shown that of the four test organisms used in the EN1276 disinfectant test, *P. aeruginosa* ATCC 15442 showed the greatest variability between tests, often as much as 2 log cycles difference (Payne *et al.*, 1999). However, the test concluded that the strain's high degree of resistance to disinfectants made it an appropriate test organism.

3.5 Conclusions

It was impossible to separate compound 48/80 in separate chain length oligomers to examine their separate antimicrobial activity. The peptides synthesised based upon viral peptides show a degree of activity, more so when truncated in length. The novel acridine based molecules show a range of activity alone and when combined with BKC. This activity ranges from synergy and up to 4 log cycle kills to antagonism and no antibacterial action. However a number of compounds do show reproducible levels of kill alone and synergy with BKC.

<u>Chapter 4: Generation of resistance to BKC by serial passage of *P. aeruginosa* cells</u>

4.1 Introduction

4.1.1 Aims

Workers have, by growing *Pseudomonas* species in increasing concentrations of disinfectants, selected resistant organisms (Jones *et al.*, 1989; Russell *et al.*, 1998; Méchin *et al.*, 1999). However, few comparisons have been made between laboratory-passaged strains and those isolated from the environment. This work examines whether both environmental and laboratory strains of *P. aeruginosa* can be adapted to resist the action of BKC. In addition, any co-resistance of such adapted strains to a range of 11 other disinfectants and antibiotics was determined and the effect upon the organisms' survival in the EN1276 disinfectant test was investigated.

4.12 Serial passage

Disinfectant and antibiotic resistant strains of *P. aeruginosa* have been recognised for many years (Adair *et al.*, 1969; Hoffman *et al.*, 1973; Joynson *et al.*, 1999). The examination of such strains requires a means of selecting for resistant cells within a population. There are two main methods used to achieve this. A population of cells can be challenged with a MIC or higher concentration of an antimicrobial and resistant cells isolated by plating onto agar containing the same antimicrobial. Alternatively populations of cells can be grown in increasing concentrations of an antimicrobial to select for cells with increased resistance to the agent. This method is commonly known as serial passage.

4.13 Determination of culture purity

It is essential when studying disinfectant resistant strains derived by serial passage to determine that contamination has not occurred during the selection process. Such contamination can occur either from environmental sources or from the other strain cultures. When examining each strain for alterations in phenotype due to adaptation it is vital to ensure that changes are not the result of such contamination. Contamination by other bacterial species such as skin carried staphylococci or micrococci are readily identifiable by examining the colonial morphology of suspect cells on an agar plate. However, when dealing with different strains of the same organism, other means are required.

4.13.1 Serotype

As described in the introduction, the LPS of *P. aeruginosa* consists, in part, of a polysaccharide region made up of different sugar monomers which results in two forms of LPS (Rivera *et al.*, 1988). One, known as A band polysaccharide, is shorter in length and in strain PAO1 is made up predominantly of uncharged sugars such as rhamnose (Rivera & McGroarty, 1989; Yokoto *et al.*, 1987). The second, known as B band or O antigen polysaccharide, is much longer and made up of many different sugars. It is largely anionic in nature and is the primary antigen for the organism, although attached to only 10% of LPS molecules (Wilkinson, 1975; Wilkinson, 1983). The heterogeneous nature of the sugars in O antigen polysaccharide, coupled with its antigenicity, allow it to be the basis for separation of *P. aeruginosa* into subclasses known as serotypes. There are currently 20 O serotypes (Habs nomenclature) of *P. aeruginosa*, the structures of which have each been chemically determined (Wilkinson, 1983; Knirel *et al.*, 1988). The O serotype is still the primary

means of classifying a strain of *P. aeruginosa* and has been used to track the origins of multi-resistance strains in the past (Pascale *et al.*, 1994; Patzer & Dzierzanowska, 1994).

4.1.3.2 Pulsed field gel electrophoresis and other molecular methods

In the past two decades new techniques have been used to both identify *P. aeruginosa* strains and to discover how related strains are to each other. This search for techniques appears to have been stimulated by the limitation of serotyping when examining strains present in cystic fibrosis patients. Most strains present in such patients are of the "rough colony" type, and as such have no LPS chain polysaccharides of either A or B band. Therefore strains from patients cannot always be separated into serotypes.

At present there are two main methods currently used for both determination of relatedness of isolates and perhaps more importantly in a clinical sense, the determination of sources of hospital acquired infections. The first is the randomly amplified polymorphic DNA (RAPD) fingerprinting method (Kersulyte *et al.*, 1995; Renders *et al.*, 1996). This involves sample genomic DNA being used as a template for a polymerase chain reaction (PCR) based upon primers of arbitrarily chosen sequence. The DNA template need not be of a high molecular weight, of high purity, or of a double stranded nature. In addition only a few nanograms of DNA are required and, while one or two primers are generally sufficient for distinguishing unrelated strains, if more sensitivity is required then more primers can be used, thus utilising a larger portion of the genomic template.

A second technique that has been compared favourably with RAPD is that of pulsed field gel electrophoresis (PFGE) (Kersulyte *et al.*, 1995; Renders *et al.*, 1996). In this procedure the genome of an organism is cut with a selected restriction enzyme and the pattern of DNA fragments compared with that produced by the same enzyme treatment upon another isolate. This method utilises restriction enzymes that have cutting sites that occur infrequently in the genome of the organism to be investigated, so that the number of fragments produced can be visualised relatively easily. The choice of enzyme is usually based upon %GC content of the genome, as well as previous work published. With *P. aeruginosa* the enzymes that have been used include *Dra*I (5'-TTTAAA), *Spe*I (5'-ACTAGT), *Xba*I (5'-TCTAGA), *Asn*I (5'-ATTAAT), and *Ssp*I (5'-AATATT). Research has been completed into the frequency at which enzymes cut when compared to the theoretical frequency based upon %GC content and codon usage. This led to the discovery that enzymes such as *Dra*I and *Asn*I cut more frequently than would be expected from data acquired from codon usage in the organism, suggesting that the enzyme preferentially cuts at sites outside of open reading frames. In contrast an enzyme such as *Ssp*I has a recognition site that appears to be randomnly distributed throughout the genome and shows no preference for cutting within or without an open reading frame (Grothues & Tümmler, 1991). Often an isolate is digested separately by two different enzymes to produce two profiles that can be used to improve the discrimination of the method. Conventional agarose electrophoresis can separate fragments of DNA of up to approximately 50kbp in size, but to separate the larger fragments generated by infrequent cutting enzymes pulsed-field electrophoresis is required. In this process the fragments are resolved into a discrete band pattern in the gel by apparatus that switches the direction of current according to a predetermined pattern (Tenover *et al.*, 1995).

The work of Tenover *et al.* (1995) provides a set of criteria by which isolates can be compared upon the similarity of their band profile. These criteria are most appropriate if PFGE resolves at least 10 distinct fragments since the discrimination drops if fewer fragments are compared. Isolates presenting identical profiles can be defined as "genetically related" and the epidemiological interpretation is that they represent the same strain and are unlikely to be distinguishable by other typing techniques. Isolates are determined to be "closely related" if the banding profile differs in such a manner that can be attributed to a single genetic event such as a point mutation, insertion, deletion or chromosomal inversion. That is, if the bands differ by 2-3 bands by the insertion of a new restriction site, one larger band disappears to be replaced by two smaller bands. Isolates separated by two independent genetic events are termed "possibly related" and are recognised by a band difference of 4-6 fragments. Isolates separated by 3 or more separate genetic events will have a band difference of 7 or more and are considered unrelated. This technique has been used to map the chromosomes of *Pseudomonas* species, (Ratnaningsih *et al.*, 1990; Schmidt *et al.*, 1996) but is primarily used to examine the epidemiological spread of disease, where isolates from separate cases can be compared and genetic relatedness determined.

PFGE has been successful, in conjunction with RAPD, in discriminating between isolates of multi-drug resistant *P. aeruginosa* serotype O:12, which were indistinguishable by other methods (Bingen *et al.*, 1996). However, on other occasions it has been unsuccessful in discriminating between clinically related and epidemiologically unrelated isolates of these multi-resistant strains (Misfud *et al.*, 1997). In addition, the technique has been used on several occasions when

determining relatedness of isolates from cystic fibrosis patients in the cases of both *P. aeruginosa* (Grothues *et al.*, 1988; Struelens *et al.*, 1993) and *Burkholderia cepacia* (Anderson *et al.*, 1991; Livesley *et al.*, 1998) and also in evaluating commercial serotyping tests (Rautlein & Hänninen, 1999). In the present work however the technique was used to check the culture purity of strains of *P. aeruginosa* that had been passaged in disinfectant rich media for up to 6 weeks.

4.14 Stability of BKC resistance

The process of growing bacteria in increasing concentrations of an antimicrobial has been shown to result in bacteria more resistant to the agent (Jones *et al.*, 1989; Russell *et al.*, 1998; Méchin *et al.*, 1999). The mechanism behind this resistance could be one of two possibilities. The presence of such a selection pressure can select for those organisms in a population that, by spontaneous mutation, have a higher resistance to the antimicrobial and gradually these organisms will become the dominant phenotype by a process of natural selection. The second possibility is that the organisms already possess the means for resistance but the mechanism would place them at a disadvantage in an environment without the antimicrobial. For example, the overproduction of a membrane stabilising protein that protects against the antimicrobial, but is results in significant energy expenditure for the organism, and is only "switched on" in the presence of the antimicrobial. In order to determine which of the two mechanisms is responsible for changes in resistance, cells can be grown in antimicrobial free media to determine if the absence of selective pressure removes the resistance phenotype form the population of cells.

4.15 Cross-resistance to disinfectants and antibiotics

Resistance to one antimicrobial can often confer resistance to others, either structurally related (for example β-lactamases confer resistance to a range of β-lactam antibiotics), or not linked by structure at all (for example the multi-drug resistance pumps of *P. aeruginosa* are thought to efflux a range of unrelated compounds not all of which are antimicrobials). Therefore, by determining whether the organisms resistant to BKC also share a resistance to other agents it is possible to determine what type of resistance mechanisms may be involved.

4.2 Materials and Methods

4.2.1 Strains of P. aeruginosa

Sixteen *P. aeruginosa* strains were used in the generation of BKC resistant strains. These strains and their origins are listed in table 4.1

Table 4.1 Strains of *P. aeruginosa* used in the production of BKC resistant organisms and details of their history and pure culture serotype.

Name	Habs Serotype	Supplied by	Isolated where and when
CL 7	1	M. Noy	Selly Oak Hospital. 06/84
CL 8	3	M. Noy	Selly Oak Hospital. 06/84
CL 9	4	M. Noy	Selly Oak Hospital. 06/84
CL 10	7	M. Noy	Selly Oak Hospital. 06/84
CL 11	8	M. Noy	Selly Oak Hospital. 06/84
CL 12	9	M. Noy	Selly Oak Hospital. 06/84
CL 13	13	M. Noy	Selly Oak Hospital. 06/84
CL 14	16	M. Noy	Selly Oak Hospital. 06/84
CL 16	10	M. Noy	Selly Oak Hospital. 06/84
17TS	17	T. Pitt	Colindale 06/84
0014	12	T. Pitt	Colindale 06/84
0072	5α	T. Pitt	Colindale 06/84
9766	15	T. Pitt	Colindale 06/84
9481	14	T. Pitt	Colindale 06/84
ATCC 15442	1	M. Jones	Beloian, animal room water bottle
PAO1 ATCC 15692	Poly-agglutinable	ATCC	Holloway, Infected wound

Organisms were maintained as frozen cultures at –70°C for up to 36-months and on agar plates for no more than 1-month. Unless otherwise stated, all overnight cultures were passaged from a plate culture.

4.2.2 Minimum inhibitory concentration (MIC) determination

The MICs of all antimicrobial agents used were determined by the broth dilution method using twofold dilutions of each agent. Approximately 1 x 10^6 bacteria in stationary culture were inoculated into test tubes of nutrient broth containing various concentrations of each agent. The bacteria were grown as static broth cultures for 16-18 hours at 37°C. The MIC was determined as the lowest concentration of agent that inhibited visual growth of the organism in broth.

4.2.3 Minimum bactericidal concentration (MBC) determination

The MBC of antimicrobial agents was determined by plating a loopful of culture from those tubes containing no visual growth onto plates of nutrient agar. These were incubated at 37°C for 18-hours. The MBC was determined as the lowest concentration of agent that prevented growth on the agar plates.

4.2.4 Adaptation of strains to BKC

Strains were inoculated to static cultures containing 0.00078% (w/v) BKC, a concentration that was 50% lower than that of the lowest MIC of the strains. The cultures were grown for 16-hours at 37°C in a static environment after which 100μl of each culture was transferred to a fresh tube of nutrient broth containing the same concentration of BKC. This was repeated every day for 5-days. On the sixth day, the MIC of each strain was determined as described in section 4.2.2. If the strain did not produce an MIC of at least double that of the solution it was grown in, it was discarded. Of those remaining strains, a sample was taken from each and frozen at –70°C in nutrient broth containing the same concentration of BKC and 10% v/v glycerol. Using bacteria from each strain that grew at double the original concentration of BKC (0.00078% w/v), a new set of 5 passages was begun at 0.00156% (w/v) BKC.

This process was repeated, producing strains passaged at BKC concentrations of 0.00078%, 0.0015%, 0.003%, 0.006%, 0.0125% and 0.025% w/v. These passages, and bacteria adapted by them, are referred to as P1, P2, P3, P4, P5 and P6 respectively. The original cell cultures are referred to as WT (Wild Type).

4.2.5 Determination of culture purity

4.2.5.1 Serotype

The Habs serotype of each passage of each bacterial strain was determined by slide agglutination using both polyvalent and monovalent sera (Sanofi). The results were compared with those of the non-adapted organisms to ascertain if contamination had occurred during the passaging of the strains.

4.2.5.2 Pulsed field gel electrophoresis

4.2.5.2.1 Preparation of agarose blocks for pulsed field gel electrophoresis

Cells from an overnight culture were centrifuged at 13,500 rpm for 10-minutes in an Eppendorf microcentrifuge, and the pellet resuspended to a minimum concentration of 20mg/ml wet weight in 1ml NET-100 (0.1M NaCl, 0.1M EDTA pH 8.0, 0.01M Tris-HCl pH 8.0). The cells were vortexed until they were an even suspension, then recentrifuged at 13,500 rpm for 10-minutes and again resuspended in 1ml Net-100 to 20mg/ml wet weight. A stock solution of 0.9% w/v chromosomal grade agarose in NET-100 was mixed, autoclaved for 5-minutes at 15psi and kept molten at 60°C. The bacterial suspension was kept warmed at 60°C and 0.5ml samples mixed with 0.5ml of molten agarose. The agarose and cell mixture was briefly vortexed and transferred into a 9mm Perspex block mould (Biorad) maintained at 4°C on ice. The mould was left on ice for at least 20-minutes to allow the agarose and cell mixture to solidify fully. Solid agarose blocks were carefully removed from the mould and transferred to 5ml plastic bijou bottles containing 3ml lysis solution (6mM Tris-HCl pH 7.6, 1M NaCl, 0.1M EDTA pH 8.0, 0.5% w/v Sarkosyl, 1mg/ml molecular biology grade lysozyme). The bottles were sealed and rolled on a rotating platform for 24-hours at 37°C to ensure complete cell lysis. After

24-hours the lysis solution was replaced with 3ml ESP solution (0.5M EDTA pH9.0, 1% w/v Sarkosyl, 1.5mg/ml proteinase K). The bottles were resealed and statically incubated for 48-hours at 50°C to allow full degradation of remaining cellular debris. Blocks were then stored in fresh ESP at 4°C for further treatment.

4.2.5.2.2 Pre digestion treatment

Prior to restriction enzyme digestion of the genomic DNA, the ESP solution was replaced with 3ml of TE (10mM Tris pH 8.0, 1mM EDTA pH 8.0). The bottles were rolled for 2-hours at room temperature. The TE was replaced with fresh TE and the bottles rolled for a further 2-hours at room temperature. Finally, the TE was removed and the blocks washed 3 times for 20-minutes each, at room temperature, with fresh TE. Washed blocks were then stored in a further 3ml of TE at 4°C until required.

4.2.5.2.3 DNA digestion

A washed block was placed on a piece of parafilm and excess TE blotted with filter paper. A slice, (approximately 1mm x 1mm x 9mm) was cut from the block using a microscope slide glass coverslip. The slice was transferred to a sterile 1.5ml microcentrifuge tube, with the remainder of the block returned to the bottle of TE and stored again at 4°C. The slice was then bathed with 200μl of the buffer appropriate to the enzyme used. The buffer had been diluted with sterile ddH_2O to the manufacturer's specifications and was incubated with the agarose slice on ice for 15-minutes. The buffer was replaced with a mixture of 160μl of fresh buffer and 40μl of restriction enzyme, *Dra*I (cutting at 5'-TTTAAA-'3). This was thoroughly

mixed, stored on ice for 15-minutes, and then incubated at 37°C for 20-hours to allow full digestion of the DNA within the block.

4.2.5.2.4 Electrophoresis of genomic fragments

A stock solution of 10xTBE (1.0M Tris, 0.89M boric acid, 0.02M EDTA pH 8.0) was prepared in advance. 100ml of molten agarose was produced by melting 1.2g of molecular biology grade agarose in 100ml 0.5xTBE using an 850W microwave oven at full power, with numerous pauses for mixing the solution. Once the agarose had cooled to approximately 50°C, a gel was cast onto a supporting plate using the BioRad CHEF DR-III casting mould. A levelled platform was used to produce a uniform, flat gel.

The CHEF DR-III electrophoresis cell was prepared by filling with 2L of freshly made 0.5xTBE which was pre-cooled by setting the apparatus pump to maximum (100), and the cooling unit to 10°C.

The restriction enzyme solution was carefully removed and replaced with 200μl of ES (0.5M EDTA pH 9.0, 0.1% w/v Sarkosyl). The agarose slice was then incubated at 50°C for 15-minutes to quench the activity of the restriction enzyme. The ES was replaced by 1.0ml TE and left at room temperature for 15-minutes prior to loading into the gel. The slices of agarose were loaded one into each lane of the gel. Each slice sealed into place with a coating of molten 0.5% w/v molecular biology grade agarose in 0.5xTBE. The final lane on each gel was loaded with a 2mm thick slice of DNA size standard, commercially produced for PFGE (BioRad Lambda ladder containing concatamers of cl857 Sam7 providing DNA bands in increasing units of 48.5Kb up to approximately 1000kb).

Once the sealing agarose had solidified, the electrophoresis cell buffer flow rate was reduced to 70% of maximum. This gave a flow of approximately 1L/minute. The gel was transferred from the mould to the locating bracket within the electrophoresis cell. In this position the gel was now submerged in cooled 0.5xTBE. The lid to the cell was placed in position and the gel left to equilibrate to the temperature of the running buffer for 5-minutes. The DNA fragments were then separated electrophoretically using running conditions that were specific to each enzyme. *Dra* I required an initial pulse time of 2-seconds, a final pulse time of 25-seconds, a total run time of 22-hours, at a temperature of 10°C, with an electric field intensity of 6 V/cm and at an electrode angle of 120°.

Once the run time had elapsed the gel was carefully removed from the electrophoresis cell, submerged in 500ml of ethidium bromide staining solution (5µg/ml in 500ml ddH_2O), and gently agitated to allow visualisation of DNA bands. After 60-minutes the gel was carefully removed from the staining solution and destained by submerging in 1L of ddH_2O for up to 2-hours.

Banding patterns were visualised by placing the gel on an ultraviolet transilluminator and an image captured using UVP products and software.

Those strains that showed no change in banding pattern after passaging in BKC for a number of weeks were examined in reference to their resistance to other antimicrobials.

4.2.6 Determination of resistance properties

4.2.6.1 Preparation of antimicrobial agents

Solutions of antimicrobial agents were prepared as stated in table 4.2 and sterilised by membrane filtration using a 0.2µm cellulose acetate pore filter.

Table 4.2 Details of preparation of antimicrobial agents and storage prior to use.

Antimicrobial agent	**Preparation**	**Storage**
Benzalkonium chloride (BKC)	Water	4°C
Cetylpyridium chloride (CPC)	Water	Room temperature
Dodecyl trimethyl-ammonium bromide (DBC)	Water	4°C
Thymol (THY)	Water	Made fresh
Chlorhexidine (CHX)	Water	4°C
Triclosan (TLN)	Water	4°C
Cetrimide (CET)	Water	4°C
Polymyxin B (PMB)	Water	4°C
Tobramycin (TOB)	Water	4°C
Ceftazidime (CEF)	Water	Made fresh
Ciprofloxacin (CIP)	Water	4°C
Chloramphenicol (CPCL)	10%v/v ethanol	Made fresh
Imipenem (IMP)	Water	Made fresh

Antimicrobial agents were prepared and stored according to protocols from Journal of Antimicrobial Chemotherapy (1991 Supl. D Vol.27).

The MIC for each antibacterial agent was determined in triplicate as described in section 4.2.2. Similarly the MBC was determined as described in section 4.2.3 for those agents which precipitated at higher concentrations, rendering an MIC impossible to visualise. These MBC values were used instead of MIC values where appropriate.

4.2.6.2 Stability of BKC resistance

To determine the stability of BKC resistance the most resistant passaged cells of PAO1 (passage P6) and OO14 (passage P5) were passaged in BKC-free broth for the same number of passages used to generate the resistance. This was 6x5days passage in the case of PAO1 and 5x5days for the OO14 strain. After each 5-day period the MIC for BKC was determined for each strain in the manner described in section 4.2.2.

4.2.7 European disinfectant test EN1276

The disinfectant test used in chapters 2 and 3 used an ATCC strain of *P. aeruginosa* (15422) to simulate strains that disinfectants are likely to encounter in their use. This test was performed as described in section 3.2.5 with BKC as the sole active agent used at a final concentration of 0.025%w/v and 0.0125%w/v. Instead of the test strain ATCC 15422, OO14 and PAO1 adapted cells were used in triplicate.

4.3 Results

4.3.1 Adaptation of P. aeruginosa strains to BKC

As strains were passaged in increasing concentrations of BKC, their MIC to the disinfectant was noted. The results are shown in table 4.3

Table 4.3 MICs of BKC against *P. aeruginosa* strains adapted by multiple passage in the disinfectant.

Strain	**MICs (% w/v) to BKC following multiple passages (P1-P6) in BKC.**						
	0 **WT**	**0.00078** **P1**	**0.00156** **P2**	**0.00312** **P3**	**0.00625** **P4**	**0.0125** **P5**	**0.025** **P6**
CL7	0.003	0.003	0.006	0.05	>0.05	>0.05	>0.05
CL8	0.003	0.006	0.0125	>0.05	>0.05	-	-
CL9	0.003	0.003	0.0015	0.0015	-	-	-
CL10	0.003	0.003	0.025	>0.05	>0.05	-	-
CL11	0.0015	0.003	0.003	-	-	-	-
CL12	0.0015	0.003	0.025	>0.05	>0.05	>0.05	>0.05
CL13	0.0015	0.003	0.025	>0.05	>0.05	-	-
CL14	0.003	0.003	0.0125	>0.05	>0.05	>0.05	>0.05
CL16	0.0015	0.003	0.025	>0.05	>0.05	>0.05	>0.05
17TS	0.0015	0.006	0.025	>0.05	>0.05	-	-
OO14	0.0015	0.025	>0.05	>0.05	>0.05	>0.05	>0.05
OO72	0.0015	0.003	0.025	>0.05	>0.05	>0.05	>0.05
9766	0.0125	0.025	>0.05	>0.05	>0.05	-	-
9481	0.0015	0.003	0.025	>0.05	>0.05	>0.05	>0.05
ATCC15442	0.003	0.003	0.025	0.025	>0.05	-	-
PAO1	0.003	0.006	0.0125	0.025	0.05	>0.05	>0.05

>0.05 indicates an MIC > 0.05% w/v (>500µg/ml) where accurate determination was not possible due to restricted solubility of BKC. MBC of these strains was 0.1%w/v (1mg/ml) BKC – indicates loss of viability of the strain.

