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Antimicrobial Resistance of CF Pathogens; Mechanisms of Biocide Resistance and Action

Thesis presented for the Degree of Philosophiae Doctor

By

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Scientific Conferences and Publications

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Summary

Cystic fibrosis (CF) patients are predisposed to a number of bacterial infections, including *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex (Bcc). Both groups of bacteria have been associated with contamination of products containing biocides, leading to concerns that the over use of biocide products could select for multi drug resistant organisms.

This investigation examined the susceptibility profile of panels of *P. aeruginosa* and Bcc strains to a range of biocides including chlorhexidine and cetylpyridinium chloride (CPC). It was found that certain epidemic strains that had spread among individuals with CF, such as the *B. cenocepacia* J2315 strain lineage and the *P. aeruginosa* Liverpool strain were less susceptible to chlorhexidine than other strains representative of the same species. Although minimum inhibitory concentration (MIC) screens gave an overall view of biocide susceptibility, minimum bactericidal concentrations (MBC) for these bacteria were higher. For CPC 27 out of 40 strains required more than 20 fold more biocide than the MIC to achieve a bactericidal effect. Suspension tests were performed on two commercial biocide formulations, a chlorhexidine-based wash, Hibiscrub™ and a triclosan-based hand gel Cuticura™. The epidemic *B. cenocepacia* strain J2315 was capable of surviving in both these products after 20 minutes of exposure and viable bacteria were isolated after 60 minutes of exposure in Hibiscrub™. The results from this investigation suggest that certain commercial biocides are not effective against the Bcc. Therefore to assist in the future development of biocides, three highly resistant Bcc strains were proposed as suitable reference strains to use in challenge testing for biocide efficacy.

The molecular basis of biocide resistance was determined using a microarray approach to profile global gene expression of *B. cenocepacia* in response to chlorhexidine. *B. cenocepacia* J2315 was exposed to sub-inhibitory levels of chlorhexidine (5 µg/ml) and expression compared to cells not exposed to biocide. The microarray analysis demonstrated significant alterations in expression at $P < 0.05$, with a > 1.5 fold change; with 98 up-regulated and 76 down-regulated genes. Two chlorhexidine up-regulated genes were selected for further analysis, a response regulator (BCAM 0924) potentially involved with efflux and a novel transport related gene (BCAL 2553). Site directed mutagenesis of these genes was carried out in *B. cenocepacia* strain K56-2 and a reduction in chlorhexidine MIC was observed for each respective mutant compared to the wildtype. 66 out of 76 (87%) of the down-regulated genes were involved with motility related functions. This led to the hypothesis that sub-inhibitory levels of chlorhexidine inhibited swarming motility and induced biofilm production. This question was tested for Bcc strains using soft-agar swarming tests and 96-well plate biofilm assays. A total of 6 of 10 strains screened exhibited both biofilm induction and swarming inhibition in response to chlorhexidine. A potential conserved regulatory binding motif was observed upstream of all gene sets down-regulated chlorhexidine in the microarray analysis. This suggested that swarming inhibition and biofilm induction in *B. cenocepacia* may be controlled by a coordinated regulatory pathway controlled by a two component system sensor-regulator system. A transposon mutagenesis approach was used to identify *B. cenocepacia* mutants that lacked the inhibitory response. The screen identified several mutations involved in the phenomenon including *cheY*-like receiver genes and a glycosyl transferase encoding gene. In conclusion, the molecular analysis of biocide resistance in Bcc bacteria demonstrated it was multifactoral, involving efflux pumps, transport related genes, membrane proteins and regulatory genes. The ability of chlorhexidine to inhibit swarming and switch Bcc to a non motile biofilm lifestyle was identified as a novel biocide survival response. With further research this regulatory pathway may be a potential target for the development of a novel biocides and therapeutics which overcome the antimicrobial resistance of these bacteria pathogens.

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Abbreviations

AAC	**Aminoglycoside acetyltransferase**
aa-dUTP	**Amino allyl – dUTP**
ABC	**ATP dependent binding cassette**
AHL	**N – acyl – homoserine lactone**
ANT	**Aminoglycoside nucleotidyltransferase**
AP	**Ampicillin**
APH	**Aminoglycoside phosphoryltransferase**
Ara4N	**4 – amino – 4 deoxyarabinose**
Arr	**Aminoglycoside response regulator**
ATCC	**American type culture collection**
ATP	**Adenosine 5' tri-phosphate**
AZ	**Azithromycin**
Bcc	***Burkholderia cepacia* complex**
BCSA	***B. cepacia* selective media**
BLAST	**Basic Local Alignment Search Tool**
BSA	**Bovine serum albumin**
BZK	**Benzalkonium chloride**
$CaCl_2$	**Calcium chloride**
cAMP	**Cyclic adenosine monophosphate**
CCCP	**Carbonyl cyanide – 3 - chlorophenylhydrazone**
CF	**Cystic Fibrosis**
CFTR	**Cystic fibrosis transmembrane conductance regulator**
CFU/ml	**Colony forming units per millilitre**
Chx	**Chlorhexidine**

CI	**Ciprofloxacin**
Ci-di-GMP	**Cyclic di-guanosine monophosphate**
CL	**Chloramphenicol**
CoA	**Coenzyme A**
COG	**Clusters of orthologous groups**
CPC	**Cetylpyridinium chloride**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxyribonucleotide tri phosphate (including adenine, guanine, cytosine and thymine**
EDTA	**Ethylenediaminetetraacetic acid**
EPS	**Exopolysaccharide**
E-test	**Epsilometer test**
HAMP	**Histidine kinases, adenylyl cyclases, methyl binding proteins and phosphotases**
Hz	**Hertz**
IP	**Imipenem**
IPTG	**Isopropyl-β-D-thiogalactopyranoside**
JGI	**Joint genome institute**
KDO	**3–deoxy-D-manno-oct-2-ulosonic acid**
KM	**Kanamycin**
KO	**D-glycero-α-D-taloct-2-ulopyranosylonic acid**
LB	**Luria Bertani**
LBA	**Luria Bertani agar**
LES	**Liverpool epidemic strains**
LPS	**Lipopolysaccharide**

MATE	**Multidrug and toxic compound extrusion family**
MBC	**Minimum bactericidal concentration**
MEME	**Multiple EM for elicitation**
MFS	**Major facilitator superfamily**
$MgCl_2$	**Magnesium chloride**
$MgSO_4$	**Magnesium sulfate**
MIC	**Minimum inhibitory concentration**
MLST	**Multilocus sequence typing**
MP	**Meropenem**
MRSA	**Methicillin resistant *Staphylococcus aureus***
NaOH	**Sodium hydroxide**
NCBI	**National centre for Biotechnology information**
NCTC	**National collection of type cultures**
NHS	**N – hydroxy-succinimidyl**
OD	**Optical density**
OMP	**Outer membrane protein**
PAGE	**Polyacrylamide gel electrophoresis**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PFAM	**Protein family database**
PFGE	**Pulse field gel electrophoresis**
PMX	**Polymyxin**
PP	**Piperacillin**
PSI-BLAST	**Position specific iterated BLAST alignment**
QAC	**Quaternary ammonium compounds**

QPCR	**Quantitative real time PCR**
QS	**Quorum sensing**
RAPD	**Random amplified polymorphic DNA**
RE	**Restriction enzyme**
RFLP	**Restriction fragment length polymorphism**
RNA	**Ribonucleic acid**
RND	**Resistance nodulation division**
RT	**Reverse transcription**
SDS	**Sodium dodecyl sulfate**
SE	**Standard error of the mean**
SMR	**Small multidrug resistance pump**
TAD	**Tight adherence loci**
TAE	**Tris acetate EDTA buffer**
Taq	***Thermus aquaticus* polymerase**
TBE	**Tris borate EDTA buffer**
TC	**Tetracycline**
TCDB	**Transport classification database**
TM	**Tobramycin**
TMHMM	**Transmembrane hidden Markov model**
TMO	**Temocillin**
TP	**Trimethoprim**
TPR	**Tetratricopeptide repeat**
Tris	**2-amino-2-(hydroxymethyl)-1, 3-propanediol**
TS	**Trimethoprim / sulfamethoxazole**
TSA	**Tryptone soya agar**

TSB	**Tryptone soya broth**
TTS	**Type three secretion system**
UV	**Ultra violet light**
X-gal	**5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside**

Chapter 1.0

Introduction

Chapter 1 Introduction

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is the most common autosomally inherited recessive disease in Caucasian populations. It occurs in approximately 1 in 2500 births and has a carrier frequency of 1 in 25 (Tummler and Kiewitz, 1999). Patients diagnosed with cystic fibrosis face a life of hospital visits, bacterial infections and clinical treatment cycles. They also face the prospect of a much shortened life span and the consequences that arise from having a genetically inherited disease. Thankfully due to advances in treatment, patients now have a life expectancy of 40 years and can lead fulfilling lives. A major advance in the understanding of cystic fibrosis was the identification of the gene that caused the disease. CF sufferers have a mutation in a cAMP regulated chloride channel called the cystic fibrosis transmembrane conductance regulator (CFTR) (Chmiel and Davis, 2003). These mutations cause the CFTR to not function properly leading to the symptoms of CF. CFTR is expressed at the apical surface of epithelial cells and therefore although predominantly associated with lung disease, cystic fibrosis effects many other epithelial cells in the body (Chmiel and Davis, 2003). These include sweat glands, airways, vas deferens in males, bile ducts, pancreas and intestines. This leads to many other CF associated problems such as diabetes, malnutrition and infertility (Chmiel and Davis, 2003). However, it is the progressive lung disease that causes increased morbidity and mortality in CF patients (Tummler and Kiewitz, 1999).

1.11 The Cystic fibrosis transmembrane conductance regulator

Cystic Fibrosis is caused by mutations in the CFTR gene, which is encoded on chromosome 7. Approximately 900 disease causing mutations have been identified, but the most common, occurring in 70% of patients is an in frame 3 bp deletion of a phe codon (phenylalanine encoding), known as the delta F508 mutation (Vankeerberghen *et al.*, 2002). CF has a variable phenotype, with severity of disease depending on the mutations carried. A patient carrying two delta F508 mutations would be classed has having a severe disease and would usually be

pancreatic deficient, whereas a patient carrying two mild mutations (e.g. R117HH or R334W) or one mild and one severe mutation may have a less severe phenotype, retaining some pancreatic function (Sheppard and Ostedgaard, 1996).

CFTR belongs to the ATP binding cassette (ABC) transporter family and contains two sets of membrane spanning segments, two nucleotide binding domains and a regulatory cellular domain. This regulatory domain contains sensor kinases, which phosphorylate the regulator domain in a cAMP dependent manor leading to the channel opening (Tummler and Puchelle, 1997). The CFTR is predominately a chloride ion channel, thus CF patients with defective CFTR have a lack of secretion of chloride ions across epithelial cells and a lack of reabsorption of chloride ions leading to the characteristic salty sweat that CF patients produce. The sweat test is commonly used by clinicians as a diagnostic tool for CF. In addition to its role as a chloride channel, the CFTR also acts as a conductance regulator by affecting the function of other channels in the epithelium, particularly the amiloride sensitive epithelial sodium channel (ENaC) (Chmiel and Davis, 2003). This channel transports salt and water and is involved with the reabsortion of sodium from airway surface liquid, with water then following. Balanced CFTR and ENaC mediated ion transport is required to maintain a constant airway surface fluid depth. Functional CFTR down regulates the ENaC channel, so consequently in CF patients lacking CFTR function sodium reabsorption is increased (Chmiel and Davis, 2003). The excess reabsorption of sodium and thus water from the airway fluid and the decreased secretion of chloride ions across the airway leads to a reduced airway fluid depth and the production of a sticky mucus (Chmiel and Davis, 2003, Tummler and Puchelle, 1997) (Figure 1.1).

The dehydrated mucus produced by CF patients leaves them susceptible to bacterial infections. The sticky mucus leads to an impaired mucillary clearance, meaning bacterial infections are more likely to persist in the CF lung (Tummler and Kiewitz, 1999). An additional factor for the acquisition of bacterial infections is that CFTR may be a receptor for bacterial infections such as *Pseudomonas aeruginosa*, so patients with defective CFTR may have impaired endocytosis (Pier *et al.*, 1997). However, this is a controversial study with little follow up work in the last ten years.

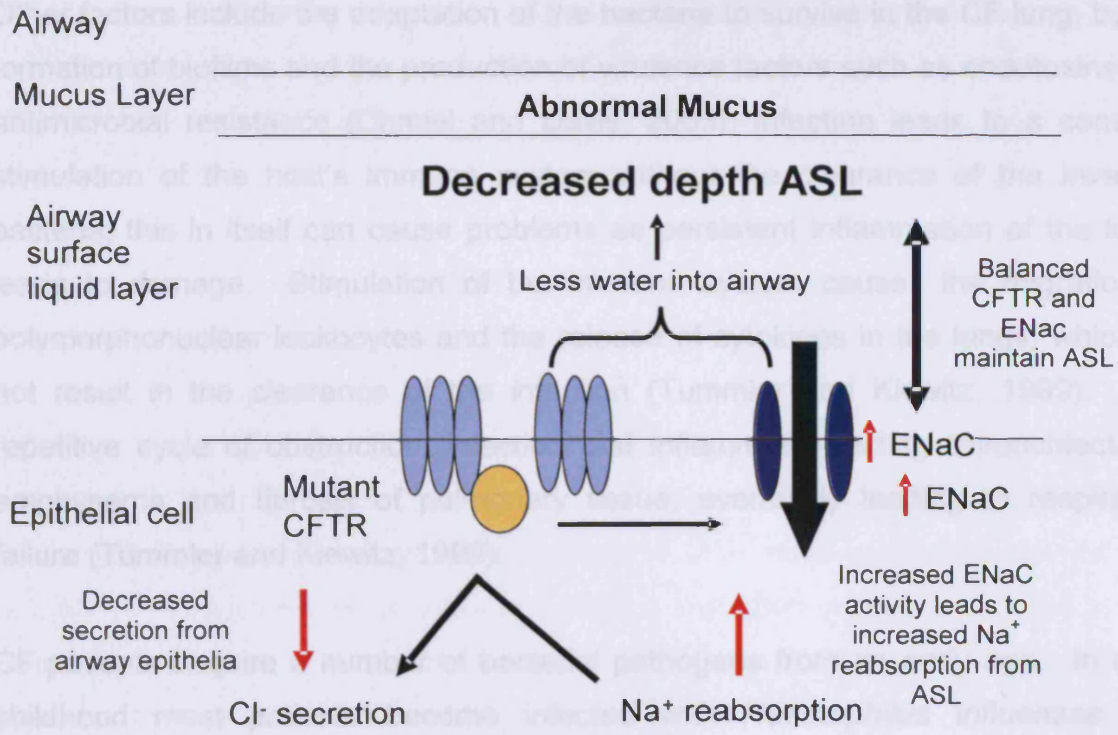

Figure 1.1 The effect of abnormal CFTR on the airways of CF patients

Defective CFTR leads to decreased chloride secretion across the epithelial cells into the CF airway. In CF patients CFTR channels are no longer able to open to allow secretion due to the lack of phosphorylation of the CFTR regulator domain. As well as effecting the chloride secretion, sodium reabsorption is increased by pleiotrophic effects on a sodium transporter channel, ENaC. The combination of reduced chloride secretion and increased sodium reabsortion leads to less water in the CF airway, causing a lower surface airway fluid depth and consequently and abnormal, sticky mucus. (Figure adapted from Chmiel and Davis, 2003).

Other factors include the adaptation of the bacteria to survive in the CF lung, by the formation of biofilms and the production of virulence factors such as endotoxins and antimicrobial resistance (Chmiel and Davis, 2003). Infection leads to a constant stimulation of the host's immune system without the clearance of the invading bacteria; this in itself can cause problems as persistent inflammation of the lungs leads to damage. Stimulation of the immune system causes the migration of polymorphonuclear leukocytes and the release of cytokines in the lungs, which do not result in the clearance of the infection (Tummler and Kiewitz, 1999). The repetitive cycle of obstruction, infection and inflammation lead to bronchiectasis, emphysema and fibrosis of pulmonary tissue, eventually leading to respiratory failure (Tummler and Kiewitz, 1999).

CF patients acquire a number of bacterial pathogens from an early age. In early childhood most patients become infected with *Haemophilus influenzae* and *Staphylococcus aureus*, then as the patient gets older they frequently become infected with *P. aeruginosa* and to a lesser extent the *Burkholderia cepacia* complex (Bcc) (Pilewski and Frizzell, 1999). Recurrent bacterial infections are a major cause of respiratory problems and are the main cause of increased morbidity and mortality in CF patients. Not only do bacterial infections have a detrimental effect on the patients health, they are also responsible for increased hospital stays, treatment with a combination of potent antibiotics and the stigma of having infections such as the Bcc which may result in segregation. This investigation focuses on two of the most common pathogens infecting CF patients, *P. aeruginosa* and the Bcc.

1.2 Multidrug resistant infections in CF patients

1.21 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram negative rod that has polar flagella. Pseudomonads usually have very simple nutritional requirements and grow chemoorganotrophically. It can utilise a wide range of organic compounds which are then used as carbon sources, which allows *Pseudomonas* species to grow in a

variety of environments, that other bacteria may not be able to grow in due to the limited nutrients (Madigan *et al.*, 2001b). *P. aeruginosa* can make use of many different sugars, fatty acids, dicarboxylic acids, tricarboxylic acids, alcohols and polyalcohols as well as many others. *P. aeruginosa* is an important organism in the soil environment as it may be responsible for the degradation of many soluble compounds from plant and animal materials (Madigan *et al.*, 2001b). It is also involved in the nitrogen cycle as a denitrifyer (Tummler and Kiewitz, 1999).

Although this organism is important in environmental situations it is also an opportunistic pathogen, infecting the urinary tract and respiratory tract in humans. *P. aeruginosa* is also associated with patients who have severe burns and skin damage. *P. aeruginosa* is often associated with nosocomial infections and is difficult to treat due to its innate resistance to many antibiotics, such as ß-lactams, some quinolones, chloramphenicol, tetracycline, macrolides, trimethoprim / sulfamethoxazole and rifampin (Rossolini and Mantengoli, 2005) This leaves limited treatment options for patients infected with *P. aeruginosa* and often results in potent, toxic antibiotics being used (Tummler and Kiewitz, 1999). The most important anti-pseudomonal agents include some modified ß-lactams (ticarcillin, ureidopenicillins, piperacillin, cefoperazone, ceftazidime, aztreonam, imipenem and meropenem), aminoglycosides (gentamicin, tobramycin, netilmicin and amikacin) and fluoroquinolones (ciprofloxacins) (Rossolini and Mantengoli, 2005).

Polymyxins, such as polymyxin B and colistin have high activity against Pseudomonads, but have a higher toxicity so are reserved to treat multidrug resistant strains (Rossolini and Mantengoli, 2005). Multi drug resistance is often conferred by a resistance transfer plasmid (R plasmid), which carries genes coding for detoxification of many of the antibiotics in day to day use (Tummler and Kiewitz, 1999). These plasmids may also encode enzymes called extended spectrum ß-lactamases (ESBL). These enzymes hydrolyse and cause resistance to oxyimino-cephalosporins and aztreonam. The majority of ESBLs are derived from mutations of other broad spectrum ß-lactamases such as TEM-1 and contributes to the innate resistance of *P. aeruginosa* to many antimicrobial agents (Bradford, 2001).

P. aeruginosa is the most common infection found in CF suffers, with up to 70% of patients becoming chronically infected with it by adulthood (Speert *et al.*, 2002). *P. aeruginosa* causes infections involved in the later stages of a CF patients life and the mechanisms of it's colonisation of the respiratory tract have been well documented. The CF lung is already prone to bacterial infections due to dehydrated mucus and abnormal pulmonary clearance (Chmiel and Davis, 2003). It may become more susceptible to *P. aeruginosa* infections due to previous colonisation with other infections such as *S. aureus* and *H. influenzae*. Infection with these bacteria will increase lung damage and may aid the colonisation of *P. aeruginosa* (Govan and Deretic, 1996). The *P. aeruginosa* phenotype is quite distinctive when colonizing the CF respiratory tract. Initially when *P. aeruginosa* colonises the respiratory tract, pilli, non pilus adhesions and motility are involved. However as it moves into chronic infection, *P. aeruginosa* appears to adapt to the CF host environment by switching to a mucoid form. This is where the bacteria secrete exopolysaccharides, exotoxins, proteases, and siderophores, which allow it to form biofilms and to effectively evade the hosts defence system (Govan and Deretic, 1996). (Figure 1.2). Other adaptations that allow *P. aeruginosa* to maintain infection is the conversion of lipopolysaccharides to rough from smooth and the loss of motility (Mahenthiralingam *et al.*, 1994). These changes in the phenotype of *P. aeruginosa* appear to be due to adaptations by the bacteria to chronic colonisation and not the replacement of one strain with another (Govan and Deretic, 1996).

The epidemiology of *P. aeruginosa* is a significant factor for research as it allows correct infection control procedures to be put into place. Nearly all CF patients become colonised with *P. aeruginosa* and this infection is usually chronic and difficult to eradicate. To see if patient to patient spread was a problem typing systems such as a random amplified polymorphic DNA (RAPD) typing and pulsed field gel electrophoresis were developed (Mahenthiralingam *et al.*, 1996a). The RAPD typing method uses PCR primers of arbitrary DNA sequences, which produce different band patterns when separated by gel electrophoresis. Those with the same patterns can be said to be the same strain. The RAPD analysis indicated that patient to patient transmission was not a problem as most patients were colonised with a single strain that they never lost (Mahenthiralingam *et al.*, 1996a). The only exceptions were in CF siblings, where prolonged contact led to cross

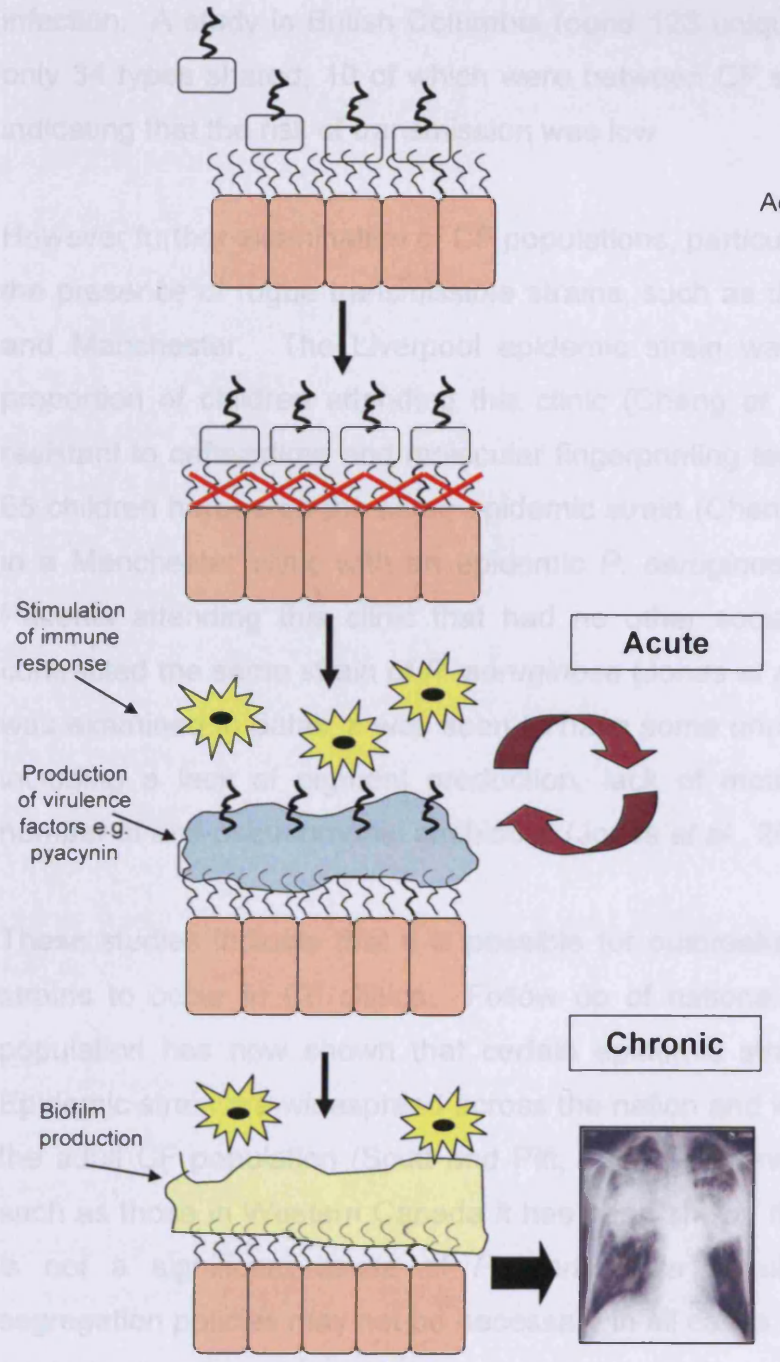

Figure 1.2 *P. aeruginosa* infection in CF patients

The progression of *P. aeruginosa* infection from acute to chronic. Initially cells adhere to the epithelial cells by binding to mucin. The CF airways impaired pulmonary clearance leads to aggregation of cells and the stimulation of the host immune system. The ineffective immune system causes inflammation and the production of virulence factors by *P. aeruginosa*. As the infection progresses to a chronic state *P. aeruginosa* loses motility and becomes mucoid and sessile.

infection. A study in British Columbia found 123 unique *P. aeruginosa* strains and only 34 types shared, 10 of which were between CF siblings (Speert *et al.*, 2002), indicating that the risk of transmission was low.

However further examination of CF populations, particularly in the UK demonstrated the presence of rogue transmissible strains, such as the strains found in Liverpool and Manchester. The Liverpool epidemic strain was found to colonise a high proportion of children attending this clinic (Cheng *et al.*, 1996). This strain was resistant to ceftazidime and molecular fingerprinting techniques indicated 55 out of 65 children harboured the same epidemic strain (Cheng *et al.*, 1996). An outbreak in a Manchester clinic with an epidemic *P. aeruginosa* strain had similar results. Patients attending this clinic that had no other social contact outside the clinic contracted the same strain of *P. aeruginosa* (Jones *et al.*, 2001b). When this strain was examined in detail, it was seen to have some unusual phenotypic differences, including a lack of pigment production, lack of motility and was resistant to a number of anti-pseudomonal antibiotics (Jones *et al.*, 2001b).

These studies indicate that it is possible for outbreaks of epidemic *P. aeruginosa* strains to occur in CF clinics. Follow up of national surveillance of the UK CF population has now shown that certain epidemic strains, such as the Liverpool Epidemic strain are widespread across the nation and infect approximately 1 in 4 of the adult CF population (Scott and Pitt, 2004). However, in other CF populations, such as those in Western Canada it has been shown that patient to patient spread is not a significant cause of *P. aeruginosa* acquisition and thus draconian segregation policies may not be necessary in all cases (Speert *et al.*, 2002). RAPD analysis also demonstrated that the phenotypic changes in *P. aeruginosa* are due to adaptation to chronic colonisation and not due to replacement of other strains (Mahenthiralingam *et al.*, 1996a). There is also some concern that *P. aeruginosa* may be acquired from the environment. This hypothesis is feasible because it is found in many habitats including environmental, such as soil and clinical such as hospitals (Govan, 2000).

1.22 The *Burkholderia cepacia* complex (Bcc)

The genus *Burkholderia* is a group of Gram negative, aerobic rods from the beta proteobacteria group (Madigan *et al.*, 2001b). These species can occupy diverse ecological niches ranging from bacteria that inhabit the soil to those that colonize the respiratory tract of humans (Coenye *et al.*, 2001). They can also have varied host ranges. Some *Burkholderia* species are restricted to occupying only one type of host; for example *B. mallei,* which causes glanders in horses, where as other species can occupy a range of hosts. These interactions can be pathogenic or symbiotic or may even be both (Coenye *et al.*, 2001). The *Burkholderia* genus can occupy many niches and are particularly associated with the environment. *Burkholderia cepacia* was originally described as a plant pathogen that caused sour skin of onion (Burkholder, 1950), but it was soon discovered that *Burkholderia* did not only form pathogenic interactions with plants but also beneficial associations. Many species of *Burkholderia* can be isolated from the rhizosphere of plants, such as rice, peas, tomatoes and maize, where they are involved in nitrogen fixation. There is evidence that *Burkholderia* can form endosymbiotic relationships with plants and these may also have beneficial properties to the plant (Coenye *et al.*, 2001).

Burkholderia are also found in the soil where they have commercial and ecological importance as they can be used in agriculture as bio-pesticides and plant growth promoters (Govan and Vandamme, 1998). The ability of members of the Bcc to metabolize a wide range of substrates means that they could also be used as a bioremediation agent to break down pollutants such as trichloroethylene (O'Sullivan and Mahenthiralingam, 2005). Therefore these desirable properties of the *Burkholderia* species mean that it is an important group for the agricultural industry. However, there has been restrictions placed on the use of *Burkholderia* species in agriculture because many species can also form pathogenic interactions in humans (Govan and Vandamme, 1998), especially the Bcc complex, which is a group of at least nine closely related species that all have the ability to infect immuno-compromised patients, especially patients with cystic fibrosis (Mahenthiralingam and Vandamme, 2005). As a result there is concern that the environment may be a source of infection for cystic fibrosis patients and consequently accurate tests are

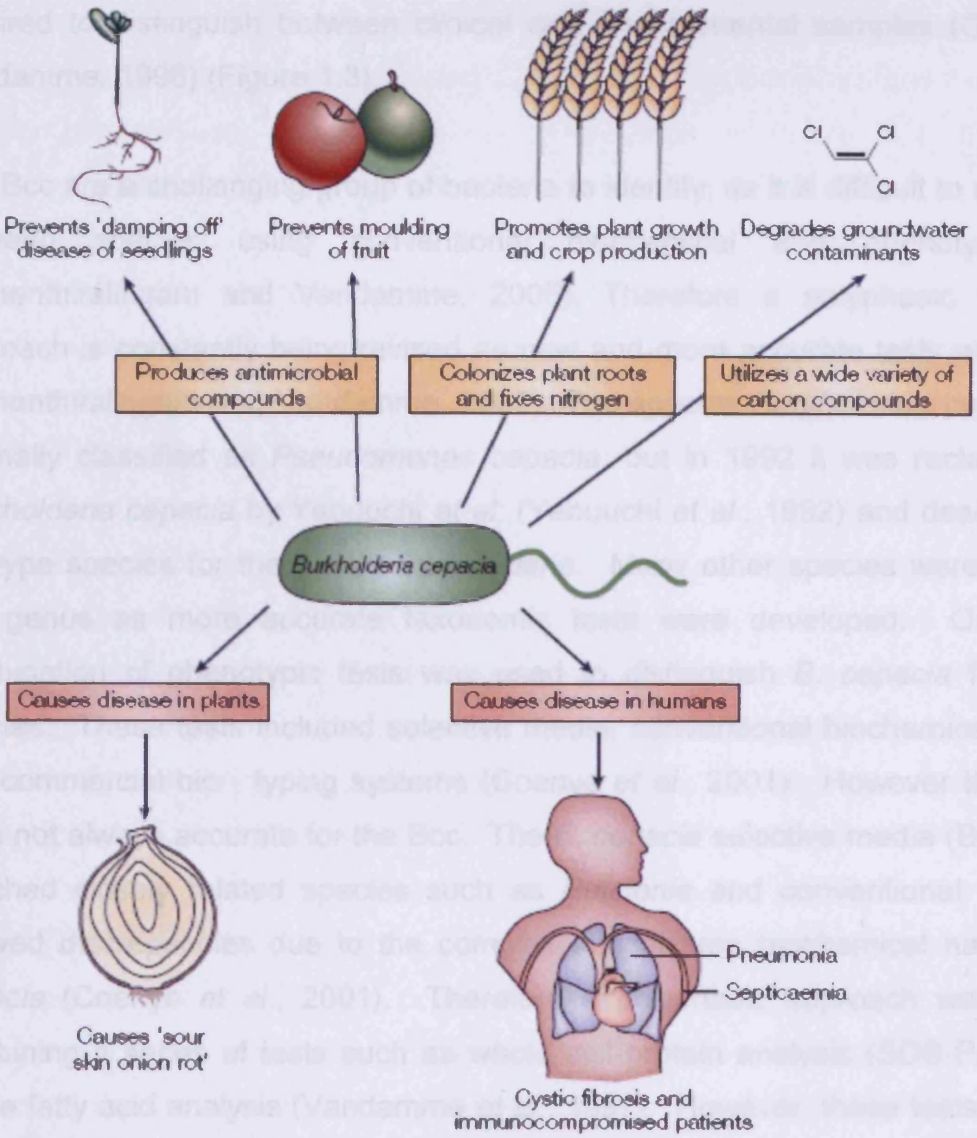

Figure 1.3 Interaction of the Bcc with plants and humans

The Bcc was originally designated as a pathogen of onions; however it is now recognised as having beneficial interactions with plants as well. They produce secondary metabolites that can protect the plant and also aid plant growth. In addition, the Bcc are capable of degrading a number of compounds, including pollutants. The Bcc are also opportunistic pathogens in cystic fibrosis patients. Figure taken from (Mahenthiralingam *et al.*, 2005).

required to distinguish between clinical and environmental samples (Govan and Vandamme, 1998) (Figure 1.3).

The Bcc are a challenging group of bacteria to identify, as it is difficult to distinguish between species using conventional biochemical and phenotypic tests (Mahenthiralingam and Vandamme, 2005). Therefore a polyphasic taxonomic approach is constantly being revised as new and more accurate tests are devised (Mahenthiralingam and Vandamme, 2005). The species *Burkholderia cepacia* was originally classified as *Pseudomonas cepacia*, but in 1992 it was reclassified as *Burkholderia cepacia* by Yabuuchi *et al.* (Yabuuchi *et al.*, 1992) and designated as the type species for the genus *Burkholderia*. Many other species were added to this genus as more accurate taxonomic tests were developed. Originally a combination of phenotypic tests was used to distinguish *B. cepacia* from other species. These tests included selective media, conventional biochemical analysis and commercial bio - typing systems (Coenye *et al.*, 2001). However these tests were not always accurate for the Bcc. The *B. cepacia* selective media (BCSA) also enriched closely related species such as *Ralstonia* and conventional tests also showed discrepancies due to the complex and diverse biochemical nature of *B. cepacia* (Coenye *et al.*, 2001). Therefore a polyphasic approach was devised combining a series of tests such as whole cell protein analysis (SDS-PAGE) and whole fatty acid analysis (Vandamme *et al.*, 1997). However, these tests could not distinguish between the entire Bcc, which lead to the development of genetic assays based on the polymerase chain reaction (PCR), which allowed the quick and accurate identification of the Bcc (Mahenthiralingam *et al.*, 2000a).

The first assay developed was the 16S rRNA based assay. The 16S rRNA gene has been used for the identification of species of other bacteria, so a PCR based assay was developed (Coenye *et al.*, 2001). LiPuma *et al.* (1999) investigated species-specific signature sequences in the 16S rRNA gene. However it was discovered, using restriction fragment length polymorphism (RFLP) analysis of the amplified 16S rRNA gene, that there was a high degree of similarity between sequences, thus species – specific primers could not be designed for most of the five members of the complex that were known at this time (LiPuma *et al.*, 1999). RFLP analysis involves the use of restriction enzymes, which recognize specific

short sequences of double stranded DNA and cleave the DNA at these sites. The number of recognition sites and their position varies, so the gene may be cut into a number of fragments of varying sizes. The variation in the 16S rRNA gene sequence between different species often allows RFLP analysis to be used as a rapid speciation approach. The 16S rRNA gene was capable of identifying *B. multivorans* and, *B. vietnamiensis*, but it could not discriminate between *B. cepacia*, *B. cenocepacia* and *B. stabilis* (LiPuma *et al.*, 1999). Therefore, although the 16S rRNA gene improved the diagnosis of infection with Bcc, a more accurate method was required to identify all the species of the Bcc.

RecA a conserved bacterial protein is involved with DNA repair and is encoded on the largest chromosome within the *Burkholderia* genome. RecA is essential to three biological processes. The first is general genetic recombination, which allows the re-assortment of the genome. RecA also mediates DNA strand exchange between two homologous strands of DNA. The second process it is involved with is the error prone replicative bypass of DNA lesions. This allows DNA to keep functioning even when the damage is too great to repair. The most important function of RecA is its role in the SOS response. Here RecA regulates genes that are involved with cleavage of proteins required to repair DNA in response to damage. Since the *recA* gene is found in all proteobacteria it has been used as a systematic marker and provides taxonomical resolution compared to 16S rRNA.

It was found that there was only a single copy of the *recA* gene in the *B. cepacia* genome and within the gene there was sufficient nucleotide sequence variation to enable the discrimination between all known species of the Bcc (Mahenthiralingam *et al.*, 2000a). PCR primers were designed to specifically amplify the *recA* gene and RFLP analysis indicated that these were specific to the Bcc and did not amplify other species of *Burkholderia* or other closely related species of bacteria. Therefore it was concluded that *recA* was a more suitable target for accurate, rapid PCR based diagnostic tools (Mahenthiralingam *et al.*, 2000a). The use of the *recA* gene assay allowed the identification of all genomovars of the Bcc, expanding the complex from five members to nine (Mahenthiralingam and Vandamme, 2005). A *recA* based assay was developed which was specific for the *Burkholderia* genus (Payne *et al.*, 2005) and subsequently assays were designed that used *recA* in a

non culture approach. This approach identified a number of novel *Burkholderia* species groups which had not been isolated using a culture dependent approach (Payne *et al.*, 2006).

Single gene sequence based identification has now been surpassed by a relatively new technique, multi locus sequence typing (MLST). MLST examines nucleotide polymorphisms in seven housekeeping genes located on the first and second chromosome of Bcc bacteria (Baldwin *et al.*, 2005). This provides an allelic profile which is then used to assign a clonal type to each strain (Mahenthiralingam *et al.*, 2008). MLST provides strain type and species identification that is unequivocal as it is based on sequence data. This data can also be placed into a database making it easily accessible to the whole scientific community (Mahenthiralingam *et al.*, 2008). MLST is becoming the gold standard to use for Bcc strain and species identification and has resulted in the classification of several novel species groups (Vanlaere *et al.*, 2008, Vanlaere *et al.*, 2009).

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The development of *recA* based assays originally distinguished nine closely related *B. cepacia* species that are all associated with the infection of immuno-compromised patients such as cystic fibrosis patients and patients with chronic granulomatous disease. These were formally named as *B. cepacia*, *B. multivorans*, *B. cenocepacia, B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia* (Mahenthiralingam *et al.*, 2000b). All nine species have been isolated from patients with CF, but some species are more prevalent than others. *B. cenocepacia* is the most predominant infecting between 50 – 80% of cases. *B. multivorans* is the next most predominant infecting 9 – 37% of cases (Mahenthiralingam and Vandamme, 2005). The rest cause less than 10% of CF infections. The Bcc complex was expanded to include *B. ubonensis* as the tenth genomovar (Vermis *et al.*, 2002). Two recent studies have expanded the complex to 17 members by the addition of *B. latens* sp. nov, *B. diffusa* sp. nov., *B. arboris* sp. nov., *B. seminalis* sp. nov., and *B. metallica* sp. nov (Vanlaere *et al.*, 2008) and *B. lata* and *B. contaminans* (Vanlaere *et al.*, 2009).

Members of the Bcc were important CF pathogens in the 1980's when Isles *et al.* described high levels of mortality with patients infected with members of the Bcc in

an outbreak in a CF clinic (Isles *et al.*, 1984). They noted that some patients appeared to be infected chronically and may have asymptomatic carriage, whilst other patients rapidly deteriorated with a necrotizing pneumonia which was characterised by high fever, severe progressive respiratory failure, leukocytosis and elevated erythrocyte sedimentation (Mahenthiralingam and Vandamme, 2005). This became known as "cepacia" syndrome and little is known about why some patients develop this whilst others carry Bcc infections chronically, although it is thought that both host and strain factors contribute (Mahenthiralingam and Vandamme, 2005).

Disease caused by the Bcc is very difficult to manage clinically and this was compounded by the emergence of patient to patient spread, first documented in the 1990's (Mahenthiralingam *et al.*, 2000a). The most transmissible strain appeared to be *B. cenocepacia* especially lineage ET-12 which has caused the most epidemic outbreaks in Canada and the United Kingdom (Mahenthiralingam *et al.*, 1997) (Govan *et al.*, 1993). MLST analysis of ET-12 strains found that they fell into four very closely related strain types (Baldwin *et al.*, 2005), which only differed at one of seven loci (single locus variants). ST 29, 30 and 31 were dominant in Canada, where as ST 28 was specific to UK CF patients (Mahenthiralingam *et al.*, 2008). Worryingly, *B. cenocepacia* also appeared to be able to replace other species such as *B. multivorans* (Mahenthiralingam *et al.*, 2001), which lead to the incorporation of stringent segregation polices in CF centres and CF camps. This can leave CF patients feeling isolated and discriminated against and can be very difficult to uphold within or outside the clinical setting.

Members of the Bcc have large genomes, which contain at least three replicons. This extensive genomic composition accounts for its versatility that sets it apart from other CF pathogens, such as its high transmissibility and inherent resistance to antimicrobial compounds (Mahenthiralingam *et al.*, 2005). There are several mechanisms that some species of the Bcc employs to allow it to infect CF patients. Many of these traits are poorly understood and much research is being carried out on these virulence factors in the hope of uncovering novel therapeutic targets. These virulence factors include lipopolysaccharides, biofilm formation, quorum sensing, the presence of genomic islands, the cable pillus, flagella and intrinsic

resistance to antibiotics and disinfectants (Govan and Deretic, 1996, Mahenthiralingam *et al.*, 2005). Antibiotic and disinfectant resistance is an important factor as some members of the Bcc are difficult to treat due to intrinsic and acquired resistance. Much research has been carried out into the mechanisms of antibiotic resistance, but little is known about disinfectant (biocide) resistance. Biocide resistance is becoming more and more common due to the increasing domestic and clinical use of biocides and thus this study will attempt to investigate the mechanisms of biocide resistance.

1.3 Biocides

Biocides are inorganic or synthetic organic molecules that are used to disinfect, sanitize or sterilize objects and surfaces. They can also be used to preserve materials from microbiological deterioration (Chapman, 2003a). Biocides are usually broad spectrum chemical agents that inactivate microorganisms. Biocides can be spilt into different groups, such as antiseptics which destroy and inhibit the growth of microorganisms on living tissues, and disinfectants which are used on inanimate objects or surfaces (Russell, 2002c). Antiseptics and disinfectants are used extensively in hospitals, for topical and hard surface applications. The domestic use of biocides as disinfectants has rapidly increased as concerns of bacterial contamination have been made more widely known by the media and press. These products contain a variety of biocides and are used in things such as antibacterial sprays, e.g. kitchen and bathroom cleaners, antiseptic wipes, mouthwashes and antiseptic creams to apply to skin wounds (McDonnell and Russell, 1999).

Biocides have an important role in hospitals where they are used as an essential component of infection control procedures and are used to aid prevention of nosocomial infections (Russell, 2002b). Although many biocides have been in use for hundreds of years, relatively little is known about their modes of action compared to antibiotics, which have been studied intensively. It is known that

Table 1.1 Biocide classes and their uses

Biocide Class	Name	Uses
Alcohols	Ethanol Isopropanol	Antisepsis Disinfection Preservation
Anilides	Triclocarbon	Antisepsis
Biguanides	Chlorhexidine	Antisepsis Antiplaque agents Preservation Disinfection
Bisphenols	Triclosan	Antisepsis Antiplaque agents Deodorants Preservation
Phenols	Phenol	Disinfection Preservation
Quaternary Ammonium Compounds	Cetrimide Benzalkonium chloride Cetylpyridinium chloride	Disinfection Preservation Antisepsis Cleaning

Table 1.2 Biocide "In use" concentrations

Name	Concentration (w/v)	Use
Chlorhexidine	0.5% - 4%	Disinfection
	0.02% - 4%	Antisepsis
	0.0025% - 0.01%	Preservation
Benzalkonium chloride	0.1 – 0.2%	Disinfection of skin
	0.1%	Application on mucus membrane
	0.005%	Bladder irrigation
	0.001 - 0.01%	Throat lozenges, contact lens solutions, eye drop
Cetrimide	1 - 3%	Shampoo
Cetylpyridinium chloride	0.1 – 0.5%	Skin disinfection and antiseptic treatment of wounds
	0.05% - 0.1%	Cosmetics – deodorants and face lotions
Povidone - iodine	> 10%	Wound disinfection

biocides tend to have multiple targets in the cells, where as antibiotics tend to have one specific intracellular target (McDonnell and Russell, 1999). The major classes of biocides used are: alcohols, anilides, biguanides, bisphenols, phenols and quaternary ammonium compounds. Table 1.1 summarizes the clinical use of these classes of biocides and Table 1.2 provides "In use" concentrations for common biocides (McDonnell and Russell, 1999). There is increasing concern that the over use of biocides may lead to an increase in bacterial resistance, which raises the question of whether biocides used in hospitals could become a source of an outbreak themselves? Thus it is important to study the mechanisms of action and resistance of biocides, which will aid the development of novel therapeutic targets.

1.31 Biguanides – Chlorhexidine

Chlorhexidine is a 1, 6 – di-chlorophenyl-biguanide hexane cationic bisbiguanide (Figure 1.4a). It was first synthesized in the 1950's and is now one of the most widely used biocides. Chlorhexidine has a high level of antibacterial activity and low mammalian toxicity. It has a strong binding affinity to the skin and mucus membranes and has a low rate of irritation, although there are some cases of irritation documented (McDonnell and Russell, 1999). It is therefore widely used in antiseptic products such as hand washing solutions and oral products, and is an important chemical used to prevent nosocomial infections (McDonnell and Russell, 1999). Chlorhexidine is an odourless, white crystalline powder that can be obtained as dihydrochloride, diacetate or gluconate salts. The activity of chlorhexidine is dependant on the pH, with its optimum pH being 5-7 and concentration. Solutions that are made up using diacetate salts have an odour of acetic acid and all solutions using chlorhexidine have a bitter taste, which has to be masked for oral use (McDonnell and Russell, 1999). Chlorhexidine is compatible with cationic materials and quaternary ammonium compounds, but its activity is significantly reduced in the presence of organic substances such as serum, blood and pus. It is often formulated as a solution with ethanol and in conjunction with detergents, which are used as surgical scrubs. It is also used in eye drops, antiseptic creams, mouthwashes, dental gels, catheter sterilisation, disinfectant

solutions for contact lenses, medical dressings and in cleaning sprays for hard surfaces (McDonnell and Russell, 1999).

Chlorhexidine targets cell components such as the outer envelope layers, although this is not sufficient to cause complete cell lysis. The chlorhexidine may then pass into the cell and attacks the cytoplasmic membranes and causes cell leakage (McDonnell and Russell, 1999). A high concentration of chlorhexidine causes coagulation of intracellular constituents. It is also thought that chlorhexidine targets the enzyme ATPase, although it appears not to be a primary target, with membrane disruption causing the most extensive damage (McDonnell and Russell, 1999). Chlorhexidine is active against both Gram positive and Gram negative bacteria, but is not sporicidal (McDonnell and Russell, 1999). Organisms, such as *P. aeruginosa* and *B. cepacia* have high innate resistance to chlorhexidine.

1.32 Alcohols

Alcohols are widely used for disinfection purposes, such as hard surface disinfection and antiseptic creams. There are many classes of alcohols that show antimicrobial activity. However, ethyl alcohol, isopropyl alcohols and n-propanal are the most widely used (McDonnell and Russell, 1999). Alcohols have a rapid broad spectrum effect on microbes and are active against most bacteria, viruses and fungi, but are not sporicidal (McDonnell and Russell, 1999). They are often used in conjunction with other biocides especially chlorhexidine to potentiate their activity. Antimicrobial activity is significantly lower at alcohol concentrations of 50% and optimal concentrations are in the range of 60-90% (McDonnell and Russell, 1999). The mode of action of alcohols is poorly understood, although it is thought that they cause membrane disruption and rapid denaturation of proteins which leads to interference with metabolism and cell lysis (McDonnell and Russell, 1999).

1.33 Quaternary Ammonium Compounds (QACs)

The quaternary ammonium compounds are a large class of biocides that include cetylpyridinium chloride (CPC) (Figure 1.4b), benzalkonium chloride (BZK) (Figure 1.4c) and cetrimide (Figure 1.4d). They are cationic agents and are used mainly for

antiseptics and disinfectants (McDonnell and Russell, 1999). The positive charge of these biocides allows a strong binding affinity to the bacterial cell membrane which has an overall negative charge, and causes displacement of divalent cations. This results in cellular leakage and the loss of osmoregulatory and physiological functions (Gilbert and Moore, 2005). QACs are used for a variety of clinical purposes such as preoperative disinfection of skin, application to mucus membranes and disinfection of non critical surfaces. As well as their antimicrobial properties, QACs have hard surface cleaning and deodorizing properties (McDonnell and Russell, 1999).

QACs have been postulated to target the cytoplasmic membrane of bacteria in the following manner (Salton, 1968): (1) adsorption and penetration of the agents into the cell wall; (2) reaction with the cytoplasmic membrane, followed by membrane disorganization; (3) leakage of intracellular low molecular weight material; (4) degradation of proteins and nucleic acids and (5) wall lysis. It is also thought that QACs damage the outer membrane of Gram negative bacteria, enhancing their own uptake into the periplasmic space, inner membrane and cell (Salton, 1968, McDonnell and Russell, 1999).

1.34 Phenols and Anilides

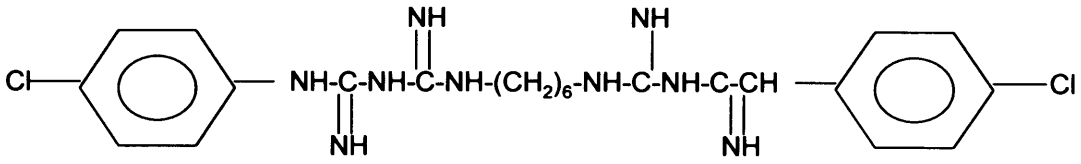
Phenols are used for their antiseptic, disinfectant and preservative properties. They are known as general "protoplasmic" poisons, but they also have membrane active properties. Phenols induce leakage of intracellular constituents, including K^+. There is also evidence that high concentrations of phenols causes cytoplasmic coagulation which subsequently leads to irreversible cellular damage (McDonnell and Russell, 1999).

Triclosan (2, 4, 4 – trichlor-2-hydroxydiphenylether, also known as Irgasan) (Figure 1.4e) is classed as a bis phenol. It is only sparingly soluble in water thus is diluted in alkalis and organic solvents in most applications (Moore and Payne, 2004). Its activity is not affected by the presence of most soaps, surfactants and organic solvents, but is affected by ethoxylated surfactants such as polysorbate 80 (Moore

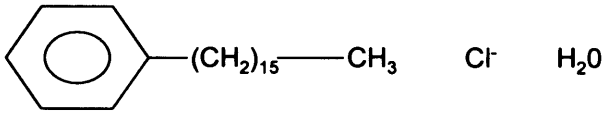
and Payne, 2004). Triclosan is generally bacteriostatic against Gram positive organisms, but has varying effects on Gram negatives, although its activity against these bacteria can be enhanced by adding a chelating agent such as EDTA (McDonnell and Russell, 1999). Some Gram negatives such as *P. aeruginosa* exhibit extremely low susceptibility to triclosan requiring over 1000 µg/ml for inhibition (Moore and Payne, 2004).

Triclosan is found in many personal care products such as toothpaste, handwashes, deodorants and has recently been incorporated into other household products such as in plastics and fabrics. Triclosan is suited to these applications as it is non toxic and can be applied to the skin (Moore and Payne, 2004). However, the increased use of triclosan, and the association between triclosan resistant bacteria and antibiotic resistance has lead to concerns about the use of this biocide (McMurry *et al.*, 1998a).

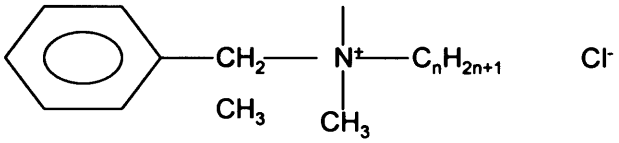
Triclosan was found to act on a specific target in the bacterial cell. This target was fatty acid synthesis and was particularly associated with the *fabI* gene. This gene encodes an enoyl reductase which was essential for fatty acid synthesis (McMurry *et al.*, 1998b). McMurry *et al.* found that triclosan blocks this gene by mimicking its substrate, thus preventing fatty acid synthesis. When mutants in this gene were created they were no longer inhibited by triclosan (McMurry *et al.*, 1998b). This specific mode of action is unusual for biocides, which usually have a broad spectrum of cellular targets. More recently, evidence has suggested that as well as targeting the fatty acid synthesis pathways triclosan may also have additional effects. At high concentrations, triclosan killing of the bacterial cells was rapid and fast acting, where as at low concentrations bacterial growth was effected. It has been suggested that at lower concentrations fatty acid synthesis is effected but at higher concentrations a more general damaging effect may be taking place, such as membrane disruption (Gomez Escalada *et al.*, 2005). Although, there may be other modes of action of triclosan the specific *Fab*I gene target may be a key factor in the emergence of resistance to this biocide as mutations to change this target site may lead to a less susceptible strain.



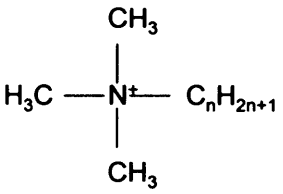
A - Chlorhexidine



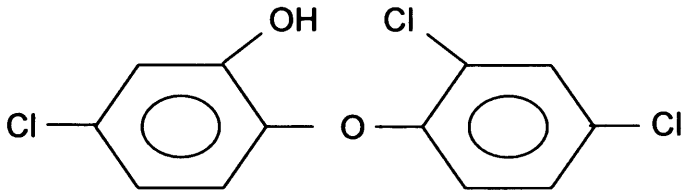
B – Cetylpyridinium chloride



C – Benzalkonium chloride



D - Cetrimide



E - Triclosan

Figure 1.4 Chemical structure of common biocides

The chemical structures of five common biocides including A: chlorhexidine, biguanide, B: Cetylpyridinium chloride, a QAC, C: benzalkonium chloride, a QAC and D: cetrimide, a QAC, E: Triclosan, bis phenol.

Anilides are generally used for their antiseptic properties and the most widely used anilide is triclocarbon. Triclocarban is used in commercial soaps and deodorants. It is most active against Gram positive organisms, but it shows significantly less activity against Gram negative bacteria and fungi (McDonnell and Russell, 1999). It is also not very effective when used on the skin and for these reasons is rarely used in clinical environments. Anilides appear to target the cytoplasmic membrane by adsorbing and destroying the semi-permeable character of the cytoplasmic membrane which leads to cell death (McDonnell and Russell, 1999).

1.35 Iodophors

Iodophors are surface active agents that can solubilize iodine, such as povidone-iodine. These are more desirable than free iodine as they retain bactericidal properties but eliminate the undesirable properties such as staining and toxicity. Different concentrations of iodophors are used depending on their application, either for antiseptic or disinfection purposes (Table 1.2). The concentration of free iodine is the active component of the disinfectant and is released when the iodophor is diluted. They have an activity over a wide range of pHs and the presence of a surface active carrier increases the wetting capacity. Iodophors are used in a wide range of applications such as in industry for sterilisation of farm equipment and for clinical uses such as pre surgical hand sanitisation and skin and wound disinfection (Moore and Payne, 2004). The mode of action of iodophors is poorly understood, however bactericidal action is rapid even at low levels. Iodophors penetrates the bacterial cell rapidly and attacks key proteins such as the free sulphur amino acids cysteine and methionine, nucleotides and fatty acids, which rapidly results in cell death (McDonnell and Russell, 1999). Although iodine containing solutions have been useful for wound disinfection, there have been a number of cases where solutions of povidone – iodine have been contaminated causing outbreaks. Berkelman *et al.* (Berkelman *et al.*, 1981) attributed the Bcc to an outbreak caused by contamination of a povidone-iodine solution.

1.4 Biocide resistance mechanisms

Biocides are increasingly being used in domestic and clinical situations. They play a large role in clinical situations, where they are often used in disinfection procedures of patient's rooms, equipment and for antiseptic purposes, including hand hygiene, cleansing of wounds and in surgical scrubs (McDonnell and Russell, 1999). Biocides now make up the primary ingredients of many domestic products, which are used to disinfectant household surfaces. In a community which is becoming increasingly concerned with bacterial infection these antibacterial products are being used more frequently and in larger quantities. This increased usage of biocides in both clinical and domestic environments has raised concerns that incidence of biocide resistance may be rising. This can cause many problems with bacteria which have multiple antibiotic resistance and possible biocide resistance, therefore making it very difficult to treat and eradicate these infections.

The minimum inhibitory concentration (MIC), which is the lowest dosage of the antibacterial that is inhibitory to growth for an organism, is used to determine how resistant an organism is (Russell, 2003). Members of the Bcc are examples of multi drug resistant bacteria, which have been found to contaminate benzalkonium chloride and other QACs as well as chlorhexidine. This is a worrying discovery as these biocides are frequently used in hospitals as disinfectants (McDonnell and Russell, 1999). Much research has been carried out into antibiotic resistance and ways to reduce its emergence. However, little is known about the mechanisms of biocide action and therefore the mechanisms of biocide resistance are also poorly understood. Biocides in general lack target specificity but different organisms vary in their susceptibility to each biocide, suggesting that biocides exert different effects on different target cells (Russell, 2001). Generally bacterial spores are the most resistant, then mycobacteria, followed by Gram negatives, with Gram positive bacteria the most susceptible (Russell, 2001). The mechanisms of resistance can be put into two classes, intrinsic resistance, which is resistance that the bacteria already posses or acquired resistance which the organism can gain by plasmid acquisition or mutation (McDonnell and Russell, 1999). Table 1.3 shows some examples of intrinsic and acquired resistance.

Table 1.3 Bacterial mechanisms of biocide resistance

Type of resistance	Possible mechanism	Example
Intrinsic	Impermeability	Gram negatives – several biocides
	Efflux	Gram negatives – several biocides
	Inactivation	Triclosan, chlorhexidine
Acquired	Inactivation / modification	Formaldehyde
	Insensitive target site	Triclosan
	Decreased accumulation (plasmid mediated)	Several biocides
	Overproduction of target	Triclosan
	Absence of enzyme metabolic pathway	Isoniazid

1.41 Acquired Resistance

1.41.1 Non plasmid mediated resistance

Acquired non-plasmid mediated resistance can occur when bacteria are "trained" to become resistant to a certain substance. This can occur when a particular antimicrobial is continuously present in the environment. The organism adapts to grow in gradually increasing concentrations of the biocide, although this resistance may not always be stable and can be lost if the antibacterial agent is removed from the environment (Russell, 2001). For example *P. stutzeri* can develop stable resistance to increasing concentrations of chlorhexidine and QACs resulting in changes to the outer membrane profile (Tattawasart *et al.*, 2000). Although temporary resistance by phenotypic adaptation has been shown it is not thought to play an important role in resistance to biocides (Russell, 2001).

1.41.2 Plasmid mediated acquired resistance

There has been much debate about the role of plasmid mediated resistance and its role in biocide resistance. There have been many documented cases of plasmids encoding resistance to antibiotics such as β lactams, tetracyclines, macrolides and chloramphenicol (Russell, 1997). However, there is little evidence of plasmid mediated biocide resistance (Russell, 1997). The RP1 plasmid in *P. aeruginosa* was examined and was found not to alter the resistance to QACs, chlorhexidine iodine, or phenols (McDonnell and Russell, 1999). Many studies demonstrated that it was difficult to transfer chlorhexidine or QAC resistance by plasmid mediated transfer and that the increase in resistance to these biocides was a result of mutation (McDonnell and Russell, 1999)

Nonetheless, in methicillin resistant *Staphylococcus aureus* (MRSA) plasmid pSAJ1 was found to be involved in biocide resistance. Plasmid pSAJ1 conferred resistance to kanamycin, gentamicin, tobramycin, amikacin, benzalkonium chloride, acriflavin, ethidium chloride and chlorhexidine. In addition when this plasmid was transferred to an *E. coli* host all of these resistance phenotypes were conserved, and resistance to acriflavine was also observed (Yamamoto *et al.*, 1988). MRSA

also have plasmids that encode *qac* genes which are found on pSK1 plasmid. The *qac* genes encode export proteins and confer resistance to a number of biocides including QACs (McDonnell and Russell, 1999).

1.41.3 Target Alterations

Target alteration is a well known adaptation technique by bacterial cells to resist the action of antibiotics. Biocides usually have multiple targets, hence target site mutations are not often seen (McDonnell and Russell, 1999). Triclosan however is the exception. As mentioned above, triclosan has a specific target, the *fabI* gene, which encodes an enoyl reductase involved in fatty acid synthesis (McMurry *et al.*, 1998b). Mutations in this gene in some bacterial species such as *P. aeruginosa* are no longer inhibited by triclosan, thus supporting evidence that this gene is an important target of triclosan (Poole, 2004).

1.42 Intrinsic resistance

Intrinsic resistance is a natural property of the bacterial cell that renders the biocide inactive. Unlike for antibiotics where acquired resistance is more prevalent, biocide resistance is frequently due to intrinsic resistance (White and McDermott, 2001). Intrinsic resistance is usually associated with cellular impermeability due to the composition of the cell wall, but can also be due to chromosomally encoded efflux pumps and biocide inactivation. Physiological adaptation is another type of intrinsic resistance and is observed when the organism is under a certain growth condition (White and McDermott, 2001) (Table 1.3)

1.42.1 Cellular impermeability and the Gram negative outer membrane

One of the main causes of biocide resistance is the reduction in biocide uptake by the bacterial cell. In general, Gram negative organisms are more resistant to biocides than Gram positive organisms due to the presence of an outer membrane. The outer membrane acts as a barrier that limits the entry of many chemically unrelated compounds (McDonnell and Russell, 1999). The structure of the Gram negative outer membrane is a lipid bilayer and consists of an outer membrane, a

thin layer of peptidoglycan and a periplasmic space between the outer membrane and the cytoplasmic membrane (Denyer and Maillard, 2002). The outer envelope bilayer consists of lipopolysaccharides and phospholipids which contain porins embedded within them (Figure 1.5) (Denyer and Maillard, 2002) (Hancock, 1997). There are a number of contributions the outer membrane makes to resistance which are indicated on Figure 1.5 and described below.

1.42.2 Lipopolysaccharides (LPS)

The LPS is a hydrophobic high molecular weight, strongly negative charged molecule, which can be divided into three sections, the lipid A portion inserted into the membrane, the core region (which the lipid A is attached to), containing a region of 8 – 12 variable sugars, that include negatively charged 2 – keto – 3 – deoxyoctanate and 3 – 8 phosphate residues. This region is covalently linked to the O antigen which consists of 3 – 5 sugar units that are repeated a variable number of times (Hancock, 1997). Some bacterial strains have the O antigen missing and these are designated as rough strains (for a diagram of the LPS structure see Figure 5.1). Cationic biocides bind to the negatively charged membrane to penetrate the cell. To reduce the uptake of the biocide, modifications of the LPS can occur. The Bcc modify the composition of the LPS to produce a less negatively charged membrane thus reducing the binding affinity of cationic compounds (Cox and Wilkinson, 1991). This includes increased Mg^{2+} content which neutralises the negative charge and causes strong LPS-LPS links making diffusion into the cell difficult (Hancock, 1997) (McDonnell and Russell, 1999). The modifications of the LPS is discussed in more detail in Chapter 5.1.

1.42.3 Porins

The Gram negative outer membrane serves as a molecular "sieve" which allows the entry of small hydrophilic molecules via porins (Hancock, 1987). Porins are hydrophilic channels that can be classed as either non specific diffusion porins or specific porins that mediate the entry of a particular solute (Denyer and Maillard, 2002). Resistance to a number of biocide agents can be enhanced by the loss of

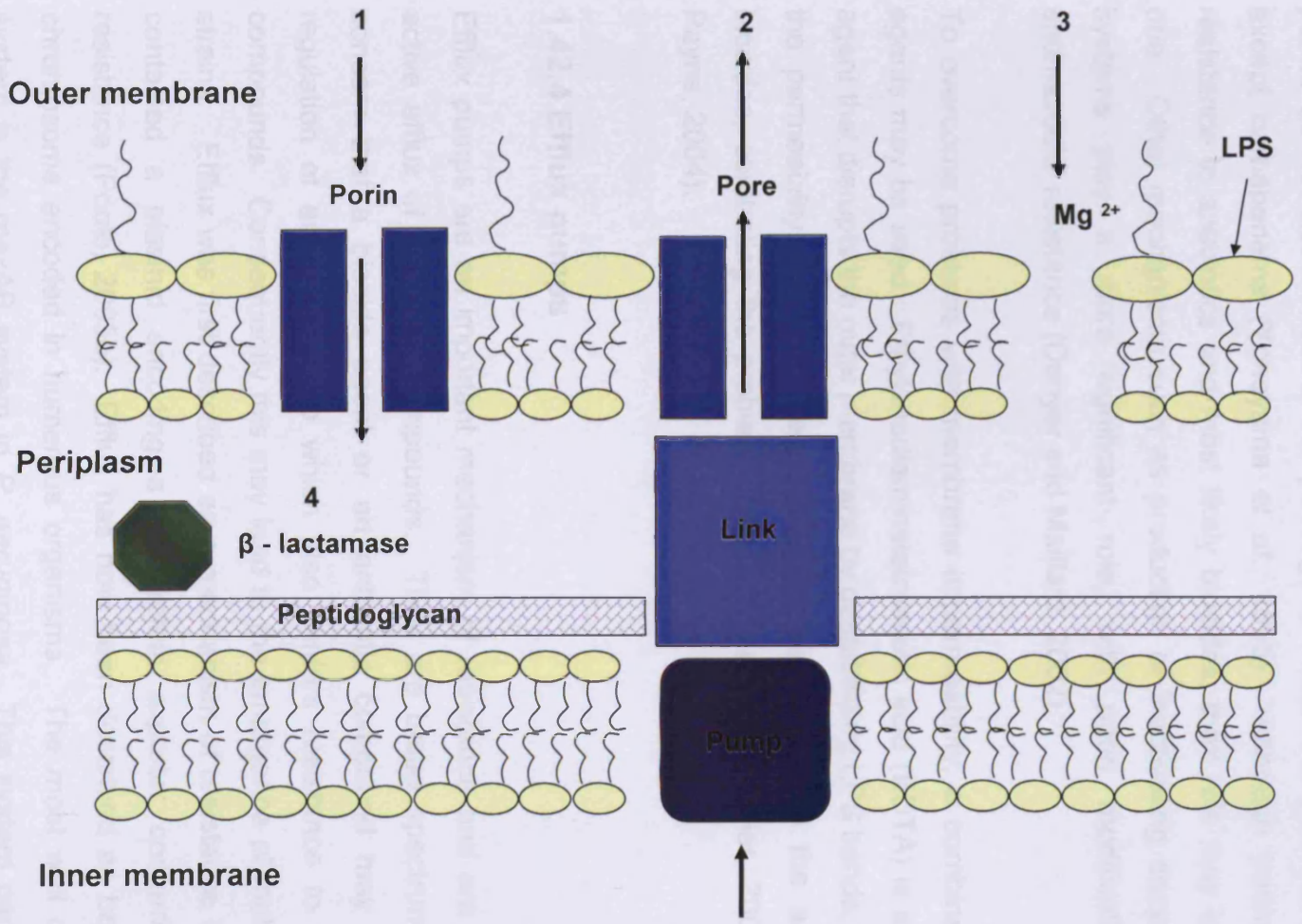


Figure 1.5 The Gram negative cell wall and resistance mechanisms

1: Porin pathway for small hydrophilic molecules, 2: Three component efflux system, 3: Self promoted uptake pathway for small polycations and 4: periplasmic β lactamases. Figure adapted from (Hancock, 1997).

porin proteins or alteration of porin size (Denyer and Maillard, 2002). In the Bcc, some strains were found to have a small porin size which contributed to the reduced susceptibility to the β lactam nitrocefin (Parr *et al.*, 1987). In *P. aeruginosa* porins do not accommodate the passage of most anti pseudomonal antibiotics except carbapenems (Yoneyama *et al.*, 1995). Although porins contribute to resistance to antibiotics and most likely biocides, their role may only be a minor one. Other mechanisms such as production of inactivating enzymes and efflux systems play a more significant role, with porin modification supporting antimicrobial resistance (Denyer and Maillard, 2002).

To overcome problems with membrane impermeability, a combination of biocide agents may be used. Ethylenediaminetetraacetic acid (EDTA) is a permeabilizing agent that disrupts the outer membrane by destabilising LPS bonds, thus increasing the permeability of cell. Therefore EDTA can potentate the activity of many biocides, combating the problem of reduced uptake (Stickler, 2004) (Moore and Payne, 2004).

1.42.4 Efflux pumps

Efflux pumps are an important mechanism of resistance and are involved in the active efflux of unrelated compounds. They are broad spectrum, thus there is concern that a biocide agent or an antibiotic compound may induce the up regulation of an efflux pump which also confers resistance to other antibiotic compounds. Consequently this may lead to the emergence of multi drug resistant strains. Efflux was first described as a mechanism of resistance in *E. coli*, which contained a plasmid encoding a tet protein exporter conferring tetracycline resistance (Poole, 2005b). Efflux has now been described as both plasmid and chromosome encoded in numerous organisms. The most well described efflux system is the mexAB system in *P. aeruginosa*. This system can accommodate both antibiotics and biocides thus causing many related clinical issues (Poole, 2004). There are five classes of efflux pumps, with the resistance nodulation division occurring most frequently (Poole, 2004). The structure and function of efflux pumps are discussed more thoroughly in Chapter 5 and Figure 5.9.

1.42.5 Inactivation / modification

Another method of preventing killing by antimicrobials is inactivation. This can occur by a number of methods. The periplasmic space contains systems that inactivate toxic compounds. The periplasm contains a number of enzymes which can inactivate compounds, such as β lactamases (Denyer and Maillard, 2002) (Figure 1.5). This is most well known for antibiotics, with the inactivation of β lactamases and the modification of aminoglycosides by phosphotransferases.

1.42.6 Production of a biofilm

Many bacteria will actually grow in biofilms rather than planktonically. A biofilm is a cohort of bacterial cells, which may or may not be a mixed population that has adhered to a surface. They usually produce an exopolysaccharide matrix which surrounds the bacterial cells (Stickler, 2004). Cells growing in biofilms are notoriously more difficult to eradicate and cause problems not only in clinical situations, but in industrial settings as well (Stickler, 2004). The biofilm growth state renders the antimicrobial ineffective due to reduce permeation of the biofilm, thus many cells are protected from contact with the substance. A slower metabolic growth rate may be observed and hence a reduced uptake by the cells of the biocide follows (Stickler, 1999). Biofilms result in many problems for clinicians and can result in treatment failure.