A number of strains showed an increase in MIC to BKC as they were passaged in increasing concentrations of BKC. However, other strains became non-viable. For example, CL11 became non-viable at 0.003% w/v BKC after showing a slight increase in resistance. In addition, strains that were grown in more than 0.0125% w/v BKC and frozen in glycerol for storage, were found to be increasingly difficult to recover from this frozen state.

4.3.2 Serotypes of passaged strains

In order to check the purity of the cultures undergoing multiple passage each strain was serotyped at each stage of adaptation. The results are shown in table 4.4

Table 4.4 Serotype of each strain of *P. aeruginosa* tested at each stage in passage in BKC.

Strain & O serotype when first isolated.	Serotype of organism at each passage concentration						
	0% WT	0.00078 P1	0.00156 P2	0.00312 P3	0.00625 P4	0.0125 P5	0.025 P6
CL7 1	1	1	1	1	1	1	1
CL8 3	1/3	3	3	3	12	-	-
CL9 4	4	4	4	ND	-	-	-
CL10 7	7	7	7	12	12	-	-
CL11 8	8	8	F	F	-	-	-
CL12 9	9	ND	4	4	12	-	-
CL13 13	13	ND	12	12	12	12	12
CL14 16	16	16	16	16	12	12	12
CL16 10	4	4	4	4	12	12	12
17TS 17	ND	ND	ND	ND	12	-	-
OO14 12	12	12	12	12	12	12	12
OO72 5α	E	E	12	12	12	12	12
9766 15	15	15	15	15	15	-	-
9481 14	13	ND	4	4	11/12	12	12
ATCC15442 1	1	1	1	1	12	-	-
PAO1 ATCC15692 PA	PA	PA	PA	PA	PA	PA	PA

ND: not detectable by agglutination.
PA: polyagglutinating.
E: agglutinated by mixture of sera containing O:2, O:5, O:12, O:15
F: agglutinated by mixture of sera containing O:7, O:8, O:11, O:12

Of the strains examined only four showed the same serotype throughout the passages in BKC (CL7, OO14, 9766 and PAO1). All of those that showed a sustained altered serotype changed to serotype O:12. These results suggest that contamination may have occurred between passages P3 and P4 for strains CL8, CL12, CL14, CL16, 17TS, 9481 and ATCC 15442, although the serotype for Cl12 also altered at P1 and P2. Contamination may have occurred between passages P2 and P3 for CL10 and between P1 and P2 for strain 0072 and possibly CL11 and CL13. CL9 became untypable between passages P2 and P3.

4.3.3 Pulsed field gel electrophoresis

Since PAO1 remained untypable by O-serology, it was further examined by PFGE in order to determine its purity. A number of strains that showed an unchanging serotype were included as controls (CL7 and OO14). A strain that exhibited altered serotype, ATCC 15442, was also examined to determine if the changes were due to contamination by another strain.

Figure 4.1 shows PFGE DNA patterns of passages WT to P5 of PAO1 strain of *P. aeruginosa.*

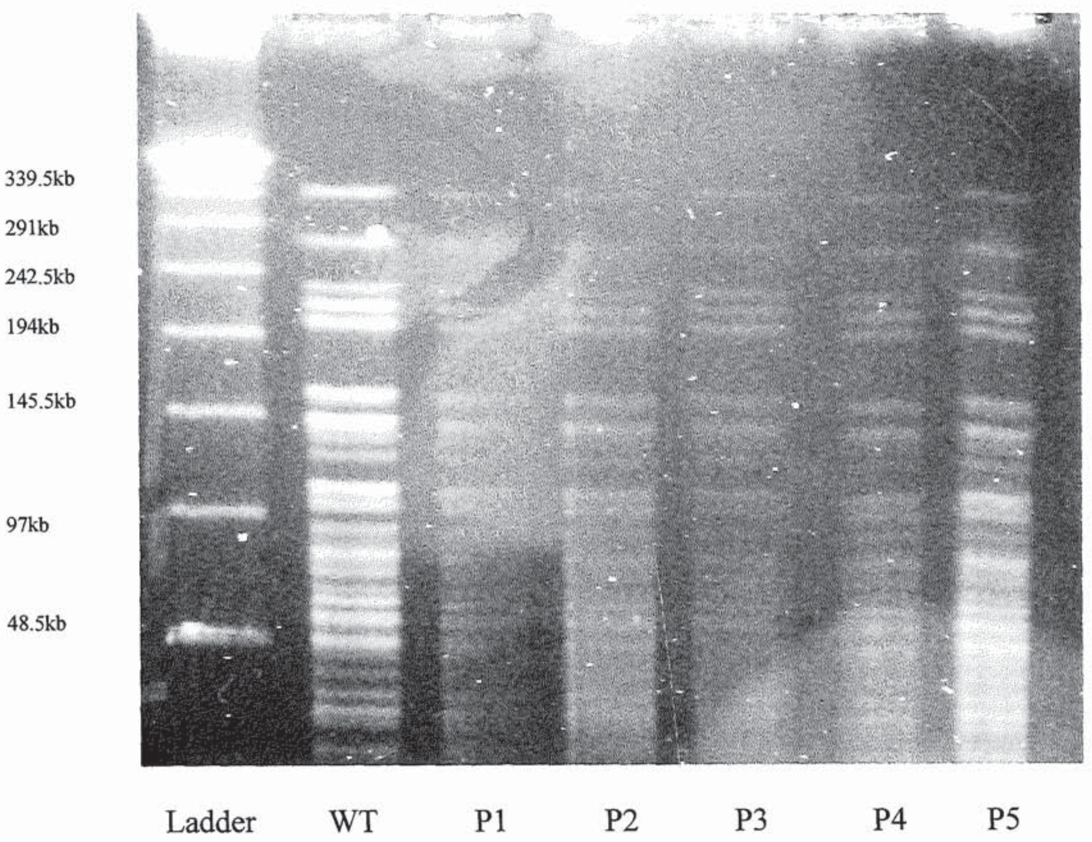


Figure. 4.1 PFGE banding patterns of PAO1 adapted cells to determine purity of passaged cultures.

There was no visible alteration in the banding pattern of the digested chromosomal DNA of PAO1 adapted cells. While it was not possible to regrow P6 cells from frozen culture it would appear than no contamination of PAO1 occurred in the

process of adaptation, thus supporting the view that alteration in resistance was due to adaptive changes in the PAO1 cells themselves.

Figure 4.2 shows PFGE DNA patterns of passages WT, P1, P2, P3 and P5 of OO14 strain of *P. aeruginosa*.

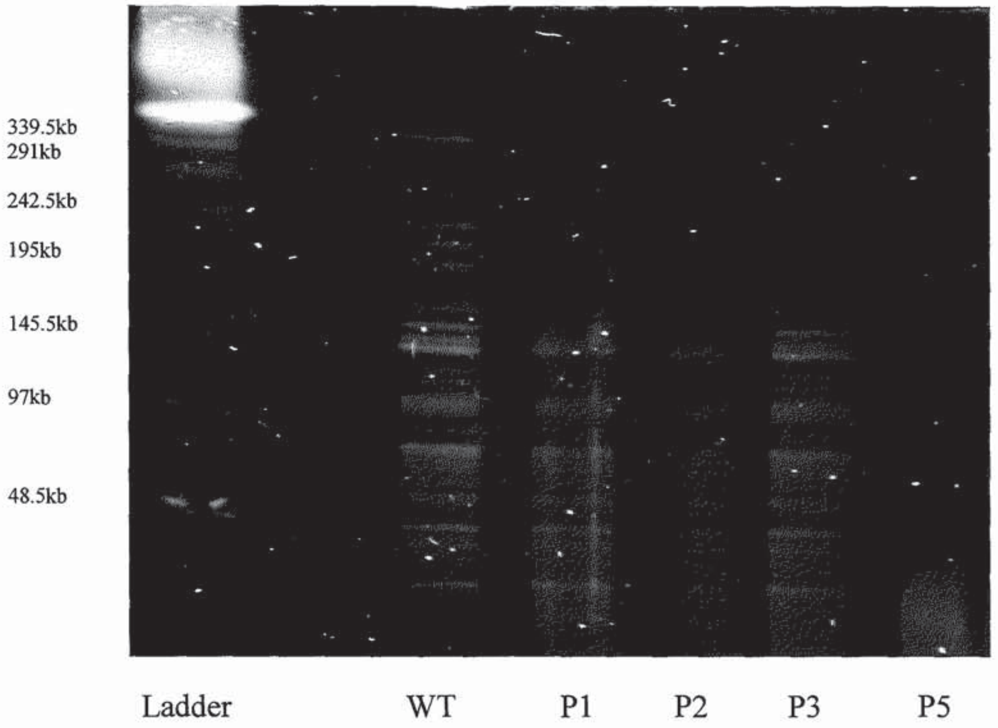
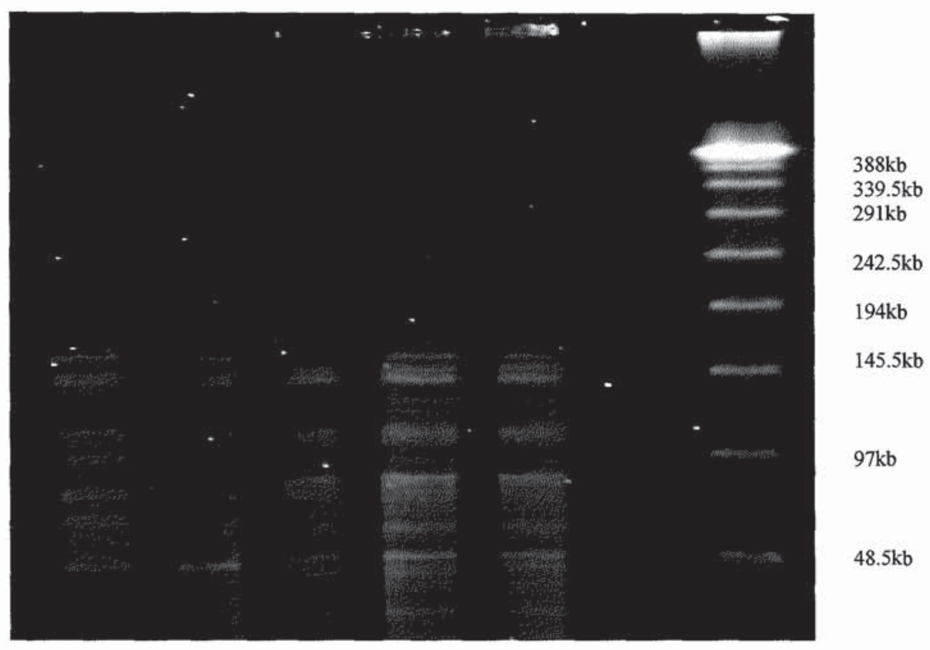


Figure 4.2 PFGE banding patterns of OO14 adapted cells to determine purity of passaged cultures.

There was no visible alteration in the banding pattern of the digested chromosomal DNA of OO14 adapted cells although the banding pattern of P5 in unclear. While it was not possible to regrow P4 and P6 cells from frozen culture it would appear that no contamination of OO14 occurred in the process of adaptation, thus supporting the view that alteration in resistance was due to changes in the OO14 cells themselves. There is also a degree of similarity between the banding pattern of PAO1 and OO14 although not enough bands are clearly shown to interpret degree of relatedness.

Figure 4.3 shows PFGE DNA patterns of passages WT, P1, P2, P3 and P5 of ATCC 15442 strain of *P. aeruginosa.*



WT P1 P2 P3 P4 Ladder

Figure 4.3 PFGE banding patterns of ATCC 15442 adapted cells to determine purity of passaged cultures.

There was a clear alteration in banding pattern between P1 and P2, including the addition of a band in the 388kb region and the change in pattern in the 194kb-291kb region. This indicated either an alteration in the chromosome of the organism or contamination of culture P2 with another organism. In addition, it would appear that the banding pattern of the "contaminant" was identical to that of O014 which was also of the serotype that ATCC 15442 exhibited in later passages.

Figure 4.4 shows PFGE DNA patterns of passages WT, P1, P2, P3, P4 and P5 of CL7 strain of *P. aeruginosa.*

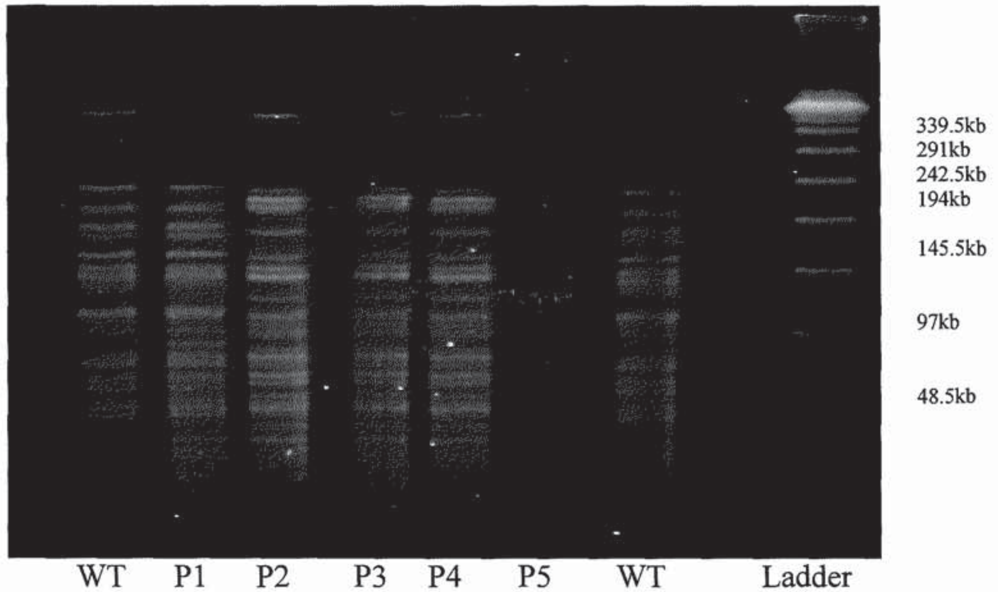


Figure 4.4 PFGE banding patterns of CL7 adapted cells to determine purity of passaged cultures.

Again there was a clear alteration of banding pattern between P1 and P2 indicating either an alteration in the chromosome of the organism or contamination of culture P2. However the "new" banding pattern did not resemble that of OO14.

The alterations in PFGE pattern in CL7 and ATCC 15442 indicated a change in the genotype of the culture. This was likely to be due to contamination of the culture by another *Pseudomonas* strain although the serological data indicated that in the case of CL7, the contaminating strain had the same serotype as CL7 and therefore must be an organism outside of those used in this experiment. Strain ATCC 15442 had an altered PFGE pattern and an altered serotype, although these alterations did not occur at the same time.

The lack of alteration of PFGE pattern for both OO14 and PAO1, combined with serological data for OO14, indicated that these two strains had not been contaminated during their passage in BKC.

4.3.4 Co-resistance to other antimicrobial agents

Strains OO14 and PAO1 were examined to determine if the passage in BKC altered their resistance to a range of antimicrobial agents. The MIC values for PAO1 and OO14 are shown in tables 4.5 and 4.6 respectively with those compounds that cells developed resistance to being highlighted. Those compounds where the MBC was recorded, instead of the MIC, are marked *. Abbreviations for agents used are those used in table 4.2.

Table 4.5 MIC values (n=3) for PAO1 cells passaged in BKC.

	Passage and MIC						
Disinfectant	WT	P1	P2	P3	P4	P5	P6
BKC % w/v	0.003	0.006	0.006	0.0125	0.025	0.05	0.05
*Thym % w/v	0.0125	0.0125	0.0125	0.0125	0.0125	0.0125	0.0125
CPC % w/v	0.003	0.006	0.0125	0.0125	0.0125	0.0125	0.0125
DBC %w/v	0.025	0.025	0.025	0.05	0.05	0.05	0.05
CHX µg/ml	10	5	2.5	10	10	10	10
*TLN % w/v	0.5	0.5	0.5	0.5	-	-	-
CET % w/v	0.0035	0.00925	0.0125	0.0125	0.0125	0.0125	0.0125
PMB µg/ml	0.45	1.25	1.35	1.785	1.25	3.5875	5
TOB µg/ml	1.35	1.25	0.716	1.25	1.25	1.25	1.25
CEF µg/ml	1.25	1.25	1.25	1.25	0.6	0.6	0.3
CIP µg/ml	0.6	0.6	0.6	0.6	0.6	0.6	1.25
CPCL µg/ml	12.5	18.75	18.75	18.75	18.75	25	25
IMIP µg/ml	2.5	1.25	1.25	1.25	1.25	2.5	1.25

Table 4.6 MIC values (n=3) for OO14 cells passaged in BKC.

	Passage and MIC				
Disinfectant	WT	P1	P2	P3	P5
BKC % w/v	0.015	0.025	0.05	0.05	0.05
*Thym % w/v	0.0125	0.0125	0.0125	0.0125	0.0125
CPC % w/v	0.05	0.05	0.05	0.1	0.3
DBC %w/v	0.1	0.1	0.1	0.05	0.05
*TLN % w/v	0.5	0.5	0.5	0.5	-
CHX µg/ml	5	6.25	5	3.75	6.25
CET % w/v	0.0125	0.0125	0.0375	0.075	0.075
PMB µg/ml	0.3	0.15	0.3	0.3	0.3
TOB µg/ml	133	66.6	56	30	20
CEF µg/ml	1.25	0.6	0.6	1.25	0.6
CIP µg/ml	0.3	0.3	0.387	0.189	0.189
CPCL µg/ml	500	500	400	550	550
IMIP µg/ml	1.416	2.5	2.5	1.25	1.25

The OO14 passage 4 was not recoverable from frozen culture for these experiments.

Any resistance alterations in the passages of strains OO14 and PAO1 are shown also in figures 4.5 and 4.6.

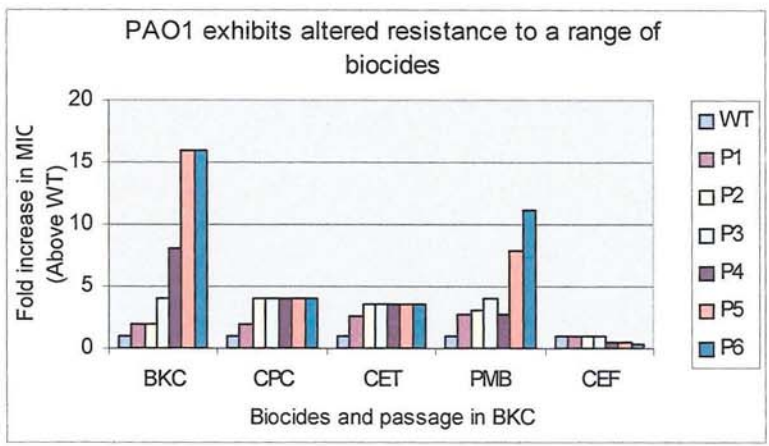


Figure 4.5 Fold increase above Wild Type of resistance to biocides in strain PAO1.

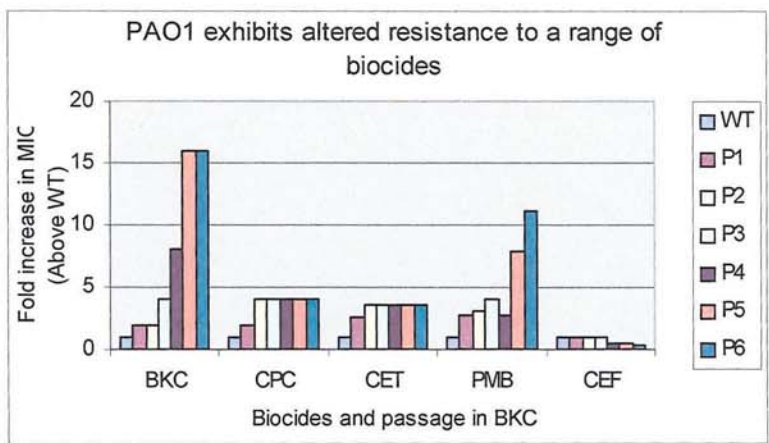


Figure 4.6 Fold increase above Wild Type of resistance to biocides in strain OO14.

PAO1 adapted cells showed an increase in resistance to BKC, to the quaternary ammonium compounds, cetrimide and cetylpyridium chloride and to the membrane active antibiotic polymyxin B. There was a slight (2-fold) increase, between WT and P5 and P3 respectively, in the MIC for chloramphenicol and dodecyltrimethyl-ammonium bromide, and a decrease in resistance to the cephalosporin ceftazidime. For the remaining biocides there was no trend in resistance alteration, or no change at all.

OO14 adapted cells showed increases in resistance to BKC, cetrimide and cetylpyridium chloride, but not to polymyxin B. The only other observable trend in resistance alteration was to the aminoglycoside, tobramycin. The OO14 strain was initially highly resistant, but the MIC to the antibiotic decreased in a steady fashion as resistance to BKC increased.

4.3.5 Stability of BKC resistance.

The MIC for BKC of the PAO1 P6 and OO14 P5 strains was recorded after multiple passage in disinfectant free broth.

Table 4.7 Stability of resistance to BKC in passaged cells of PAO1 and OO14

Strain	MIC (%w/v) of BKC after passage in BKC free media for a number of days						
	0days	5days	10days	15days	20days	25 days	30 days
PAO1(P6)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
OO14 (P5)	0.05	0.1	0.1	0.1	0.1	0.1	0.1

Both strains showed a stability of resistance to BKC in their adapted cells even when grown in BKC-free media. The serotype of OO14 remained the same throughout these passages and PAO1 remained polyagglutinable.

4.3.6 Effect of adaptation to BKC in strains PAO1 and OO14 performance in the EN1276 disinfectant test

The EN1276 disinfectant test, described in section 3.2.5, used BKC at concentrations of 0.0125%w/v and 0.025%w/v. By replacing the test strain ATCC 15442 with adapted cells from PAO1 and OO14 it was possible to show what effect adaptation of cells to BKC had on the relevance of the EN1276 test.

The results for PAO1 are illustrated in figures 4.8 and 4.9.

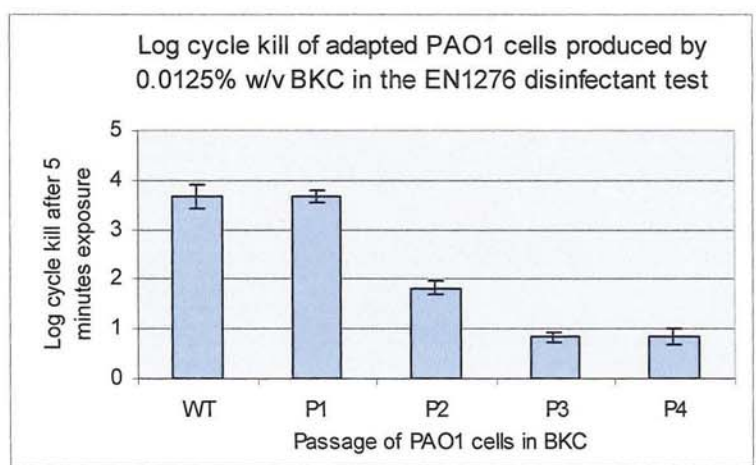


Figure 4.7 Log cycle kill of adapted PAO1 cells following 5-minutes exposure to 0.0125% w/v BKC as part of the EN1276 disinfectant test.

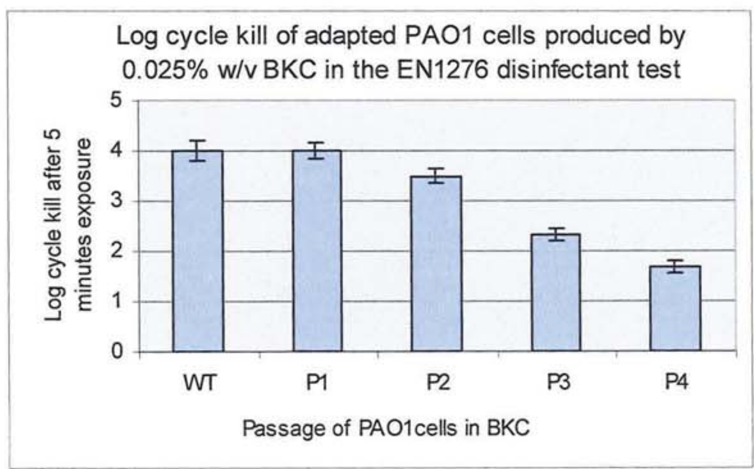


Figure 4.8 Log cycle kill of adapted PAO1 cells following 5-minutes exposure to 0.025% w/v BKC as part of the EN1276 disinfectant test.

The results for OO14 are shown in figures 4.9 and 4.10.

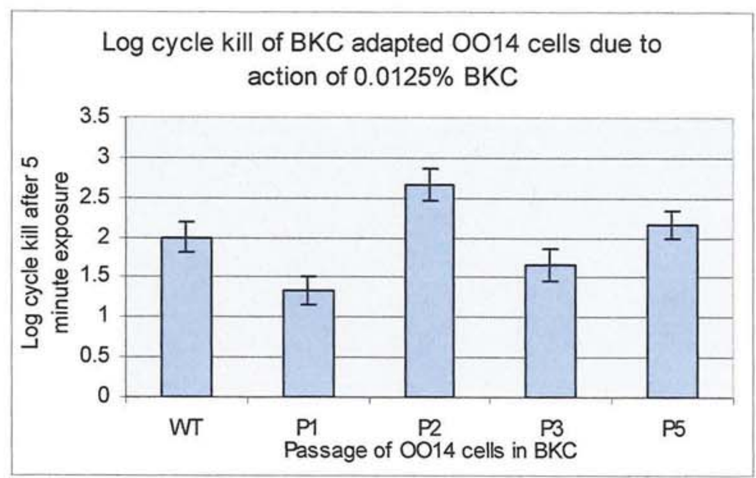


Figure 4.9 Log cycle kill of adapted OO14 cells following 5-minutes exposure to 0.0125% w/v BKC as part of the EN1276 disinfectant test.

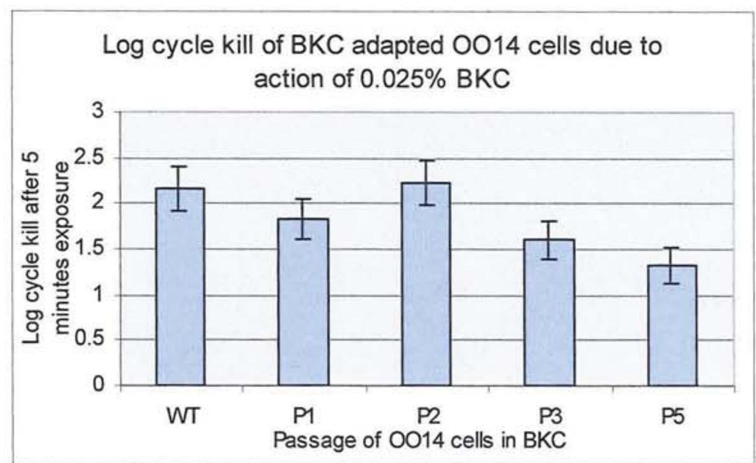


Figure 4.10 Log cycle kill of adapted OO14 cells following 5-minutes exposure to 0.025% w/v BKC as part of the EN1276 disinfectant test.

As PAO1 cells adapted to BKC they showed a reduced level of log cycle kill in the EN1276 test at both concentrations of the disinfectant. At the 0.0125% w/v concentration of BKC there appeared to be little link between the level of adaptation of OO14 cells and the log cycle kill in the EN1276 test. However, at the higher (0.025% w/v BKC) concentration there was a reduction in the log cycle kill of the cells as they became adapted to BKC.

4.4 Discussion

4.4.1 Adaptation to BKC

In accordance with previous research (Joynson *et al.*, 1999; Méchin *et al.*,1999; Méchin *et al.*,1999) it was possible to generate resistance to BKC by serial passage of bacteria in increasing concentrations of disinfectant. Many strains showed an increase in resistance to BKC when exposed to a concentration of the disinfectant 4-fold lower than the MIC. Two strains became non-viable after a low number of passages, indicating an inability to adapt to the disinfectant. The remaining strains showed an ability to grow in BKC concentrations of 0.025% w/v, a higher concentration than that in appropriately diluted disinfectants. The most resistant of these cells have an MIC of more than 0.05% w/v BKC. This is a similar concentration to that reached by *P. aeruginosa* cells in the work of Joynson *et al.* (1999), and far higher than that reached by cells of the ATCC15442 strain used in the work of Méchin *et al.* (1999), although the fold-increase in resistance in all cases was comparable. In addition, the BKC resistant phenotype was found to be stable in both PAO1 and OO14 strains in disinfectant-free media over a period equal to that required to generate the phenotype.

4.4.2 Serotype alterations

When strains were examined by means of serotyping it was found that many of the strains exhibited an alteration of serotype as they were passaged. This alteration in the O-antigen of the strains could indicate contamination. Alternatively, in becoming resistant to BKC, an alteration in the LPS, specifically involving the stricture of the O-antigen may have occurred. Most of the strains that underwent serotype change, altered to serotype O:12, perhaps indicating contamination by

another strain, such as OO14, a strain used in the work with a serotype of O:12. Examination of the structure of serotype O:12 (Wilkinson, 1983; Knirel *et al.*, 1988) has shown that it is unlikely that an alteration in the O-antigen occurred in the strains showing serotype alteration, as the O:12 antigen is not a truncated form of another antigen, and is unlikely to be the result of modification due to increase in BKC resistance. Alterations in serotype as a result of antipseudomonal agents have been reported in the past, (Kobayashi *et al.*, 1994) when cells exposed to various antibiotics exhibited numerous alterations in serotype although there appeared no link between type of antibiotic and nature of alteration. Lack of the O-antigen, or B band LPS, has been shown to be associated with resistance to aminoglycosides (Kadurugamuwa *et al.*, 1993) which are thought to have an action upon the outer membrane in a not-dissimilar fashion to BKC, however such alterations would have led to a complete loss of serotype, not the acquisition of another.

4.4.3 Pulsed field gel electrophoresis

To determine culture purity in the face of such serotypical alterations pulsed-field gel electrophoresis was used to determine if strains with altered serotype were the result of contamination. While strains PAO1 and OO14 had shown viability, unaltered serotype (or lack thereof) and an unchanged genotype (at least as detectable by this method) ATCC15442 showed both an alteration in serotype to O:12 and a change in banding pattern to that identical to the O:12 strain OO14. While this indicated contamination by such a strain it is interesting to note that alteration in serotype and electrophoretic band patterns did not occur at the same time.

By using strain CL7, that had not altered serotype, as a control it was discovered that it had an altered genotype, not resembling that of OO14. It may be that such examples are due to a small proportion of a heterogeneous culture population, since these were environmental isolates, containing an altered genotype and it was these organisms that survived the passages in greater and greater numbers, eventually replacing the "weaker" sub-strain as the dominant genotype. However, since the original "wild type" passage of cells was grown from an isolated colony, it may be unlikely that such heterogeneity would be present. In addition, certain resistance characteristics have been carried by bacteriophages, which may have been present in a fraction of the environmental isolate culture (Blahova *et al.*, 1992, 2000). The insertion of DNA in this manner would certainly alter the banding pattern of a pulsed-field gel without necessarily altering the serotype of the cell.

It is difficult to apply the criteria set by Tenover (1995) to the results obtained in this work, as at least 10 clearly defined bands are required in each of the strains to be compared. In addition, comparison of the two strains PAO1 and OO14 showed that while having clearly differing serotype, they shared an undistinguishable pulsed-field fragment pattern. This illustrates the limitations of such techniques when using only one restriction enzyme. However, pulsed-field gel electrophoresis did allow determination of strain purity without relying on components such as the O-antigen that may be affected by the process of generating resistance.

4.4.4 Cross-resistance

Cross-resistance between disinfectants and antibiotics in bacteria caused to become resistant by exposure to disinfectants is an area of research of growing interest. The disquieting thought that the hospital environment, already known to

generate resistant bacteria due to use of antibiotics, may be harbouring bacteria that disinfectant use has made resistant has been examined in recent work by Russell *et al.* (1998). Studies by Schweizer (1998) and Chuanchuen (2001) have shown links between resistance in *P. aeruginosa* to the disinfectant triclosan and antibiotics such as ciprofloxacin, by means of efflux.

4.4.4.1 Cross-resistance to other disinfectants

Quaternary ammonium compounds (QAC) such as BKC have a membrane active bactericidal action and have been seen to strip the outer membrane from *P. aeruginosa* cells (Richards & Cavill, 1976). Therefore resistance to such compounds has been thought to involve the membranes surrounding bacteria and resistance to BKC would logically confer resistance to other membrane active agents. In the case of *P.aeruginosa* strains PAO1 and OO14 this has been shown to be true. PAO1 showed co-resistance to cetylpyridinium chloride, cetrimide and dodecyl trimethyl-ammonium bromide, all QACs while OO14 showed resistance to cetrimide and cetylpyridinium chloride only. Neither strain showed any resistance to the bisbiguanide chlorhexidine or the phenolic disinfectant triclosan.

Such resistance is not unheard of as *P. aeruginosa* ATCC 15442 cells adapted to dodecyl trimethyl-ammonium bromide by Méchin *et al.* (1999) showed co-resistance to other QACs and to chlorhexidine, which is also membrane active. Similarly Jones *et al.* (1989) adapted *P. aeruginosa* CMC 2730 to a QAC and showed cross-resistance to other QACs and to chlorhexidine. Much earlier work by Adair *et al.* (1971) showed that BKC resistant "mutants" of *P. aeruginosa* ATCC 9027 cells were cross-resistant to other QACs. However, the lack of resistance in the PAO1 and

OO14 strains to chlorhexidine is unusual as research where *Pseudomonas stutzeri* cells were adapted to chlorhexidine showed co-resistance to QACs and other membrane active agents (Russell *et al.*, 1998). However, it appears more and more that cross-resistance to other disinfectants is a very strain specific phenomenon and caution must be taken not to generalise results to cover whole species.

4.4.4.2 Cross- resistance to antibiotics

It is usual to examine co-resistance to other disinfectants such as chlorhexidine when examining resistance attributed to growth in quaternary ammonium compounds such as BKC, but usually the interest in antibiotic resistance in such cases is limited to the membrane active polymyxins and the aminoglycoside group of agents. Indeed work by Joynson *et al.* (1999) adapted *P. aeruginosa* cells to BKC and looked for resistance only to aminoglycoside antibiotics. However, research has been carried into a wider range of antibiotics, leading this study to examine co-resistance to antibiotics that reflected the common therapies used in treating *P. aeruginosa* in conditions such as CF.

PAO1 showed enhanced resistance to both polymyxin B and chloramphenicol, reduced resistance to the β-lactam ceftazidime and no alteration in resistance to imipenem, ciprofloxacin and surprisingly the aminoglycoside tobramycin. OO14 showed no enhanced resistance to any antibiotic and a marked stepwise reduction in resistance to tobramycin.

Polymyxin resistance and sensitivity in disinfectant adapted cells has been observed before. Adair *et al.* (1971) showed that *P. aeruginosa* strains able to grow in

0.1%w/v BKC were up to 20 times more sensitive to polymyxin B than non-adapted cells, while Russell *et al.* (1998) showed a variable alteration in resistance in *Pseudomonas stutzeri* cells adapted to chlorhexidine, in some cases an up to 500 fold increase in polymyxin B MIC was observed.

Resistance to aminoglycosides has been associated with polymyxin resistance in the past in *P. aeruginosa*, especially when resistance is mediated by overproduction of the outer membrane protein, OprH (see chapter 5). Indeed the membrane active nature of aminoglycosides has been shown in the past (Hancock & Wong, 1984; Loh *et al.*, 1984) by enhancing the uptake of hydrophobic probes. The putative link between BKC adaptation and aminoglycoside resistance was strong enough for Joynson *et al.*(1999) to test only for co-resistance to aminoglycosides in their BKC adapted cells. In their work no increase in resistance was detected. Therefore it seems likely that, however attractive the link between QAC resistance and aminoglycoside resistance is, they are not connected. However, Russell *et al.* (1998) did observe in *P. stutzeri* cells adapted to chlorhexidine increases in resistance to both cetylpyridinium chloride and to gentamicin although the highest increases in resistance to these compounds did not occur in the same strains. The drop in resistance of OO14 adapted cells to tobramycin has no precedent and will be examined further in chapter 5.

Links between disinfectant adaptation and resistance to those antibiotics not regarded as membrane-active are plentiful, if largely explained by the catch-all statement of "reduced permeability". With some antibiotics such as novobiocin, nalidixic acid and erythromycin this is likely to be true as the outer membrane poses a considerable

barrier for them. The work of Russell *et al.* (1998) showed chlorhexidine adapted *P. stutzeri* cells were more resistant to ampicillin, erythromycin and nalidixic acid in addition to the resistances already described. This implies a general alteration in permeability to these agents as it is unlikely that separate resistance mechanisms would have been acquired in the process of adaptation.

In *E. coli*, resistance to pine oil is associated with co-resistance to the antibiotics tetracycline, nalidixic acid, ampicillin and chloramphenicol (Moken *et al.*, 1997), although this is thought to be linked to the AcrAB efflux system present in *E.coli* and shows no co-resistance to phenolic disinfectants or QACs. However, in addition to this example of disinfectant resistance linked to antibiotic resistance there is the more recent work by Chuanchuen *et al.* (2001). Based upon an observation noted in a previous paper by a member of the same research group (Schweizer, 1998), it was discovered that wild-type *P. aeruginosa* cells were resistant to the disinfectant triclosan despite having the non-resistant form of the disinfectant's target enzyme. Resistance was as a result of the MexAB-OprM efflux system that is thought to be responsible for much of the intrinsic resistance *P. aeruginosa* shows to antiobiotics. By using a MexAB-OprM deficient mutant exposed to triclosan it was possible to generate disinfectant-resistant bacteria that utilised a different efflux pump system. The hyper-expression of this alternate pump system caused the increase in MIC of a number of antibiotics, including the commonly therapeutic ciprofloxacin. With the advent of research showing that efflux has an ever greater role in the "intrinsic" resistance phenotype of both wild type and "mutant" *P. aeruginosa* strains the mechanisms for such resistance appear complex and difficult to generalise upon.

4.5 Conclusions

It is possible to generate stable adaptive resistance to the disinfectant BKC by serial passage in almost all *P. aeruginosa* strains examined, both laboratory and environmental isolates. In a number of cases alteration in serotype observed can be traced to contamination by another strain, although alterations in serotype do not always occur at the same point as alterations in genotype, if they occur at all. Co-resistance is observed to other quaternary ammonium compounds and the membrane active antibiotic polymyxin B although such changes differ from strain to strain. For example, a stepwise reduction in resistance to tobramycin is observed in strain OO14 only, while increased resistance to polymyxin B is present in strain PAO1 alone.

Chapter 5: Determining mechanisms of resistance

5.1 Introduction

5.1.1 Aims

Having determined that cells from *P. aeruginosa* strains PAO1 and OO14 become more resistant to BKC and other antimicrobials following passage in BKC, the aim was to characterise any changes in the strains that could be linked to their resistance properties.

5.1.2 Efflux of biocides

As mentioned in section 1.5.2 efflux of disinfectants and antibiotics is commonly seen as a mechanism of resistance in *P. aeruginosa* and other bacteria (Nikaido, 1994). The action of certain efflux pumps can be retarded by the addition of the alkaloid reserpine (Gill *et al.*, 1999) and has been used in the past to determine whether such pumps have a role in bacterial resistance.

5.1.3 Outer membrane proteins

The biocide efflux pumps mentioned above and more completely in section 1.52 all have an outer membrane component. Over expression of this outer membrane component is often associated with antimicrobial resistance. In addition, outer membrane proteins such as H1 of *P. aeruginosa* have an important role in maintaining the structure of the outer membrane, and are associated with antibiotic resistance (Nicas *et al.*, 1980; Bell *et al.*, 1991). Therefore the outer membrane material of the adapted strains of *P. aeruginosa* was examined for changes in expression of these proteins. Certain of the outer membrane proteins of many bacteria, including *P. aeruginosa*, are subject to a phenomenon known as heat

modifiability. When prepared for SDS PAGE examination the proteins apparent molecular weight depends upon the temperature and duration of heating involved in the denaturing of the protein with sample buffer (Hancock & Carey, 1979; Lambert & Booth, 1982).

Therefore by heating samples at different temperatures and for different durations it is possible to identify more accurately some proteins due to the altered apparent molecular weight.

5.1.4 Lipopolysaccharide

Alterations in the LPS of *P. aeruginosa* have been linked to changes in resistance to antimicrobial agents including polymyxin B and complement (Moore *et al.*, 1984; Tateda *et al.*, 1994). Therefore the LPSs of the two strains were examined to determine any alterations associated with the resistance produced by multiple passage. There are two main methods for producing LPS to be analysed by SDS-PAGE and silver staining. One involves the isolation of LPS by phenol extraction (Westphal & Jann, 1967; Galanos *et al.*, 1969) while the other treats outer membrane material with a proteinase to digest proteinaceous material leaving LPS unaffected (Darveau & Hancock, 1983). Since this latter method utilises the same outer membrane material used in the examination of outer membrane proteins it is more appropriate to be used to compare the two results. Some strains of *P. aeruginosa* produce LPS lacking in the sugar moieties involved in the process of silver staining (Kropinski *et al.*, 1985). Consequently LPS patterns on SDS-PAGE gels cannot be reliably studied by silver staining. It is still possible to determine gross changes in quantity of LPS due to resistance to BKC by measuring a component of LPS not found in other parts of the cell. The compound 2-keto-3-deoxyoctonate (KDO) is

present only in LPS and can be detected by a colourimetric assay (Karkhanis *et al.*, 1978). This assay has the advantage that it is unaffected by DNA contamination.

5.1.5 Fatty acids

The alteration of the fatty acids of *P. aeruginosa* when adapted to growth in antimicrobial agents has been examined on several occasions (Moore *et al.*, 1984; Conrad & Galanos, 1989). However, this work aims to examine alterations in the outer and cytoplasmic membrane separately, and of the whole of the outer membrane, not just the fatty acids of the LPS as has been achieved in the past (Jones *et al.*, 1989; Guérin-Méchin *et al.*, 1999; Méchin *et al.*, 1999). The separation of outer and cytoplasmic membranes of Gram-negative bacteria is usually achieved by differential solubilisation using the ionic detergent sodium N-lauroyl sarcosine. However, the detergent has a detrimental effect when samples are examined by gas chromatography (GC) for fatty acids. It produces a peak on the GC trace, much as a fatty acid would, that masks the signature of the fatty acids present in the sample.
By lysing cells in a French press and subjecting them to sedimentation through a sucrose gradient, it is possible to separate the outer and cytoplasmic membranes successfully without the need for such a detergent (Hardie & Williams, 1998). It is recognised however, that certain areas of the outer and cytoplasmic membrane are linked and may not be able to be separated successfully (Ishidiate *et al.*, 1986).

5.1.6 Whole cell polar lipids

Phospholipids are an essential part of both the cytoplasmic and outer membrane of bacteria. Their composition affects the structure and permeability of both membranes and changes have been observed in the amount and type of phospholipids present in organisms subjected to antimicrobial agents (Anderes *et al*., 1971; Conrad & Gilleland, 1981; Gilleland & Conrad, 1982; Champlin *et al*., 1983; Moore *et al*., 1984; Gilleland *et al*., 1984). Their isolation from cells is based upon the methods of Bligh and Dyer (1959). Specific lipids are identified by thin layer chromatography where the mobility of each phospholipid component of the bacterial sample is compared with those of pure phospholipid samples. Visualisation of phospholipids is achieved by spraying the plate containing the lipids with molybdenum blue reagent (Dittmer & Lester, 1964). Further characterisation can be achieved by spraying plates with ninhydrin and heating for a short time. This practice causes purple colouration of samples containing free amino groups such as phosphotidylethanolamine and phosphotidylserine. This also allows detection of lyso (partially deacylated) forms of phospholipids containing free amino acids.

5.1.7 Hydrophobicity

At the bacterial cell surface there are a number of non-specific forces involved in the interaction between the cell and the surrounding environment. This environment can include: the cell surface of other micro-organisms, host cells, antimicrobial agents, non-organic surfaces, or more simply the media that supports the growth of the organism. One force that is important in the ability of an organism to interact with the environment is hydrophobicity. The measurement of hydrophobicity has been dealt with in several reports (Rosenberg & Doyle, 1990;

Van der Mei *et al.*, 1991). Hydrophobicity reflects the inability of water to accommodate non-polar chemical species. In liquid water, a hydrogen bond network extends throughout the liquid. In the case of polar or charged species, it is possible for the chemical to interact with the network by either donating or accepting hydrogen bonds from the water. Non-polar molecules, however, cannot interact so easily with the network as they have no groups to donate or accept hydrogen bonds. This results in the network rearranging itself around the non-polar molecule rather than interacting directly with it. This "lack of attraction" between a molecule and water forms the basis of the term "hydrophobic", whereas such charged, polar compounds that form hydrogen bonds easily with water are described as "hydrophilic" or "water loving". The cell surfaces of bacteria are neither completely hydrophobic nor hydrophilic, but are made up of species contributing to both these characteristics, species known as hydrophobins, or hydrophilins respectively (James, 1991).

There are a number of methods used to determine hydrophobicity and recently they have been examined to determine the effect each has upon the cells examined (Pembrey *et al.*, 1999). The microbial adhesion to hydrocarbon (MATH) assay measures the proportion of cells in a suspension that, when mixed with a hydrocarbon, partitions into the hydrophobic phase. The higher the proportion of cells that partitions into the hydrophobic phase the more hydrophobic the cells are considered. While the reproducibility of this assay has been seen to be dependant upon the pH of the suspension used and there is evidence that cell surfaces are disrupted by the use of hydrocarbons (Pembery *et al.*, 1999), it is still a common assay used to examine alterations of hydrophobicity in a range of organisms. These include Gram-positive and Gram-negative bacteria, protozoa and fungi (Van

Loosdrecht *et al.*, 1987a,b; Van der Mei *et al.*, 1988; Van der Mei *et al.*, 1997; Smith *et al.*, 1998). The test has also been used in the past to determine any correlation between hydrophobicity and resistance of *P. aeruginosa* to disinfectants (Jones *et al.*, 1989) and the effect of growth conditions on the cell surface hydrophobicity of *Pseudomonas fluorescens* (Jana *et al.*, 2000).

5.1.8 Cell surface charge

Another non-specific force present on the surface of micro-organisms is cell surface charge. This is common to all liquid-solid and liquid-gas interfaces and in bacteria is usually negative in nature. This charge is separate to the potential found between the bulk of a solid particle and the bulk of the solution, known as the Nerst potential. The cell surface charge is derived from the ionization of components of the cell wall and, while it cannot be measured directly, it is possible to measure a charge equivalent to that of the surface, known as the zeta potential. Measuring this charge takes advantage of one of the properties of ions at interfaces such as the solid-liquid interface found at the surface of a cell in liquid media. Ions at such an interface will associate primarily with one phase or another depending upon the sign of the charge, negative ions to one phase, positive ions to the other phase. If an electric charge is applied across such a system then there is a resultant movement of one phase relative to the other at a velocity that relates to the charge at the point of shear. This charge at the point of shear is known as the zeta potential. The position of the point of shear is not fully understood but is believed to be at some point beyond the Stern layer, an area surrounding the cell with a thickness of approximately 0.5nm. This Stern layer contains ions that are held by specific chemical adsorption or by localised electrostatic interaction and hence are not free to move. The zone beyond

this layer extends into the liquid phase and is diffuse, permitting free movement of ions. However, ion distribution here is not uniform since the electrostatic field present at the cell surface attracts ions of an opposite charge.

Measurement of the zeta potential is commonly achieved by a procedure known as particle microelectrophoresis. This procedure measures the rate and direction of movement of particles or cells suspended in a medium within an applied electrical field, velocity being dependant upon both the magnitude and nature of the charge. Since such surface charge is produced by the ionisation of cell surface chemical moieties, the pH of the medium and its ionic concentration will have a large impact on the charge. However, the medium used can be specified and cells are often examined in a range of pHs and concentrations to determine what effect such conditions would have on zeta potentials (Van Loosdrecht *et al.*, 1987b; Van der Mei *et al.*, 1988; Smith *et al.*, 1998). Often zeta potential is measured with reference to the interaction of bacteria with other organisms or adhesion to non-organic surfaces. Such forces as cell surface charge act as a repellent force between cells due to the almost universally negative nature of bacterial cell charge, but can act as an attractive force when promoting adhesion to other surfaces (Makin & Beveridge, 1996). In this work, cell surface charge may either act as a mechanism of resistance, attracting or repelling charged antimicrobial agents, or reflect alterations in the outer membrane that alter the organism's susceptibility to antimicrobial agents.