Resistance to ciprofloxacin and ceftazidime, two agents used in the treatment of CF infection was enhanced in *B. cepacia* biofilms up to 15 times more than planktonically growing cells (Desai *et al.*, 1998) and evidence that 0.2% chlorhexidine was not sufficient to kill a mixed biofilm population after an hours exposure has been described (Wilson *et al.*, 1998). Biofilms cause particular problems for patients with indwelling catheters and indeed any implanted device. Strains of *P. aeruginosa* and *Proteus mirabilis* can encrust catheters and cause blockages. Once biofilms have formed it appears that even previous sensitive strains can become resistant to biocides and that there may be a cross over with antibiotic resistance (Stickler, 2002). Stickler found that chlorhexidine was ineffective at eliminating biofilms; Williams *et al.* found similar results with triclosan (Williams and Stickler, 2008). Consequently, disinfection routines may not be

adequate to prevent infections when bacteria are growing as a biofilm and may in fact promote resistance to a number of antimicrobials. Biofilms are also discussed in Chapter 5.

1.5 Biocide rotation policy

In light of the evidence presented above, disinfection policy, particularly in clinical settings should be carefully examined and implemented. Biocide rotation involves the alternative use of two active chemical agents with different modes of action. This should reduce the possibility of selecting for multi drug resistant organisms and to increase the possibility of killing all organisms (Murtough *et al.*, 2001). There are many factors involved when implementing rotation, including frequency of rotation and period of contact (Murtough *et al.*, 2001). There has been much debate over whether biocide rotation is a good idea or not, with arguments for including the evidence that adaptive resistance can be trained to low levels of biocides present in the environment (Tattawasart *et al.*, 1999). These adapted strains also exhibited cross resistance to antibiotics causing concern that multi drug resistant strains may emerge (Tattawasart *et al.*, 1999). However, the reverse argument states that this resistance is usually unstable and that microbial contamination may be due to poor hygiene by personnel, thus it may be more prudent to improve cleanliness and personal hygiene rather than rotating biocides (Murtough *et al.*, 2001).

There has been little evidence that biocides, when used appropriately are causing resistant organisms to occur. There is also concern that the over use of biocides and residuals in the environment may lead to cross resistance to some antibiotics (Chapman, 2003b). The potential cross resistance of biocide and antibiotic resistance mechanisms are discussed in Chapter 3.13. However, it may still be beneficial to rotate biocides in certain situations where particularly problematic organisms such as the Bcc and *P. aeruginosa* may be present (Murtough *et al.*, 2001). To prevent contamination of biocides infection control policies should be in place and cleanliness, incorporating biocide disinfection should be stringent.

1.6 Infection control in cystic fibrosis clinics

With the emergence of multi drug resistant organisms, infection control has played a vital role in reducing the occurrence of nosocomial infections in patients. In CF patients it is particularly important as their disease leaves them vulnerable to many, difficult to treat infections. Some members of the Bcc cause infection control problems as they are transmissible between patients (Jones *et al.*, 2001a), hence clinics often implement draconian segregation polices, which can lead to CF patients feeling isolated and discriminated against. Epidemic strains of *P. aeruginosa*, such as the Liverpool (Cheng *et al.*, 1996) and Manchester epidemic strains (Jones *et al.*, 2001b) also require attention to prevent other CF patients from acquiring these infections The increasing incidence of MRSA in hospitals is also causing problems in CF clinics and stringent guidelines must be put into practice to reduce the acquisition of these infections.

The Cystic Fibrosis Trust have released guidelines for preventing Bcc infections, which contains information on segregation policies and disinfection practices: "The *Burkholderia cepacia* complex: suggestions for Prevention and Control" (www.cftrust.org.uk). There are many ways that infections such as *B. cenocepacia* can be spread from patient to patient or health care worker to patient (Table 1.4). The standard precautions to prevent infections are as follows: hand washing, barrier precautions, e.g. wearing gloves, gown and mask and sterilization of respiratory equipment (Saiman and Siegel, 2004).

Those patients that carry highly transmissible infections have further transmission based precautions implemented. These are categorised as contact, droplet, airborne infection and protective environment (Saiman and Siegel, 2004). Hand washing is implemented before and after all patient contact and alcohol based hand rubs are used to reduce bacterial contamination. Respiratory equipment such as nebulizers, bronchoscopes, spirometers and lung function test machines can be reservoirs of infection; therefore careful attention must be paid when disinfecting this equipment. Ideally all equipment should be single use only, but this is rarely feasible in a hospital environment, so equipment needs to be sterilised appropriately (Saiman and Siegel, 2004). To prevent infections being spread in hospitals, including out patient clinics, those infected with the Bcc complex and other infectious organisms should attend separate clinics on different days to

Table 1.4 Factors associated with acquisition of infections in CF patients

Risk factors in Non Health Care settings	Social Contact	Risk Factors in health care settings
Attendance at CF summer camps	Kissing	Inpatient exposure
Sleeping in the same cabin	Intimate contact	Recent hospitalization
Sharing a personal item	Prolonged car rides	Use of specific shower
Dancing or hugging a camper infected with Bcc	Fitness classes	Sharing a hospital room with another infected patient
Attendance at summer educational groups	Sharing drinking utensils	Use of respiratory equipment
Participation in a support group	Handshaking	Sharing equipment
	Sibling with infection	Hospital nebulizers
		Spirometer
		Mouthpiece filters

(Adapted from Saiman and Siegel, 2004)

uninfected patients. Also those patients infected with *B. cenocepacia* should attend clinics on different days to those patients infected with other Bcc strains to avoid replacement of less virulent strains with more virulent strains (Saiman and Siegel, 2004). Acquisition of *B. cepacia* infections has been associated with hospitalization, poor adherence to hand washing, contaminated respiratory equipment and contaminated hospital showers; therefore regular disinfection of sinks and showers also needs to take place (Saiman and Siegel, 2004). Table 1.5 highlights some of the methods used to help reduce the acquisition of infections, nevertheless to truly prevent patients from acquiring these life threatening infections, the cooperation of the patient is vital and regrettably this is often difficult to implement and maintain. Therefore it is important to reduce these factors, which unfortunately usually means keeping CF patients with Bcc infections away from other CF patients

1.7 Aims

The aims of this project were three fold:

1) To characterise the biocide susceptibility for a number of biocides, particularly focusing on chlorhexidine and cetylpyridinium chloride for a panel of representative strains that encompasses all species of the Bcc.

2) To determine the genetic basis of biocide resistance mechanisms to chlorhexidine using a whole genome expression approach.

3) To further characterise genes that may be involved in resistance to chlorhexidine.

An overall summary of the aims and the experimental approach taken is shown in Figure 1.6

Table 1.5 Measures to prevent the acquisition of infections in CF patients

Prevention method	Intervention
Education	Emphasis on hand hygiene
	Educate patients families and health care workers about risk factors for transmission
Intervention in health care settings	Use single patient rooms with separate showers
	Eliminate socialising between CF patients infected with Bcc and other CF patients in hospital
	Place hospitalized patients with infections on contact precautions
	Inpatients and outpatients should wear masks Inpatients should wear gloves
	Segregate outpatient clinics e.g. *B. cepecia* clinics attend on different days
	Ban patients with *B. cepecia* infection from attending CF conferences
Environmental decontamination	Decontaminate environment including respiratory equipment
	Monitor environmental decontamination i.e. drains, showers, physiotherapy equipment
Laboratory practices	Improve microbiological detection
Intervention in non health settings	Reduce social contact between patients infected with the *B. cepacia* complex and patients in non health care facilities
	Provide separate summer camps for CF patients with virulent infections

(Adapted from Saiman and Siegel, 2004)

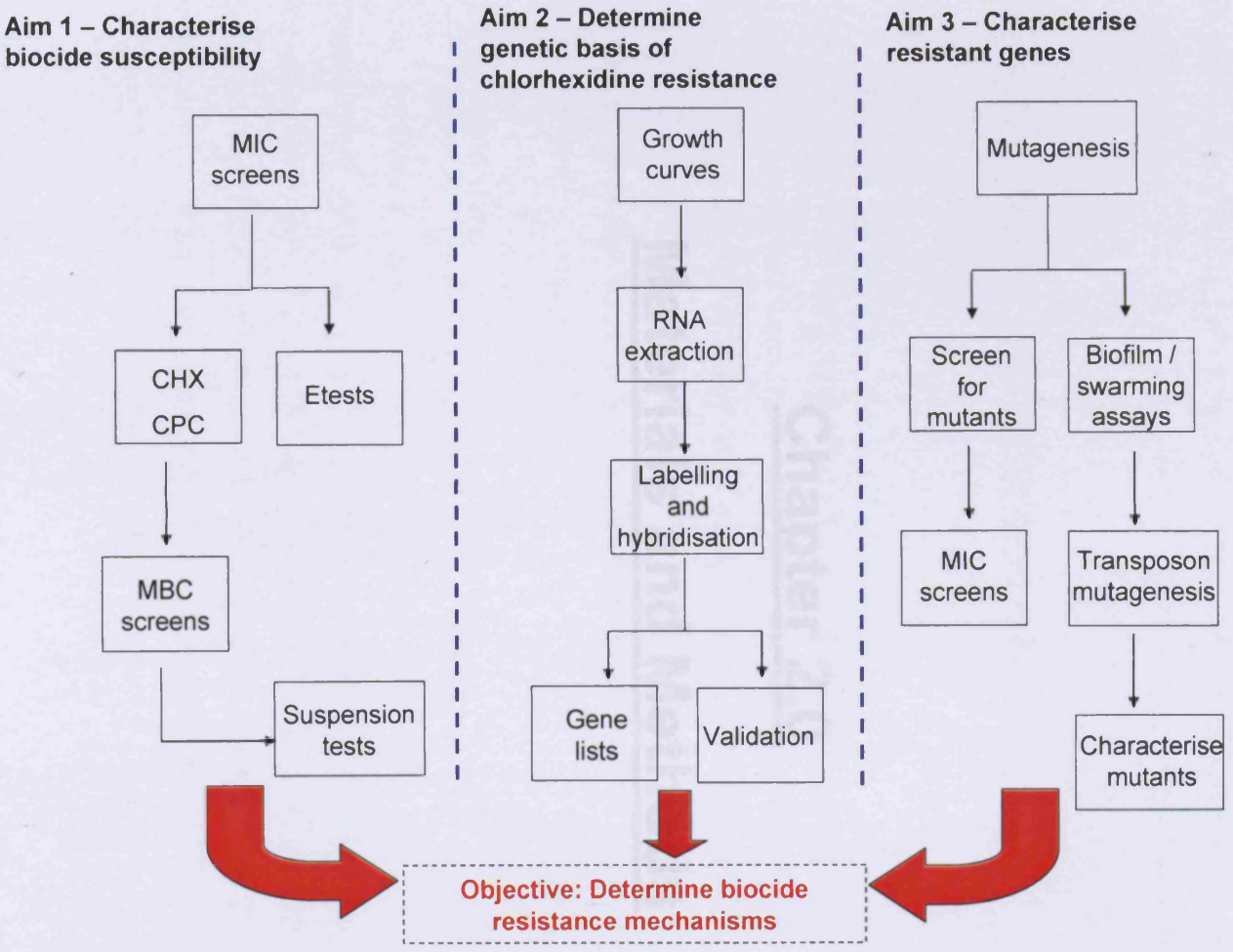


Figure 1.6 Experimental method used for each aim

Experimental methods used to determine aims of this investigation. The aims were three fold and were used to achieve the main objective; to determine biocide resistance mechanisms and possible targets for novel therapeutics.

Chapter 2.0

Materials and Methods

Chapter 2.0 Materials and Methods

2.1 Chemicals

The chemicals used in this study, unless otherwise stated were obtained from Sigma-Aldrich, Poole, UK, ICN Biomedicals Ltd, Oxon, UK and Fisher Scientific Ltd, Loughborough, UK. All aqueous solutions were made in double deionised water.

2.2 Preparation of antimicrobial agents

2.21 Biocides

The composition of the biocides used in this study are presented in Table 2.1 All aqueous biocide stock solutions were heated to 50°C to aid the dissolving process and left on a rocker platform until a clear solution was obtained and were filter sterilised using a 0.2 µm mini filter (Sartorius AG, Goettingen, Germany). These stock solutions were subsequently used to create further biocide concentrations when needed.

2.22 Antibiotics

The antibiotics used in this study were ampicillin (Ap), kanamycin (Km), meropenem (Mp), polymyxin (Pmx) tetracycline (Tc), tobramycin (Tm) and trimethoprim (Tp). All antibiotics were made up to a 100 mg/ml stock except polymyxin (120,000 units/ml). Antibiotics were dissolved in sterile polished water, apart from meropenem, tetracycline and trimethoprim which were dissolved in dimethyl sulfoxide (DMSO) due to insolubility in water.

2.3 Bacterial Strains and Plasmids

Bcc organisms, *P. aeruginosa* organisms and non Bcc species such as *Staphylococcus aureus*, *Burkholderia gladioli*, *Stenotrophomonas maltophilia*, *Ralstonia mannitolilytica*, *Pseudomonas stutzeri*, *Ralstonia pickettii*, *Pseudomonas putida*, *Achromobacter xylosoxidans* and *Escherichia coli* were drawn from the

Table 2.1 Biocide stock solution compositions

Biocide	Class	Stock Solution	Solvent
Chlorhexidine	Biguanide	10 mg/ml	Water
Cetylpyridinium chloride	Quaternary Ammonium Compound	10 mg/ml	Water
Triclosan	Phenol	10 mg/ml	DMSO
Benzalkonium Chloride	Quaternary Ammonium Compounds	10 mg/ml	Water
Povidone	Iodine releasing agent	20 % (w/v)	Water

Mahenthrialingam lab collection, unless otherwise stated. Species and strain designations for all bacterial isolates are shown within specific tables in each chapter. Plasmids, strains and genetic constructs used for cloning are shown in Table 2.2.

2.31 Bacterial Growth conditions

All bacterial strains used were revived from frozen stocks by plating on either Tryptone Soya Agar (TSA) or Luria Bertani agar (LBA) at 37°C overnight; environmental isolates often required a lower temperature of 30°C. Overnight cultures were made by taking a single colony from the revival plates and inoculating 3 ml of tryptone soya broth (TSB) or Luria Bertani broth (LB), and grown with shaking in an incubator (200 rpm) at 37°C for 18 hours.

2.32 Storage of bacterial strains

All strains used in this study were held in an ultra low temperature freezer. Fresh colony material from a pure culture plate was inoculated into TSB containing 8% DMSO and mixed to produce a bacterial suspension. These stocks were then maintained in a -80°C freezer.

2.33 Enumeration of bacteria

To enumerate bacterial suspensions viable drop counts were used. A serial dilution of the bacterial suspension was made in TSB and 10 µl drops were placed in triplicate onto TSA plates and incubated at 37°C overnight. Individual colonies were then counted, the number of bacteria calculated and expressed as colony forming units per ml (cfu/ml).

2.4 Preparation of DNA from bacterial cells

2.41 Bead beater method

Table 2.2 Strains, plasmids and genetic constructs used in this investigation

Strain / Plasmid	Features	Reference / source
Strains		
B. cenocepacia K56-2	ET-12 CF strain used for allelic exchange mutagenesis	(Mahenthiralingam *et al.*, 2000b)
B. cenocepacia AU1054	Environmental *B. cenocepacia* III B strain used for random transposon mutagenesis	(Mahenthiralingam *et al.*, 2000b)
E. *coli* – HB101	Contains plasmid pRK2013 – helper strain for tri parental mating, self transmissible strain with mobilisation genes	(Figurski and Helinski, 1979)
E. coli – JM109	Contains plasmid pTnMod-OTp – trimethoprim resistant plasposon used for random mutagenesis	(Dennis and Zylstra, 1998)
E.coli – omnimax cells	Standard cloning strain	Invitrogen co
Plasmids		
pGEM – T easy	PCR product cloning vector	Promega corporation inc
pEGM105Tc	pJQ200SK with tetracycline resistant cassette, suicide vector used for allelic exchange mutagenesis	(Quandt and Hynes, 1993)
pUC-Tp	1.1 Kb trimethoprim resistant cassette	(Sokol *et al.*, 1999)
p0924-Tp	pEGM105Tc carrying 0924 response regulator gene interrupted with Tp resistant cassette	This study
p2353-Tp	pEGM105Tc carrying 2353 transport gene interrupted with Tp resistant cassette	This study

Overnight cultures (TSB) of the bacterial strains to be harvested were centrifuged at 900 x g for 10 minutes. The supernatant was decanted and the bacterial pellet was re-suspended in 100 µl of TE buffer (10mM Tris-Cl pH8, 1mM EDTA pH8).

The re-suspension was then added to 500 µl of lysis buffer (50 mM Tris-Cl pH8, 70 mM EDTA pH8, 1% SDS) containing 0.5 mg/ml of pronase (Boehringer/Roche) in a screw cap tube containing approximately 500 µl of 0.1 mm diameter Zirconium glass beads (Biospec Products Inc). The bacterial cells were then placed in a bead beater machine (Biospec Products Inc) at the lowest speed setting (25) for 5 to 10 seconds and then incubated at 37°C for one hour. After incubation, cells are briefly centrifuged and 200 µl of saturated ammonium acetate added to the lysate. The lysate was pulsed on the bead beater for 5 seconds and 600 µl of chloroform added. This was then centrifuged for 5 minutes at maximum speed to separate phases and 400 µl of the clear aqueous phase was removed and placed into a tube containing 1 ml of 100% ethanol. The tubes were inverted to clot DNA and centrifuged for 5 minutes to form a pellet. The ethanol was removed and the DNA pellet washed with 70% ethanol. Once removed, the pellet was dried under a vacuum for 10 minutes. The DNA was dissolved in low EDTA TE buffer (10 mM Tris-Cl, pH8, 0.1 mM EDTA) with 0.5 µg/ml RNase A and incubated for one hour at 37°C to remove any RNA.

2.42 Chelex DNA preparation

Bacterial strains were streaked to single colony; one colony was picked with a pipette tip and placed into sterile 5% chelex 100 solution (Sigma – Aldrich). This was then placed in a thermal cycler and heated to 98°C for 5 minutes. After incubation, it was then placed on ice for a further 5 minutes. This cycle was repeated and then the tube was centrifuged for 5 minutes at 700 x g. DNA within the cleared supernatant could then be used in further applications.

2.43 Plasmid preparation for bacterial cells

To isolate plasmids from bacterial cells the Wizard Plus SV mini prep kit (Promega corporation) was used. Overnight cultures were harvested by centrifugation at 900 x g for 10 minutes. A cleared lysate was produced by adding 250 µl of cell re-suspension solution to the cell suspension. The cell re-suspension was mixed with 250 µl of cell lysis solution and incubated until the cell suspension cleared (approximately 1- 5 minutes). 10 µl of alkaline protease solution was then added, mixed and incubated for 5 minutes. The alkaline protease digested endonucleases and other proteins released during the lysis of the bacterial cells, therefore improving the quality of the eluted plasmid. After incubation, 350 µl of Wizard plus neutralisation solution was added and immediately mixed by inversion. The suspension was then centrifuged for 10 minutes at maximum speed.

A series of centrifugations were carried out to remove unwanted products and to obtain the plasmid. The cleared lysate was decanted into a prepared spin column and centrifuged for 1 minute. The flow through from the collection tube was discarded. Then 750 µl of column wash solution was added to the spin column and centrifugation was carried out for 1 minute and again the flow through was discarded. This wash procedure was repeated with 250 µl of column wash solution and was then centrifuged for 2 minutes. The spin columns were transferred to a low DNA binding tube and the plasmid eluted by adding 100 µl of nuclease free water. This was centrifuged for 1 minute and the spin column discarded. All plasmid solutions were stored at -20°C

2.44 Visualisation and quantification of DNA

To visualise DNA and plasmid preps gel electrophoresis was used. Agarose gels were made to a percentage depending on the size of the product; 1% for general use (1 – 12 Kb), 1.8% or 2.5% for fragments below 3 Kb. A DNA stain, SafeView (NBS Biologicals) was added to both the gel and the buffer so no post staining was required. Loading dye was added to the samples and the gel ran at 80 volts for approximately an hour. A 1 Kb molecular weight ladder was also run to determine the size of the products. Once the gel had run, pictures were taken using a UV GelDoc machine (Syngene)

To determine the amount of DNA or plasmid in a sample, 1.2 µl of the sample was placed on a Nanodrop (Thermo Scientific), which was calibrated to the buffer used to dissolve the sample in. The Nanodrop measured the amount of DNA in the sample in ng/µl (using UV absorbance at 260 nm / 280 nm) and provided a graph to indicating the quality of the sample.

2.5 Restriction digestion of DNA

Restriction enzyme digests of DNA were required for several cloning steps. All enzymes were obtained from Promega and can be seen in Table 2.3. The digest protocol used the following: 0.2 – 1.5 µg of substrate DNA, with a two to ten fold excess of enzyme. A typical reaction was set up with: sterile deionised water, 16.3 µl, RE 10 x buffer 2 µl, acetylated BSA (10 µg/µl), 0.2 µl, DNA (1 µg/µl), 1 µl and restriction enzyme (10 units/µl), 0.5 µl, to give a final reaction total of 20 µl. Digests were incubated at the appropriate temperature (Table 2.3) for approximately 4 hours. Once incubation was complete termination of the reaction was required. Many of the enzymes were susceptible to heat inactivation (Table 2.3), where the reactions were heated to 65°C for 20 minutes. However, several enzymes could not be heat inactivated so to terminate the reaction chloroform extraction was carried out.

2.53 Chloroform extraction

DNA volume was made up to 200 µl and then one third of saturated ammonium acetate was added. To extract the DNA, 200 µl of chloroform was added to the tube and shaken vigorously. The extract was centrifuged for 1 minute at maximum speed and the upper aqueous phase was collected. Three volumes of 100% ethanol was added, mixed by inversion and then placed at -80°C for one hour. After freezing, the DNA was collected by centrifugation for 20 minutes at maximum speed, the supernatant removed and the pellet washed with 100 µl of 70% ethanol. This was then dried in a vacuum for 10 minutes and dissolved in approximately 10 µl of water. Restriction digests can then be visualised by gel electrophoresis.

2.6 DNA extraction from gels

DNA extraction from agarose gels was carried out using a QIAquick spin kit (Qiagen). DNA bands were excised under a UV lamp and weighed in a 1.5 ml tube. Three volumes of Buffer QG was added to one volume of gel (e.g. 100 mg of gel assumed to be 100 µl) and incubated at 50°C for 10 minutes until the gel had dissolved completely. During the incubation period the tube was mixed every 2-3 minutes. One volume of isopropanol was then added to the sample and mixed, to increase the yield of DNA. DNA was bound by applying the sample to a QIAquick spin column and centrifuged at maximum speed for 1 minute. Flow through was discarded and 500 µl of Buffer QG was added to column and centrifuged for 1 minute. To wash 750 µl of Buffer PE was added to QIAquick column and centrifuged for 1 minute. Flow through was discarded and tube centrifuged for an additional 1 minute. Column was placed into a clean tube and DNA eluted by adding 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5) to the centre of the QIAquick membrane and centrifuging for 1 minute.

2.7 Preparation of RNA from bacterial cells

To extract RNA for microarray analysis, a Qiagen kit was used and the manufactures protocol followed as stated. Bacterial cells were harvested as described in section 2.13. The bacterial pellet was loosened by flicking the tube and then re-suspended in 100 µl of TE buffer containing lysozyme by vortexing. This was then incubated at room temperature for 10 minutes. An additional sonication step was carried out to aid lysis of bacterial cells and increase RNA yield. Samples were sonicated at 5 Hz for 10 seconds on, then 10 seconds off 10 times using a Sanyo Soniprep 150 Watt sonicator. 350 µl of buffer RLT containing β-mercaptoethanol was added to the sample and mixed by vortexing. Homogenization was then carried out using the QIAshredder homogeniser. The lysate was transferred into the QIAshredder spin columns, which were held within a 2 ml collection tube. This was then centrifuged for 2 minutes at maximum speed and the homogenized lysate collected. 250 µl of 100% ethanol was added and mixed by pipetting. The sample was then applied to an RNeasy mini column held in

a 2 ml collection tube. This was centrifuged for 30 seconds at 7000 x g and the flow through discarded.

An optional RNase free DNase step was used to remove residual traces of DNA. 350 µl of buffer RW1 was added to the RNase mini column and centrifuged at 7000 x g for 30 seconds and the flow through discarded. Then 10 µl of the DNase 1 stock solution was added to 70 µl of buffer RDD and mixed by inversion. This was pipetted directly onto the RNeasy silica gel membrane and left at room temperature for 15 minutes. Buffer RW1 (350 µl) was added to the RNeasy column and centrifuged for 30 seconds at 7000 x g and the flow through discarded.

The RNeasy column was transferred to a new 2 ml collection tube and 500 µl of buffer RPE was added. The sample was centrifuged at 7000 x g for 30 seconds and the flow through discarded. The previous step was repeated and centrifuged for 2 minutes to dry the silica membrane. The column was placed into a new tube and centrifuged for 1 minute to eliminate carry over. The RNA was eluted by transferring the column to a 1.5 ml collection tube and pipetting 30 – 50 µl of RNase free water directly onto the silica gel membrane. The sample was centrifuged for 1 minute at 7000 x g and the eluted RNA collected in the tube. The RNA concentration was determined using the Nanodrop as previously described.

After RNA extraction was completed, the RNA was concentrated using precipitation with 2.5M of lithium chloride. Lithium chloride (100 µl) was added for every 200 µl of RNA and then chilled at -20°C for 30 minutes; the precipitated RNA was then collected by centrifugation at 700 x g for 15 minutes. The supernatant was discarded and the pellet washed with ice cold 70% ethanol. The pellet was dissolved in nuclease free water. The concentration of RNA was determined using the Nanodrop and 4.5 – 15 µg were used to synthesise and form labelled cDNA (section 2.14).

2.8 Preparation of protein from bacterial cells

Table 2.3 Restriction enzymes used in this investigation

Restriction enzyme	Recognition site	Buffer	Temperature (°C)	Termination
*Eco*RI	GAATCC	H	37	Heat inactivation (65°Cfor 20 minutes)
*Bam*HI	GGATCC	E	37	Heat inactivation
*Xho*I	CTCGAG	D	37	Heat inactivation
*Bsa*MI	GAATGCN	D	65	Chloroform extraction

2.81 Whole cell protein lysis

Overnight cultures of bacterial cells (10 ml) were grown and harvested by centrifugation at 900 x g for 10 minutes. The supernatant was decanted and the pellets re-suspended in 1 ml of PBS. From this point onwards, everything was kept on ice. The re-suspension was centrifuged for 2 minutes at 7000 x g and the pellet re-suspended in 1 ml of PBS containing a protease inhibitor (1 µl per 100 µl). The tubes were placed on ice for 5 minutes and transferred to 2 ml screw cap top tubes containing 500 µl of zirconium beads. The tubes were placed in a bead beater for approximately 1 minute at lowest setting (25) and placed back on ice. The tubes were centrifuged for 3 minutes at maximum speed to produce a thick, clear supernatant. The protein sample was aliquoted into clean tubes. For visualisation by protein polyacrylamide gel electrophoresis, a 1:1 ratio of sample buffer was added to the tubes. The tubes were boiled for 5 minutes and centrifuged for 3 minutes to remove cellular debris. The supernatant was removed to a fresh tube and 15 µl was loaded on the gel. Protein concentration was determined before visualisation by SDS PAGE (see below).

2.82 Determination of protein concentration

The Bio-Rad Quick Start Bradford protein assay was used to determine protein concentration. The kit was supplied with bovine serum album pre diluted standards ranging from 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2 mg/ml. For determination of protein concentration, 20 µl of protein sample and the seven standard concentrations were added to clean disposable cuvettes. Then 1 ml of dye reagent was added to each sample (containing Coomassie blue). The tubes were incubated at room temperature for 5 minutes and the samples read at 595 nm. A standard curve was produced from the standard OD readings and protein concentrations read off this graph. To determine an exact protein concentration the linear regression formula was used. A linear regression = $y = a + bx$. In this case the appropriate values can be entered to give: $OD = a + b_{conc}$. The values of a and b can be determined from the graph using the Minitab version 15 software. To find the concentration the equation was rearranged and the values of a and b substituted in to give:

conc = OD – a (0.1039) / b (0.635).

Approximately 20 µg was required for each sample and thus proteins were diluted to the appropriate concentration.

2.83 Membrane protein extraction from bacterial cells

To extract membrane proteins a Bio-Rad ReadyPrep protein extraction kit (membrane 1) was used. The kit is based on the separation of membrane proteins by temperature dependent phase partitioning using a detergent. Overnight cultures were harvested by spinning for 10 minutes at 900 x g. Buffers M1 and M2 were pre chilled for at least 15 minutes before use. 0.5 ml of buffer M1 was added per 50 µl of bacterial cell pellet. A protease inhibitor was also added at 1 µl per 100 µl to prevent protein lysis. The cell suspension was placed in a tube containing approximately 500 µl of Zircomium beads and placed in a bead beater for 1 minute. This was repeated at least 5 times and tubes were placed on ice between bead beatings. The suspension was spun for 3 minutes at maximum speed to produce a clear supernatant. An equal volume of chilled buffer M2 was added to the cell extract and mixed on a vortex for 60 seconds, 4 – 5 times, taking care to keep tubes chilled between mixes. At the end of this mixing the tube was placed on ice for 10 minutes. The tube was transferred to a 37°C water bath and incubated for 30 minutes. The suspension was then mixed for 30 seconds periodically (3 – 4 times) and the tube centrifuged at maximum speed for 5 minutes at room temperature.

After centrifugation two layers were present and the top layer removed to a clean tube and labelled hydrophilic proteins. 500 µl of buffer M2 was added to the tube containing the bottom phase and the mixing steps and chilling steps repeated as described above. The tubes were incubated at 37°C and centrifugation steps repeated so that two layers were produced. Again the top layer was removed as previously described and pooled with the hydrophilic proteins collected earlier. The bottom phase was collected and transferred to a clean tube. This was labelled the

hydrophobic protein fraction. An insoluble pellet was also present and this was frozen and stored as the hydrophilic and hydrophobic fractions.

An RC DC protein assay (Bio-Rad) was used to determine protein concentration. This assay was compatible with both reducing agents and detergents. 5 µl of DC reagent S was added to each 250 µl of DC reagent A that will be required for the run. This reagent is referred to reagent A' and each sample requires 127 µl of reagent A'. Dilutions of the BSA standard were made ranging from 0.2, 0.5, 1 and 1.5 mg/ml. 25 µl of each standard and protein samples were placed into clean centrifuge tubes and 125 µl of RC reagent 1 was added to them and incubated at room temperature for 1 minute. Then 125 µl of reagent 2 was added to each tube, mixed and centrifuged for 3 – 5 minutes at maximum speed. The supernatant was discarded and 127 µl of reagent A' was added, mixed and incubated for 5 minutes at room temperature, until the precipitate had completely dissolved. Before proceeding the tubes were vortexed and then 1 ml of DC reagent B was added to the sample and mixed. This was incubated at room temperature for 15 minutes. After the incubation step absorbance was read at 750 nm and a standard curve produced. Protein concentrations can then be determined from the graph as described in section 2.82.

2.84 Membrane protein clean up

Before proteins could be visualised, samples were cleaned using the Bio-Rad ReadyPrep 2D cleanup, to remove detergents that may interfere with SDS – PAGE. The clean up procedure was carried out as described by the manufacture. First, 1 – 500 µg of protein in a final volume of 100 µl was transferred to a clean tube and 300 µl of precipitating agent 1 was added and mixed well. This was then incubated on ice for 15 minutes and then 300 µl of precipitating agent 2 was added and mixed well. The samples were centrifuged for 5 minutes at maximum speed to form a pellet and without disturbing the pellet, the supernatant removed. The tubes were then centrifuged for a further 15 to 30 seconds and any residual liquid removed. 40 µl of wash reagent 1 was added to the pellet and centrifuged for 5 minutes at maximum speed. The wash was removed and discarded and 25 µl of ultrapure water was added and mixed. 1 ml of wash reagent 2 (prechilled at -20°C for at

least an hour) and 5 µl of wash 2 additive was added to the pellet and vortexed for 1 minute. The samples were incubated at -20°C for 30 minutes, with mixing every 10 minutes during the incubation period. After incubation, the sample was centrifuged for 5 minutes and the supernatant removed. A brief centrifugation was repeated to collect and remove any residual liquid and the pellet was then air dried at room temperature for no longer than 5 minutes. Pellets were re-suspended in Laemmli buffer.

Before samples were run on the gel, sample buffer was added to the proteins. A reducing agent β mercaptoethanol was added to the sample buffer (50 µl to 950 µl of buffer). The sample buffer was then added to the sample at a 1:1 ratio and mixed until the sample had completely dissolved. The sample was then heated for 5 minutes at 95 – 100 °C and was then ready for SDS-PAGE.

2.9 Visualisation of proteins by SDS-PAGE

To visualise proteins, SDS – PAGE was performed using a Bio-Rad Criterion Cell. For whole cell protein analysis and initially for membrane protein analysis, a 4 – 20%, Tris-HCl gradient gel (Bio-Rad) was used. For outer membrane proteins to look for changes at a particular protein size, a 12.5% Tris-HCl gel was used instead. Proteins were prepared as described above. The gel was placed into the tank and 10 X Tris/Glycine/SDS (Bio-Rad) buffer, diluted to a 1 X working solution was added. The gel was loaded with the protein samples (20 µg) and run at 80 volts for approximately one hour.

To stain the gels, Bio-safe Coomassie blue (Bio-Rad) was used. First the gel was washed three times in 200 ml of deionised water for 5 minutes. The water was removed and 50 ml of Coomassie stain was added and left with rocking for 1 hour. The stain was removed and gels washed with 200 ml of deionised water for 30 minutes. Gels were then photographed. However, due to the lack of sensitivity with the Coomassie stain, silver staining was also evaluated.

2.91 Silver staining of protein gels

Silver staining of gels was carried out using Bio-Rad silver stain kit (Bio-Rad). First the gel was fixed in 400 ml fixative (40% methanol, 10% acetic acid v/v) for 30 minutes with rocking. It was then placed in a second fixative solution (400 ml; 10% ethanol, 5% acetic acid v/v) for 15 minutes. The fixative was removed and the gel was placed in the second fixative again for 15 minutes. The gel was then placed in 200 ml of an oxidizer, for 5 minutes and washed in a series of deionised water steps of 400 ml for 5 minutes, repeated until all the yellow colour has been removed from the gel. 200 ml of silver reagent was then added for 20 minutes. This was removed by washing with 400 ml of deionised water for 1 minute. Developer solution (200 ml) was then added until the solution turned yellow. The developer was removed and fresh developer (200 ml) was added for approximately 5 minutes. Developing was stopped when bands were clearly visible by the addition of 400 ml 5% acetic acid (v/v) for 5 minutes.

2.10 Polymerase chain reaction (PCR)

PCR reactions were used extensively in this investigation for a number of applications, including strain identification, cloning and mutagenesis. Promega PCR reagents were mainly used (Promega Corporation). For a 1x PCR mix the following Promega reagents were used: PCR buffer (5x), 2.5 µl, Mg^{2+} (25 mM) 2 µl (final concentration 2mM), dNTPs (10 mM), 0.5 µl (final 0.2 mM), 1:1 primer mix (20 µM), 0.6 µl, Taq (5 units/µl), 0.18 µl. 1 µl of template DNA was added to give a final reaction volume of 25 µl.

2.10.1 Reverse transcriptase PCR (RT)

RT PCR was used to investigate the expression of genes in the presence of chlorhexidine. RNA was harvested from cells in the presence of 5 µg/ml chlorhexidine and from cells grown in LB broth as previously described (Chapter 2, section 2.7 and 2.13). RNA was then converted to cDNA using the ImProm-II Reverse transcription system (Promega). RNA was first treated with RQ1, RNase free DNase (Promega), to remove any DNA present in the sample. The RNA sample (5 µl) was added to 1 µl RQ1 RNase free 10 x buffer (400 mM Tris-HCl, pH8, 100 mM $MgSO_4$ and 10 mM $CaCl_2$) and 1µl RQ1 RNase free DNase

(containing 10 mM $MgCl_2$, 10 mM HEPES, 50% glycerol and 10 mM $CaCl_2$). The reaction was incubated at 37°C for 30 minutes, and then 1 µl of RQ1 DNase stop solution was added to terminate reaction. After incubation at 65°C for 10 minutes to inactivate DNase, the RNA was used in the reverse transcriptase reaction.

The ImProm – II reverse transcriptase kit, required several controls, including a negative control, no RNA and a positive control, with template and control PCR primers which were included in the kit. A no RT control was also included to check that no DNA activity was present as this may affect results. DNase treated RNA (2 µl) was added to 1 µl of random primers for the experimental samples and 1 µl of control primers for the positive control. This was made up to 5 µl with sterile deionised water. The negative control contained no RNA. Tubes were placed into a preheated thermal cycler at 70°C for 5 minutes, and then chilled on ice for 5 minutes and each tube spun in a bench top centrifuge for 10 seconds. The seconds part of the reaction was prepared as follows: for experimental, negative and positive control, 4 µl of buffer, 4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 0.5 µl of RNasin inhibitor, 4.5 µl of nuclease free water and 1 µl of ImProm reverse transcriptase was added to give a final volume of 15 µl. The no RT control was made as above with the exception of no reverse transcriptase being added and water making up the final 20 µl. This reverse transcription reaction was added to the 5 µl RNA and primer reaction tubes and place in a thermal cycler. The following programme was used to synthesise cDNA: annealing at 25°C for 5 minutes, extension at 42°C for 1 hour, and inactivation of RT at 70°C for 15 minutes.

cDNA was then analysed via PCR. PCR primers used are shown in Table 2.4. A control housekeeping gene (*phaC*) was included which had a constant level of expression across both conditions. The programme used was: 94°C for 5 minutes, initial denaturation, 94°C for 30 seconds, annealing at specific primer temperature (Table 2.4), extension, 72°C for 1 minute, for 40 cycles. $MgCl_2$ concentration was adjusted to compensate for carryover from the reverse transcription reaction (6mM) to give a final volume of 2 mM. To determine when the PCR product appeared in both the control and experimental condition, a 40 cycle PCR reaction was set up. After the initial 10 cycles, a PCR tube was removed every 5 cycles, allowing the

determination of product appearance. PCR products were analysed via gel electrophoresis to look for up regulation of genes in the experimental condition.

2.10.2 Real time PCR (QPCR)

Real time PCR was used to validate the microarray results. Real time PCR is a quantitative method compared to RT PCR which is only semi quantitative. Q PCR incorporates a fluorescent dye called SYBR green (Thermo scientific), which only fluoresces when bound to double stranded DNA. As DNA is synthesised the dye is incorporated and fluorescence recorded. In this manor, DNA synthesis can be recorded in "real time", thus those genes that may be up-regulated in the experimental condition would start to appear at earlier cycles than those in the control condition and vice versa.

cDNA was made from RNA as mentioned previously and 0.5 µl of cDNA template was added to: 10 µl Absolute SYBR green mix, 0.5 µl of primer mix (20 µM) and sterile deionised water to make a final volume of 20 µl. To prepare a standard curve, dilutions of the cDNA was made ranging from 1, 1 in 5, 1 in 25 and 1 in 125. Before adding cDNA to the sample it was essential to determine the concentration of cDNA and adjust it so that the same amount of cDNA was added to each reaction. The controls included were a negative control with no cDNA and a no RT control, containing no reverse transcriptase. Also included in every reaction was the housekeeping gene *phaC*, to check that the real time PCR had worked as expected. Once the reactions were prepared, the tubes were placed in a real time thermal cycler (Opticon 2, MJ Research). The programme used was as follows: 95°C for 15 minutes, 95°C for 15 seconds, and specific annealing temperature for primer (64°C) for 30 seconds, 72°C for 15 seconds for 50 cycles. To check that the correct product was formed and that controls were negative, a melting curve was performed after the 50 cycles, this ranged from 50 – 95°C with a reading taken at every 0.5°C for 1 second. This determines the melting point for the product. All PCR products for each primer pair should have a similar melting temperature, hence the same peak should be observed. The negative controls should have a lower melting point indicating no contamination or DNA activity in the no RT control.

Table 2.4 Primers used for RT and Q PCR

Primer Name	Primer (Forward and Reverse) 5' – 3'	Annealing temperature °C	Size bp
Control gene BCAL 1861 (*phaC*)	AGACGGCTTCAAGGTGGT	66	470
	ACACGGTGTTGACCGTCA		
BCAL 0114 RT	GCACAGCAGAACCTCAAC	64	519
	GTTGTTGTCGAGCGACA		
BCAL 0126 RT	CAAGGAAGGCACGCTGAC	64	366
	CCGTACGACAGCAGGATG		
BCAL 0143 RT	GAGCGACTTCATCGACGG	64	404
	GCCAGTACAACAGGTCGG		
BCAL 2353 RT	GCAACTCGGCAGCTTCTC	65	457
	GAATGCGGTGAACGATCC		
BCAL 2370 RT	GCATCATCCAGCACATGC	64	518
	CACCAGTGTGCAGACGAG		
BCAM 0924 RT	CACCTGAAACCCGACCTG	65	473
	CTGCAGATGTGGCTGTCG		
BCAM 0925 RT	CGACACGCAGTCGAATCC	64	540
	GCGACGAGGTTCTGTTCG		
BCAM 0927 RT	GGCTGTTCGAACAAGGCAC	64	394
	CACGTCCACGTAGACCTG		
BCAS 0081 RT	GCACTGCACACGATGGTC	65	323
	AGACGCGGCACGAAATAC		
BCAS 0083 RT	GCTTCCATGCGACGTCCAC	64	358
	CTTCAGGCTCCCGTCGATC		

Once the programme was completed the Opticon monitor analysis software was used to produce a graphical representation of the reaction as well as the melting curve. The threshold value (ct) is the point at which the fluorescence crosses the horizontal line set by the user. The ct value can be used to calculate fold change by two separate methods. The Delta – delta method does not take into account the efficiency of the reaction and assumed 100% efficiency. The reactions were carried out in triplicate and an average taken. The ct value of the control gene was subtracted from the ct value of the control housekeeping gene and the ct value of the experimental gene was subtracted from the ct value of the experimental housekeeping gene. Then the experimental gene delta ct value was subtracted from the control gene delta ct value and the fold change calculated using the equation: Power (2delta delta ct value).

A more accurate method, which took into account the reaction efficiency, was the Pffaffl method (Pfaffl, 2001). Standard curves were produced using the dilutions of the cDNA for each gene and the reference gene. The efficiency of the reaction was calculated by determining the slope of the curve and by then using the efficiency calculator provided by Stratagene. (http://www.stratagene.com/techtoolbox/calc/qpcr_slope_eff.aspx). The ratio was calculated using the Pfaffl equation: (Pfaffl, 2001)

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

where E_{target} = the efficiency of the target gene transcript, E_{ref} = the efficiency of the reference gene, e.g. *phaC*, DCt target, which is the Ct value for the target gene experimental condition subtracted from the Ct value of the target gene in the control condition and DCt ref is the Ct value for the reference gene in the experimental condition subtracted from the Ct value of the reference gene in the control condition.

2.11 PCR purification

For some applications, such as cloning and sequencing, DNA within PCR reactions was purified to remove excess primers, enzymes and dNTPs that interfered with downstream processes. The QIAquick PCR purification protocol was used to achieve this (Qiagen). Five volumes of buffer PB1 was added to one volume of PCR sample and mixed. A QIAquick spin column was placed into a collection tube and the DNA bound to the column by applying the sample and centrifuging for 30 – 60 seconds. The flow through was discarded and the samples washed with 750 µl of buffer PE. This was centrifuged at maximum speed for 30 – 60 seconds and flow through was discarded. The column was centrifuged for an additional 1 minute and the QIAquick column was placed in a clean tube. DNA was eluted by adding between 30 µl and 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) to the membrane and centrifuging for 1 minute. Eluted DNA was then quantitated or visualized by gel electrophoresis.

2.12 Random amplified polymorphic DNA analysis (RAPD)

To determine if mutants were the same strain as the wildtype, a PCR band DNA fingerprinting method was used. Random amplified polymorphic DNA analysis uses arbitrary primers that anneal randomly to the genome, creating a unique phenotype for that particular strain. Chelex DNA preparations were performed to obtain DNA (Chapter 2, section 2.42). Primers used were designed as previously described (Mahenthiralingam *et al.*, 1996a) and are as follows: Primer 270, 5' -TGC GCG CGG G- 3' (MWG Biotech, Covent Garden, London) and Primer 272, 5' – AGCGGGCCAA-3' (MWG Biotech, Covent Garden, London). A PCR mix was set up using Qiagen reagents (Qiagen) as follows: 2.5 µl of 10 x coralload buffer, 5 µl of Q solution, 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP (200 µM final concentration), 4 µl primer (10 pmol/µl working solution), 10.3 µl of sterile water and 0.2 µl of Taq polymerase. 2 µl (approximately 50 – 100 ng) of template DNA was added to 23 µl of mix to give a final volume of 25 µl. The PCR thermal cycles were carried out on a Flexigene Thermal Cycler (Techne Ltd., Newcastle, United Kingdom) as follows (ramping time between temperatures): (i) 4 cycles of 94°C for 5 minutes, 36°C for 5 minutes (70 seconds cooling time), and 72°C for 5 minutes. (70 seconds heating time), (ii) 30 cycles of 94°C for 1 minute (55 seconds to heat from 72°), 36°C for 1

minute (60 seconds to cool), 72°C for 2 minutes. (70 seconds. to heat); and (iii) a final extension of 72°C for 6 minutes

Once the reaction had completed, the products were separated on a 1.5% high resolution gel (Sigma) with TBE buffer. 12 µl of product was placed on to the gel and run at 80 V for approximately 3 hours or until it has run to 8 cm. SafeView DNA stain was added to both the gel and the buffer so no post staining was required. Once the run was complete, a picture was taken using the UV Gel Documentation machine.

2.13 *B. cenocepacia* growth curve analysis

To prepare bacteria for RNA extraction and use in microarray experiments, growth curve experiments were performed. The bacteria needed to be harvested at mid log phase for consistent RNA profiling. LB broth (Sigma) was made with polished water and autoclaved. *B. cenocepacia* J2315 was revived on TSA agar and an overnight culture was set up in 3 ml of TSB. This was left for 18 hours. 25 ml of LB broth was added to a sterile 250 ml flask. One flask contained normal LB, the second flask contained LB and chlorhexidine at a concentration of 5 µg/ml. 500 µl of bacterial suspension was added to the flasks and placed into a 37°C incubator with shaking at 150 rpm. An OD reading was taken at 600 nm every 90 minutes, until exponential growth was observed and then a reading was taken every 60 minutes. Once both bacterial cultures have reached an OD of 0.5 the bacteria were harvested.

To harvest bacteria, 5 ml was placed into a snap top tube and rapidly cooled for 10 seconds in liquid nitrogen. The tube was then placed in a centrifuge cooled to 2°C and spun for 5 minutes at 3000 x g. The supernatant was discarded and the pellet frozen in liquid nitrogen. The pellet was stored at -80°C until RNA extraction was performed.

2.14 Microarray experiments

A two colour microarray of *B. cenocepacia* J2315 was used to determine the global gene expression in response to chlorhexidine. The 2 x 11 K microarray was provided by Agilent (Agilent technologies, Santa Clara, California) and was a 60 mer probe design containing 7251 genes, 1489 intergenic regions from strain J2315 and genes from 610 control strains. An overview of the microarray experiment is shown in Figure 2.1.

2.14.1 Cy3/Cy5 labelling design

Once the RNA was concentrated (section 2.7) it was converted to cDNA and labelled with fluorescent dyes. The design used in the microarray experiment was a direct comparison with 4 replicates; therefore a dye swap method was used to counter for any dye effects. Spike-in controls were also used according to the manufactures instructions and this was taken into account when using the dye swap method. Microarray labelling was carried out as follows; tube A contained control J2315, spike A and dye Cy3, tube B contained experimental J2315, spike B and Cy5, tube C contained control J2315, spike B and Cy5 and tube 4 contained experimental J2315, spike A and Cy3. This process was repeated for the other 2 replicates.

2.14.2 Labelling first strand cDNA with amino allyl-dUTP

Labelled cDNA was made using the CyScribe post labelling kit (an indirect amino allyl procedure) following the manufactures instructions (Amersham). Amino allyl dUTP (AA-dUTP) aliquots were first centrifuged for 15 seconds and 30 µl of nuclease free water added. The following reagents were added to PCR tubes: Spike controls (A or B), 2 µl, mRNA (500ng), 6µl, random nonamers, 2 µl, anchored oligo dt, to give a total volume of 11 µl. These were mixed by gentle pipetting and then incubated at 70°C for 5 minutes. The reactions were then cooled for 10 minutes at room temperature, so that primers and mRNA can anneal. The reactions were then centrifuged for 15 seconds. For the extension reaction, the tubes were placed on ice and the following was added, leaving the enzyme until last: 5 x Cyscript buffer, 4µl, 0.1 M DTT, 2 µl, nucleotide mix, 1 µl, AA-dUTP, 1 µl

and Cyscript reverse transcriptase to give a total reaction volume of 20 µl. These were mixed by pipetting and centrifuged for 15 seconds. The reactions were then incubated at 42 °C for 1.5 hours and then placed on ice.

2.14.3 Purification of amino allyl modified cDNA

To degrade the mRNA, 2 µl of 2.5M NaOH was added to each reaction; these were mixed by vortexing and spun in a centrifuge for 15 seconds. The reactions were then incubated in a water bath at 37°C for 15 minutes. 10µl of 2M HEPES free acid was added and mixed by vortexing. The reaction was then centrifuged for 15 seconds. The cDNA was then ready for purification by ethanol precipitation. 3 µl of 3 M sodium acetate was added to each reaction and then 105 µl of absolute ethanol was added. This was mixed and centrifuged for 15 seconds. The reactions were then incubated in -80°C freezer for 1 hour. The reaction was then centrifuged at 13,000 x g for 15 minutes and the supernatant was removed with a pipette. The pellet was then air dried for approximately 30 minutes and fully re-suspended in 40 µl of 0.1 M sodium bicarbonate buffer.

2.14.4 Labelling of amino-allyl modified cDNA with CyDye

The purified amino allyl cDNA was added directly to one aliquot of CyDye NHS (N-hydroxy-succinimidyl) ester in a 1.5 ml tube. The NHS ester was completely re-suspended by pipetting several times and then centrifuged at 13 000 x g for 1 minute to collect the liquid at the bottom of the tube. The reactions were incubated at room temperature in the dark for 90 minutes. 15 µl of 4 M hydroxylamine was added to each reaction tube and mixed by pipetting. This was then incubated in the dark at room temperature for 15 minutes.

2.14.5 Purification of labelled cDNA with autoseq G-50 columns

Two autoseq columns should be used to purify one labelling reaction as the final volume of the reaction before purification should be approximately 45 µl. The

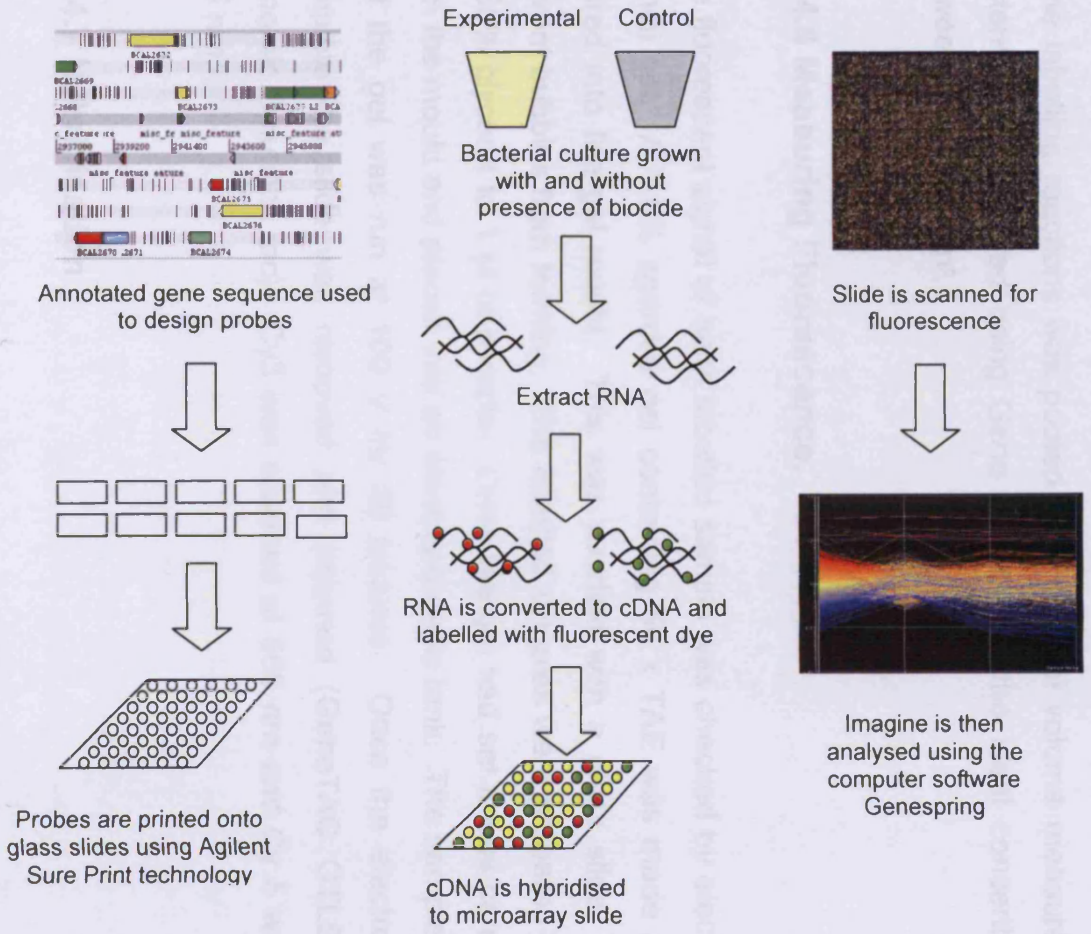


Figure 2.1 Overview of microarray experiment

Agilent designed probes using the annotated gene sequence and printed probes onto glass slides. *B. cenocepacia* J2315 was grown with and without the presence of chlorhexidine; RNA was extracted, converted to cDNA and labelled with fluorescent dyes, Cy3 and Cy5. cDNA was printed onto slides and unbound probes washed off. The slide was scanned using an Agilent scanner and data put into the computer software Genespring for analysis.

columns were prepared by vortexing gently, to re-suspend the resin. The caps were loosened and the bottom closures were snapped off. The columns were placed in 2 ml screw cap microcentrifuge tubes and spun for 1 minute at 1500 x g. The columns were placed into a new 1.5ml tube, without a cap and 23 µl of the labelling reaction was slowly added to the centre of each column. The sample was applied to the angled surface of the resin bed taking care not to disturb the resin. The columns were spun for 1 minute at 1500 x g and the purified samples were collected at the bottom of the microcentrifuge tube. The recovered material from the same labelling reactions was pooled and the total volume measured. The DNA content was measured using Gene Quant with the ideal concentration ranging between 20 – 50 µg/ml.

2.14.6 Measuring Fluorescence.

The fluorescent signal of each labelled sample was checked by electrophoresis on a mini gel. A 1.5% agarose gel containing 50 x TAE was made and 3 ml was poured into the gel mould. This was overlaid with a glass slide, taking care to prevent bubbles from forming. The labelled samples were prepared by adding 1 µl of 50% glycerol to 1 µl of sample. Once the gel had set it was carefully removed from the mould and placed into an electrophoresis tank. The samples were loaded and the gel was run at 100 V for 20 minutes. Once the electrophoresis was complete the slide was removed and scanned (GeneTAC GTLS IV Scanner, Genomic Solutions Inc). Cy3 was scanned at 560 nm and Cy 5 was scanned at 675 nm.

2.14.7 Hybridisation

The amount of sample used for each microarray was adjusted according to the fluorescence and concentration in respect to one another. The total amount of sample that could be analysed on a microarray slide was 500 ng. Cy3 and Cy5 labelled samples were combined to give a concentration of approximately 500 ng and was re-suspended in 80 µl of nuclease free water. The samples were heat denatured at 98°C for 3 minutes and then allowed to cool at room temperature.

The cDNA target hybridization solution was prepared by adding the following reagents: 80 µl of combined cDNA targets, 25 µl of 10 x control targets and 105 µl of 2 x hybridisation buffer. This was mixed by pipetting taking care not to introduce bubbles. The sample was then centrifuged briefly. The hybridization chambers were then prepared by loading a clean gasket slide into the hybridization chamber base. The entire sample was then drawn up into a pipette and the solution was dispensed slowly into the centre of the gasket well. The Agilent microarray was then placed onto the top of the gasket slide making sure that the number labelled side was facing upwards. The chamber cover was placed onto the sandwiched slides and clamped together using the clamp assembly. To check that the samples would hybridize evenly, the chamber assembly was held vertical and rotated clockwise. Once a large mixing bubble had formed that could move freely when rotated the chamber assembly could be loaded into the hybridisation oven. The hybridisation oven was set to rotate at 4 rpm at 65°C and was left to hybridise for 17 hours.

2.14.8 Washing slides

Before the incubation period was finished four staining dishes were set up, the first contained 250 ml of wash solution 1, the seconds contained 250 ml of wash solution 2, and the third contained 250 ml of acetonitrile and the fourth contained 250 ml of wash solution 3. The staining dishes containing wash solution 1, 2 and 3 were placed onto magnetic stirrers that contained a stirring bar. Once the hybridisation was complete, the chamber assembly was removed from the oven and the clamp assembly loosened and removed. The sandwiched slides were removed and transferred to the rack in the first staining dish. The gasket slide was then removed from the microarray slide using tweezers to gently prise it away. The magnetic stirrers were then switched on and washing could begin. Slides were washed in washing solution one for 1 minute the rack was then removed and placed into wash solution 2 for 1 minute. The timing of this step was critical to success. The rack was then removed from wash solution two and excess liquid poured off. It was then transferred to the acetonitrile for a couple of seconds and then transferred to wash solution 3 for 30 seconds. The slide rack was removed

from wash solution three at a slow and constant rate to minimise the chance of precipitates forming on the slide.

2.15 Scanning Microarray

The microarray was scanned using an Agilent scanner and data extracted using the Feature Extractor V.8.1 software. Quality control data was observed to check that the microarrays were sufficient for analysis. These consisted of: checking the microarray by eye for even spread of fluorescence and hybridisation, background check controls, checking that control spots were present in all 4 corners of the array, outliers evenly distributed, Mvs plots close to 1, even distribution of up regulated and down-regulated features and linear spike-in controls. The microarray data was then saved to disk and analysed further using the GeneSpring software.

2.16 Microarray data analysis – Genespring

The computer program Genespring was used to analyse the microarray data. All 4 replicates were uploaded into GeneSpring. The data were checked to make sure that all arrays were sufficient to use in analysis. This was assessed visually by the graph produced by Genespring, by the production of a condition tree and a principal component analysis. Control genes were then filtered out to produce a sub set of genes used for analysis. These genes could then be filtered on confidence level at P = 0.05 and P = 0.01, allowing genes that were significantly up-regulated or significantly down-regulated to be determined.

2.17 Biofilm assays

To asses the production of biofilms by members of the Bcc, a 96 well plate assay was used. The assay was adapted from that of Stepanovic *et al.* (Stepanovic *et al.*, 2000). First, 96 well polypropylene plates (Sterlin Ltd, Fisher Scientific) were coated with 1 mg per well of mucin (Porcine mucin – Sigma) (200 µl of a 5 mg/ml stock solution). The coating of wells with mucin was to aid adherence of bacterial cells. This was left at 37°C overnight and then washed with 250 µl of PBS – Tween

(0.1% v/v). Each well is washed three times and then left to dry at room temperature for 10 minutes. Plates were stored at 4°C until use.

Bacterial suspensions were made by inoculating a 3 ml nutrient broth culture and leaving to grow at 37°C overnight. Bacterial suspensions were then adjusted to OD 1 at 630 nm and diluted to approximately 10^6 cfu/ml. 200 µl of this suspension was added to the wells and left to grow for 72 hours statically at 37°C. Planktonic growth was removed and plates washed three times with PBS buffer.

Bacteria were fixed by adding 200 µl of 99% methanol for 15 minutes. This was then removed and wells left to dry at room temperature for 10 minutes. The bacteria were stained for 5 minutes with 1% crystal violet solution. Excess stain was removed and plates washed with tap water until the wash water ran clear. The wells were left to dry at room temperature for 10 minutes and then biofilms were re - solubilised by adding 160 µl of 33% glacial acetic acid. To quantify the biofilm material, an OD reading was taken at 570 nm. Each assay was carried out in triplicate.

To see if biocides induced the formation of biofilms, a similar plate assay was performed, but 10 µl of the 10^6 cfu/ml bacterial suspension was added to 990 µl of nutrient broth containing the biocide at a range of concentrations (chlorhexidine and CPC, ranging from 0 – 5 µg/ml). The exact same staining method as described above was then carried out after 72 hours of growth at 37°C.

2.18 Swarming assays

To asses the ability of the Bcc to swarm over a surface, a swarming screen was developed. Semisolid agar plates were made using 0.3% nutrient agar with the addition of 0.5% glucose. Bacteria were grown in overnight cultures and OD to 1. The suspension was diluted to approximately 10^6 cfu/ml and a 1 µl spot of this suspension was spotted onto the agar and left to dry. Plates were incubated at 37°C and swarming zones measured at 24 and 48 hours.

To determine if chlorhexidine inhibited swarming, semisolid swarm plates were made as described, with the addition of chlorhexidine at concentrations, 0, 0.1, 0.3, 0.6, 1.25, 2.5 and 5 µg/ml.

2.19 Mutagenesis

2.19.1 Sub - cloning into pGEM - T easy vector

PCR primers were designed for the target genes, with the appropriate restriction enzymes tails (Table 2.5). These gene products were amplified and then sub cloned into pGEM – T easy vector (Promega) before carrying out site directed mutagenesis. pGEM – T easy vector contains multiple restriction cloning sites and are prepared with T overhangs for easy cloning (Figure 2.2).

Once PCR products were amplified they were ligated into pGEM-T easy vector. The vector and control insert were centrifuged briefly and the reaction set up as follows: 5 µl of 2 x rapid ligation buffer, 1 µl of vector (50 ng), PCR product X µl and 1 µl of T4 DNA ligase. The amount of PCR product added was determined using the equation (ng vector X Kb size of insert / Kb size of vector) x insert: vector ratio (3:1). The final volume of 10 µl was made up using sterile deionised water. The reactions were mixed by pipetting and then incubated at 4°C overnight.

Transformation into *E. coli* was then performed. High efficiency competent cells (Invitrogen) were used for transformation. LB plates with 100 µg/ml of ampicillin, 0.5 mM IPTG and 80 µg/ml of X-gal were made as the selection agar. 2 µl of the ligation was added to 50 µl of competent cells. The tubes were gently flicked to mix and the incubated on ice for 20 minutes. After incubation the cells were heat shocked for 45 – 50 seconds at 42°C and then immediately placed on ice for 2 minutes. 950 µl of SOC medium was added and the tubes incubated at 37°C with shaking (150 rpm) for 1 hour. After incubation, a 1 in 10 dilution of the original transformation suspension was made. 100 µl of both neat and diluted suspension was spread onto selection plates and left to dry. Plates were then incubated at 37°C for 24 – 48 hours and white colonies were picked and placed into 96 - well

Table 2.5 PCR primers used for Mutagenesis

Name	Primer (5' – 3')	Annealing temperature	Size (bp)	Function
BCAM0924 (*Eco*RI)	TTACGAATTCCCTGATACTGATTG	65	622	pGPΩTp mutagenesis
	TATAGAATTCGAGCTTCTTGCGCAGCCTG			
BCAL 2353 (*Eco*RI)	TATAGAATTCCGTTCGCCTTCGGCATTG	65	1028	pGPΩTp mutagenesis
	AATTGAATTCGAGCACGTCGTGATCGATG			
BCAM 0924 Mutant	GACATCGCACGGCACACTCC	68	657	Checking pGPΩTp mutants
	CATCACCTCGGGCATGCCCG			
BCAL 2353 Mutant	CCTGCTGCTGATCGTGAAGC	57	1051	Checking pGPΩTp mutants
	GCGACGAACGCATCGAGCAG			
POM – Tp	GTCTGACGCTCAGTGGAACG	62°C	650	Specific primer for Tp cassette pGPΩTp
	CGCTTAGGCCACACGTTCAAG			
BCAM 0924 (*Bam*HI)	TATAGGATCCGATGGTTCGACATCGC	65	896	pEGM105Tc mutagenesis
	AATGGATCCAGGTAAGGGGACGCAC			
BCAL 2353 (*Bam*HI)	TATAGGATCCCGACGACGACAATTCC	58	2500	pEGM105Tc mutagenesis
	AATGGATCCGAGGAGTCGGCGCTCGC			
pUCTp (*Bsa*MI)	AAGAATGCCGCTTGGTTCGATTGTGAG	60	529	Amplifying Tp cassette for cloning into 2353
	AAGAATGCCTCGGGCATCCAAGCAGCAAG			

pUCTp (*Xho*I)	TATACTCGAGGAATTCCCACAGCAG	60	509	Amplifying Tp cassette for cloning into 0924
	AATCTCGAGCAGCAACGATGTTAC			
BCAM 0924 check	GACGACTACATCGTCAAG	65	300	Checking pEGM105Tc mutants
	CATGCACGCATCGAGCAAC			
BCAL 2353 check	CGTTGCTGGTGGCATGGG	60	340	Checking pEGM105Tc mutants
	CGCTTCGAGGCTCAGCAG			

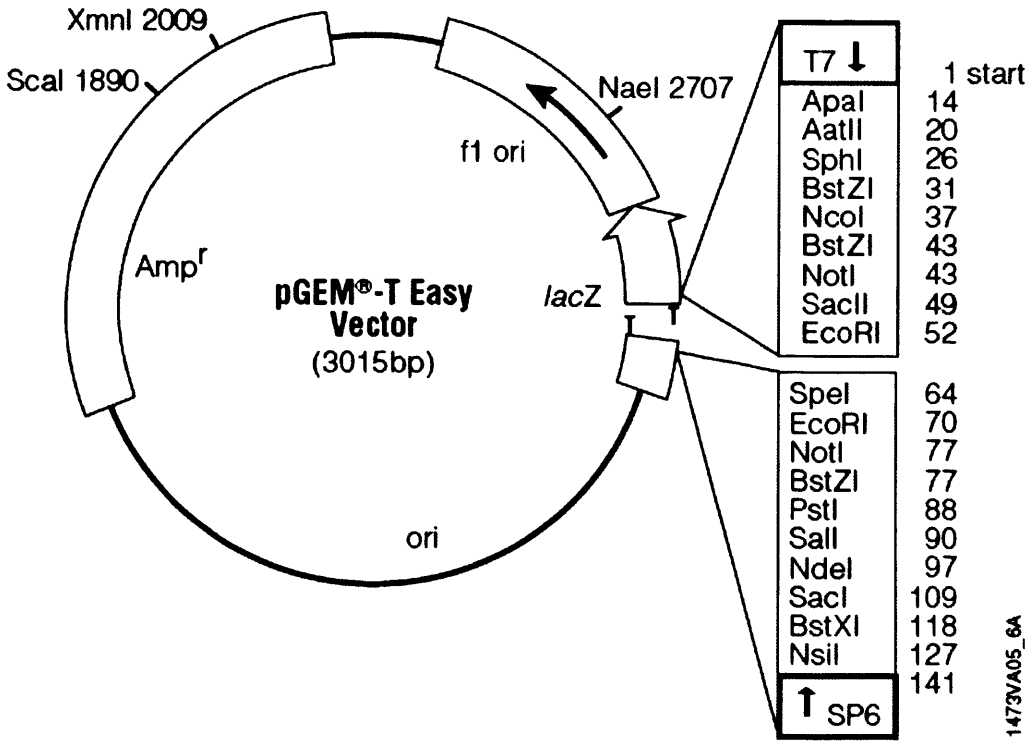


Figure 2.2 pGEM– T easy vector map (Promega inc).

Vector map of pGEM-T easy plasmid, showing size of plasmid, resistance genes carried out the multi cloning restriction site.

plates and grown overnight. Plasmid extractions were carried out on clones (Section 2.43) and checked for the correct insert by PCR (primers shown in Table 2.2). If the correct insert was present, the site directed mutagenesis was carried out.

2.19.2 Mutagenesis - pGPΩTp

Once the PCR products had been sub - cloned into pGEM-T easy vector, the products were then cloned into the suicide vector provided by Valvano *et al* (Flannagan *et al.*, 2007). The suicide vector contains a Tp resistant cassette, flanked by omega fragments (Table 2.2), which terminates the transcription of genes either side of the plasmid. The PCR products were digested from the pGEM easy vector and the pGPΩTp was linearized using the *Eco*RI restriction enzyme as described in 2.5. The excised fragments were removed from the gel using the QIAquick spin column as described previously (section 2.6).

Ligation was carried out using T4 DNA ligase (Promega). The amount of PCR product added was determined using the equation as described in section 2.18.1 and the reaction was set up as follows: 100 ng vector DNA, PCR product adjusted to correct volume as determined by equation (Section 2.19.1), 1 µl of ligase 10 x buffer, 1 µl of T4 DNA ligase and sterile water to make a final volume of 10 µl. The reactions were then incubated at 4°C overnight.

Transformation was then performed. The pGPΩTp plasmid does not contain Lambda Pir, which was required for replication, JM109 and Omnimax *E.coli* competent cells also do not contain these genes. Therefore, *E. coli* strain S.17 was made competent, using a calcium chloride extraction method (Section 2.19.3). Transformation was carried out as described in section 2.19.1 and transformants plated out onto Tp 100 µg/ml selective agar and left for 24 – 48 hours.

Transformants were picked and left to grow overnight. PCR checks were carried out using primers as shown in Table 2.5 to check that the gene was correctly disrupted. Once correctly inserted transformants were selected, tri parental mating was carried out to insert the mutation into *B. cenocepacia* K56-2 (Section 2.21).

2.19.3 Making cells competent using calcium chloride

Overnight cultures of *E.coli* S.17 cells were grown at 37°C with shaking overnight in 5 ml of LB broth. 100 µl of this overnight culture was placed in 10 ml of LB broth and left to grow at 36 °C for 2- 3 hours until OD 0.5 at 600 nm was reached. The cells were harvested by centrifugation at 700 x g for 10 minutes and the supernatant was discarded. The pellet was re-suspended in 1 ml of $CaCl_2$ that had been prechilled at -20°C and aliquoted into 1.5 ml tubes. This was then centrifuged for 1 minute at 13 000 x g. The supernatant was discarded and the pellet washed with 1 ml of $CaCl_2$. This wash step was repeated 2 – 3 times. Finally the pellet was re-suspended in 200 µl of $CaCl_2$ and left on ice for 30 minutes.

2.19.4 Mutagenesis using pEGM105Tc

The second mutagenesis method used a suicide vector pEGM105Tc, as described by Quandt *et al* (Quandt and Hynes, 1993). First unique restriction sites were identified in the two genes of interest (Table 2.5) and primers designed with *Bam*HI restriction tails. A trimethoprim resistant cassette was amplified from plasmid pUC, with primers with the restriction tails that correspond to the restriction site within the gene of interest (Table 2.5). Both, the gene targets and the Tp resistant cassette were sub - cloned into pGEM-T easy vector as described in section 2.19.1; an overview of the method can be seen in Figure 2.3. Once the transformants had been selected, plasmids were extracted (Section 2.43) and checked using PCR (Table 2.5).