5.1.9 BKC uptake or binding

Benzalkonium chloride acts upon the cytoplasmic cell membrane causing both membrane disorganisation and protein damage. It is thought to attack these

sensitive sites by first binding to and traversing the outer membrane (McDonnell & Russell, 1999). The uptake of quaternary ammonium compounds such as BKC has been determined by using ^{14}C labelled agents and measuring the quantity of agent unbound after centrifugation (Jones *et al.* 1989). A non-radioactive method can be based upon the work of Scott (1968) who developed a colourimetric assay to determine quantities of cationic surfactants such as BKC.

5.1.10 Permeability assay

As described in section 2.1.3 and more fully in section 1.5.1 reduced permeability of the bacterial cell to antimicrobials is one mechanism of resistance. The NPN permeability assay often used to assay permeability of the outer membrane of Gram-negative bacteria is described in full in section 2.1.3 and is used here to determine if there are alterations in the permeability of BKC adapted cells that may be responsible for the resistance alterations observed in chapter 4.

5.1.11 Aminoglycoside resistance

Results from strain OO14 showed that the resistance to tobramycin fell at a steady rate as the cells were passaged in higher and higher concentrations of BKC. Resistance to tobramycin in *P. aeruginosa* is due to efflux, enzymatic degradation or alteration of the target site. Since there was no evidence of increase in any outer membrane proteins associated with efflux (section 5.3.2.2) or reduced permeability, in the adapted cells, it was considered to be due to one of the other two mechanisms.

5.2 Materials and Methods

5.2.1 MIC determination for BKC with addition of reserpine

The MICs for both OO14 and PAO1 were determined for BKC in the presence of 50mM reserpine. These experiments were carried out in triplicate in the same manner as described in section 4.2.2.

5.2.2 Preparation of outer membrane material

A 200ml over night culture of bacteria was centrifuged at 9630 x *g* for 10-minutes and resuspended in 10ml saline. This solution was recentrifuged at 7840 x *g* for 10-minutes and the pellet resuspended in 3ml ddH_2O. Samples were placed in an ice bath and sonicated 4 times, each for a period of 30-seconds with a 30-second gap in between. N-lauroyl sarcosine was added to the sonicated cells to a final concentration of 2% w/v. The lysate was then centrifuged at 7840 x *g* for 20-minutes to remove unbroken cells. Outer membrane material was recovered as a pellet by further centrifugation at 31,400 x *g* for 45-minutes. These pellets were lyphopilised over night, weighed and resuspended in ddH_2O to a final concentration of 1mg/ml outer membrane material.

5.2.2.1 Examination of outer membrane proteins

The method used for separating the different outer membrane proteins isolated in section 5.2.2 followed by SDS-PAGE was that of Lugtenberg *et al.* (1975). Outer membrane proteins were denatured in sample denaturing buffer (table 5.1) by heating for 10-min at 100°C or for 30-minutes at 37°C. A 10-20μl sample

was added to each well of a 12% w/v acrylamide gel (table 5.1), with one well containing a sample of pre-stained molecular weight marker (table 5.2). Samples were electrophoresed for 50-min at 200 volts (BioRad miniProtean II system).

Table 5.1 Components for preparation of SDS-PAGE gels.

	Volume for stacking gel (3%)	Volume for running gel (12%)	Sample denaturing buffer	Electrode buffer
Stock 1		5ml		
Stock 2	2.5ml			
10% SDS	0.15ml	0.5ml	5ml	20ml
1.5M Tris pH 8.8		6ml		
0.5M Tris pH 6.8	3.75ml		2.5ml	
Water	8ml	8ml	5ml	to 2L
10% ammonium persulphate	50μl	70μl		
Temed	40μl	50μl		
Glycerol			2.5ml	
2-mercaptoethanol			0.25ml	
5% w/v Bromophenol blue			0.2ml	
Tris				6.0g
Glycine				28.8g

Notes: Stock I: solution of 44% w/v acrylamide and 0.8% w/v bis (N, N-methylene) bisacrylamide;

Stock II: solution of 30% w/v acrylamide and 0.8% w/v bis (N, N-methylene) bisacrylamide.

Table 5.2 Contents of prestained marker used.

Protein	Source	Apparent MW (Da)
MBP-β-galactosidase	*E.coli*	175,000
MBP-paramyosin	*E.coli*	83,000
Glutamic dehydrogenase	Bovine liver	62,000
Aldolase	Rabbit muscle	47,500
Triosephosphate isomerase	Rabbit muscle	32,500
β-Lactoglobulin A	Bovine milk	25,000
Lysozyme	Chicken egg white	16,500
Aprotinin	Bovine lung	6,500

Gels were stained by immersion in 0.1% coomassie brilliant blue R-250 in a solution of 20% v/v methanol, 10% v/v glacial acetic acid for 1-hour and excess stain

removed by overnight immersion in a solution of 20% v/v methanol, 10% v/v glacial acetic acid. Gels were rinsed in double distilled water and stored in distilled water. The gels were visualised under normal light, using a UVP scanner. The image visualised on the scanner was saved to disk and this was used in the Phoretix 1D Advanced gel analysis program (version 4.01, Nonlinear Dynamics, Newcastle upon Tyne). The program compared the position of bands in the test lanes with those in the molecular marker lane, which were of a known size to determine the size of bands in the strains. In addition, the software allowed densitometry measurements to determine relative amounts of each protein.

5.2.3 Examination of LPS

5.2.3.1 Preparation of samples

Outer membrane material was prepared as previously stated in section 5.2.2. 50μl samples were mixed with 30μl sample buffer and heated at 100°C for 10-minutes. Then 20μl of sample buffer containing 2.5mg/ml of proteinase K was added to each sample and incubated at 60°C for 1-hour. A 20μl portion of each sample was loaded onto a 12% acrylamide gel as described in section 5.2.2.1. The stacking and separating gels also contained urea to a final concentration of 4M. Samples were electrophoresed for 50-minutes at 200 volts (BioRad miniProtean II system). Due to the sensitive nature of the stain used in this method, the gels were handled as little as possible and only with a washed, gloved hand. Gels were stored overnight in a solution of 40% v/v ethanol and 5% v/v acetic acid. Gels were oxidised in a solution of 40% v/v ethanol, 5% v/v acetic acid and 1% w/v periodic acid by immersion and gentle shaking for 1-hour. The gel was then washed for 3 x 30-minutes in ddH_2O to

remove excess acid. Staining agent was freshly made from 28ml 0.1M NaOH, 2ml NH_4OH (33% NH_3), 5ml 20% w/v silver nitrate and ddH_2O to a final volume of 150ml. The washed gel was immersed and shaken in the staining solution for 45-minutes. The stained gel was washed for 3 x 30-minutes in ddH_2O. The washed gel was then developed in a solution of 0.5ml formaldehyde (37% w/v), 50mg citric acid and ddH_2O to a final volume of 1L. Once bands had developed fully the process was halted by replacing the developing solution with 1l of 40% v/v ethanol, 5% v/v acetic. Gels were then stored in ddH_2O (Tsai & Frasch, 1982).

5.2.3.2 2-keto-3-deoxyoctonate (KDO) assay

Quantification of KDO in outer membrane samples was carried out based upon the method of Karkhanis *et al.* (1977). A 2ml sample of 1mg/ml outer membrane suspension, produced as described in section 5.2.2, was added to a 10ml Pyrex glass hydrolysis test tube. 1ml of 0.1M H_2SO_4 was added and the sealed tube heated at 100°C for 30-minutes. The solution was cooled to room temperature for 5-minutes and 0.5ml of the clear solution transferred to a clean test tube. 0.25ml of 0.04M HIO_4 in 0.006M H_2SO_4 was added, vortexed and allowed to stand for 20-minutes. 0.25ml of 2.6% w/v $NaAsO_2$ in 0.5M HCl was added and the tubes vortexed until the brown colouration disappeared. Once clear 0.5ml of 0.6% w/v thiobarbituric acid was added, the tube vortexed and heated at 100°C for 15 minutes. While the tube was still hot 1ml DMSO was added and left to cool to room temperature. Absorbance was read at 548nm using water treated as above as a blank. A calibration curve was generated using the ammonium salt of KDO as a standard (Sigma).

5.2.4 Examination of fatty acid content of cytoplasmic and outer membranes

5.2.4.1 Non sarkosyl preparation of outer and cytoplasmic membranes

Cells from 500ml overnight cultures were centrifuged for 10-minutes at 18900 x *g* and resuspended in 10ml of ddH_2O. The suspended cells were broken by three passages through a French Pressure cell at 5-ton per square inch. Unbroken cells were removed by centrifugation at 31,400 x *g* for 5-minutes and the supernatant centrifuged at 31,400 x *g* for 180-minutes to produce a pellet containing cell envelope material. The pellet was resuspended in 2.5ml of sterile ddH_2O.

5.2.4.2 Separation of outer and cytoplasmic membranes by sucrose density gradient centrifugation

Stepped sucrose gradients were prepared in polyallomer tubes (14 x 89mm, Beckman) by layering 1.6ml each of the following % w/w aqueous sucrose solutions: 65, 60, 55, 50, 45, 40, 35, taking care not to mix the layers. The membrane samples (0.5ml) were mixed with 0.5 ml of 20% w/w aqueous sucrose solution, layered onto the top of the gradient and covered with 0.5ml ddH_20. The tubes were centrifuged at 38000rpm for 20-hours at 5°C in a Beckman SW40 rotor to produce separate bands (Fig 5.1).

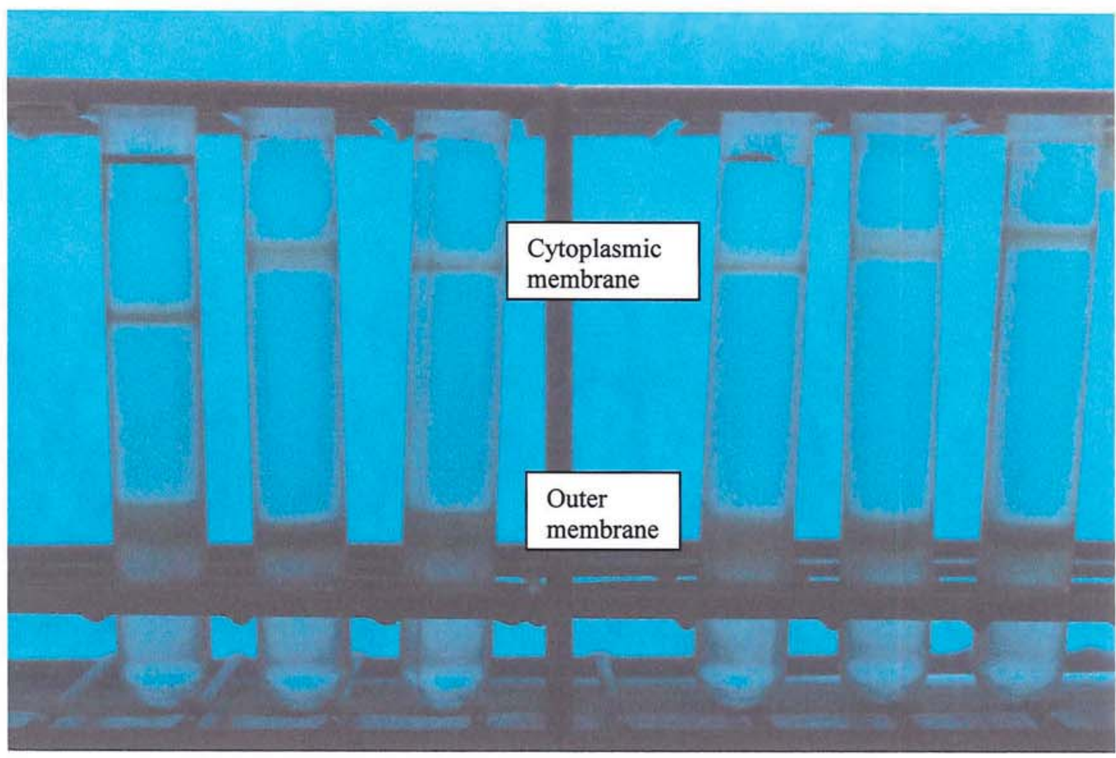


Fig. 5.1 Sucrose gradients after centrifugation showing outer membrane and cytoplasmic material separated into distinct bands, lower and upper respectively.

The base of the tube was pierced with a sterile syringe needle and fractions of approximately 0.2ml collected in a 96-well microtitre plate. The absorbance of each fraction was measured at 340nm (Anthos plate reader) and two separate peaks detected (see figure 5.2). Fractions making up each peak were pooled, diluted 20-fold in ddH_2O, centrifuged at 31,400 x *g* for 10-minutes to remove the sucrose and resuspended in water.

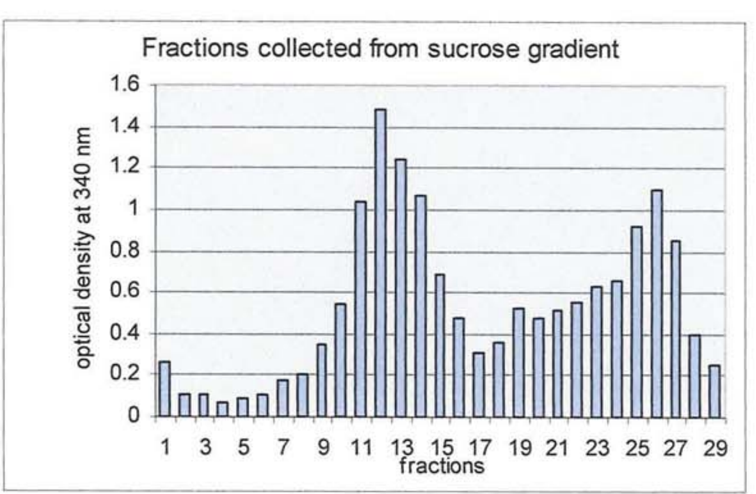


Figure 5.2 Optical density of sucrose gradient fractions after centrifugation showing outer membrane and cytoplasmic material separated into distinct bands.

5.2.4.3 Fatty acid extraction

All glassware and caps were prepared by washing in acetic acid followed by rinsing in deionised water and drying overnight at 65°C. Samples of 100µl separated membrane material were transferred to 10ml Pyrex glass hydrolysis tubes and mixed, by vortexing, with 1ml of 3.8M NaOH in 50% v/v aqueous methanol. The tubes were sealed with Teflon lined caps, and incubated at 100°C for 5-minutes. Tubes were then revortexed and incubated for a further 25-minutes at 100°C in the same manner as before. Following this tubes were removed from heat and left to cool to room temperature. 6ml of a (1:1) solution of 6M HCl and methanol was added and the unsealed tubes heated at 80°C for 10-minutes to form the methyl esters of the free fatty acids that had been released from lipids in the bacterial membranes by the alkaline hydrolysis. The samples were then removed from heat at left to cool to room temperature. A volume of 1ml of (1:1) hexane/diethyl ether mixture was added and repeatedly inverted for 10-minutes. This allowed partitioning of the fatty acid methyl esters (FAMEs) from the aqueous phase to the organic phase and samples were allowed to stand for 10-minutes to allow the two phases to separate. The upper

organic layer was removed carefully by glass pipette and mixed with 3ml of 0.3M NaOH in a sealable glass tube. The mixture was then repeatedly inverted for 10-minutes to allow extraction of any unwanted residues from the organic to the aqueous phase. The upper organic phase was then transferred to a 2ml glass tube with a foil-lined cap. The organic solvent was evaporated by the passage of compressed nitrogen gas into each tube at room temperature. The completed preparations were sealed and stored at –20°C until analysed.

5.2.4.4 Chromatographic analysis of fatty acid methyl ester profiles

A volume of 1µl of each sample was loaded onto a Hewlett-Packard HP-1 capillary column on a Unicam 610 series Gas Chromatograph (GC). Conditions used are shown in table 5.3. In addition a second column (Supelco Omegawax™ 320) was used to confirm the reproducibility of this technique and to aid identification of the fatty acids. Again the conditions used are found in table 5.3. The peaks detected were integrated and the data analysed using the Unicam software package. Fatty acids were identified by comparing the retention times of the sample peaks with those of a standard bacterial FAME mix containing 26 FAMEs, commonly found in bacteria (CP™ Mix), prepared according to manufacturer's instructions. When the Omegawax column was used, four standard bacterial fatty acid mixes were used to identify sample fatty acids.

Table 5.3 Conditions used in gas chromatography of fatty acid samples

Conditions	Used in column HP1	Used in column Omegawax
Sample split	1:50	1:50
Mobile gas phase	Helium	Helium
Column length (m) x column diameter (mm)	25 x 0.32	30 x 0.32
Film thickness	0.17μm	0.25μm
Linear velocity (hexane) cm/sec	20.8	35.78
Initial temperature	150°C	200°C
Programme rate	4°C/min	4°C/min
Means of peak detection	Flame ionisation detector	Flame ionisation detector.
Standard used	Bacterial acid methyl esters CP™ Mix	RM-1 1084, NHI-F 1092, GLC-60, GLC-100.

5.2.4.5 Identification of unknown peaks

To identify those peaks that did not correspond with any FAME present in either of the two standards used, samples were reanalysed using a GC 5890 series II+ (Hewlett & Packard) chromatograph with the same HP-1 column. This time however, the GC was used in conjunction with a Hewlett Packard 5989B Quadrapole mass spectrophotometer, to identify peaks by electron impact mass spectrometry. Conditions for the HP-1 column were as detailed in table 5.3 with the exception of the flow rate being controlled by a constant pressure sensor. The source temperature was 260°C (max 375°C) and a quad temperature of 100°C (max 150°C). The mass:charge ratio range scanned was between 95 and 320, a full 50 higher than the largest fatty acid likely to be present.

5.2.5 Whole cell lipids

5.2.5.1 Preparation of whole cell lipids

A volume of 500ml of overnight culture from each strain was centrifuged at 9630 x *g* for 10-minutes and resuspended in 30ml of ddH_2O. Chloroform and methanol were mixed with the bacterial suspension in the ratio of 1 part chloroform, 2 parts methanol and 0.8 parts bacterial suspension. This solution was mixed, covered with aluminium foil and left to stand overnight. Chloroform and water were added to the solution in the proportions; 1 part water, 1 part chloroform and 1 part solution. This was mixed and allowed to separate into organic and aqueous phases. The bottom phase was carefully removed and dried using a rotary evaporator. The lipid residue was dissolved in a 1ml solution of 2 parts methanol, 1 part chloroform and stored at –20°C in a glass vial with a foil lined cap.

5.2.5.2 Separation of whole cell lipids

The lipids were separated by thin layer chromatography. Spots of approximately 100µl of each lipid solution were spotted onto the bottom of a silica coated aluminium chromatography plate (20 x 20cm, layer thickness 200µm, particle size 2-25µm, pore size 60Å, Sigma) that acted as the stationary phase, using a microcapillary tube. In addition, samples of authentic common phospholipids were spotted at equal concentrations. The plate was allowed to dry fully at room temperature. The mobile phase (25 parts methanol, 65 parts chloroform and 4 parts water) was left to equilibrate in the glass chromatography tank, sealed with a glass lid for at least an hour. This allowed the atmosphere within to saturate with mobile phase vapour.

The dry plate was placed with the glass tank, the mobile phase settling below the spotted samples. The tank was sealed until the solvent front had progressed nearly to the top of the plate.

5.2.5.3 Visualisation of lipids.

Two methods of visualisation were used. For lipids containing amino groups, plates were sprayed with 0.2% v/v ninhydrin in ethanol and dry heated to 110°C for up to 15-minutes. Phospholipids were visualised by spraying with molybdenum blue spray, a solution of 1.3% w/v molybdenum oxide in 4.2M H_2SO_4 (Sigma), onto the dry plates. Once the plates had been developed an image of them was recorded using a UVP scanner. The image visualised on the scanner was saved to disk and this was used in the Phoretix gel analysis program to determine the proportions of each lipid in the cell samples. Those plates sprayed with ninhydrin were not used for determining proportions of lipids, but were used to confirm the presence of phosphotidylethanolamine and to putatively identify its lyso form.

5.2.6 Hydrophobicity assay

Cells from overnight cultures were centrifuged at 10,000 x g for 5 minutes and resuspended in PUM buffer (2.22% w/v K_2HPO_4, 0.726% w/v KH_2PO_4, 0.18% w/v Urea, 0.02% w/v $MgSO_4$). Cells were re-centrifuged and resuspended in PUM buffer to a final optical density of 0.5 at 470nm. Samples of 1.25ml were taken in triplicate and transferred to acid washed test tubes. A volume of 200µl of hexadecane was added and left at room temperature for 10-minutes. Each tube was vortexed for 45-seconds and left to stand for a further 15-minutes at room temperature. The top layer containing hexadecane and any cells that had partitioned from aqueous to organic phase, was carefully removed by pipetting. The tubes were then incubated at 5°C for 15-minutes. While still at 5°C the hardened crust of residual hexadecane was removed from the top of each tube by a wire loop. The tubes were allowed to remain at room temperature for 15-minutes after which the optical density of the remaining solution was measured at 470nm and compared to that of cells left untreated by hexadecane.

5.2.7 Cell surface charge

Each strain was grown for between 19-23 hours in nutrient broth then diluted to 2-4x10^7cells/ml in 10mM KCl. This cell solution was injected into the measuring cell of a Zetamaster Particle Electrophoresis Analyser, (Malvern Instruments). Measurements of zeta potential and electrophoretic mobility were taken of the bacterial cells according to the manufacturer's information. In addition, the wild type strain was examined at a range of pHs by carefully adding HCl or KOH to 10mM KCl.

5.2.8 Uptake/Binding of BKC

Cells from overnight cultures were centrifuged at 10,000 x *g* for 10-minutes and resuspended to a concentration of approximately $1x10^7$ cells/ml in 0.1M PB, pH 7.6. Cells were treated with 0.003% w/v BKC for 10-minutes at room temperature. Cells were then removed by centrifugation at 10,000 x *g* for 10-minutes and 5ml of the supernatant was transferred to a clean sealable test tube. 0.33ml of ethanol was added to the tube and vortexed for 10 seconds. An aliquot of 1.7ml of 0.2M sodium bicarbonate buffer, pH 9.25, was added and the tube vortexed. A 1ml volume of 0.5mg/ml orange II (sodium p- (2-hydroxy-1-naphthylazo) benzenesulphonate) was added and mixed. A 4ml volume of chloroform was added, vortexed for 1 minute and left to stand at room temperature for 10-minutes for the organic and aqueous phases to separate. The upper organic phase was removed by careful pipetting and its absorbance at 485nm measured using chloroform as a blank. PB containing 0.003% BKC alone and cells not treated with disinfectant were used as controls.

5.2.9 Permeability assay using N-phenyl-naphthalene (NPN)

NPN was dissolved in acetone to a concentration of 500μM. It was used at a final concentration of 10μM when added to stationary phase bacteria ($5x10^7$ cells/ml) in 0.1M phosphate buffer, pH 7.6. The final concentration of acetone was 2% v/v. These cells had been pre-treated for 30-min with either 0.003% w/v BKC or EDTA at 400μg/ml. Control experiments showed no change in viability or permeability of the outer membrane due to the added acetone alone. After a 15-min incubation at 22-25°C with NPN, the fluorescence of each sample was measured using a Perkin Elmer spectrophotometer. Excitation and emission wavelengths were set at 350 and 420nm

respectively with slit widths of 5nm (Lambert, 1991). Both compounds tested were examined for fluorescence in the absence of cells.