When the correct inserts had been isolated, restriction digests were performed using the unique restriction enzyme for the target genes to remove the Tp resistant cassette from the pGEM-T easy vector and to lineralize pGEM-T easy vectors with the gene insert. The Tp cassette was extracted from the gel (section 2.6) and ligated into the pGEM-T easy vector as described previously (section 2.19.2).

After ligation, the plasmid was transformed into *E.coli* Omnimax competent cells as described previously (section 2.19.1) and transformants selected on Tp 100 µg/ml plates. Transformants were picked and grown up and plasmids extracted. To

check that the transformants were correct, PCR using primers specific for the Tp resistant cassette was carried out (Table 2.5). A restriction digest was also performed to check for the presence of Tp resistant cassette.

bamH1 digests were performed on the pGEM plasmid containing the target genes interrupted with the Tp cassette (Table 2.2) and on the pEGM105Tc plasmid. The digest was visualised on a gel and the disrupted gene was excised using the QIAquick spin kit (section 2.6). The disrupted gene was then ligated into the linearized pEGM105Tc plasmid and transformed into *E. coli* Omnimax competent cells. Transformants were selected on Tp 100 µg/ml plates, picked, regrown and frozen down at -80°C. Plasmid extraction was carried out and PCR checks performed to check that the Tp cassette had inserted correctly. Once transformants with the correct insert were isolated, triparental mating with *B. cenocepacia* K56-2 was carried out (section 2.21).

2.20 Random transposon mutagenesis

Random transposon mutagenesis was carried out on *B. cenocepacia* AU1054, to examine the chlorhexidine swarming phenotype. Preliminary experiments were carried out to determine the antibiotic selection marker required. *B. cenocepacia* overnight suspensions were spread onto plates containing various concentrations of kanamycin, tetracycline and trimethoprim. The plasposon containing a trimethoprim resistant cassette (Dennis and Zylstra, 1998) was selected to use and tri parental mutagenesis was carried out to transfer the plasposon into *B. cenocepacia* AU1054.

2.21 Conjugal mating into Bcc strains

Conjugal mating was carried out to transfer: (i) mutant constructs into *B. cenocepacia* K56-2 via site directed mutagenesis, and (ii) transposons into *B. cenocepacia* AU1054. J2315 was not used as a mutagenesis strain as it is notoriously difficult to do genetic work on and K56-2 is a closely related single locus variant of this strain, so can be used instead. All matings required the helper plasmid pRK2013. The strains required were grown in 3 ml overnight culture.

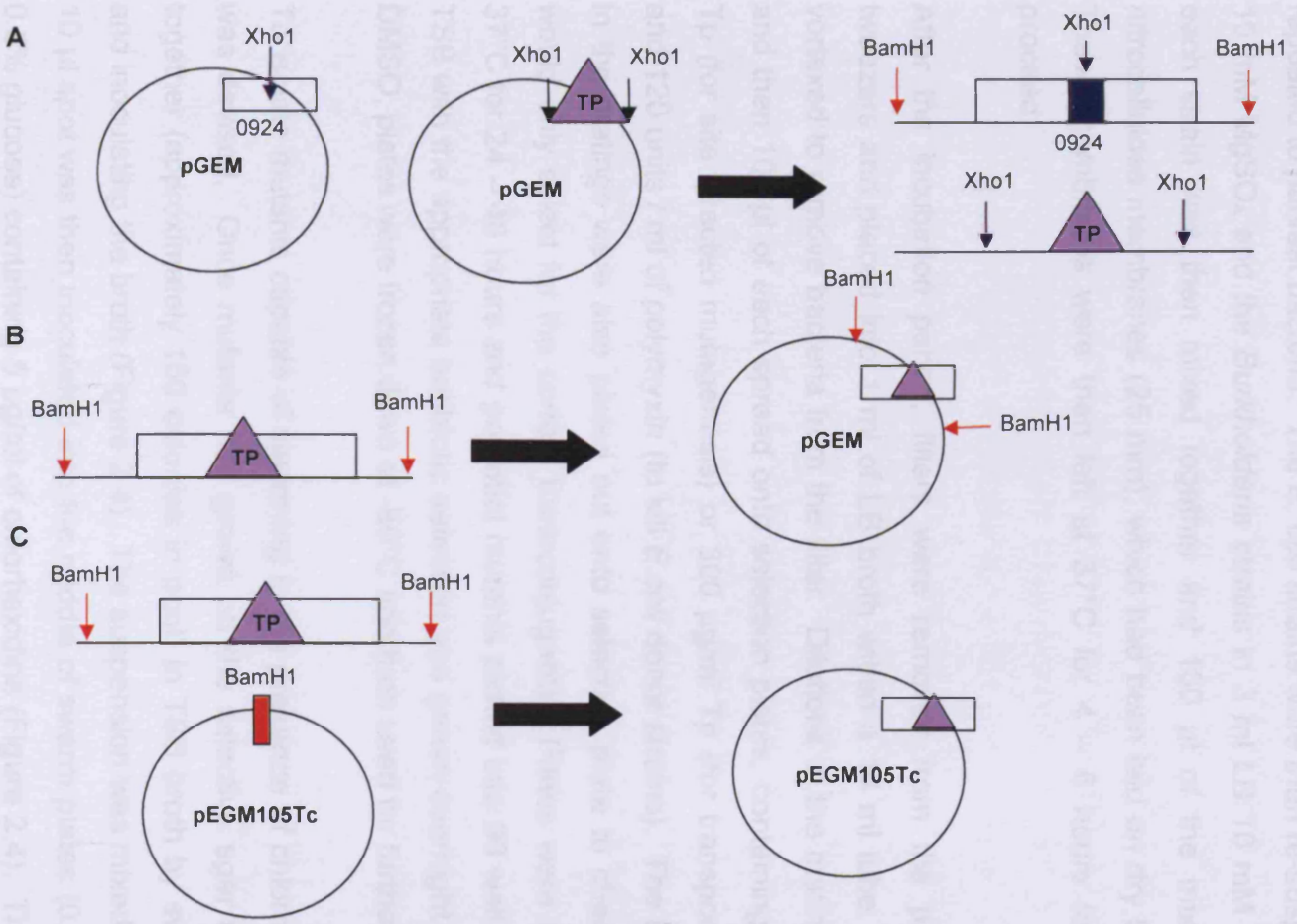


Figure 2.3 Mutagenesis of the *B. cenocepacia* BCAM 0924 gene

A – BCAM 0924 with *XhoI* site and Tp resistant cassette with *XhoI* tails B cloned into pGEM vector. BCAM 0924 and Tp cassette digested with *XhoI*. B – Tp cassette cloned into BCAM 0924. BCAM 0924 disrupted with Tp cassette digested with *BamHI* and suicide vector pEGM105Tc also digested with *BamHI*. C -disrupted gene cloned into suicide vector pEGM105Tc and tri parental mating then carried out with *B. cenocepacia* K56-2

These strains included the *B. cenocepacia* strain to be mutated, *E. coli* strains containing plasmid/plasposon and *E. coli* strain carrying helper plasmid (Km 50 selection) (Table 2.2). The cells were harvested by centrifugation at 1200 x g for 10 minutes and the pellet was washed in 5 ml LB 10 mM $MgSO_4$. The spin was then repeated to harvest bacteria. The *E. coli* strains were then re-suspended in 4 ml LB 10 mM $MgSO_4$ and the *Burkholderia* strains in 3 ml LB 10 mM $MgSO_4$. 50 µl of each strain was then mixed together and 100 µl of the mixture placed onto nitrocellulose membranes (25 mm), which had been laid on dry LB $MgSO_4$ plates. These membranes were then left at 37°C for 4 – 6 hours to allow mating to proceed.

After the incubation period, filters were removed from the plate using sterile tweezers and placed into 1 ml of LB broth within a 14 ml tube. The tubes were vortexed to remove bacteria from the filter. Dilutions of the mating mix were made and then 100 µl of each spread onto selection plates, containing either 100 µg/ml Tp (for site directed mutagenesis) or 300 µg/ml Tp (for transposon mutagenesis) and 120 units / ml of polymyxin (to kill *E.coli* donor strains). The three strains used in the matings were also plated out onto selection plate to check that the plates would only select for the correct transconjugants. Plates were then incubated at 37°C for 24 – 48 hours and potential mutants picked into 96 well plates containing TSB with the appropriate antibiotic selection and grown overnight. After addition of DMSO, plates were frozen down at -80°C and then used for further analysis.

To isolate mutants capable of swarming in the presence of chlorhexidine, a screen was devised. Once mutants had grown on the selective agar they were pooled together (approximately 100 colonies in pool) in TSB broth by swabbing the plate and inoculating the broth (Figure 2.4). The suspension was mixed thoroughly and a 10 µl spot was then inoculated into the middle of swarm plates (0.3% nutrient agar, 0.5% glucose) containing 5 µg/ml of chlorhexidine (Figure 2.4). The plates were left to grow for 24 – 48 hours at 37°C. Those mutants that could swarm were picked by stabbing the agar at the edge of the swarm zone and re-plating onto standard TSA with Tp 300 µg/ml (Figure 2.4). Once the mutants had grown freezer stocks were made and the genes involved determined.

2.22 Determining genes involved in swarming regulation

2.22.1 Two step PCR to amplify flanking DNA

To determine the genes knocked out by the random transposon mutagenesis screen a two step nested PCR was performed. The two step PCR used four sets of primers (Table 2.6). The first round of PCR used a primer specific for the plasposon and a primer that randomly annealed to the Bcc genome. This primer also has a conserved tail added for subsequent PCR reactions. After the first round of PCR a second reaction is set up using two new primers, one specific for the plasposon, which annealed just inside of the first primer (Figure 2.5 and Table 2.6) and a primer that is specific for the conserved tail on the second primer (Figure 2.5). A chelex preparation of DNA from the mutants was performed as described in section 2. 42. A 1X PCR reaction was set up as follows using Promega reagents: 2 µl 10 x buffer, 1.2 µl of $MgCl_2$, 0.4 µl of dNTPs, 1 µl primer 1 (20 pmol/µl stock), 1 µl of primer 2b, 0.2 µl Taq polymerase, 13.2 µl sterile water and 1 µl of template DNA to make a final volume of 20 µl. These reactions were placed in a thermal cycler and the programme used is shown in Figure 2.6. After this reaction, a second PCR mix was made as follows: 5 µl of 10 x buffer, 2 µl of $MgCl_2$, 1 µl of dNTPs, 2.5 µl primer2.5 µl primer 4, 0.5 µl Taq polymerase, 33 µl of sterile water and 2.5 µl of PCR product from first reaction to make a final volume of 50 µl. This was placed into a thermal cycler and the programme used is shown in Figure 2.6.

Once the reaction was complete the reactions were cleaned up using a QiaQuick spin kit (section 2.11) and then 2 µl of loading dye was added to 8 µl of sample and analysed on a 2% gel at 80 volts.

2.22.2 Sequencing mutant PCR products

Once the nested PCR had been carried out, the products were sent for sequencing to determine the site of transposon insertion. The DNA quantity was measured and recorded. Primer 3 was used for sequencing as this was specific for the plasposon (1.6 pmol/µl stock). The sequencing reaction was carried out by Applied

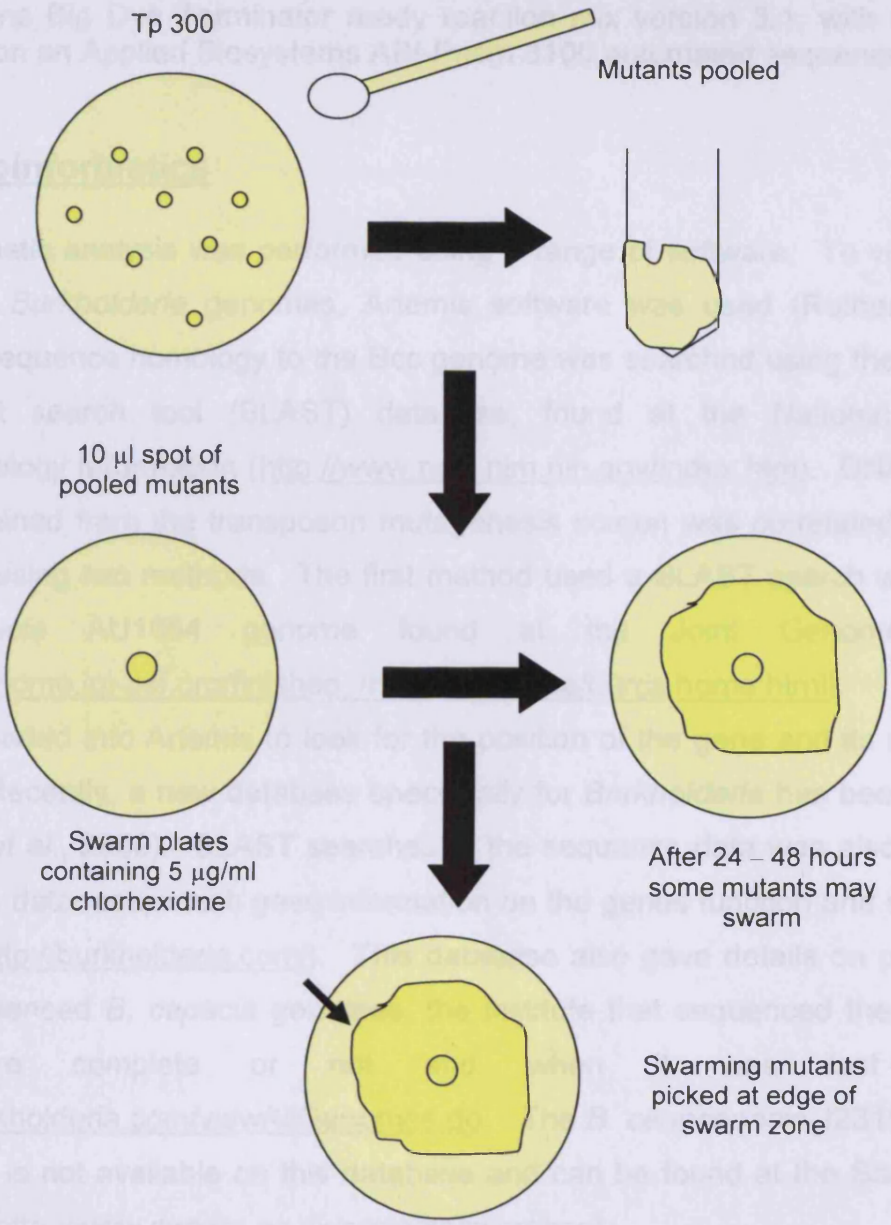


Figure 2.4 Mutagenesis screen to isolate swarming mutants

Mutants were swabbed from the selective plate and pooled in broth. Then 10 µl of the pooled mixture was spotted onto swarming agar plates containing 5 µg/ml of chlorhexidine and left for 24 – 48 hours. After this time non chlorhexidine responding mutants may swarm. To isolate these mutants a pick was made at the edge of the swarm zone, so as to avoid any non swarming mutants.

Biosystems Big Dye Terminator ready reaction mix version 3.1, with subsequent analysis on an Applied Biosystems ABI-Prism 3100 automated sequencer.

2.23 Bioinformatics

Bioinformatic analysis was performed using a range of software. To visualise and annotate *Burkholderia* genomes, Artemis software was used (Rutherford *et al.*, 2000). Sequence homology to the Bcc genome was searched using the basic local alignment search tool (BLAST) database, found at the National centre of Biotechnology Information (http://www.ncbi.nlm.nih.gov/index.htm). DNA sequence data obtained from the transposon mutagenesis screen was correlated to the Bcc genome using two methods. The first method used a BLAST search within the *B. cenocepacia* AU1054 genome found at the Joint Genome Institute (http://genome.jgi-psf.org/finished_microbes/burca/burca.home.html). This was then uploaded into Artemis to look for the position of the gene and its surrounding genes. Recently, a new database specifically for *Burkholderia* has been produced (Winsor *et al.*, 2008). BLAST searches of the sequence data was also performed using this database, which gave information on the genes function and surrounding genes (http://burkholderia.com/). This database also gave details on partially and fully sequenced *B. cepacia* genomes, the institute that sequenced them, whether they are complete or not and when it was last updated. http://burkholderia.com/viewAllGenomes.do. The *B. cenocepacia* J2315 sequence however, is not available on this database and can be found at the Sanger centre website (http://www.sanger.ac.uk/projects/microbes)

To further analyse the sequence data, derivatives of BLAST were used. PSI BLAST (position specific Iterated) (Altschul *et al.*, 1997) and BLAST P were used to identify protein domains. Protein family information was obtained from the protein family database (PFAM http://pfam.sanger.ac.uk/) and from the Clusters of Orthologous Groups database (COG http://www.ncbi.nlm.nih.gov/COG/). To predict transmembrane helices within the sequence data, the Transmembrane Hidden Markov Model database was used. (TMHMM http://www.cbs.dtu.dk/services/TMHMM-2.0/). Homology to transport related

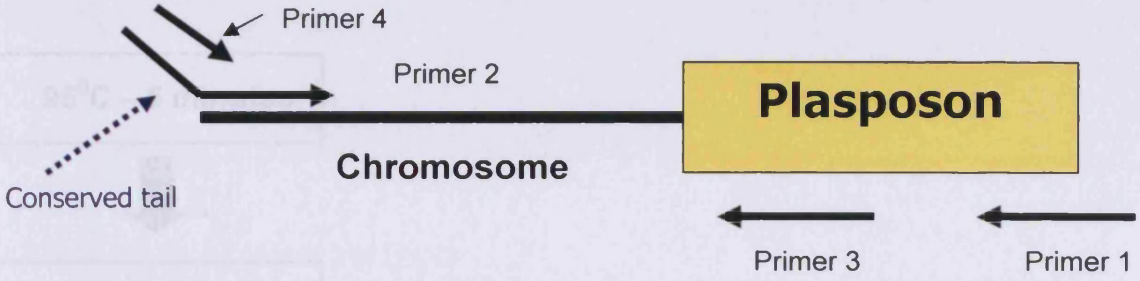


Figure 2.5 Schematic drawing of how the two step plasposon PCR works.

Primer 1 was specific for the plasposon and primer 2 randomly anneals to the *Burkholderia* chromosome. Primer 2 also has a conserved tail added to the end. After the first round of PCR, a second reaction was performed using primer 3 (which was specific for the plasposon) and (primer 4, which was specific for the conserved tail on primer 2). Once the reactions were complete the products were sent for sequencing using Primer 3.

Table 2.6 PCR primers for Random transposon mutagenesis

Name	5' – 3'
Primer 1	AGGCTCAGTGCAAATTTATCC
Primer 2	GGCCACGCGTCGACTAGTACN_{10}ACGCC
Primer 3	TTGAACGTGTGGCCTAAGCGAGC
Primer 4	CGTCACCATTTGGGAGCACATGC

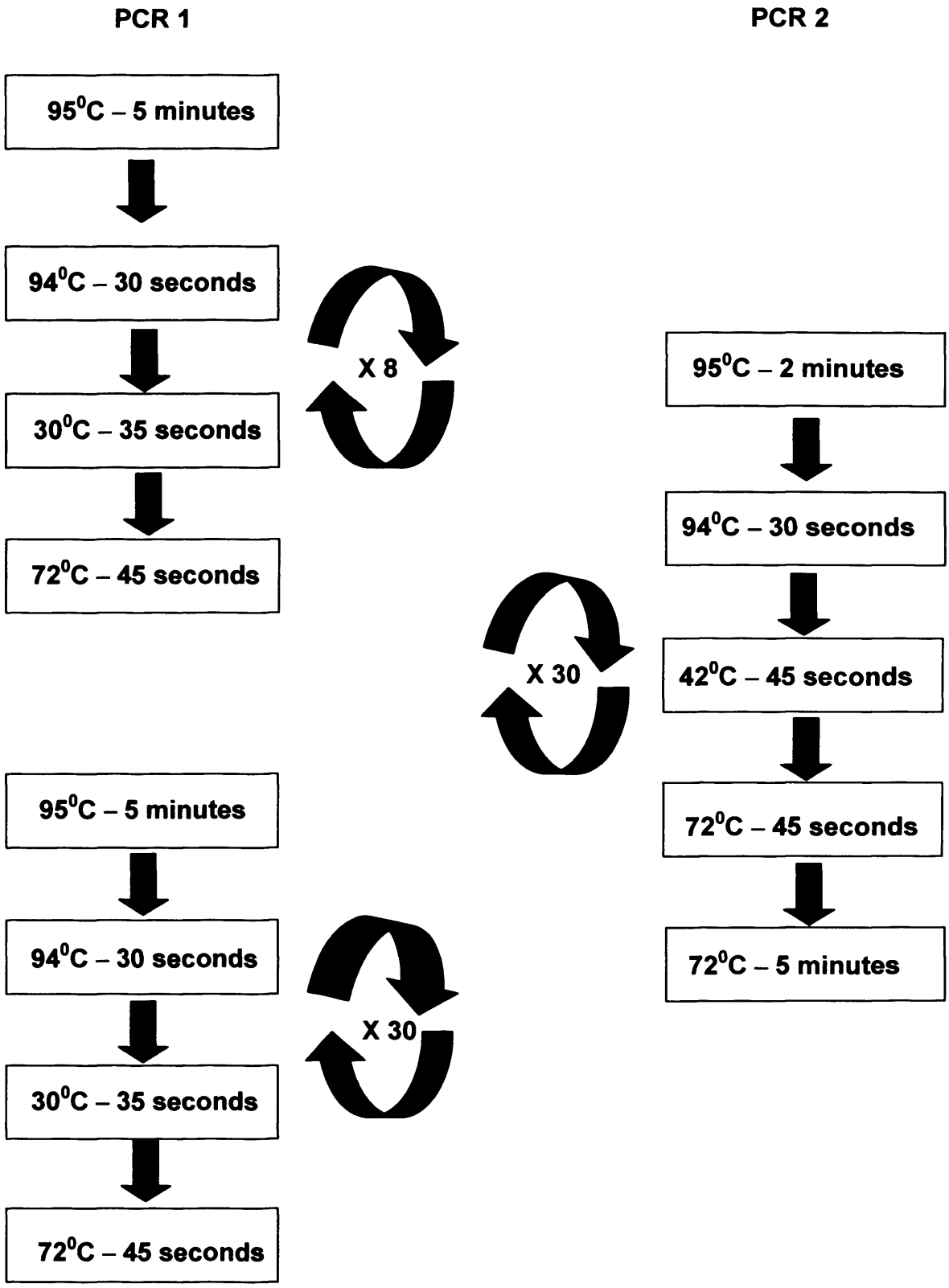


Figure 2.6 PCR programmes for nested PCR

PCR 1 was carried out on 2 µl of template DNA from mutants. Once reaction was completed 2.5 µl of PCR product was added to the reaction and PCR 2 was carried out. Once the reactions were complete, products were visualised on a gel and sent for sequencing.

proteins was determined using the Transport Classification database (TCB http://www.tcdb.org/).

2.24 Minimum inhibitory concentrations (MIC)

2.24.1 Broth dilution method

A broth dilution assay adapted to examine biocide susceptibility of Bcc bacteria was carried out as follows. Filtered sterilised aqueous solutions of chlorhexidine (10 mg/ml) and cetylpyridinium chloride (5 mg/ml) were used to make up stock concentrations of 100 µg/ml and 1000 µg/ml biocide. These solutions were then added to TSB to make up biocide concentrations ranging from: 0-100 µg/ml for chlorhexidine and 0-200 µg/ml for cetylpyridinium chloride. We found that growth in liquid medium as opposed to growing bacteria on slopes of solid agar produced consistent numbers of viable cells for the Bcc strains studied. Therefore, each Bcc strain was grown overnight (18 hours) in 3 ml TSB at 37°C (within a 14 ml tube, shaken horizontally at 200 rpm); the culture was diluted to an optical density of 1 (630 nm) corresponding to a viable count of approximately 1 x 10^8 cfu/ml. Approximately 1 x 10^5 cfu of each test Bcc strain was then added to 1 ml of each biocide concentration and 200 µl of this was added to 3 replicate wells within a 96 well plate. The microplates were incubated with shaking (200 rpm) at 37°C for 24 hours and the OD was read using a Dynex technologies MRX® microplate absorbance reader with Revelation application. An endpoint reading at 630 nm was taken with ten seconds of shaking beforehand.

B. multivorans LMG 13010 was included in all assays as a control strain as preliminary experiments with this strain exhibited a reproducible effect. Addition of biocides to the growth media resulted in changes to optical density. Therefore, the MIC value was designated as the concentration of biocide that resulted in an 80% knockdown of culture OD in comparison the control wildtype growth with no added biocide. Viable counts performed on the control strain after running the broth dilution MIC assay demonstrated the presence of 9.7 x 10^5 cfu/ml at a chlorhexidine concentration of 20 µg/ml, while no viable cells were detected above this concentration. Using the 80% knockdown in culture OD, the MIC of the control

strain was found to be 30 – 40 µg/ml, which corresponded well to the absence of viable cells in concentrations of chlorhexidine above 20 µg/ml. The results were subjected to statistical analysis to determine levels of susceptibility.

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2.24.2 Agar dilution method

To screen the subset of 38 strains against the three additional biocides, povidone, benzalkonium chloride and triclosan, an agar dilution method was used. The method allowed for higher concentrations of biocides to be examined where the broth dilution assay was prevented due to the biocides altering the OD reading. Stock solutions of the three biocides were made up as follows: Triclosan, 10 mg/ml in DMSO (this solvent did not inhibit the growth of Bcc bacteria at the concentrations used); povidone, 20 % in water, and benzalkonium chloride, 10 mg/ml in water. After autoclaving, Tryptone soya agar was cooled to 50°C and the appropriate amount of each biocide was added to 40 ml of agar for concentration ranges of 0 – 500 µg/ml for triclosan, 0 – 400 µg/ml for benzalkonium chloride and 0 – 5 % for povidone. The bacterial strains were cultured and diluted to 1 x 10^8 cfu per ml as described above; 200 µl of this suspension was then added to a 96-wells plate to create a master plate containing eight replicates of each test strain. The strain suspensions from the master plate were then replicated onto the biocide containing agar plates using a 96-well replicator and left to grow for 48 h at 37°C. The MIC was designated as the concentration of biocide where no visible growth was apparent.

2.25 MIC determination using the Bioscreen C

Chlorhexidine susceptibility of the site directed mutants was determined using an automated microbiology growth analysis system, the Bioscreen C (Thermo Fisher scientific). MIC experiments were set up as described in section 2.24.1 and placed into 100 - well honeycomb plates (Thermo Fisher Scientific). Two replicates of each mutant and wildtype strain, and a control containing only broth were included in each experiment. Incubation conditions were set up using the EZExperiment software supplied with the Bioscreen C. The Bioscreen C was set to take an OD

reading every 5 minutes, with 10 seconds of shaking beforehand for 48 hours, using the wideband filter with a range of 420 – 580 nm. After the experiment was completed, data was exported into Excel, the control blank checked and subtracted and growth curves produced.

2.26 Minimum bactericidal concentrations (MBC)

2.26.1 Neutraliser efficiency tests

Before minimum bactericidal concentrations could be performed a neutraliser which was effective at terminating biocide activity but which was non toxic to the bacterial cells was required. The neutraliser chosen was 5% Tween 80 and 0.75% azolectin, which was filter sterilised. The neutraliser was selected on the basis of previous studies which had successfully employed this neutraliser (Lear *et al.*, 2002).

The strain tested was *B. cenocepacia* K56-2. A cell suspension of this strain was made by diluting an overnight culture to approximately 10^6 cfu/ml. Firstly, to check the neutraliser was not toxic to bacterial cells, 100 µl of bacterial suspension was added to 900 µl of neutraliser and left in contact for 5 minutes. Viable cells were enumerated by viable counts and compared to a control where the neutraliser was replaced with water.

To ensure the neutraliser was effective at terminating the activity of the biocide, 100 µl of biocide at a lethal concentration was added to 800 µl of neutraliser solution. Immediately, 100 µl of bacterial suspension was added and left in contact for 5 minutes. Bacterial counts were enumerated by viable counts and compared to a control, whereby both the biocide and the neutraliser was replaced with water. If the neutraliser was efficient at terminating activity no reduction in viable cell count was observed.

2.26.2 MBC assays

Bacterial suspensions were made as described previously (section 2.24) and diluted to approximately 10^6 cfu/ml. Biocide solutions were made ranging from 0 – 1000 µg/ml for chlorhexidine and 0 – 5000 µg/ml for CPC. 10 µl of the bacterial suspension was added to 990 µl of the biocide solution and 200 µl placed into the well of a 96-well plate in triplicate. The plates were incubated at 37°C with shaking for 24 hours and then 20 µl of the bacterial suspension was placed into 180 µl of neutraliser solution. This was left in contact for 5 minutes and then replicated onto TSA agar plates. The plates were incubated at 37°C for 24 – 48 hours and the MBC recorded when growth ceased.

2.27 Antibiotic MICs – E - test®.

To determine the antibiotic MIC values for the Bcc, commercial E-test® strips were used (AB biodisk). These strips contain a gradient of the antibiotic and an MIC can be read directly from the strip. Eleven antibiotics were screened, that encompassed a range of classes of antibiotics, including some that are important for treatment of CF infections, e.g. meropenem, ciprofloxacin and tobramycin. The bacterial strains were revived on TSA and colonies placed into iso – sensitest broth (Oxoid) and diluted to an OD of 0.5 at 630 nm. The cultures were then swabbed onto iso sensitest agar to cover the whole plate and left to dry for 15 minutes. The E-test® strip was then placed onto the lawn of bacteria using sterile tweezers and the plates incubated at 37°C for 24 hours. The MIC was read off the strip as a zone of clearing on the lawn of bacteria (Figure 2.7). To determine if the bacteria was classed as resistant, sensitive or intermediate, the MIC was compared to a chart of MIC breakpoints, provided by the manufacturer (Table 2.7).

2.28 Suspension tests

Suspension tests were carried out on two commercial biocides, the chlorhexidine based Hibiscrub™ and triclosan based Cuticura™. Suspension tests were carried out as described by Lear *et al.* (Lear *et al.*, 2002). Bacterial cultures were prepared as previously described (section 2.24). Cells were harvested by centrifugation at 700 x g and supernatant removed. The cells were re suspended in PBS and

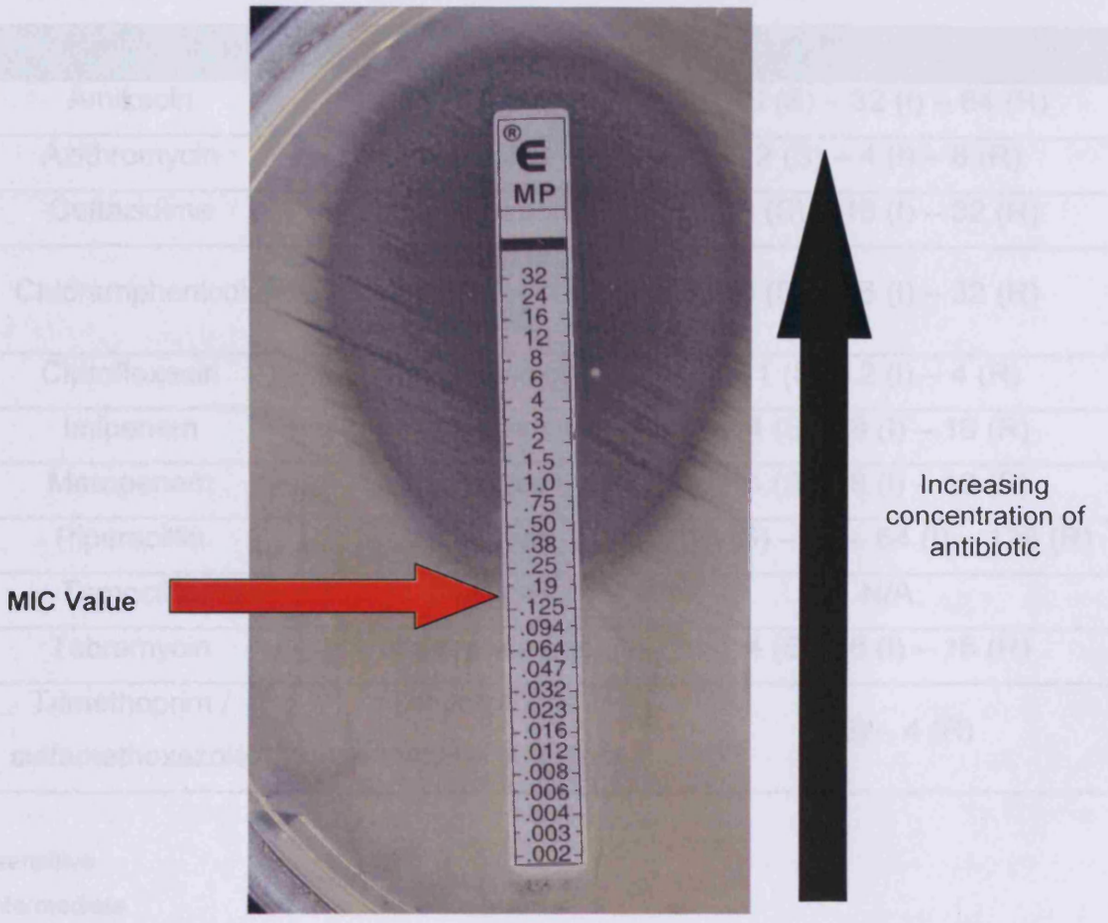


Figure 2.7 Antibiotic E-test® strip

The strip was placed onto a lawn of bacterial cells and left to grow for up to 48 hours. A zone of inhibition may be present and the MIC value read from the strip, where the zone of clearing intersects the strip.

Table 2.7 E- test® MIC breakpoints

Antibiotic	Class	MIC (µg/ml)
Amikacin	aminoglycoside	16 (S) – 32 (I) – 64 (R)
Azithromycin	macrolide	2 (S) – 4 (I) – 8 (R)
Ceftazidime	cephalosporin	8 (S) – 16 (I) – 32 (R)
Chloramphenicol	Protein synthesis inhibitor	8 (S) – 16 (I) – 32 (R)
Ciprofloxacin	fluoroquinolone	1 (S) – 2 (I) – 4 (R)
Imipenem	β lactam	4 (S) – 8 (I) – 16 (R)
Meropenem	β lactam	4 (S) – 8 (I) – 16 (R)
Piperacillin	β lactam	16 (S) – 32 – 64 (I) – 128 (R)
Temocillin	β lactam	N/A
Tobramycin	aminoglycoside	4 (S) – 8 (I) – 16 (R)
Trimethoprim / sulfamethoxazole	Dihydrofolate reductase inhibitors	2 (S) – 4 (R)

S = sensitive

I = intermediate

R = resistant

diluted to approximately 10^6 cfu/ml. 1 ml of the cell suspension was added to 9 ml of commercial biocide and left in contact for 5, 10, 20 and 60 minutes. At every time point 100 µl of suspension was removed and placed into a neutraliser solution (5 % Tween 80 and 0.75% azolectin). The neutraliser was checked as previously described (section 2.26.1). After 5 minutes of neutralisation, serial dilutions were made in PBS. Viable cells were enumerated by drop counts onto TSA and compared to a control where the biocide was replaced with water.

2.29 Efflux inhibitor assays

To examine the potential role of a mutant in efflux, an inhibitor assay was employed (Guglierame *et al.*, 2006). Bacterial suspensions of the mutant and the *B. cenocepacia* K56-2 wildtype were grown in an 18 hour overnight culture and diluted to an OD of 0.5. The bacteria were harvested and washed twice with PBS. The suspension was added to glass cuvettes and ethidium bromide added to a final concentration of 20 µM. The fluorescence was recorded over a period of 10 minutes, with a reading taken at 2 minute intervals using a Cary Eclipse fluorimeter. After 10 minutes the energy uncoupler carbonyl cyanide 3 chlorophenylhydrazone (CCCP) was added to a final concentration of 30 µM and fluorescence recorded for a further 16 minutes at 2 minutes intervals. Two replicates of each strain were performed and graphs were produced.

2.3 Statistical analysis

Due to the multifactorial mechanisms of biocide action, the MIC values obtained in replicate experiments spanned a range; therefore the results were split into a mean minimum value and a mean maximum value. All the results were analysed using the statistical software Minitab V.14. Kruskal–Wallis and Mann-Whitney statistical tests were carried out on the data sets and significant differences were determined ($p < 0.05$ for Kruskal-Wallis and $P < 0.05$ for Mann-Whitney). An antibiotic profile score was also calculated as a measure of overall antibiotic resistance within a Bcc species as follows: (i) for a given antibiotic, the mean MIC of all the strains in

species tested was calculated; (ii) the mean for all ten of the antibiotics tested was then calculated to produce an overall score value for the Bcc species. Mann-Whitney tests were applied to determine the significance of the species mean antibiotic profile score ($P < 0.05$); however, the significance of the score was not determined for Bcc species represented by less than two strains. The antibiotic profile score did not correlate to clinically defined break points for resistance; it just served as a measure of the overall multiple drug resistance observed in each Bcc species.

Chapter 3.0

Characterising Antimicrobial Susceptibility in the *Burkholderia cepacia* Complex

3.0 Characterising antimicrobial susceptibility in the *Burkholderia cepacia* complex

Published in part; in the Journal of Antimicrobial Chemotherapy – Biocide Susceptibility of the Burkholderia cepacia complex. (See appendix 1)

3.1 Introduction

3.11 Antibiotic resistance in the Bcc

Since the first cases of *B. cepacia* infections in cystic fibrosis patients were identified, incidences of antimicrobial resistance, often multidrug resistance, have been observed (Mahenthiralingam and Vandamme, 2005). Some members of the Bcc are particularly problematic due to innate resistance to multiple antibiotics, such as aminoglycosides, polymyxins and beta lactams (Chernish and Aaron, 2003). Clinically, this innate resistance is a problem as isolates are resistant to combinations used for treatment (Mahenthiralingam *et al.*, 2005). Often this results in an aggressive treatment regime for the patient, using double or triple antibiotic combinations before eradication can occur (Chernish and Aaron, 2003). Chernish *et al.* found that 50% of *B. cepacia* isolates were resistant to all single antibiotics, which included meropenem, tobramycin, chloramphenicol and ceftazidime. When double combinations such as meropenem plus either amikacin, ceftazidime, chloramphenicol or tobramycin were tested 8% of isolates were resistant to these. This leaves only triple drug combination therapy as an option, such as meropenem plus tobramycin and either ceftazidime, trimethoprim / sulfamethoxazole, chloramphenicol or aztreonam (Chernish and Aaron, 2003).

Members of the Bcc are also intrinsically resistant to trimethoprim via a trimethoprim resistant dihydrofolate reductase. As mentioned previously (Chapter 1) efflux pumps can have broad specificity and lead to resistance to a wide range of antimicrobials, in the Bcc group this includes trimethoprim, chloramphenicol and quinolones (Burns *et al.*, 1996). The *ceo* operon in *B. cenocepacia* encodes a RND efflux pump that confers resistance to chloramphenicol, trimethoprim and ciprofloxacin, therein is a vital mechanism of multidrug resistance (Nair *et al.*, 2004).

The outer membrane of the Bcc enhances the resistance phenotype due to its lack of permeability and the structure of the LPS confers resistance to β lactams and cationic peptides. Consequently infection with the Bcc, particularly multidrug epidemic strains can be difficult to treat, leaving clinicians and patients with little options. Therefore much research has been carried out into understanding resistance to antimicrobials and to find an effective treatment to prevent and eradicate Bcc infections.

3.12 Biocide resistance and the Bcc

In contrast, the molecular basis of biocide resistance in the Bcc bacteria has been poorly studied despite these organisms being linked to many cases of contamination in disinfectants and preservative solutions (Jimenez, 2007). Such contamination is problematic and may act as a reservoir for infection in CF patients and in vulnerable non-CF patients. Infection with Bcc outside of CF is not well characterised, however cases of specific Bcc infections have been isolated. Reik *et al.* found that in 90 non-CF patients 25.6% were infected with *B. cenocepacia*, 18.9% with *B. cepacia* and 15.6% with *B. multivorans* (Reik *et al.*, 2005). A study by Garcia-Erce *et al.* reported an outbreak of *B. cepacia* in blood transfusion patients was due to contamination of a 0.5% chlorhexidine solution that was used to disinfect the skin (Garcia-Erce *et al.*, 2002). These patients suffered from septic shock and were seriously ill. Pharmaceutical formulations containing benzalkonium chloride (Frank and Schaffner, 1976), cetylpyridinium chloride (Kutty *et al.*, 2007) and povidone-iodine (Panlilio *et al.*, 1992) have also been contaminated by Bcc bacteria. CF patients often use nebulizers to deliver aerosols of antibiotics and other drugs. These can become contaminated if disinfection is not carried out adequately and may be a primary route of infection. Bcc isolates frequently contaminate home use nebulizers and DNA fingerprinting indicated that the contaminating isolate was indistinguishable from the patients sputum isolate (Hutchinson *et al.*, 1996). Thus, disinfection of nebulizers should be carried out frequently to prevent patients from acquiring infections.

The potential contamination of disinfectant solutions may have serious consequences for patients, especially those with cystic fibrosis. A patient who has

been hospitalised can acquire a serious infection from these contaminated solutions and this may lead to increased hospital stays, increased antibiotic use and an increase in morbidity and mortality. This not only impacts the patient but also has economical outcomes. Hospital acquired infections can put an enormous strain on clinics due to increased hospital costs from replacing contaminated solutions to the cost of keeping a patient in hospital for a longer period of time. Therefore preventing the contamination of antinfectives is an important area of research. However, the only reported Bcc outbreak in CF patients due to contaminated products was in a Portuguese CF clinic and this outbreak was attributed to the contamination of non sterile saline solutions used in inhalant therapy, not contamination of a disinfectant solution (Cunha *et al.*, 2007). Therefore there appears to be a low risk of infection outbreaks in CF clinics from contaminated biocide products. Nonetheless, care should be taken as members of the Bcc are capable of surviving in biocide solutions and could potentially be a hazard for vulnerable patients.

The Bcc are a group of closely related bacteria that can be isolated from many sources including the environment. Preventing patients from acquiring these infections can be difficult due their abundance in nature, thus disinfection procedures are important in both clinical and domestic situations. Although there are guidelines to hospital disinfection, there are no standardised polices that are implemented by all hospitals (Maillard, 2005). There may also be a lack of compliance to outlined procedures, which leads to ineffective disinfection and sterilization (Rutala and Weber, 2004). This can be particularly problematic with surgical implants and equipment such as endoscopes, which can lead to the spread of infection (Rutala and Weber, 2004). There have also been conflicting opinions on the indiscriminate use of biocides and the use of biocides in preservative formulations at lower concentrations (Russell, 2002a). There is a disagreement whether disinfection of non-critical surfaces, such as walls and floors should be carried out as over use of biocides may pave the way for resistance to occur. Generally it is agreed that disinfection should occur in areas where hospital acquired infection is a high risk, such as showers, baths and toilets, where as other lower risk areas may only require cleaning (Maillard, 2005).

3.13 Biocide resistance and antibiotic resistance

The increased usage of biocides not only in clinical situations but also in domestic situations has raised the issue of whether residues of biocides in the environment selects for antibiotic resistance (Russell, 2000). Although biocides and antibiotics have different modes of actions, because of the broad specificity of biocides due to their action on multiple targets these mechanisms may overlap (Fraise, 2002) (Figure 3.1). Efflux pumps are a good example of an overlapping mechanism of resistance. Efflux pumps are often broad spectrum and can pump out a number of unrelated compounds from the cell conferring resistance. The use of triclosan has been seen to select for low level resistance to some antibiotics in *E. coli* due to efflux pumps (McMurry *et al.*, 1998a). The MexAB-OprM efflux system in *P. aeruginosa* can also pump out triclosan. Exposure to triclosan selected for multidrug resistant strains, which included resistance to antipseudomonal drugs such as ciprofloxacin. This resistance was due to the over expression of the MexAB-OprM efflux pump due to exposure to triclosan (Chuanchuen *et al.*, 2001).

The latter mechanisms of resistance are all intrinsic. Although acquired resistance is a major cause of antibiotic resistance in the Bcc, rendering some strains untreatable, in biocide resistance acquired mechanisms are less important. However, acquired mechanisms may lead to cross resistance with biocides and antibiotics. Yammamato *et al.* found a plasmid from a methicillin and gentamicin resistant strain of *Staphylococcus aureus* conferred resistance to kanamycin, gentamicin, tobramycin, amikacin, benzalkonium chloride, acriflavin, ethidium bromide and chlorhexidine (Yamamoto *et al.*, 1988). In addition this plasmid could be transferred to other bacterial species (Yamamoto *et al.*, 1988). This is a cause for concern as broad spectrum resistance plasmids could be spread and selected for in clinical situations. However, although there is much debate on whether the indiscriminate use of biocides does select for multidrug resistant bacteria, there is also another side to the argument. Many of these studies use low levels of the biocide. In reality the in use concentration (Table 1.2) is often used at much higher levels that are bactericidal and therefore selection of resistance mechanisms will not occur ` (Russell, 2000), although persistence of residuals of disinfectants may be a cause

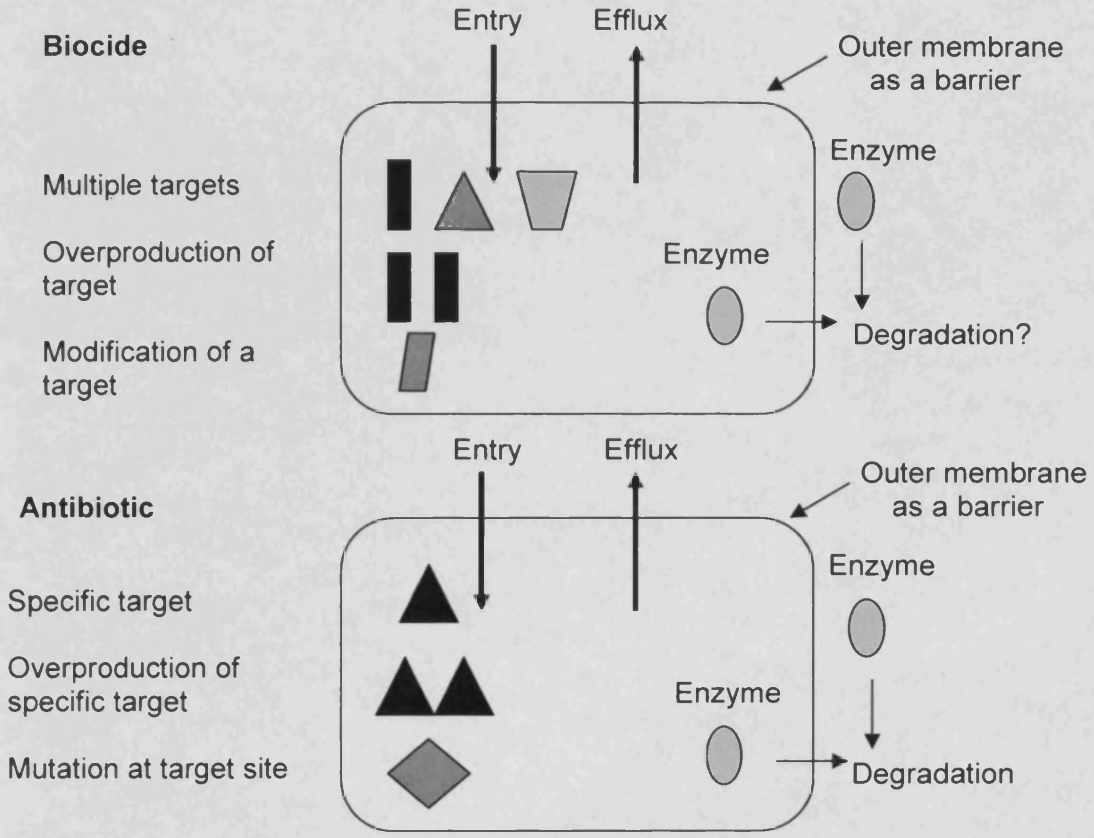


Figure 3.1 Biocide and antibiotic mechanisms of resistance.

Biocide resistance mechanisms include overproduction of the target, modification of target site, active efflux of the compound, enzyme degradation and low permeability. Antibiotic resistance mechanisms include overproduction of the target, mutations of targets, active efflux, enzyme degradation and low permeability. The potential for cross resistance between antibiotics and biocides is clearly a possibility, with some mechanisms overlapping, such as low permeability and efflux. Adapted from (Russell, 2003)

for concern.

3.14 Measuring biocide susceptibility

Routine antibiotic susceptibility screening is carried out in laboratories. However for biocides, routine susceptibility testing is more difficult and are often not carried out as the in use concentration of disinfectants are at such a high level they should be bactericidal. Due to increasing incidences of biocide contamination, susceptibility testing of biocides should be performed regularly. A major problem with biocide susceptibility testing is the lack of a standardized reproducible test. This means that a disinfectant that passes in one country may not pass in another (Lambert, 2004). The British testing standards BS EN 1276 consist of 3 stages of testing. Stage 1, where suspension tests are carried out to look for surviving bacteria, with two reference species, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, after exposure to the disinfectant. Stage 2, involves testing possible in use scenarios using capacity tests, where the in use dilution is inoculated for a certain amount of time and sampled for surviving bacteria, then a fresh inoculum was added to the biocide. Finally in stage 3, the disinfectant is tested in the field e.g. surface testing (Lambert, 2004). Although these tests are recognised as the gold standard to use, they come with problems. Many of these tests have poor reproducibility and a study by Taylor *et al.* found that 7 out of 18 commercial disinfectants failed the suspension test (Taylor *et al.*, 1999). This indicates that more rigorous, standardized tests are required.

Minimum inhibitory concentration (MIC) screens are often used to gauge the susceptibility of a bacterial strain to an antimicrobial. The minimum inhibitory concentration is the lowest concentration where growth is inhibited (Lambert, 2004), but is only a measure of bacteriostatic activity. Although it would appear that MIC's are unrelated to the much higher in use concentrations, these screens provide important preliminary information and can aid our understanding of the action of biocides as well as the effect lower concentrations have on bacterial strains (Russell, 2001). Minimum bactericidal concentration (MBC) screens are used to determine the killing action of a biocide in support of MIC data.

The aim of this study was to provide an up to date survey of the biocide susceptibility profiles of a representative panel of Bcc species which accounted for both taxonomic and genetic diversity. To ensure that a wide genetic diversity of Bcc was sampled isolates were selected on the basis of their MLST type (Baldwin *et al.*, 2005). Susceptibility data also related to the clinical use of disinfectants by looking at minimum bactericidal concentrations for the most resistant and susceptible strains and investigating the efficacy of commercial biocides against members of the Bcc.

3.2 Results

3.21 Chlorhexidine and Cetylpyridinium chloride susceptibility

Minimum inhibitory concentrations for two biocides, the bisbiguanide chlorhexidine and the quaternary ammonium compound cetylpyridinium chloride were initially determined for 101 strains of the Bcc via a broth dilution assay (Chapter 2, section 2.24.1). These two biocides were chosen as they are widely used in clinical situations and are also soluble and easily incorporated into the MIC screens. However, limitations with the broth dilution method meant a maximum value of 100 µg/ml of chlorhexidine and 500 µg/ml of CPC was used as at higher concentrations these biocides precipitated out of solution, skewing OD readings. After replicate analysis the mean minimum and the mean maximum MIC values for each species was calculated (Table 3.1). Despite rigorous standardization of the MIC assay an absolute end point for the biocides MIC were not obtained, due to the fluctuations observed between the replicates of the assay. An internal *B. multivorans* control strain, LMG 13010 was included in every assay. Although this strain exhibited the desired effect, slight differences were observed between experiments and the standard error of the mean was quite large in some cases (Figure 3.2). Therefore a range was used as the MIC value to compensate for these differences.

The species screened included the recently classified species, *B. lata*, *B. contaminans* and novel Bcc group K isolates, which have been associated with contamination of preservative products (Vanlaere *et al.*, 2008). Preservatives are

used to prevent the spoilage of commercial products and are incorporated into personal care products which can be directly applied to the surface of the body or used internally, like shampoos, conditioners, toothpaste and mouthwashes and to home use products such as washing powders (Russell, 2006). Biocides may be incorporated into products as a preservative agent at lower concentrations, hence issues with bacterial resistance are abundant with Bcc group K being particularly problematic (Vanlaere *et al.*, 2009). For chlorhexidine, a variable range of growth inhibition was observed around the Bcc mean of 33 to 42 µg/ml. This ranged from 15 µg/ml for *B. lata* to 57 µg/ml for *B. cepacia* (Table 3.1). Although there were no significant differences observed between the MIC's for chlorhexidine, strains of *B. cepacia*, *B. vietnamensis* and *B. cencepacia* possessed the highest MIC values. For CPC, the susceptibility of the Bcc was much less, with approximately 3 fold larger amounts of CPC required for inhibition (mean MIC range 129 µg/ml to 144 µg/ml); Table 3.1). Species differences were also observed; with *B. cepacia*, *B. cenocepacia*, B. *lata*, *B. contaminans* and novel Bcc group K being significantly less susceptible to CPC than other members of the Bcc (Table 3.1).

The collection of isolates examined contained nine examples of Bcc strains that were involved in outbreaks in multiple CF patients. The *B. cenocepacia* ET-12 epidemic strain J2315 and two additional single locus variants strains (K56-2 and LMG 18827) had high chlorhexidine MIC values (>100 µg/ml). In comparison, only one other *recA* group III-A outbreak strains (Mu1) and a *recA* III-B group isolated from an urinary tract infection (LMG 18832) possessed a chlorhexidine MIC value greater than 100 µg/ml. Other epidemic strains within *B. cenocepacia* III-B, such as the PHDC and Mid Western strains were prevented from growing at < 60 µg/ml of chlorhexidine.

Interestingly, *B. multivorans* isolate, LMG 16660 was observed to have a high chlorhexidine MIC value (90-100 µg/ml) compared to other *B. multivorans* strains (Table 3.2). This strain was part of an outbreak in CF patients in Glasgow. To determine if this was a feature of the outbreak strains, 19 additional Glasgow

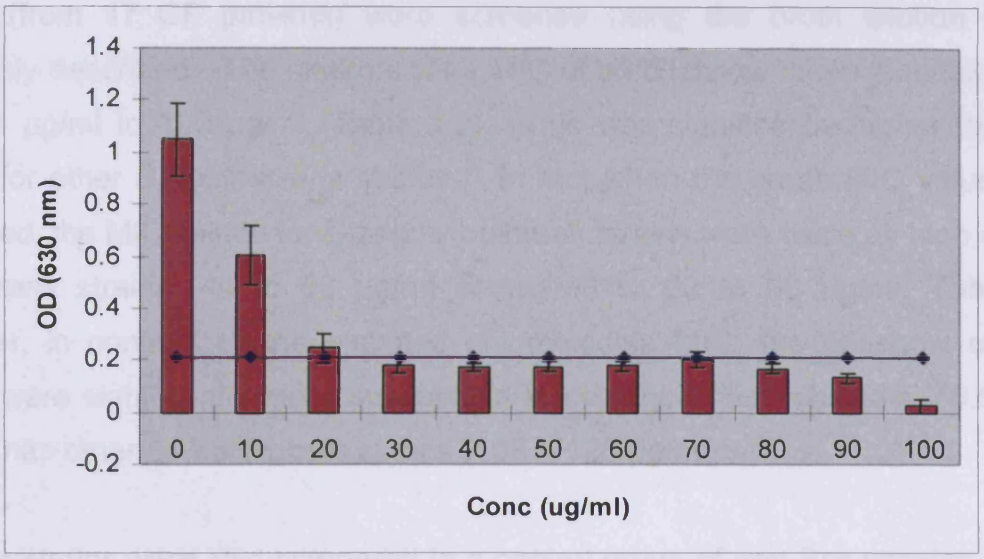


Figure 3.2 Mean MIC range for control B. *multivorans* strain, LMG 13010 over ten experiments in chlorhexidine.

The MIC was taken as an 80% knockdown from growth at zero concentration of chlorhexidine (blue line). Mean data obtained from ten separate experiments and standard error of mean was calculated (error bars). MIC value = 30 – 40 µg/ml. Fluctuations were observed over the ten experiments, with the MIC ranging from 20 – 50 µg/ml.

strains (from 17 CF patients) were screened using the broth dilution method previously described. The chlorhexidine MIC of all Glasgow strain isolates ranged from 44 µg/ml to 100 µg/ml (Table 3.2). This was significantly higher than MIC values for other *B. multivorans* strains. In fact when the mean MIC values were examined, the MIC values for Glasgow outbreak strains were twice as high other *B. multivorans* strains (44 to 53 µg/ml compared to 22 to 33 µg/ml, Table 3.2). However, in contrast to the elevated chlorhexidine MIC, the Glasgow outbreak strains were significantly more susceptible to cetylpyridinium chloride (76.6 - 89.5 µg/ml) than other *B. multivorans* strains (105 – 126 µg/ml).

The Bcc strains were also compared to a control group of non-Bcc species that are commonly encountered in CF infections, such as *S. aureus*, *P. aeruginosa* and *Ralstonia pickettii*. For chlorhexidine there were no significant differences between the MIC values for the Bcc compared to the non Bcc species, although 6 of the 10 species tested were inhibited by < 20 µg/ml of chlorhexidine (Table 3.3). Cetylpyridinium chloride resistance was elevated in several Bcc species when the means were compared (Table 3.1 and 3.3). *B. cepacia*, *B. cenocepacia* and Bcc novel group K were significantly less susceptible ($p<0.05$) than the non-Bcc species total. *P. aeruginosa* was the only CF pathogen that was comparable to Bcc bacteria for susceptibility profiles to both biocides (see chapter 4.0 for more details on *P. aeruginosa* biocide susceptibility).

In addition to screening non – Bcc strains, three control reference strains that are often used in clinical laboratories for antibiotic susceptibility tests were also screened for comparison. These strains were from the NCTC (National collection of Type Cultures) collection and consisted of an *E. coli* NCTC 12241, *P. aeruginosa* NCTC 12903 and a *S. aureus* NCTC 12981. All three strains were very susceptible to chlorhexidine with MIC values of 10 – 20 µg/ml (Figure 3.3a), however CPC MIC susceptibilities were more comparable with Bcc MIC's with all three strains having an MIC of >200 µg/ml (Figure 3.3b).

3.22 Minimum Bactericidal Concentrations

Table 3.1 Susceptibility of Bcc and other species to chlorhexidine and CPC

Species (No. of Strains tested)	Chlorhexidine Mean Min. MIC (µg/ml)	Chlorhexidine Mean Max. MIC (µg/ml)	CPC Mean Min. MIC (µg/ml)	CPC Mean Max. MIC (µg/ml)
B. cepacia (10)	57	65	170[a]	175[b]
B. multivorans (30)	36.6	46	77.7	101.3
B. cenocepacia (31)	47.4	54.8	175.5[c]	184.5[c]
B. stabilis (3)	40	46.7	146.7	166.7
B. vietnamiensis (5)	52	58	90	106
B. dolosa (3)	26.7	36.7	66.7	86.7
B. ambifaria (6)	33.3	41.7	98.3	116.7
B. anthina (2)	10	20	95	125
B. pyrrocinia (2)	15	25	50	70
B. lata (4)	15	25	183.3[d]	200[d]
B. contaminans (3)	13.3	23.3	200[e]	200[f]
Novel Bcc Group K (2)	50	60	200[g]	200[g]
Mean Bcc (101)	33.03	41.9	129.4	144.3

[a] At the mean minimum CPC MIC, *B. cepacia* was significantly more resistant than *B. multivorans, B. dolosa, B. ambifaria* and *B. pyrrocinia* ($p < 0.05$).

[b] At the mean maximum CPC MIC, *B. cepacia* was significantly more resistant than *B. multivorans, B. dolosa* and *B. pyrrocinia* ($p < 0.05$).

[c] At the mean minimum and mean maximum CPC MIC, *B. cenocepacia* was significantly more resistant than *B. multivorans, B. vietnamensis, B. dolosa, B. ambifaria, B. anthina* and *B. pyrrocinia* ($p < 0.05$).

[d] At the mean minimum and mean maximum CPC MIC, *B. lata* was significantly more resistant that *B. multivorans* ($p < 0.05$).

[e] At the mean minimum CPC MIC, *B. contaminans* was significantly more resistant than *B. multivorans* and *B. ambifaria* ($p < 0.05$).

[f] At the mean maximum CPC MIC, *B. contaminans* was significantly more resistant than *B. multivorans* ($p < 0.05$).

[g] At the mean minimum and mean maximum CPC MIC, Bcc novel group K significantly more resistant than *B. multivorans* ($p < 0.05$).

Table 3.2 Susceptibility of *B. multivorans* Glasgow outbreak strains and *B. multivorans* strains to chlorhexidine and CPC

Species	Chlorhexidine Mean Min. MIC (µg/ml)	Chlorhexidine Mean Max. MIC (µg/ml)	CPC Mean Min. MIC (µg/ml)	CPC Mean Max. MIC (µg/ml)
B. multivorans (10)	22	33	105*	127.3*
Glasgow Outbreak strains (20)	44*	53*	76.5	89.5

* Denotes those species that are significantly different as determined by Kruskal-Wallis and Mann-Whitney tests ($P < 0.05$).

In addition to MIC screening, the chlorhexidine and CPC MBC of 38 strains from the original panel of 101 strains was determined. Twenty two strains were selected with high MIC's to chlorhexidine and CPC, while 16 were selected with low MICs to both biocides. The MBC screen was carried out as previously described (Chapter 2, section 2.26.1). To terminate the action of the biocides after the given exposure period a neutraliser was used (0.75% azolectin and 5% Tween 80). Before MBC experiments could be performed, control experiments to check that the neutraliser was efficient at terminating activity and not toxic to bacterial cells were carried out as described in chapter 2.

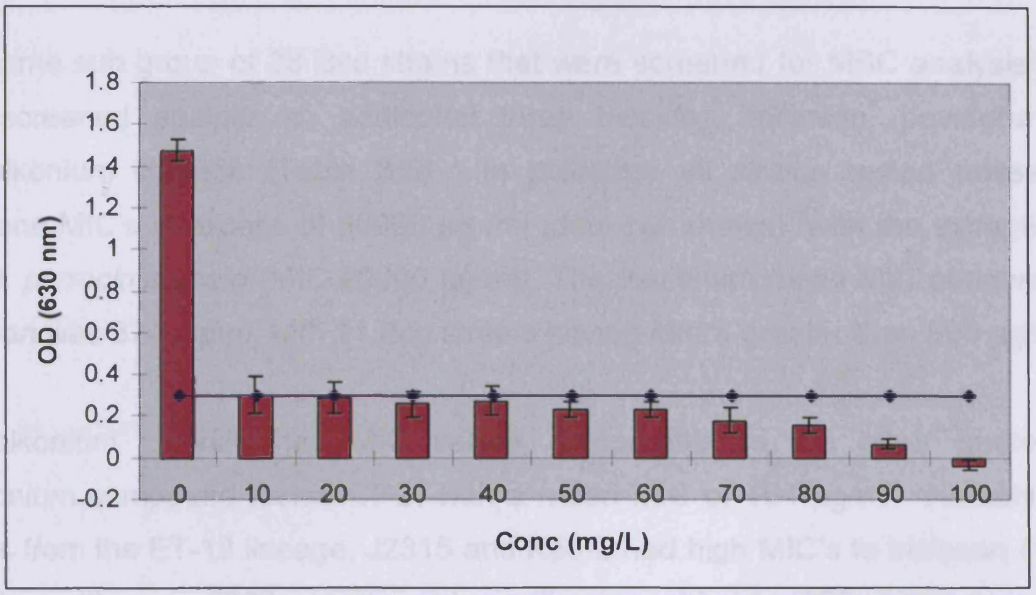
To check that the neutraliser was efficient at terminating activity viable counts for bacteria in a control (biocide and neutraliser replaced with water) were compared to viable counts of bacteria exposed to chlorhexidine and CPC which had been exposed to the neutraliser for five minutes. There were no log differences between the number of viable bacteria in the control and the two neutralised biocide solutions, thus indicating that the neutraliser solution was capable of terminating activity of the biocide (Table 3.4). To check that the neutraliser was non toxic to bacterial cells it was inoculated directly with bacterial suspension and viable counts indicated that the neutraliser was not lethal to the bacteria (Table 3.4). Therefore this neutraliser solution was used for both chlorhexidine and CPC screens.

The mean Bcc chlorhexidine MBC was 229 µg/ml (Table 3.5) which was approximately six fold greater than the MIC value for this biocide. The majority of strains tested (35/38) required no more than 300 µg/ml for a bactericidal outcome. A *B. multivorans* strain, C1607 and two *B. cenocepacia* strains, DN and X100 had MBC values of 500 µg/ml and only two *B. cenocepacia* strains, LMG 18832 and Mu1 needed at least 1000 µg/ml chlorhexidine to achieve killing, a value ten fold above their MIC. In CPC, 27 out of the 38 strains tested required over 20 fold more CPC than the MIC for a bactericidal effect to be seen, with a mean CPC MBC of 2998 µg/ml (Table 3.5). In addition 13 Bcc strains were still viable after 24 hours of exposure to 5000 µg/ml of CPC.

Table 3.3 Chlorhexidine and cetylpyridinium chloride susceptibilities of non Bcc bacterial species.

Species	Isolate	Chlorhexidine MIC (µg/ml)	CPC MIC (µg/ml)
Staphylococcus aureus	MRSA - 224005	10 - 20	150 - 180
	MRSA - 223021	90 - 100	150 - 180
	LMG 22203	90 - 100	10 - 30
	MRSA 223054	90 - 100	10 - 30
	MSSA	90 - 100	10 - 30
	NCTC 12981	90 - 100	10 - 30
Burkholderia gladioli	LMG 2216	10 - 20	150 - 180
Stenotrophomonas maltophilia	LMG 958	10 - 20	100 - 120
Pseudomonas aeruginosa	PA01	20 - 30	>200
Ralstonia mannitolilytica	LMG 6866	80 - 90	150 - 180
Pseudomonas stutzeri	LMG 11199	10 - 20	120 - 150
Burkholderia dolosa	AU1069	20 - 30	50 - 70
Ralstonia pickettii	LMG 5942	40 - 50	120 - 150
Pseudomonas putida	LMG 2257	10 - 20	150 - 180
Achromobacter xylosoxidans	LMG 1863	10 - 20	100 - 120
Escherichia coli	NCTC 12241	10 - 20	10 - 30
	Mean Non – Bcc (16)	42.5 – 52.5	93.1 – 116.3

a.



b.

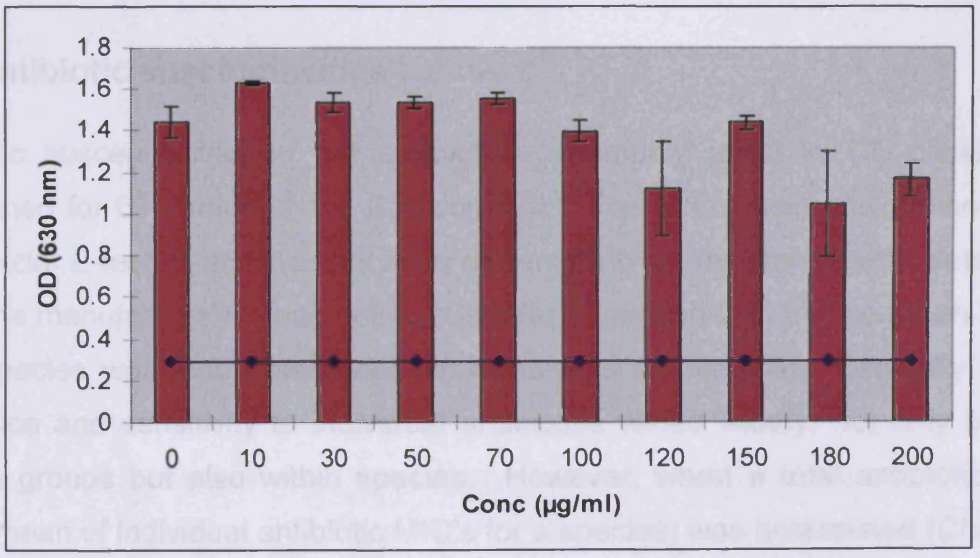


Figure 3.3 *E. coli* NCTC 12241 (± SE) MIC values to a. chlorhexidine and b. CPC.

E. coli antibiotic reference strain NCTC 12241 has a MIC of 10 – 20 µg/ml in chlorhexidine and >200 µg/ml in CPC. MIC values are taken as an 80% knockdown from growth at zero concentration, as indicated by the blue line.

3.23 Triclosan, povidone and benzalkonium chloride susceptibility

The same sub group of 38 Bcc strains that were screened for MBC analysis were also screened against an additional three biocides, triclosan, povidone and benzalkonium chloride (Table 3.5). In povidone all strains tested possessed povidone MIC's in excess of 50000 µg /ml (data not shown), with the exception of one *B. pyrrocinia* strain (MIC 20000 µg/ml). The maximum mean MIC observed for triclosan was 374 µg/ml, with 11 Bcc strains having MIC's greater than 500 µg/ml.

Benzalkonium chloride had MIC values comparable to the other quaternary ammonium compound tested CPC, with a mean MIC of 164 µg/ml. Notably both strains from the ET-12 lineage, J2315 and K56-2 had high MIC's to triclosan (> 500 µg/ml), povidone (> 5000 µg/ml) and benzalkonium chloride (350 µg/ml) as well as possessing high chlorhexidine and CPC MIC's.

3.24 Antibiotic susceptibilities

Antibiotic susceptibilities to ten antibiotics commonly used in CF clinics were determined for 60 strains of the Bcc complex. The MIC's were determined using commercial E-tests® and classification of sensitivity or resistance was determined using the manufacture's break points (Chapter 2, section 2.27). The mean MIC for each species was also determined and analysed (Table 3.6). Clinically defined resistance and sensitivity to individual antibiotics varied widely, not only between species groups but also within species. However, when a total antibiotic profile score (mean of individual antibiotic MIC's for a species) was determined (Chapter 2, section 2.10), differences were observed. *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis* and *B. dolosa* possessed the highest levels of multiple antibiotic resistances (Table 3.6).