5.2.10 Aminoglycoside resistance in strain 0014

5.2.10.1 Plasmid Preparation

A 500ml sample of overnight culture was centrifuged at 5000 x g for 10-minutes at 25°C. The pellet was resuspended in 15ml of cell resuspension solution (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100μg/ml RNase A). A 15ml aliquot of cell lysis solution (0.2M NaOH, 1% w/v SDS) was added and mixed gently, without vortexing. Cell lysis was considered complete after approximately 20-minutes, when the solution became clear and viscous. A further 15ml of neutralisation solution (1.32M potassium acetate, pH 4.8) was added and mixed by gentle inversion. The neutralized solution was centrifuged at 14,000 x g for 5-minutes at room temperature. The supernatant was filtered using Whatman® No.1 paper and its volume measured. Room temperature isopropanol was added to 0.5 volume of the filtered solution and mixed by inversion. The solution was centrifuged at 14,000 x g for 15-minutes at room temperature. The pellet of DNA was resuspended in 2ml of TE buffer (10mM Tris-HCl, pH7.5; 1mM EDTA). The DNA solution was mixed with 10ml of Wizard™ Maxipreps DNA Purification Resin. A Maxicolumn Wizard™ was attached to a vacuum source and the resin/DNA mix transferred into the column. The mix was pulled into the column by applying a vacuum. A 25ml aliquot of column wash solution was added to the maxicolumn and pulled through by again applying a vacuum. The resin was rinsed by drawing 5ml of 80%(v/v) ethanol through the column by applying a vacuum for 1-minute. The column was centrifuged

for 5-minutes at 1,300 x g. The Maxicolumn resin was dried by drawing a vacuum through it for 5-minutes. The column was placed in the supplied 50ml screw cap tube. 1.5ml of 65-70°C water was added to the column and left to stand for 1 minute. Centrifuging the column at 1,300 x g for 5-minutes eluted the DNA. The elute was filtered using a 0.22μm syringe filter and stored at –20°C.

5.2.10.2 Spectrophotometric Quantitation of Plasmid DNA

Accurate measurement of the DNA concentration of samples was performed by measuring the optical density of 10μl of the sample diluted into 1ml of sterile deionised water. The concentration was determined by measuring the optical density at 260nm based on an OD_{260} of 1.0 for 50μg/ml DNA and the purity of the DNA was measured by calculating the ratio of the optical density at 260nm and 280nm. A pure sample of DNA had a 260:280 absorbance ratio of 1.8.

5.2.10.3 Determination of plasmid size

A stock solution of TAE was produced in advance (0.04M Tris acetate, 0.001M EDTA). A 75ml volume of 1.25% w/v molten agarose was produced by melting 9.375g of molecular biology grade agarose in 75ml of 1x TAE using an 850W microwave oven at full power, with pauses to mix the solution. Once the agarose had cooled to approximately 50°C ethidium bromide was added to a final concentration of 0.5μg/ml and the solution cast as a gel and left to cool at 4°C until solid. Samples of DNA were mixed with loading buffer and deionised water as shown in table 5.4. A 15μl volume of each sample was loaded on the gel, which was electrophoresed in 1xTAE buffer at 65volts for 1-hour. The gels were visualised under ultraviolet light, using a UVP scanner. The image visualised on the scanner

was saved to disk and this was used in the Phoretix 1D 4.01 gel analysis program. The program compared the position of the bands in the test lanes with those in the molecular marker lane, which were of covalently closed circular DNA of known sizes to determine the size of bands in the strains. Other lanes containing plasmids of known sizes were used for further comparison. In addition the software allowed densitometry measurements to determine relative amounts of each DNA band.

Table 5.4 Sample contents for plasmid preparations run on 1.25% w/v agarose gel.

Sample	WT	P125	WT	P125	Ladder	Ladder	λ Hind III
Volume DNA	5μl	5μl	10μl	10μl	2μl	4μl	8μl
Volume Buffer	4μl	4μl	4μl	4μl	4μl	4μl	4μl
Volume deionised water	5μl	5μl	0μl	0μl	8μl	6μl	2μl

5.3 Results

5.3.1 MIC determination for BKC with addition of reserpine

The addition of reserpine to media containing BKC used in an MIC experiment had no significant effect upon the MIC when compared to a parallel experiment without reserpine. This was the case for all passages of strains OO14 and PAO1. The data are recorded in tables 5.5 and 5.6 and alterations in MIC/MLC recorded in table 5.7.

Table 5.5 Effect of reserpine on the MIC (% w/v) and MLC (% w/v) of BKC to adapted cells of PAO1.

	MIC/MLC in % w/v no reserpine added				MIC/MLC in % w/v reserpine added			
Passage	MIC1	MIC2	MLC1	MLC2	MIC1	MIC2	MLC1	MLC2
Wt	0.0125	0.0125	0.05	0.05	0.025	0.0125	0.05	0.025
P1	0.0125	0.0125	0.05	0.025	0.025	0.0125	0.1	0.025
P2	0.0125	0.0125	0.025	0.025	0.025	0.0125	0.025	0.025
P3	0.025	0.0125	0.025	0.05	0.025	0.0125	0.025	0.025
P5	0.025	0.006	0.05	0.0125	0.025	0.003	0.025	0.0125

Table 5.6 Effect of reserpine on the MIC and MLC of BKC to adapted cells of OO14.

	MIC/MLC in % w/v no reserpine added				MIC/MLC in % w/v reserpine added			
Passage	MIC1	MIC2	MLC1	MLC2	MIC1	MIC2	MLC1	MLC2
WT	0.025	0.025	0.1	0.05	.025	0.025	0.1	0.05
P1	0.0125	0.05	0.025	0.1	.0125	0.025	0.1	0.05
P2	0.0125	0.05		0.1	.0125	0.025	0.025	0.05
P3	0.05	0.05	0.1	0.1	0.0125	0.025	0.025	0.1

Table 5.7 Collation of data shown in tables 5.5 and 5.6.

	Effect on MIC/MLC of reserpine on passages of PAO1				Effect on MIC/MLC of reserpine on passages of OO14			
	MIC1	MIC2	MLC1	MLC2	MIC1	MIC2	MLC1	MLC2
WT	↑2	Equal	Equal	↓2	Equal	Equal	Equal	Equal
P1	↑2	Equal	↑2	Equal	Equal	↓2	↑4	↓2
P2	↑2	Equal	Equal	Equal	↑2	↓2	Equal	↓ 2
P3	Equal	Equal	Equal	↓2	↓4	↓2	↓ 4	Equal
P5	Equal	↓2	↓2	Equal	ND	ND	ND	ND

"↑" denotes a fold increase in MIC/MLC, "↓" denotes a fold decrease in MIC/MLC and "Equal" denotes no change in MIC/MLC from that of cells without reserpine added.

It would appear from the results in table 5.7 that there was very little effect of reserpine on the sensitivity of PAO1 or OO14 cells to BKC suggesting no involvement of efflux systems inhibited by reserpine.

5.3.2 Outer membrane proteins

Samples from passages of strains PAO1 and OO14 were prepared for electrophoresis by either heating in sample buffer for 10-minutes at 88°C or 30-minutes at 37°C as detailed in section 5.2.2.1. SDS electrophoresis gels were run, stained with Coomassie blue and the better resolved of the gels produced used for further analysis (figures 5.3 and 5.4). However, care was taken when identifying outer membrane components due to the heat modifiable nature of certain outer membrane proteins.

5.3.2.1 PAO1

175kD
83kD
62kD
47.5kD
32.5kD
25kD
16.5kD
6.5kD

Unk A
Unk C
Unk D
OprF
OprG
H1/H2
Unk F
Unk G

Ladder WT P1 P3 P4 P5 P6

Figure 5.3 SDS PAGE gel illustrating outer membrane material of strain PAO1 prepared by heating in denaturing at 37°C for 30-minutes, the gel stained with Coomassie blue. Where possible bands are identified according to their molecular weight, other bands are denoted Unk.

Outer membrane material prepared by heating in denaturing buffer at 88°C for 10-minutes and run on an identical gel to that observed in figure 5.3 is shown in figure 5.4

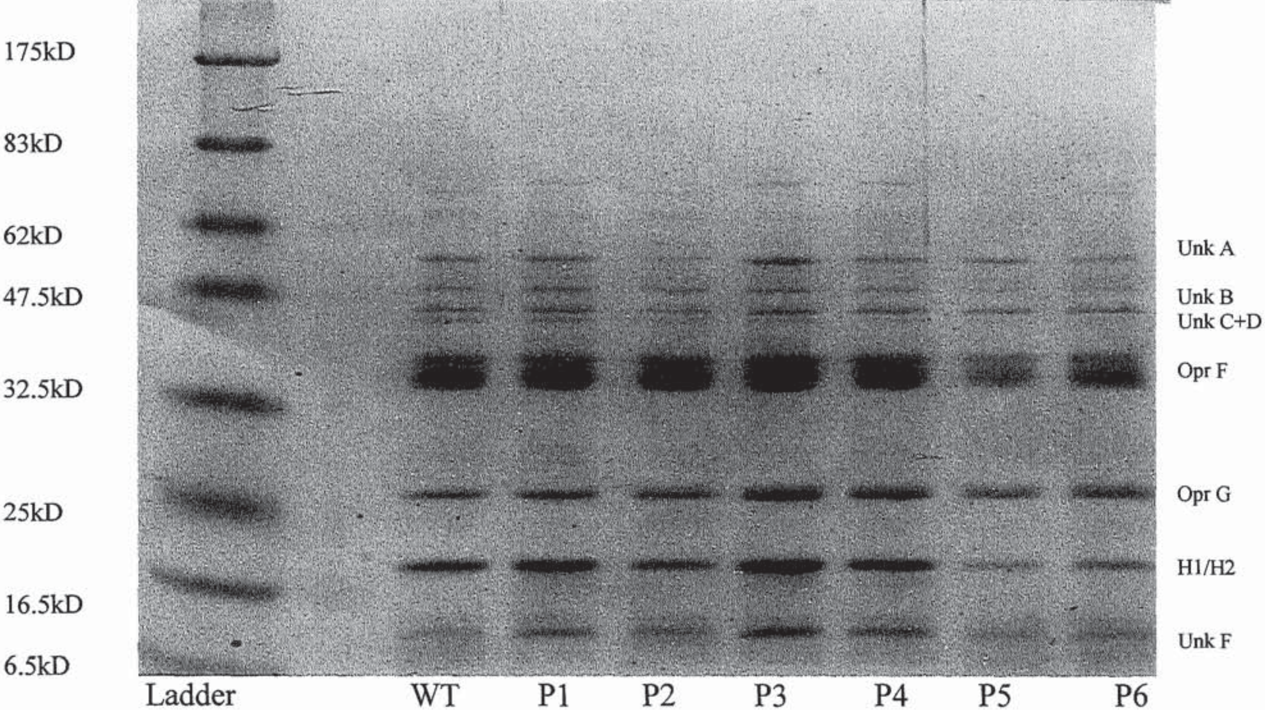


Figure 5.4 SDS PAGE gel illustrating outer membrane material of strain PAO1 prepared by heating in denaturing buffer at 88°C for 10-minutes, the gel stained with Coomassie blue. Where possible bands are identified according to their molecular weight, other bands are denoted Unk.

Each lane of the gels was examined by use of Phoretix software to determine the proportion of outer membrane material each band made up in each sample. In addition, comparisons were made between the protein ladder and the bands present to determine the molecular weights. An example of such analysis is presented in figure 5.5.

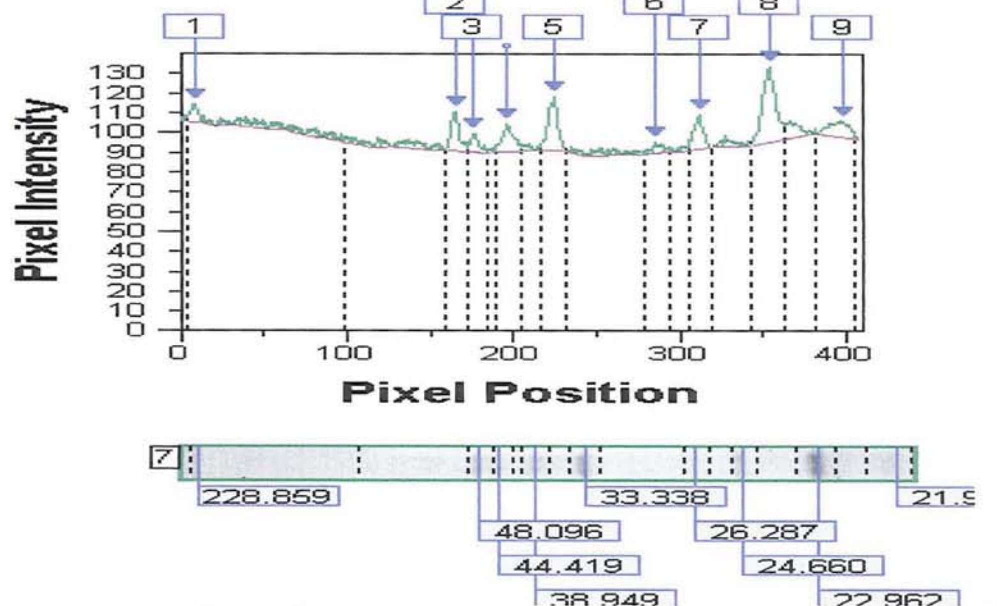


Figure 5.5 Example of densitometric analysis of protein profile for strain PAO1 (figure 5.3 lane containing sample P6) using Phoretix software.

This was repeated for all gels produced. Sample data is shown in table 5.8

Table 5.8. Data showing the molecular weight and relative band density of proteins in outer membrane preparations of strain PAO1 prepared at 37°C for 30-minutes. Band density is recorded as the relative peak area (RPA) of each band as determined in figure 5.5.

WT		P1		P2		P3		P4	
RPA	Mw	RPA	Mw	RPA	Mw	RPA	Mw	RPA	Mw
9192	139	14807	48.2	27524	114.8	14569	46.7	6615	48.6
15052	48.2	4877	37.3	8346	36.12	6267	37.1	6526	38.6
6420	37.8	6325	35.4	9050	30.1	5074	34.9	4885	35.6
9181	35.6	5387	30.6	30990	28.2	10214	30.3	8591	30.6
11683	30.8	19100	28.2	5295	20.9	26283	28.2	30018	28.8
29354	28.8	6058	18.4	7827	18.4	4937	22.7	2274	26.2
49186	18.2	25590	18.1	20142	18.1	9212	20.9	3661	22.8
16323	17.3	10453	17.2	8223	17.26	14665	18.4	10564	21.0
						35372	18.2	39991	18.2
						18939	17.3	15540	17.4

P5		P6		WT MP *	
RPA	Mw	RPA	Mw	RPA	Mw
8457	48.9	3671	50.9	3518	117.6
6693	38.1	3468	44.6	5993	81.37
6450	31.1	7859	38.9	4384	65.4
17822	28.7	10047	31.1	2646	49.163
6565	20.9	25622	29.3	7403	42.1
17785	18.4	8548	21.1	5654	37.43
12314	17.4	7183	18.6	17472	27.01
		25638	18.3	13101	20.6
		15614	17.4	43247	18.6

MP refers to P6 after passage for 6-weeks in BKC free media as described in section 4. *indicates sample run on a gel other than that shown in figure 5.3. Mw refers to molecular weight of each band in KDa

Bands of above 50 KDa were ignored when calculating the approximate proportion of each samples outer membrane proteins.

By comparing the molecular weights of bands observed to those in the literature (Hancock & Carey, 1979; Hancock *et al.*, 1990) it was possible to identify a number of the outer membrane proteins present as shown in figures 5.3 and 5.4. These data, and the proportion of each protein as a percentage of the whole outer membrane protein material, are shown in table 5.9 and 5.10 for sample denaturing conditions of 37°C for 30-minutes and 88°C for 10-minutes respectively.

Table 5.9 Proportion of outer membrane proteins denatured at 37°C for 30-minutes, in strain PAO1, their molecular weight and, where possible, putative identity.

Protein	WT	P1	P2	P3	P4	P5	P6	MP
	Proportion % of total outer membrane proteins.							
Unkn A	10.28	15.99		10.01	5.14	11.11	3.4	2.96
Unkn C+D	6.27	6.83	9.2	7.78	8.86	8.79	7.3	6.31
OprF	20.05	20.63	34.5	18.06	23.3	23.42	23.8	19.52
OprG	-	-	5.89	6.33	8.21	8.63	7.94	14.63
H1/H2	41.59	39.99	41.18	41.39	37.75	31.85	39.8	48.3
Unkn F	11.15	11.29	9.15	13.01	17.36	16.18	14.5	
Unkn G	6.28	-	-	-	-	-	-	

Unknown A =MW 46-50
Unknown C+D = MW 35-38
Unknown F = MW 17.3 possibly OprI
Unknown G= MW 13.9

The results indicated an alteration in the proportion of the band putatively identified as OprG in the outer membrane of bacteria of increasing resistance to BKC. The proportion of OprG rose from 0% in the Wild Type to 14% in the most resistant strain following passage in BKC free media for 6-weeks.

Table 5.10 Proportion of outer membrane proteins denatured at 88°C for 10-minutes, in strain PAO1, their molecular weight and, where possible, putative identity.

	WT	P1	P2	P3	P4	P5	P6	MP
	Proportion (%) of total outer membrane proteins.							
UnkA	5.2	5.2	1.7	5.5	3.7	7.4	3.3	3.0
UnkB	3	3.2	4.3	3.1	2.4	4.5	3.8	8.3
UnkC	4.6	4.6	3.9	4	4.2	8.7	6.9	6.3
UnkD	2.6	1.2	2.4	1.3	1.2	0	0	0
OprF	41.8	35.1	40.45	32.9	38.5	35.7	46.4	19.5
OprG	12	14.7	14.8	16.1	15.7	20.8	19.58	14.6
H1/H2	21.2	23.8	12.8	25.1	19.1	10.9	16	48.3 **
Unkn F	9.6	11.1	19.5	11.8	15.0	11.7	4	

Unknown A= 49-51 MW
Unknown B= 42-44 MW
Unknown C= 38-40 MW
Unknown D= 36-37MW
Unkn F = 17 MW possibly OprI
** this value refers to H1/H2 and Unknown F, separation of values was not possible.

In samples of PAO1 denatured at 88°C for 10 minutes OprG showed an increase in proportion from 12% to 20% but after 6-weeks of passaging in BKC-free broth the proportion fell to 14.6%. In addition, there was a reduction in a protein of approximately 36-38kDa (Unk D) from 2.6% of the total protein bands, to a complete absence. This second alteration was visible in samples prepared at 37°C for 30-minutes but not discernable by the software.

5.3.2.2. Strain OO14

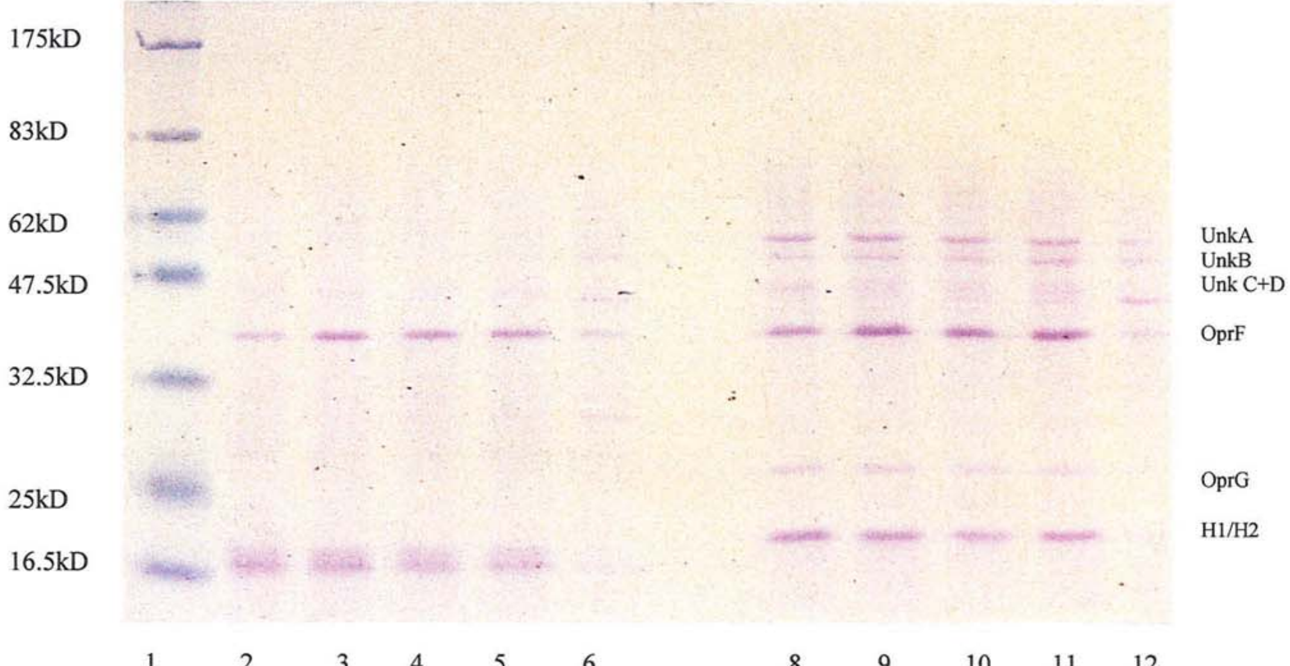


Figure 5.6. SDS PAGE gel illustrating outer membrane material of passaged cells of strain OO14 prepared by heating at 88°C for 10-minutes, stained with Coomassie blue (lanes 2-6) and 37°C for 30-minutes (lanes 8-12) Where possible bands are identified by their molecular weight, other bands are termed Unk.

The density of bands in lanes 8-12 (WT, P1, P2, P3, P5) was calculated by determining the area of the peak corresponding to each band as described when using Phoretix software, and the identity and proportion of proteins bands determined as with PAO1. These data are shown in table 5.11. Lanes 2-6 did not give sufficiently clear results to determine band proportions even upon repetition.

Table 5.11 Proportion of outer membrane proteins in strain OO14, their molecular weight and, where possible putative identity.

Protein	WT	P1	P2	P3	P5	P6*	MP*
Proportion % of total outer membrane proteins.							
Unkn A	10.3	9.76	9.45	8.05	9.56		11.67
Unkn B	4.87	5.46	5.25	7.89	12.09	14.18	14.7
Unkn C	9.57	8.07	4.29	1.278	15.83	24.37	19.34
Unk D	3.216	2.7					
OprF	16.42	26	21.65	24.36	18.12	29.63	22.14
Unk E		1.7	3.74	8.18			
Opr G	10.1	12.5	20.2	11.09	18.06		
H1/H2	31.5	27.57	32.7	32.37	26.3	31.82	32.13

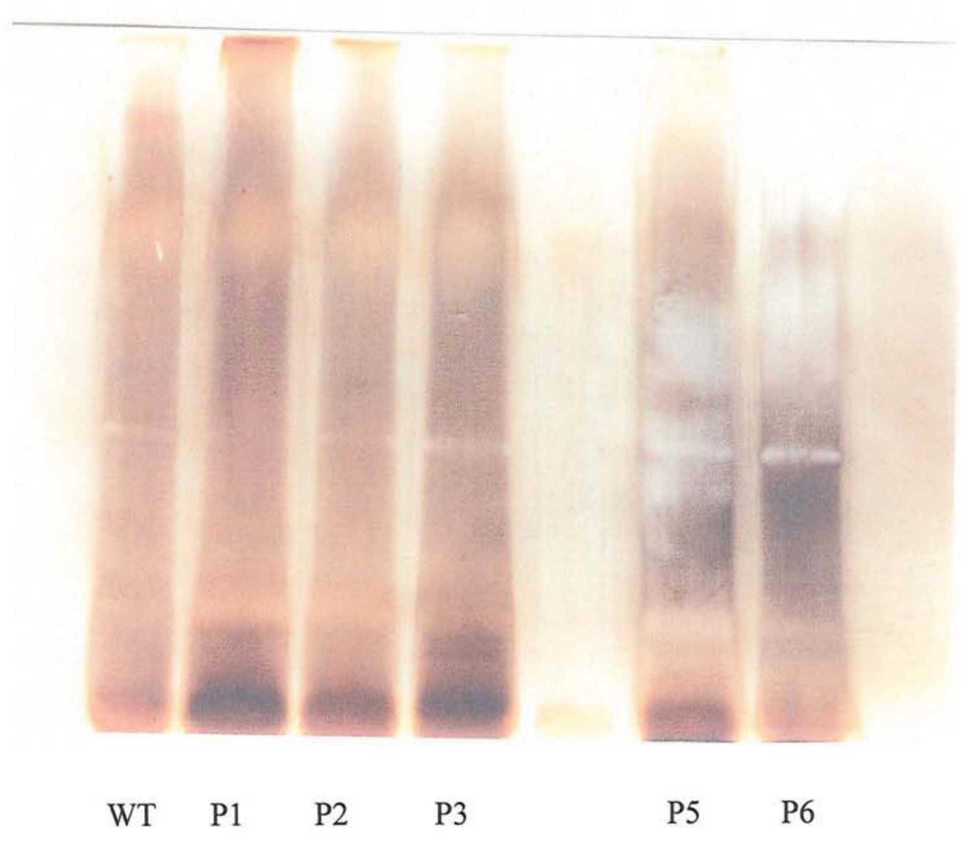
Unknown A =MW 46-50
Unknown B = MW 44
Unknown C = MW38-39
Unknown D = MW 35.88-38
Unknown E = MW 26.2

* indicates data collected from gel photo not shown.