This did not always correlate with biocide resistance. For example a *B. dolosa* strain involved in an outbreak in a hospital in Boston (Kalish *et al.*, 2006) had low MIC's to chlorhexidine and CPC (20 – 30 µg/ml and 50 – 70 µg/ml respectively), but was defined as clinically resistant to all ten of the antibiotics. Generally, all species tested appeared to show high levels of resistance to azithromycin and tobramycin,

Table 3.4 Neutraliser efficiency tests for minimum bactericidal concentrations

Condition	Viable counts of K56-2 (cfu/ml to 10^7 ± SE)
Control*	3.53 (± 6.6)
Chlorhexidine exposed	3.75 (± 22.5)
CPC exposed	7.6 (± 0.3)
Neutraliser control#	13.3 (± 3.3)

* = biocide and neutraliser replaced with water

= neutraliser inoculated with bacterial suspension

SE = standard error of the mean

Table 3.5 Biocide susceptibility and minimal bactericidal concentrations for Bcc

Species	Strain name	Chlorhexidine		CPC		Triclosan MIC (µg/ml)	Benzalkonium chloride MIC (µg/ml)
		MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)		
B. cepacia (7)	LMG 17997	100	100	70 - 100	2000	400	200
	ATCC 49709	100	100	>200	500	300	50
	IST431	90 - 100	-	>200	3000	400 - 450	200
	LMG18821	90 - 100	300	>200	2000	>500	100
	AVC1717	70 – 80	300	>200	>5000	500	200
	ATCC 17759	20 – 30	100	>200	3000	>500	100
	Bcc0176	10 – 20	50	30 - 40	50	500	100
B. multivorans (5)	LMG 16660	90-100	300	> 200	>5000	>500	200-250
	ATCC 17616	10-20	300	50 - 150	1000	50	150
	C1582	100	300	> 200	1000	450-500	150
	C1607	100	500	200	>5000	500	250
	LMG 18825	20-30	100	50-100	50	50	100
B. cenocepacia (12)	J2315	>100	100	>200	>5000	>500	350
	X100	90 – 100	500	70 - 100	>5000	>500	350
	LMG18827	>100	-	>200	-	500	150
	MU1	>100	1000	>200	>5000	10	350
	WH1	10 – 20	100	150 - 180	4000	450-500	200
	LMG 16654	80-90	300	>200	>5000	>500	50
	DN	100	500	>200	2000	450 - 500	50
	K56-2	90-100	300	>200	>5000	>500	150
	PC002	100	300	>200	>5000	>500	>400
	ATCC 25609	50 – 60	300	>200	>5000	400	350
	LMG 18832	>100	1000	>200	>5000	>500	350
	PC523	10 – 20	100	150 - 190	3000	500	200
B. stabilis (2)	ATCC 35254	10 – 20	100	200	>5000	500	200
	LMG 14294	100	100	180 - 200	>5000	400	200
B. vietnamiensis (4)	LMG 16232	100	100	180 - 200	4000	450-500	100
	G4	10 – 20	300	30 - 50	500	50	50
	J1738	20 – 30	100	10 - 30	50	50	50
	LMG 10929	100	100	30 - 50	300	100	50
B. dolosa (2)	LMG 18943	20-30	300	50 - 70	2000	450-500	200
	AU0090	40 – 50	100	100 - 120	5000	450-500	100
B. ambifaria (3)	MC40-6	20 – 30	50	100 - 120	2000	50	50
	ATCC 53267	100	-	180 - 200	5000	>500	50
	LMG 19467	20 – 30	50	70 - 100	1000	>500	150
B. anthina (2)	LMG 16670	10 – 20	50	70 - 100	500	150-200	50
	LMG 20980	10 – 20	50	120 - 150	2000	150	50-100
B. pyrrocinia (1)	LMG 14191	10 – 20	50	50 - 70	2000	50	50
	Mean maximum value (µg/ml)	**74**	**229**	**155**	**2998**	**374**	**164**

Table 3.6 Mean MIC values (µg/ml) for Bcc species to ten antibiotics

Organism (No. of strains tested)	AK	AZ	TZ	CL	CI	IP	MP	PP	TM	TS	Antibiotic Profile Score
B. cepacia (5)	109.6	125.3	5.98	13.3	5.2	17.6	0.9	13.1	88.23	0.5	37.9 [a]
B. multivorans (24)	81.6	110.4	52.9	101.6	1.976	25.8	2.2	21.4	30	0.4	42.8 [b]
B. cenocepacia (14)	111.5	81.2	2.6	12.2	1	35.6	3.1	23.4	37.5	9.1	31.7 [c]
B. stabilis (2)	8	32.7	1.6	22	16.3	3	1.5	3	4.5	2	9.5 [d]
B. vietnamiensis (4)	6.3	13.5	2.5	8	0.2	0.4	0.5	1.25	4.6	0.3	3.8
B. dolosa (4)	224	256	66.7	192.7	10.3	26.2	17.5	82.3	118	12	100.6 [e]
B. ambifaria (4)	9.8	23.6	1.2	3.25	0.1	1.1	0.2	1.1	6.6	0.1	4.7
B. anthina (1)	16	32	1.5	4	0.19	2	0.38	1	8	0.19	6.5
B. pyrrocinia (1)	0.094	24	0.75	1.5	0.003	0.19	0.002	0.25	2	0.002	2.9
B. contaminans (1)	1	16	1.5	8	0.25	24	0.75	1	0.38	0.125	5.3

[a] *B. cepacia* significantly more resistant than *B. vietnamensis*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia* and *B. contaminans*.

[b] *B. multivorans* significantly more resistant than *B. ambifaria*, *B. anthina*, *B. pyrrocinia* and *B. contaminans*.

[c] *B. cenocepacia* significantly more resistant than *B. vietnamensis*, *B. ambifaria*, *B. anthina* and *B. contaminans*.

[d] *B. stabilis* significantly more resistant than *B. pyrrocinia* and *B. contaminans*

[e] *B. dolosa* significantly more resistant than *B. stabilis, B. vietnamensis, B. ambifaria, B. anthina, B. pyrrocinia* and *B. contaminans*.

Key: AK = amikacin, AZ = azithromycin, TZ = ceftazidime, CL = chloramphenicol, CI = ciprofloxacin, IP = imipenem, MP = meropenem, PP = piperacillin, TM = tobramycin and TS – trimethoprim / sulfamethoxazole

but were overall more susceptible to the β lactams, such as meropenem, piperacillin and imipenem. The Bcc were also susceptible to the trimethoprim/ sulfamethoxazole combination, although the epidemic ET-12 *B. cenocepacia* strain J2315 was resistant to this combination.

The 20 *B. multivorans* Glasgow outbreak strains were also screened against the ten antibiotics and compared statistically to the four other *B. multivorans* strains (Table 3.7). The Glasgow outbreak strains were significantly less susceptible to amikacin, azthromycin, ceftazidime, ciprofloxacin, meropenem, piperacillin and tobramycin than other *B. multivorans* strains. The *B. multivorans* strains were only significantly less susceptible than Glasgow outbreak strains to the β lactam, imipenem. There is a lack of correlation between biocide and antibiotic resistance as *B. multivorans* Glasgow outbreak strains with both low and high resistance to chlorhexidine, having multidrug antibiotic resistance. For example, *B. multivorans* Glasgow outbreak strain C1579 has a chlorhexidine MIC of 80 – 90 µg/ml and is resistant to amikacin, azithromycin, ceftazidime, chloramphenicol, ciprofloxacin, meropenem and tobramycin. In comparison, C1636 has a lower chlorhexidine MIC of 20 – 30 µg/ml, but was resistant to the same antibiotics as the strain with a higher chlorhexidine MIC.

As well as screening ten antibiotics commonly used in CF treatment, temocillin a β lactam antibiotic derived from ticarcillin (Lekkas *et al.*, 2006) was also screened against 20 representative strains of the Bcc (Table 3.8). Although there are currently no breakpoint values for temocillin, it can clearly be seen that most strains had low MIC values, apart from J2315, which has a higher MIC of 48 µg/ml (Table 3.9). When comparing J2315 and the Tp mutant, this was the only antibiotic where the MIC decreased in the mutant (Table 3.8).

The production of spontaneous resistant colonies was observed within the zone of inhibition after 48 hours for several antibiotics, assessed by E-strips® (Figure 3.4). The species that produced the most spontaneous resistant colonies were *B. cenocepacia* and *B. multivorans* strains (Figure 3.5) compared to strains such as *B. cepacia* which produced them less often. β lactam antibiotics induced the most spontaneous resistant colonies (Figure 3.5), with *B. multivorans* (41%) and *B.*

cenocepacia (33%) strains generating the greatest proportion of these resistant colonies (Figure 3.6). All other Bcc species demonstrated low rates of this phenomenon with *B. vietnamensis* (at 11% of strains tested) being the next highest observed.

3.25 Training resistance to trimethoprim

Resistance to the dihydrofolate reductase inhibitor trimethoprim was induced in *B. cenocepacia* J2315 by successive culture passage onto increasing concentrations of trimethoprim in TSA plates. Resistance could be induced up to 1100 µg/ml of trimethoprim, approximately five fold higher than the normal MIC of between 150 – 200 µg/ml. To see if this resistance was stable, the resistant mutant was plated out onto normal TSA plates and passaged three times before re plating onto Tp 1100 µg/ml plates. The Tp mutant grew after re-plating in 48 hours, thus suggesting that the acquired resistance was stable. The adapted resistant mutant was also screened for antibiotic susceptibilities using E tests®. Interestingly, as well as the increased resistance to trimethoprim, the mutant also had increased resistance to azithromycin, ceftazidime, ciprofloxacin, chloramphenicol, and meropenem making the J2315 Tp mutant clinically resistant to nine out of ten antibiotics (Table 3.9)

Previous studies have suggested that TP resistance may be due to changes in the outer membrane, with the loss of major protein bands (Rajyaguru and Muszynski, 1997). This was investigated for our mutants by SDS-page. From whole protein extracts no differences could be observed between the wild type J2315 and the resistant mutant (Figure 3.7). When outer membrane protein extraction was performed, again no obvious differences were observed (data not shown).

3.26 Susceptibility to commercial biocide formulations

To determine if commercial biocide formulations were bactericidal for Bcc strains, four strains with high MIC and MBC values to specific biocides were selected and suspension tests were performed. Two commercial biocide products were evaluated. Hibiscrub™, a chlorhexidine based disinfectant used in clinical settings

Table 3.7 Mean MIC values (µg/ml) for Glasgow outbreak strains and *B. multivorans* strains to ten antibiotics

	Mean Antibiotic MIC (µg/ml)									
Name	**AK**	**AZ**	**TZ**	**CL**	**CI**	**IP**	**MP**	**PP**	**TM**	**TS**
B. multivorans	38	74	2.1	95	0.97	32*	1.7	4.8	21.5	0.3
Glasgow outbreak strains	225.2*	197.1*	216.2*	98.1	11.7*	7.4	18.1*	73.2*	75.6*	0.4

* - denotes significantly difference (P<0.05)

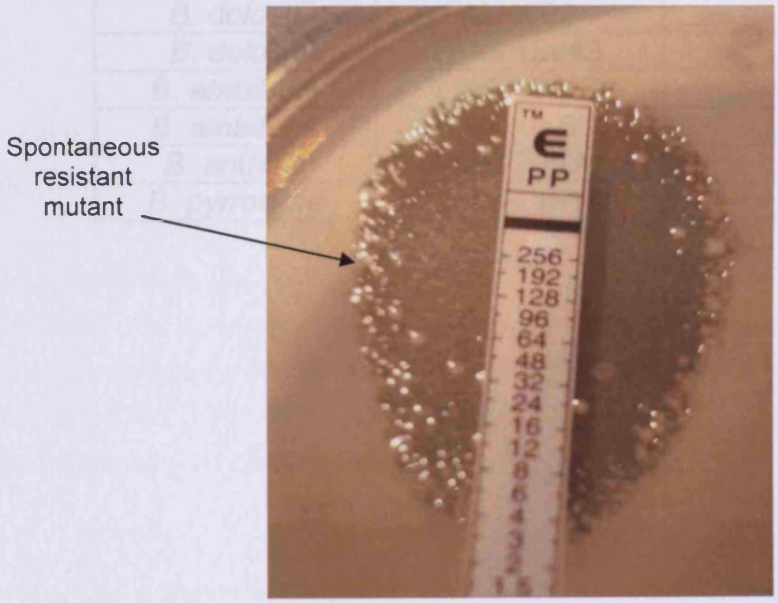


Figure 3.4 Production of spontaneous resistant colonies to piperacillin

B. cenocepacia, ATCC 17765, spontaneous resistant colonies produced in response to piperacillin, after 48 hours of growth.

Table 3.8 Temocillin susceptibility of selected Bcc strains

Species	Name	MIC (µg/ml)
B. cepacia K	LMG 23255	3
B. cepacia	IST431	4
B. cepacia	AVC1717	6
B. multivorans	LMG 16660	16
B. multivorans	ATCC 17616	8
B. multivorans	C1607	12
B. cenocepacia	J2315	48
B. cenocepacia	LMG 18832	2
B. cenocepacia	LMG 18829	0.75/1.5
B. cenocepacia	AU1054	4
B. cenocepacia	K56-2	16
B. stabilis	ATCC 35254	6
B. vietnamiensis	G4	3
B. vietnamiensis	J1738	4
B. dolosa	AU1069	12
B. dolosa	LMG 18943	12
B. ambifaria	LMG 19467	0.38
B. ambifaria	MC40-6	3
B. anthina	LMG 20980	6
B. pyrrocinia	LMG 141	1.5

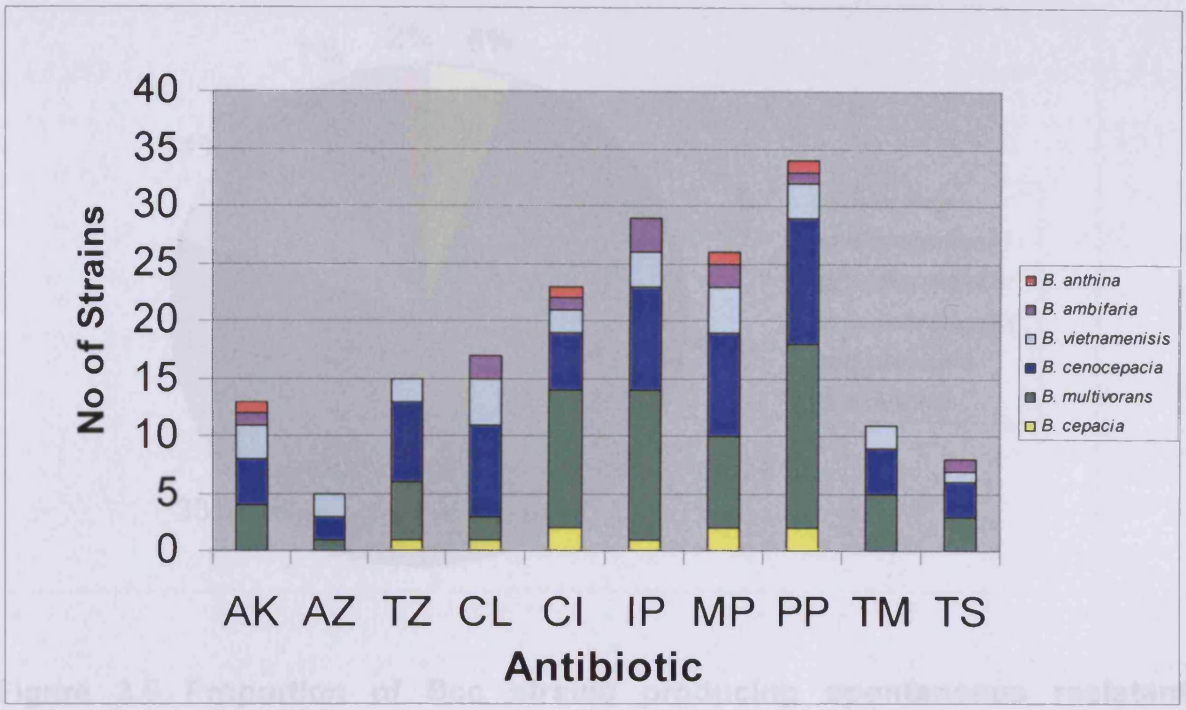


Figure 3.5 Proportion of strains producing spontaneous resistant colonies to ten antibiotics

Spontaneous resistant colonies were defined as those that appeared within the zone of inhibition after 48 hours. Not all species produced spontaneous resistant colonies. *B. cenocepacia* and *B. multivorans* strains produced the most spontaneous resistant colonies compared to other species such as *B. anthina*. The β lactam antibiotics imipenem, meropenem and piperacillin induced adaptive resistance most often.

Key

AK = amikacin, AZ = azithromycin, TZ = ceftazidime, CL = chloramphenicol, CI = ciprofloxacin, IP = imipenem, MP = meropenem, PP = piperacillin, TM = tobramycin and TS – trimethoprim / sulfamethoxazole

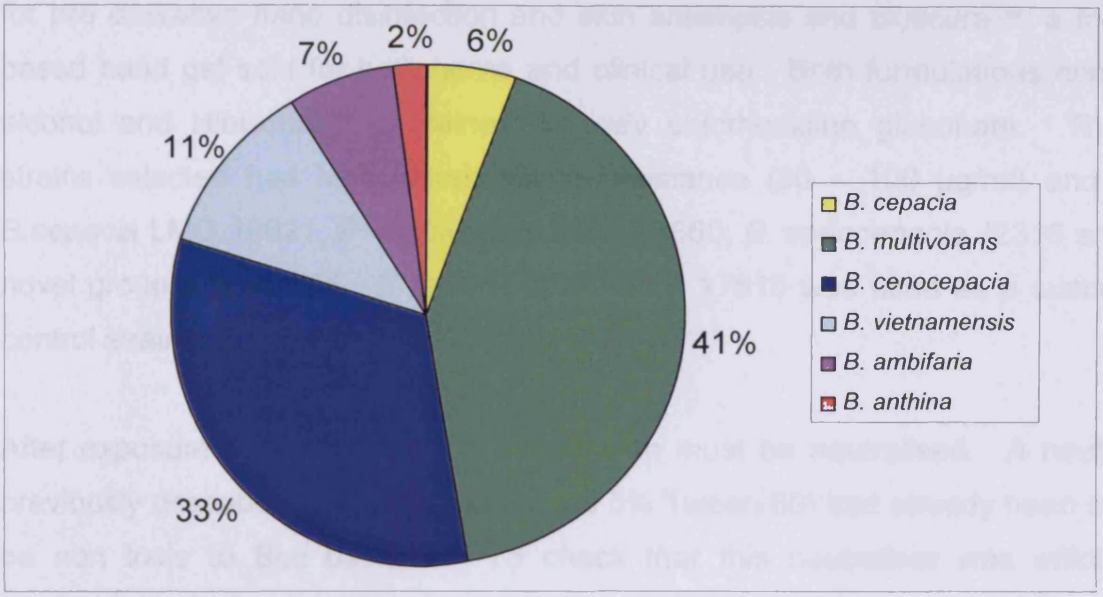


Figure 3.6 Proportion of Bcc strains producing spontaneous resistant colonies to the β lactam antibiotics, meropenem, imipenem and piperacillin

B. multivorans strains and *B. cenocepacia* strains produced the largest proportion of spontaneous resistant colonies to the three β lactam antibiotics, compared to other species. 41% of *B. multivorans* strains produced spontaneous resistant colonies compared to 7% of *B. ambifaria* strains.

for pre operative hand disinfection and skin antisepsis and Cuticura™, a triclosan based hand gel sold for both home and clinical use. Both formulations contained alcohol and Hibiscrub™ contained 4% w/v chlorhexidine gluconate. The four strains selected had high chlorhexidine resistance (90 – 100 µg/ml) and were: *B.cepacia* LMG 18821, *B. multivorans* LMG 16660, *B. cenocepacia* J2315 and Bcc novel group K strain 24. *B. multivorans* ATCC 17616 was used as a susceptible control strain.

After exposure to the biocide the suspension must be neutralised. A neutraliser previously described (0.75% azolectin and 5% Tween 80) had already been seen to be non toxic to Bcc bacteria. To check that this neutraliser was efficient at terminating activity of commercial biocides, neutraliser efficiency tests were carried out (Chapter 2, section 2.26.1). In Hibiscrub™ a small knockdown from the control where the biocide and neutraliser was replaced with water was observed (Table 3.10). However, using the equation: $LogN_c$ - $LogN_b$, where N_c is the neutraliser control and N_b is the biocide suspension, the log difference was only 0.11 which was sufficient to allow use of this neutraliser. In Cuticura™ there were no differences observed between the control and the biocide suspension (Table 3.10). As expected the sensitive control strain was killed after 5 minutes of exposure to both biocides. Five minutes of exposure to both biocides was also sufficient to produce a bactericidal effect for *B. multivorans* LMG 16660 and *B. cepacia* novel group K strain 24. In contrast *B. cenocepacia* J2315 were able to survive after an hour exposure with only a 1 log knockdown from the control observed (Table 3.11). J2315 was more susceptible to Cuticura™ with complete killing observed after 20 minutes exposure (Table 3.11). *B. cepacia* LMG 18821 was killed after five minutes of exposure to Hibiscrub™, but was viable after an hour exposure to Cuticura™ with a one log knockdown observed (Table 3.11 and Figure 3.8). According to the British Standard: BS EN 13727, chemical disinfectants and antiseptics, quantitative suspension test for evaluation of bactericidal activity of chemical disinfectants, a disinfectant product will only pass the standard if it demonstrates at least a five log reduction of viable bacteria within 60 minutes or less (13727, 2003). Two of the strains tested, J2315 in Hibiscrub™ and LMG 18821 in Cuticura™ would not pass this criteria.

Table 3.9 Comparison of antibiotic susceptibilities between *B. cenocepacia* J2315 and the J2315 Tp mutant

Name	AK	AZ	TZ	CI	CL	IP	MP	PP	TM	TS	TMO
J2315	>256 (r)	96 (r)	8 (s)	3 (I)	12 (s)	>32 (r)	1 (s)	>256 (r)	128 (r)	>32 (r)	48
Tp 1100	-	>256 (r)	12 (s)	>32 (r)	>256 (r)	>32 (r)	24 (r)	>256 (r)	128 (r)	>32 (r)	32

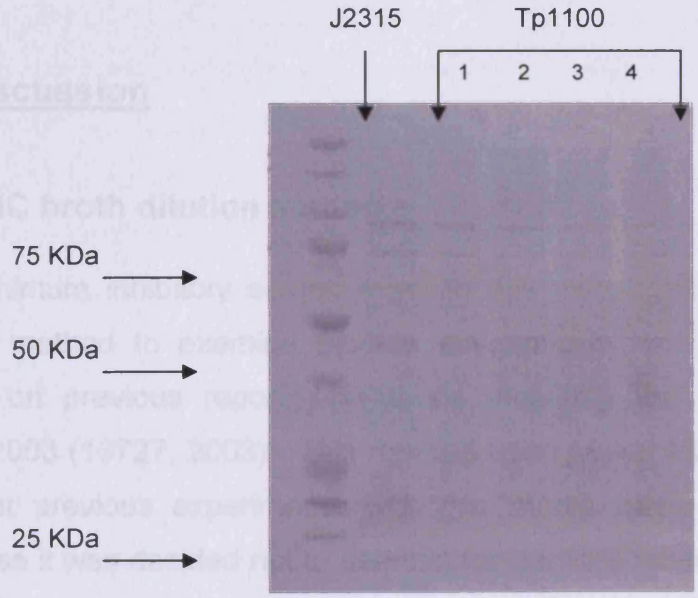


Figure 3.7 Whole cell protein extraction of J2315 and the resistant mutant Tp1100

First lane – J2315 control protein extract

1 = Tp1100 grown in TSB

2 = Tp1100 grown in TSB and Tp 100

3 = Tp1100 grown on Tp 100 agar

4 = Tp1100 grown on TSA

Tp1100 mutant was grown in trimethoprim to select for mutations. Approximately 20 µg of protein was loaded onto the gel.

3.26 Biofilm production

Bacteria within biofilms are less susceptible to killing by antimicrobial agents than those grown planktonically (Stickler, 1999). To determine if there was a correlation between biofilm formation and biocide resistance in a planktonic state, 11 Bcc strains were screened for their ability to form biofilms. Six strains were selected with low susceptibilities to chlorhexidine (>80 µg/ml) and five with high susceptibility (< 30 µg/ml). No correlation between innate ability to form biofilms and chlorhexidine resistance was observed (Figure 3.9). The strain that produced the best biofilm was *B. multivorans* ATCC 17616 one of the most sensitive to chlorhexidine (MIC 10 – 20 µg/ml) (Figure 3.9). In contrast, the highly transmissible epidemic strains, associated with clinical infections, with a high chlorhexidine MIC (> 100 µg/ml) were poor biofilm producers in the assay used (Figure 3.9).

3.3 Discussion

3.31 MIC broth dilution screen

The minimum inhibitory screen used in this investigation was an adapted broth dilution method to examine biocide susceptibility. This method was modelled closely on previous reported methods including the British Standard, BS EN 13721:2003 (13727, 2003). This method used Muller Hinton broth as the medium. However previous experiments with this media have found that the Bcc grow poorly, so it was decided not to use this for our MIC screens. Iso-sensitest medium was also evaluated, but a starch precipitate was often produced that could interfere with optical density readings. Thus it was decided to use TSB as the medium for this screen as the Bcc can grow well and consistently in this medium.

An 80% reduction in OD from the control with no biocide was designated as the MIC value. This value was chosen for a number of reasons. The addition of chlorhexidine and CPC alters the optical density in a variable manner depending on the concentration of the biocide. This meant that a direct comparison to the control culture with no biocide could not be made. Therefore an optical density cut-off that was above the baseline noise produced by the biocides needed to be adopted.

Furthermore, the variability of the Bcc growth made it difficult to determine an exact endpoint. For example, the internal control strain LMG 13010 was chosen as it represented a good biocide inhibition effect. The MIC was determined at 30 – 40 µg/ml using the 80% endpoint (Figure 3.12). However above this biocide concentration the optical density remained higher than the media blank which was used to subtract all reading in the plate reader. This variation made it difficult to determine an exact MIC value. When viable counts were performed (data not shown), no viable bacteria were isolated after 30 µg/ml, which correlates well with the MIC determined by the 80% endpoint, therefore this endpoint was used to designate an MIC range for all strains screened.

3.32 Biocide resistance and the Bcc

Biocides are used to sterilise a range of surfaces and have an integral role in infection control procedures. Disinfection procedures have greatly aided the reduction of nosocomial infections, however the over use of biocides have raised concerns that bacteria will adapt and become resistant to multiple antinfectives including widely used biocides (McDonnell and Russell, 1999). This concern extends to bacteria that infect cystic fibrosis patients, as it is known that many of these bacteria, such as *P. aeruginosa* and members of the Bcc are innately resistant to many antimicrobials (Govan and Deretic, 1996). Biocides have been used for many years in clinical situations for hand washes, surgical scrubs, antiseptic creams and surface disinfection, however in recent years there has been an increasing demand for the inclusion of biocides in everyday domestic products to satisfy consumers fears of bacterial contamination (Bloomfield, 2002). Many biocides are incorporated into daily life, such as surface decontamination of food preparation areas, for cleaning toilets, sinks and drains and for general cleanliness of the home (Bloomfield, 1978). Some biocides have now been incorporated into household products, for example the inclusion of biocides such as triclosan in chopping boards, knife handles, paint and socks (Gilbert and McBain, 2001). There is concern that the publics over zealous use of biocides may lead to residues of biocides in the environment, which in turn may select for resistant organisms (Fraise, 2002).

Table 3.10 Neutraliser efficiency tests for Hibiscrub™ and Cuticura™

Condition	Viable counts cfu/ml (± SD)
Control*	1.03×10^8 (0.7)
Hibiscrub™#	8×10^7 (1.0)
Cuticura™#	1.5×10^8 (0.9)
Neutraliser control+	1.2×10^8 (1.5)

* = control – biocide and neutraliser replaced with water

= neutralised biocide test suspensions

+ = neutraliser control – bacterial suspension inoculated in neutraliser

Table 3.11 Log Reduction in viable cell count for *B. cepacia* LMG 18821 and *B. cenocepacia* J2315 after exposure to Hibiscrub™ and Cuticura™

	Log Reduction *B. cepacia* LMG 18821		Log Reduction *B cenocepacia* J2315	
Time (min)	Cuticura™	Hibiscrub™	Cuticura™	Hibiscrub™
0	*	*	*	*
5	0.5	~	1	1.2
10	0.9	~	1.7	1.2
20	1	~	4.7~	1.1
60	1.1	~	4.7~	1

* - No inhibition

~ - No growth

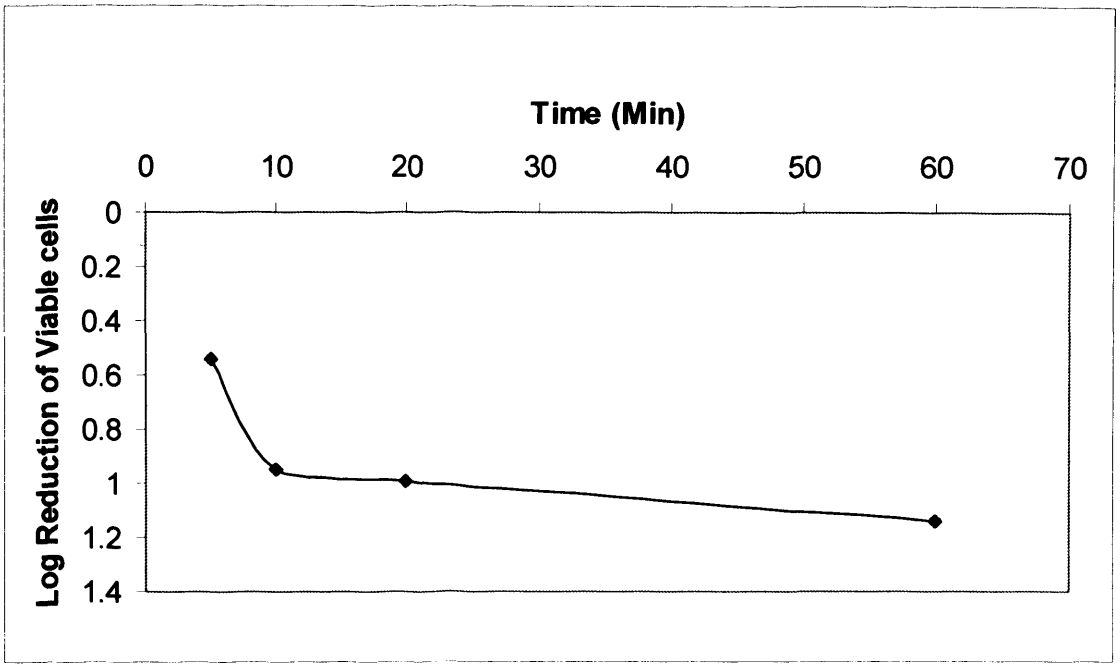


Figure 3.8 Log Reduction of *B. cepacia* LMG 18821 cells in Cuticura

Cuticura™ a triclosan based hand gel, caused a one log reduction in viable *B. cepacia* LMG 18821 cells after 20 minutes of exposure. After one hour viable bacteria were still present and the criteria of a five log reduction was not fulfilled.

Members of the Bcc are known to have resistance to multiple antibiotics. However an up to date survey of their biocide resistance, which is related to recent taxonomic changes has not been carried out. The aim of this study was to analyse the susceptibilities of the Bcc to a range of biocides. This analysis revealed that resistance levels varied widely within and between species to chlorhexidine, cetylpyridinium chloride, triclosan and benzalkonium chloride. Species dependent differences were only observed for CPC where *B. cenocepacia*, *B. cepacia*, *B. lata*, *B. contaminans* and Bcc novel group K were significantly more resistant ($P<0.05$, Table 3.1) than other Bcc species. This is interesting as *B. cenocepacia* strains are often associated with serious clinical infections that are often transmissible and are the most prevalent infections in CF patients (Reik *et al.*, 2005).

In chlorhexidine, *B. cepacia*, *B. cenocepacia* and *B. vietnamensis* species had elevated MICs compared to other species. This is not surprising for *B. cepacia* and *B. cenocepacia* strains as these are relatively common pathogens in both CF and non-CF patients (Mahenthiralingam *et al.*, 2008). However, *B. vietnamensis* is a less common Bcc member found to infect CF patients and is more commonly encountered in the environment. *B. vietnamensis* can colonise the rhizosphere of plants and has the potential for use in bioremediation (O'Sullivan *et al.*, 2007). Nevertheless, *B. vietnamensis* can infect CF patients, albeit with a lower frequency than *B, cenocepacia* and *B. multivorans* strains. For example from a survey of 606 US patients, 50% were infected with *B. cenocepacia*, 38% with *B. multivorans* and 5% with *B. vietnamensis* (Parke and Gurian-Sherman, 2001). There has been much debate about whether clinical strains are more resistant than environmental strains, with some studies indicating that clinical isolates are more antibiotic resistant than environmental isolates (Butler *et al.*, 1995, Wigley and Burton, 1999). However these studies were performed before Bcc species epidemiology was characterised and other more recent studies indicate that environment and clinical isolates may be similar. For example Bevivino *et al.* found that some of the candidates for transmissibility and virulence were also present in environmental isolates (Bevivino *et al.*, 2002). Baldwin *et al.* found that > 20% of 381 clinical isolates were indistinguishable from environmental isolates via MLST (Baldwin *et al.*, 2007). Another investigation found a *B. cenocepacia* isolate in agricultural soil identical to that found in CF clinics, signifying that there may be a risk to CF

patients from environmental sources (LiPuma *et al.*, 2002). In this study clinical isolates, including CF and non CF isolates were significantly less susceptible ($P<0.05$, Mann-Whitney)) to chlorhexidine than environmental strains (clinical mean = 485 – 58.6 µg/ml compared to environmental mean = 31.4 – 40 µg/ml). In contrast no differences were found in CPC susceptibilities ($P>0.05$, Mann-Whitney) between environmental and clinical isolates (clinical mean = 130.3 – 143.4 µg/ml compared to environmental strains 145 – 160.6 µg/ml). Thus this indicates that the environment could be a reservoir for infection. Hence, there needs to be careful consideration of the risks before Bcc species can be used as bioremediation. This may be an example of residuals of biocides in the environment leading to mutational resistance. Recently, new species of the Bcc have been classified (Vanlaere *et al.*, 2008). In particular, *B. lata* and *B. contaminans*, as well as a novel Bcc group K strains have been associated with the contamination of various commercial products. Therefore, it is interesting to note that these strains have higher MIC values to CPC than other Bcc bacteria (approx 200 µg/ml, Table 3.1).

Maintaining product sterility is a problem faced by manufacturing industries on a daily basis. A recent survey of the pharmaceutical industry has shown that *B. cepacia* are one of the leading causes of product recalls (Jimenez, 2007). Jimenez *et al.* analysed FDA recall data and found that *B. cepacia* was the most frequently isolated contaminant in both non sterile and sterile products (22% and 2.5% respectively). These included mouth washes, antiseptic creams, body sprays, shampoo, hand disinfection detergents and soaps and ophthalmic solutions (Jimenez, 2007). The MBC data obtained in this study indicates that some Bcc bacteria may need more than 25 times more biocide than the MIC value to achieve killing. The concentration of the biocide is an important factor in it's efficacy against bacteria and varies for different applications. For example chlorhexidine is used for surface disinfection at 0.5 – 4% (w/v), for antisepsis at 0.02 – 4% (w/v) and for preservation at 0.0025% - 0.01% (w/v) (Maillard, 2005). At the lowest concentrations this is not sufficient to kill some of the more resistant strains. *B. cenocepacia* strains, LMG 18832 and Mu1, both CF infection isolates, required more than 1000 µg/ml before a bactericidal effect was seen.

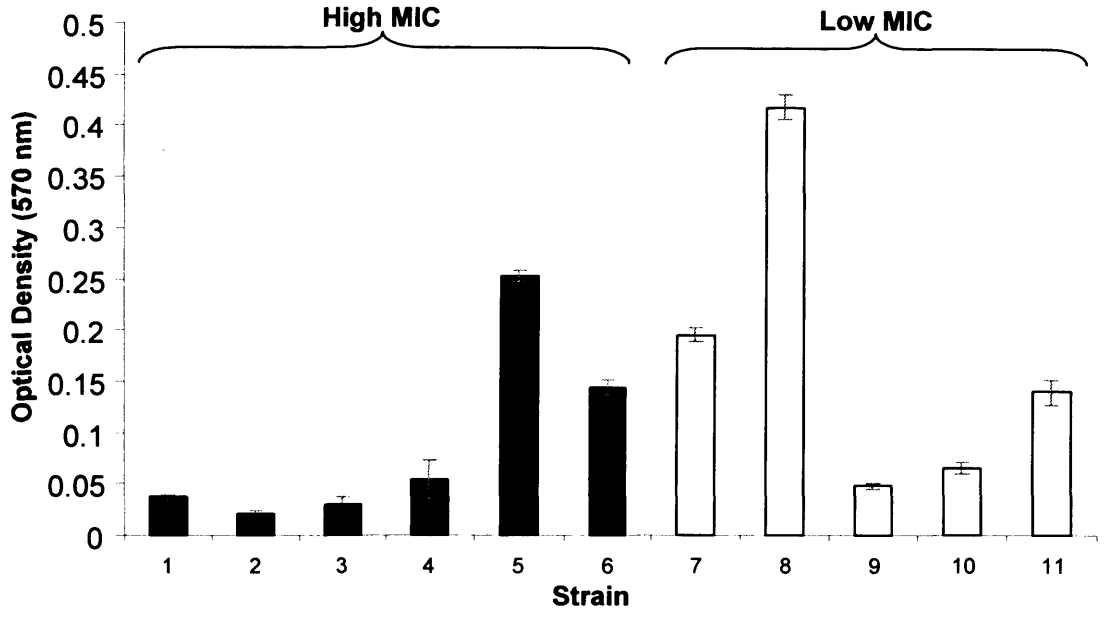


Figure 3.9 Biofilm production by selected Bcc strains

Six strains with high (black bars) and low (open bars) chlorhexidine were tested for biofilm formation. The biofilm biomass readout is shown on the vertical axis. Strains are as follows: 1 – *B. cepacia* LMG 18821, 2- *B. multivorans* LMG 16660, 3 – *B. cenocepacia* LMG 18832, 4 – *B. cenocepacia* J2315, 5 – *B. cenocepacia* C4455, 6 – *B. cepacia* novel group K, 24, 7– *B. multivorans* LMG 13010, 8 – *B. multivorans* ATCC 17616, 9 – B. *dolosa* LMG 18943, 10 *B. ambifaria* AMMD, 11 – *B. vietnamensis* G4.

The data from this investigation suggests that MIC data can be misleading and irrelevant in real life situations where the in use concentrations of biocides are often much higher compared to MIC levels (Maillard, 2007). Nonetheless, it is useful to investigate MIC values as it may indicate a trend towards emerging resistance (Maillard, 2007). Formulations such as preservatives are used to prevent a product from spoiling, so contain lower concentrations of biocides, as they do not need to be bactericidal. At this level MIC data can be valuable, as at lower concentration bacterial contamination is likely to be more frequent (Russell, 2004).

3.33 Contamination of commercial biocides by the Bcc

Considering that in use concentrations of biocides are often quite high, it would be expected that the incidences of bacterial contamination would be fairly low. Nevertheless, there have been many recorded instances of contamination of antimicrobial formulations, many of which resulted in outbreaks in patients. Chlorhexidine has been widely used in hospitals as a major disinfectant, however dilute solutions appear to be particularly prone to contamination. Sobel *et al* (Sobel *et al.*, 1982) carried out a retrospective study due to the increase in *B. cepacia* isolates recovered from patients in the hospital. Most of these isolates were from urinary origin, especially catheterised patients, although the outbreak was widespread in the hospital with *B. cepacia* being recovered from wound, vaginal and oral samples. The outbreak was traced to a mouthwash solution that contained 0.2% (v/v) chlorhexidine as well as a 0.05% (v/v), and a 0.1% (v/v) chlorhexidine solutions used for swabbing the perineum and genitalia before catheters were inserted. The chlorhexidine was provided at a 5% (w/v) solution and diluted with non sterile water and it was determined that the chlorhexidine was contaminated during the dilution process (Sobel *et al.*, 1982). This corroborates data from our study, where in lower concentrations of chlorhexidine many strains of the Bcc could easily survive (*B. cenocepacia* LMG 18832, Chlorhexidine MBC > 1000 µg/ml, Table 3.5).

Incidences of bacteraemia linked to the contamination of biocide solutions include bloodstream infections caused by the use of contaminated water to dilute alcohol

skin antisepsis solutions (Nasser *et al.*, 2004), contamination of a 0.5% (v/v) chlorhexidine solution used for skin cleansing (Garcia-Erce *et al.*, 2002), contamination of 0.02% (v/v) benzalkonium chloride and 0.02% (v/v) and 0.05% (v/V) of chlorhexidine gluconate (Oie and Kamiya, 1996). Both Berkelman *et al.* (Berkelman *et al.*, 1981) and Panlilio *et al.* (Panlilio *et al.*, 1992) reported an outbreak of *Burkholderia cepacia* in their hospitals due to contamination of a 10% (v/v) povidone-iodine solution that was used to disinfect the skin before veno puncture. Similarly 37 out of 38 strains screened in this investigation were able to survive in 5% (v/v) povidone. These studies indicate that biocides can harbour resistant organisms and may be a source of infection themselves. Worryingly, there are increasing reports of products not necessarily just used in clinical settings that are becoming contaminated with problematic pathogens. Kutty *et al.* (Kutty *et al.*, 2007) reported a multi-state outbreak of *B. cepacia* which was associated with a contaminated alcohol free mouthwash. This mouthwash contained an unknown amount of CPC and was contaminated during the manufacturing process. The outbreak involved 22 hospitals across nine states and two patients died as a direct result of the outbreak (Kutty *et al.*, 2007). Similarly, moisturizing body milk used in hospitals was found to be contaminated with *B. cepacia* causing infection in an intensive care unit (Alvarez-Lerma *et al.*, 2008). These are obviously serious issues that need to be addressed as contamination of these products are causing infection in already vulnerable people. There is also concern that contaminated products, such as moisturisers and cosmetics that contain preservatives and low levels of disinfectants may cause infections in people such as CF sufferers if they use them in their homes.

Data obtained in this study supports the observation that Bcc bacteria are adept at contaminating commercial products. Two biocides were tested to see if strains with high MIC values to chlorhexidine could survive. The two biocides were Hibiscrub™, a chlorhexidine based disinfectant and Cuticura™, a triclosan based hand gel. Both are used for skin antisepsis. Five strains were tested including a sensitive *B. multivorans* strain as a control. It was observed that two strains, *B. cepacia* LMG 18821 and *B. cenocepacia* J2315 were capable of surviving at these in use concentrations. Although *B. cepacia,* LMG 18821 was killed by Hibiscrub™, only a one log reduction was seen after an hour of exposure to Cuticura™. *B.*

cenocepacia J2315 was equally capable of surviving in Hibiscrub™ after an hour of exposure, although was killed after 20 minutes exposure to the triclosan based hand gel (Table 3.11). The lack of rapid killing may still be problematic as in day to day situations hand gels may not be left on the skin for this length of time. This data also indicates that more than one mechanism of resistance is employed as survival in one disinfectant does not necessarily confer survival in a different disinfectant. These results are concerning as *B. cenocepacia* and *B. cepacia* strains are prevalent in clinical infections and *B. cenocepacia* J2315 is representative of a transmissible strain, that appears to be able to survive in high concentrations of chlorhexidine (Hibiscrub™ 4% v/v). Hibiscrub™ may not be used at the concentration provided as it is often diluted to use as a pre operative body wash or scrub, thus lending itself to further contamination by strains that may be inhibited at the higher concentration. Although this data leans towards the conclusion that problematic, epidemic Bcc strains are capable of contaminating commercial biocide formulations, it is worth noting that the *B. multivorans* Glasgow outbreak strain, LMG 16660 was killed after five minutes exposure to both biocides.

3.34 Antibiotic susceptibility of the Bcc

As well as characterising the susceptibility of the Bcc to biocides, 60 strains were also screened for resistance to ten antibiotics commonly used to treat CF infections (Table 3.6). Generally the antibiotic resistance varied widely within and between species, however when an antibiotic profile score was calculated for each species *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis* and *B. dolosa* were significantly more resistant (Table 3.6). In contrast Nzula *et al.* (Nzula *et al.*, 2000) found that there were no significant trends in antimicrobial resistance to strains in six of the Bcc species, although this study was carried out eight years ago, when the Bcc taxonomy was not completely defined. However, with recent changes in the taxonomic status of the Bcc and the larger collection of isolates examined in this study, it is perhaps not surprising to find some significant differences.

B. cenocepacia strains possessed high levels of resistance to both biocides and antibiotics and interestingly are one of the most common cause of infections in CF

patients (Mahenthiralingam *et al.*, 2005), although recently *B. multivorans* infections have become more frequent in the UK (Govan *et al.*, 2007). *B. cenocepacia* strains had high resistance to the trimethoprim / sulfamethoxazole combination, possibly due to fact that *B. cenocepacia* strains are intrinsically resistant to this combination by the production of different dihydrofolate reductase inhibitors (Rajyaguru and Muszynski, 1997). Almost all strains tested were resistant to azithromycin, a macrolide antibiotic. This is concerning as macrolides are increasingly being used as a long term treatment in cystic fibrosis patients. Saimen *et al.* carried out a randomized placebo controlled trial in CF patients and found a 6.2% improvement in lung function when using azithromycin and that it may also reduce the frequency of pulmonary exacerbations (Saiman *et al.*, 2005). Thus it is worrying that the use of an antibiotic as an anti inflammatory drug may be selecting for resistant species such as the Bcc.

Most strains were more susceptible to β lactam antibiotics, such as meropenem, imipenem and piperacillin, however it was these antibiotics that produced the most spontaneous resistant colonies. Spontaneous resistant colonies are colonies that appear within the zone of inhibition after 48 hours of growth (Figure 3.4). These colonies indicate that in the presence of the antibiotic the strain may adapt or mutate to become resistant to this substance and thus are able to grow (See chapter 1 for more details on bacterial resistance mechanisms). This may involve the induction of enzymes such as β-lactamases or the up-regulation of efflux pumps. The species that produced the most spontaneous resistant mutants to the three β lactam antibiotics were *B. multivorans* (41%) and *B. cenocepacia* (33%) strains. This is particularly worrying as these strains are most commonly associated with clinical infections that are most problematic for CF patients (Mahenthiralingam *et al.*, 1996b). The appearance of these apparently resistant colonies indicates that even antibiotics that are fairly effective against the Bcc complex, can become less potent if the species are exposed to it for a period of time. In this case, resistance was observed after only 48 hours of exposure.

The ability of bacteria to be able to adapt to their environment and survive antibiotic treatment, is a cause for concern as many patients may not complete their antibiotic regime and the inappropriate use of antibiotics has led to drug resistance. For

example, clinicians often prescribe antibiotics unnecessarily. Data from surveys have indicated that treatment with antibiotics is needed 20% of the time, yet are often prescribed in 80% of cases (Madigan *et al.*, 2001a). In this study resistance to the antibiotic trimethoprim was trained in *B. cenocepacia* J2315 by successive passages onto increasing concentrations of the antibiotic. Resistance was trained up to 1100 µg/ml, indicating that in the presence of the antibiotic resistance can be induced and that prolonged therapy with a single antibiotic can have serious consequences with respect to resistance. This mutant was also resistant to nine out of ten antibiotics screened, compared to six in the wildtype, indicating that the mutation has a broad spectrum that is not just specific for the dihydrofolate reductase, indicating that several changes have occurred.

Consequently this is indicative that the outer membrane may play a role. This is supported by a previous study by Rajyaguru *et al* (Rajyaguru and Muszynski, 1997). They isolated chloramphenicol and trimethoprim / sulfamethoxazole mutants of *B. cepacia* strains ATCC1394, Per, CAS and D4 by stepwise exposure. Resistance to trimethoprim / sulfamethoxazole was attributed to the absence of a major outer membrane protein (39 – 47 kDa) and changes to the LPS profile. Additional outer membrane proteins were also seen in the mutants. It was suggested that the missing outer membrane protein may directly be involved in the passage or uptake of these antibacterials (Rajyaguru and Muszynski, 1997). Thus, this indicated that a similar mechanism may be occurring in the J2315 trimethoprim mutants, especially as resistance to other antibiotics are also seen in the mutant (Table 3.9). Whole cell protein extraction and outer membrane protein extraction was carried out (Chapter 2, section 2.8) and visualised by SDS-PAGE. However, no differences could be clearly observed (Figure 3.7), signifying that changes to the outer membrane may not be involved in adaptive resistance, or that the method of extracting the protein was not sufficient to visualise changes.

Many of the antibiotics are not effective against the Bcc, epidemic strains being particularly problematic with resistance to some of the extended spectrum β lactams being observed, for example J2315 resistance to piperacillin (MIC >256 µg/ml). This poses a problem when trying to treat CF patients with these infections. Recently, the antibiotic temocillin, a semi synthetic derivative of the β lactam

ticarcillin has been suggested as an alternative treatment for patients infected with the Bcc. Temocillin fails to induce β lactamase production so is generally more active than other β lactams. Retrospective studies of the use of treatment with temocillin have produced positive results, with one centre reporting a 56.25% improvement in patients infected with Bcc (Lekkas *et al.*, 2006). Kent *et al.* found that temocillin given in conjuncture with an aminoglycoside had equivalent activity for the Bcc when compared to standard combination therapy for an acute exacerbation (Kent *et al.*, 2008). A small representative panel of Bcc strains were screened for susceptibility to temocillin in this study. The E-test® strips for temocillin have no break points for resistance as of yet, however all of the strains screened apart from *B. cenocepacia* J2315 had low MIC values (Table 3.8) ranging from 0.38 – 16 μg/ml. J2315 had a three times greater MIC (48 μg/ml), than the previous highest MIC. Interestingly, temocillin was the only antibiotic to show a decrease in the MIC value for the trimethoprim mutant (Table 3.9), although it still has a higher MIC to temocillin than any other strain tested. Consequently, temocillin appears to be more active against the Bcc and may be an appropriate alternative when treating patients.

3.35 Biocide and antibiotic resistance

To explain the controversy of cross resistance between antibiotics and biocides we correlated MIC's for both. There were no obvious relationships between biocide or antibiotic resistances, although many of the strains tested were less susceptible to two or more agents. Certain epidemic strains, such as the ET-12 strains possessed among the highest levels of resistance to both classes of antimicrobial agents. For example, *B. cenocepacia* J2315 had high MIC's to chlorhexidine, CPC, triclosan, benzalkonium chloride, povidone and was classed as resistant to six out of ten antibiotics. The *B. multivorans* Glasgow epidemic strain (Whiteford *et al.*, 1995) had chlorhexidine MIC values nearly double that of other *B. multivorans* strains and also was significantly more resistant to seven out of ten antibiotics (Table 3.7). This indicates that there may be cross resistance in the Bcc.

Previous investigations have found similar results in other pathogen species. One such study found that resistance to ciprofloxacin, imipenem, cefotaxime, ceftazidime, gentamicin and aztreonam indicated increased chlorhexidine resistance in some Gram negative species, such as *E. coli* and *P. aeruginosa* (Koljalg *et al.*, 2002). Akimitsu *et al.* reported that methicillin resistant *Staphylococcus aureus* mutants resistant to benzalkonium chloride also exhibited increased resistance to some β lactam antibiotics (Akimitsu *et al.*, 1999). This was corroborated by another study which suggests that methicillin resistant *S. aureus* are also resistant to chlorhexidine and QACs such as CPC and benzalkonium chloride compared to methicillin sensitive *S. aureus* (Suller and Russell, 1999). These investigations indicate that there may be some overlapping mechanisms of resistance, such as permeability and efflux. There should be concerns over residues of biocides, causing a gradient to form, leading to the selection of bacteria that have low levels of susceptibility to disinfectants and antibiotics (White and McDermott, 2001).

Given the well known phenotypic diversity of the Bcc, it was not surprising that in this study there was also contradictory evidence of cross resistance. One epidemic strain, *B. dolosa* LMG 18943, (Kalish *et al.*, 2006) possessed low chlorhexidine and CPC MIC's but were resistant to all ten antibiotics tested. There was also a lack of correlation between planktonic biocide resistance and the ability to form biofilms (Figure 3.9). Although the ability to form a biofilm is a well know resistance mechanism and despite the advantages it confers in terms of survival under antimicrobial stress (Parsek and Fuqua, 2004), not all Bcc have adapted this phenotype, but instead have other enhanced resistance mechanisms. However, two of the strains tested, both *B. cenocepacia* strains (K56-2 and C4455) have the potential to be particularly problematic as they both have the ability to form good biofilms and have high intrinsic resistance to biocides.

3.36 European guidelines strain recommendation

The efficacy of disinfectant products and procedures may be tested according to Standard European guidelines. A set of reference strains are recommended for

inclusion in these tests, including *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus hirae* (Marchetti *et al.*, 2003). Considering the potential of contamination by the Bcc complex of disinfectants and preservatives, only one strain, *B. cepacia* type strain ATCC 25416 is included in the reference strain panel (Lambert, 2004). Although this strain has the maximum CPC MIC (> 200 µg/ml), its chlorhexidine MIC was relativity low (20 – 30 µg/ml) and was not among the most resistant encountered. Given the major role that Bcc organisms may play in contamination of disinfectant products (Jimenez, 2007), future testing should include representative strains of the Bcc. Three well defined strains stand out as being resistant to multiple biocides (Table 3.5): (i) *B. cenocepacia* LMG 18832 (ATCC 17765), a urinary tract infection isolate, (ii) *B. cenocepacia* J2315 (LMG 16656), an epidemic CF isolate, and (iii) *B. multivorans* LMG 16660 (C1576) a CF isolate from the Glasgow outbreak. All the latter strains were included in a historical reference panel of Bcc strains (Mahenthiralingam *et al.*, 2000b) and are available form recognised strain collections.

3.4 Conclusions

In summary, characterisation of the antimicrobial susceptibility of an up to date survey of the Bcc, focusing on resistance of two biocides, the cationic bisbiguanide chlorhexidine and the quaternary ammonium compound cetylpyridinium chloride, revealed that many of the Bcc strains possessed high resistance to biocides and commercial formulations of these agents. Some of the clinical epidemic strains that have been involved in outbreaks in numerous hospitals are in particular resistant to multiple biocides and antibiotics, which may be problematic for vulnerable patients, particularly CF sufferers. From this investigation it appears that QAC's such as CPC, triclosan and povidone are ineffective against Bcc bacteria and therefore their use should be restricted in situations where these bacteria may be encountered.

There was inconclusive evidence that the over use of biocides leads to cross over with antibiotic resistance, but there appears to be the potential for this to occur so caution should be exercised when using disinfectants in the home and in clinical situations. The ability of the Bcc bacteria to adapt to harsh environments, including

exposure to antinfectives, means that stricter guidelines should be implemented for cleaning and sterilisation in hospitals, particularly those that deal specifically with CF and particularly vulnerable patients such as intensive care units. In addition, the three Bcc strains recommended in this study should be included as reference strains in standard testing methods, in the hope that this will reduce incidences of contamination and lead to the production of new antinfectives capable of killing these bacteria.

In addition, contrary to belief, we found that biofilm formation is not linked to intrinsic antibiotic and biocide resistance, therein other resistance mechanisms, such as decreased permeability and efflux are more important for intrinsic resistance than biofilm production.

Chapter 4.0

Characterising Antimicrobial Susceptibility in *Pseudomonas aeruginosa*

4.0 Characterising the antimicrobial susceptibility in *Pseudomonas aeruginosa*

4.1 Introduction

4.11 Antimicrobial resistance

Pseudomonas aeruginosa is the most common infection found in CF sufferers, with up to 80% of patients becoming infected chronically by the time they reach adulthood (Currie *et al.*, 2003). Thus, *P. aeruginosa* is a problematic pathogen that causes significant problems in both the management and treatment of CF patients. Like the Bcc *P. aeruginosa* strains are difficult to treat and eradication is almost impossible due to its innate resistance to multiple drugs, such as β-lactams, older quinolones, chloramphenicol, tetracycline, macrolides, trimethoprim / sulfamethoxazole and rifampicin (Rossolini and Mantengoli, 2005). This leaves very few treatment options and possibly the use of more potent drugs that could have serious side effects (Tummler and Kiewitz, 1999). The most important anti pseudomonal treatments are β-lactams (ticarcillin, piperacillin, cefoperazone, ceftazidime, cefepime, aztreonam, imipenem and meropenem), aminoglycosides (gentamicin, tobramycin and amikacin) and fluoroquinolones (ciprofloxacin). Polymyxins and colistin are also active but are reserved for multidrug resistant strains due to increased toxicity (Rossolini and Mantengoli, 2005).

Intrinsic antimicrobial resistance is mediated by a number of well known mechanisms. *P. aeruginosa* strains possess an AmpC chromosomally inherited β lactamase that confers resistance to ampicillin and most cephems. This enzyme is inducible by the presence of the antibiotics it degrades (Rossolini and Mantengoli, 2005). A general decrease in permeability due to the outer membrane also increases antibiotic resistance and the interplay between this low permeability and broadly specific drug efflux pumps leads to the emergence of multiple drug resistant strains (Li *et al.*, 2000). One such efflux pump is the well characterised MexA-MexB-OprM pump, which can export a number of unrelated compounds such as antibiotics, biocides, dyes and detergents (Poole *et al.*, 1993). *P. aeruginosa* can also acquire resistance through the acquisition of multi drug plasmids, such as the

transferable R plasmid, which carries genes for the detoxification of many antibiotics (Tummler and Kiewitz, 1999). Resistance to aminoglycosides, which are often used in combination with β lactams as the primary treatment for *P. aeruginosa* infections have also become more common with the advent of modifying enzymes. There are many enzymes which modify the structure of aminoglycosides which cause the inactivation of that antibiotic. These modifications include phosphorylation (aminoglycoside phosphoryltransferase [APH]), acetylation (aminoglycoside acetyltransferase [AAC]) or adenylation (aminoglycoside nucleotidyltransferase [ANT]). Often *P. aeruginosa* strains will carry multiple modifying enzymes that confer broad aminoglycoside resistance (Poole, 2005a). Thus, the acquisition of *P. aeruginosa* in a CF patient can be devastating for the patient and lead to increased hospital stays and the application of multiple antibiotics.

A strategy to eradicate early *P. aeruginosa* infection, before it becomes chronic may prevent the rapid decline of lung function. During early infection in the CF lungs *P. aeruginosa* demonstrates a non mucoid, smooth lipopolysaccharide and sensitivity to anti-pseudomonal antibiotics. Often chronic infections are established with the conversion to a mucoid, alginate producing phenotype that are almost impossible to eradicate and that lead to a poor prognosis for the patient (Govan and Deretic, 1996). Eradication therapy involves the use of nebulised antibiotics either alone or in combination with oral or intravenous anti-pseudomonal antibiotics (Jones, 2005). The Cystic Fibrosis Trust produced a booklet on infection control in CF patients and suggested the combination of nebulised colistin or tobramycin and oral ciprofloxacin, which produced eradication of *P. aeruginosa* in 80% of newly infected individuals (Littlewood, 2002). Therefore understanding the mechanism of resistance to antimicrobials is important so that new therapeutic treatments can be developed to reduce the emergence of resistant *P. aeruginosa* and to potentiate existing therapies.

4.12 Biocide susceptibility

P. aeruginosa is a CF pathogen that like the Bcc is multidrug resistant and difficult to treat. Similarly, both are associated with contamination of disinfectant products that should contain high enough concentrations to be bactericidal. As the threat of bacterial infection is highlighted by the media, the use of disinfectants in the home environment is becoming more and more widespread. Coupled with the copious use of biocides in hospital surroundings, cases of bacterial contamination of these products and resistance to them are becoming more and more common.

Triclosan is an example of a biocide where bacterial resistance has developed. It is a broad spectrum antimicrobial that is used in products such as soaps and oral hygiene products (Gomez Escalada *et al.*, 2005). One of its targets in bacteria is the FabI, an enoyl acyl carrier protein reductase that is involved with fatty acid biosynthesis (McMurry *et al.*, 1998b). It acts as a potent inhibitor by mimicking its substrate (Gomez Escalada *et al.*, 2005). It is unusual for a biocide to have a specific target in the bacterial cell, although at higher concentrations it is thought that triclosan has a broader activity by causing membrane damage (Villalain *et al.*, 2001). This specific target may be a factor in resistance occurring and *P. aeruginosa* isolates are well known to be triclosan resistant, with the presence of multiple resistance nodulation division (RND) efflux pumps (Mima *et al.*, 2007) and the low permeability of the *P. aeruginosa* outer membrane for hydrophobic compounds (Ellison *et al.*, 2007).

This intrinsic resistance may cause issues in clinical settings as *P. aeruginosa* is an opportunistic pathogen, that as well as being a common infection in CF patients also infects many other vulnerable people. For example any patient that has a urinary catheter inserted is likely to become infected. Catheters are particularly problematic due to the formation of crystalline biofilms that encrust the catheter. *P. aeruginosa* is a common pathogen found to form these encrustations. In a study by Williams *et al.* the use of triclosan to prevent encrustation found that *P. aeruginosa* was not reduced and blockages could still form (Williams and Stickler, 2008). Stickler *et al.* (Stickler, 2002) also found that catheter associated bacteria were biocide resistant. The in use concentration was not effective at killing strains of *Proteus mirabilis* and *P. aeruginosa* and the chlorhexidine resistant strains were multidrug resistant. It was determined that chlorhexidine was not an effective agent

to use in catheter care and that alternatives should be sort (Stickler, 2002). Once *P. aeruginosa* infection is acquired it is often impossible to eradicate and has a serious, detrimental impact on the patient's outcome. Adequate and efficient hygiene regimes to prevent the acquisition of infections from the hospital environment is particularly important for multi-drug resistant organisms and increasing reports of contamination of disinfectant and cosmetic products should be addressed to reduce this problem.

The objectives of this chapter were to characterise the susceptibility of a panel of *P. aeruginosa* strains obtained from a range of sources to a number of commonly used biocides. Biocide susceptibility was then correlated to their antibiotic susceptibilities and recommendations made for disinfection procedures.

4.2 Results

4.21 Chlorhexidine and cetylpyridinium chloride susceptibility

As previously described (section 2.24) a panel of 61 *P. aeruginosa* strains were screened for chlorhexidine and CPC susceptibility (Table 4.1). The panel included the following: (i) environmental strains (ii) catheter strains (iii) clinical CF strains (iv) Liverpool epidemic strains and (v) other epidemic strains. This gave a broad diversity of strains from various sources to give a rounded overview of the susceptibility of *P. aeruginosa* strains. In chlorhexidine, susceptibility was much higher, with most strains inhibited at approximately 25 µg/ml. There were no significant differences between epidemic, catheter, clinical CF and environmental strain groups. However, Liverpool epidemic strains were approximately four times less susceptible to chlorhexidine than other strains (Table 4.1) with an approximate MIC of 80 µg/ml compared to 25 µg/ml. This was also significantly higher than other epidemic strains which included the Manchester epidemic strain and Melbourne epidemic strain. There were no significant differences between environmental isolates and clinical isolates, with all having comparable MIC values to chlorhexidine. In the quaternary ammonium compound, CPC all strains tested were capable of growth, with an MIC of > 200 µg/ml, the maximum tested, and indicated intrinsic resistance.

4.22 Susceptibility to other biocides

Twenty four strains from the original panel of 61 were selected for further biocide analysis. These strains were representative of chlorhexidine sensitive strains (18) and resistant strains (6). MICs were determined for three biocides in conjunction with chlorhexidine and CPC. These were triclosan, benzalkonium chloride and povidone (Table 4.2). All *P. aeruginosa* strains screened were resistant at the highest level of triclosan (MIC >500 µg/ml) and povidone (>5%) (Table 4.2). Benzalkonium chloride susceptibility was more variable, with MIC's ranging from 200 µg/ml to 400 µg/ml. The Liverpool epidemic strain LES B40 exhibited low levels of susceptibility to chlorhexidine (70 – 80 µg/ml), CPC (200 µg/ml), triclosan (>500 µg/ml), povidone (>5%) and benzalkonium chloride (>400 µg/ml), indicating a multiple biocide resistant phenotype for this problematic strain.

4.23 Minimum bactericidal concentrations

The panel of 24 strains described above were also screened for biocide bactericidal efficacy as described previously (Chapter 2.26). For CPC the MBC's were approximately 15 times higher than the MIC value with all five groups having similar MBC's, with no significant differences seen between them (Table 4.3). Chlorhexidine MBC's were similar to the MIC values with a 1.5 fold change between the values. Some strain group differences were seen, with Liverpool epidemic strains being significantly less susceptible than other epidemic strains (85 µg/ml and 50 µg/ml respectively) or other clinical strains (Table 4.3, Mann Whitney $P<0.05$).

4.24 Antibiotic susceptibilities

The antibiotic susceptibilities of 33 strains from the panel of 61 were tested and mean values calculated. In general there were high incidences of resistance to azithromycin and chloramphenicol and low levels of resistance to the β-lactams, such as meropenem. When the susceptibility of individual antibiotics is examined, clinically defined resistance varies across and within the strain groups. As described for the Bcc complex, an antibiotic profile score was determined that encompasses all ten antibiotics and was analysed statistically (Table 4.4). Unlike

Table 4.1 Mean *P. aeruginosa* MIC values in chlorhexidine

Strains (61)	Mean min Chlorhexidine (µg/ml)	Mean max Chlorhexidine (µg/ml)
Liverpool epidemic (15)	80*	83.3*
CF Epidemic strains (8)	12.9	22.9
Catheter strains (8)	18.3	28.3
Clinical CF Strains (26)	14.4	24.4
Environmental strains (4)	12.5	22.5
Total Mean	27.6	36.3

* Denotes the species that are significantly different as determined by Kruskall Wallis and Mann Whitney Tests ($P<0.05$)

Table 4.2 Mean MIC values for benzalkonium chloride

Strains (24)	Mean Benzalkonium chloride MIC (µg/ml)
Liverpool epidemic (7)	271.4
CF Epidemic strains (5)	300
Catheter strains (3)	333.3
Clinical CF Strains (7)	328.6
Environmental strains (2)	200
Total mean	286.7

the Bcc, the antibiotic profile scores were similar for each group. However, Liverpool epidemic strains were significantly less susceptible to the antibiotics than both the clinical and environmental strains (39.16 µg/ml compared to 37.69 µg/ml and 34.1 µg/ml respectively). The antibiotic MIC values also demonstrated no significant differences between clinical and environmental strains.

The production of spontaneous resistant colonies within the zone of inhibition was also observed for several *P. aeruginosa* strains. The Liverpool epidemic strains produced the most resistant colonies to all antibiotics tested, 49% compared to 24% of clinical strains, 15% of epidemic strains, 6% of catheter strains and 6% of environmental strains (Figure 4.2). The β lactam antibiotics were among the most potent against the strains tested, but after 48 hours produced the most spontaneous resistant mutants out of all of the antibiotics (Figure 4.1) and again the Liverpool epidemic strains produced the largest proportion of spontaneous colonies to this class of antibiotic. The combination of trimethoprim / sulfamethoxazole and ciprofloxacin also produced numerous resistant mutants.

4.3 Discussion

4.31 Biocide susceptibility

P. aeruginosa infections in patients with CF are often chronic and are difficult to eradicate leading to increased morbidity and mortality (Tummler and Kiewitz, 1999). *P. aeruginosa* strains are notoriously difficult to treat due to their intrinsic resistance to many antimicrobials. This concern stretches to disinfectant solutions, where contamination of these products has been frequently observed. *P. aeruginosa* strains have been isolated from the surface of hospital soap dispensers, containing a 2% (v/v) chlorhexidine based hand soap. The *P. aeruginosa* strains isolated were also resistant to 14 antibiotics, raising concerns that the soap was not bactericidal and that it was selecting for multidrug resistant organisms (Brooks *et al.*, 2002). Similarly, from urinary catheters in spinal cord patients, *P. aeruginosa* resistant to chlorhexidine and other cationic biocides were found. These strains cause

Table 4.3 Biocide susceptibility and minimum bactericidal concentrations in *P. aeruginosa* strains

		Chlorhexidine (* = significant difference as determined by Mann Whitney (P<0.05)			CPC		
Name	**Type (source)**	**MIC (µg/ml)**	**MBC (µg/ml)**	**Mean MBC (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**	**Mean MBC (µg/ml)**
CF Epidemic strains	Epidemic Manchester (CF)	20 – 30	50	50	>200	3000	3400
	Clone C epidemic (CF)	10 – 20	50		>200	3000	
	Midland 1 epidemic (CF)	10 – 20	50		>200	3000	
	Midland 1 epidemic (CF)	80 – 90	50		>200	3000	
	Melbourne epidemic (CF)	20 – 30	50		>200	5000	
Liverpool Epidemic	Liverpool Epidemic (CF)	10 – 20	50	85.7*	>200	5000	3428.5
	Liverpool Epidemic (CF)	70 – 80	100		>200	3000	
	Liverpool Epidemic (CF)	20 – 30	100		>200	4000	
	Liverpool Epidemic (CF)	20 – 30	50		>200	3000	
	Liverpool Epidemic (CF)	100	100		>200	4000	
	Liverpool Epidemic (HV +) (CF)	100	100		>200	4000	
	Liverpool Epidemic (HV -) (CF)	100	100		>200	1000	
Catheter Strains	Catheter (clinical)	10 – 20	50	50	>200	5000	4666.7
	Catheter (clinical)	20 – 30	50		>200	4000	
	Catheter (clinical)	20 – 30	50		>200	5000	
Clinical Strains	Non - epidemic Manchester CF (CF)	10 – 20	50	50	>200	5000	3857.1
	Non - epidemic Manchester CF (CF)	10 – 20	50		>200	5000	
	Random Unique (clinical)	10 – 20	50		>200	5000	
	Random Unique (clinical)	10 – 20	50		>200	5000	
	Random Unique (clinical)	20 – 30	50		>200	3000	
	Random Unique (clinical)	10 – 20	50		>200	3000	
	Random Unique (clinical)	10 – 20	50		>200	1000	
Environmental strains	Random Unique (Env)	30 – 40	50	75	>200	100	1050
	Random Unique (Env)	80 - 90	100		>200	2000	

particular problems in patients with catheters and can lead to blockages (Stickler *et al.*, 1981). *P. aeruginosa* strains tolerant to chloroxylenol and triclosan have also been recovered from industrial sources (Lear *et al.*, 2002). The ubiquitous nature of these bacteria in the environment indicates that the environment is a key reservoir of infection and leads to infection control issues (Balfour and Elborn, 2007). There is considerable debate about the use of biocide rotation in hospitals to combat the problem of resistance. Currently there is little evidence to support biocide rotation and the need for hygiene and cleanliness should be emphasised. However, as some bacteria, such as *P. aeruginosa* are intrinsically resistant to biocides such as chlorhexidine, the precautionary rotation of biocides could be implemented to prevent contamination of biocide stock solutions (Murtough *et al.*, 2001). As the public become more aware of the possibility of infection, the use of biocides as disinfectants in the home has increased dramatically. The over use of biocides could lend itself to the possibility of residues of the biocide in the environment which may confer a selective advantage for resistant organisms, although there is limited evidence that this is a major problem (Russell, 2002a).