SDS PAGE of outer membrane material indicated an alteration in the proportion of a protein of MW 44 KDa, tentatively identified as OprE, in the outer membrane of bacteria of increasing resistance to BKC. The proportion of OprE rose from 4% in the Wild Type to 14.18% in the most resistant passage. This strain showed no detectable trend in the proportion of OprG in the outer membrane although a protein of the same MW as UnkD in PAO1 also reduced in proportion, 3.26% to absence.

5.3.3 Alterations in lipopolysaccharide

No discernible alteration in banding patterns was observed in LPS samples of proteinase K-digested outer membrane material of OO14 from wild type to passage 5 (figure 5.7). However, it was not possible to visualise a banding pattern for LPS from PAO1, despite numerous attempts. This has been reported by other workers (Kropinski *et al.*, 1985) and it is thought to be due to the lack of periodate cleavable sugar residues on the LPS of strain PAO1 since these sugar residues are the basis for the silver staining technique used to visualise LPS.



WT P1 P2 P3 P5 P6

Figure 5.7 Banding pattern of LPS of strain OO14 proteinase K-digested outer membranes on SDS-PAGE revealed by silver staining.

There appeared no discernible alteration in the banding pattern of the LPS of strain OO14 as it became more resistant to BKC.

5.3.4 Keto-3-deoxyoctonate (KDO) assay.

A calibration curve was generated in order to quantify how much KDO was present in each sample of outer membrane protein harvested from the passaged strains PAO1 and OO14. Each sample of outer membrane examined was a volume of 2ml of 1mg/ml outer membrane material. Therefore the quantity of KDO detected was measured in µg/2mg outer membrane material. Results are shown in figures 5.8 and 5.9 for PAO1 and OO14 respectively.

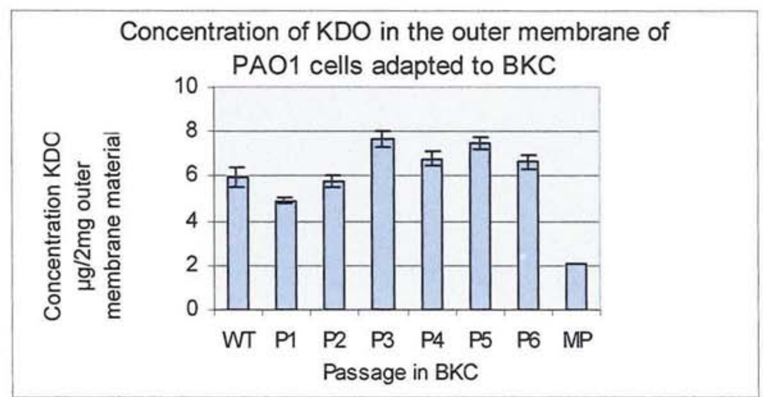


Figure 5.8 Concentration of KDO present in outer membrane preparations of BKC adapted PAO1 cells

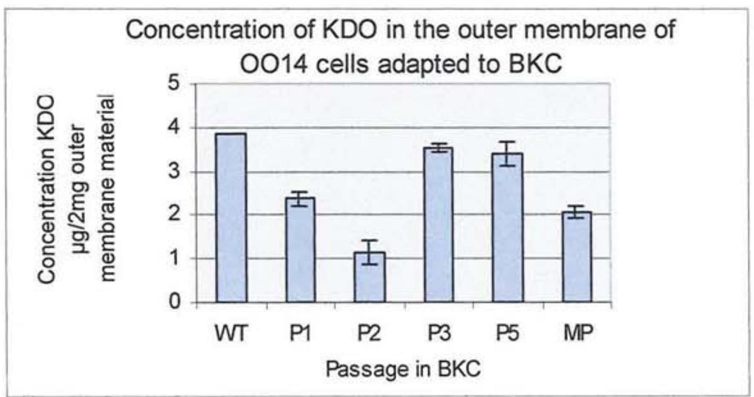


Figure 5.9 Concentration of KDO present in outer membrane preparations of BKC adapted OO14 cells

No significant change in the quantity of KDO contained in the outer membrane material of strain OO14 or PAO1 was observed, as they became more resistant to BKC. The outer membrane material of those resistant cells grown in disinfectant-free media showed a reduction in the quantity of KDO present in both cases.

5.3.5 Fatty acids of outer and cytoplasmic membranes of strains PAO1 and OO14 of P. aeruginosa

The proportion of each fatty acid was determined by examining the area of peaks produced by gas chromatography. Proportions were calculated from peaks between a retention time of 3-minutes and approximately 25-minutes. Figure 5.9 shows the standard trace while fig 5.10 shows an example trace. The fatty acid methyl esters used in the standard are listed in table 5.14 and those identified in fig. 5.10 are shown in table 5.15.

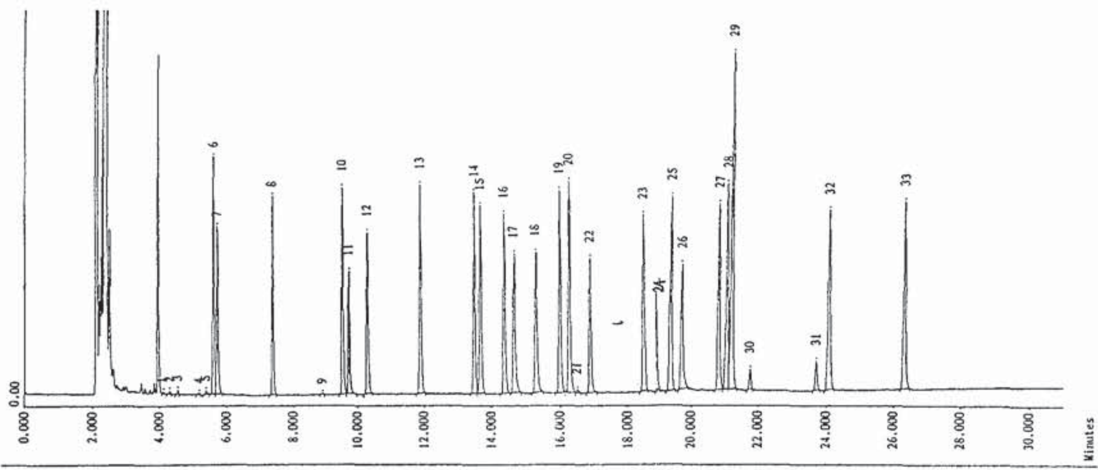


Figure 5.10 Gas Chromatography trace of Standard Bacterial fatty acid methyl esters CP^{TM} Mix. The X-axis indicates retention time in minutes and the Y-axis indicates relative concentrations of each fatty acid methyl ester.

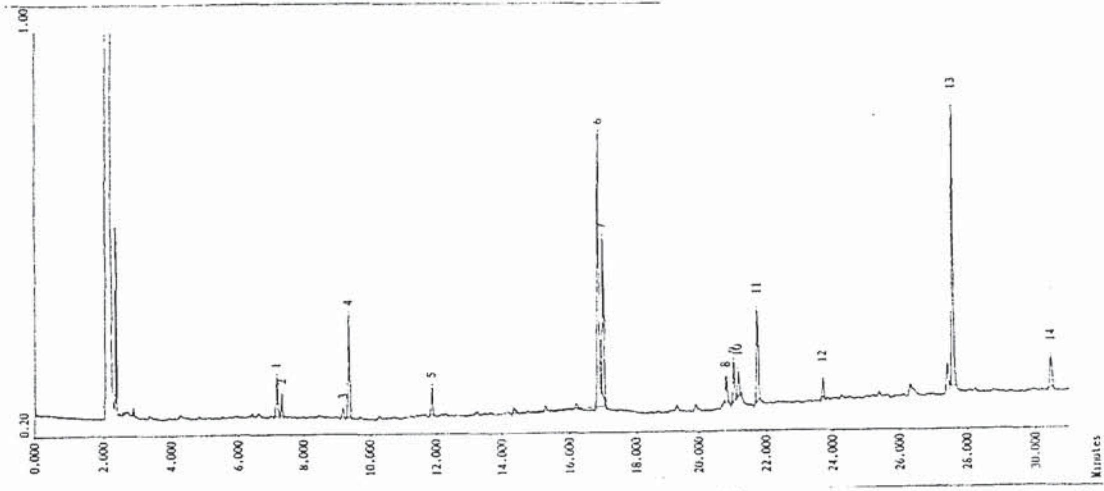


Fig 5.11 Sample trace showing methyl esters of fatty acids present in the cytoplasmic membrane of PAO1. The X-axis indicates retention time in minutes and the Y-axis indicates relative concentrations of each fatty acid methyl ester.

Table 5.12 List of fatty acid methyl esters used in the standard shown in figure 5.10.

Peak	Fatty acid methyl ester	Peak	Fatty acid methyl ester
1	False peak	18	**3-OH 14:0** 3- hydroxytetradecanoate
2	False peak	19	**i-16:0** 14-methylpentadecanoate
3	False peak	20	**16:1** cis-9-hexadecenoate
4	False peak	21	False peak
5	False peak	22	**16:0** hexadecanoate
6	**11:0** undecanoate	23	**i-17:0** 15-methylhexadecanoate
7	**2-OH 10:0** 2-hydroxydecanoate	24	**17:0Δ**cis-9,10-methyleneheaxadecanoate
8	**12:0** dodecanoate	25	**17:0** heptadecanoate
9	False peak	26	**2-OH 16:0** 2-hydroxyhexadecanoate
10	**13:0** tridecanoate	27	**18:2** cis-9,12octadecadienoate
11	**2-OH 12:0** 2-hydroxydodecanoate	28	**18:1** cis-9 octadecenoate
12	**3-OH 12:0** 3-hydroxydodecanoate	29	**18:1***trans-9octadecenoate & cis-11 octadecenoate
13	**14:0** tetradecanoate	30	**18:0** octadecanoate
14	**i-15:0** 13-methyltetradecanoate	31	**19:0Δ** cis-9,10methyleneoctadecanoate
15	**a-15:0** 12-methyltetradecanoate	32	**19:0** nonadecanoate
16	**15:0** pentadecanoate	33	**20:0** eicosanoate
17	**2-OH 14:0** 2-hydroxytetradecanoate		

Table 5.13 List of fatty acids identified from gas chromatography trace in figure 5.11

Peak number	Fatty acid	Peak number	Fatty acid
1	Unknown retention time 7.118	8	18:2
2	12:0	9	18:1
3	13:0	10	18:1*
4	2-OH 12:0	11	18:0
5	14:0	12	19:0Δ
6	16:0	13	
7	Unknown termed X	14	

The data gathered from the gas chromatograph traces of PAO1 outer and cytoplasmic membrane preparations are collected in tables 5.14 and 5.15.

Table 5.14 Relative proportions (% total) of fatty acids present in the outer membrane of PAO1 passaged strain.

Fatty acid	WT	P1	P2	P3	P4	P5	P6	MP
Rt 7.188	21.0	16.5	19.6	26.3	22.5	19.2	2.47	4.48
12:0	4.28	3.1	1.61	<0.7	2.94	2.9	3.25	3.04
13:0	2.03		2.72	<0.7	3.04			
14:0	3.42	12.9	2.82	4.47	3.99	5.8	4.55	5.28
16:0	18.6	5.74	7.36	8.94	2.49	8.6	42.8	41.0
X	48.5	57.7	46.8	57.5	49.14	61.0	11.2	19.8
18:1			3.63				8.45	4.32
18:1*							4.91	4.46
18:0	1.93	3.95	2.12	<0.7	5.145	2.4	22.1	17.1
19:Δ			13.3					

Rt 7.188 refers to an unknown fatty acid that ran at that retention time, X refers to an unknown fatty acid that ran at a retention time just later than that of 16:0. Details of fatty acid nomenclature is found in table 5.12.

Table 5.15 Relative proportions (% total) of fatty acids present in the cytoplasmic membrane of PAO1 passaged strain.

Fatty acid	WT	P1	P2	P3	P4	P5	P6	MP
Rt 7.188	24.2	21.7	24.3	20.9	11.2	17.9	6.99	19.1
12:0	3.43	3.55	5.78	6.22		6.84	3.65	3.73
13:0	2.71	2.54	2.46	2.31		1.89		3.96
14:0	3.84	3.55	3.94	4.01	6.6	4.01	6.52	8.82
16:0	15.4	15.7	21.9	23.3	25.4	29.9	38.8	31.16
X	46.1	52.4	34.4	41.9	46.2	31.0	20.3	13.17
18:1							<0.8	3.16
18:1*				0.6			4.13	5.26
18:0	4.29		3.97	0.6	10.6	5.19	18.4	11.4
19:Δ		<0.6	3.32			3.18		

Rt 7.188 refers to an unknown fatty acid that ran at that retention time, X refers to an unknown fatty acid that ran at a retention time just later than that of 16:0. Details of fatty acid nomenclature is found in table 5.12.

Following examination by mass spectrometry the fatty acid running at 7.188 was identified as having two peaks at a mass:charge ratio of 205 and 220. This indicates that Rt 7.188 is a fatty acid methyl ester no larger than C12:0. Those fatty acids with mass:charge ratios approaching 205 and 220 and are not among the standards shown in table 5.12 are C10:0 3-OH (m/c ratio 202) and C11:0 3-OH (m/c ratio 216).

In the PAO1 strain of *P. aeruginosa* there were different alterations in the outer and cytoplasmic membrane fatty acids as the strain became adapted to BKC.

In the outer membrane the largest changes occurred between P5 and P6 where there was an increase in proportion of 16:0 and 18:0, with a reduction in proportion of RT 7.188 and X.

In the cytoplasmic membrane there was a general stepwise increase in the proportion of 14:0 and 16:0 with a reduction in X. Indeed 16:0 and X were the main constituents of the cytoplasmic membrane of this strain and their relative proportions altered together as is shown in figure 5.12.

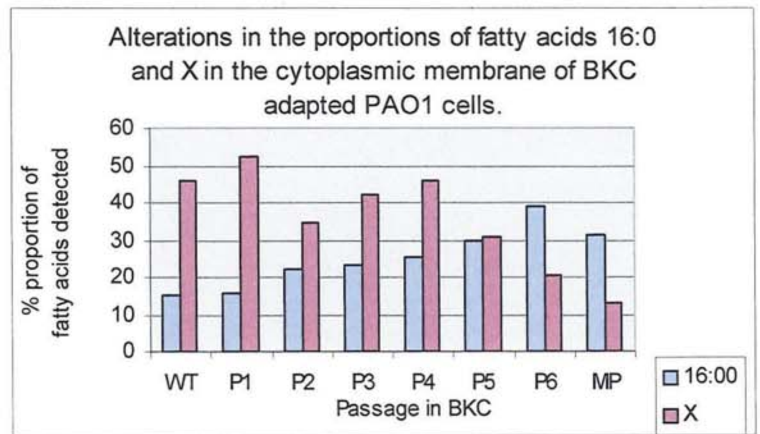


Figure 5.12 Specific alterations in the fatty acid proportions of the cytoplasmic membrane of BKC adapted cells of the PAO1 strain of *P. aeruginosa*.

The data gathered from the gas chromatograph traces of OO14 outer and cytoplasmic membrane preparations are summarised in tables 5.16 and 5.17.

Table 5.16 Relative proportions (% total) of fatty acids present in the outer membrane of OO14 passaged strain.

Fatty acid	WT	P1	P2	P3	P5	MP
Rt 7.188	3.12	11.05	9.646	7.452	3.0475	3.465
12:0 2-OH					1.6675	
12:0 3-OH			<0.91		1.84	
12:0	6.24	10.66	6.006	6.966	5.6925	2.805
13:0	46.32	29.25	24.57	17.982	6.6125	16.5
14:0	4.44	6.63	<0.91		2.76	2.64
15:0	2.88					
16:0	33.72	25.09	22.75	32.4	36.86	17.49
X	<0.6	11.31	23.114	14.58	12.36	<0.825
18:1	<0.6	<0.65	4.186	7.938	6.67	4.125
18:1*	<0.6	<0.65			2.846	
18:2						39.6
18:0	<0.6		<0.91	<0.81	9.66	12.54
19:△	<0.6	3.77	7.098	11.988	6.9	<0.825

Rt 7.188 refers to an unknown fatty acid that ran at that retention time, X refers to an unknown fatty acid that ran at a retention time just later than that of 16:0.

Table 5.17 Relative proportions (% total) of fatty acids present in the cytoplasmic membrane of OO14 passaged strain.

Fatty acid	WT	P1	P2	P3	P5	MP
Rt 7.188		12.067	6.18	4.26	10.9	9.89
12:0				3.22		
13:0	43.8	37.966	15.19	10.5	27.97	57.8
14:0	11.83	14.612	1.78	2.989		8.31
16:0	10.496	11.03	23.87	35.3	20.77	12.6
X	31.135	20.27	31.61	16.39	22.29	1.159
18:1		<0.82	3.45	5.75	<0.99	
18:1*		<0.82	2.21	5.56		
18:2		<0.82	2.62			
18:0		<0.82	9.086	12.5	14.03	8.365
19:△			2.565	2.397	<0.99	<1.159

Rt 7.188 refers to an unknown fatty acid that ran at that retention time, X refers to an unknown fatty acid that ran at a retention time just later than that of 16:0.

There appears to be no trend in the proportion of fatty acids in either cytoplasmic or outer membranes of *P. aeruginosa* strain OO14 as it became adapted to BKC.

5.3.6 Whole cell lipids.

The TLC plates were photographed (Fig. 5.13 and 5.14) and scanned for use with Phoretix software to calculate the identity of each lipid resolved and the proportion of each in the organism. The raw data and proportions calculated from the photographs are shown in tables 5.18 and 5.19.

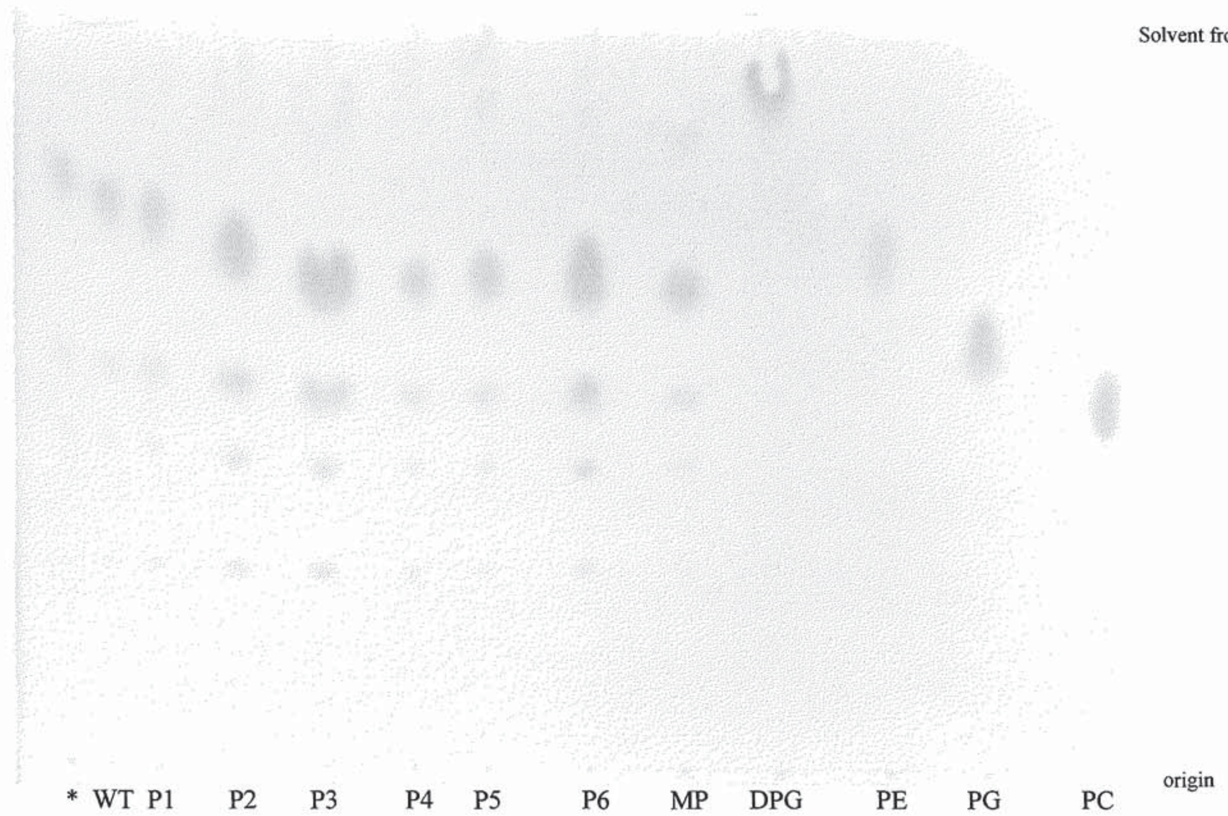


Figure 5.13 Whole cell phospholipids of adapted cells of *P. aeruginosa* strain PAO1 visualised with molybdenum blue spray. WT, P1-P6 refer to passages of strain PAO1, MP refers to passage P6 after 6 weeks of passage in BKC-free media. * refers to WT ATCC 15442 strain. PE refers to phosphatidylethanolamine, DPG refers to diphosphatidylglycerol, PG refers to phosphatidylglycerol and PC refers to phosphatidylcholine.

Table 5.18 Data collated from Phoretix software analysis of fig. 5.13 and identification of phospholipids present in BKC adapted cells of PAO1. RPA refers to the relative peak area corresponding to the density of colour of the separated phospholipids as determined by Phoretix software.

	DPG		PE		PG		PC		Lyso PE	
	RPA	%	RPA	%	RPA	%	RPA	%	RPA	%
WT	1221.5	10.5	5956.5	51.4	2469.5	21.3	905	7.8	1027.5	8.87
P1	1083	6.62	9635.63	58.9	2240.5	13.7	1783	10.9	1611	9.85
P2	2297	7.63	16313.1	54.2	5496.55	18.2	3072.5	10.2	2936.46	9.75
P3	4260	10.86	21786	55.53	7246	18.4	3133.66	7.98	2804.1	7.14
P4	595.18	3.84	8711	56.16	2316.3	14.93	1455	9.38	2433.4	15.68
P5	2148	9.46	11823.5	52.05	2471.92	10.88	1674	7.37	4595	20.23
P6	2903.7	8.86	18792.2	57.35	5880.35	17.94	2732.15	8.33	2458	7.5

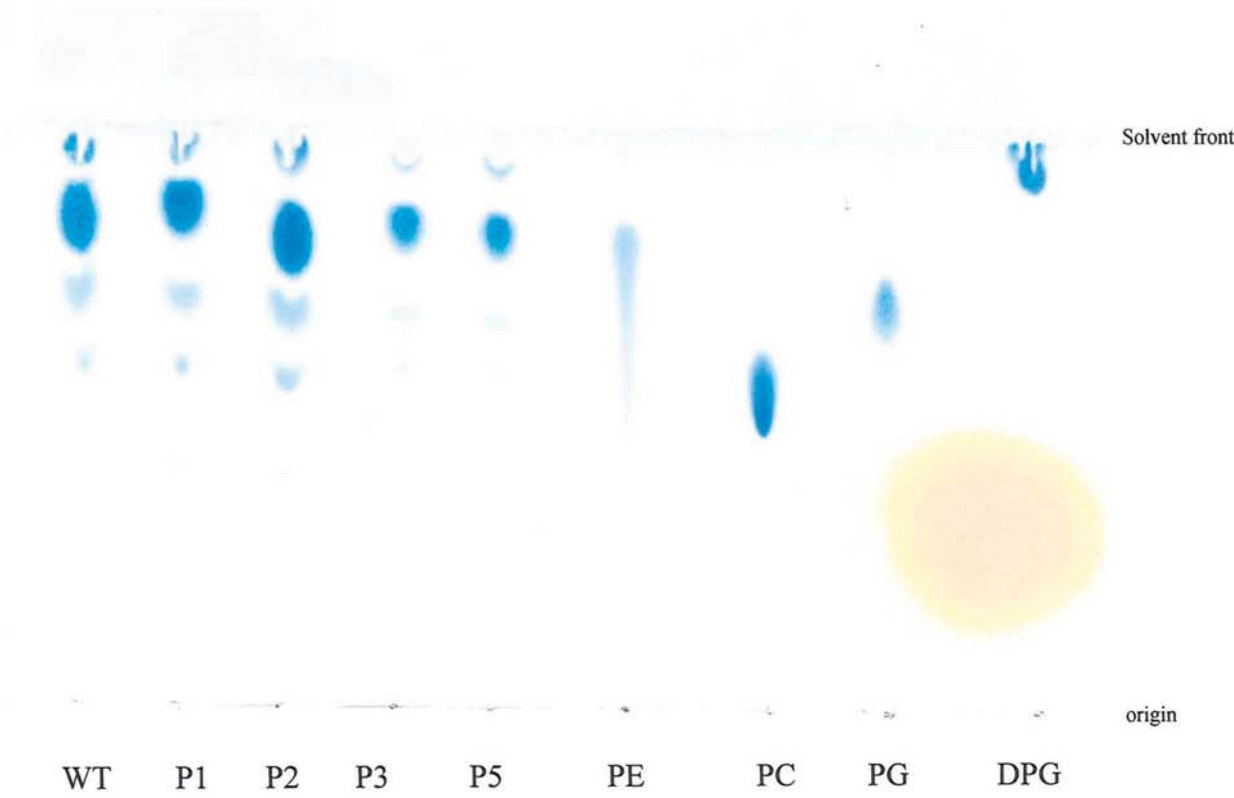


Figure 5.14 Whole cell phospholipids of adapted cells of *P. aeruginosa* strain OO14 visualised with molybdenum blue spray. WT, P1-P6 refers to passages of strain PAO1, PE refers to phosphatidylethanolamine, DPG refers to diphosphatidylglycerol, PG refers to phosphatidylglycerol and PC refers to phosphatidylcholine.

Table 5.19 Data collated from Phoretix software analysis of figure 5.14 and identification of phospholipids present in BKC adapted cells of OO14. RPA refers to the relative peak area corresponding to the density of colour of the separated phospholipids as determined by Phoretix software.

	DPG		PE		PG		PC		Lyso PE	
	RPA	%	RPA	%	RPA	%	RPA	%	RPA	%
WT	7814.52	9.84	36062.1	45.41	17877.4	22.51	16696.1	21.03	9517.5	11.98
P1	6085.22	4.44	71979.0	52.5	37063.2	27.03	15158.6	11.05	6809	4.96
P2	21979.3	10.77	90075.6	44.15	49114.0	24.07	29150.1	14.28	13710.5	6.7
P3	9937.88	7.93	70733.5	56.44	24852.9	19.83	9731.45	7.76	10073.5	8.03
P5	14744.5	11.22	71266.0	54.26	27821	21.18	8656.32	6.59	8843.75	6.730

In strain OO14 there appeared to be a reduction in the proportion of phosphatidyl choline as the cells became adapted to BKC, graphically shown in figure 5.15.

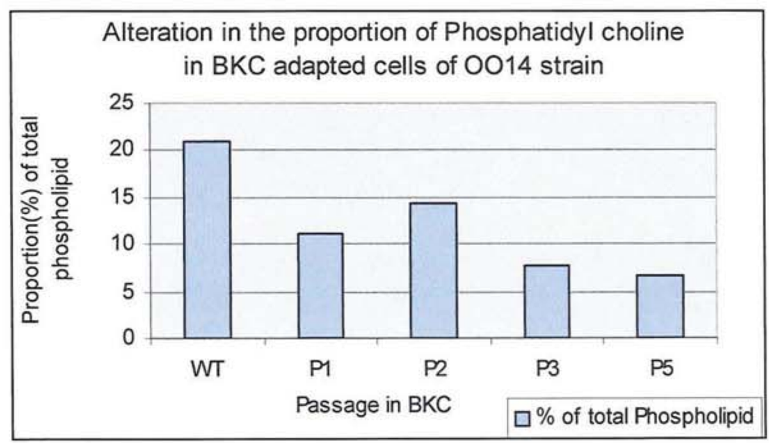


Figure 5.15 Alteration in phosphatidyl choline content of OO14 cells adapted to BKC.

No other trends of alteration in phospholipid proportions were observed in OO14 or PAO1 strains adapted to BKC.

5.3.7 Uptake binding of BKC

The uptake or binding of BKC to strain PAO1 at different stages of resistance to BKC is shown in figure 5.16.

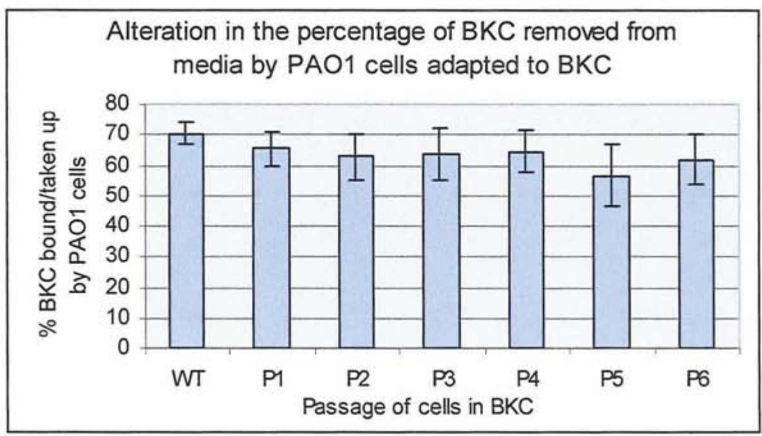


Figure 5.16 Alterations in the % of total (0.003% w/v) BKC removed from media by PAO1 cells after 10-minutes exposure and subsequent centrifugation.

Overall there was a trend that uptake or binding of BKC was decreased as PAO1 cells became more resistant to BKC although this trend was very slight.

The uptake or binding of BKC to strain OO14 at different stages of resistance to BKC is shown in figure 5.17.

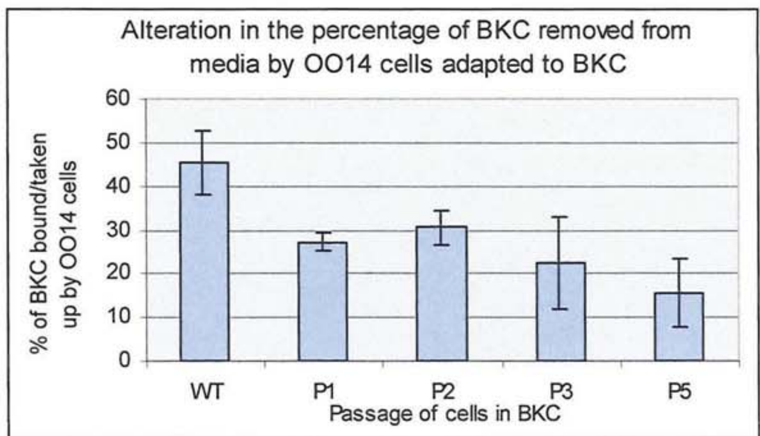


Figure 5.17 Alterations in the % of total (0.003% w/v) BKC removed from media by OO14 cells after 10-minutes exposure and subsequent centrifugation

Overall there was a trend that uptake or binding of BKC decreased as the OO14 cells became more resistant to BKC.

5.3.8 Hydrophobicity

The cell surface hydrophobicity of PAO1 and OO14 cells was examined by a MATH assay measuring % cell partitioning from an aqueous to a hydrophobic (hexadecane) phase. Results are shown in Figures 5.18 and 5.19.

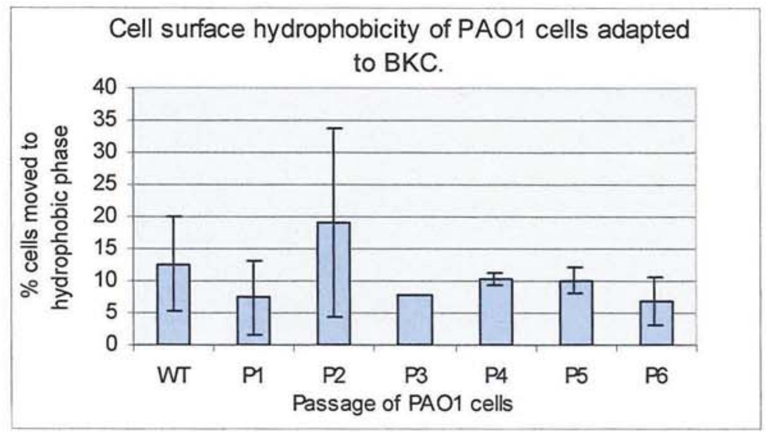


Figure 5.18 Cell surface hydrophobicity of PAO1 cells adapted to BKC.

There would appear to be little connection between cell surface hydrophobicity of PAO1 and its resistance to BKC.

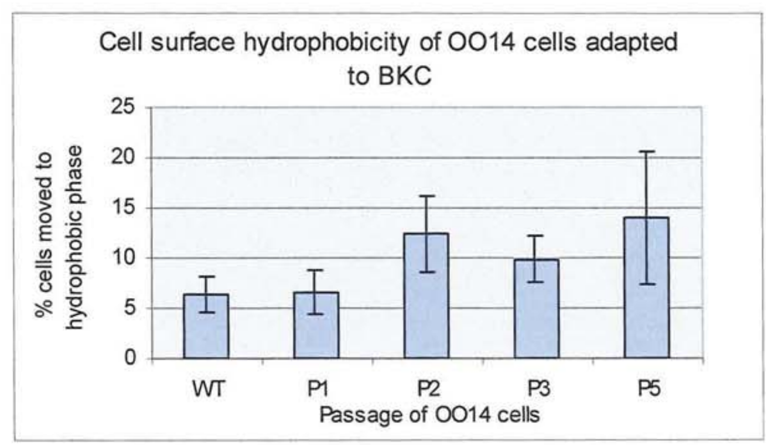


Figure 5.19 Cell surface hydrophobicity of OO14 cells adapted to BKC.

Overall it would appear that increased resistance to BKC in strain OO14 was accompanied by an increase in cell hydrophobicity measured by this method.

5.3.9 Cell surface charge

The cell surface charge of cells of strains OO14 and PAO1 adapted to varying levels of BKC, was determined. These data are displayed graphically in figures 5.20 and 5.21.

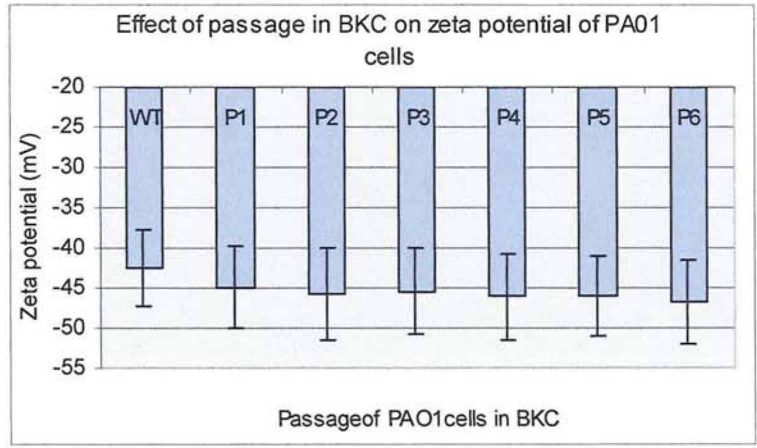


Figure 5.20 Zeta potential of PAO1 cells adapted to BKC.

While there were differences in the range of zeta potential in each replicate experiment, in each case as the cells were adapted to BKC the zeta potential became more negative. Alterations between experiments may have been due to slight differences in the pH of the 10mM KCl media used to suspend the cells in (investigated further in section 5.3.9.1).

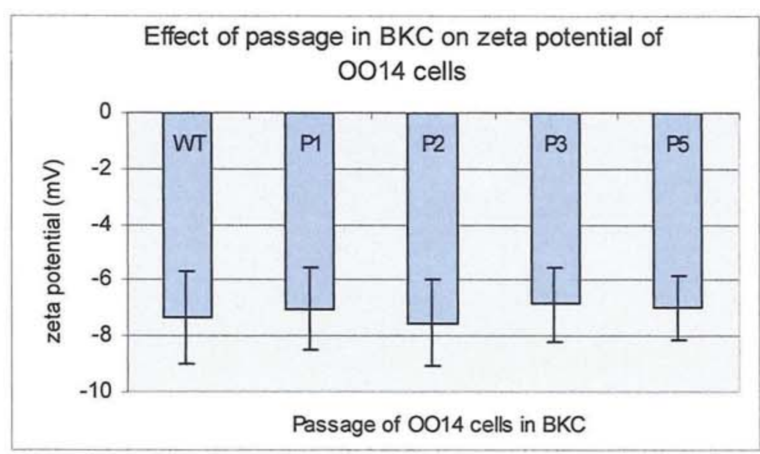


Figure 5.21 Zeta potential of OO14 cells adapted to BKC.

There appeared to be no link between zeta potential of OO14 cells and the level of adaptation to BKC that they had achieved.

5.3.9.1 Effect of pH on cell surface charge.

It is known that pH of the media has a profound effect upon the cell surface charge of a micro-organism (James, 1991). In order to determine the effect in this work non-adapted cells of the two strains were resuspended in media of various pHs. The surface charges of these cells were recorded and the data graphically illustrated in figure 5.22 and 5.23.

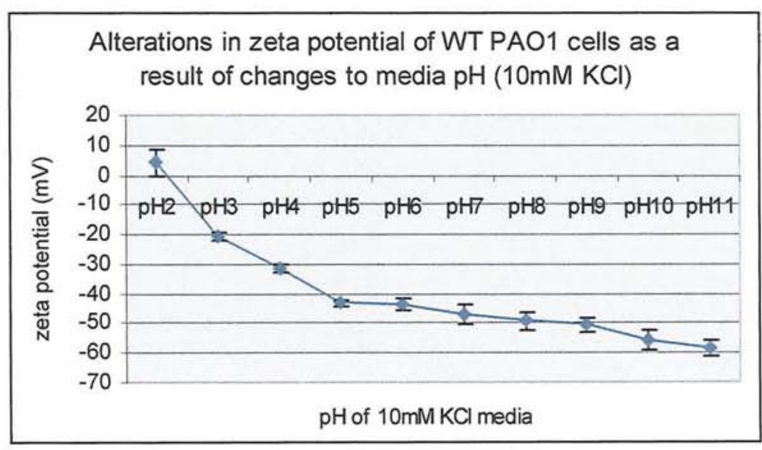


Figure 5.22 Effect of pH on zeta potential of non-adapted PAO1 cells.

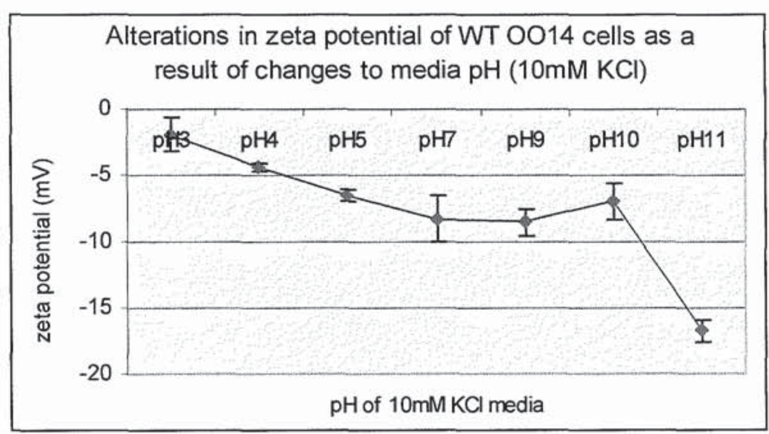


Figure 5.23 Effect of pH on zeta potential of non-adapted OO14 cells.

The pH of the 10mM media used in the preparation of adapted cells to be examined in this work (figures 5.20 & 5.21) was between pH 5 and pH 6. In both strains, PAO1 and OO14 the Zeta potential over this range remained fairly constant.

5.3.10 Resistance of outer membrane to permeabilisation by EDTA and BKC

Adapted cells from both PAO1 and OO14 strains were treated with EDTA and BKC separately and examined to see if these agents increased the permeability of the cells to the hydrophobic probe NPN and how passage of the cells in BKC may affect this.

The results for PAO1 cells adapted to BKC are presented graphically in Figures 5.24 and 5.25 showing the action of EDTA and BKC respectively.

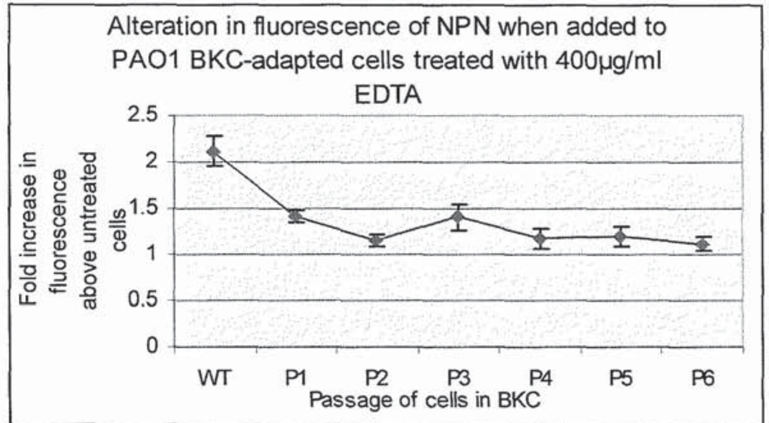


Figure 5.24 Alterations in permeability of the outer membrane of PAO1 cells adapted to BKC when treated with EDTA (400µg/ml).

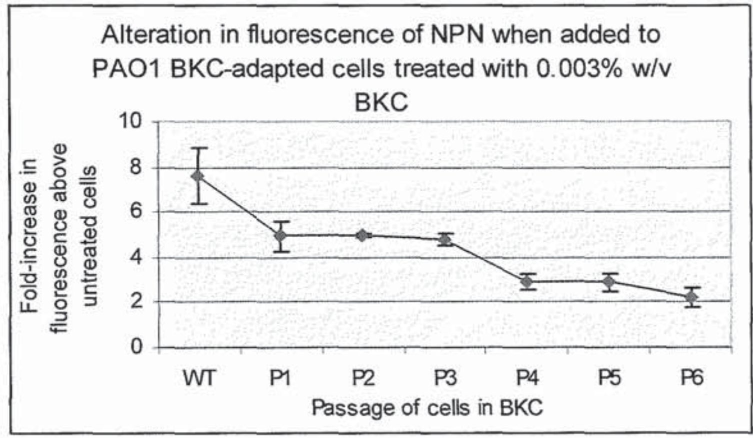


Figure 5.25 Alterations in permeability of the outer membrane of PAO1 cells adapted to BKC when treated with 0.003%w/v BKC.

The permeability produced by both agents was reduced as the PAO1 cells became adapted to BKC.

The results for OO14 cells adapted to BKC are presented graphically in Figures 5.26 and 5.27 showing the action of EDTA and BKC respectively.

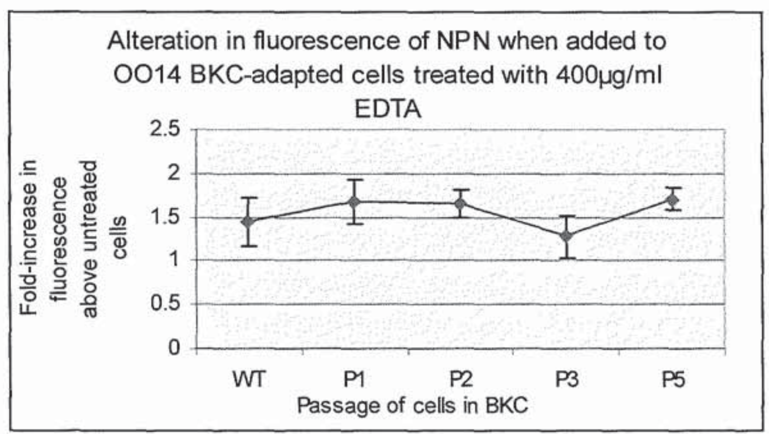


Figure 5.26 Alterations in permeability of the outer membrane of OO14 cells adapted to BKC when treated with EDTA (400µg/ml).

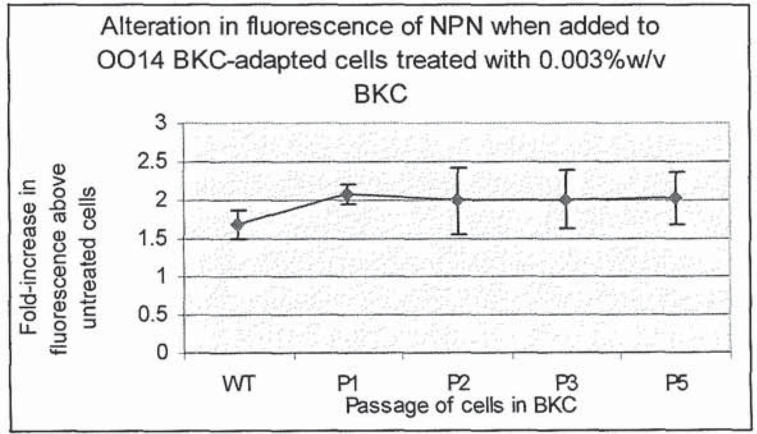


Figure 5.27 Alterations in permeability of the outer membrane of OO14 cells adapted to BKC when treated with 0.003% w/v BKC.

There appears to be no pattern in the alteration of the action of BKC or EDTA upon permeabilising OO14 cells to NPN, connected to the degree of adaptation the cells have to BKC.

5.3.11 Possible source of aminoglycoside resistance

The determination of relative quantities of plasmid DNA in strain OO14 WT and P5 was achieved by two methods. Maxiprep DNA was examined by spectrophotometric quantitation and also electrophoresed on an agarose gel to determine size of plasmid and approximate quantitation.

5.3.11.1 Spectrophotometric quantitation.

The optical densities of the plasmid DNA isolated from Wild Type and Passage 5 cells of the OO14 strain are recorded in table 5.20.

Table 5.20. Optical densities at 260nm and 280nm for plasmid DNA isolated from WT and P5 cells of strain OO14, and calculated concentrations and purity of DNA.

Passage of strain OO14	OD260nm	OD280nm	OD260/OD280nm	Concentration of DNA	% Purity of DNA
Wild type	0.114	0.069	1.652	285 ng/µl	91.7
Passage 5 (P5)	0.051	0.032	1.594	127.5 ng/µl	88.6

It would appear that cells of P5 contained less than 50% of the plasmid DNA than did non-adapted cells of the same strain. Since the purity of DNA had a value of lower than 1.8 it would indicate that any contamination was due to protein (Sambrook *et al.*, 1989) while a value of 1.8 indicates pure DNA.

5.3.11.2 Quantitation and plasmid size determination by agarose gel electrophoresis

Samples of WT and P5 plasmid DNA were electrophoresed on a 1.25% w/v agarose gel to confirm relative concentrations of DNA and to estimate the size of the plasmid isolated.