This investigation explored the susceptibility of a panel of *P. aeruginosa* strains to a range of biocides. The panel of strains included epidemic, clinical (CF and non CF) and environmental strains. All the strains tested were intrinsically resistant to three of the biocides screened, povidone, triclosan and CPC, with growth above the threshold (> 80% knockdown from zero biocide control) still being observed at the maximum concentration tested. Resistance to triclosan is well documented, with many instances of triclosan being ineffective for the disinfection of *P. aeruginosa* (Williams and Stickler, 2008, Ellison *et al.*, 2007). CPC is a quaternary ammonium compound, which causes membrane damage and coagulation of cell components (McDonnell and Russell, 1999). It is concerning that *P. aeruginosa* strains appear to be intrinsically resistant to CPC. QAC's are common biocides used in many disinfectant products such as preoperative skin disinfection (0.1% - 0.2% (v/v)), in antiseptic creams (0.01 – 0.5% (v/v)), in throat lozenges (0.01% (v/v)) and in contact lens disinfection solutions (0.001 – 0.01% (v/v)) (Moore and Payne, 2004). For a more comprehensive list of biocides and their uses see Table 1.2. Considering the degree to which *P. aeruginosa* strains are resistant to this QAC, it would raise the issue of cross resistance with other QAC biocides. Loughlin *et al.*

Table 4.4 Mean antibiotic susceptibilities of P. *aeruginosa*

Strain	AK	AZ	TZ	CL	CI	IP	MP	PP	TM	TS	Antibiotic profile score
Liverpool epidemic (15)	33.9	188.8	48.6	24.4	1.5	15.7	12.1	58.4	3.4	4.8	39.16*
Epidemic Strains (4)	7.5	256	3.3	160	2.1	6.6	0.2	1.6	3.9	32	47.32
Catheter strains (3)	2.1	234.6	2	149.3	0.1	0.9	0.4	2.5	0.3	1.8	39.4
Clinical strains (7)	3.0	191.1	4.78	141.5	0.7	0.4	0.2	31.0	0.5	3.7	37.69
Environmental strains (2)	1.5	256	2.5	76	0.1	0.5	0.3	2.2	0.3	1.5	34.1

* Liverpool epidemic strains significantly less susceptible than clinical and environmental strains

Key

AK = amikacin, AZ = azithromycin, TZ = ceftazidime, CL = chloramphenicol, CI = ciprofloxacin, IP = imipenem, MP = meropenem, PP = piperacillin, TM = tobramycin and TS – trimethoprim / sulfamethoxazole

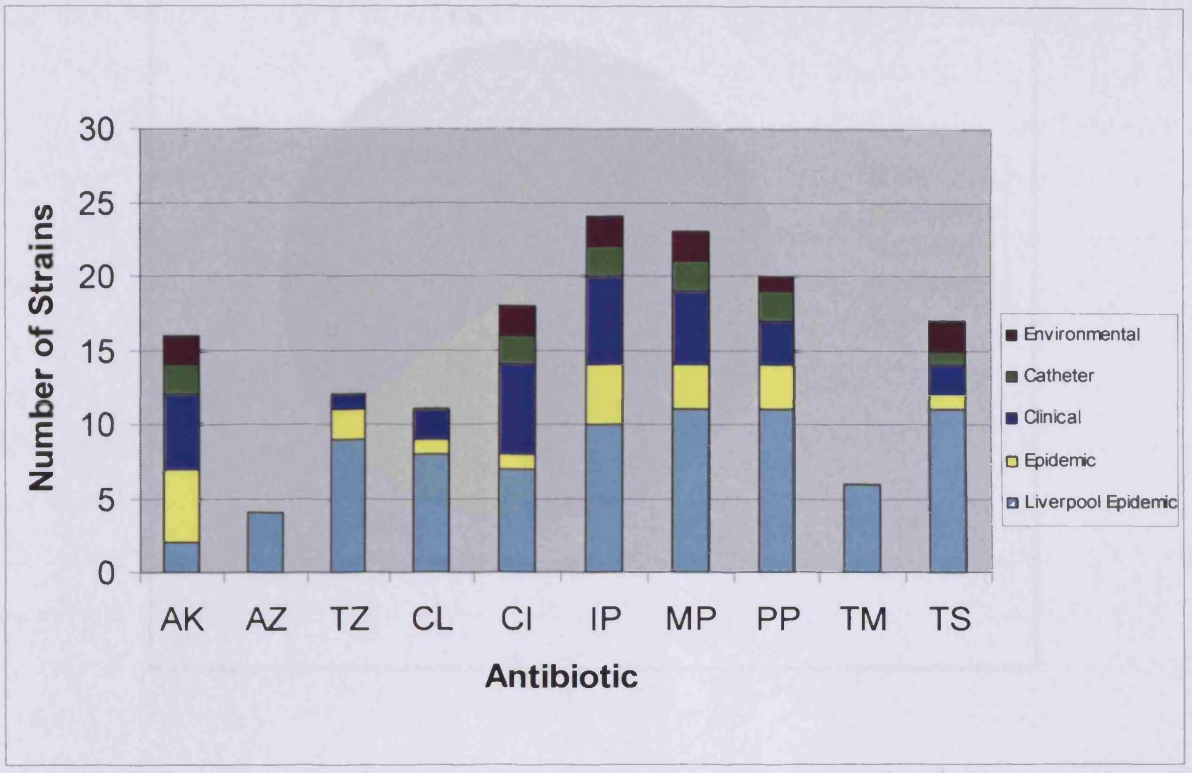


Figure 4.1 Production of spontaneous resistant colonies in *P. aeruginosa* strains.

Not all strain groups produced spontaneous resistant colonies (spontaneous resistant colonies = colonies seen within the zone of inhibition after 48 hours of growth). The Liverpool epidemic strains consistently produced resistant colonies to all antibiotics, followed by clinical strains. The β lactams, imipenem, meropenem and piperacillin induced the most adaptive resistant mutants compared to the other antibiotics.

Key

AK = amikacin, AZ = azithromycin, TZ = ceftazidime, CL = chloramphenicol, CI = ciprofloxacin, IP = imipenem, MP = meropenem, PP = piperacillin, TM = tobramycin and TS – trimethoprim / sulfamethoxazole

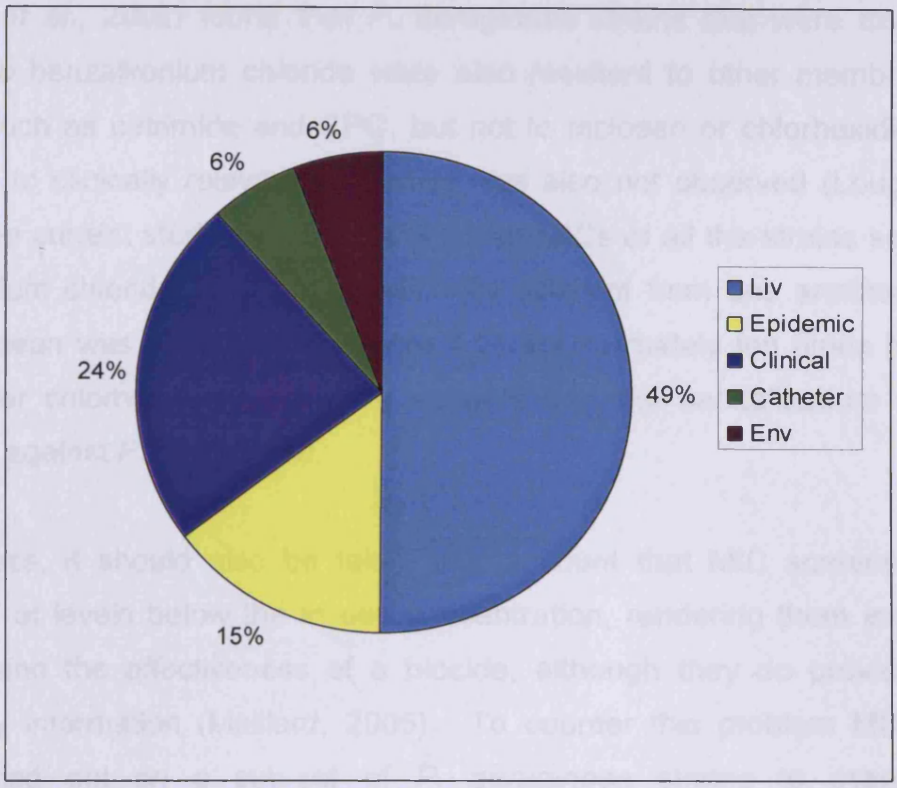


Figure 4.2 Percentage of *P. aeruginosa* strain groups producing spontaneous resistant colonies to all ten antibiotics

Liverpool epidemic strains produced the largest proportion of spontaneous resistant colonies after 48 hours of exposure to all ten antibiotics (49%). This was followed by clinical strains (24%), epidemic strains (15%), catheter and environmental strains (6%).

(Loughlin *et al.*, 2002) found that *P. aeruginosa* strains that were trained to be resistant to benzalkonium chloride were also resistant to other membrane acting biocides such as cetrimide and CPC, but not to triclosan or chlorhexidine. Cross resistance to clinically relevant antibiotics was also not observed (Loughlin *et al.*, 2002). The current study found that the mean MICs of all the strains screened for benzalkonium chloride were not significantly different from one another, although the total mean was 286.7 µg/ml, (Table 4.2) approximately ten times higher than the MIC for chlorhexidine. Thus, it appears that the benzalkonium chloride is ineffective against *P. aeruginosa*.

Nevertheless, it should also be taken into account that MIC screens are often conducted at levels below the in use concentration, rendering them inappropriate for assessing the effectiveness of a biocide, although they do provide valuable preliminary information (Maillard, 2005). To counter this problem MBC screens were carried out on a sub-set of *P. aeruginosa* strains to asses whether chlorhexidine and CPC were suitable biocides to use in infection control procedures. Out of the biocides screened chlorhexidine was the most effective growth inhibitor, with fairly low MIC values (Mean 27.6 – 37.3 µg/ml, Table 4.1). MBC analysis reveals that for a bactericidal affect approximately three times more chlorhexidine is required, thus the MIC and MBC values are concurrent with one another. Unfortunately this was not the case for CPC. The MBC values are much higher than the MIC, with a 16 fold greater requirement of CPC needed to achieve a bactericidal effect, although both screens indicate that CPC is a poor biocide for use against *P. aeruginosa* (Table 4.3). In general it would appear that QACs are not suitable for disinfection of places where *P. aeruginosa* are encountered frequently. Similarly, triclosan and povidone are also ineffective. Although the "in use" concentration of biocides should be high enough to affect rapid killing of the bacteria, there is much speculation on the effect of residues of biocides in the environment. In situations where biocides are used to sterilise surfaces or are diluted with water for handwashing or equipment sterilisation, bacteria will be exposed to sub-inhibitory residues (Gilbert and McBain, 2001). This may induce resistance to this biocide and consequently cross resistance between unrelated compounds may occur. However, Thomas *et al.* found that *P. aeruginosa* strains with elevated MIC values to chlorhexidine that were exposed to chlorhexidine

residues were no less susceptible to chlorhexidine and antibiotics, suggesting that biocides residuals are not selecting for multi drug resistant organisms (Thomas *et al.*, 2000).

4.32 Antibiotic susceptibilities

The antibiotic susceptibilities were also determined using commercial E-test® strips. In general *P. aeruginosa* strains were sensitive to all of the β-lactams tested (imipenem, meropenem and piperacillin) and to ciprofloxacin, amikacin, ceftazidime and tobramycin (Table 4.4, for MIC break points see Table 2.7). It is interesting that many of the strains tested were sensitive to β-lactams as it has been well documented that *P. aeruginosa* have β-lactamases, which degrade the antibiotic. Extended spectrum β-lactamases have also arisen and are spread on plasmids. The PER-1 type extended spectrum β-lactamase confers resistance to several antibiotics as well as ceftazidime, piperacillin and ticarcillin, which usually retain their activity (Vahaboglu *et al.*, 1997). In this investigation approximately 45% of *P. aeruginosa* strains were resistant to the β-lactams (15 out of 33), however after 48 hours of exposure spontaneously resistant colonies were observed (Figure 4.1). Thus, it may be that increased contact with the antimicrobial leads to the selection of resistant organisms. There were no significant differences between environmental and clinical strains of *P. aeruginosa*, indicating that there is little differentiation between strains found in clinical hospital situations and strains found in the environment, thus vulnerable patients should be cautious as the environment may pose as a source of infection.

4.33 The Liverpool Epidemic strains

One of the striking observations made from this investigation were the elevated chlorhexidine MIC values in the Liverpool epidemic strains compared to all other strains. This included other epidemic strains, such as the Manchester and Melbourne epidemic strains. Most strains were inhibited at 20 μg/ml of chlorhexidine, but the Liverpool epidemic strains required four times this amount to achieve the same effect (Table 4.1).

Historically it was observed that a unique *P. aeruginosa* strain was acquired by the patient and they then progressed to a chronic infection with this strain. Isolates from CF patients were phenotypically different from environmental isolates as they exhibited loss of motility and mucoidy. It was shown by RAPD profiling that this was due to the adaptation of the strain to a chronic state, not replacement with a new strain (Mahenthiralingam *et al.*, 1996a). A surveillance study carried out in Vancouver found that out of 174 CF patients, 123 carried a unique strain that was not seen in any other patient. 34 strains were shared, with ten of these patients being siblings (Speert *et al.*, 2002). Therefore it was concluded that *P. aeruginosa* carried a very low risk of transmission and prolonged contact with an infected individual such as in the case of siblings was the greatest cause of cross infection (Speert *et al.*, 2002), thus consequently segregation of patients was not recommended.

However, in the UK a different epidemiology was observed with the emergence of epidemic transmissible strains. Cheng *et al* (Cheng *et al.*, 1996) found that an unusually high number of children (70.6%) in a Liverpool clinic were colonised with a ceftazidime and β-lactam resistant strain of *P. aeruginosa.* They recommended that this strain may be an antibiotic resistant induced transmissible strain and that those patients harbouring it should be segregated from other patients (Cheng *et al.*, 1996). In a Manchester CF clinic the emergence of a different epidemic strain became apparent. Fourteen percent of patients carried an epidemic strain that had an unusual phenotype (non-pigmented and non-motile). This strain was genetically distinct from the transmissible strain isolated in Liverpool and was called the Manchester epidemic strain (Jones *et al.*, 2001b).

A more recent survey of over 31 CF centres in England and Wales, identified the Liverpool epidemic strain as the most common with 48% of CF centres harbouring it (Scott and Pitt, 2004). This implies that cross infection can occur and that these epidemic strains may be more aggressive and able to replace a patient's original strain (Scott and Pitt, 2004). The possibility of cross infection with healthy individuals may also be a concern. Although very few cases have been documented, McCallum *et al.* (McCallum *et al.*, 2002) reported a CF patient infected with the Liverpool epidemic strain, which consequently infected her parents.

Although her father had a pre-existing lung condition, he was generally in good health and the patient's mother was healthy with no medical conditions. The colonisation of non- CF healthy people is a serious issue, particularly for parents with CF children and although cross infection is rare, it is perhaps pertinent to screen people who are in close contact with colonised patients for these organisms (McCallum *et al.*, 2002).

The emergence of the Liverpool and Manchester epidemic strains is worrying as they demonstrate increased antibiotic resistance and are more virulent, causing serious health implications for the patients. Due to the transmissibility of these strains there are also infection control issues, where patients harbouring a transmissible strain may be segregated (Scott and Pitt, 2004).

In this study, the Liverpool epidemic strains were significantly less susceptible than other strains to chlorhexidine, including other epidemic strains (Table 4.1). This is interesting as in previous studies it has been noted that the LES may have enhanced virulence contributing to its transmissibility. It was reported that LES strains had multi drug resistance to β-lactams such as ceftazidime and imipenem (Cheng *et al.*, 1996). This was probably due to selection by the use of monotherapy to treat *P. aeruginosa* infection. However, it has also been determined that multiple drug resistance can be attributed to specific mutations in efflux pumps. LES isolates also have up regulation of the AmpC β-lactamase gene and in quorum sensing genes (Salunkhe *et al.*, 2005). In particular, strain LES 431, was deemed hyper virulent due to it's high levels of β-lactamase activity, coupled with up regulation of quorum sensing, thus LES 431, which also has the ability to infect non CF parents of CF patients, is a particularly problematic strain for CF patients (Salunkhe *et al.*, 2005). Many of the LES isolates have this unusual phenotype, which is thought to contribute to their increased virulence. This includes up regulation of quorum sensing genes, such as the over production of pyocyanin, a blue green pigment which has many toxic effects and the LasA protease (Fothergill *et al.*, 2007). However, not all isolates of the LES strain have this phenotype and the LES genotype appears to be unstable with many deletions, insertions and rearrangements occurring (Fothergill *et al.*, 2007). The current investigation found that not all LES strains had increased chlorhexidine MICs,

although the majority did show this phenotype (78.6% had a chlorhexidine MIC of 70 µg/ml or above compared to 21.4% of strains with low chlorhexidine MIC).

The antibiotic susceptibility profiles for individual strains varied, but overall the Liverpool epidemic strains had higher MIC's to the β-lactams, ceftazidime, imipenem, meropenem and piperacillin (Table 4.4), than all other strains, including other epidemic strains. This could be attributed to the up regulation of the AmpC β lactamase gene and also to the up regulation of efflux pumps. It is interesting to note that the hypervirulent strain LES 431 was resistant to chlorhexidine and six out of ten antibiotics screened, correlating to previous data that this isolate in particular is more virulent than other strains (Salunkhe *et al.*, 2005). All strains tested were resistant to CPC, triclosan and povidone and there were no significant differences between benzalkonium chloride MIC values, indicating that *P. aeruginosa* strains are less susceptible to these agents. Most strains were very susceptible to chlorhexidine, except the LES strains. This may be due to the up regulation of efflux pumps, such as the MexAB OprM, which can have broad specificity for many unrelated compounds (Li *et al.*, 2000), but as of yet the mechanism of the LES increased chlorhexidine resistance has not been determined.

Evidently the Liverpool epidemic strains are a group of strains that have increased virulence and multiple antimicrobial resistance which extends to antibiotics and disinfectants. Not only does this make it difficult to treat patients infected with these strains, but the ability of the LES to super infect patients and its transmissibility generates infection control problems that need to be resolved to prevent the spread of these epidemic strains. *P. aeruginosa* strains have high tolerance to a number of biocides and this could be a contributory factor to its continued spread across CF clinics. Understanding the mechanism of resistance so that new therapies can be developed to target these strains is unmistakably an important area of research.

4.34 Biocide resistance mechanisms

The results obtained in this investigation correlate with other literature that suggest *P. aeruginosa*, especially transmissible epidemic strains are multidrug resistant to antibiotics. A new finding of this work is that epidemic strains are resistant to many

classes of biocides, including QACs, phenols and povidone. Due to the action of biocides on multiple targets, specific resistance mechanisms are poorly understood, but a brief overview is provided here and Table 4.5 provides a summary of the antibiotic and biocide resistant mechanisms. The outer membrane of *P. aeruginosa* plays an important part in the resistance to many biocidal agents, particularly hydrophobic agents that cannot diffuse across the outer membrane due to LPS molecules (McDonnell and Russell, 1999). The high Mg^{2+} content leads to strong LPS – LPS links, preventing access of the biocide to the cell (McDonnell and Russell, 1999).

Efflux is a well known mechanism involved with both antibiotic and biocide resistance and is well defined in *P. aeruginosa*. One of the main efflux pumps, the RND MexAB-OprM , is known to induce resistance to a number of antibiotics and biocides (Poole, 2004). Recently an efflux pump was described that actively effluxes triclosan. This pump was one of five that is known to effectively efflux triclosan and indicates that efflux is one of the main resistant mechanisms for triclosan (Mima *et al.*, 2007). The latter mechanisms are all intrinsic to *P. aeruginosa*.

Although not thought to play a central role in resistance in Gram negative bacteria, acquired resistance can occur. This includes the adaptation of bacteria to become resistant to a certain antimicrobial due to increased exposure to the agent. This can lead to up regulation of efflux pumps or enzymes and may also lead to outer membrane changes that leave the bacteria less susceptible (Loughlin *et al.*, 2002). The acquisition of plasmids is also known to increase bacterial resistance. The acquisition of plasmids conferring antibiotic resistance has not been well described in *P. aeruginosa* and it is not thought to play a central role in resistance to biocides (Russell, 1997).

The lifestyle of *P. aeruginosa* could contribute to resistance to both antibiotics and biocides. In the CF lung, *P. aeruginosa* congregate and forms biofilms, which produce an exopolysaccharide glycocalyx. Biofilms are difficult to penetrate with antimicrobials as they are protected by the exopolysaccharide and cells within the

Table 4.5 Antibiotic and biocide resistant mechanisms in *P. aeruginosa*

Antimicrobial Agent	Resistant mechanisms	Reference
Antibiotics		
β-lactams	Enzyme inactivation e.g. β - lactamases Efflux	(Bradford, 2001, Russell, 2000)
Aminoglycosides	Enzyme modification Reduced uptake Efflux	(Russell, 2000, Poole, 2005a)
Chloramphenicol	Efflux	(Poole *et al.*, 1993)
Fluoroquinolones	Efflux	(Russell, 2000)
Biocides		
QACs	Reduced uptake Efflux	(Maillard, 2005) (McDonnell and Russell, 1999)
Chlorhexidine	Reduced uptake Efflux	(Maillard, 2005, McDonnell and Russell, 1999)
Triclosan	Reduced uptake Efflux	(Maillard, 2005, McDonnell and Russell, 1999, Mima *et al.*, 2007)

biofilm are often growing more slowly reducing the uptake (Govan and Deretic, 1996). Biofilm grown isolates were perceived to be less susceptible to single and double combinations of antibiotics compared to planktonically grown strains (Hill *et al.*, 2005). Stickler (Stickler, 2002) found that patients with catheters often had blockages due to the formation of biofilms. These biofilms were made up of bacteria from a number of species including *Proteus mirabilis* and *P. aeruginosa*. Chlorhexidine was used to disinfect the area before the catheter was applied and many of the isolates obtained from these biofilms were chlorhexidine resistant Other studies have also found that 80% of *P. aeruginosa* cells in biofilms were not killed by in use concentrations of benzalkonium chloride (1% w/v), chlorhexidine gluconate (4% w/v) and triclosan (1% w/v) (Smith and Hunter, 2008). *P. aeruginosa* strains adapt to the CF lung and become mucoid producing a sticky alginate that contributes to the biofilm production and therefore the antibiotic and biocide resistant profile that this species are well known for (Govan and Deretic, 1996). In this study, mucoidy and biocide resistance was not investigated. Mucoidy may have a key role in biocide resistance thus to expand this work, *P. aeruginosa* strains grown as biofilms could be screened for biocide MICs and this data compared with planktonically grown cells.

The many and varied mechanisms of resistance that *P. aeruginosa* employs to avoid being killed and promote survival in hostile environments generates a species that is particularly difficult to treat once a patient is infected. Infection control and the prevention of patients acquiring *P. aeruginosa* is an important area of research, although the abundance of this organism in the environment may make this an impossible task. New therapeutics should be developed to target mechanism of resistance, such as modification of existing agents to prevent enzyme inactivation and the combination of antimicrobials to produce a synergistic effect, such as increased permeability.

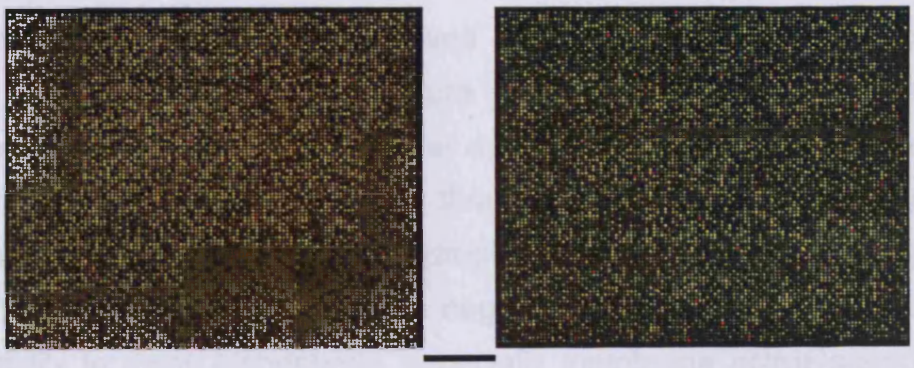
4.4 Conclusions

In summary, this chapter expands other data that *P. aeruginosa* strains are multidrug resistant and are also intrinsically resistant to many biocides, such as

triclosan, CPC and benzalkonium chloride. These are agents that are often used in clinics for a multitude of purposes and this in itself may select for resistant organisms and lead to nosocomially acquired infections. In addition, the Liverpool epidemic strains, a transmissible strain, now recognised as one of the most common clones in the UK (Scott and Pitt, 2004), was observed to be more chlorhexidine resistant compared to all other strains tested. The LES strains are known to posses multi-drug resistance and increased virulence factors which may contribute to the higher resistance seen in these strains to chlorhexidine. Liverpool epidemic strains pose a particular problem due to their transmissibility, therefore it is especially important that efficient disinfection practices are employed to prevent infection and spread of these organisms. In general, QACs, triclosan and povidone should not be used against *P. aeruginosa* strains as all strains regardless of the habitat they came from or whether they were epidemic or not, were intrinsically resistant to them. Chlorhexidine should be used with caution, as most strains were susceptible to it, but it should be noted that resistance can be trained and the LES were more resistant to it. Further investigations should be carried out to determine if commercial biocides are bactericidal for *P. aeruginosa* and additional disinfection guidelines can then be recommended. Although in this investigation the genetic basis of biocide resistance in *P. aeruginosa* was not studied, it is still an important area for research, as the genetic understanding of biocide resistance may help prevent CF patients becoming infected with multidrug *resistant P. aeruginosa* that have a large consequence on their morbidity and mortality.

Chapter 5.0

Gene Expression of *B. cenocepacia* J2315 in response to Chlorhexidine



Chapter 5.0 – Gene expression of *B. cenocepacia* J2315 in response to chlorhexidine

5.1 Introduction

5.11 The *Burkholderia cepacia* complex – mechanisms of biocide resistance

The Bcc are a group of intrinsically antimicrobial resistant organisms, which are capable of contaminating disinfectant and preservative formulations, hence making infection control exceedingly difficult. Their ability to contaminate commercial products are particularly problematic for cystic fibrosis (CF) and intensive care (ITU) patients as they may acquire these serious infections from the contaminated product (Frank and Schaffner, 1976, Berkelman *et al.*, 1981), leading to a poor outcome. Evidently it is important to understand the mechanisms by which pathogens become resistant to certain antimicrobials so that new therapies may be devised to combat these life-threatening infections.

The general mechanisms of biocide and antibiotic resistance were outlined in Chapter 1. The outer membrane structure is an important aspect of resistance for both classes of antimicrobial. The outer membrane gives limited permeability so many antimicrobials cannot penetrate through to the inner cell. It consists of lipopolysaccharides, porins that form hydrophilic channels and efflux pumps (Burns, 2007). The LPS composition of Gram negative bacteria is a major cause of the impermeability to many compounds, especially membrane active agents such as chlorhexidine (Denyer and Maillard, 2002). Bcc species have a very different LPS composition compared to other Gram negative species. The core oligosaccharide of the Bcc LPS contains less phosphate and 3-deoxy-D-manno-octo-2-ulosonic acid (KDO) than other Gram negative species (Cox and Wilkinson, 1991). The Bcc LPS also contains 4 amino-4 deoxyarabinose (Ara4N) residues attached to phosphate groups of glucosamine disaccharide in the lipid A backbone (Isshiki *et al.*, 1998) (Figure 5.1). The presence of Ara4N and the fact that the LPS contains less phosphate and –deoxy-D-manno-oct-2-ulosonic acid contributes to antimicrobial resistance as it reduces the negative charge of the outer membrane, effectively

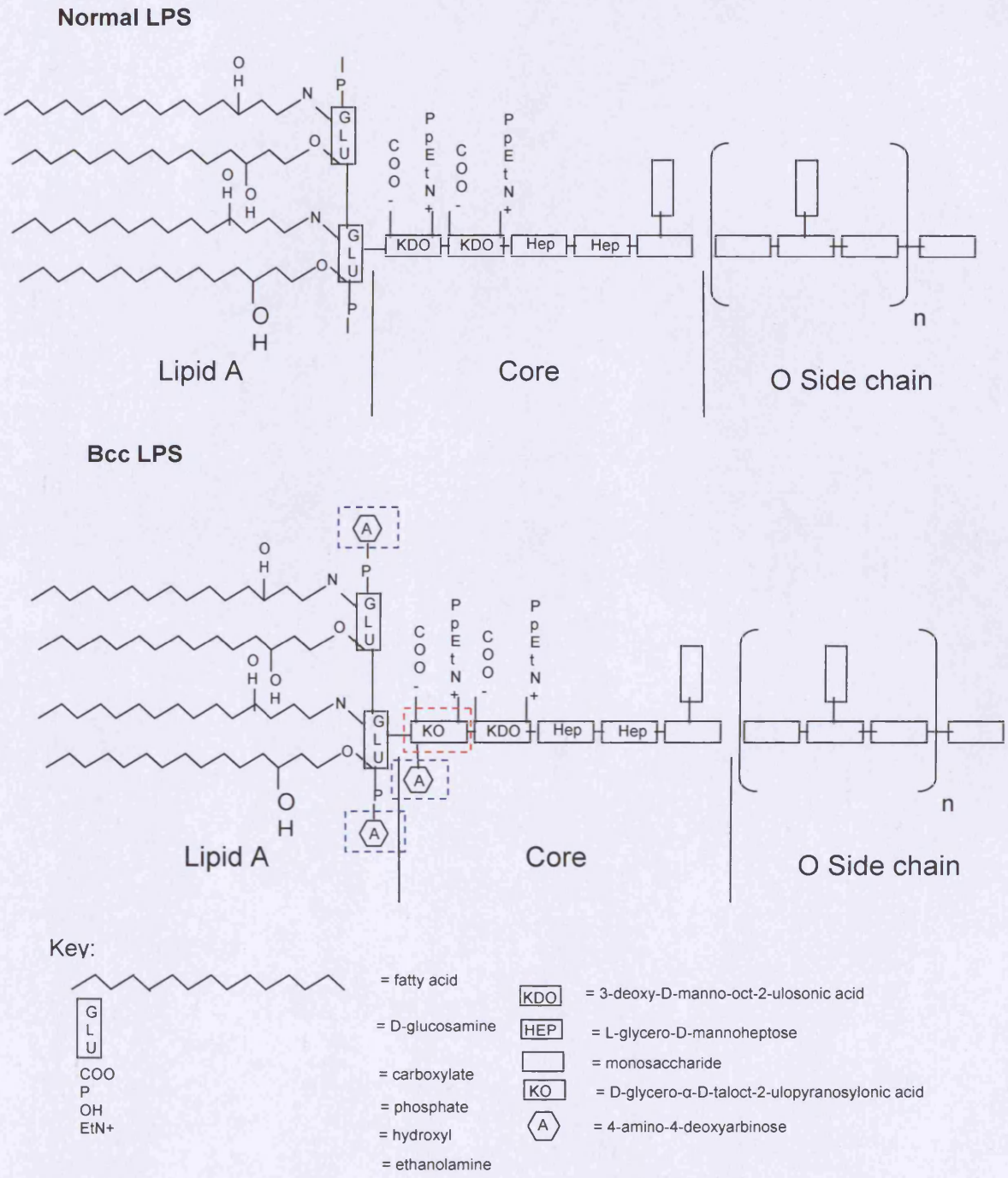


Figure 5.1 The structure of LPS in (i) Gram negative bacteria and (ii) Bcc bacteria.

Bcc LPS structure contains 4 – amino-4-deoxyarbinose (highlighted in blue) and substitutes a KDO module for a KO (highlighted in red). This reduces the negative charge of the membrane, concealing binding sites for cationic compounds. Adapted from (Russell and Chopra, 1996)

concealing the binding sites for cationic agents (Goldberg, 2007, Burns, 2007).

Porins are also contributing factors to resistance. Porins form channels through the outer membrane that allow the free diffusion of hydrophilic compounds which include many antibiotics and some biocides. The Bcc notably have a lower permeability for many of these compounds, particularly β lactam antibiotics. It has been suggested that the low permeability of the outer membrane contributes significantly to this intrinsic resistance and that the porins play a major role. The porins of the Bcc have been found to have low conductance for hydrophilic compounds, due to the small channel size they form (Parr *et al.*, 1987). This restricts access across the membrane for many antimicrobials.

Also part of the outer membrane are efflux pumps, an essential component to the resistance machinery. Efflux pumps use the proton motive force to expel cytotoxic compounds (Borges-Walmsley and Walmsley, 2001). These compounds may be unrelated and can include antibiotics, dyes, detergents and biocides (Piddock, 2006a). There are five classes of efflux pumps: the resistance nodulation division (RND), the major facilitator superfamily (MFS), the small multi drug resistance (SMR), multidrug and toxic compound extrusion (MATE) family and the ATP binding cassette (ABC) transporters. Members of the Bcc have a number of efflux pumps in their outer membranes, including RND and MFS drug pumps. Wigfield *et al.* (Wigfield *et al.*, 2002) described an immunodominant drug efflux pump called BcrA, which was specific for tetracycline and nalidixic acid. This efflux pump belongs to the major facilitator superfamily class of efflux pumps and although did not increase resistance to a number of detergents and biocides (e.g. Tween 20, ethidium bromide and chlorhexidine, it still pumped out non related classes of antibiotics (Wigfield *et al.*, 2002). RND pumps have also been identified, with some showing homology to the MexAB-OprM pump described in *P. aeruginosa* (Guglierame *et al.*, 2006) and an efflux operon (*ceoA*) that is induced by the siderophore salicylate and confers resistance to ciprofloxacin, chloramphenicol and trimethoprim (Nair *et al.*, 2004). The presence of these efflux pumps contributes to the multidrug resistance of the Bcc and possibly gives rise to cross resistance due to the ability of the pumps to remove unrelated classes of compounds.

Members of the Bcc also have the ability to form biofilms which are thought to be associated with increased resistance due to the lack of penetration of the biofilm by the antimicrobial. Cells growing in a biofilm have an altered growth rate compared to planktonically growing cells, reducing uptake of the antimicrobial. Biofilms are formed by the production of exopolysaccharides which surround colonies growing within the biofilm. Approximately 80% of the Bcc produce an exopolysaccharide, cepacian which contributes to the persistence and colonisation of the Bcc (Cunha *et al.*, 2004). There are three stages to the formation of a mature biofilm, adhesion, aggregation and formation of mature biofilm (Figure 5.2). The production of a mature biofilm is not only associated with increased antimicrobial resistance but also other virulence factors, such as ability to withstand the hosts immune system and inflammation (Riedel and Eberl, 2007).

Biofilm production is mediated by the cell density dependent quorum sensing system, a cell to cell communication system which allows the coordination of many regulatory mechanisms in a number of cells at the same time (Riedel and Eberl, 2007). The quorum sensing system uses small signalling molecules, N-acyl-homoserine lactones (AHL) to mediate coordination. The Bcc has such a system, the CepI / CepR system, where CepI makes the autoinducer, which when a threshold level is reached binds to the regulator protein (CepR), which then activates the required genes (Lewenza *et al.*, 1999). As well as mediating biofilm production, the QS system also mediates the expression of extracellular proteases chitinases and swarming motility (Riedel and Eberl, 2007), thus in itself it is an important virulence factor and may contribute to the biocide resistance displayed by many members of the Bcc. In addition some transmissible *B. cenocepacia* strains have genomic islands that contain virulence factors. The *cenocepacia* island (cci), encodes a predicted N-acylhomoserine lactone synthase gene and a response regulator, indicating that this may contribute to quorum sensing and therefore biofilm production (Malott *et al.*, 2005).

The above mechanisms of resistance all have a role in the Bcc resistance to biocides. Although these mechanisms have been determined the underlying genetic pathways and their role in resistance have yet to be elucidated. It is important to determine these pathways, so that resistance can be fully understood

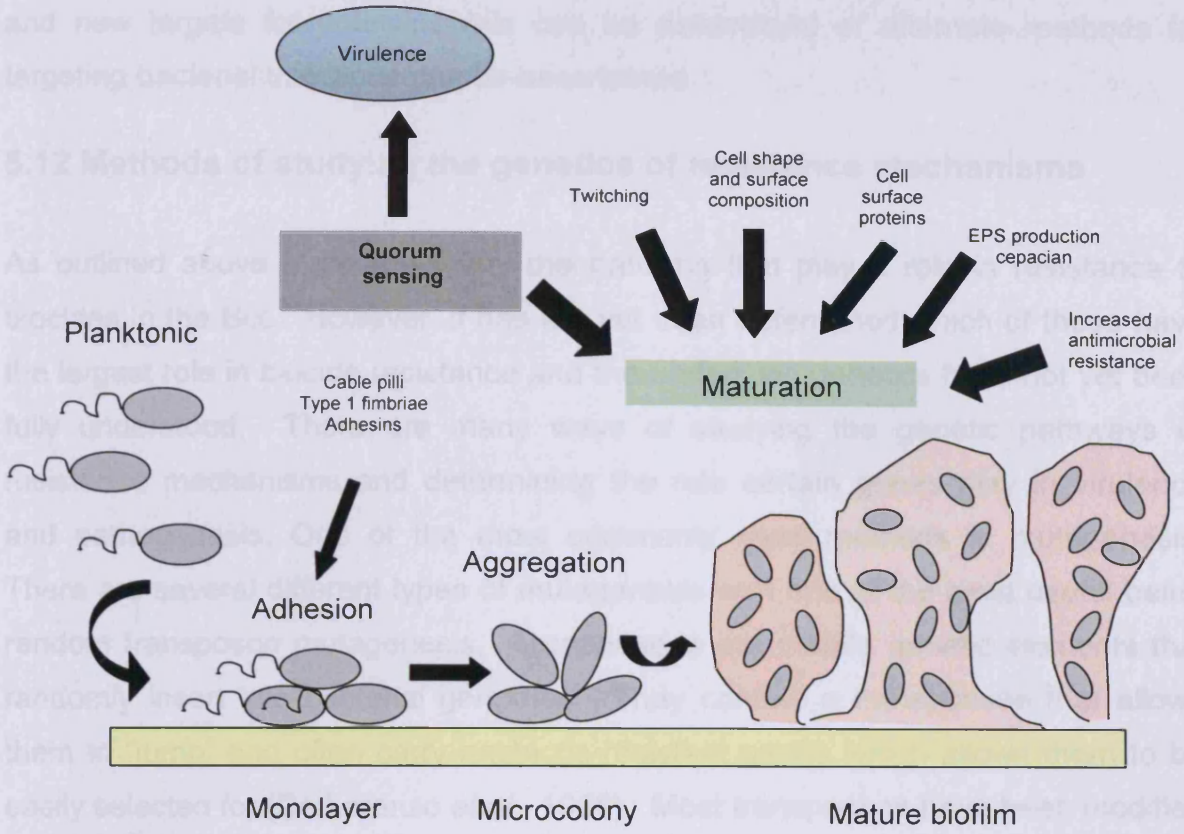


Figure 5.2 The development of a mature biofilm in Bcc

Planktonic bacteria swim towards the substratum using flagella and then attach loosely. They then migrate over the surface forming micro-colonies. These micro-colonies differentiate into a mature biofilm, where cells are embedded in a thick EPS matrix (cepacian). Maturation involves quorum sensing, twitching, cell surface proteins and an increase in antimicrobial resistance. Adapted from (Riedel and Eberl, 2007).

and new targets for antimicrobials can be determined or alternate methods for targeting bacterial infections can be ascertained.

5.12 Methods of studying the genetics of resistance mechanisms

As outlined above there are many mechanisms that play a role in resistance to biocides in the Bcc. However, it has not yet been determined which of these have the largest role in biocide resistance and the underlying genetics have not yet been fully understood. There are many ways of studying the genetic pathways of resistance mechanisms and determining the role certain genes play in virulence and pathogenesis. One of the most commonly used methods is mutagenesis. There are several different types of mutagenesis with one of the most useful being random transposon mutagenesis. Transposons are mobile genetic elements that randomly insert into bacterial genomes. They contain a transposase that allows them to "jump" and often carry antibiotic resistant genes which allows them to be easily selected for (De Lorenzo *et al.*, 1998). Most transposons have been modified such as the mini Tn5 and Tn10 transposons, which are specialised transposons that are stable and contain antibiotic selection markers for rapid cloning (de Lorenzo *et al.*, 1990).

Random transposon mutagenesis works by mating a strain containing the transposon on a suicide delivery plasmid with the bacterial strain of interest. The transposon will then insert into genes and possibly knockout their function. A screen can then be developed to identify a loss of function in a given mutant. For example to look for loss of resistance to an antibiotic, mutants would be plated out onto agar containing the antibiotic in question. Those that didn't grow would be selected from a master plate and further analysis carried out, e.g. sequencing and bioinformatics. Although this approach produces many mutants, it can be time consuming to screen for mutants, and the process is greatly facilitated by having a genome to determine the location of each mutated gene. The insertion of transposons may induce polar effects, terminating the transcription of genes downstream of the insertion point (Hensel and Holden, 1996).

Other mutagenesis techniques include directed mutagenesis, where a known identified gene is knocked out to confirm function. This is achieved either by gene disruption or gene replacement. Gene replacement involves replacing a gene with previously cloned DNA that contains insertions, deletions or base replacements. This effectively alters the gene, so that it is no longer functional (Quandt and Hynes, 1993). Gene disruption inactivates the gene by insertion of a marker, usually an antibiotic resistant cassette into the gene. This has the added bonus of allowing the mutants to be selected for easily, as long as they are originally susceptible to the antibiotic. This technique is useful to confirm gene function however it is not useful if the genes involved are not already known.

Gene transfer can also be used to determine gene function. Genes possibly involved in resistance can be transferred to a susceptible strain which is then screened to see if resistance has been induced by the presence of the new gene. This instantly provides information on the importance of that gene in resistance. Nevertheless, there are many limitations to this approach. Genes in operons may not show a significant effect if only one component is transferred to the susceptible host. This technique is generally applied to systems where known genes are deleted. There may also be issues if the resistant host and the susceptible host are evolutionary different as the recipient may not contain the necessary regulatory machinery to express the genes transferred into it (Hensel and Holden, 1996).

Investigating gene expression is another way to determine genes that may have a role in resistance. This approach is based on the hypothesis that genes important in pathogenesis will be expressed during infection and may only be activated when in this state. This technique can also be applied to resistant genes as generally they are switched off until placed in a situation where they are required. Often maintaining expression of resistant genes can have a large metabolic cost to the organism, thus it is beneficial to only switch them on in response to particular situations. To study gene expression RNA is extracted from bacteria present in an infection state or exposed to the antimicrobial and then converted to cDNA. Those genes present in the infection state can then be screened for using subtractive hybridization, where labelled probes are synthesised and hybridized to cDNA

(Hensel and Holden, 1996). Again a major limitation of this method is the need to know the gene involved so a suitable probe can be designed.

5.13 Whole genome approach to studying gene expression

Recently the whole genome microarray approach has become more popular for studying gene expression. A microarray allows the observation of the transcriptional response of the whole genome to an environmental stimulus (Ehrenreich, 2006). This produces a large data set that allows the researcher to get an overview of the genes that are important in the conditions they have set. A microarray is a microscopic slide that contains a series of samples, usually DNA or RNA from a particular organism. These are placed or synthesised on the slide in an ordered manor so that any data obtained can be traced back to the original gene (Pasanen *et al.*, 2003). RNA is extracted and converted to cDNA, labelled with fluorescent dye and then hybridised onto the microarray. The ratio of fluorescence is then determined and expression changes can be ascertained quantitatively. Microarray approaches are becoming more favourable as the whole transcriptomic expression can be elucidated in as little as one experiment. This provides a massive bank of data that can be further investigated and may also pin point important genes that may otherwise not have been seen due to small changes in their expression. Not only does a microarray approach provide data on genes that are up-regulated in their expression in response to the stimulus, but it also highlights genes that are repressed in response to the stimulus. Thus the experimenter gets an overall view of the important pathways involved in response to certain conditions.

Microarrays have been used to look at gene response to a number of different environmental stimuli. For example, oxidative stress is a contributory factor to killing of bacterial species. In the lung of CF patients, phagocytic reactive oxidants are present, thus protection against these radicals is important. Chang *et al.* investigated the response of *P. aeruginosa* to oxidative stress by exposing *P. aeruginosa* to hydrogen peroxide. Genes important in the antioxidant defence system included DNA repair proteins and catalyses. Interestingly, it was seen that

pyocin production was up-regulated, indicating that this may be a novel defence mechanism against oxidative killing (Chang *et al.*, 2005).

Other studies have also focused on the CF lung as an interesting environment for investigation. Drevinek *et al.* used a *B. cenocepacia* microarray to look at the gene expression in response to cystic fibrosis sputum. Genes involved in iron uptake, protection against reactive oxidative stress, secretion and motility and antimicrobial resistance were significantly altered (Drevinek *et al.*, 2008). Due to the continuous nature of antibiotic treatment in CF patients, it was perhaps not surprising that some efflux genes were up-regulated in CF sputum, however not all efflux genes were up-regulated and some appeared to be repressed in CF sputum depending on the antibiotic treatment each patient received (Drevinek *et al.*, 2008).

Studying expression profiles of genes exposed to antibiotics can aid our understanding of resistance mechanisms and expose new targets for antimicrobials. Comparison studies of resistant and sensitive strains could elucidate genes that play a vital role in resistance (Brazas and Hancock, 2005). Biocides have a broad spectrum of activity compared to antibiotics, which generally have one target. Thus mechanisms of resistance are more complex and may be more difficult to determine due to the involvement of many genes. Consequently using a microarray approach may overcome some of these problems, by providing a whole genome analysis. A study investigating the effects of triclosan on *S. aureus,* using a microarray found that triclosan affects many components of metabolism. These included the down regulation of virulence related genes and genes involved in energy metabolism, such as amino acid, carbohydrate and lipid transport and metabolism, where as up regulated genes were involved in multi drug resistance (Jang *et al.*, 2008). The study implied that triclosan may kill *S. aureus* by interfering with its ability to form cell membranes. Genes involved with fatty acid metabolism were also down-regulated which may have consequences, as triclosan targets fatty acid synthesis (McMurry *et al.*, 1998b). However, the *fabI* enoyl reductase gene involved in resistance had no change in expression after exposure to triclosan (Jang *et al.*, 2008).

Previous studies have therefore shown a microarray approach to be appropriate for the discovery of important genes in response to the biocide triclosan. In this study, a microarray was carried out to investigate genes that have a vital role when exposed to the biocide chlorhexidine. It was hypothesised that genes involved in resistance would have a change in expression and that this may lead to the identification of novel antimicrobial targets. *B. cenocepacia* J2315 was used as model organism.

5.14 *B. cenocepacia* J2315 as a model organism

B. cenocepacia J2315 was used as a model organism for this study as its genome has already been sequenced (Pathogen Sequencing Group at the Sanger Institute, Hinxton, Cambridge, http://www.sanger.ac.uk/projects/B_cenocepacia). Recently the genome paper containing detailed annotation has been published (Holden *et al.*, 2009). Hence, any genes determined by the microarray could be linked back to the sequence and functions determined. J2315 is a pathogenic strain of *B. cenocepacia*, from the ET-12 lineage which is known to be an epidemic transmissible strain. Therefore it is interesting to study this strain, as it may display traits involved with virulence, transmissibility and antimicrobial resistance. This pathogen is already known to be multidrug resistant to a number of antibiotics and from preliminary work carried out in this study (Chapter 3.0), able to survive within biocides routinely used in clinical situations. Consequently, a microarray looking at the change in gene expression in response to chlorhexidine may highlight genes involved in resistance to chlorhexidine.

As well as the above reasons, J2315 was used as a model organism in this study as a microarray had already been produced for this organism. The microarray was a custom 2 x 11 K microarray, developed using Agilent's two colour 60 mer SurePrint™ ink jet synthesis platform and contained all the genes in the J2315 genome as well as some control strains (see Chapter 2, section 2.14). The array and labelling procedures had been validated previously (Leiske *et al.*, 2006). Therefore, *B. cenocepacia* J2315 was a good model to use, due to the extensive

genome data available and the availability of a validated, commercially produced microarray.

5.15 – Aims

The aim of this investigation was to determine genes involved in chlorhexidine resistance, by (i) Developing growth conditions to examine the effect of sub MIC levels of chlorhexidine on *B. cenocepacia* J2315 and (ii) performing a microarray experiment to look for genes with altered levels of expression under these conditions. These genes can then be characterised further and their role in biocide resistance determined.

5.2 Results

5.21 J2315 growth curves and RNA extraction

Preliminary experiments were conducted to select the best concentration of chlorhexidine to use. Ideally exposure to the in use concentration of chlorhexidine would be used, however these concentrations are usually bactericidal thus rapid killing would occur and very few interesting genes expressed. Bacteria are often exposed to sub lethal concentrations of biocides, particularly in clinical situations where disinfectants are employed regularly. Therefore a microarray on sub MIC values would show altered expression in genes that may aid bacterial survival in chlorhexidine. Originally, J2315 was grown in the presence of 10 µg/ml of chlorhexidine. However, with this concentration J2315 took over 10 hours to reach mid log phase, thus it was decided to use 5 µg/ml of chlorhexidine. J2315 exposed to 5 µg/ml of chlorhexidine took 8 hours to reach mid log phase (Figure 5.3), compared to six hours when grown in LB broth. Once mid log phase was reached, cells were harvested and RNA was extracted and concentration determined.

5.22 Microarray performance

Four microarray experiments were carried out, four technical replicates and two biological replicates. The transcriptomic profiles produced in the four replicates were excellent, although the second two arrays had a greater coverage of the slide

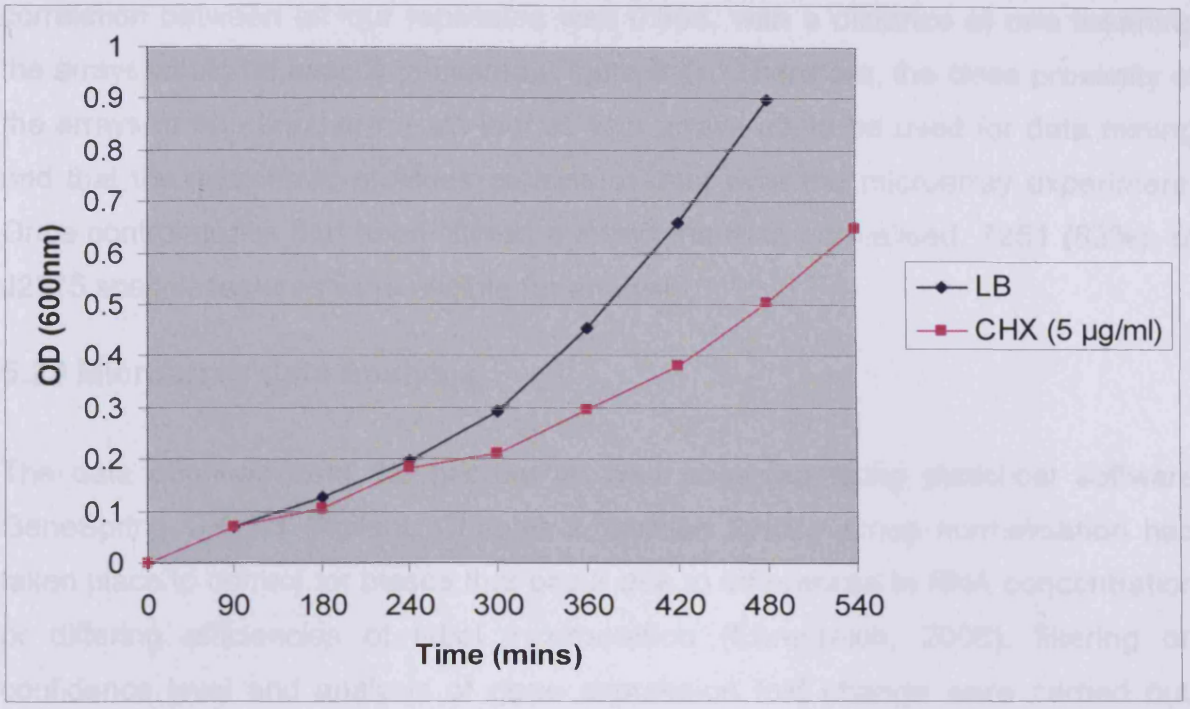


Figure 5.3 Growth curves for J2315 grown in LB broth (blue) and grown in the presence of 5 µg/ml chlorhexidine (pink).

B. cenocepacia J2315 reached mid log phase after 6 hours of growth with approximately 3 hour lag phase. In the presence of chlorhexidine, although a similar lag phase was seen, mid log phase took approximately 2 hours longer to reach mid log phase (8 hours). Once mid log phase was reached (designated as an OD reading of 0.5) bacterial cells were harvested for expression analysis.

with a more even hybridisation than the first two arrays. The array to array correlation between all four replicates was 0.898, with a distance of one meaning the arrays would be exactly the same (Figure 5.4). Therefore, the close proximity of the arrays to one another meant that all four arrays could be used for data mining and that the microarray provides consistent data over the microarray experiment. Once control genes had been filtered out and the data normalised, 7251 (83%), of J2315 specific features were eligible for analysis.

5.23 Microarray data analysis

The data obtained from the microarray was analysed using statistical software GeneSpring GX 7.3 (Agilent, Chapter 2, section 2.16). Once normalisation had taken place to correct for biases that occur due to differences in RNA concentration or differing efficiencies of label incorporation (Ehrenreich, 2006), filtering on confidence level and analysis of gene expression fold change were carried out. This produced a reduced list of significantly changed genes to work with. Filtering on confidence level $p < 0.05$ reduced the list of significantly altered genes to 339. In GeneSpring the data can be represented in various formats, including line graphs and block diagrams. One signifies no change in expression in both conditions (yellow colour) and anything above one represents genes up-regulated in response to chlorhexidine (red colour) and below one indicates genes down-regulated in response to chlorhexidine (blue colour) (Figure 5.5).

Fold change differences were looked for by filtering on the $p<0.05$ gene list. A greater than two fold change gave a gene list of 32 up-regulated genes and 41 down-regulated genes. Although a two fold threshold is fairly low, to further prevent missing false negatives, the threshold was lowered even further to a > 1.5 fold change. Whilst this may seem an insignificant change, it has been seen for efflux proteins, that a change as low as 1.3 fold change in expression has a dramatic effect on the activity of the pump (Eaves *et al.*, 2004). Therefore this data was included in the original analysis and produced a list of 98 up-regulated and 76 down-regulated genes (Table 5.1 and 5.2). These genes were spread across the genome of J2315 and often clusters of genes were seen to be affected. As

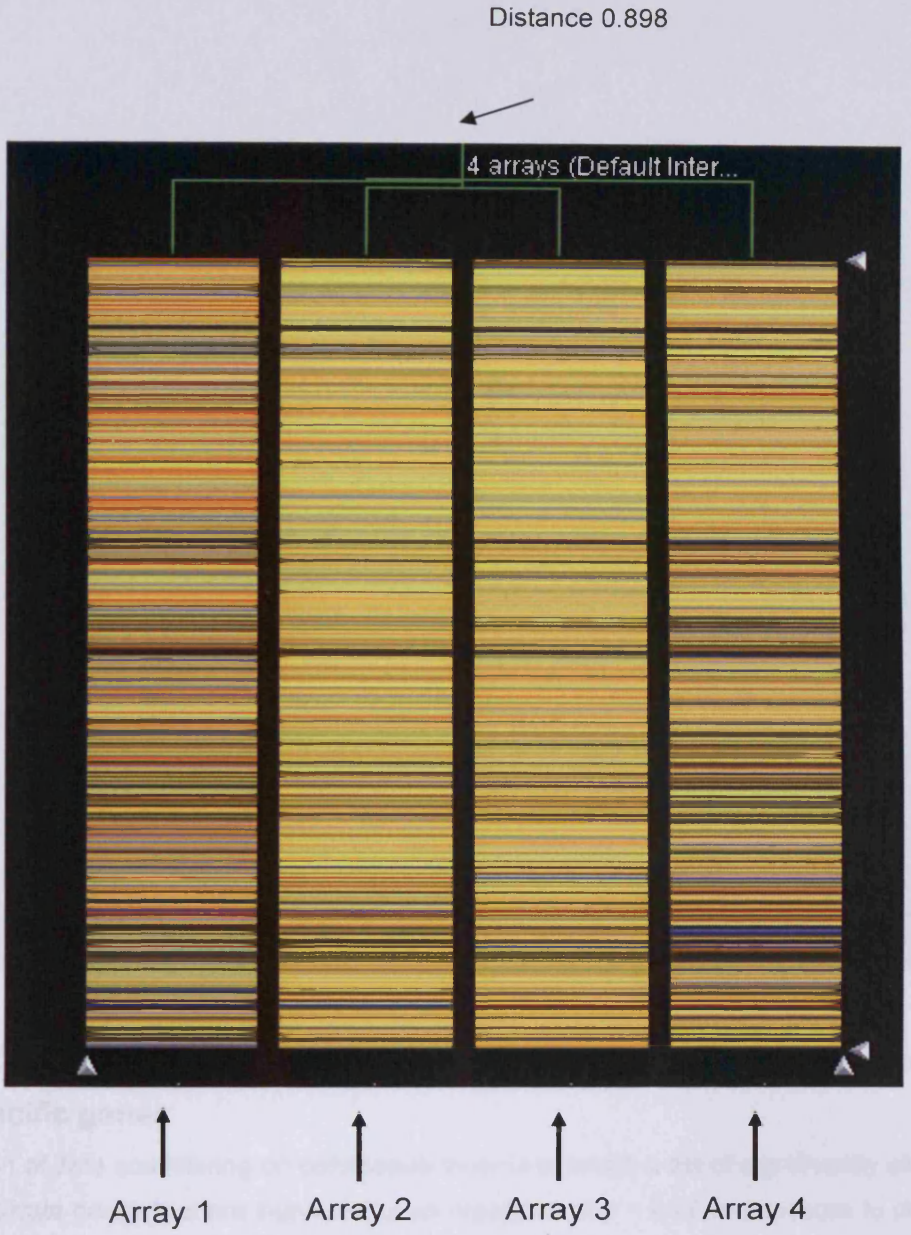


Figure 5.4 Condition tree analysis of all four replicates showing an 0.898 array to array correlation

Condition tree analysis to compare reproducibility of the four microarray replicates. The array to array correlation was 0.898, with a distance of 1 representing arrays that are exactly the same. Due to the close proximity of the four microarrays, all of replicates were included in further analysis.

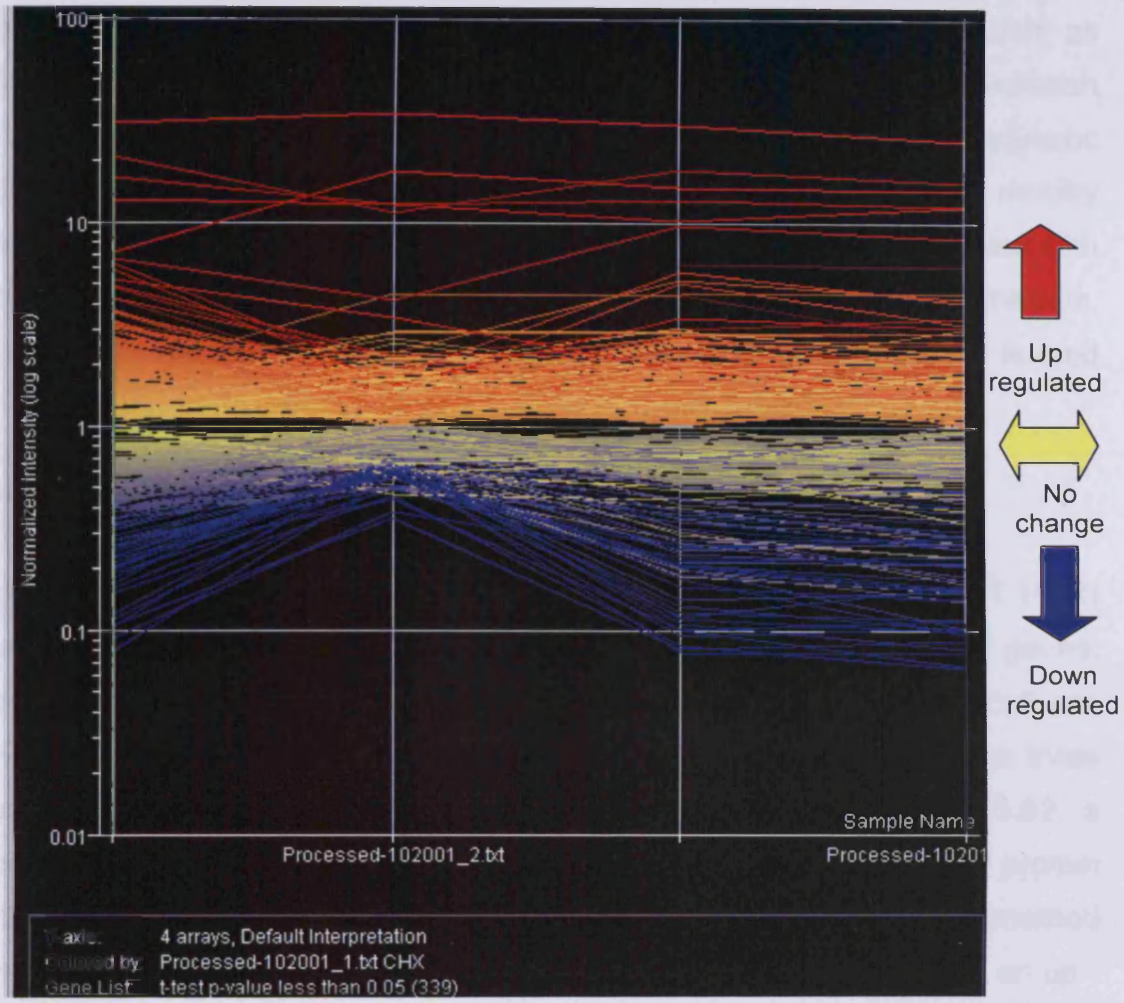


Figure 5.5 Filtering on confidence level p<0.05 gives 339 genes out of 7251 J2315 specific genes

Normalisation of data and filtering on confidence level to produce a list of significantly altered genes. Red lines indicate genes that are significantly up-regulated at $p < 0.05$ in exposure to chlorhexidine. Blue lines indicate genes that are significantly repressed at $p < 0.05$ in response to chlorhexidine and yellow lines indicate genes with no alteration in gene expression in response to chlorhexidine.

expected many of the genes up-regulated were involved in efflux, such as resistance nodulation division pumps. Genes involved with transport and secretion were also up- regulated, which may also be involved in the removal of cytotoxic compounds from the cell. Down-regulated genes were mostly involved in motility and chemotaxis, with only 10 genes (13%) of the 76 repressed genes involved with other processes. Two up-regulated genes were selected for further analysis, BCAM 0924, a putative response regulator and BCAL 2353 a transport related gene.

5.24 Validating microarray data

To validate the microarray data, semi quantitative reverse transcriptase (RT PCR) and quantitative Real Time PCR (QPCR) was carried out on a selection of genes. The growth curve experiments and RNA harvesting was repeated on fresh cultures before validation was performed. Semi quantitative PCR was performed on three up-regulated genes, a response regulator (BCAM 0924), fold change 15.92, a secretion protein (BCAM 0927), fold change 13.14 and a transport related protein (BCAL 2353), fold change 3.453 (primers Table 2.4). The semi quantitative method compares the cycle with which the product appeared in both conditions. In an up - regulated gene you would expect a product to appear earlier. For comparison, a housekeeping gene, *phaC* (BCAL 1861) was selected which had a constant level of expression in both conditions. In the control experiment, the product appears after 30 cycles in both conditions (Figure 5.6), indicating that there is a constant level of expression and that cDNA concentrations are not affecting the PCR outcome. For the three up-regulated genes, it was seen that in each experimental condition (Figure 5.6), the product appears earlier compared to the control condition. BCAL 2353 appears after 25 cycles compared to 30 cycles in the control condition. BCAM 0924 and BCAM 0927 have very low intensities in the control condition, but it can clearly be seen that the product is appearing at 30 cycles compared to 35 cycles in BCAM 0924 and 35 cycles compared to 40 cycles in BCAM 0927. This indicates that the microarray experiment produced accurate results and that transcription of these three genes was genuinely up-regulated in response to chlorhexidine.

Table 5.1 Up-regulated genes in J2315 exposed to Chlorhexidine (P<0.05, >1.5 fold change)

Gene Name	Fold change	Function
BCAL0010	2.6	phenylalanine-4-hydroxylase
BCAL0042	1.6	bifunctional PutA protein [includes: proline
BCAL0057	1.8	putative membrane protein
BCAL0355	1.7	putative transmembrane protein (fragment)
BCAL0428	1.6	putative tRNA modification GTPase
BCAL0500	1.9	ATP-dependent Hsl protease ATP-binding subunit
BCAL0659	2.3	conserved hypothetical protein
BCAL0660	1.9	biotin carboxylase
BCAL0661	2.3	putative biotin-binding protein
BCAL0662	1.9	LamB/YcsF family protein
BCAL0874	1.8	putative membrane protein
BCAL0961	1.7	putative lipoprotein
BCAL1664	1.5	conserved hypothetical protein
BCAL1665	1.7	conserved hypothetical protein
BCAL1689	1.5	conserved hypothetical protein
BCAL1725	1.55	precorrin-8X methylmutase
BCAL1726	1.7	putative oxidoreductase
BCAL1808	1.7	putative membrane protein
BCAL1853	1.5	putative cystathionine beta-lyase
BCAL1857	11.7	putative membrane protein
BCAL1858	12.7	conserved hypothetical protein
BCAL1919	1.8	ClpB heat-shock protein
BCAL1938	1.5	family C40 unassigned peptidase
BCAL1952	1.8	conserved hypothetical protein
BCAL2022	1.9	conserved hypothetical protein
BCAL2023	1.5	putative membrane protein
BCAL2024	1.5	large-conductance mechanosensitive channel
BCAL2116	1.5	putative acyl-CoA-binding protein
BCAL2352	3.3	putative carbonic anhydrase
BCAL2353	3.4	putative transport-related membrane protein
BCAL2370	2.6	putative membrane protein
BCAL2420	1.6	putative depolymerase/histone-like protein
BCAL2441	1.8	conserved hypothetical protein
BCAL2442	1.692	chaperone protein HtpG
BCAL2451	2.7	putative membrane protein
BCAL2462	1.6	putative RNA polymerase sigma factor, sigma-70
BCAL2472	1.7	alpha,alpha-trehalose-phosphate synthase
BCAL2607	1.6	putative exported protein
BCAL2820	1.8	outer membrane efflux protein
BCAL2821	2.1	putative RND family acriflavine resistance
BCAL2822	2.1	putative RND family acriflavine resistance
BCAL2823	2.0	TetR family regulatory protein
BCAL2828	1.9	putative exported protein
BCAL3003	2.2	hypothetical protein
BCAL0010	2.6	phenylalanine-4-hydroxylase
BCAL0042	1.6	bifunctional PutA protein [includes: proline
BCAL0057	1.8	putative membrane protein
BCAL0355	1.7	putative transmembrane protein (fragment)
BCAL0428	1.6	putative tRNA modification GTPase
BCAL0500	1.90	ATP-dependent Hsl protease ATP-binding subunit
BCAL0659	2.3	conserved hypothetical protein
BCAL0660	1.9	biotin carboxylase

Table 5.1 cont

Gene Name	Fold change	Function
BCAL3148	1.9	conserved hypothetical protein
BCAL3152	1.8	putative RNA polymerase sigma factor
BCAL3153	1.6	putative lipoprotein
BCAL3524	1.6	putative general secretory pathway protein
BCALr2006	1.8	tRNA Leu anticodon TAG, Cove score 75.32
BCAM0025	1.7	putative membrane protein
BCAM0085	3.1	putative regulatory protein - TetR family
BCAM0269	1.7	hypothetical protein
BCAM0301	1.6	putative membrane protein
BCAM0398	1.5	hypothetical protein
BCAM0459	2.2	cysteine desulfurase
BCAM0476a	1.5	hypothetical protein
BCAM0493	1.7	putative membrane protein
BCAM0893	1.6	putative membrane protein
BCAM0924	15.9	putative response regulator
BCAM0925	8.0	putative outer membrane protein OprM precursor
BCAM0927	13.1	putative secretion protein - HlyD family
BCAM1010	2.3	putative UTP-glucose-1-phosphate
BCAM1017_J_1	1.7	formate dehydrogenase, major subunit
BCAM1239	1.7	hypothetical protein
BCAM1411	1.5	putative short-chain dehydrogenase
BCAM1726	1.8	putative exported protein
BCAM1742	2.9	putative exported protein
BCAM2052	1.7	putative type III secretion system protein
BCAM2053	2.0	putative type III secretion system protein
BCAM2073	1.8	conserved hypothetical protein
BCAM2152	1.6	putative transmembrane protein
BCAM2400b	2.3	putative exported protein
BCAM2418	1.5	putative membrane-anchored repetitive
BCAM2444	2.3	putative exported protein
BCAM2487	2.0	conserved hypothetical protein
BCAM2562	1.6	putative succinate-semialdehyde dehydrogenase
BCAM2623	1.8	conserved hypothetical protein
BCAMr0727	1.8	tRNA Pseudo anticodon GAA, Cove score 36.58
BCAS0018	1.5	putative transcriptional regulator, MarR family
BCAS0081	6.8	ABC transporter
BCAS0082	2.1	putative exported protein
BCAS0083	3.5	putative transcriptional regulator - TetR
BCAS0167	1.7	squalene-hopene cyclase
BCAS0203	1.6	putative membrane protein
BCAS0219	1.6	putative exported protein
BCAS0221	1.5	putative ABC transporter (pseudogene)
BCAS0223	1.5	possibly involved in production of antifungal
BCAS0235	1.5	putative two-component response regulator
BCAS0246	1.6	conserved hypothetical protein
BCAS0412	1.6	putative membrane protein

Gene name in bold = > 2 fold change

Underlined = neighbouring genes

Table 5.2 Down-regulated genes in J2315 exposed to Chlorhexidine (P<0.05, >1.5 fold change)

Gene Name	Fold change	Function
BCAL0110	2.8	putative aminotransferase, O-antigen related
BCAL0111	2.7	putative TPR repeat protein
BCAL0112	4.8	conserved hypothetical protein
BCAL0113	7.2	flagellar hook-associated protein
BCAL0114	7.8	flagellin
BCAL0124	1.5	flagellar regulon master regulator subunit FlhD
BCAL0126	2.4	chemotaxis protein MotA
BCAL0127	2.3	chemotaxis protein MotB
BCAL0128	3.4	chemotaxis two-component response regulator
BCAL0129	3.4	chemotaxis two-component sensor kinase CheA
BCAL0130	1.8	chemotaxis protein CheW
BCAL0132	2.3	chemotaxis protein methyltransferase
BCAL0133	3.7	putative chemotaxis protein
BCAL0134	4.5	chemotaxis protein-glutamate methylesterase
BCAL0135	2.7	chemotaxis protein CheY2
BCAL0136	2.7	chemotaxis protein CheZ
BCAL0137	1.9	conserved hypothetical protein
BCAL0142	2.7	flagellar biosynthesis protein FlhF
BCAL0143	2.9	flagellar biosynthesis protein FlhG
BCAL0144	2.5	RNA polymerase sigma factor for flagellar
BCAL0349	1.5	putative outer membrane protein
BCAL0350	1.6	conserved hypothetical protein
BCAL0520	4.0	putative flagellar hook-length control protein
BCAL0521	3.1	flagellar fliJ protein
BCAL0523	2.7	flagellar assembly protein
BCAL0524	1.8	flagellar motor switch protein
BCAL0525	2.2	flagellar M-ring protein
BCAL0527	4.2	flagellar protein
BCAL0528	1.7	conserved hypothetical protein
BCAL0561	2.0	putative flagella synthesis protein
BCAL0562	3.4	putative negative regulator of flagellin
BCAL0564	4.2	putative flagellar basal-body rod protein
BCAL0565	5.9	flagellar basal-body rod protein
BCAL0566	4.7	putative basal-body rod modification protein
BCAL0567	5.2	putative flagellar hook protein
BCAL0568	6.8	flagellar basal-body rod protein
BCAL0569	5.1	flagellar basal-body rod protein
BCAL0570	4.3	flagellar L-ring protein precursor
BCAL0571	3.9	flagellar P-ring protein precursor
BCAL0572	3.9	putative peptidoglycan hydrolase
BCAL0575	2.97	conserved hypothetical protein
BCAL0576	4.9	putative flagellar hook-associated protein
BCAL0577	6.2	putative flagellar hook-associated protein
BCAL0910	1.5	putative PhoH-family protein
BCAL1063	1.6	succinylarginine dihydrolase
BCAL1069	1.6	putative cyclic-di-GMP signaling protein
BCAL1213	2.2	2-oxoisovalerate dehydrogenase beta subunit
BCAL1252	1.5	putative proline/betaine transporter
BCAL1262	1.5	carbamoyl-phosphate synthase large chain
BCAL1668	1.7	putative amino acid transport system, exported
BCAL1859	1.6	putative dihydrofolate reductase
BCAL2682	1.7	putative sulfate adenylyltransferase subunit 2

Table 5.2 cont

Gene Name	Fold Change	Function
BCAL2905	2.1	hypothetical protein
BCAL3244	1.5	putative glycosyl transferase
BCAL3336	1.6	bifunctional purine biosynthesis protein
BCAL3502	1.5	flagellar biosynthetic protein
BCAL3505	5.3	probable flagellar motor switch protein
BCAL3506	1.5	flagellar motor switch protein FliM
BCALr2994	1.5	tRNA Thr anticodon CGT, Cove score 87.03
BCAM0548	1.8	60 kDa chaperonin (protein cpn60) (groel
BCAM0987	2.0	putative flagellar basal body rod protein
BCAM1503	4.4	putative methyl-accepting chemotaxis protein
BCAM1744	3.3	putative exported peptidase
BCAM2193	1.6	putative 3-hydroxyisobutyrate dehydrogenase
BCAM2342	1.6	putative betaine aldehyde dehydrogenase
BCAM2395	1.6	putative dehydrogenase/oxidoreductase protein
BCAM2564	3.7	putative aerotaxis receptor
BCAM2837_J_0	1.7	putative response regulator, pseudogene
BCAM2837_J_1	2.5	putative response regulator, pseudogene
BCAS0130	1.6	putative ABC transporter substrate-binding
BCAS0520	1.9	hypothetical phage protein
BCAS0521	1.8	hypothetical phage protein
BCAS0523	1.7	hypothetical phage protein
BCAS0524	1.9	hypothetical phage protein
BCAS0717	1.5	hypothetical protein
fliE	3.1	fliE

Bold gene name = > 2 fold change

Underlined = neighbouring genes

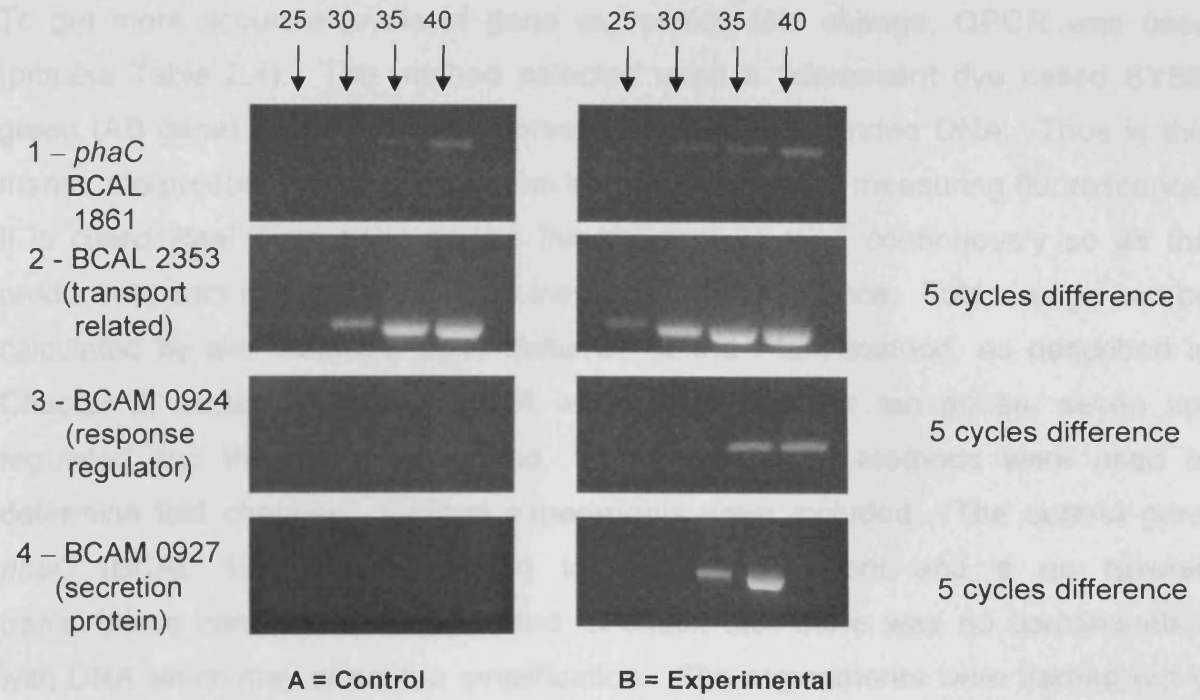


Figure 5.6 Validation of microarray data by semi quantitative PCR

Results of semi quantitative PCR on cDNA derived from *B. cenocepacia* grown in A - control conditions (LB broth only) and B experimental conditions (5 µg/ml chlorhexidine). The number of cycles at which the product appeared are labelled above the panel.