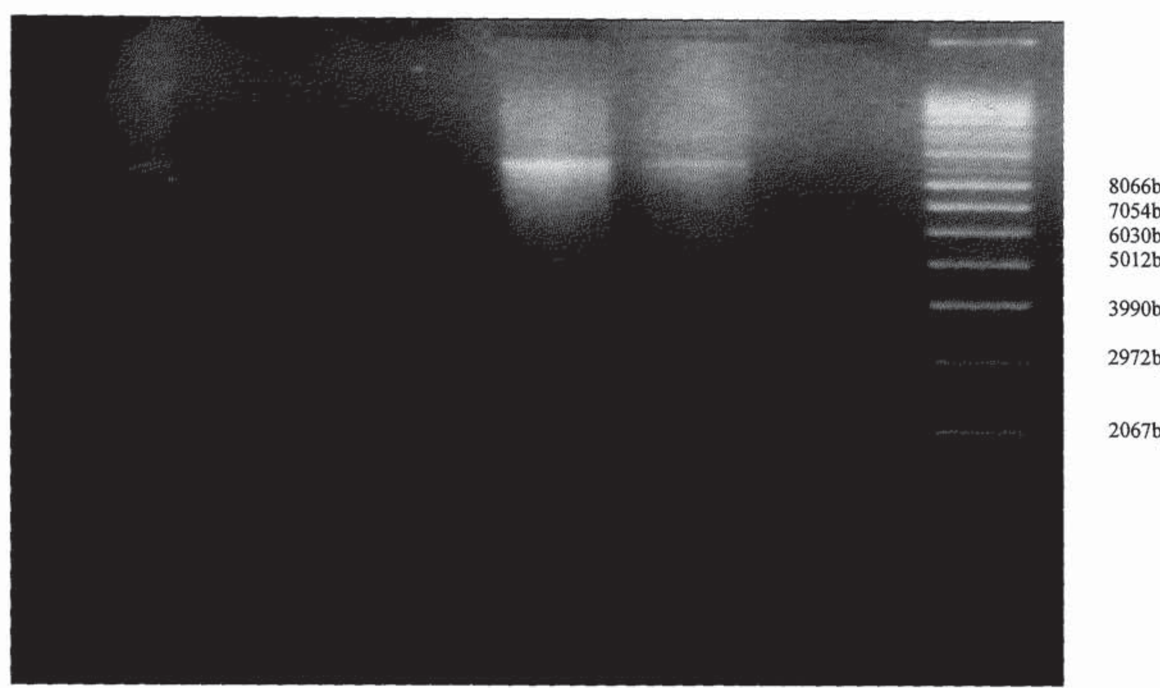


Figure 5.28 Plasmid DNA extracted from OO14 Wild type and P5 and separated on a 1.25% agarose gel. Size comparisons are made with a supercoiled ladder containing 11 fragments. (16210bp, 14174bp, 12138bp, 10102bp, 8066bp, 7045bp, 6030bp, 5012bp, 3990nbp, 2972bp & 2067bp).

The agarose gel was analysed using Phoretix 4.01 software to produce the data on size and intensity of plasmid DNA visualised shown in table 5.21

Table 5.21. Relative band intensity and size of plasmid DNA extracted from OO14 strain Wild Type and P5.

Passage of strain OO14	Size of plasmid bp/Kda	Peak intensity(arbitrary units)
Wild Type	9319.5bp/6150.8	13801
Passage 5 (P5)	9319.5bp/6150.8	4725

It would appear that a plasmid of 9319.5bp in size was present in strain OO14. The concentration of the plasmid was less in passage 5 than WT.

5.4 Discussion

The title of this chapter, "Determining mechanisms of resistance" could be considered somewhat of a misnomer. In order to determine such a mechanism it must be present in a resistant organism and absent in both the wild type "parent" organism and in the revertant organism having lost the resistant phenotype. While many physiological changes accompanied the development of a BKC resistant phenotype in the two strains of *P. aeruginosa* examined, it is impossible to state that they are responsible for resistance, especially in the absence of any revertants to wild type resistant phenotype. In addition, while certain alterations, outer membrane protein proportions for instance, remained after growth in BKC-free media, the difficulties in growing the more resistant passaged cells from frozen stock meant that the stability of other changes in such cells could not be determined. In conclusion, the changes described in the results section and discussed here, can only be said to be associated with development of resistance not responsible for the alteration in resistance phenotype observed.

5.4.1 Efflux

The addition of reserpine to cells adapted to BKC appeared to have no effect upon their resistance to the disinfectant in a manner that would have reflected the presence of an efflux system. Reserpine has been used in the past to inhibit those efflux pumps associated with Gram-positive organisms such as pneumococci (Gill *et al.*, 1999), pumps such as NorA. There has been no evidence that reserpine has an inhibitory action upon such RND efflux pumps as are found in *P. aeruginosa* and it could be that its addition to cells of PAO1 and OO14 had no effect because the alkaloid truly has no action on such pumps. In that case treating cells with proton

conductors such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) has shown to be effective in inhibiting the action of efflux pumps in *P. aeruginosa* (Li. *et al.*, 1995). In addition, recent work by Lomovskaya *et al.* (2001) has described the development of new efflux pump inhibitors for use against *P. aeruginosa* strains.

In the case of OO14 and PAO1 cells there is no evidence of a co-resistance to antibiotics normally associated with efflux in *P. aeruginosa* cells, such as aminoglycosides or fluoroquinolones, and so it is unlikely that such a system was affected by passage in BKC. However, there is evidence that RND efflux pumps were responsible for the disinfectant resistance of *P. aeruginosa* (Schweizer, 1998; Chuanchuen *et al.*, 2001) and associated antibiotic resistance, so the possibility of links between passage of disinfectants and possible up regulation of efflux should not be ignored.

5.4.2 Outer membrane proteins

One of the earliest studied antibiotic resistance mechanisms in *P. aeruginosa* involves expression of OprH and its effect upon the action of polymyxin B, EDTA and aminoglycosides (Nicas & Hancock, 1980). As described in chapter 1, polymyxin antibiotics are currently used successfully in the therapy of cystic fibrosis and are lipopeptide antibiotics with outer membrane permeabilising properties. Much work has been done on adapting strains of *P. aeruginosa* to polymyxin B and examining mechanisms of resistance. The relevance of this to this research project is detailed in the mode of action of the antibiotic. Of all antibiotics polymyxin B is the most dependant upon membrane permeabilising properties to kill bacteria, and so is the closest in mode of action to surfactants such as BKC. Indeed the results detailed in Chapter 4 show that PAO1 cells adapted to BKC showed an increase in resistance

to polymyxin B although Adair *et al.* (1971) reported contradictory results. The only protein associated with polymyxin B resistance is OprH, a 21kDa protein which is thought to replace the divalent cations positioned between phosphate groups on adjacent LPS molecules in the outer membrane (Bell & Hancock, 1989). In this way OprH also acts to increase resistance to EDTA and aminoglycosides such as gentamicin by stabilising the outer membrane (Bell *et al.*, 1991). Such increases in OprH appear to be due to either chemically induced mutation (Nicas & Hancock, 1980) or by growth in Mg^{2+} deficient media (Nicas & Hancock, 1980; Nicas & Hancock, 1983b). Since OprH acts to stabilise the outer membrane, it is unexpected that its proportion in the outer membrane of BKC and polymxin resistance cells generated does not increase in either PAO1 or OO14 strains. However, there have been reports of polymyxin resistant PAO1 that have shown no increase in OprH, (Shand *et al.*, 1988) which is discussed more fully in section 5.4.3.

The only protein in PAO1 whose expression was found to increase in proportion to BKC resistance was OprG, a 25kDa protein associated with alterations in the LPS of the organism and linked with iron transport and magnesium deficiency (Hancock *et al.*, 1990). The gene encoding this protein has recently been sequenced, although the DNA sequence homologies found with proteins of *Vibrio cholerae* shed little light on its role, if any, in resistance to BKC (Gensberg *et al.*, 1999). The only protein to decrease in proportion in PAO1 was an unknown 36-37kDa protein visible in both gels, but discernable by Phoretix 4.0 software only in samples denatured at a higher temperature.

In strain OO14 a 36-37kDa protein also reduced its proportion as cells became adapted to BKC, no OprG alterations were observed and a 44kDa protein postulated as being OprE increased in proportion. None of these changes match any other observed alterations in outer membrane proteins that have been associated with antibiotic, or disinfectant resistance. However, in the absence of available monoclonal antibodies it is impossible to discount that, since some changes were seen in the outer membrane of PAO1, it is likely that proportions of OprG were altered because of such changes in the LPS and not as a direct mechanism of resistance.

5.4.3 Phospholipid, lipopolysaccharide and fatty acid content of cell membranes

The contents of bacterial membranes have always been of interest to those examining the mechanism of resistance to membrane active agents such as quaternary ammonium compounds (QAC), and polymyxin B. In this work, cells of *P. aeruginosa* strain PAO1 showed an increase in resistance to both BKC and to polymyxin B when grown in increasing concentrations of the QAC. These increases in resistance were accompanied by an increase in the proportion of the fatty acid hexadecanoate (16:0) and tetradecanoate (14:0) in the cytoplasmic membrane accompanied by a decrease in a fatty acid with a retention time just beyond that of 16:0 but not identifiable by either comparison with gas chromatography standards or by examination by mass spectrometry. No alterations were observed in the proportions of fatty acids in the outer membrane. In cells of strain OO14 similarly adapted to BKC there were no such alterations in either membrane. It should be noted that only those fatty acids linked to lipid A by ester bonds were examined, attempts to release amide-linked fatty acids were not successful.

In previous research examining fatty acid alteration of cell membranes of *P. aeruginosa* cells similarly adapted to QAC, no attempt was made to examine the two cell membranes separately. In work by Méchin *et al.* (1999) Guérin-Méchin *et al.* (1999) and Jones *et al.* (1989) examining cells adapted to QACs such as BKC and amphoteric disinfectants, there was evidence of alterations in the total fatty acid content of the cells including reductions in the proportion of 16:0 and increases in 12:0 and certain hydroxy fatty acids thought to be present in the outer membrane alone. Earlier work by Anderes *et al.* (1971) showed that with resistance to BKC came an increase in proportion of the fatty acids 16:0 and 18:1 in both of what were termed phospholipid and free fatty acid fractions, although such alterations were reversed if cells were harvested in media containing the QAC. The work by Jones *et al.* (1989) was completed in media containing the QAC and this may explain the difference between those results and those presented here. This would indicate that alterations in fatty acid proportions of the bacterial membranes of *P. aeruginosa* cells adapted to QACs are specific to the strain of *P. aeruginosa* used, the QAC used to adapt the cells and the presence or absence of the QAC in the media in which the cells are harvested prior to fatty acid isolation.

In this work the increase in fatty acid 16:0 in the cytoplasmic membrane and reduction in proportion of the unknown fatty acid termed X, may act to rigidify the cytoplasmic membrane. This could act as a resistance mechanism to BKC whose site of action is the cytoplasmic membrane and would explain the reduction in cold resistance in PAO1 cells adapted in higher concentration of BKC illustrated by the decrease in viability of such cells in cold storage in this work. The lack of alteration of fatty acid proportions in OO14 cells further supports the specific nature of such

resistance. Ernst *et al.* (1999) have recently shown alterations in the fatty acid content of *P. aeruginosa* LPS associated with growth in the CF lung. These alterations involve substitution of palmitate (16:0) onto the LPS of strains from the CF lung, and those grown *in vitro* in low Mg^{2+} conditions. These alterations were associated with increased resistance to both polymyxin B and certain cationic antimicrobial peptides. This work, and its importance to the understanding of CF as a whole, has recently been reviewed by Pier (2000). Another well-studied alteration in fatty acids as a response to biocidal agents is the *Pseudomonas putida* resistance to toluene and other organic solvents that appears due to the cis-trans isomerisation of cis-oleic acid (C16:1, C-9) (Weber *et al.*, 1994; Junker & Ramos, 1999).

Proportions of phospholipids in such adapted cells have not been widely examined with reference to resistance to QAC or other disinfectants but has been examined with reference to resistance to the membrane active antibiotic polymyxin B, as have alterations of fatty acid proportions of cell membranes. A very brief exposure, 10-minutes, of *P. aeruginosa* to polymyxin-rich media produced alterations similar to those in polymyxin B adapted strains (Gilleland *et al.*, 1984) namely reduction in phosphatidyl ethanolamine and phosphatidyl glycerol and increase in diphosphatidyl glycerol that had been reported by others (Conrad and Gilleland, 1981; Gilleland & Conrad, 1982; Champlin *et al.*, 1983). There is no evidence for reductions in phosphatidyl choline being reported previously as associated with polymyxin resistance. This may indicate that the reduction in this phospholipid observed in strain OO14 in this work is connected to BKC resistance alone, as unlike PAO1 adapted cells of this strain showed no co-resistance with polymyxin B.

Work by Gilleland and Farley (1982) indicated that the cytoplasmic membrane played no role in resistance to polymyxin B and only alterations in the outer membrane reduced access of the antibiotic to its target. Work by Conrad and Gilleland showed polymyxin adapted *P. aeruginosa* cells to have an increased content of unsaturated fatty acids, yet a decreased content of cyclopropane fatty acids when compared to polymyxin-susceptible strains. These two alterations would serve to increase and decrease the fluidity of the cell membrane respectively, although the method of harvesting makes it difficult to determine what proportion of cytoplasmic and outer membranes were being examined. Later work by Conrad and Galanos (1989) specifically examining the fatty acids of LPS, found resistant cells had reductions in the hydroxy fatty acids 2-hydroxydodecanoic and 3-hydroxydecanoic acid (2OH-12:0 and 3-OH 10:0 respectively).

The only work that has examined polymyxin resistant strains and action of detergents is that of Moore *et al.* (1984). This showed that *P. aeruginosa* strains isolated as polymyxin resistant mutants rather than adaptively generated, showed reductions in whole cell levels of dodeccanoic acid (12:0) and an increased susceptibility to detergents with a negative or neutral charge, when grown in polymyxin B.

5.4.4 Hydrophobicity, cell surface charge and uptake of BKC

While no trend was observed in the surface hydrophobicity of PAO1 cells adapted to BKC, as OO14 cells underwent adaptation their surfaces became more hydrophobic, a change also observed by Jones *et al.* (1989) in *P. aeruginosa* cells adapted to either QAC or amphoteric disinfectants. In addition more recent work has shown a decrease in the cell surface hydrophobicity of *Acinetobacter baumanni*

associated with subinhibitory concentrations of aminoglycosides (Hostacka, 2000a) and β-lactam antibiotics (Hostacka, 2000b) although this is likely to be as a result of the antibiotics acting upon the outer membrane rather that adaptation of the organism to their presence.

The hydrophobic nature of a cell surface can be due to a number of chemical species. The work of Makin and Beveridge (1996) has shown links between the hydrophobicity of *P. aeruginosa* cell surfaces to the presence or absence of A and B band oligosaccharide portion of LPS. The presence of the long chain B band (O-antigen) confers a hydrophillic nature while mutants expressing only A band or neither oligosaccharide have a much more hydrophobic cell surface. However, this alteration in A and B band LPS also produced clear differences in the appearance of LPS examined by silver stained SDS PAGE (Makin & Beveridge, 1996), differences absent in the LPS isolated from OO14 cells showing such alterations in hydrophobicity. In addition, the removal of B band LPS would have an effect on the identification of the strain by serological methods, a change that was not observed in OO14 cells.

The uptake of BKC from media containing the disinfectant decreased as OO14 cells were adapted to the biocide, but not when PAO1 cells were similarly adapted. The uptake of quaternary ammonium compounds was not observed in cells adapted in the work of Jones *et al.* (1989) or in the work of El-Falaha *et al.* (1985) which compared wildtype and outer membrane mutants of both *E. coli* and *P. aeruginosa* in resistance to and uptake of both BKC and chlorhexidine. However, the LPS of *P. aeruginosa* cells adapted to the membrane active agent polymyxin B bound less of the antibiotic

than the parental wild type (Conrad & Galanos, 1989). However, the alteration in cell surface hydrophobicity in OO14 cells may be linked with reduced uptake in BKC as BKC is thought to bind to charged moieties on the cell surface, reduction of charged moieties may lead to a reduction in cell surface hydrophobicity as observed in OO14. Mutants of *E. coli* resistant to organic solvents, showed both a reduction in binding to the solvent and a reduction in cell-surface hydrophobicity, mirroring those changes observed in OO14 (Aono & Kobayashi, 1997).

5.4.5 Outer membrane permeability

The outer membrane of PAO1 cells showed a marked reduction in susceptibility to both EDTA and BKC as permeabilising agents. Resistance to EDTA is usually associated with production of OprH and linked with polymyxin and aminoglycoside resistance. While there was evidence of polymyxin resistance in PAO1 cells there was no evidence of either an increase in OprH or in resistance to the aminoglycoside tobramycin. Other membrane alterations associated with polymyxin resistance (see section 5.4.3) were absent from adapted PAO1 cells. EDTA acts to remove Mg^{2+} from between adjacent LPS molecules in the outer membrane, so whatever alteration occurred must have prevented this. The only alteration in cells of PAO1 that was associated with LPS and was not present in OO14 was the increase in OprG, a protein not known to be associated with antimicrobial resistance.

5.4.6 Aminoglycoside resistance in strain OO14

Strain OO14 cells not adapted to BKC showed a very high resistance to the aminoglycoside tobramycin. Resistance to aminoglycosides in *P. aeruginosa* is due to a number of mechanisms including over-expression of OprH, efflux and modifying enzymes such as acetyltransferases. There was no evidence of either OprH or outer membrane proteins such as OprM or OprN associated with efflux being over-expressed in strain OO14 wild type or passaged cells. Therefore, it was considered that resistance might be due to the production of modifying enzymes. These enzymes are encoded upon plasmids and plasmid DNA was detected in the cells of both wild type and BKC-adapted cells of OO14. The quantity of plasmid DNA in wild type, tobramycin resistant cells was twice that present in BKC-adapted, tobramycin sensitive cells. This plasmid was 9319bp (6150kDa) in size, a size not normally associated with resistance plasmids of *P. aeruginosa* (Boronin, 1992). Whether this plasmid DNA contains an operon for expression of an aminoglycoside-modifying enzyme is unknown, but the reduction in quantity of plasmid DNA is associated with an increased sensitivity to tobramycin. In addition, it is unknown whether the passage in BKC affected the quantity of plasmid DNA present in P5 cells or whether the two changes are unconnected.

5.5 Conclusions

The properties associated with resistance to BKC vary between the strains examined. They include alterations in outer membrane proteins, fatty acid proportions of cytoplasmic and outer membranes, phospholipid content of cells, uptake of BKC from media, sensitivity of cells to permeabilisation by BKC and EDTA, cell surface hydrophobicity and charge and quantity of plasmid DNA present. This suggests that resistance is due to alterations in the structure of outer and cytoplasmic membranes, alterations that are specific for each strain adapted in this way. Such alterations bring with them co-resistance properties unique to each strain, making it impossible to generalise about a general method of resistance to the disinfectant BKC.

Chapter 6: Conclusions

Broadly, the aim of this study was two-fold. Firstly, to identify and assay possible outer membrane permeabilisers and, from any structures common to active compounds, design and synthesise novel agents for use in conjunction with BKC in a disinfectant. Secondly, to determine how prevalent the ability to adapt to BKC is among environmental isolates of *P. aeruginosa*, noting any co-resistance with relevant antibiotics and determining mechanisms of resistance where possible.

While membrane permeabilising, "non-antibiotic" agents have been examined as antibacterial agents in previous work, this is the first where they have been investigated for synergy with disinfectants, although the assays developed here would also be useful in determining synergy with antibiotics. Certain of the compounds had no history of reported antimicrobial action and were chosen on their structure alone, allowing examination of compounds that might otherwise not have been considered. Work not included in this project has shown synergy between certain of these compounds and common anti-pseudomonal antibiotics, possibly indicating a role for them as therapeutic agents. While the novel agents produced showed lower levels of activity than hoped some did show synergy with BKC, fulfilling the purpose of the project. The basis for much of the original work in this field was based upon anecdotal observations (Kristiansen, 1990) as was the recent discovery of resistance to triclosan in *P. aeruginosa* being linked to efflux (Schweizer, 1998). Where resistance to antimicrobial agents is becoming more commonplace, the search for truly novel agents, whose action is unlikely to provoke swift resistance in its target, is more and more important. This is likely to involve examination of other “non-antibiotics” or unusual agents in the future.

Resistance to BKC was observed in all but one of the strains serially passaged in the disinfectant. This indicates that the ability to adapt to disinfectants is common among strains of *P. aeruginosa*, although not present in all. Since many of the strains used were environmental isolates this may mean that hospitals relying on just one active disinfectant agent may select for resistant strains. The alteration in serotype and genotype observed may be due to contamination during the project or by the environmental cultures not being genotypically pure, however this explanation may have far reaching consequences for the isolation of strains from the environment.

Co-resistance to antibiotics has been shown with *P. stutzeri* adapted to chlorhexidine (Russell *et al.*, 1998) so it is not wholly unexpected to see resistance to antibiotics in the PAO1 adapted cells examined in this work. However, with the exception of polymyxin B, no clinically important antibiotics had reduced activity against adapted cells. Development of resistance due to disinfectant use, is a new and worrying development, especially since resistance to polymyxins has not been encountered when used to treat *P. aeruginosa* infections in CF

While most explanations for cross-resistance in disinfectant adapted cells involve reduced permeability of the cell wall, the recent discovery of the phenolic disinfectant triclosan acting as a substrate for a number of efflux systems in *P. aeruginosa* indicates that resistance mechanisms are likely to be more complex. The range of differences between two strains adapted in an identical manner clearly shows that resistance development is a highly strain specific matter, involving a combination of alterations in the outer and cytoplasmic membranes, the examination of which this project sought to begin. It appears that the fatty acid palmitate (16:0)

has an important role to play in adaptation of *P. aeruginosa* to its environment whether that be the CF lung or disinfectant rich media, as it increases in proportion in both environments, in the LPS and the cytoplasmic membrane respectively.

Alterations in hydrophobicity and cell surface charge as observed in this work are likely to be as a side effect of other changes in the outer membrane associated with resistance to BKC, as it is unlikely that such alterations would significantly effect action of the disinfectant. The reduction in uptake or binding of BKC observed in strain OO14 is perhaps simpler to reconcile with resistance since, if less BKC is coming to close contact with the cell membranes, then the bactericidal action is reduced. The mechanism for this reduction in binding is unknown but does suggest a specific binding motif for BKC, that is altered in the process of adaptation of cells to the disinfectant.

From the evidence presented in this work it appears that the stable resistance to BKC shown by strains OO14 and PAO1 is derived from alterations in both cytoplasmic and outer membranes. The associated changes in hydrophobicity and cell surface charge indicate alterations in the outer membrane, perhaps undiscovered by this work. Further examination of the nature of the LPS of these strains may lead to more definite answers as to the nature of the resistance observed. In addition, use of the recently discovered efflux inhibitors used with *P. aeruginosa* (Lomovskaya *et al.* 2001) may determine if the efflux mechanisms responsible for the organism's resistance to so many agents may also play a role in its resistance to BKC.

Appendix 1 Chemical structures of agents tested

Name	Structure	Selection Process
Promazine		Structural similarity
Chlorpromazine		Rajyaguru & Muszynski 1997
Prochlorperazine		Rajyaguru & Muszynski 1997
Imipramine		Structural similarity
Iministolibene		Structural similarity
Amitryptiline		Bourlioux *et al.* 1992
Chlorprothixene		Bourlioux *et al.* 1992
Lidocaine		Rajyaguru & Muszynski 1998
Tetracaine		Labedan 1988

Dibucaine		Rajyaguru & Muszynski 1998
Famotidine		Rajyaguru & Muszynski 1998
Quinacrine		Structure similarity
2(2-methylaminoethyl) pyridine		Structure similarity
4 (ethylaminomethyl) pyridine		Structure similarity
2-methyl aminomethy 1,3, dioxolone		Structural similarity
3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate		Structural similarity
6-(dimethylamino fulvene)		Structural similarity

2-dimethylamino-9-fluorenone		Structural similarity
2-dimethylamino fluorene		Structural similarity
9-methylamino methyl anthracene		Structural similarity
2-chloro-3-ethylbenzoxazolium tetrafluoroborate		Structural similarity
[3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethyl-ammonium chloride		Structural similarity
Theophylline		Rajyaguru & Muszynski 1998
Theobromine		Rajyaguru & Muszynski 1998

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