To get more accurate levels of gene expression fold change, QPCR was used (primers Table 2.4). The method selected used a fluorescent dye called SYBR green (AB gene) that binds and fluoresces to double stranded DNA. Thus is this manor, the product can be detected as it is synthesised by measuring fluorescence. It is called Real Time PCR as the fluorescence is read continuously so as the product appears it is recorded by an increase in fluorescence. Fold change can be calculated by two methods, delta delta C_T or the Pfaffl method, as described in Chapter 2, section 2.10.2). QPCR was carried out for ten genes, seven up-regulated and three down-regulated. Both calculation methods were used to determine fold changes. Control experiments were included. The control gene *phaC* (BCAL 1861) was included in every experiment and a no reverse transcriptase control was incorporated to check that there was no contamination with DNA which may effect the amplification. The experiments were carried out in triplicate so an average could be calculated (Figure 5.7). To check that the right product has been amplified and that all replicates were the same a melting curve analysis was carried out (Figure 5.8). All products should have the same melting point, so a melting curve as observed in Figure 5.8 suggests that the QPCR results are correct. The QPCR calculations corroborated the microarray data, with all genes showing the correct up or down regulation (Table 5.3).

5.3 Discussion

5.31 Whole genome analysis using microarrays compared to traditional methods

In this investigation a microarray experiment was conducted to determine the effects of sub inhibitory levels of chlorhexidine on the gene expression in the model *B. cenocepacia* pathogen J2315. Chlorhexidine is a disinfectant often used in clinical situations, but is often found to be contaminated by many species of bacteria including members of the Bcc (Oie and Kamiya, 1996). Several Bcc species in particular cause many problems due to contamination of both clinical and domestic products, which may be the source of infection in vulnerable patients (Jimenez, 2007). Consequently it is important to understand the underlying mechanisms of resistance to help prevent infections occurring and possibly

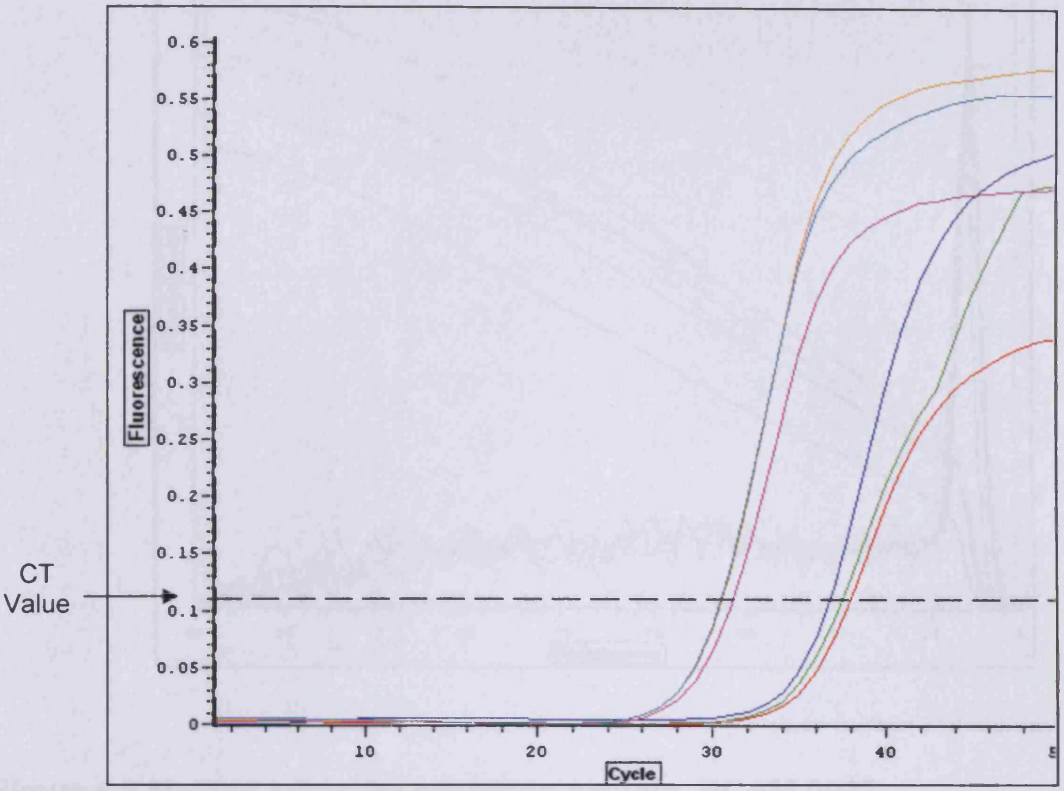


Figure 5.7 Real time PCR results for secretion protein BCAM 0927

QPCR screen of the secretion protein BCAM 0927. Red, green and blue lines represent replicates of the control condition and orange, turquoise and purple lines represent replicates of the experimental condition (exposure to chlorhexidine). Products appeared at approximately 30 cycles in the experimental conditions compared to 36 cycles in the control condition, corresponding to an up regulation of this gene. The CT value is an arbitrary threshold level set by the experimenter, which allows cycle number to be determined.

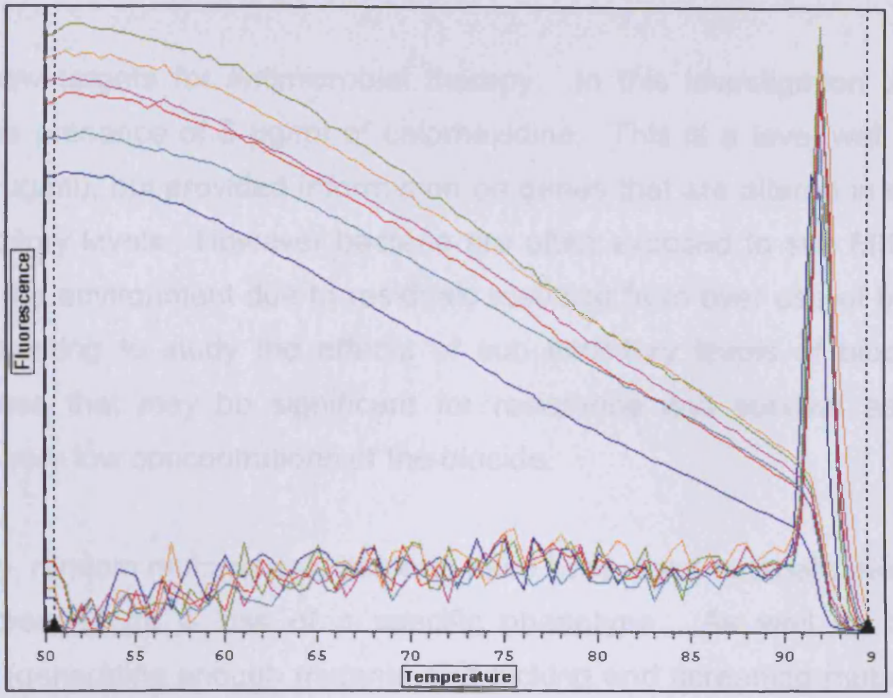


Figure 5.8 Melting curve for secretion protein, BCAM 0927

Melting curves are performed after the QPCR run has taken place as a quality control step. Products of the same size should have similar melting points as shown in this graph. Lower temperature melting points may indicate the formation of primer dimers and suggest the formation of an incorrect product.

elucidate new targets for antimicrobial therapy. In this investigation J2315 was grown in the presence of 5 µg/ml of chlorhexidine. This is a level well below the MIC (>100 µg/ml), but provided information on genes that are altered in expression to sub inhibitory levels. However bacteria are often exposed to sub MIC levels of biocides in the environment due to residuals resulting from over use of biocides. It is also interesting to study the effects of sub inhibitory levels of biocides as it reveals genes that may be significant for resistance and survival as they are affected by very low concentrations of the biocide.

Traditionally, random mutagenesis screens have been used to create mutant banks that are screened for a loss of a specific phenotype. As well as being time consuming (generating enough mutants and picking and screening mutants), there are limitations with the screening process as they may miss some genes that play a role due to no mutant being generated and it may be difficult to see the interactions of certain genes in pathways. The microarray provides data on all genes that are altered in expression and can rapidly distinguish the role they play in the experimental conditions. As well as providing functional information (for annotated genomes), a microarray experiment also provides data on genes that are not only switched on due to the environmental stimulus but also repressed. Clusters of genes are also revealed, indicating operons which are altered in expression, thus giving an in depth view of the bacterium's response in relation to the stimulus, in this case chlorhexidine.

A microarray experiment has the added bonus of allowing the comparison of two conditions simultaneously, generating a vast amount of data (Ehrenreich, 2006). Expression profiling using microarrays has the capacity to produce a large bank of data. However, this is also one of the drawbacks of using a microarray. Extensive data mining needs to be carried out, which can be time consuming and the sheer volume of data produced can make it difficult to select which genes to analyse further. Other issues that arose from the microarray experiment are the cost of production and reagents for the experiments. Specialist equipment, such as the scanner are also required and extensive technical training is also needed

Table 5.3 Confirmation of microarray data by real time PCR

Gene	Putative Function	Microarray fold change	Delta delta CT method	Pfaffl method	Up / Down regulated
BCAL 0114	Flagellin	-7.8	-238.1	-15873	↓
BCAL 0126	Chemotaxis proton MotA	-2.4	-3.3	-1.8	↓
BCAL 0143	Flagellar biosynthesis protein FilG	-2.9	-8.9	-5.9	↓
BCAL 2353	Transport related protein	3.4	11.7	7.4	↑
BCAL 2370	Membrane protein	2.6	2.9	7.7	↑
BCAM 0924	Response regulator	15.9	10.6	8.1	↑
BCAM 0925	Outer membrane protein OprM	8.0	106.4	62.7	↑
BCAM 0927	Secretion protein HlyD	13.1	150.5	71.2	↑
BCAS 0081	ABC transporter	6.8	10.2	37.9	↑
BCAS 0083	Transcriptional regulator - TetR	3.5	34.6	7.7	↑

Fold change as determined by delta – delta method and Pfaffl method (Chapter 2, 2.10.2). Delta – delta method uses the control gene (*phaC*) for normalisation. The Pfaffl method also takes into account the efficiency of the reaction, determined by a standard calibration graph.

(Ehrenreich, 2006). Nonetheless, microarray experiments are being used more frequently and provide the researcher with a comprehensive whole genome view of the expression profile of the bacterium in the experimental conditions. In this investigation the microarray produced a list of genes with altered expression in the presence of chlorhexidine. These genes were distributed across the genome of *B. cenocepacia* J2315. The largest proportion of altered genes was found on the large chromosome which was not surprising as this chromosome contains most genes, including essential housekeeping genes. For up-regulated genes, 57 were found on the large chromosome, 29 on the second chromosome and 12 on the small chromosome. Down-regulated genes were distributed as follows: 59 on the large chromosome, 10 on the second chromosome and 6 on the small chromosome.

5.32 Validation of microarray data

The *B. cenocepacia* J2315 microarray had only been recently designed, therefore to fully validate it the data was confirmed by both reverse transcriptase PCR and real time PCR (QPCR). Three up-regulated genes were selected for reverse transcriptase PCR and ten genes for QPCR. These genes were all compared to a housekeeping gene which expressed consistently in both condition (*phaC*). The genes were selected to include: up-regulated and down-regulated genes, those from different functional classes and those with a range of fold changes. Reverse transcriptase PCR on three up-regulated genes indicated that the microarray data was correct (Figure 5.6). All three gene products appeared in the experimental condition (5 µg/ml of chlorhexidine), at least five cycles earlier than in the control condition. The *phaC* housekeeping gene appeared at 30 cycles in both conditions demonstrating that equal concentrations of cDNA was added in both conditions, so that the results were not biased.

Reverse transcriptase PCR is a semi quantitative method of validating the microarray results, so to further confirm these results a quantitative method can be used. Real time PCR measures the production of double stranded DNA by scanning for fluorescence and gives a numerical value that allows fold change to be calculated. There are a number of ways to calculate fold change. The delta delta CT method is a quick method that assumes 100% efficiency (see Chapter 2 for

equation). Although this is not the most accurate method it still gives a close estimation of fold change. Alternatively the Pffaffl method can be used (Pfaffl, 2001). This takes into account the efficiency of the reaction by creating a standard curve and then calculating the efficiency of the reaction from the slope. Both methods were used in this investigation and compared to the microarray data (Table 5.3). Both methods of calculating fold change in the QPCR experiment gave an appropriate answer and correlated well to one another.

Most of the genes had a fairly consistent fold change across the three separate methods, although the QPCR method generally produced higher fold changes than the microarray experiment. For example a putative ABC transporter had a 6.8 fold change in the microarray, but had a 10.2 and 37.9 fold change from the QPCR analysis. The flagellin gene (BCAL 0114) was the only gene that demonstrated a large discrepancy in fold change between the microarray data and Real time PCR data, although it still confirmed the microarray data by indicating this gene was down-regulated (Table 5.3). Although absolute values were different they still corroborate the microarray data (Table 5.3). Thus the semi quantitative reverse transcriptase and Real time PCR have an excellent correlation with the microarray data, demonstrating that the J2315 microarray was performing well and that data obtained via the microarray could be used for further analysis.

5.33 Up-regulated genes involved with biocide resistance

The microarray experiment produced a list of genes with altered expression in response to chlorhexidine. Filtering at $p<0.05$ with a fold change of > 1.5, gave a list of 90 up-regulated genes. These genes can be spilt into three major categories that encompass genes involved in biocide resistance: (i) efflux proteins, (ii) transport and secretion proteins and (iii) membrane related proteins (Table 5.4).

5.33.1 Efflux pumps

Table 5.4 Major Gene Groups in *B. cenocepacia* involved in biocide resistance

Gene Number	Fold Change	Putative Gene function
Transport / Secretion		
Up-regulated		
BCAL 2353	3.7	Transport related
BCAL 2821	2.1	RND acriflavine resistance
BCAL 2822	2.1	RND acriflavine resistance
BCAM 0924	16.3	Response regulator (efflux)
BCAM0925	7.9	Outer membrane protein OprM precursor
BCAM 0927	13.2	Secretion protein HlyD
BCAM 2053	2.0	Type III secretion system
BCAS 0081	7.1	ABC transporter
Down-regulated (none)		
Regulation and Stress		
Up-regulated		
BCAL 1919	1.8	Heat shock ClpB
BCAL 2823	2.0	TetR regulatory protein
BCAS 0018	1.5	MarR transcriptional regulator
BCAS 0083	3.5	TetR transcriptional regulator
BCAS 0235	1.5	Two component response regulator
Down-regulated (none)		
Motility and Chemotaxis		
Up-regulated (none)		
Down-regulated		
BCAL 0110 – 0114	2.7 – 7.8	Flagellin
BCAL 0126 – 0137#	1.8 – 4.5	Chemotaxis
BCAL 0142 – 0144	2.5 – 2.9	Flagellar biosynthesis
BCAL 0520 – 0528*	1.7 – 4.2	Flagellar assembly
BCAL 0561 – BCAL 0577+	1.5 – 6.8	Flagellar assembly / proteins
Other		
Up-regulated		
BCAL 1857	11.7	Membrane associated protein
BCAL 2370	2.6	Membrane associated protein
BCAL 2451	2.7	Membrane associated protein
Down-regulated (none)		

- excluding BCAL 0231, * - excluding BCAL 0522 and 0526, + - excluding BCAL 0563, 0573 and 0574

Efflux pumps are well known devices of antimicrobial resistance and are often associated with multidrug resistance. The most commonly found efflux pump is the RND family, which have a tripartite structure (Piddock, 2006a). The three components are a transporter protein embedded in the inner membrane, a periplasmic accessory protein which links the inner and outer components and the outer membrane protein channel (Figure 5.9) (Poole, 2004). The RND efflux pumps work by the proton motive force, using the proton gradient across the membrane to exchange one hydrogen ion for one drug molecule (Piddock, 2006a). MFS, MATE and SMR pumps also use the proton motive force, where as ABC transporters are driven by the hydrolysis of ATP (Figure 5.9).

Efflux pumps work by the transporter or efflux protein in the inner membrane binding to the substrate, most likely through hydrogen bonding or electrostatic interactions and then transporting them to the external medium via the outer membrane protein channel. This interaction between the efflux protein and the outer membrane is mediated by the periplasmic accessory protein (Piddock, 2006a, Paulsen, 2003). All types of pump apart from MATE pumps have a tripartite structure similar to RND pumps.

Efflux pumps can either be chromosomally encoded or may be transferred on mobile genetic elements. Most bacteria carry more than one efflux pump, which confers resistance to a number of substrates that may be structurally unrelated. An efflux pump may have broad specificity, due to a flexible hydrophobic cavity which can bind unrelated substrates (Paulsen, 2003). A strain is classed as multidrug resistant (MDR) if it is resistant to at least three different classes of antimicrobial such as antibiotics, disinfectants, dyes and detergents (Piddock, 2006a). Many efflux pumps have been identified in bacterial species, especially those that efflux antibiotics, such as the ArcA-ArcB-TolC pump in *E. coli* and MexA-MexB-OprM pump in *P. aeruginosa* (Poole, 2004). Biocides are also inducers of efflux systems. The qac genes efflux quaternary ammonium compounds and can be found in Gram positives (usually plasmid encoded) and Gram negatives (chromosomally encoded). An efflux gene *cepA* has been isolated from *Klebsiella pneumoniae* which confers resistance to chlorhexidine (Fang *et al.*, 2002) and was found to have putative homologs in many other bacterial species indicating that an

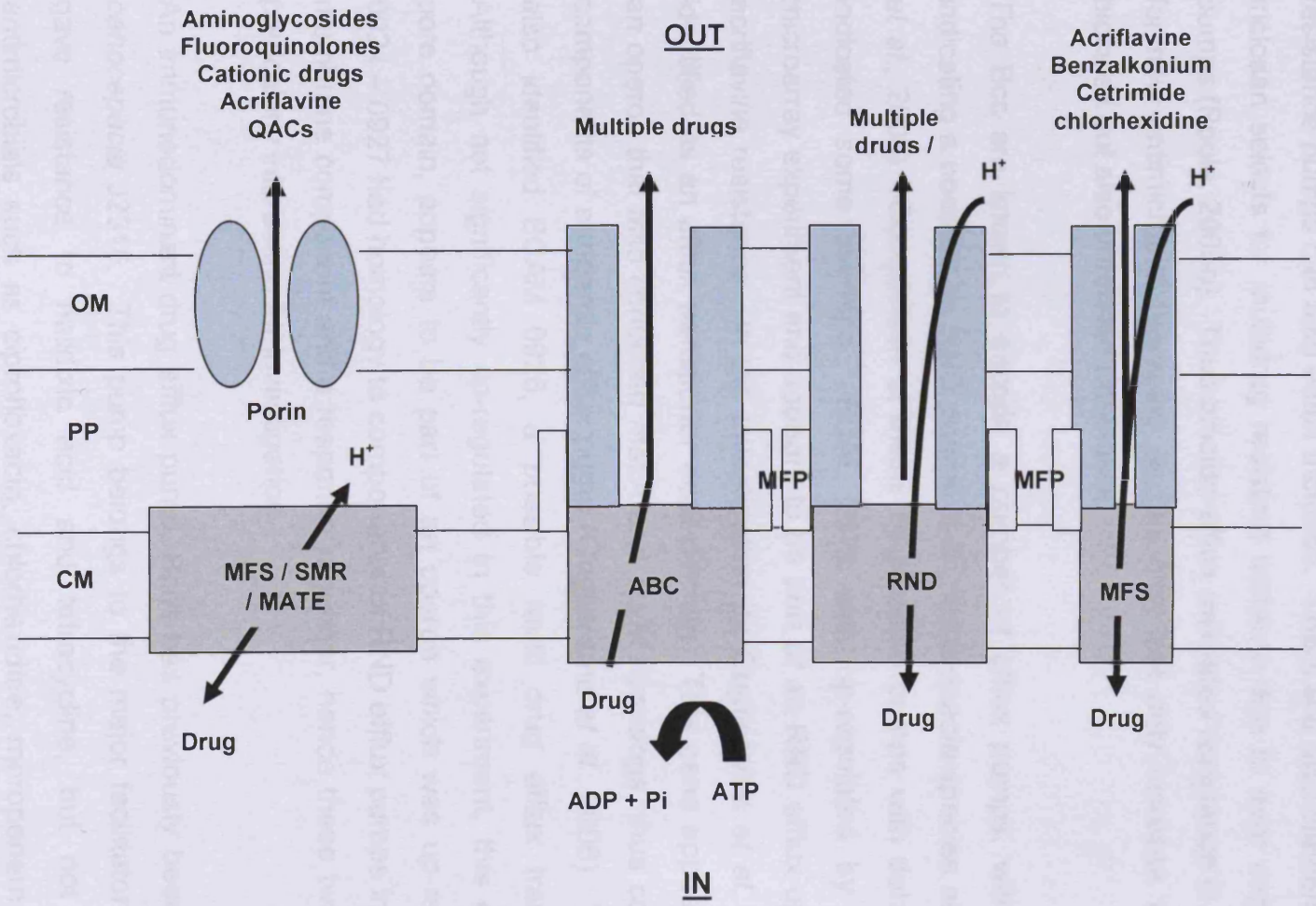


Figure 5.9 Classes of Efflux pumps and their mechanism of action

OM = outer membrane, PP = periplasmic space, CM = cytoplasmic membrane, MFP = membrane fusion protein, MFS = multifacilitator superfamily, SMR – small multidrug resistance, MATE = multidrug and toxic compound extrusion, ABC = ATP binding cassette, RND = resistance nodulation family and QAC = quaternary ammonium compound. Adapted from (Poole, 2004).

chlorhexidine efflux pump may be widely distributed amongst Gram negative pathogenic bacteria (Poole, 2005b). Triclosan resistance has been widely reported and efflux plays a major role (Mima *et al.*, 2007). Many RND antimicrobial resistance pumps can also efflux triclosan. There are also concerns that the use of triclosan selects for multidrug resistant mutants due to over expression of efflux pumps (Poole, 2005b). Thus biocide efflux mediated resistance is a potential target for new antimicrobial therapies as this may not only increase susceptibilities to biocides but also unrelated antibiotics.

The Bcc are known to encode a number of efflux pumps, with a recent study indicating a possible 14 RND pumps in *B. cenocepacia* species alone (Guglierame *et al.*, 2006). Comparison of these 14 possible pumps with data from this study indicated some overlaps. BCAL 2821 was up-regulated by 2.1 fold in this microarray experiment and appears to be part of an RND efflux operon involved in acriflavine resistance. In the investigation by Guglierame *et al.* BCAL 2821 was identified as an efflux transporter *acrB* domain. This gene appeared to be part of an operon that also contained *mexA* and *oprM* homologs, thus contained all three components of a tripartite efflux pump (Guglierame *et al.*, 2006). This investigation also identified BCAM 0926, a possible multi drug efflux transporter protein. Although not significantly up-regulated in this experiment, this efflux transporter pore domain, appears to be part of an operon which was up-regulated. BCAM 0924 – 0927 had homology to components of RND efflux pumps including the outer membrane component and a response regulator, hence these two pumps may be particularly interesting for investigation.

An immunodominant drug efflux pump, BcrA has previously been described in *B. cenocepacia* J2315. This pump belongs to the major facilitator superfamily and gave resistance to nalidixic acid and tetracycline, but not to many other antimicrobials such as ciprofloxacin, chlorhexidine, meropenem, gentamicin and detergents such as Tween (Wigfield *et al.*, 2002). Arguably the ceo operon (BCAM 2549-2552, 2554) is the most well characterised efflux pump in *B. cenocepacia*. This is a RND efflux cluster that can remove chloramphenicol, trimethoprim and ciprofloxacin and is induced by salicylate, an important siderophore (Nair *et al.*, 2004). Interestingly, the *ceo* operon or the BcrA pump was not altered in

expression in response to chlorhexidine. The microarray indicated that other less well understood pumps such as the two RND operons up-regulated in this experiment (BCAM0924 – 0927 and BCAL 2820 – 2823) play a larger role in disinfectant resistance.

Also up-regulated in the microarray experiment was an ABC transporter, BCAS 0081. ABC transporters are types of efflux pumps that use ATP to actively efflux compounds. ABC transporters are involved with the removal of cytotoxic compounds, including antimicrobials. They have a high affinity for binding antimicrobials and thus contribute to multidrug resistance (Sheps and Ling, 2007). The ABC transporter may be an interesting gene to select for further analysis as to our knowledge, an ABC transporter involved with biocide resistance in the Bcc has not yet been identified.

5.33.2 Secretion and Membrane proteins

Transport and secretion related genes were seen to be up-regulated in this experiment, such as the type three secretion system, BCAM 2052 and BCAM 2053. Type three secretion systems (TTS) are well known virulence factors and have a variety of functions within the bacterium. They deliver excreted proteins to target cells including toxins and other cytotoxic compounds that may aid pathogenesis. They do this via ATP hydrolysis, allowing secretion across the inner membrane (Winstanley and Hart, 2001) Type three secretion systems are linked with the host cell response and are often induced by the host interactions. Type three secretion systems can protect the bacterium from the host's immune system by killing macrophages. They also aid invasion of host cells and transport other proteins involved in virulence, such as elastase, protease, α haemolysin and exotoxins directly into host cells (Glendinning *et al.*, 2004) .

Secretion systems have been identified in members of the Bcc, including type three secretion systems BCAM 2052 and BCAM 2053 (Table 5.1), which are usually found on pathogenicity islands. These two genes are components of a characterised type three secretion system which is possibly involved in pathogenesis but not necessarily invasion of host cells (Holden *et al.*, 2009).

Bacterial strains that have mutations in type three secretion systems have attenuated virulence and are thus easily killed by the host's immune response, implying that TTS are important for the bacterium's survival within the host (Tomich *et al.*, 2003). After exposure to chlorhexidine, a TTS system (BCAM 2052 – BCAM 2053) was up-regulated in *B. cenocepacia* J2315, suggesting that sub inhibitory levels of chlorhexidine are causing an increase in virulence factors that may allow the persistence of Bcc species in the host.

Also observed in this experiment was the up regulation of membrane proteins such as BCAL 1857 and BCAL 2370 (Table 5.1 and 5.4). The outer membrane is associated with antimicrobial resistance due to its limited permeability to many antimicrobials, especially cationic compounds. As previously mentioned (Chapter 5, section 5.1) the Bcc has a membrane composition that lends itself to being particularly resistant to a multitude of compounds. Therefore it is not surprising that in response to chlorhexidine, many membrane proteins are over expressed. Tattawasart *et al.* conducted a study to investigate outer membrane changes in chlorhexidine sensitive and chlorhexidine resistant *P. stutzeri*. They found that chlorhexidine resistant strains had a different outer membrane profile compared to the sensitive strains, with the appearance of three dominant bands, signifying that these bands are being over expressed (Tattawasart *et al.*, 2000). Thus, in *B. cenocepacia* J2315 a similar effect may be happening, with chlorhexidine inducing expression of membrane proteins to prevent chlorhexidine entering the cell. Although studies such as Tattawasart *et al.* have shown changes in the membrane structure in resistant bacteria very few specific genes have actually been implicated in these changes. This study found three membrane proteins up-regulated (Table 5.4) in response to chlorhexidine and could account for membrane profile changes.

5.33.3 Other genes involved in antimicrobial resistance

When bacterial cells are exposed to environmental stress, such as fluctuations in temperature or exposure to antimicrobials they need to adapt rapidly to survive. Many of these adaptive responses are controlled by regulatory proteins, which respond to the stimulus and then modulate transcription and translation of genes

that are required for survival (Ramos *et al.*, 2005). Two interesting transcriptional regulators were observed as up-regulated in response to chlorhexidine, BCAS 0018, a MarR transcriptional regulator (1.5 fold change) and BCAS 0083 and BCAL2823, TetR transcriptional regulators (3.5 and 2 fold change respectively).

The MarR regulator is part of a regulon found in *E. coli* that is involved with multiple antibiotic resistance (multiple antibiotic resistance). The *mar* locus alters gene expression of multiple genes involved in resistance on the chromosome, thus conferring resistance to not only antibiotics, but also organic solvents, oxidative stress agents and disinfectants (Alekshun and Levy, 1999). MarA, an activator is usually repressed by MarR, however when the bacterial cell is exposed to stress, MarR is inactivated and MarA can be over expressed and activate the expression of resistance genes. Resistance is induced by the decrease of influx and increase of efflux of toxic chemicals, hence the expression of membrane proteins are effected and resistance to oxidative stress is achieved by increased expression of enzymes that counteract the damage to the cell (Alekshun and Levy, 1999). The *mar* operon is induced by tetracycline, chloramphenicol and confers resistance to a range of antibiotics including norfloxacin, ampicillin, nalidixic acid and rifampicin. As well as resistance to antibiotics over expression of MarA gives resistance to disinfectants including phenols (chloroxylenol), QACs and triclosan (Alekshun and Levy, 1999). In this experiment, MarR regulatory protein was over expressed in response to sub inhibitory concentrations of chlorhexidine, indicating that it can also be induced by biguanides, however the role MarR plays in resistance may be different in *B. cenocepacia* J2315 as it is the repressor that is being over expressed. The over expression of efflux pumps, such as the RND acriflavine pump and a heat shock protein (BCAL 1919) involved in protection against oxidative stress, suggests that the mar locus may be involved in the regulation of these important resistance genes. There may also be an indication of cross resistance as the *mar* locus is involved in the regulation of a broad spectrum of resistance mechanisms.

The second transcriptional regulator up-regulated was a homolog of TetR, which is part of a family of proteins that control the expression of *tet* genes whose products are involved in tetracycline resistance (Ramos *et al.*, 2005). Like the *mar* operon, the *tet* locus may regulate efflux pumps. In the chlorhexidine experiment, two of the

TetR family regulators were adjacent to genes involved with efflux, BCAL 2820 – 2823, RND efflux and BCAS 0081 – 0083, ABC transporter. Thus this suggests that the TetR family of regulators are intricately involved with efflux regulation and that chlorhexidine is capable of inducing this regulatory system.

5.34 Down-regulated genes are involved in motility and chemotaxis

An interesting observation from the microarray experiment was that the majority of genes repressed after exposure to chlorhexidine were involved with the production of flagellar and chemotaxis proteins (Table 5.4). Many of these genes were adjacent to one another suggesting that whole operons were being repressed in response to chlorhexidine, indicating that chlorhexidine is sensed by the bacterial cell and a global regulation mechanism is employed (Figure 5.10). The down regulation of genes involved in motility leads to the conclusion that this phenomenon must play an important role in bacterial survival to biocides like chlorhexidine, raising many interesting questions. Before these questions can be addressed, the role of flagella and chemotaxis should be discussed.

Flagella biosynthesis is complicated with the hierarchical transcription of over 40 genes usually conforming to the order the proteins are assembled. The coordination of transcription is controlled by master regulons such as FilD (McCarter, 2006). The structure of the flagella comprises of an basal body (containing an MS ring, rod and a periplasmic P ring), the flagellar motor, regulatory switch which is activated by environmental signals, a flagellar hook, flagellar filament, capping proteins and export apparatus (Macnab, 2003) (Figure 5.11). Flagella are associated with host invasion, adhesion, chemotaxis, swarming, biofilm production and interactions with the host defence system. Chemotaxis is the coordinated migration of bacterial cells towards a chemical signal. For example planktonic cells migrate towards nutrients or other cells attached to a surface (Pratt and Kolter, 1998), thus chemotaxis may promote biofilm formation, a lifestyle which allows bacteria to persist in unfavourable environments.

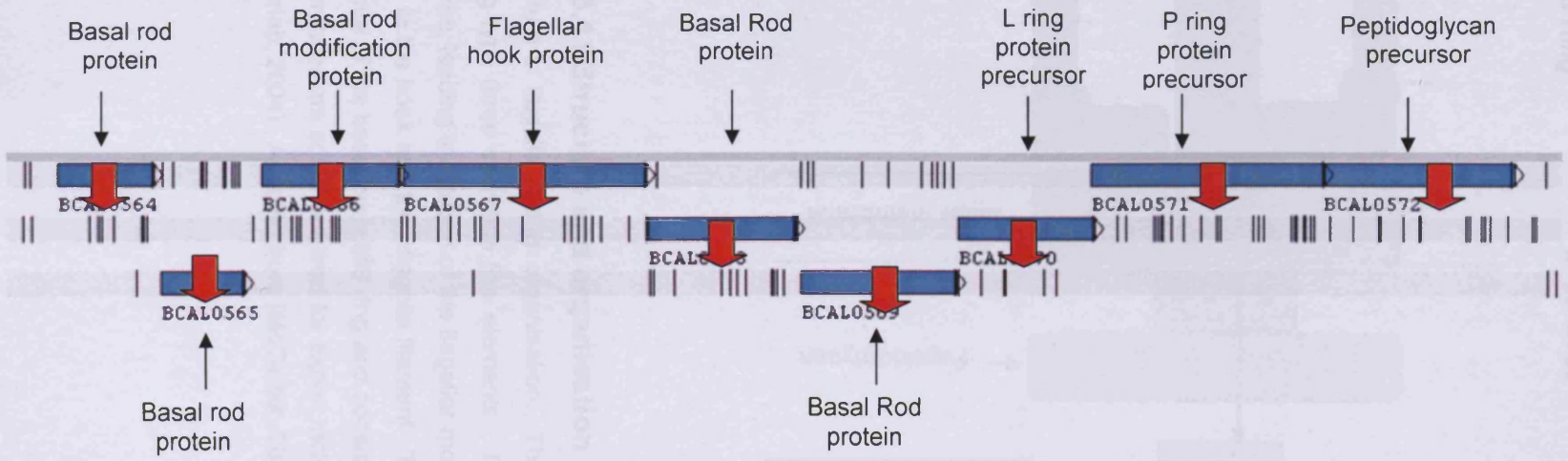


Figure 5.10 Flagellar operon down-regulated in response to chlorhexidine

Artemis view of part of the flagellar operon down-regulated in response to chlorhexidine and their putative functions.

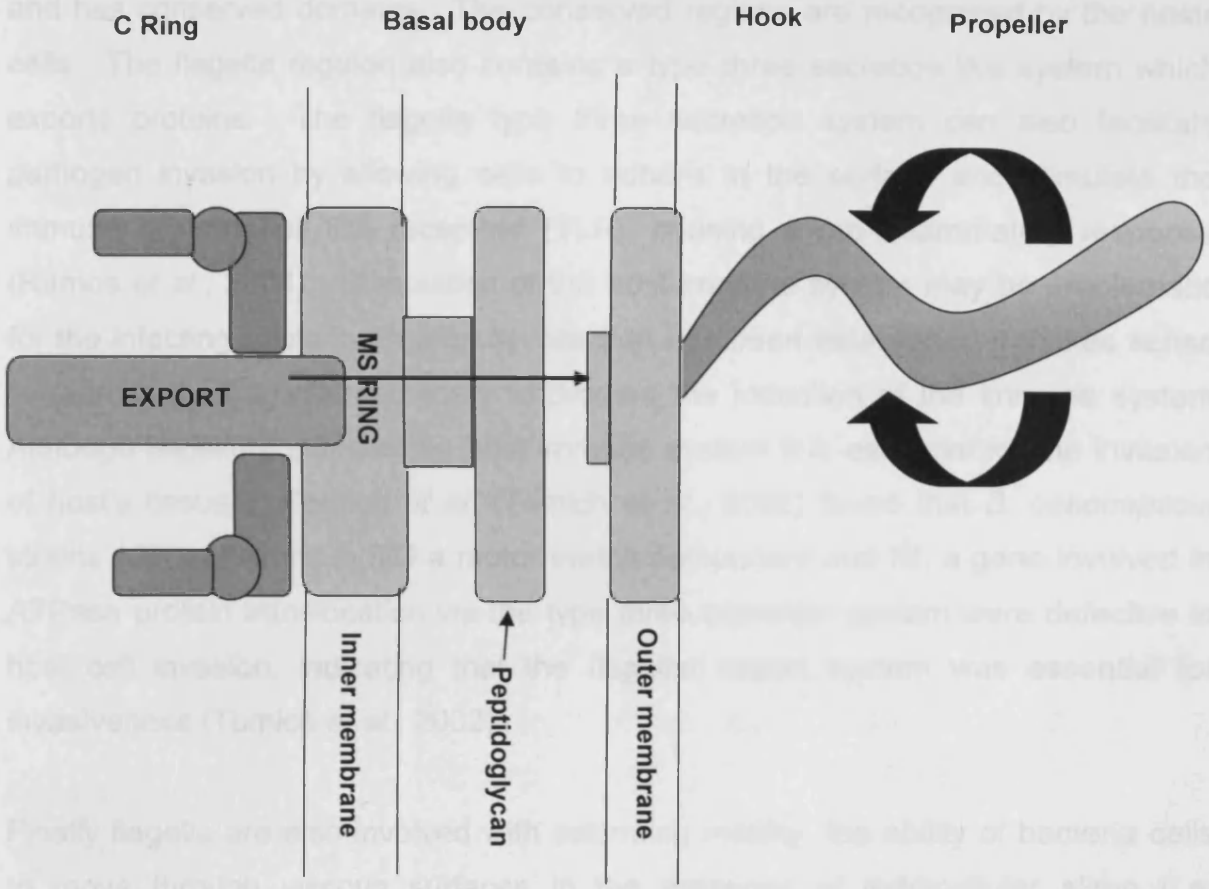


Figure 5.11 Structure and organisation of bacteria flagella.

The structure of flagella and its organisation. The flagella plays a role in both motility and export containing type three secretion like elements. The bacterial flagellum is powered by the proton motive force, leading to rotation of the flagellar motor, which is attached via the MS ring to the basal body rod, to the hook and then flagella filament. The type three secretion export apparatus is found at the centre of the basal body MS ring and consists of ATPase components that drive export. Nine flagellar proteins are solely required for export including FliH, FliI, FliJ, FilA, FilB, FilO, FilP, FilQ and FilR (Macnab, 2004). Adapted from (McCarter, 2006)

Flagellin is the structural protein that forms a major portion of flagellar filaments, and has conserved domains. The conserved regions are recognised by the hosts cells. The flagella regulon also contains a type three secretion like system which exports proteins. The flagella type three secretion system can also facilitate pathogen invasion by allowing cells to adhere to the surface and stimulate the immune system Toll like receptors (TLR), causing a pro-inflammatory response (Ramos *et al.*, 2004). Stimulation of the host immune system may be problematic for the infecting bacteria, thus once infection has been established it makes sense to repress the flagella machinery to prevent the induction of the immune system Although flagellum induces the host immune system it is essential for the invasion of host's tissues. Tomich *et al.* (Tomich *et al.*, 2002) found that *B. cenocepacia* strains with mutations in *filG* a motor switch component and fill, a gene involved in ATPase protein translocation via the type three secretion system were defective in host cell invasion, indicating that the flagellar export system was essential for invasiveness (Tomich *et al.*, 2002).

Finally flagella are also involved with swarming motility, the ability of bacteria cells to move through viscous surfaces in the presence of extracellular slime (i.e. exopolysaccharides) (Daniels *et al.*, 2004). Swarming allows the spreading of biofilms across surfaces and non motile strains appear to have attenuated virulence. Swarming may be regulated by quorum sensing, which is a population dependent response to an environmental stimulus (Daniels *et al.*, 2004). CF patients infected chronically with *P. aeruginosa* tend to have isolates that are non motile and resistant to phagocytosis, making eradication difficult (Mahenthiralingam *et al.*, 1994). Although this has never been described in Bcc species, it appears that a similar, non motile sessile lifestyle is being entered in the presence of chlorhexidine. This could lead to the emergence of resistant strains present in a biofilm that are difficult to treat, thus the regulation of the expression of motility genes after exposure to chlorhexidine should be elucidated to determine novel antimicrobial targets.

5.4 Conclusions

The microarray experiment used in this investigation provided a large bank of data which was mined to determine *B. cenocepacia* genes with altered expression after exposure to 5 µg/ml of chlorhexidine. Prior to data analysis, the microarray was validated by real time PCR methods. The good correlation between quantitative PCR and microarray methods indicated that the microarray was working well and the data obtained could be used for further analysis.

The hypothesis that genes involved in antimicrobial resistance such as efflux pumps and membrane proteins was fulfilled. Two RND efflux pumps were significantly up-regulated (BCAL 2820 – 2823 and BCAM 0924 – BCAM 0927), as well as an ABC transporter (BCAS 0081 – 0083). Other transcriptional regulators involved in antimicrobial resistance and stress were up-regulated (MarR and TetR) and numerous membrane and transport related proteins were also up-regulated (Table 5.1). These genes were the most important in relation to chlorhexidine resistance and two genes, BCAM 0924 (efflux response regulator) and BCAL 2353 (previously un-described transport related gene) were selected for further investigation (Chapter 6).

In addition to determining up-regulated genes, genes that were repressed were also examined. These genes followed a pattern and were involved in the production of flagellar and the process of chemotaxis. Flagella are important virulence factors associated with adhesion, invasion, biofilm production and swarming. It is interesting to note that whole operons appear to be affected by the presence of chlorhexidine, pointing to a global regulation response. To our knowledge this is the first time that a motility repression response has been described in the Bcc, which may be associated with a non motile, biofilm lifestyle that leads to biocide resistance. This phenomenon was investigated further (Chapter 6) as a potential target for novel antimicrobial targets.

Chapter 6.0

Characterising the function of *B. cenocepacia* J2315 genes regulated by exposure to chlorhexidine

Chapter 6.0 Characterising the function of *B. cenocepacia* J2315 genes regulated by chlorhexidine

6.1 Introduction

As outlined in the previous chapter, there are many complex mechanisms involved, in resistance to the biocide chlorhexidine. Gene expression determined using a microarray, identified genes which were either up-regulated or down-regulated in response to chlorhexidine (Table 5.1, 5.2 and 5.4). These genes involve RND efflux pumps, transcriptional regulators, secretion proteins and membrane proteins. Less obviously involved with biocide resistance was the repression of genes involved with the biogenesis of flagella and chemotaxis. Hence, the microarray experiment provided an extensive data bank, from which to select interesting genes for further analysis. The microarray also raised a number of questions that were explored in more detail in this chapter:

1) What is the role of efflux and transport genes in resistance to chlorhexidine?

2) Do sub-inhibitory levels of chlorhexidine inhibit swarming motility?

3) Do sub-inhibitory levels of chlorhexidine induce biofilm production?

4) Is biocide induced biofilm production a coordinated regulatory phenotype?

5) Are any of these genes potential targets for antimicrobial therapy?

6.11 Investigating efflux and transport genes

Efflux pumps are an important mechanism of antimicrobial resistance and may also contribute to cross resistance between unrelated classes of compounds such as dyes, detergents, antibiotics and biocides, due to their broad spectrum of activity (Chapman, 2003b). For example the acquisition of qac genes, found on a transmissible plasmid can mediate cross resistance between quaternary

ammonium compounds and antibiotics in both Gram negative (*P. aeruginosa*) and Gram positive (*S. aureus*) organisms (Chapman, 2003b). Bacteria can express more than one efflux pump, both within the same family (i.e. RND) or members of different families, increasing the amount of substrates an individual strain may be resistant to. Efflux attributed resistance is most likely when there is a simultaneous increase in MIC's to three or more compounds compared to a sensitive strain (Piddock, 2006b). Efflux pumps are problematic and can render treatment ineffective, if they are over expressed. They also contribute to the multidrug resistance of a bacterial species.

As well as playing a major role in antimicrobial resistance there has also been speculation on the role of efflux in bacterial pathogenesis. Several studies have indicated that efflux pumps not only remove antimicrobials but may also export virulence factors, such as toxins and also help the bacteria to evade host defences by removing secondary metabolites produced by the host which may be toxic to the bacterium (Piddock, 2006b). Studies have shown that the lack of expression of efflux pumps have a deleterious effect on the invasiveness of some species. *P. aeruginosa* mutants lacking components of the MexAB-OprM could not invade epithelial cells, thus exhibited attenuated pathogenesis. The ability to invade epithelial cells was restored by complementation with MexAB-OprM (Hirakata *et al.*, 2002). Therefore there is evidence that efflux pumps may also be important for virulence and pathogenesis of clinically relevant bacteria.

Data obtained from the microarray experiment revealed the up regulation of two RND efflux pumps in *B. cenocepacia* J2315 after exposure to chlorhexidine (Table 5.1 and 5.4). The efflux of triclosan (Mima *et al.*, 2007) has been well described in many bacterial species, however efflux pumps that are specific for chlorhexidine resistance have not been determined, thus this data suggests that these two pumps may be important in chlorhexidine resistance. As well as influencing the MIC of *B. cenocepacia* to chlorhexidine, there is the potential that up-regulated pumps may be involved in the *B. cenocepacia* pathogenesis. Thus exposure to chlorhexidine may also contribute to virulence in *B. cenocepacia* species however this speculation can only be tested by the creation of mutants in each efflux pump, followed by analysis in models of *B. cenocepacia* virulence.

To conclude the investigation into the molecular basis of chlorhexidine resistance in *B. cenocepacia* J2315, an efflux pump gene was chosen for further analysis. BCAM 0924 was found on the second chromosome of the *B. cenocepacia* genome and is a response regulator for a MexA like efflux pump. This gene was selected as it is involved in the regulation of efflux and thus may be a reasonable target for antimicrobial therapy as disrupting its function should have polar effects on the rest of the genes in the efflux operon.

As well as a response regulator, a transport related gene was also chosen for further investigation. BCAL 2353 was found on the first chromosome in the genome and little is known about its specific function. Transport related genes could be involved with a number of traits, such as an undefined efflux pump, secretion systems, virulence, metabolism and pathogenesis. This gene was selected as it was up-regulated in sub inhibitory levels of chlorhexidine and its function in biocide resistance has not yet been elucidated. Thus this may be an novel mechanism of resistance.

To investigate the role of these two genes, detailed bioinformatic analysis was carried out to fully determine the function of the genes. Then site directed mutagenesis was used to delete these genes and the phenotypic effect on the chlorhexidine susceptibility determined. If these genes are involved in chlorhexidine resistance then it would be expected to see a reduction in the MIC of *B. cenocepacia* J2315 to chlorhexidine, which at > 100 µg/ml is representative of the highest level of resistance seen within the Bcc.

6.12 Is motility important for survival in chlorhexidine?

An interesting observation from the microarray experiment was that genes down-regulated in expression after exposure to chlorhexidine were involved in motility and chemotaxis. It appears that the flagella operon is completely repressed in response to chlorhexidine (Figure 5.10). This signifies that a coordinated regulation of this phenotype is occurring and that chlorhexidine induced a non motile state in *B. cenocepacia*. This non motile lifestyle may be essential for survival in the presence

of chlorhexidine. The down regulation of motility genes pointed to a decrease in swarming motility and the induction of a biofilm lifestyle. Swarming and biofilms are implicated in many important processes in the bacterial cell, including invasion of host cells, virulence and antimicrobial resistance. Swarming is the coordinated movement of bacterial cells across a surface in the presence of an extracellular slime (Daniels *et al.*, 2004). This is advantageous if there are nutrient limitations in the environment and in addition can spread biofilms across a surface (Daniels *et al.*, 2004). Flagella are required for attachment of bacterial cells during the formation of biofilms. However, in this investigation it was observed that flagella were repressed in response to chlorhexidine, indicating that initial attachment of cells may occur by other mechanisms and that repression of flagella leads to maturation of the biofilm (Daniels *et al.*, 2004)

The regulation of this non motile phenotype may be an essential component of *B. cenocepacia's* ability to survive in chlorhexidine, thus understanding this mechanism should aid the development of new antimicrobials that can kill this organism. A well described swarming regulation system in the Bcc is quorum sensing. Quorum sensing is a cell density dependent regulatory mechanism that is controlled by the production of sensing molecules, N-acylhomoserine lactones (N-acyl-HSLs) which can bind to specific proteins and regulate transcription of genes (Lewenza *et al.*, 1999). The quorum sensing system in the Bcc involves two proteins, CepI, which produces the autoinducer N-acyl-HSLs and CepR, which the N-acyl-HSLs bind to and activate transcription (Lewenza *et al.*, 1999). Quorum sensing is known to regulate virulence factors, such as the production of extracellular proteases and chitinase, but also regulates swarming and biofilm production (Subsin *et al.*, 2007). Huber *et al.* found that mutants defective in CepI and CepR were incapable of forming biofilms, due to lack of maturation of the biofilm. Swarming was also seen to be QS regulated, through the control of biosurfactant (extracellular slime) production (Huber *et al.*, 2001). Consequently QS may be intrinsically linked to the regulation of biofilms and motility and may be involved in the regulation that has been observed in this investigation.

In prokaryotes, there is an abundance of two component regulatory systems, which regulate gene transcription in response to environmental signals. A bacteria's

environment is always changing, hence for the bacteria to survive it must be able to readily adapt to its environment. Two component regulatory systems regulate bacterial adaptation via a signalling system that involves a histidine kinase and a response regulator. The histidine kinase is a sensor molecule, which contains a signal recognition domain. The environmental signal is recognised and it autophosphorylates a histidine residue, by ATP hydrolysis. The response regulator then catalyses the transfer of the phosphoryl group to one of its own aspartine residues. This activates an effector domain in the response regulator, such as a GGDEF domain, which elicits a specific response, such as the switching on of transcription of specific genes (West and Stock, 2001, Hoch, 2000) (Figure 6.1). Potentially, a two component system could have a role in the regulation of flagella and the transition to a non motile, sessile lifestyle in response to chlorhexidine.

The connection between sub inhibitory levels of chlorhexidine and the repression of flagella genes was explored further in this chapter. Does chlorhexidine induce bacteria to form biofilms and in turn become less susceptible to the biocide? Is *B. cenocepacia* adapting to the environment via a two component regulatory system and can this system be targeted by antimicrobial therapy? To investigate this phenomenon a swarming screen was performed to determine if chlorhexidine inhibits swarming. Biofilm assays were carried out to establish if sub inhibitory levels of chlorhexidine induced biofilm production. Finally a mutagenesis screen was performed to try and uncouple the swarming down regulation mechanism to reveal genes that may be central to the expression of the swarming / biofilm phenotype.

6.2 Results

6.21 Bioinformatic analysis on BCAM 0924 and BCAL 2353

Two genes with up-regulated expression in response to chlorhexidine were chosen for further bioinformatic analysis. As well as analysing the two selected genes, the

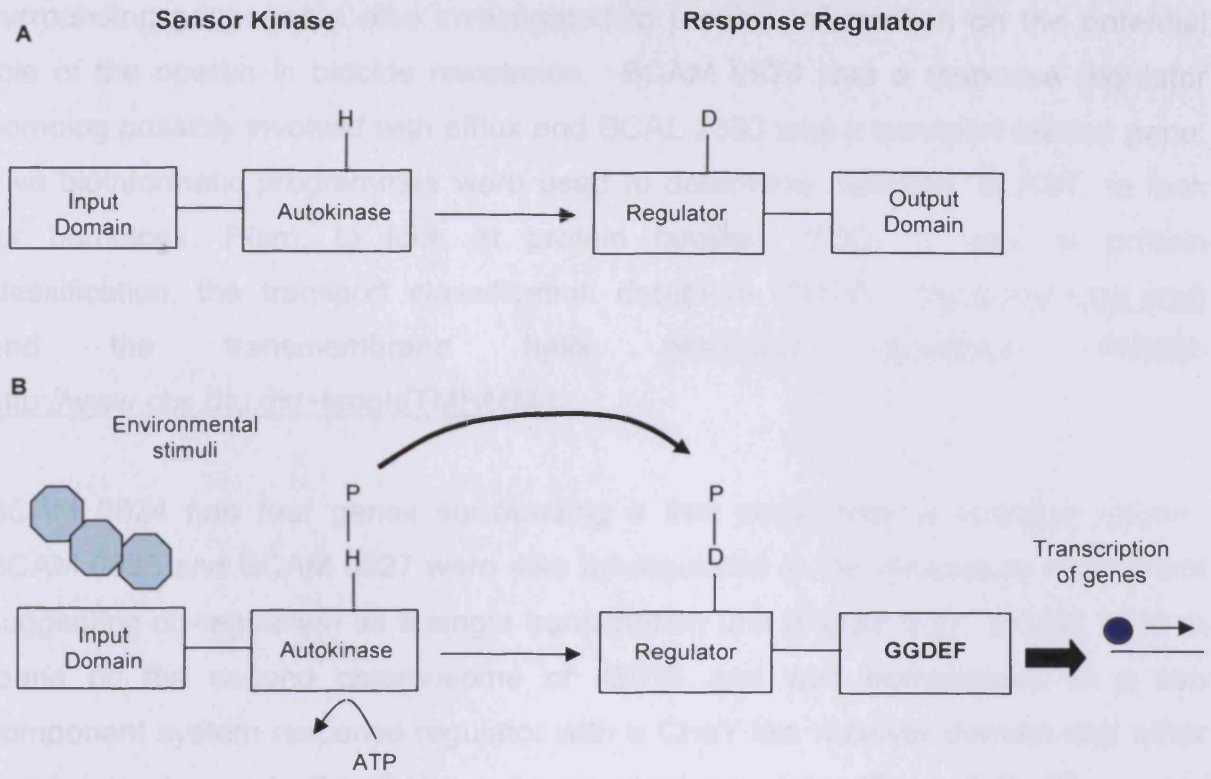


Figure 6.1 Structure of a two component regulator system: a without an environmental stimulus and b, with an environmental stimulus

The environmental stimulus binds to the signal recognition site in the sensor kinase and causes the ATP dependent autophosphorylation of a histidine residue (H). The phosphoryl (P) group is then transferred to a specific aspartine residue (D) located on the response regulator. Phosphorylation of the response regulator activates an effector domain such as the GGDEF domain, which elicits a cellular response such as the transcription of genes, i.e. GGDEF synthesis of signal molecule cyclic diguanylate monophosphate (ci-diGMP).

surrounding genes were also investigated to provide information on the potential role of the operon in biocide resistance. BCAM 0924 was a response regulator homolog possibly involved with efflux and BCAL 2353 was a transport related gene. Five bioinformatic programmes were used to determine function, BLAST, to look for homologs, Pfam, to look at protein families, COG, to look at protein classification, the transport classification database (TCDB: http://www.tcdb.org/) and the transmembrane helix prediction database (THMM: http://www.cbs.dtu.dk/~krogh/TMHMM/).

BCAM 0924 had four genes surrounding it that could form a possible operon. BCAM 0925 and BCAM 0927 were also up-regulated in the microarray experiment suggesting co-regulation as a single transcription unit (Figure 6.2). BCAM 0924 is found on the second chromosome of J2315 and was homologous to a two component system response regulator with a CheY like receiver domain and other motifs homologous to OmpR like, outer membrane proteins (Table 6.1). The genes that surround BCAM 0924 are potentially involved in secretion, efflux and were homologous to a MexAB like RND efflux pumps. BCAM 0923 had no significant Pfam or COG matches but was homologous to a HAMP sensor kinase which would link with the response regulator as part of a potential two component system (Table 6.1). BCAM 0924 was aligned with other *Burkholderia* genomes via the BLAST search tool on the *Burkholderia* genome database. It was conserved across the 20 available genomes, indicating that this response regulator is a vital gene required in many species.

BCAL 2353 had three genes surrounding it that could be a possible operon (Figure 6.3). BCAL 2352 was also up-regulated in the microarray experiment. BCAL 2353 was homologous to a transport related gene predicted to be involved in inorganic ion transport such as sulphate and could potentially be involved with a MFS superfamily of transporters which are multidrug facilitator efflux pumps (Table 6.2). BCAL 2352 was a carbonic anhydrase and BCAL 2351 was a hypothetical protein with an unknown function (Table 6.2). Similarly to BCAM 0924, BCAL 2353 was conserved across the 20 Bcc species, again indicating an important conserved gene.

6.22 Mutagenesis of BCAM 0924 and BCAL 2353

Mutagenesis was performed to delete the response regulator and transport related gene to see if they were phenotypically involved with chlorhexidine resistance. The first method attempted was based on an insertional inactivation method using a suicide plasmid constructed by Flannigan *et al.* (Flannagan *et al.*, 2007). The pΩGPTp plasmid contains a trimethoprim cassette and omega fragments. The two genes of interest were amplified with primers with EcoRI tails (Table 2.5) and cloned into the pΩGPTp plasmid. The omega fragments cause polar mutations effectively knocking out down stream genes as well as the gene of interest. The genes were cloned into the suicide vector, transformed into *E. coli*, and the correct plasmid constructs identified by PCR. Once constructs were created, tri-parental matings were carried out to insert the plasmid into the gene in *B. cenocepacia* K56-2. PCR was used to identify the correct gene insertions (Table 2.5, Chapter 2, section 2.19.2). When PCR checks were carried out on potential mutants, the correct insertion of the pΩGPTp constructs had not occurred. Therefore a second strategy was employed to generate mutants.

A second insertional inactivation method was used where a trimethoprim cassette was inserted into the gene to disrupt expression (Chapter 2, section 2.19.4). Both genes and the trimethoprim cassette were successfully cloned into the pGEM easy vector. The trimethoprim cassette was then digested and cloned into single restriction sites within the BCAM 0924 and BCAL 2353 gene constructs to form plasmids pGEM0924Tp and pGEM2353Tp (trimethoprim resistant) (Table 2.2 and 2.5). PCR was performed to check that the insert was correctly inserted. Once the construct was complete and stored, the plasmid was extracted and digested with *bamHI.* The suicide vector pEGM105Tc (Quandt and Hynes, 1993), was also digested with *bamHI* and the gene construct ligated into this plasmid to form plasmid pHR0924Tp and pHR2353Tp. PCR confirmed that the construct was correct and tri-parental mating was then carried out to transfer the deleted gene construct into *B. cenocepacia* strain K56-2.

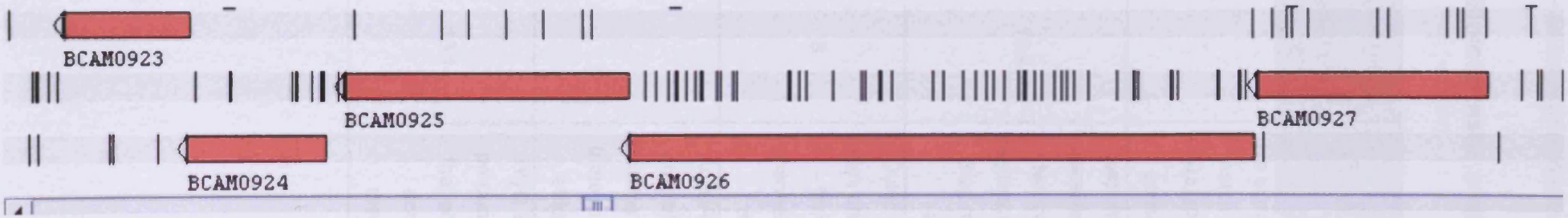


Figure 6.2 BCAM 0924 operon

BCAM 0924 putative operon: BCAM 0923 possible sensor kinase, BCAM 0924, putative response regulator, BCAM 0926, putative efflux pump and BCAM 0927 putative HlyD secretion protein

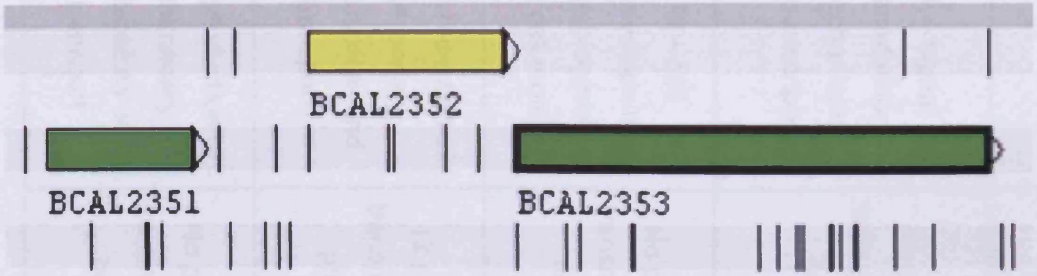


Figure 6.3 BCAL 2353 operon

BCAL 2353 putative operon: BCAL 2351 hypothetical protein, BCAL 2352 carbonic anhydrase and BCAL 2353 putative transport related gene.

Table 6.1 Bioinformatic analysis of BCAM 0924 and surrounding genes

Gene Name	Fold Up regulation	Pfam function	COG	THMM	TCDB
BCAM 0923	-	No significant Pfam match – closest – HAMP domain – sensor kinase	No significant matches	2 predicted transmembrane helices	No transport related proteins
BCAM 0924	15.9	Response regulator receiver domain – two component regulatory systems – drug resistance - efflux	T – signal transduction mechanisms K - transcription	No predicted transmembrane helices	No transport related proteins
BCAM 0925	8	Outer membrane efflux protein – OprM – form trimeric channels that allow export	M – cell wall/envelope/membrane biogenesis N – cell motility	No predicted transmembrane helices	Outer membrane proteins – RND efflux proteins
BCAM 0926	-	Acrd/AcrB/AcrF – integral membrane proteins – part of the trimeric structure of efflux pumps	Q – production of secondary metabolites and transport	12 predicted transmembrane proteins	RND efflux transport
BCAM 0927	13.1	HlyD secretion – produces proteins that are secreted across the membrane	Q – production of secondary metabolites and transport	No predicted transmembrane helices	RND family efflux proteins

Table 6.2 Bioinformatic analysis of BCAL 2353 and surrounding genes

Gene Name	Fold Up regulation	Pfam function`	COG	THMM	TCBD
BCAL 2351	-	No Pfam matches – hypothetical protein	No matches	No predicted transmembrane helices	No transport related genes
BCAL 2352	3.3	Carbonic anhydrase – catalyse reversible hydration of carbon dioxide	P – inorganic ion transport and metabolism	No predicted transmembrane helices	No transport related genes
BCAL 2353	3.4	Transport of sulphate across the membrane	P – inorganic ion transport – sulphate transport and related transporters (MFS superfamily)	10 predicted transmembrane helices	Sulphate transporters

6.23 Genetic analysis of putative mutants

After 48 hours of growth at 37°C approximately ten colonies were observed. However, the control plates which contained wildtype K56-2 also had 5 - 10 colonies growing on it, suggesting that spontaneous resistant mutants rather than true mutants may be present. The colonies were picked and left to grow for 18 hours. A direct PCR was carried out on the mutants to check for the presence of the trimethoprim cassette. In all mutants and for both genes, the Tp cassette was present (Figure 6.4), suggesting mutagenesis had been successful.

Three mutants were selected for each gene and stored frozen. Further analysis was carried out on these six potential mutants. RAPD analysis was performed to confirm that all the mutants picked were *B. cenocepacia* K56-2. All RAPD profiles were the same as the wildtype (Figure 6.5), verifying that the mutants were the correct species. Two other PCR checks were carried out. First a PCR using the forward primer of the Tp cassette and the reverse primer of the gene was used. The final product size should include the entire Tp cassette (approximately 500 bp) and half of the gene (approximately 400 bp for BCAM 0924 and 1.25 kb for BCAL2353). PCR on the mutants were positive for these products (Figure 6.6) Secondly, internal primers for each gene were designed, approximately 150 bp upstream and downstream of the restriction site where the Tp cassette had been inserted. If the Tp cassette has inserted and recombined correctly in the mutants, then a larger product would be observed. This PCR also allowed single crossovers to be detected, by the presence of both the wildtype and the larger sized band. When this PCR was carried out only the wildtype band was present, suggesting that the Tp cassette (although present as detected in the first PCR) had not correctly inserted. These PCR results suggested that a form of single cross over may have occurred at the BCAM 0924 and BCAL 2353 loci, leading to insertion of the mutation constructs, but also leaving the wildtype gene intact.

6.24 Phenotypic characterisation of potential mutants

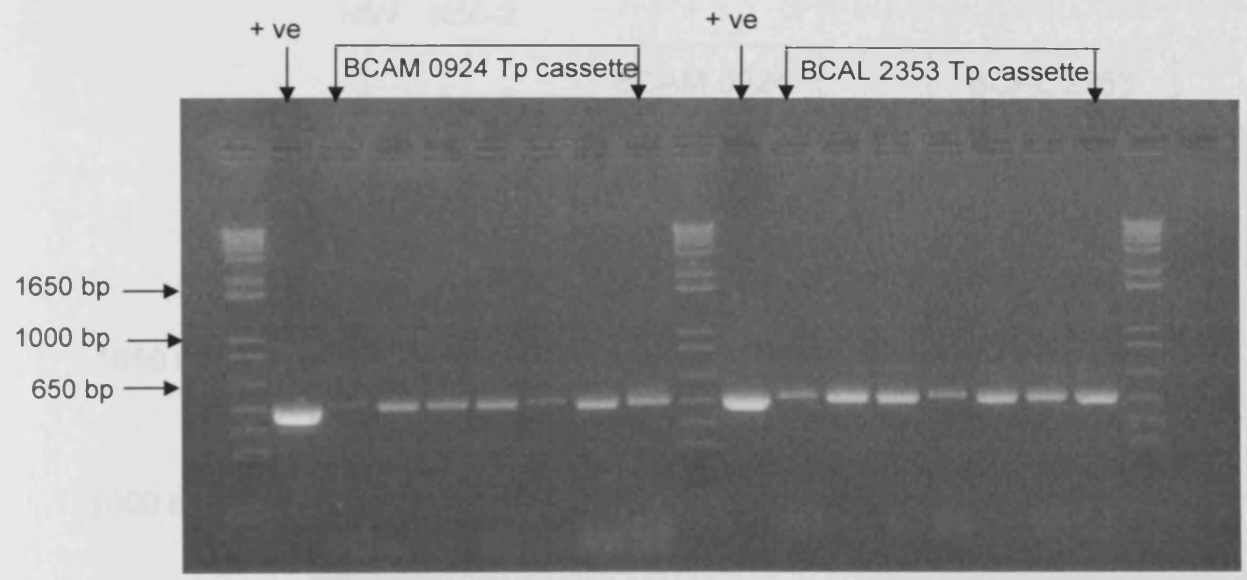


Figure 6.4 PCR amplification of the trimethoprim cassette from BCAM 0924 mutants and BCAL 2353 mutants

PCR amplification of potential mutants to confirm the presence of the Tp cassette. The pUC plasmid containing the Tp cassette was included as a positive control. All samples were positive for the Tp cassette (509 bp).

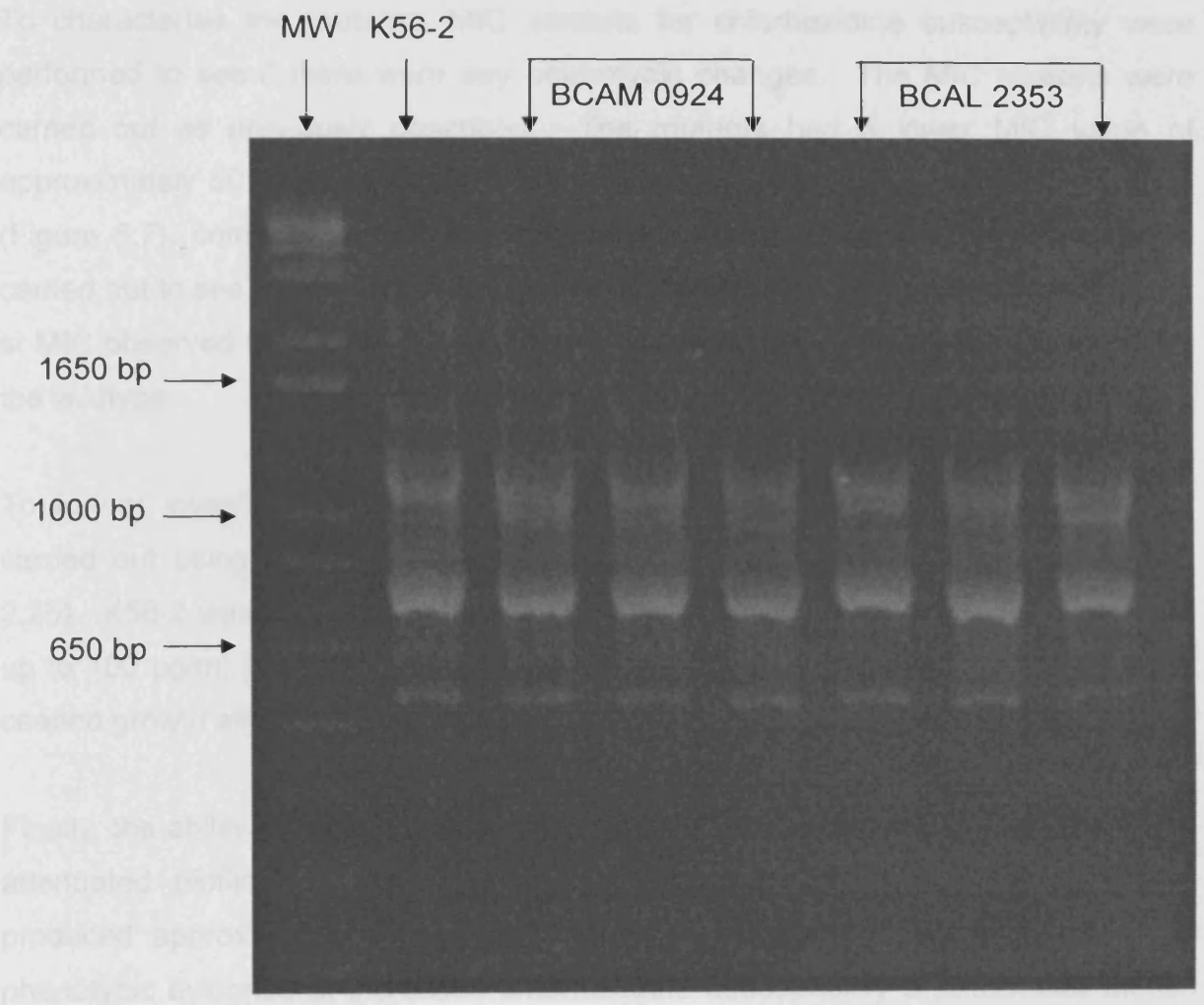


Figure 6.5 RAPD fingerprint profile of potential mutants

Three potential mutants for each gene were selected and RAPD profiling performed. K56-2 was included as the wildtype strain. All six potential mutants had the same fingerprint as the wildtype indicating that all mutants were K56-2.

To characterise the mutants, MIC screens for chlorhexidine susceptibility were performed to see if there were any phenotypic changes. The MIC screens were carried out as previously described. The mutants had a lower MIC value of approximately 50 – 80 µg/ml for BCAL 2353 and 50 – 70 µg/ml for BCAM 0924 (Figure 6.7), compared to 90 – 100 µg/ml for K56-2 wildtype. CPC MICs were carried out to see if there were any cross over effects, but there was no knock down in MIC observed with all mutants having a MIC value of > 200 µg/ml, the same as the wildtype.

To further investigate the phenotypic effect, a growth curve MIC analysis was carried out using a microbial growth analyser, Bioscreen C (Chapter 2, section 2.25). K56-2 was capable of growing in all chlorhexidine concentrations screened, up to 100 µg/ml (Figure 6.8 a). BCAM 0924 mutants and BCAL 2353 mutants all ceased growth after 40 µg/ml (Figure 6.8 b and c).

Finally, the ability of the mutants to form biofilms was assessed. The mutants had attenuated biofilm formation compared to the wildtype (Figure 6.9). K56-2 produced approximately two – four fold more biofilm than the mutants. The phenotypic evidence of increased chlorhexidine susceptibility and reduced biofilm formation in the presence of the biocide, suggested that while the wildtype genes were still detectable by PCR, the insertion of the knockout constructs may have disrupted their expression.

6.25 Biofilm production

Biofilm assays were carried out to see if the presence of sub-inhibitory levels of chlorhexidine induced biofilm formation in the Bcc. In addition, CPC was also screened for the ability to induce biofilm production. The screen was a modified plate assay and biofilms were stained with 1% crystal violet. The first screens produced variable results between replicates so the assay was optimised as follows: a range of growth media were tested for biofilm production, including TSB, nutrient broth, double strength TSB and nutrient broth and basal salts media. One test plate was coated with porcine mucin to determine if this also aided biofilm

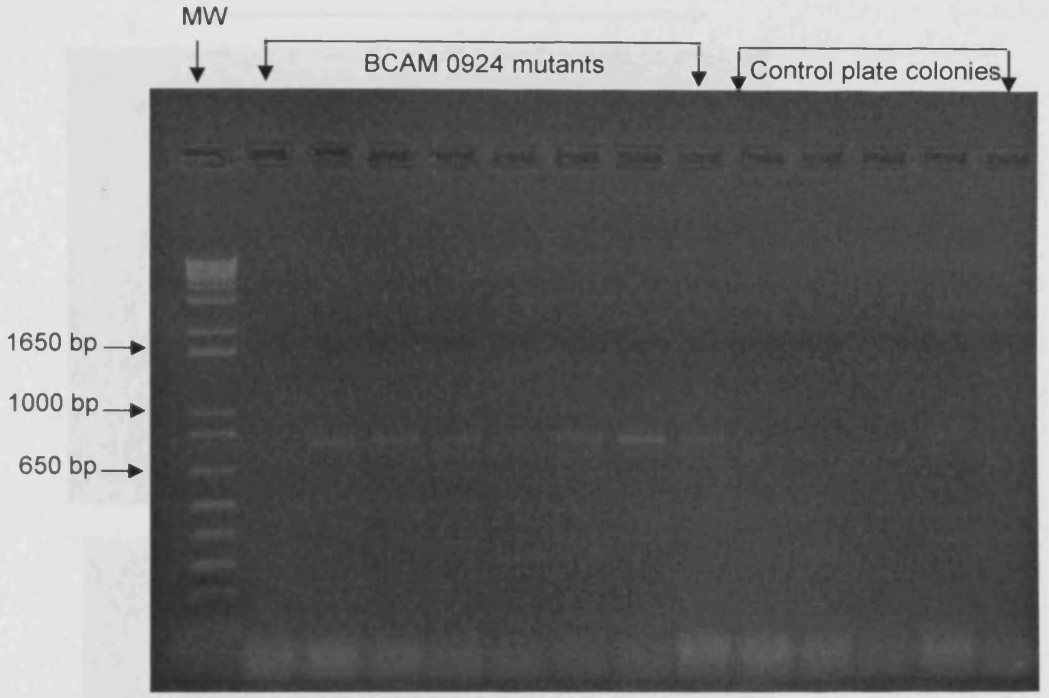


Figure 6.6 PCR checks on BCAM 0924 mutants

All but one mutant amplified. The correct product size should be approximately 850 bp. All the amplified mutants are this size, indicating they are correct. Some colonies were present on the control plate, which should have been negative. No amplification was observed on these colonies indicating that they were spontaneous resistant colonies or contamination on the plate.

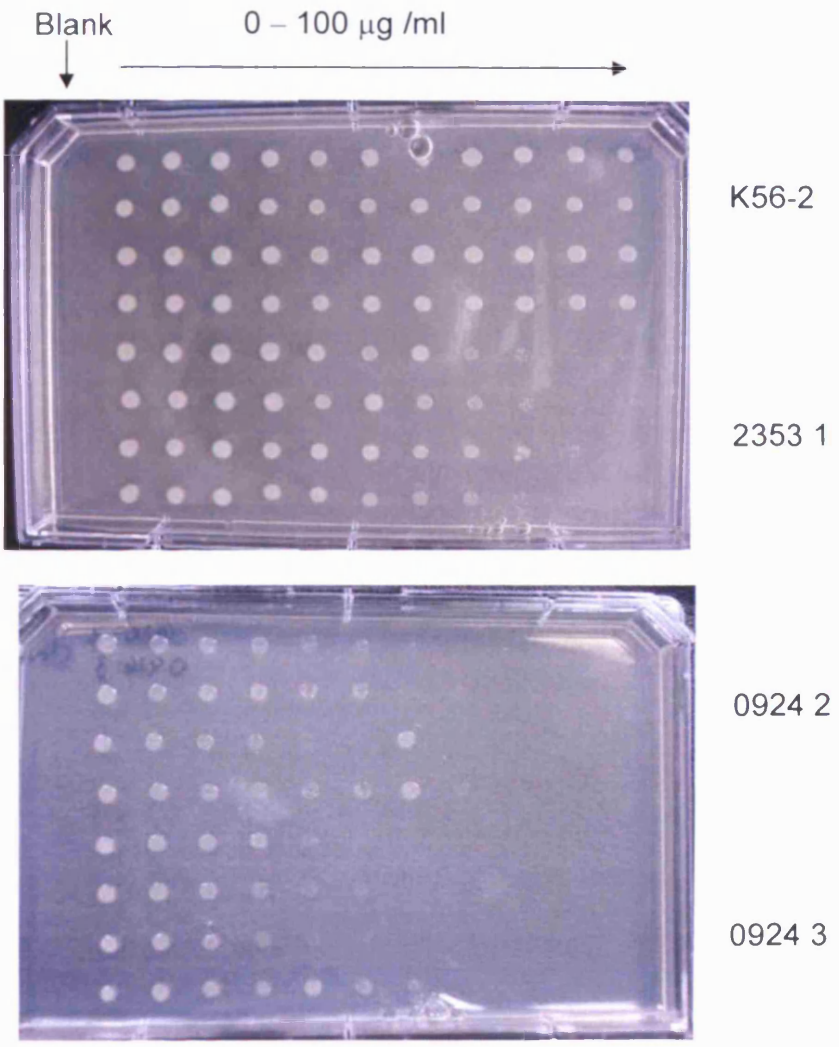


Figure 6.7 Chlorhexidine MIC's of K56-2, BCAL 2353 mutants and BCAM 0924 mutants

MIC experiments carried out as previously described (broth dilution method). After 24 hours of growth, mutants were spotted onto TSA plates using a 96 point replicator and left to grow for 48 hours. MICs were determined by visually looking for growth inhibition. K56-2 was capable of growth at 100 µg/ml, compared to 70 – 80 µg/ml for BCAL 2353 mutant 1 and 50 – 60 µg/ml for BCAM 0924 mutants 2 and 3.

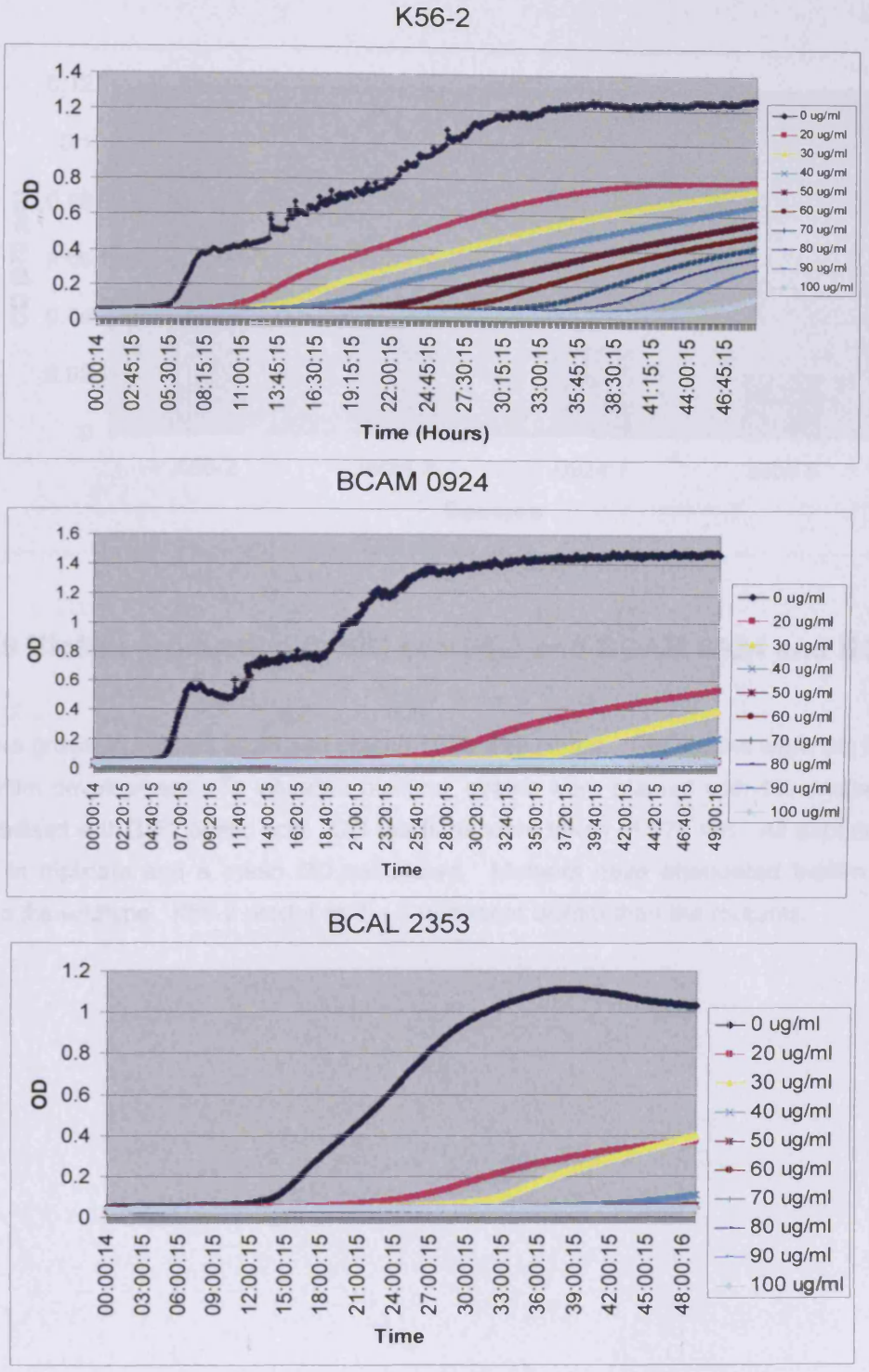


Figure 6.8 Bioscreen C analysis of BCAM 0924, BCAL 2353 mutants and the wildtype K56-2 in chlorhexidine

MIC determination was carried out in a Bioscreen microbial growth analyser. Each mutant and the wildtype were grown in TSB containing a series of concentrations of chlorhexidine. An OD reading was taken by the Bioscreen every 5 minutes for 48 hours. Each growth curve was replicated and the curves shown represent the mean analysis of this data.

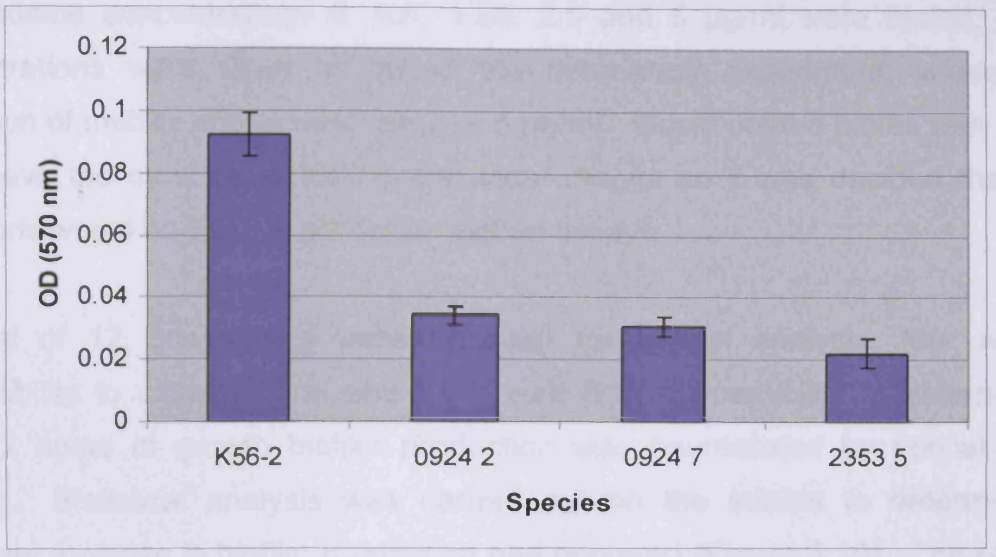


Figure 6.9 Biofilm production (± SE) of K56-2 and BCAM 0924 and BCAL 2353 mutants

Mutants were grown in nutrient broth and placed in 96 well plates. The strains were left for 72 hours to allow biofilm development. To visualise biofilms, strains were stained with 1% crystal violet and then resolubilised with 33% acetic acid. OD readings were taken at 570 nm. All experiments were carried out in triplicate and a mean OD calculated. Mutants have attenuated biofilm production compared to the wildtype. K56-2 produces 2 – 4 fold more biofilm than the mutants.

adherence. *B. cenocepacia* J2315 and C4455 were used as model strains and chlorhexidine concentrations 0, 0.6, 1.25, 2.5 and 5 µg/ml were tested. These concentrations were used to mimic the microarray experiment, where down regulation of motility genes were seen at 5 µg/ml. Mucin coated plates with nutrient broth gave the most reproducible and clear results so it was decided that these conditions would be used in all further biofilm assays.

A panel of 12 Bcc strains were selected for biofilm analysis, four with low susceptibility to chlorhexidine and eight with high susceptibility to chlorhexidine. After 72 hours of growth biofilm production was enumerated by optical density reading. Statistical analysis was carried out on the strains to determine if a significant increase in biofilm production had occurred (Figure 6.10). Out of the 12 strains, 7 strains (58.3%) showed some increase in biofilm production in chlorhexidine compared to biofilm production in zero chlorhexidine. For example, *B. cenocepacia* strain C4455, had a 7.5% increase in biofilm production at 5 µg/ml chlorhexidine (Figure 6.10). CPC exhibited a greater induction effect, with 10 (83%) of the 12 strains tested producing more biofilm in sub inhibitory levels (Figure 6.11). *B. cenocepacia* strain C4455 produced 9% more biofilm at 5 µg/m of CPC (Figure 6.11). Significant differences ($P<0.05$) were only observed for *B. cenocepacia* J2315 in CPC at 1.25, 2.5 and 5 µg/ml, *B. cenocepacia* K56-2 in CPC at 1.25 µg/ml and *B. cenocepacia* LMG 18827 in chlorhexidine at 1.25 µg/ml.

In addition, four strains, *B. cenocepacia* AU1054, C4455, J2315 and *B. multivorans* ATCC 17616, were screened against three other antimicrobial compounds, triclosan, benzalkonium chloride and the antibiotic tobramycin. Concentrations were adjusted according to the MIC values of the strains. Triclosan exhibited no biofilm effect so was not included in further analysis. Only *B. cenocepacia* J2315 had an increase in biofilm production in the presence of benzalkonium chloride, with a maximum increase of 8.7% at 2.5 µg/ml. J2315 had a significant increase in biofilm production at 1.25 µg/ml ($P < 0.0238$, Mann Whitney) and at 2.5 µg/ml ($P < 0.0019$, Mann Whitney). The other three strains all saw a reduction in biofilm production in the presence of BZK. In contrast, all four strains saw an increase in biofilm production in tobramycin (Figure 6.12). *B. cenocepacia* C4455 had the largest increase in biofilm production in tobramycin with a 20% increase at 1 µg/ml

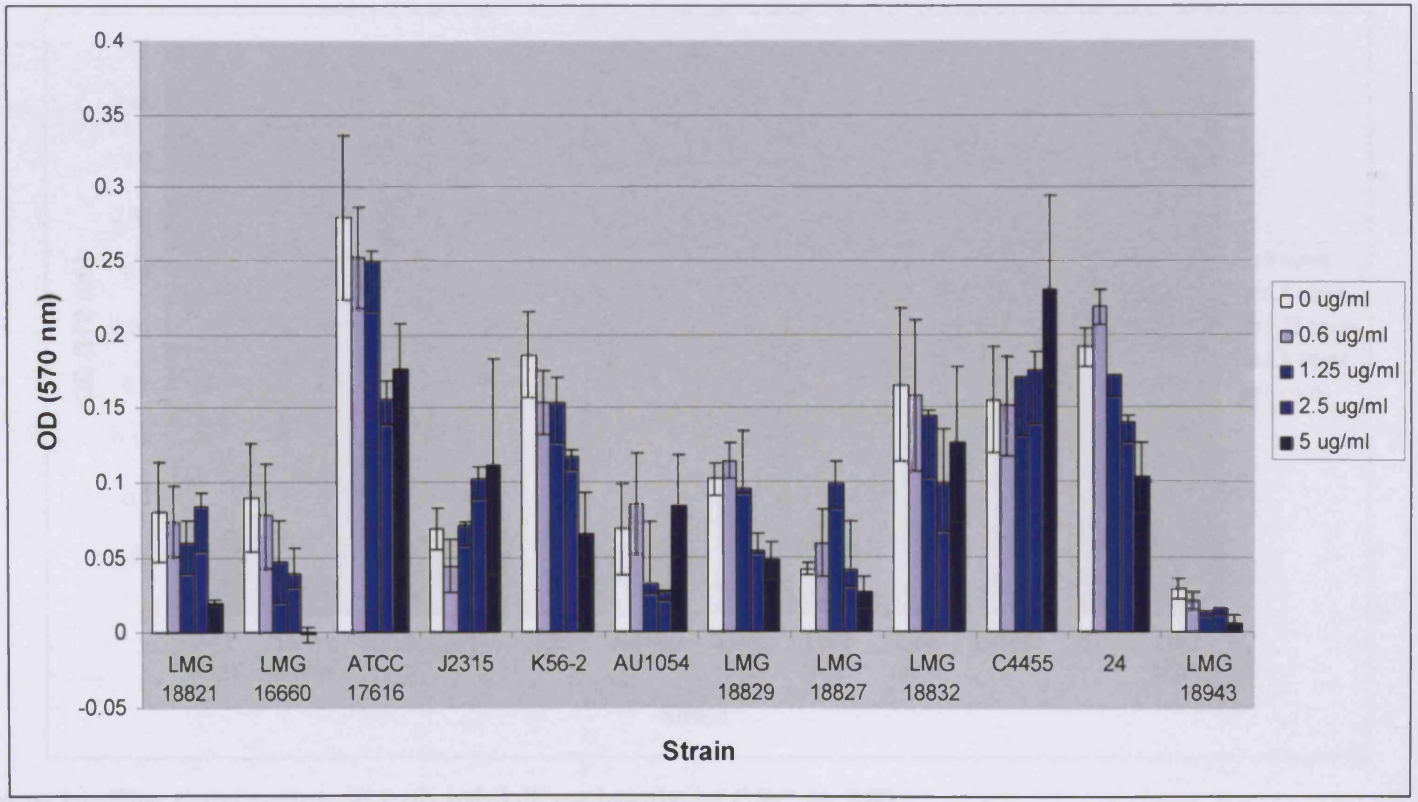


Figure 6.10 Mean biofilm production in sub inhibitory levels chlorhexidine (± SE)

Panel of 12 strains showing production of biofilm in the presence of increasing concentrations of chlorhexidine. *B. cepacia* LMG 18821 had a 0.3% increase at 2.5 µg/ml, *B. cenocepacia* J2314 had 0.3, 3.3 and 4.2% increase at 1.25, 2.5 and 5 µg/ml, *B. cenocepacia* AU1054 had a 1.8 and 1.6% increase at 0.6 and 5 µg/ml, *B. cenocepacia* LMG 18829 had 1.7% increase at 0.6 µg/ml, *B. cenocepacia* LMG 18827 had a 1.7 and 5.7% increase at 0.6 and 1.25 µg/ml, *B. cenocepacia* C4455 had a 1.5, 2 and 7.5% increase at 1.25, 2.5 and 5 µg/ml and Bcc novel group K had a 2.8% increase at 0.6 µg/ml. Biofilm production (OD) was determined after 72 hours of growth in nutrient broth containing a series of chlorhexidine concentrations, within 96 well plates. The strains were stained with 1% crystal violet and resolubilsed in 33% acetic acid. All experiments were carried out in triplicate and a mean OD calculated

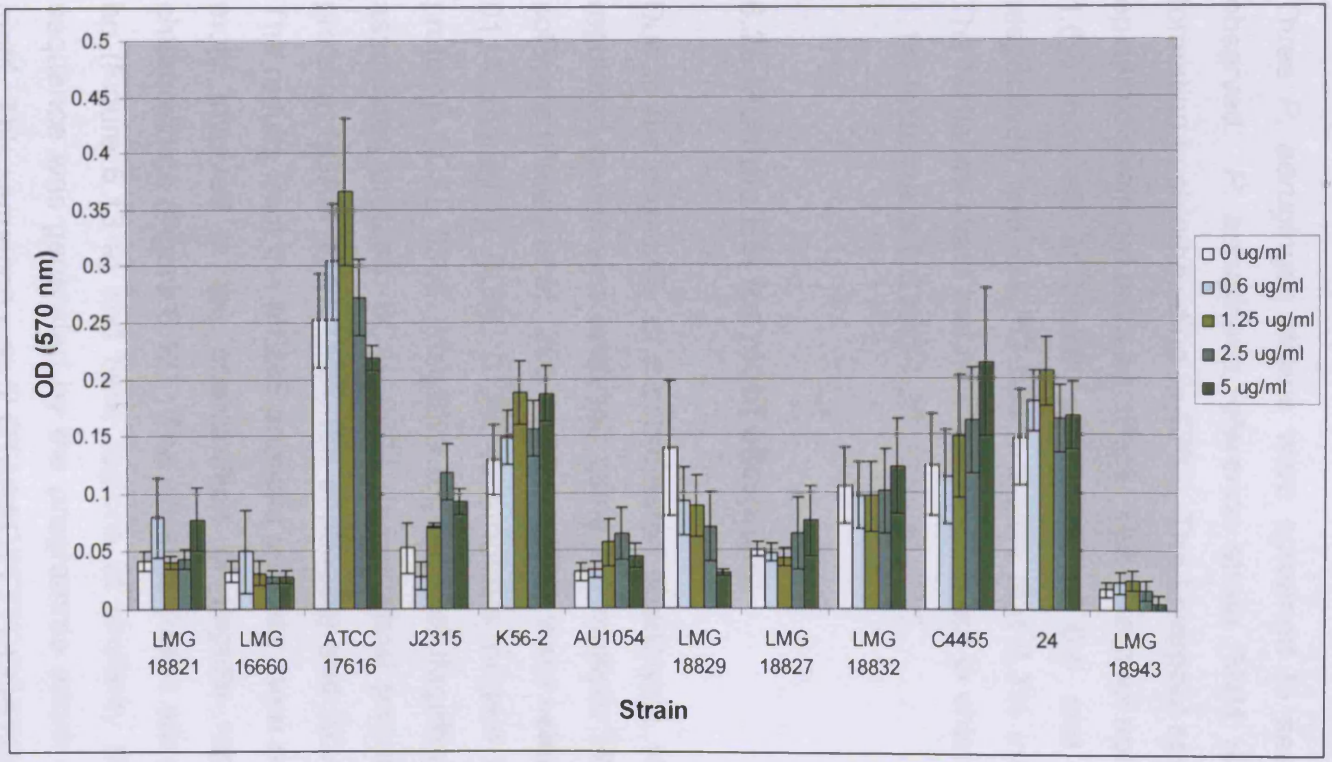


Figure 6.11 Mean biofilm production in sub inhibitory levels of CPC (± SE)

Panel of 12 strains showing production of biofilm in increasing concentrations of CPC. *B. cepacia* LMG 18821 had 3.7, 0.08 and 3.5% increase at 0.6, 2.5 and 5 µg/ml, *B. multivorans* LMG 16660 had a 1.7% increase at 0.6 µg/ml, *B. multivorans* ATCC 17616 had a 5.2, 11.4 and 2% increase at 0.6, 1.25 and 2.5 µg/ml, *B. cenocepacia* J2315 had a 1.9, 6.5 and 4% increase at 1.25, 2.5 and 5%, *B. cenocepacia* K56-2 had a 1.9, 5.8, 2.7 and 5.8% increase at 0.6, 1.25, 2.5 and 5 µg/ml, *B. cenocepacia* AU1054 had a 0.1, 2.5, 3.3 and 1.4% increase at 0.6, 1.25, 2.5 and 5 µg/ml, *B. cenocepacia* LMG 18827 had a 1.4 and 2.5% increase at 2.5 and 5 µg/ml, *B. cenocepacia* LMG 18832 had a 1.7% increase at 5 µg/ml, *B. cenocepacia* C4455 had a 2.5, 3.9 and 9% increase at 1.25, 2.5 and 5 µg/ml, Bcc novel group K had a 3.2, 5.8, 1.7 and 1.9% increase at 0.6, 1.25, 2.5 and 5 µg/ml and *B. dolosa* LMG 18943 had a 0.6 and a 0.8% increase at 0.6 and 1.25 µg/ml. Biofilm production (OD) was determined after 72 hours of growth in nutrient broth containing a series of chlorhexidine concentrations, within 96 well plates. The strains were stained with 1% crystal violet and resolubilsed in 33% acetic acid. All experiments were carried out in triplicate and a mean OD calculated

compared to the control with no antibiotic (Figure 6.12). Although all strains had an increase in biofilm formation at sub-inhibitory levels of tobramycin only *B. cenocepacia* J2315 had significant differences as determined by Mann Whitney tests at 0.3 µg/ml and 0.6 µg/ml ($P<0.05$).

Three *P. aeruginosa* strains were screened to see if the same effect could be observed. *P. aeruginosa* reference strain PA01 did not show enhanced biofilm formation in chlorhexidine or CPC. The Liverpool epidemic strain and the Midlands epidemic strain did have an effect. The Liverpool epidemic strain had a 5.6% and a 1.6% increase in biofilm production at 0.6 and 1.25 µg/ml of chlorhexidine respectively and in CPC had a 2.65% and 0.7% increase at 0.6 and 1.25 µg/ml. The Midlands strain has no effect in CPC but in chlorhexidine exhibited a 2.1% and 1.1% increase at 0.6 and 1.25 µg/ml of CPC.

6.26 Multiple EM for motif elicitation

Due to the possibility of a coordinated phenotype, regions upstream of the down-regulated genes was analysed using the multiple EM for motif elicitation (MEME) software (Bailey *et al.*, 2006). Nine genes were selected to put into MEME: BCAL 0114, (flagellin); BCAL 0126, (chemotaxis protein MotA); BCAL 0527, (flagellar protein); BCAL 0562, (negative regulator of flagellin); BCAL 0576, (flagellar hook associated protein); BCAL 2904 (hypothetical protein); BCAM 1503, (chemotaxis protein); BCAM 1744, (exported peptidase) and BCAM 2564 (aerotaxis receptor). The results from the MEME analysis indicate there may be a conserved regulatory motif involved in the coordination of flagella repression in the presence of chlorhexidine (Figure 6.13). The possible motifs are positioned between 150 – 250 bp (Figure 6.13 a) and have regions of similarity (Figure 6.13 b). A consensus sequence was generated by the programme which could be a regulatory binding motif. This sequence was 5' CTAAACTTTTCCCATCGCCCGCCGATA 3'.

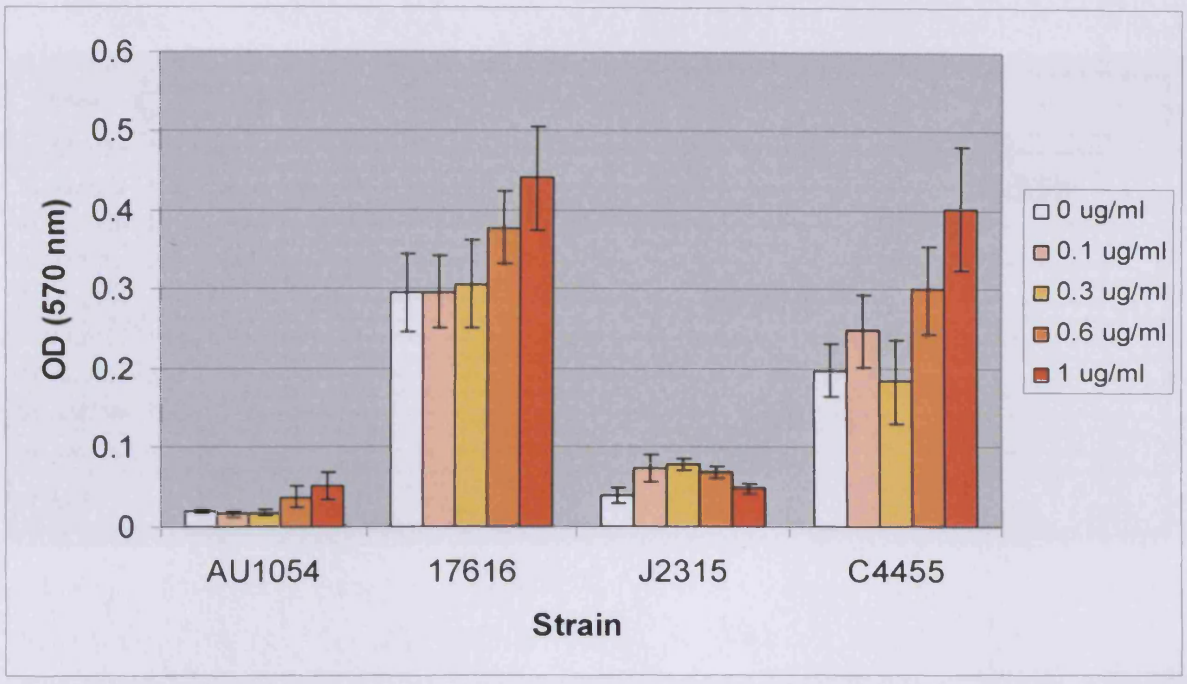
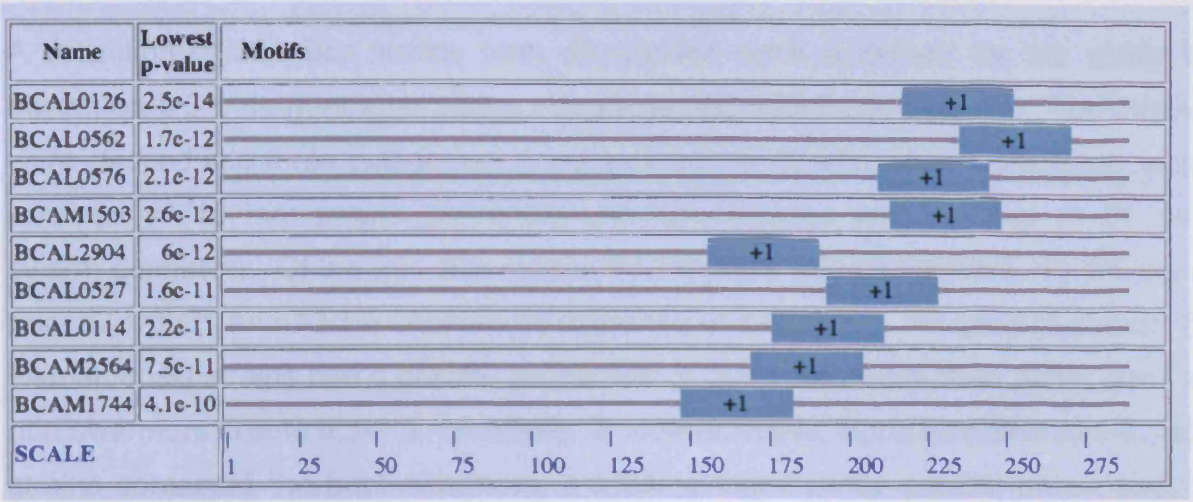


Figure 6.12 Biofilm production of four strains in the presence of tobramycin (± SE)

Biofilm production (OD) was determined after 72 hours of growth in nutrient broth containing a series of chlorhexidine concentrations within 96 well plates. The strains were stained with 1% crystal violet and resolubilsed in 33% acetic acid. All experiments were carried out in triplicate and a mean OD calculated. All strains had an increase in biofilm production at sub inhibitory levels of tobramycin. *B. cenocepacia* AU1054 had an increase of 1.8% and 3.2% at 0.6 and 1 µg/ml, *B. multivorans* ATCC 17616 had an increase of 0.09, 1.1, 8.3 and 14.5% at 0.1, 0.3, 0.6 and 1 µg/ml, *B. cenocepacia* J2315 had an increase of 3.4, 3.9, 3 and 0.9% at 0.1, 0.3, 0.6 and 1 µg/ml and *B. cenocepacia* C4455 had an increase of 5, 10.2 and 20.5% at 0.1, 0.6 and 1 µg/ml.



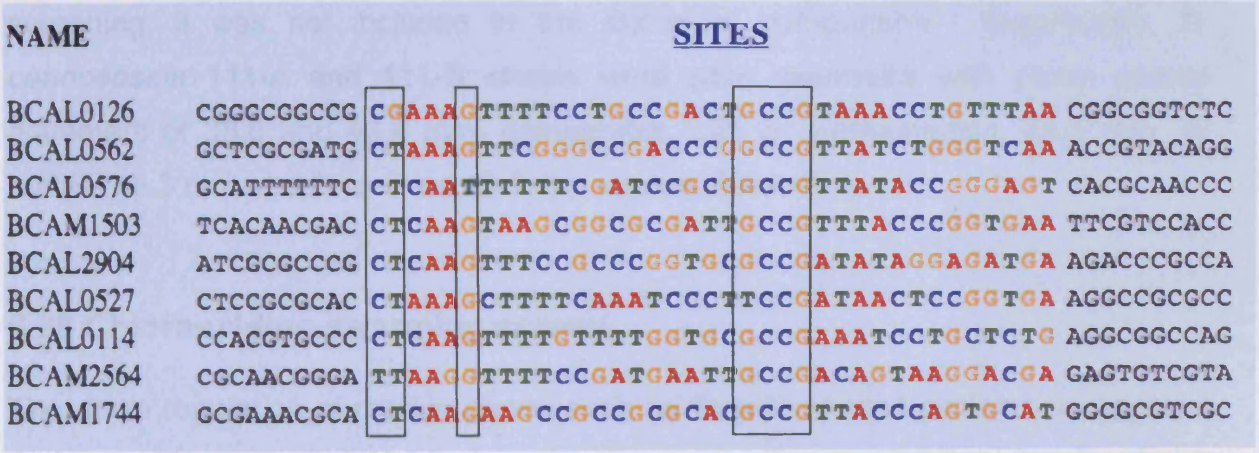


Figure 6.13 MEME analysis output; A – Block diagram of motif positions and B – alignment of potential motifis

Output from a MEME analysis: A = Position of motif alignments 150 – 250 bp upstream of genes. B = Alignment of potential motifs. Three areas are conserved, indicating a motif.

6.27 – Bcc swarming screen

A collection of 251 Bcc strains from all species were screened for the ability to swarm on 0.3% nutrient agar plates supplemented with 0.5% glucose. The strains were classed into three categories, good swarmer (> 40 mm swarm diameter), poor swarmer (< 40 mm swarm diameter) and no evidence of swarming (< 20 mm swarm diameter). Of the 251 Bcc strains 67.7% were good swarmers, 19.2% were poor swarmers and 13.1% showed no evidence of swarming. All species except *B. vietnamensis* strains had a greater proportion of good swarmers than either poor or non swarmers (Table 6.3). *B. ambifaria, B. contaminans, B. multivorans* and *B. lata* strains contained the best swarmers, exhibiting significantly greater mean swarm diameters than other species (Table 6.3). The *B. diffusa* strain also produced a large swarm diameter of 85 mm. However, as only one strain was available for screening, it was not included in the statistical comparison. Surprisingly, *B. cenocepacia* 111-A and 111-B strains were poor swarmers with mean swarm diameters of 38.6 and 43.4 mm, comparable with *B. vietnamensis,* 35.5 mm, *B. stabilis* 35.3 mm *and B. anthina* 49.1 mm strains (Table 6.3).

6.28 Chlorhexidine swarming screen

The down regulation of motility genes such as flagella related proteins, suggested that swarming may be inhibited in the presence of sub inhibitory levels of the biocide. A chlorhexidine swarming screen was devised to assess this across the Bcc. Twenty one strains representative of each Bcc species were examined. The concentration of chlorhexidine used in the screen spanned the following range: 0, 0.1, 0.3, 0.6, 1.25, 2.5 and 5 µg/ml. The maximum concentration was chosen as 5 as no strains screened had a MIC lower than 10 µg/ml, thus 5 µg/ml would indicate if motility was being inhibited without the results being biased by bacterial growth inhibition by the biocide. Twelve of he eighteen strains selected were also representative of chlorhexidine sensitive and resistant strains (Table 6.4). A total of 67% of strains exhibited reduced swarming in the presence of chlorhexidine. There was little difference between resistant and sensitive strains, with five out of seven resistant strains and five out of six sensitive strains showing the effect. *B. multivorans* ATCC 17616 had the most pronounced effect and clearly had a

Table 6.3 Bcc swarming screen

	% of species			
Species	**Good (> 40 mm)**	**Poor (< 40 mm)**	**No evidence (< 20 mm)**	**Mean swarm diameter (mm)**
B. cepacia (30)	76.7	6.7	16.7	63.3[a]
B. multivorans (24)	87.5	4.2	8.3	75.1[b]
B. cenocepacia A (27)	48.1	33.3	18.5	38.6
B. cenocepacia B (27)	44.4	33.3	22.2	43.3
B. stabilis (16)	37.5	37.5	25	35.3
B. vietnamensis (16)	37.5	43.8	18.8	35.5
B. dolosa (7)	57.1	28.6	14.3	51
B. ambifaria (35)	85.7	14.3	0	71.3[c]
B. anthina (14)	57.1	28.6	14.3	49.1
B. pyrrocinia (16)	62.5	12.5	25	48.6
B. contaminans (5)	100	0	0	83.8[d]
B. lata (10)	100	0	0	76.5[e]
Bcc (20)	55	45	0	49.6[f]
B. arboris (3)	66.7	0	33.3	51.7
B.. diffusa (1)	100	0	0	85
Mean (251)	67.7	19.2	13.1	

a = *B. cepacia* significantly different to *B. cenocepacia* A, *B. cenocepacia* B, *B. stabilis* and *B. vietnamensis*

b = *B. multivorans* significantly different to *B. cenocepacia* A, *B. cenocepacia* B, *B. stabilis*, *B. vietnamensis*, *B. dolosa*, *B. anthina*, *B. pyrrocinia* and Bcc strains

c = *B. ambifaria* significantly different to all strains except *B. cepacia*, *B. multivorans*, *B. contaminans* and *B. lata* strains

d and e = *B. contaminans* and *B. lata* strains significantly different to all strains except *B. cepacia*, *B. multivorans* and *B. ambifaria*

f = Bcc strains significantly different to *B. stabilis* and *B. vietnamensis* strains

reduction in motility as the chlorhexidine concentration increased (Figure 6.14). There was a marked decrease in swarm diameter even at low concentrations, for example at 0.1 µg/ml there was a 42% reduction in swarm zone compared to the control with no biocide (Figure 6.14).

In addition, three other biocides, triclosan, CPC and benzalkonium chloride and two antibiotics meropenem and tobramycin were screened for inhibition of swarming. The strains screened were *B. multivorans* ATCC 17616, *B. cenocepacia* J2315, and *B. cenocepacia* AU1054. *B. multivorans* ATCC 17616 was unaffected by triclosan, benzalkonium chloride and the two antibiotics. In CPC there was swarming inhibition at 2.5 and 5 µg/ml, with a 34% and 84% reduction respectively. However at the lower concentrations the results were variable. *B. cenocepacia* J2315 had no effect in any other antimicrobial agent apart from the original chlorhexidine assay, although it is worth noting that this strain was not a particularly good swarmer in the first instance. *B. cenocepacia* AU1054, also exhibited no reduction in swarming with the other antimicrobial agents, apart from triclosan, where a 75% reduction in swarming was observed at 5 µg/ml. Thus it appears that this phenomenon is more specific for chlorhexidine, but may, in certain Bcc strains and species occur for other antimicrobial agents.

6.29 Correlation of chlorhexidine biofilm induction and swarming inhibition

Correlation of the biofilm induction and the swarming inhibition phenotypes in response to sub-inhibitory levels of chlorhexidine was performed (Table 6.5). 10 strains were correlated for both biofilm induction and swarming inhibition. Out of these strains, 6 had both induction of biofilms and inhibition of swarming. Two strains (*B. multivorans* LMG 16660 and *B. cenocepacia* LMG 18832), had neither induction nor inhibition of biofilms and swarming. Two strains, *B. dolosa* LMG 18943 and *B. multivorans* ATCC 17616, did not show induction of biofilm production in chlorhexidine, but did show inhibition of swarming (Table 6.5). Although only a small panel was screened, there appears to be a tentative correlation between increase in biofilm production and swarming repression in the presence of chlorhexidine. However, four strains contradict this evidence, indicating that a

Table 6.4 Bcc swarming response to chlorhexidine

Species	Name	Chlorhexidine concentration (µg/ml)							
		0	0.1	0.3	0.6	1.25	2.5	5	R/S*
		Diameter of swarm zone (mm)							
B. cepacia	ATCC 25416	63	67	75	55	65	65	66	S
	LMG 18821	60	60	13	10	10	10	10	R
B. multivorans	LMG 13010	85	85	79	20	50	35	22	S
	ATCC 17616	85	50	38	24	15	12	10	S
	LMG 16660	29	20	29	25	30	25	20	R
B. cenocepacia	AU1054	85	85	69	49	41	20	20	S
	LMG 18832	13	15	10	15	14	14	14	R
	LMG 18827	42	53	32	32	22	25	12	R
	J2315	12	11	11	11	10	5	5	R
	C4455	12	12	5	4	4	4	2	R
B. stabilis	17-474	65	40	24	29	17	15	14	-
B. vietnamensis	CRE-7	85	45	40	25	20	23	20	-
B. dolosa	AU3556	85	15	13	85	85	85	85	-
	LMG 18943	44	42	35	23	15	12	10	S
B. ambifaria	KCO-18	85	85	85	85	85	85	85	-
	AMMD	54	49	30	35	29	32	35	S
B. anthina	AU3904	85	85	85	85	85	85	85	-
B. pyrrocinia	AU2419	80	65	60	45	45	27	25	-
B. contaminans	VP1	80	55	17	14	13	18	20	-
Bcc K	24	70	60	25	20	18	12	7	R
B. lata	ATCC 17769	85	85	85	85	85	85	80	-

* R/S = Resistant or sensitive to chlorhexidine as determined by the broth dilution MIC screen.

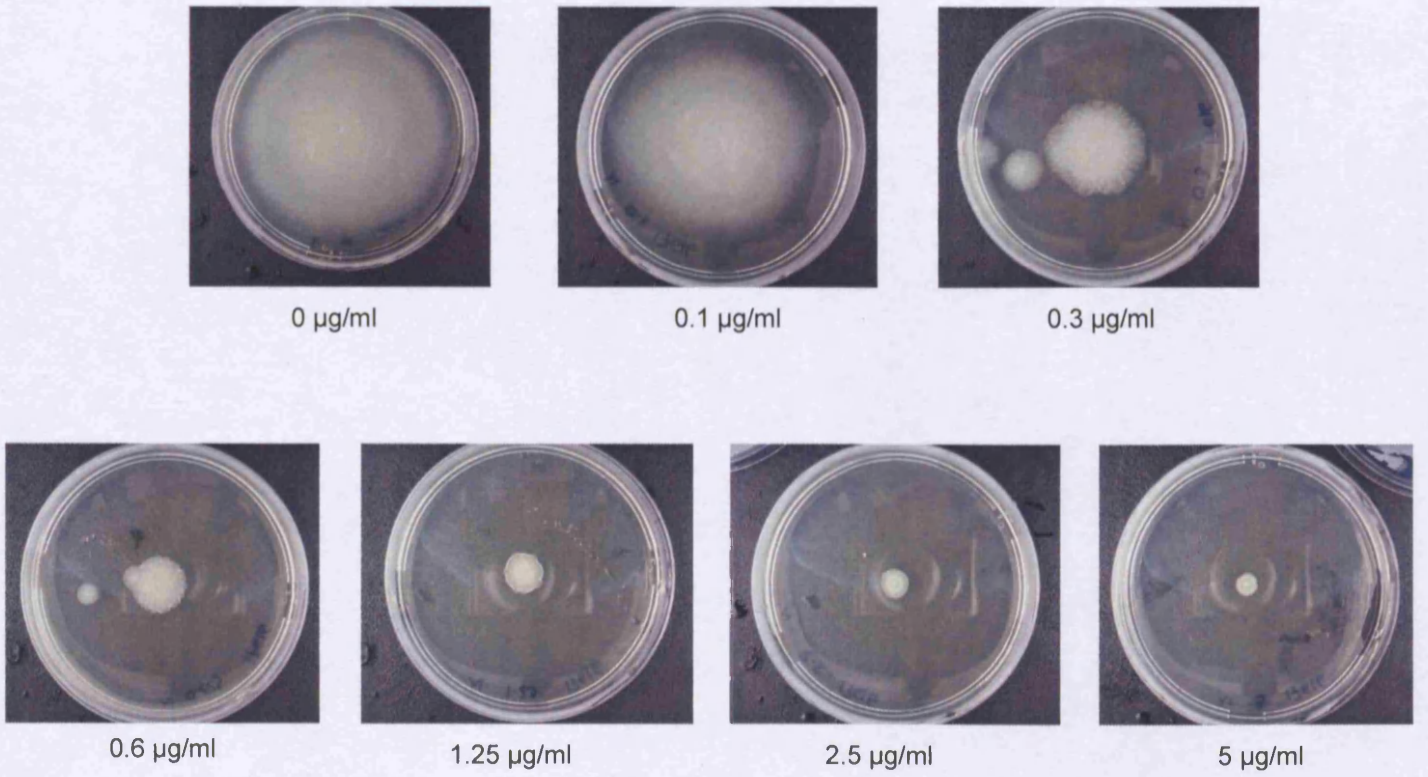


Figure 6.14 *B. multivorans* ATCC 17616 swarming inhibition in sub inhibitory levels of chlorhexidine

Swarming plates were made using 0.3% nutrient agar with the addition of 0.5% glucose. 1 µ spots were added to the centre of the plate and left to swarm for up to 48 hours.

Table 6.5 Correlation between biofilm production and swarming repression phenotypes

Strain	Species	Biofilm induction	Swarming inhibition
LMG 18821	*B. cepacia*	**Yes**	**Yes**
LMG 16660	*B. multivorans*	**No**	**No**
ATCC 17616	*B. multivorans*	**No**	**Yes**
J2315	*B. cenocepacia*	**Yes**	**Yes**
C4455	*B. cenocepacia*	**Yes**	**Yes**
AU1054	*B. cenocepacia*	**Yes**	**Yes**
LMG 18827	*B. cenocepacia*	**Yes**	**Yes**
LMG 18832	*B. cenocepacia*	**No**	**No**
24	Bcc Novel group K	**Yes**	**Yes**
LMG 18943	*B. dolosa*	**No**	**Yes**

Grey shaded areas = strains that exhibit both induction of biofilm production and inhibition of swarming in the presence of chlorhexidine.

larger panel should be screened before a definitive link can be made (Table 6.5).

6.2.10 – Transposon mutagenesis screen

The observation that swarming is inhibited at sub-lethal concentrations of chlorhexidine was explored further by creating a mutant bank and screening for mutants with uncoupled swarming inhibition. It was hoped this strategy could identify the genetic basis for sensing chlorhexidine and the repression of motility. *B. multivorans* ATCC 17616 was selected as a model organism for mutagenesis as it produced the clearest inhibition in chlorhexidine. *B. cenocepacia* J2315 would have been the ideal genetic model organism as the microarray experiment was carried out on this organism. However, although the inhibition effect can clearly be seen (Figure 6.15), J2315 is a poor swarmer and would have been difficult to use in the mutagenesis screen.

Preliminary experiments were carried out to check the antibiotic susceptibility of ATCC 17616 and to make sure insertion of the mini-Tn*5* transposon was random. Due to the high resistance of this strain to kanamycin, a transposon carrying this resistance gene could not be used (it was capable of growth at 600 µg/ml). Consequently, a tetracycline resistance transposon was utilised. A preliminary mating was carried out, but the sequence analysis of the random mutants nested PCR suggested that the transposon had inserted into the same gene. Four mutants were sent for sequencing and they all mapped to the same gene, an aldehyde dehydrogenase, indicating that the ATCC 17616 genome had an insertion hotspot for mini-Tn*5* within this gene. Hence, a plasposon with trimethoprim resistance was used, however this was also observed to insert into the same gene as the mini-Tn*5* transposon. Finally, *B. cenocepacia* AU1054, another genome sequenced strain was chosen as a model as this also had a good chlorhexidine inhibition effect. AU1054 had high resistance to kanamycin and tetracycline (300 µg/ml), so the Tp plasposon was used to create a random bank of mutants, which were then pooled and spotted onto swarming media (Chapter 2, section 2.20). Approximately 3000 mutants were screened for uncoupled swarming, producing 31 positive mutants (Table 6.6). Mutants that swarmed in the presence of chlorhexidine were picked and sequenced.

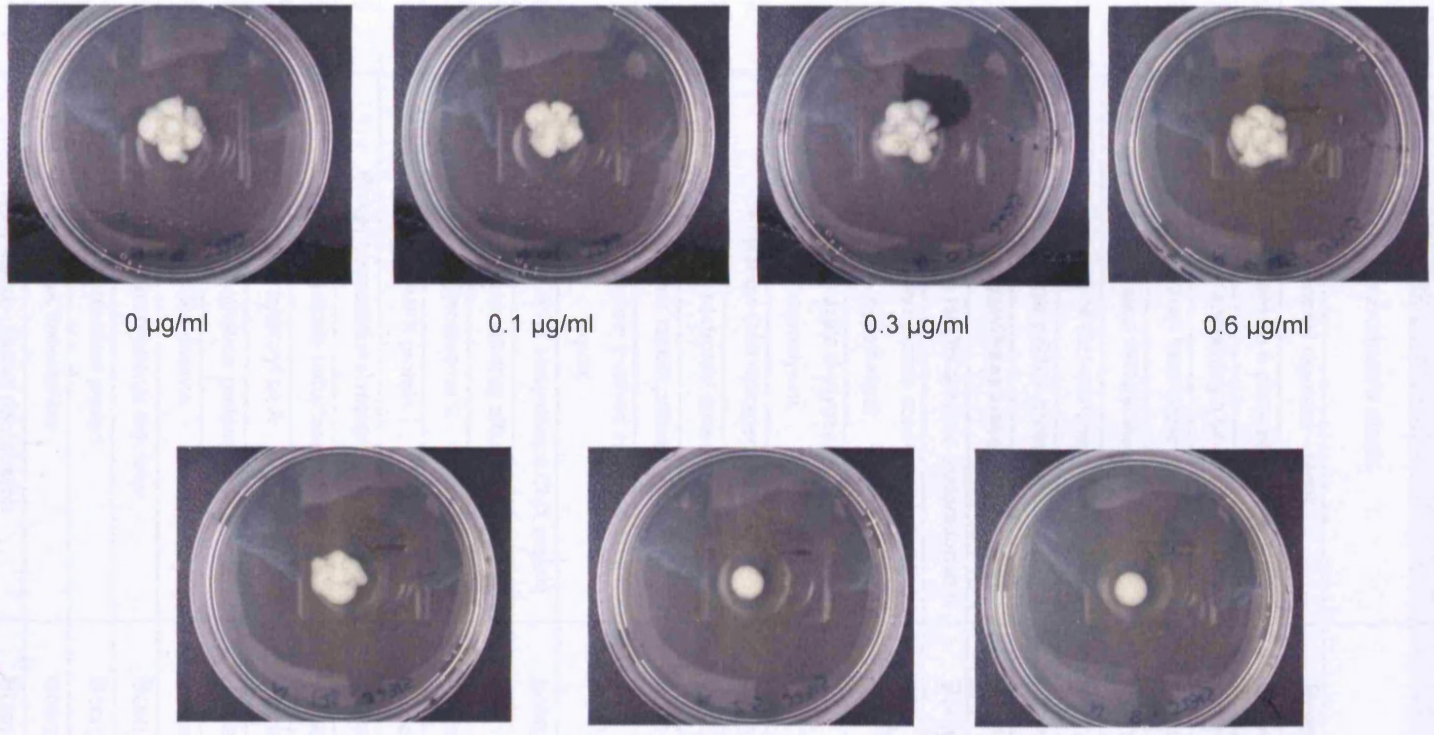


Figure 6.15 *B. cenocepacia* J2315 swarming inhibition in response to chlorhexidine

Swarming plates were made with 0.3% nutrient agar with the addition of 0.5% glucose. 1 µl spots were placed onto the centre of the plate and left to swarm for up to 48 hours.

Table 6.6 *B. cenocepacia* AU1054 swarming mutants

Mutant Name	Function	Gene Number (chromosome)	Swarming at 5 µg/ml chlorhexidine (mm)
AU1054 (control)	*B. cenocepacia strain*	-	30
HR 2	Transcriptional regulator - GntR	Bcen 5579 (3)	30
HR 3	pyroxaimnie 5 phosphate	Bcen 2826 (1)	35
HR 5	response regulator containing a CheY like receiver	Bcen 1049 (1)	40
HR 6	glycosyl transferase	Bcen_0291 (1)	50
HR 7	glycosyl transferase	Bcen_0291 (1)	30
HR 8	proton-translocating NADH-quinone oxidoreductase	Bcen_6005 (3)	35
HR 9	hypothetical signal peptide protein precursor	Bcen_5923 (3)	70
HR 10	glycosyl transferase	Bcen_0291 (1)	60
HR 11	proton-translocating NADH-quinone oxidoreductase	Bcen_6005 (3)	14
HR 12	IcmF, Uncharacterized protein conserved in bacteria - hypothetical	bcen_2620 (1)	75
HR 13	4-hydroxyphenylpyruvate dioxygenase and related haemolysins	bcen__2785 (1)	75
HR 14	Phytanoyl-CoA dioxygenase	bcen_1544 (1)	45
HR 15	IcmF, Uncharacterized protein conserved in bacteria	bcen_2620 (1)	32
HR 16	ankyrin repeat protein	bcen_6189 (3)	69
HR 17	Wza, Periplasmic protein involved in polysaccharide export	bcen_0287 (1)	80
HR 18	cobalamin (vitamin B12) biosynthesis CbiX protein	bcen_1858 (1)	85
HR 19	AcrB, Cation/multidrug efflux pump	bcen_1143 (1)	32
HR 23	Cytochrome C	Bcen_2058	35
HR 24	MFS protein	Bcen_1316	35
HR 25	Phage transcriptional regulator AlpA	Bcen_1759	65
HR 26	Ferredoxin reductase	Bcen_2439	25
HR 27	3 hydroxyl co A	Bcen_3317	60
HR 28	Hypothetical protein	Bcen_6297	72
HR 29	Cell division	Bcen_0496	39
HR 30	OmpR transcriptional regulator	Bcen_6141 (3)	60
HR 31	Hypothetical protein	Bcen_1095 (1)	55
HR 33	CoA thioesterase	Bcen_0321 (1)	30
HR 34	Hypothetical - phenol degradation	Bcen_4081 (2)	55
HR 36	Phage transcriptional regulator, AlpA	Bcen_1759 (1)	65
HR 37	OsmC like regulator	Bcen_5771 (3)	40
HR 38	Levanase	Bcen_4097	65

6.2.11 Phenotypic characterisation of swarming mutants

Minimum inhibitory concentrations in chlorhexidine were carried out for 16 of the swarming mutants to see if they had become more susceptible to chlorhexidine (Table 6.7). 13 out of 16 mutants had no difference in their MIC value to the AU1054 control. Three of the mutants had an elevated MIC value of > 100 µg/ml. None of the mutants appeared to be more susceptible to chlorhexidine.

As well as chlorhexidine MIC values, biofilm assays were performed to determine if the ability to swarm in the presence of chlorhexidine leads to attenuated biofilm formation. Eleven mutants were screened for biofilm induction on the presence of chlorhexidine compared to *B. cenocepacia* AU1054 wildtype. Out of the eleven mutants, 81% had no induction of biofilm production in chlorhexidine compared to the wildtype (Figure 6.16). Two mutants, HR5, a CheY like receiver and HR 6; a glycosyl transferase did have induction of biofilm production at sub inhibitory levels of chlorhexidine. However, the wildtype had a greater proportion of biofilm induction compared to HR 5 and HR 6 (Table 6.8).

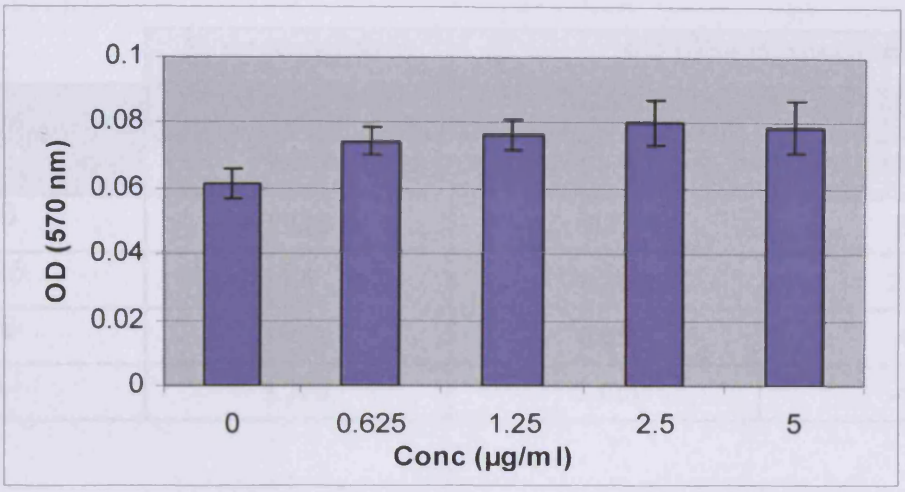
6.2.12 Bioinformatic analysis of mutants

To further elucidate the role these genes play in the swarming inhibition phenotype and the possibility of contribution to biocide resistance, two genes that produced good uncoupling mutants and their surrounding genes were analysed. These genes were, HR 5, CheY like receiver and HR 6, a glycosyl transferase. The bioinformatic analysis carried out for each gene can be seen in Table 6.9 and 6.10 and included BLAST, PFAM and COG searches. To look for transmembrane domains the TMHMM database was used and to look for transport related function the transport classification database was employed. The genes surrounding the mutated gene were selected on the basis that they may be part of an operon. BCEN_1049 was found on chromosome one and had thirteen surrounding genes. This gene mapped to a CheY-like receiver, which could be part of a two component regulator system. CheY is part of a response regulator involved with chemotaxis. The surrounding genes were involved in the synthesis and secretion of protein

Table 6.7 Chlorhexidine MIC values in *B. cenocepacia* AU1054 mutants

Mutant Name	MIC (µg/ml)
AU1054 (control)	40 – 50
HR 2	> 100
HR 3	40 - 50
HR 5	>100
HR 6	>100
HR 7	40 - 50
HR 8	40 – 50
HR 9	30 - 40
HR 10	30 - 40
HR 11	40 – 50
HR 24	40 – 50
HR 26	40 – 50
HR 29	40 – 50
HR 33	40 – 50
HR 34	40 – 50
HR 37	20 – 30
HR 38	30 - 40

a



b

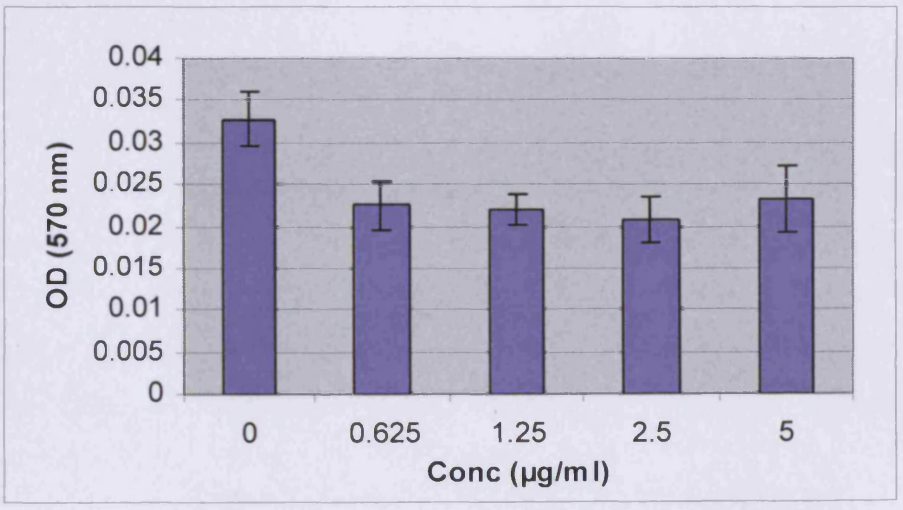


Figure 6.16 Biofilm production (± SE) in chlorhexidine in: a, B. *cenocepacia* AU1054 wildtype and b, AU1054 swarming mutant HR 2 (transcriptional regulator)

In the wildtype strain biofilm production has increased (reading taken at 72 hours after 1% crystal violent staining and resolubilisation in acetic acid) as concentration of chlorhexidine increases (Panel a). In the mutant, biofilm production decreases as chlorhexidine concentration increases.

Table 6.8 Biofilm induction in *B. cenocepacia* AU1054, swarming mutant HR 5 and HR 6

Conc (µg/ml)	*B. cenocepacia*	AU1054 derivative	
	AU1054	HR 5	HR 6
	Percentage increase in biofilm production (%)		
0.6	1.325	0.7	3.1
1.25	1.5	0.25	2.15
2.5	1.875	0.35	-0.7
5	1.775	0.125	-1.4

required for Tad pilin biogenesis. Pili are involved with bacterial adherence, as well as biofilm formation and are a well know virulence factor. The Tad genes are found on a genomic island and encode the machinery that is required for the assembly of the Flp pili. This pili is essential for colonization, adherence, virulence, pathogenesis, biofilm formation and possibly twitching motility. Originally the Tad loci was identified in the Gram negative pathogen *Aggregatibacter actinomycetemcomitans*, but homologs have also been identified in a number of Gram positive and Gram negative species such as *Yersinia pestis*, *Vibrio cholerae*, *Mycobacterium tuberculosis*, *P. aeruginosa*, *Bordetella pertussis* and *Burkholderia pseudomallei* (Tomich *et al.*, 2007).

BCEN_ 2091 was a glycosyl transferase. The mutagenesis screen had multiple hits within this gene (three), indicating it may be important in swarming. This gene was also found on chromosome one and had five possible genes within the operon. These genes were involved with the transport and secretion of sugars, cell to cell regulation and transport of cytotoxic compounds from the cell (ABC transporters).

The model strain used in transposon mutagenesis screen was *B. cenocepacia* AU1054. As the microarray experiment was carried out on *B. cenocepacia* J2315, the genes knocked out by mutagenesis were compared to the J2315 genome to look for homology and then analysed in GeneSpring to determine if any of these genes had altered expression in the presence of chlorhexidine. All of the genes had homology to the J2315 genome. Nonetheless, when searched for in GeneSpring, none of these genes had a significant change in expression in response to chlorhexidine, with values close to 1.

6.3 Discussion

6.31 Are genes BCAM 0924 and BCAL 2353 important for chlorhexidine resistance?

Table 6.9 Bioinformatic analysis of AU1054 mutant HR 5 (BCEN_1049), CheY like receiver and surrounding genes

Gene Name	BLAST PSI	PFAM	COG	TCDB	Transmembrane Prediction
BCEN_1037	Flp/Fap pilin superfamily	Flp_Fap component	COG3847 – N – Cell motility and secretion	Sodium dependent phosphate pump	1
BCEN_1038	peptidase A24A, prepilin type IV/CpaA	Peptidase_A24- essential for correct formation of pseudopilli in type 4 secretion systems (PFO 1478)	COG4960 – U - Intracellular trafficking and vesicular support	Type 4 prepillin type proteins	5
BCEN_1039	TadE family	TadE (PFO7811) – involved in pillus assembly - adherence	COG4961 – U - Intracellular trafficking and vesicular support	Type 4 – secretory pathway	1
BCEN_1040	Saf superfamily/ CpaB – pilus assembly	SAF (PFO8666) – range of proteins including flagellar FlgA and pilus CpaB proteins	COG3745 – N – Cell motility and secretion	Type 4 – secretory pathway	1
BCEN_1041	Secretin superfamily – type 2 and 3 secretion system – PulD - motility	Secretin (PFO0263) – involved with transporting exoproteins from the periplasm – secretion systems	COG4964 – U - Intracellular trafficking and vesicular support	Pillin secretion / fimbrial assembly protein	0
BCEN_1042	ABC_ATPase superfamily – response regulator receiver protein	(PF00072) – response regulator, receives the signal from the sensor partner in two component systems	COG4963 – U - Intracellular trafficking and vesicular support	0	0
BCEN_1043	VirB111-like ATpase – type 4 secretory protein/ type 2	GSP11_E (PF00437) – contains type 2 and 4 secretion proteins	COG4962 – U - Intracellular trafficking and vesicular support	0	0
BCEN_1044	Type 2 secretion system. Gsp11_F superfamily. TadB	Gsp11_F (PF00482) – type 2 secretion, type 4 pilus and archaeal flagellar	COG4965 – U - Intracellular trafficking and vesicular support	Type 4 secretary pathway	5
BCEN_1045	Gsp11_F superfamily. TadC – pilus/motility and secretion	Gsp11_F (PF00482) – type 2 secretion, type 4 pilus and archaeal flagellar	COG2064 – U - Intracellular trafficking and vesicular support	Adherence (Pilius) biogenesis	4

Name	BLAST PSI	PFAM: function	COG	TCDB	Transmembrane prediction
BCEN_1046	TadD – pilus assembly – secretion. Tetratricopeptide TPR_2	Tetratricopeptide repeat (PF07719)	COG5010 – U - Intracellular trafficking and vesicular support	No hits	0
BCEN_1047	Hypothetical protein	No matches		No hits	0
BCEN_1048	COG4655 superfamily – predicted membrane protein	TadE (PFO7811) – involved in pillus assembly – adherence – possible or domain of unknown function – transmembrane?	COG4655 – S – predicted membrane protein	No hits	1
BCEN_1049	Activates expression of genes from promoters recognised by core RNA associated with sigma factors – involved with two component regulatory system and possess a domain that can be phosphorylated by a sensor-kinase protein in their N-terminal section	Sigma 54 interaction domain	COG 2204 – response regulator containing a CheY like receiver – sigma 54 specific transcriptional regulator	Type 6 secretion system	0
BCEN_1050	Hypothetical protein	No matches – putative lipoprotein	COG4655 – D chromosome segregation ATPases	No hits	0

Table 6.10 Bioinformatic analysis of AU1054 mutant HR 6 (BCEN_0291), glycosyl transferase and surrounding genes

Name	BLAST PSI	PFAM: function	COG	TCDB	Transmembrane prediction
Bcen_0293	ABC membrane superfamily – type 2 – TagG – Carbohydrate metabolism, cell envelope biogenesis and outer membrane	ABC type 2 transporters – consists of a conserved ATP binding cassette and a transmembrane domain. Bind ATP – hydrolysis – signature motifs. Provides essential nutrients and is involved in the export of toxic substances	COG1682 – M – cell wall / membrane / envelope biogenesis	ATP binding cassette (ABC superfamily)	6
Bcen_0292	ABC transporter – involved in the export of polysaccharides. May also be involved in drug resistance	ABC transporter – same as above – responsible for the translocation of a variety of compounds across biological membranes	COG1134 – G – carbohydrate transport and metabolism	ATP binding cassette (ABC superfamily)	0
Bcen_0291	Glycosyl transferase – same as below – but contains 3 copies of tetratricopeptide repeat domain – consensus sequence in many functions – protein – protein interactions, chaperone and transport	TPR - tetratricopeptide repeat – involved in cell to cell regulation and protein folding. Also glycosyl transferase type 1 – transfers sugars – involved in many process including exopolysaccharide biosynthesis.	COG0438 – M – cell wall / membrane/ envelope biogenesis	Protein importer	0
Bcen_0290	Superfamily – Glycosyl transferase family 1 – transfer activated sugars to substrates such as lippopolysaccharides – cell envelope biogenesis and outer membrane – multi domain RfaG	Same as above – no TPR	COG0438 – M – cell wall / membrane/ envelope biogenesis	Respiratory nitrate reductase	0

Name	BLAST PSI	PFAM: function	COG	TCDB	Transmembrane prediction
Bcen_0289	Sulfotransferase domain – hypothetical protein	Sulfotransferase – transfer of sulphate groups to compounds	COG1196 – D – cell cycle control / cell division	Oxidase proteins	0
Bcen_0288	Glycosyl transferase family 2 – transfers sugars to a range of substrates. Maybe cell wall biogenesis	Glycosyl transferase family type 2 – transfers sugars to a range of substrates including cellulose, dolichol phosphate and teichoic acids.	COG0463 – M - cell wall / membrane/ envelope biogenesis	Glycosyl polymerization family	0

The microarray experiment (Chapter 5) produced a list of genes with significant changes in expression when *B. cenocepacia* J2315 was exposed to chlorhexidine. Two of these genes were selected for further analysis, BCAM 0924 a response regulator and BCAM 2353 a transport related gene.

BCAM 0924 was found on chromosome 2 of the J2315 genome and appeared to be part of an operon that was homologous to the Mex AB-OprM efflux pump found in *P. aeruginosa*. Other genes, which could be part of the efflux operon were also up- regulated, such as BCAM 0925, an outer membrane protein (OprM precursor) and BCAM 0927, a secretion protein (HlyD family). MexAB-OprM is a well characterised efflux pump in *P. aeruginosa* that is constitutively expressed (Piddock, 2006a). Although not up-regulated in this experiment, BCAM 0926 may also be part of this operon and contains 12 transmembrane helices which is characteristic of RND efflux pumps (Borges-Walmsley and Walmsley, 2001) (Table 6.1).

The MexAB-OprM pump was first described by Poole *et al.* who found a 50 KDa outer membrane protein which had homology to previously described export proteins. Mutants lacking this gene were more susceptible to tetracycline, chloramphenicol, ciprofloxacin streptonigrin and dipyridyl (Poole *et al.*, 1993). The expression of the outer membrane protein led to resistance to a number of unrelated compounds indicating that this protein was part of an efflux operon (Poole *et al.*, 1993). The MexAB-OprM efflux protein is involved with the efflux of multiple antibiotics, but may also efflux other compounds such as ethidium bromide, acriflavine, SDS, triclosan and organic solvents (Piddock, 2006a). The ability to efflux a range of substrates raised concerns that the widespread use of disinfectants may induce a number of efflux pumps that can also efflux antibiotics, leading to the emergence of strains with multi drug resistance.

In *B. cenocepacia* J2315, three genes possibly involved with RND efflux were identified with altered expression in response to chlorhexidine (Table 5.4). One of these genes (BCAM 0925) was homologous to OprM, an outer membrane channel found in *P. aeruginosa*. This channel is essential for the efflux of toxic compounds

although some Mex pumps are able to work without it (Chuanchuen *et al.*, 2002). The efflux protein seen in *B. cenocepacia* J2315 may be regulated by a response regulator (BCAM0924) and this could be part of a two component regulator system as BCAM 0923, has homology to a sensor kinase. This suggests that the efflux operon may be induced by sub inhibitory levels of chlorhexidine by interacting with the response regulator and thus allowing chlorhexidine to be removed from the cell. This observation is the first time the MexAB-OprM efflux pump has been associated with resistance to chlorhexidine. J2315 also has resistance to a number of antibiotics including β lactams such as imipenem and piperacillin, as well as tobramycin and trimethoprim / sulfamethoxazole. The induction of this efflux pump by chlorhexidine may also increase resistance to some of these antibiotics, if they are substrates for the pump.

Other Mex efflux systems have now been described in *P. aeruginosa,* such as MexCD-OprJ (Poole *et al.*, 1996), MexEF-OprN (Kohler *et al.*, 1997) and MexXY-OprM (Chuanchuen *et al.*, 2002). *P. aeruginosa*, PA01 induced expression of the MexCD-OprJ multidrug efflux pump in the presence of clinically important disinfectants, such as benzalkonium chloride, chlorhexidine gluconate and other cytotoxic agents like ethidium bromide (Morita *et al.*, 2003). When the efflux pump was induced by these compounds the strain exhibited increased resistance to norfloxacin. The MexCD-OprJ efflux pump, can also efflux tetracycline, chloramphenicol, streptomycin, erythromycin and carbenicillin (Morita *et al.*, 2003). Clearly, this resistance to a number of substrates in this strain are induced by clinically relevant disinfectants, which may be problematic in a clinical situation.

The MexXY-OprM efflux pump can also efflux triclosan, a well used disinfectant. This efflux pump is regulated by the repressor *mexL*. After exposure to triclosan, mutants containing a single nucleotide change in the repressor were selected. These mutants were able to efflux triclosan without the requirement of the OprM membrane channel, but could only efflux tetracycline and erythromycin if this channel was present (Chuanchuen *et al.*, 2002). This study provided evidence that the efflux operon was regulated by a two component system that was induced by

the presence of the disinfectant triclosan, although for efflux of other compounds, the tripartite structure, including the OprM channel was required.

BCAL 2353 was homologous to a transport related protein and was found on chromosome 1. There were 10 predicted transmembrane helices (Table 6.2), indicating that this gene could be involved in transport of organic substances and cytotoxic products out of the cell (Borges-Walmsley and Walmsley, 2001). There were 2 other genes that may be part of an operon. BCAL 2351 had no homology to known proteins and was unclassified; where as BCAL 2352 was homologous to a carbonic anhydrase and inorganic ion transport. The transport of inorganic ions, such as sulphur across the bacterial membrane is important for many processes. The Bcc produce many virulence factors, such as intrinsic resistance, production of catalase and superoxide dimutase, unusual lipopolysaccharide structure, quorum sensing, flagella, biofilm formation and production of siderophores. Production of several of these virulence factors are associated with anabolic pathways such as sulphur assimilation (Iwanicka-Nowicka *et al.*, 2007). *B. cenocepacia* produces a yellow green fluorescent siderophore, pyochelin. The production of this siderophore is dependent on sulphur availability and will not be produced if there is limited sulphur (Farmer and Thomas, 2004). This signifies that the transport of inorganic ions such as sulphur may be essential for the production of virulence factors and bacterial survival. The role of the transport related gene BCAL 2353 has not been defined in *B. cenocepacia* J2315 as of yet. The altered expression in response to chlorhexidine may suggest that this gene plays a role in transporting this substance out of the cell or it may promote transport of inorganic ions that are important for the bacterium's survival in the presence of this biocide. To further elucidate the role of these two genes in chlorhexidine, it was attempted to delete the genes by mutagenesis.

6.32 Mutagenesis of genes BCAM 0924 and BCAL 2353

Deletion of the genes was first attempted using a plasmid insertion method by Flannagan *et al.* (Flannagan *et al.*, 2007). They constructed a suicide vector that contained a resistance gene (Tp), flanked by omega fragments. The plasmid was

inserted into the target genes and polar mutations would be observed due to the presence of omega fragments (terminates RNA and protein synthesis prematurely). However, in this investigation no mutants could be isolated. Tri-parental mating was carried out, but isolates selected did not contain the trimethoprim cassette and the gene product was still present, suggesting that the plasmid had not inserted correctly. Therefore a second strategy of allelic exchange was tried. This method used a suicide vector as described by Quandt *et al* (Quandt and Hynes, 1993). Construction of this suicide vector containing the target gene with a trimethoprim resistance cassette was carried out and mated into *B. cenocepacia* K56-2. Mutants were selected and RAPD profiling carried out to check that the mutants were K56-2, not contaminants (Figure 6.5) and characterisation carried out.

To determine if the mutants of BCAM 0924 and BCAL 2353 were more susceptible to chlorhexidine MIC experiments were carried out using a Bioscreen rapid growth analyser. The Bioscreen C took an OD reading every 5 minutes for 48 hours and growth curves were calculated (Figure 6.8). The wildtype K56-2 was capable of growing at 100 µg/ml (Figure 6.8, a) although this was at a much lower rate than with no biocide present. The mutants for both genes were capable of growing up to 40 µg/ml of chlorhexidine. After this point no growth was observed (Figure 6.8, b and c), signifying that the mutants were 2.5 times more susceptible to chlorhexidine than the wildtype strain. Therefore, it appears the BCAM 0924 and BCAL 2353 do have a role in chlorhexidine resistance and that the ability to survive in higher concentrations was attenuated when these genes were not present. However, the two genes, especially BCAM 0924 may only be a small part of an operon that is involved in resistance to chlorhexidine. A greater increase in susceptibility may be observed if other genes in the operon are also deleted, for example the sensor kinase domain that is involved in the regulation of the RND efflux pump with BCAM 0924.

The ability to form biofilms was also screened to establish if these two genes played a role in other antimicrobial resistance mechanisms. The mutants for both genes had attenuated biofilm production when compared to the wildtype strain, with the wildtype producing 2 – 4 fold more biofilm than the mutants (Figure 6.9). This

suggests that the two genes may play a role in other mechanisms that allow bacterial survival in chlorhexidine such as the ability to form biofilms. In *Candida albicans*, efflux pumps were up-regulated when in biofilms promoting a multi drug resistant, protected life style (Ramage *et al.*, 2002). Consequently, a similar mechanism may be at work in the mutants, with the response regulator BCAM 0924 and transport gene BCAL 2353 playing a role in biofilm formation and efflux up regulation.

The mutants isolated were subjected to molecular analysis to establish that the Tp cassette had inserted correctly in the gene. The first check carried out was a PCR screen specific for the Tp cassette. This PCR screen was positive for all the mutants checked (Figure 6.4), producing a band of approximately 500 bp. However, when internal primers were designed that flanked the restriction cloning site for the Tp cassette, the results were less conclusive. A wildtype band (300 bp) was present from the screen for the control K56-2, but also for the mutants where a larger band of approximately 800 bp should be visible. This indicated that a double crossover had not occurred or that the Tp cassette had not inserted in the correct place. Therefore, although an increase in chlorhexidine susceptibility was observed in these mutants, the results are questionable due to the inability to obtain the correct size band for the genes with the inserted Tp cassette.

6.33 Does the presence of sub inhibitory levels of chlorhexidine induce biofilm formation and inhibition of swarming?

The observation that sub inhibitory levels of chlorhexidine caused a global down regulation of motility and chemotaxis genes raised a number of questions. Flagella are important for many processes and are a key virulence factor. For a host – pathogen interaction to occur the pathogen must first attach to epithelial cells. Pathogens use motility combined with chemotaxis to attach and colonise cells (Ramos *et al.*, 2004). Invasion of host cells is important for the establishment of the infection and attachment to surfaces may increase the chances of biofilms forming. In flagella mutants, virulence is severely attenuated, for example a non motile *B. cepacia* strain generated by random transposon mutagenesis had reduced

invasiveness of respiratory epithelial cells (Tomich *et al.*, 2002). As well as having a role in initial attachment and invasion, the flagellum machinery are similar to type three secretion systems, indicating the flagella may actually play a role in secreting virulence factors into the cytosol of host cells (Ramos *et al.*, 2004).

Although flagella are important virulence factors for pathogenic bacteria, they are also a significant stimulator of the host's immune system. To avoid this pro-inflammatory response and resist phagocytes, some pathogens can regulate their flagellar and become non motile. *P. aeruginosa* is the most common CF infection and is often carried chronically by the patient. These chronic isolates are non motile and are difficult to eradicate. Notably, the lack of motility in these chronic CF isolates are very different to environmental isolates, where only motile isolates are found (Mahenthiralingam *et al.*, 1994). These non flagellated isolates were resistant to phagocytosis by macrophages and therefore could persist in the lungs of CF patients (Mahenthiralingam *et al.*, 1994). It was suggested that flagella expression was regulated by a sigma factor (RpoN) and that the repression of flagella may be due to harsh environmental conditions (Mahenthiralingam *et al.*, 1994). This has been seen in *E. coli*, where isolates became non flagellated due to high temperatures, high concentrations of inorganic salts, high concentrations of carbohydrates, high concentrations of low molecular weight alcohols and presence of gyrase inhibitors (Shi *et al.*, 1993). The adaptation to become non flagellated and therefore non motile may have a number of advantages for the bacteria, such as evasion of the hosts immune system, biofilm production and loss of other virulence genes, aiding survival in harsh environments. Consequently, similar mechanisms may be occurring in *B. cenocepacia* J2315 in the presence of chlorhexidine.

The question "does sub inhibitory levels of chlorhexidine induce biofilm formation" was addressed in this investigation. Biofilms contribute to antimicrobial resistance as cells grown within a biofilm have an altered growth rate, making the uptake of antimicrobials difficult and penetration of the biofilm may be prevented by the accompanying production of an exopolysaccharide (Stickler, 1999). In *Burkholderia* strains biofilm production has also been linked to intracellular survival and the

invasion of host cells, via the destruction of the glycocalyx layer produced by epithelial cells (Savoia and Zucca, 2007).

The contribution of biofilms to antimicrobial resistance is well documented; however the efficacy of biocides against biofilms has also become a concern. Miyano *et al.* found that 0.1% and 0.2% chlorhexidine gluconate and 0.1% benzalkonium chloride had no effect on *B. cepacia* strains in biofilms compared to planktonic growth and that 0.5% chlorhexidine gluconate, 0.5% benzalkonium chloride and 0.1% alkyldiainoethyl glycine were barely effective against biofilms even after 60 minutes of exposure (Miyano *et al.*, 2003). This was corroborated by an investigation of common hospital biocides against biofilms of MRSA and *P. aeruginosa.* The biocides contained benzalkonium chloride (1%), chlorhexidine gluconate (4%) and triclosan (1%) (Smith and Hunter, 2008). The biocides killed 99.9% of these bacteria when grown planktonically, but when applied to *P. aeruginosa* and MRSA bioflms, viable cells were isolated after 24 hours of growth (up to 80% and 0 -11% respectively) and no biocide was 100% effective against the bioflms (Smith and Hunter, 2008). Therefore biofilms pose a real problem for hospitals and clinicians and can render some disinfection procedures useless.

A worrying observation is the possibility of induction of biofilm formation in the presence of sub-inhibitory levels of the biocide. This phenomenon has been seen in *P. aeruginosa* strains in response to the aminoglycoside tobramycin (Hoffman *et al.*, 2005). This study found that concentrations of tobramycin below the MIC caused a maximum of 3.4 fold increase in biofilm production and that this induction was regulated by a response regulator named as the aminoglycoside response regulator (arr) (Hoffman *et al.*, 2005). This regulator encoded a phosphodiesterase whose substrate was cyclic di guanosine monophosphate (ci- di – GMP), a bacterial second messenger that regulates cell adhesiveness (Hoffman *et al.*, 2005). Bioinformatic analysis did not demonstrate the presence of a significant arr gene homolog in the Bcc.

In this investigation a biofilm assay was used to determine if sub-inhibitory levels of chlorhexidine induced biofilm formation. The assay used a rapid 96 well plate

assay and a crystal violet stain. The wells of the plates were first coated with mucin, a protein component of mucus that is abundant in CF airways and aids attachment of cells to the surface of the plate, thus producing more biomass and making biofilms easier to visualise (Landry *et al.*, 2006). The plate assay revealed that 58.3% and 83% of strains had induction of biofilm production in the presence of chlorhexidine and CPC respectively. This induction did not appear to correlate with resistance as some of the more resistant strains did not show this effect, for example *B. cenocepacia* LMG 18832, chlorhexidine MIC >100 µg/ml and *B. multivorans* LMG 16660, chlorhexidine MIC – 90 – 100µg/ml, both had no induction of biofilms in chlorhexidine (Figure 6.10). Thus the induction of biofilms by biocides appears to be strain specific and is not conserved across the complex.

It also appeared that strains associated with epidemic outbreaks were not more likely to induce biofilms than those strains associated with sporadic infection. Nonetheless, a larger panel of strains that includes all members of the Bcc should be screened so a definite conclusion can be made. The biofilm induction appeared to be most effective in CPC and chlorhexidine, with other biocides not exhibiting this effect (e.g. triclosan and benzalkonium chloride), suggesting that the induction is also dependent on the type of biocide applied as well as the strain. However, in tobramycin, the same induction was seen in all 4 strains screened with a maximum increase of 20.5% in *B. cenocepacia* C4455 (Figure 6.12). This supports the study by Hoffman *et al* (2005), which found an induction by tobramycin in *P. aeruginosa* and therefore a similar regulatory mechanism may be at work in the Bcc.

Although the biofilm assays provided information on the ability to form biofilms, there were some issues with the reproducibility of the assay. Often repeats would give widely different results. This was combated by the use of mucin to coat the plates and aid adhesion, which did result in better reproducibility. However, there were still differences between the repeats, resulting in large standard errors (Figure 6.10 and Figure 6.11). To decrease fluctuations in the results a modified method could be used, where cells are left to adhere for approximately 4 hours. The plates are then washed and fresh media applied and biofilms then left to develop (Peeters *et al.*, 2008). However, the results indicate that in some strains of the Bcc including

the epidemic ET-12 lineage *B. cenocepacia* J2315, biofilms can be induced by sub inhibitory levels of biocide, which could be problematic in hospital settings, where a bacterium may be in contact with low levels of biocide frequently.

As well as the ability to form biofilms in the presence of biocides, the repression of flagella genes suggests that swarming motility may also be inhibited. Swarming is needed for a number of applications such as allowing the bacterium to reach a surface, used for chemotaxis so the bacterium can reach nutrients and for the spreading of biofilms across a surface (Pratt and Kolter, 1998). In this investigation it was seen that all species of the Bcc had strains that were capable of swarming motility, but this was not universal with some strains exhibiting no swarming or very poor swarming. Interestingly, *B. cenocepacia* strains were among the worst swarmers (Table 6.3). In chlorhexidine 67% of strains screened showed inhibition of swarming, indicating that chlorhexidine does repress flagella and inhibit motility. Nonetheless, as with biofilm induction, this inhibition was not seen in all strains, again suggesting that it may be strain dependent, although it does not appear to correlate with chlorhexidine resistance (Table 6.4). The inhibition of swarming may be part of a survival pathway, where chlorhexidine is sensed by the cell and swarming is inhibited. This may prevent contact with the biocide and biofilm production is induced to increase resistance to the compound.

There was a tentative correlation between biofilm induction and swarming inhibition in response to chlorhexidine. Out of 10 strains, 6 demonstrated both swarming reduction and biofilm induction in chlorhexidine (Table 6.5), suggesting that chlorhexidine is responsible for this behaviour and that the two processes may be linked to aid the bacterium's survival in chlorhexidine. It may also induce a lifestyle that is not only more resistant to biocides, but also to other antimicrobial agents. However, a larger panel of strains should be screened before a conclusion can be confirmed. In contrast, other studies have indicated that swarming is necessary for biofilm production as it allows the attachment of the bacteria to a surface and then disperses the biofilm across the surface (Daniels *et al.*, 2004, Pratt and Kolter, 1998). Other studies have also suggested that biofilm formation and swarming are independent of one another (Huber *et al.*, 2001), which may explain why in two

cases swarming inhibition was seen but initiation of biofilm production was not seen (Table 6.5).

6.34 Is there a coordinated regulatory pathway for this phenotype?

The investigation by Hoffman *et al.* found that the aminoglycoside antibiotic, tobramycin induced biofilm formation due to an aminoglycoside response regulator, which contained an EAL type domain (Hoffman *et al.*, 2005). This as well as GGDEF domains are linked to two component regulatory systems that regulate a number of process in the cell. It has been previously shown that the GGDEF and EAL domains are second messenger signal molecules that either produce or inhibit cyclic di-guanosine monophosphate (ci di – GMP). This signalling molecule is produced by the phosphorylation of a response regulator by a sensor kinase in response to a stimuli and is know to regulate cellulose biosynthesis, cell differentiation and motility and biofilm production (Paul *et al.*, 2004, Jenal, 2004). The involvement in motility and biofilm is particularly interesting as it may have some relevance to this investigation, where flagella were repressed in response to chlorhexidine and biofilm production enhanced and motility inhibited.

The apparent sensing of chlorhexidine and coordinated down regulation of flagella in *B. cenocepacia*, indicated that a two component system regulator may be involved. Further evidence to support this theory comes from searching for conserved sequences in upstream regions of repressed genes by MEME (Bailey *et al.*, 2006). The analysis of 9 upstream gene sequences 150 – 200 bp revealed some areas of conservation, that all had similar positions pointing to the possibility of a conserved regulatory binding motif (Figure 6.13.a and b), adding further weight to the two component regulator theory. In addition, an investigation by Simm *et al.* found that GGDEF domains produced ci di-GMP, which led to a sessile, non motile, biofilm lifestyle and that EAL domains may encode a phosphodiesterase that degrades ci di-GMP. These domains were involved with enhancement of swarming and swimming motility and the degradation of ci di-GMP (Simm *et al.*, 2004). The genes encoding the response regulator containing the GGDEF and EAL domain

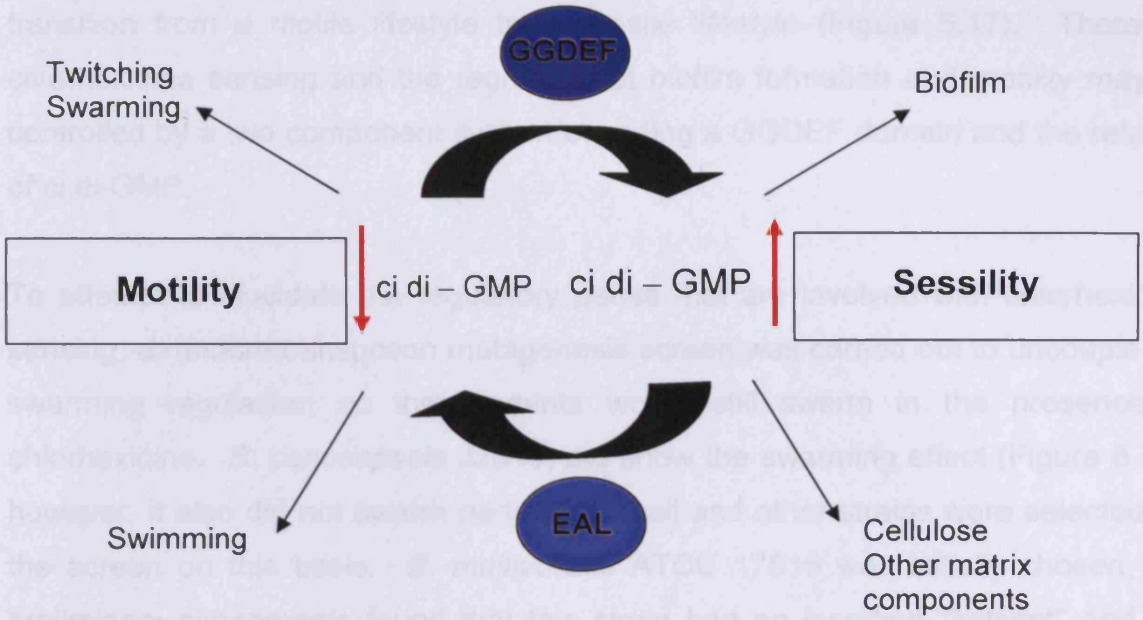


Figure 6.17 Effect of ci di-GMP on motility and biofilm production.

An increase in c di-GMP levels encoded by GGDEF domains causes a transition to a sessile lifestyle, with an increase in biofilm production and aggregation of cells onto surface. Other processes such as cellulose biosynthesis are also regulated by ci di-GMP. In contrast, the EAL domain acts as a phosphatase, degrading levels of ci di – GMP and causes a switch to a motile lifestyle, by increasing swarming, swimming and twitching motility. Figure adapted from (Simm *et al.*, 2004)

were cloned into *E. coli* and *P. aeruginosa* and the same phenotypes were observed, indicating that ci di-GMP does act as signal molecule that regulates the transition from a motile lifestyle to a sessile lifestyle (Figure 6.17). Therefore chlorhexidine sensing and the regulation of biofilm formation and motility may be controlled by a two component system encoding a GGDEF domain and the release of ci di-GMP.

To attempt to elucidate the regulatory genes that are involved with chlorhexidine sensing, a random transposon mutagenesis screen was carried out to uncouple the swarming regulation, so that mutants would still swarm in the presence of chlorhexidine. *B. cenocepacia* J2315, did show the swarming effect (Figure 6.15), however, it also did not swarm particularly well and other strains were selected for the screen on this basis. *B. multivorans* ATCC 17616 was initially chosen, but preliminary experiments found that this strain had an insertion "hotspot" and the mutagenesis was not random. Therefore *B. cenocepacia* AU1054 was used as an alternative model strain. Potential AU1054 mutants were selected on swarming agar containing chlorhexidine at 5 µg/ml and bioinformatics analysis carried out to determine function (Table 6.6). It was hoped that the mutagenesis would reveal a particular regulatory gene that was involved with the regulation of this phenotype. However, hits in many genes were observed suggesting that there may be a number of pathways involved. The genes involved included membrane protease subunits, transcriptional regulators and glycosyl transferases. Two genes were selected for further analysis, the *cheY* receiver as this is involved with chemotaxis and the glycosyl transferase as this had multiple hits. Bioinformatic analysis was carried out on these genes and their surrounding genes to determine the role they may play in swarming inhibition.

6.35 *CheY* receiver protein

One of the mutants was homologous to a CheY receiver. This receiver is part of a response regulator and is involved in the chemosensory system. This system is linked with the regulation of flagella, so was selected for further analysis as the

motility phenotype was being investigated. The surrounding genes of the CheY receiver were all involved with pilus assembly and motility related genes (Table 6.9), making this a very likely regulatory target for inhibition of motility in the presence of chlorhexidine. The CheY two component system has already been linked to biofilm production by a number of studies. Hickman *et al.* found a CheY homolog in *P. aeruginosa* (wspR), which contained a GGDEF domain and therefore catalysed the production of the signal molecule ci di-GMP. They observed that increased levels of ci di – GMP enhanced biofilm formation and aggregation of cells to surfaces (Hickman *et al.*, 2005). Another investigation found that *P. aeruginosa cheY* mutant strains (inactivated in the chemotaxis system) were deficient at cap formation and formed attenuated biofilms. Based on this evidence there is a strong indication that the *cheY* response regulator may be a key part in regulation of biofilm production and motility in response to a environmental stimuli.

6.36 Glycosyl transferase

An interesting observation from the mutagenesis screen was the multiple hits in a glycosyl transferase gene (Table 6 10.). The surrounding genes were also involved with glycosylation and were also homologous to ABC type transporters, indicating that secretion of glycogen may also be involved. Glycosyl transferases are enzymes that catalyse post translational modification of proteins by adding sugar moieties. Flagella are known to be glycosylated. Glycosylation can be involved in flagellar assembly and biological function (Logan, 2006). The glycosylation of the structural protein flagellin may also be involved in virulence of pathogenic bacteria. Mutants of *P. aeruginosa* in flagellar glycosylation, but still with active flagella were created and tested in burn wound infection models (Arora *et al.*, 2005). This investigation found that the lethal dose of the glycosylation mutants, to kill the mouse model increased 35 – 10, 000 fold, indicating that glycosylation is important for virulence (Arora *et al.*, 2005). Another study found that temperature dependent expression of flagella in *Listeria monocytogenes* was regulated by a glycosyl transferase, which acts as a repressor by inhibiting a response regulator MogR

(Shen *et al.*, 2006). Therefore the glycosylation of flagellar may be involved in the regulation of motility and may also be important in virulence.

Both the *cheY* operon and the glycosyl transferase operon contain genes that contain a tetratricopeptide repeat motif (Bcen_1046 and Bcen_2091). These repeats are important for the functioning of cell chaperone, cell cycle, transcription and protein transport complexes (Blatch and Lassle, 1999). The TPR may be involved in transcription and protein interactions, including mediating interactions between kinases (Blatch and Lassle, 1999). Broms *et al.* found that TPR repeats were essential for chaperone activity for the activity of translocator proteins PopB and PopD, involved with the secretion of virulence factors in type three secretion systems (Broms *et al.*, 2006). These tetratricopeptide repeats may be an essential component of two component regulator systems that controls the expression of the flagella operon leading to the phenotypes observed in this investigation. Subsequently the TPRs may be an interesting area for further investigation and a potential target for novel therapeutics.

6.37 Correlation of swarming genes to the J2315 genome

The microarray experiment was carried out on the model strain *B. cenocepacia* J2315, however due to constraints of using this strain for mutagenesis, a different strain, *B. cenocepacia* AU1054 was used for the transposon mutagenesis screen. To correlate these results to the microarray data, the genes highlighted in the mutagenesis screen were BLAST searched in the J2315 genome and then compared to the gene list obtained from GeneSpring. Unfortunately, there was very little correlation between genes found from the microarray and genes from the mutagenesis screen. The only gene that had a correlation was Bcen_1095, chromosome 1, hypothetical protein, which was homologous to J2315 gene BCAM 2418 (putative membrane bound repetitive protein gene), which was significantly up-regulated in the microarray by 1.5 fold change (Table 5.1 and 6.5). Thus this indicates that the regulatory mechanisms controlling the regulation may differ in the two organisms or that the screen was not sufficient to target the correct regulatory

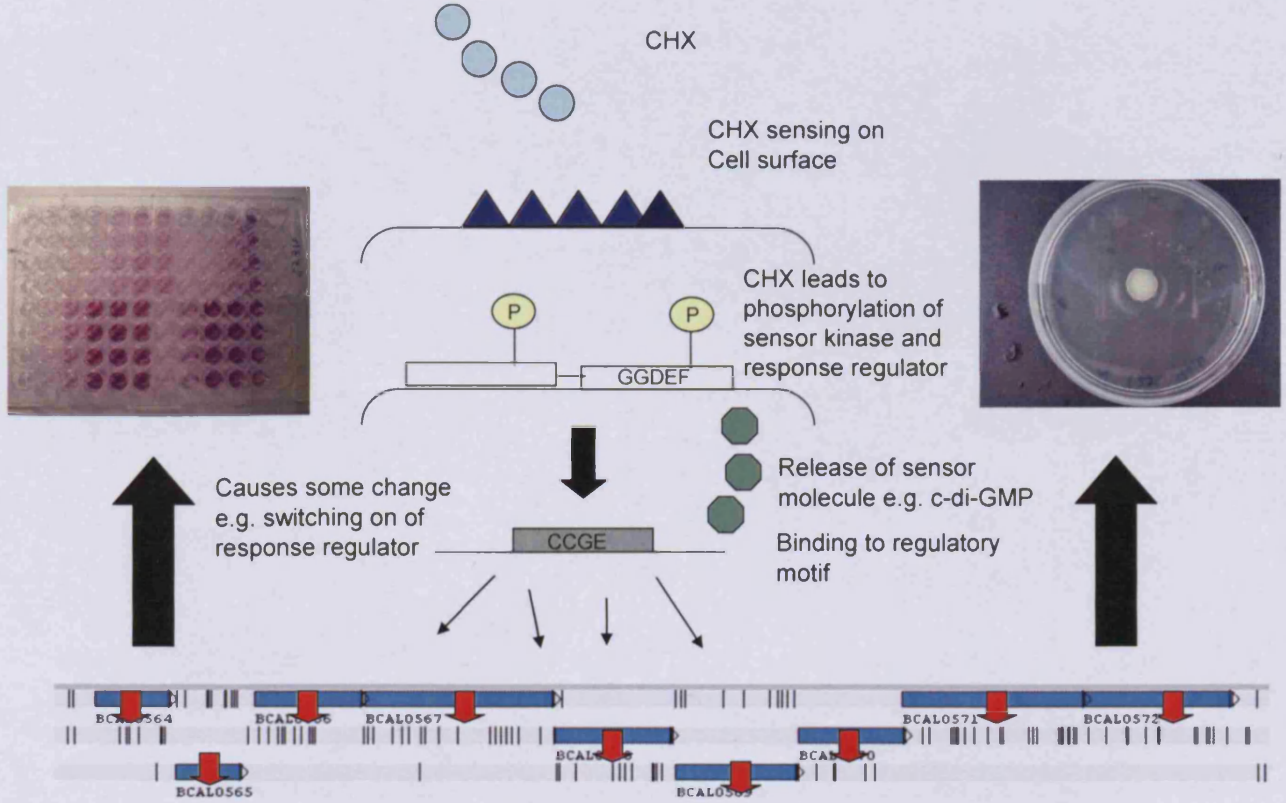
genes. This theory is supported by the amount of genes recovered from the mutagenesis screen, where only a few genes would be expected to be involved if it was a coordinated phenotype regulated by a single system.

6.38 Two component regulatory systems may be involved in chlorhexidine sensing

The evidence presented in this chapter, suggests that a coordinated regulatory pathway is involved in the sensing of chlorhexidine. This regulation is likely to be controlled by a two component regulatory system, which sense chlorhexidine at the cell membrane level. This may then cause the autophosphorylation of a sensor kinase, which is then transferred to the response regulator (possibly CheY, from the mutagenesis screen), which cause the release of a sensor molecule. From the literature a good candidate for this sensor molecule is ci di-GMP, which has already been shown to be involved with the switching between a motile lifestyle and a sessile lifestyle (Simm *et al.*, 2004). The release of ci di-GMP may bind a regulatory binding motif, such as the conserved motif found by MEME analysis which in turn leads to the repression of the flagella operon. The switching off of these genes, promotes a switch to a non motile, sessile lifestyle, with the increase of biofilm and exopolysaccharide production. Thus the Bcc strain can then survive at higher concentrations of the biocide as biofilms exhibit increased resistance to a number of antimicrobials and are also involved with persistence in the CF lung and chronic inflammation (Aubert *et al.*, 2008) (Figure 6.18).

This proposed pathway has not yet been fully elucidated and additional work would be required to determine all the genes involved and confirm that ci di-GMP was the sensing molecule. In addition, not all strains exhibit this effect with some strains only showing biofilm induction and others only showing swarming inhibition. Therefore it appears this sensing pathway may not be ubiquitous within the Bcc and further investigations should be carried out to determine if this correlates with resistance and virulence (discussed in section 6.5).

Two component systems are abundant in bacterial species and the Bcc are no exception. A novel sensor kinase response regulator hybrid, AtsR was described in



Which leads to the down-regulation of flagellar, the inhibition of swarming, an increase in biofilm production and thus a more resistant phenotype.

Figure 6.18 Possible mechanism of chlorhexidine sensing in the Bcc

Chlorhexidine is sensed by the cell membrane leading to the autophosphorylation of a sensor kinase. This phosphate group is transferred to the response regulator and the release of a sensor molecule occurs (ci –d – GMP). This binds to a conserved regulatory motif that causes the repression of the flagella operon, leading to increased biofilm production and decreased motility and hence a more resistant phenotype.

B. cenocepacia K56-2 (Aubert *et al.*, 2008). Inactivation of *atsR* led to a hyperadherent strain and increased biofilm production, indicating that this sensor kinase response regulator hybrid was important for regulating biofilm production. This gene was involved with the regulation of a type 6 secretion system. Consequently it appears that *atsR* is involved with the regulation of virulence factors in the Bcc. Therein, a number of regulatory systems may be involved in the coordination of virulence and resistant mechanisms in the Bcc and these regulatory systems may be good targets for the development of novel therapeutics. Inhibiting the ability of the Bcc to adapt to environmental stimuli such as antimicrobials would leave them more vulnerable to treatment and may prevent the acquisition of strains that are difficult to eradicate.

6.4 Conclusions

To conclude, this chapter aimed to investigate the role of genes identified from the microarray experiment described in Chapter 5. Two up-regulated genes, a response regulator and a transport related gene were deleted and the effects on chlorhexidine susceptibility examined. Although the mutants obtained from these experiments did show an increased susceptibility to chlorhexidine, the results were not conclusive as there were difficulties in confirming the phenotype of the mutants. Therefore, it cannot be certain that the mutants obtained were true mutants for the two target genes. Nonetheless, a knockdown effect was observed, indicating that these genes might be important. Further mutagenesis studies should be carried out to create true mutants. One gene could also be part of a two component regulator system (BCAM 0924, response regulator), subsequently both components may need to be deleted before a significant effect can be seen.

As well as looking at up-regulated genes, repressed genes were also investigated. All the down-regulated genes were involved with motility, raising the question of whether chlorhexidine inhibits swarming motility and induces biofilm formation. Biofilm assays on chlorhexidine and CPC and swarming assays with chlorhexidine confirmed that this hypothesis was true for the majority of strains screened (Table 6.4). However, not all strains were capable of showing these phenotypes in the presence of chlorhexidine and biofilm formation and swarming inhibition did not

always correlate (Table 6.5). Thus, chlorhexidine sensing may only be present in specific strains. Multiple EM for motif elicitation was also carried out to look for conserved regions, up stream of down-regulated genes. There was evidence for a conserved region supporting the evidence that the biofilm induction, swarming inhibition pathway may be co-ordinately regulated.

To determine genes involved in this regulation, a mutagenesis screen was performed. Unfortunately, the screen did not reveal a particular regulatory gene that could potentially be involved with the regulation of the swarming and biofilm production. A number of regulatory genes appeared to be involved, including CheY receivers and glycosyl transferases. Therefore further investigation could be carried out on these elements to determine their exact role in regulating motility and biofilm production.

Finally, the evidence obtained in this investigation point to a coordinated phenotype of the switch from a motile lifestyle to a sessile, biofilm lifestyle, regulated via a two component regulatory systems. These systems are well known to aid bacterial adaptation to environmental stimuli and may allow survival in harsh environments. In this case, it appears that sub inhibitory levels of chlorhexidine are inducing a two component system to repress flagella, leading to swarming inhibition and biofilm induction, thus allowing some strains of the Bcc complex to survive in the presence of chlorhexidine. The two component system and the efflux pumps and transport genes may be good candidates for novel therapeutics as inhibiting these mechanisms may significantly increase susceptibility not only to chlorhexidine but other antimicrobials as well.

6.5 Future work

6.51 Mutagenesis of up-regulated efflux and transport genes

Due to the inclusive results from the mutagenesis carried out in this study, further mutagenesis should be carried out to fully determine the importance of the efflux response regulator and the transport related gene for chlorhexidine susceptibility. A

new mutagenesis strategy has been devised by Flannagan *et al.* which creates targeted gene deletions specifically for *Burkholderia* (Flannagan *et al.*, 2008). This strategy is based on the homing endonuclease 1-SceI. It involves cloning the sequences flanking the chromosomal region marked for deletion into a suicide plasmid that carries the 1-SceI recognition site. This plasmid also contains a trimethoprim resistance cassette. The plasmid is transferred into *Burkholderia* by tri parental mating, resulting in a targeted insertion. A second plasmid is then introduced, which constitutively expresses the 1-SceI endonuclease, which causes a double strand break in the inserted plasmid sequence. This stimulates recombination between mutant and parent alleles. This leads to either the restoration of the wild type phenotype or deletion of the gene and the loss of the integrated plasmid and the trimethoprim resistance. To isolate the correct recombinants, PCR and southern hybridisation can be carried out on Tp sensitive mutants (Flannagan *et al.*, 2008).

6.52 Biofilm and swarming screens

To improve the biofilm and swarming screens a larger panel of strains, that encompasses all the Bcc species should be tested for swarming inhibition and biofilm induction. This would allow the correlation of the two phenotypes to be more reliable and conclusive and may aid the determination of whether biofilm formation and swarming regulation are coupled or independent of one another.

Swarming inhibition could also be observed visually by using confocal microscopy. A bacterial suspension in increasing concentrations of the biocide can be placed under the microscope and the inhibition of swarming observed. It would be expected that in higher concentrations, the bacteria would no longer be seen to be moving. It would also be interesting to see if they still produce flagella at higher concentrations or whether they are just paralyzed.

Problems with reproducibility in the biofilm screen could be overcome by using the slightly modified assay as described by Peeters *et al.* In this method, a 4 hour adhesion step was included. After this adhesion step, the supernatant was

removed and fresh medium added. Biofilms are then left to develop for 24 hours and then plates washed and stained (Peeters et al., 2008). The investigators found that this method produced reliable and reproducible results, thus the extra adhesion step may be useful to improve the assay used in this study.

The effect of sub inhibitory levels of chlorhexidine on biofilm formation could also be investigated using a lab model. Biofilms are often associated with implants and foreign devices, including urinary catheters. A bladder model has been developed that allows the formation of biofilms on catheters to be observed. The catheter is inserted into the model and inflated with a buffer, which can include the biocide to be tested. Artificial urine can be inoculated with the bacterial suspension and pumped through the model and urinary catheter. This can be run up to 7 days or until the catheter is blocked (Williams and Stickler, 2008). Comparisons can be made between a control with no biocide and the model with biocide, on time for blockage, how quick biofilm production occurs and thickness of biofilm. Catheter sections could be observed using a scanning electron microscope to look for differences as well. Flow cells can also be used to investigate the development of biofilms on silicone plates. This method has the added advantage of allowing biofilm production to be recorded visually at specific time points, so that comparisons can easily be made (Jones *et al.*, 2005) This may add to the understanding of biofilm production in the presence of biocides.

In addition, an increase in biofilm production should correlate with an increase in exopolysaccharide production. Experiments could be performed using a Congo red dye which binds to exopolysaccharides to check the correlation between biofilm production and exopolysaccharide production (Harrison-Balestra *et al.*, 2003).

6.53 Two component systems

This investigation indicated that a two component regulator system may be involved in the regulation of swarming and biofilm production. However, this has not been fully elucidated. To determine the exact nature of the regulation, further studies can be carried out. The potential of a GGDEF domain and ci di-GMP involvement is

high. BLAST searches for this domain could be carried out and correlated to the regulatory genes determined by the mutagenesis screen carried out in this investigation. This search may also highlight potential regulatory genes that were previously missed. In the investigation by Hoffman *et al.* the presence of ci di-GMP was confirmed by the addition of GTP, a ci di -GMP inhibitor. This abolished the biofilm induction seen with tobramycin (Hoffman *et al.*, 2005). Thus, GTP addition would be a appropriate test to see if biofilm induction is abolished in chlorhexidine and to confirm if ci di-GMP plays a role.

From the mutagenesis screen a number of genes were highlighted as being involved in the regulation of swarming and biofilm induction. To further elucidate their roles, targeted deletion of these genes should be performed and the effect on swarming and biofilm induction in the presence of chlorhexidine investigated. Furthermore, as flagella are an important virulence factor, mutants unable to regulate motility in response to antimicrobials may have attenuated virulence. To screen for this infection models could be used, such as the agar bead rat model of infection (Sokol *et al.*, 1999).

As this swarming inhibition and biofilm induction is not universal among Bcc species a correlation between regulatory genes, the ability to stop swarming and induce biofilms and resistance could be carried out. PCR primers specific for important two component regulatory genes could be designed and amplification in a number of strains could be carried out. Southern hybridisation would allow the determination of the presence of these genes in a panel of resistant and sensitive strains rapidly, by creating nylon membranes spotted with the genomic DNA of these strains. Hybridisation with Dig labelled probes would then be performed and chemiluminescent detection carried out to look for the presence of the genes (Baldwin *et al.*, 2004). This can then be correlated to biofilm induction and swarming inhibition.

Chapter 7.0

Genetic basis of triclosan resistance

Chapter 7.0 Genetic basis of triclosan resistance

7.1 Introduction

Using transposon mutagenesis, a previous investigation identified two *B. cenocepacia* genes that may be involved in resistance to triclosan, BCAL 2166, *tmpA* and BCAL 3474, *fadD* (Rees, 2004). Three other genes were implicated in resistance to chlorhexidine, BCAL 2716, *lytB*, BCAL 0500, *hslU* and BCAL 3128, *gltA* (Rees, 2004). These five genes were all found on the first chromosome of *B. cenocepacia* and possibly represent intrinsic mechanisms of resistance to these two biocides.

7.11 *tmpA*

The *tmpA* gene had homology to a putative outer membrane lipoprotein, however, this protein was poorly characterised and was of unknown function. Mutants of this gene had a 100 fold reduction in triclosan susceptibility compared to wildtype *B. cenocepacia* K56-2 (Rees, 2004). Investigation of the genes surrounding *tmpA* revealed homology to genes involved with transport of metal ions. Hence, this gene may be part of a metal transport efflux system. The *tmpA* gene may also encode the outer membrane component of an RND efflux pump, although this particular gene deviated from some of the characteristics typically associated with RND efflux. RND efflux pumps are approximately 1000 amino acid proteins in size, containing 12 to 14 transmembrane helices (Borges-Walmsley and Walmsley, 2001), however, *tmpA* and its surrounding genes encode a smaller amino acid product (220 amino acids), with only 6 predicted transmembrane helices (Rees, 2004).

The *tmpA* gene was also associated with cross resistance to a number of unrelated compounds, including chlorhexidine, CPC, 5 – phenyl-1-pentanol, cetrimide and benzalkonium chloride, as well as the antibiotics ciprofloxacin, nalidixic acid, novobiocin, trimethoprim, chloramphenicol, erythromycin, polymyxin B and amikacin. Most of these agents have been previously documented as substrates for efflux pumps (Nikaido, 1998, Schweizer, 2003), adding to evidence that this

gene may be an efflux pump. However, the *tmpA* gene had no homology to previously described efflux pumps in the *Burkholderia* genome, therefore it may represent a novel efflux pump that has of yet not been characterised (Rees, 2004). Consequently, *tmpA* was an interesting target for further analysis as it may contribute significantly to the resistance of *B. cenocepacia* and potentially be a target for novel therapeutics.

7.12 *fadD*

The *fadD* gene was also associated with resistance to triclosan. This gene encodes a long chain fatty acid coA-ligase (acetyl- coA synthase). It is associated with the uptake of exogenous long chain fatty acids into the cell, which are then converted into acyl Co A derivatives which are utilised as a source of energy through the β oxidation pathway or incorporated into phospholipids via a glycerol phosphate acyltransferase system (Jackowski *et al.*, 1994). It is perhaps not surprising that a gene involved with fatty acid synthesis was involved in resistance to triclosan as it is known that this pathway is a specific target of triclosan (McMurry *et al.*, 1998b). The proposed hypothesis of the interaction of *fadD* and triclosan resistance suggests that an inactivation of *fadD* leads to increased permeability of the outer membrane due to the lack of conversion of acyl Co A derivatives into phospholipids (Figure 7.1). In addition, *fadD* has been shown to be involved with the degradation of fatty acids (Olivera *et al.*, 2001) and therefore this gene may also be involved with the degradation of triclosan through the β oxidation pathway (Rees, 2004).

7.13 *lytB*

Three genes were associated with chlorhexidine susceptibility. The *lytB* gene is well characterised and involved in a number of cellular processes including biosynthesis of isoprenoids (Cunningham *et al.*, 2000), penicillin tolerance (Gustafson *et al.*, 1993), polymyxin B resistance (Burtnick and Woods, 1999), bile resistance (Begley *et al.*, 2002) and phospholipids and peptidoglycan synthesis

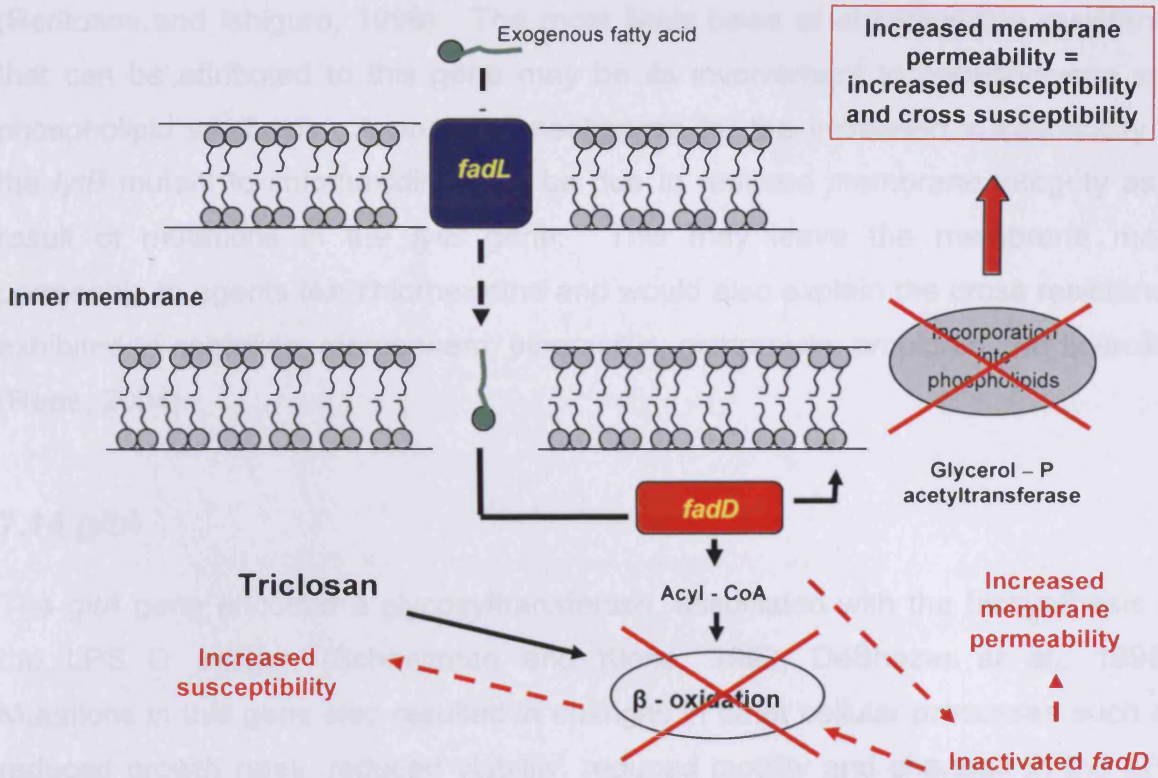


Figure 7.1The fatty acid uptake pathway and the role of *fadD*

The fatty acid pathway and the possible role of *fadD*, determined by inactivation of the gene. Exogenous fatty acids are transported across the outer membrane via the transporter protein *fadL* and then diffuse into the cytoplasm. The fatty acid is then used as a substrate for acyl coA synthase (product of *fadD*) which converts the fatty acid to acyl co-A via the β oxidation pathway. The products are then incorporated into phospholipids which make up the membrane. Inactivating the *fadD* gene prevents the β oxidation pathway and causes an increase in membrane permeability and thus an increase in susceptibility. Adapted from (Jackowski *et al.*, 1994) and (Rees, 2004).

(Rodionov and Ishiguro, 1996). The most likely basis of chlorhexidine resistance that can be attributed to this gene may be its involvement in peptidoglycan and phospholipid synthesis. A possible mechanism for the increased susceptibility of the *lytB* mutant to chlorhexidine may be due to reduced membrane integrity as a result of mutations in the *lytB* gene. This may leave the membrane more permeable to agents like chlorhexidine and would also explain the cross resistance exhibited to cetrimide, meropenem, piperacillin, gentamicin, ampicillin and ticarcillin (Rees, 2004).

7.14 *gltA*

The *gltA* gene encoded a glycosyltransferase, associated with the biosynthesis of the LPS O antigen (Schnaitman and Klena, 1993, DeShazer *et al.*, 1998). Mutations in this gene also resulted in changes in other cellular processes such as reduced growth rates, reduced viability, reduced motility and changes in the LPS profile (Rees, 2004). The LPS is well known as a virulence factor and is responsible for resistance to many antimicrobials, especially cationic antibiotics (Mahenthiralingam *et al.*, 2005). Therefore if mutations in the *gltA* gene are involved in LPS formation and membrane integrity it follows that susceptibility to cationic agents such as chlorhexidine should increase. Swarming motility was also seen to decrease in the *gltA* mutant (Rees, 2004). This may be explained by the structure of the flagellum. In Gram negative bacteria the flagellum is attached to the cell wall via an outer L ring which is anchored to the LPS layer (Madigan *et al.*, 2001b). Consequently, changes to the structure of the LPS may also lead to changes in flagella and hence motility.

7.15 *hslU*

The third gene found to be involved with chlorhexidine susceptibility was the *hslU*, heat shock gene. This gene is part of the stress response system which is up-regulated in response to an environmental stress such as pH, temperature, osmolarity, UV irradiation and the presence of toxic compounds such as antibiotics (Ramos *et al.*, 2001). Different heat shock proteins are activated depending on the stress and their function is to repair and degrade misfolded proteins, to allow the

bacteria to survive (Ramos *et al.*, 2001). The presence of an agent such as biocides may induce the stress response and therefore the heat shock type proteins aiding resistance to the antimicrobial, thus inactivation of these genes could potentially increase susceptibility.

7.16 Aims

The aim of this chapter was to explore the roles of these genes further. The triclosan susceptible mutant with an inactivated *tmpA* was of particular interest as it may represent a novel efflux pump which has yet to be described in the Bcc. Whole cell and outer membrane proteins were extracted to look for profile differences and an efflux inhibitor assay carried out to determine if any of the mutants are efflux related.

7.2 Results

7.21 MIC determination of mutants

The MIC value for all the mutants were carried out as described in Chapter 2.0 section 2.241. MICs were carried out on four biocides in total including chlorhexidine, CPC, triclosan and benzalkonium chloride. The wildtype *B. cenocepacia* K56-2 had high MIC values to all the biocides screened (Table 7.1). For example K56-2 could survive in the maximum concentration tested for chlorhexidine, CPC and benzalkonium chloride. The mutants were attenuated in their ability to survive in the biocides compared to the wildtype. All the mutants were capable of surviving in the maximum CPC concentration tested of 500 µg/ml (Table 7.1). The triclosan sensitive mutants had an MIC value of 50 µg/ml compared to >500 µg/ml for the wildtype. They also had an increased sensitivity to chlorhexidine and to benzalkonium chloride (Table 7.1). The chlorhexidine sensitive mutants had MICs of < 10 µg/ml, *lytB*, and 50 µg/ml *gltA* and *hslU* compared to >100 µg/ml for the wild type (Table 7.1). However, the chlorhexidine sensitive mutants did not exhibit increased susceptibility to triclosan or CPC and the heat shock gene did not have increased susceptibility to benzalkonium chloride

Table 7.1 MIC values for triclosan and chlorhexidine sensitive mutants

	Biocide MIC (µg/ml)					
	Wild type	Triclosan sensitive mutants		Chlorhexidine sensitive mutants		
Biocide	**K56-2**	***tmpA***	***fadD***	***lytB***	***gltA***	***hslU***
Chlorhexidine	>100	50	50	<10	50	50
Triclosan	>500	50	50	>300	>500	300
CPC	>500	500	500	500	>500	>500
BZK	>300	50 - 100	50 - 100	50 - 100	50 -100	300

Grey shaded areas indicate mutants with a knock down in MIC value from the wild type *B. cenocepacia* K56-2.

either. These results corroborated those obtained by the previous investigation and thus the next stages could be performed.

7.22 Whole cell protein lysis

To determine if any differences were present in the protein profile of the mutants that may be attributed to their increased susceptibility, whole cell protein extraction was carried out (Chapter 2, section 2.81). To determine protein concentration so a suitable amount could be run on the gel, a calibration curve using bovine serum albumin standards was produced (Figure 7.2). Protein concentrations obtained from the mutants could then be determined as described in Chapter 2, section 2.82. Approximately 20 µg of protein was required to give clear results on gel separation. The protein were run on a 4 – 20% Tris-HCl gradient gel (Bio-Rad). This gradient allows a range of protein sizes to be separated and viewed. The whole cell protein extraction produced a large number of bands which made detecting differences difficult. The Coomassie blue stain was also quite faint, meaning that once photographed some bands were difficult to visualise. However, from this analysis there appeared to be no differences in the protein profile between the wildtype and the mutants (Figure 7.3). All bands appeared to be the same size and no discernible differences were seen. This may have been due to the sheer number of bands present in a whole cell extract, therefore outer membrane extraction was performed to look for differences at particular band sizes.

7.23 Outer membrane protein extraction

Outer membrane protein extraction was carried out using a Bio-Rad Ready Prep protein kit (Chapter 2, section 2.83). This kit produced three fractions, a hydrophobic fraction, a hydrophilic section and an insoluble pellet. All three fractions were included in the analysis. Again protein quantification was carried out (Chapter 2, section 2.83) by creating a standard curve using pre determined bovine gamma globulin concentrations. Once protein concentration was determined, 1 – 500 µg was added to the Readyprep 2D clean up. Using the bioinformatics software programme Artemis, features of each gene were investigated. The

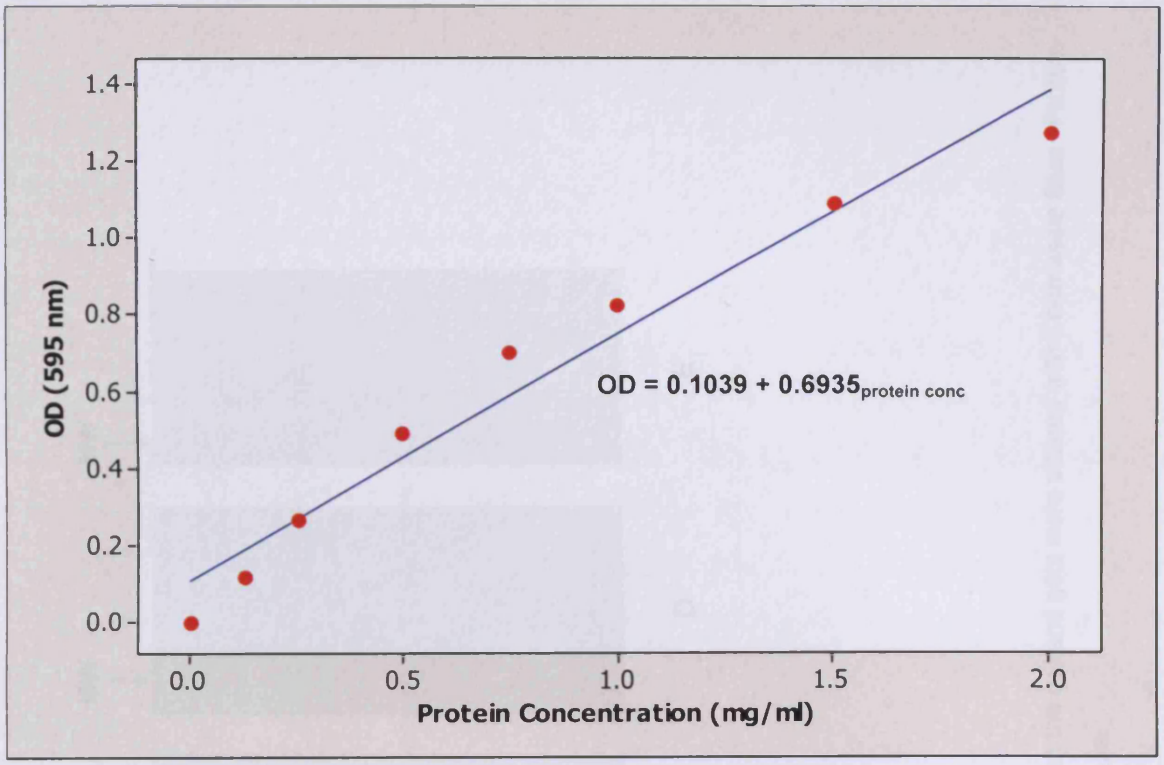
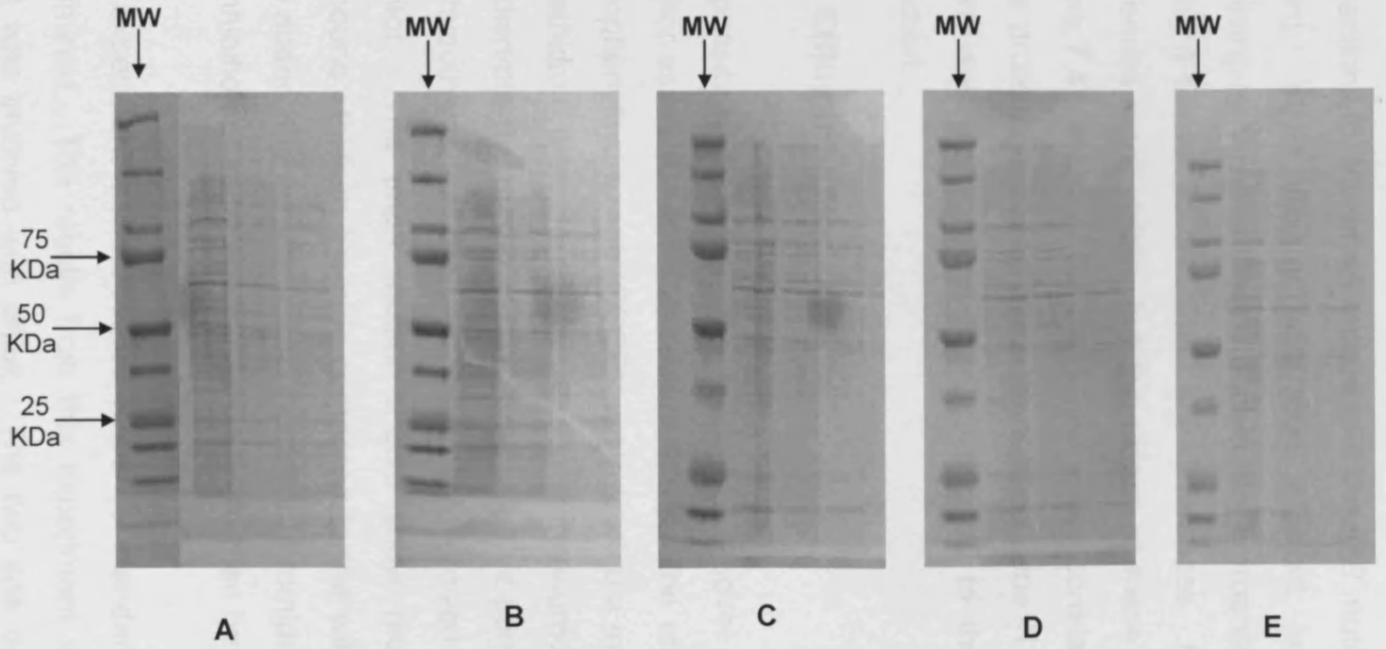


Figure 7.2 Calibration curve of bovine serum albumin standards (mg/ml)

Protein concentration was determined using a Bio-Rad Quick start Bradford assay. Standard bovine serum albumin solutions were provided ranging from 0.125 – 2 mg/ml. The standards and proteins to be tested were incubated with Coomassie blue stain and absorbance read at 595 nm. A standard curve was produced and linear regression analysis used to determine the exact protein concentrations (Chapter 2, section 2.82).



A = K56-2
B = *tmpA*
C = *lytB*
D = *hslU*
E = *gltA*

Figure 7.3 SDS PAGE of wild type *B. cenocepacia* K56-2 and mutants

Protein products were ran on a 4 – 12% Tris HCl gel at 80 volts. 20 µg of protein were run on the gel and gels were stained with Coomassie Blue. Protein marker showing the relevant protein sizes are shown in KDa on the right handside of the figure.

size of these genes ranged from 35 KDa to 60.8 KDa (Table 7.2). To enhance the resolution of the SDS-PAGE gels, a 12.5% gel was chosen as this separated bands in the region of 21.5 - 66 KDa. The gels were silver stained to increase the intensity of the bands and to make visualisation easier (Figure 7.4). As seen with the whole cell protein extraction, no differences in the band patterns were observed in any of the fractions for the *tmpA* mutant and the *lytB* mutant (Figure 7.4, pellet fraction not shown). Silver staining was more efficient, however this process may need optimising as three of the mutants in the hydrophilic fraction had a smeared profile, making it difficult to determine any differences. Nonetheless, some band pattern differences were visible, but the extent of these could not be clearly elucidated (Figure 7.4). None of the band differences correlated clearly to the expected sizes of the proteins encoded by each mutated gene (Table 7.2). The hydrophobic band pattern was also difficult to visualise due to the low yield of protein from the extraction.

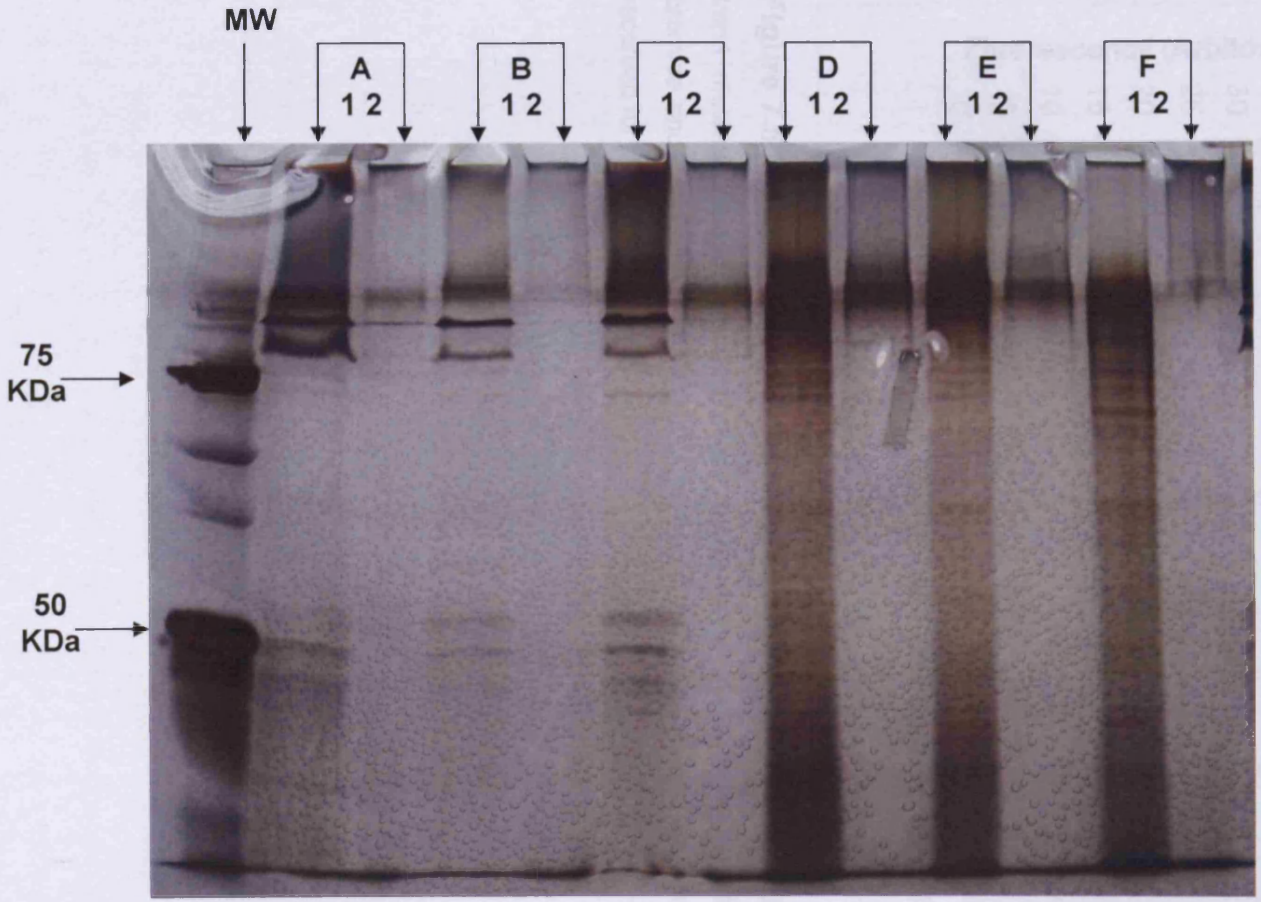
7.24 Efflux inhibitor assays

The possibility that *tmpA* may be part of a novel efflux was tested using an efflux inhibitor assay (Chapter 2, section 2.29). The efflux inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to a mixture of the bacterial suspension and ethidium bromide and fluorescence measured. CCCP is an energy uncoupler that disrupts the proton motive force in efflux pumps preventing them from acting. The hypothesis that the *tmpA* gene was involved with efflux was tested using this inhibitor. The *tmpA* mutant should show reduced efflux of the fluorescent compound ethidium bromide compared to the wild type K56-2. Once CCCP has been added, the accumulation of ethidium bromide should increase in K56-2 due to the inhibition of efflux pumps. This should then be comparable to the *tmpA* mutant.

The experiment was repeated twice so standard errors of the mean could be determined. The results from this experiment were inconclusive as to whether *tmpA* was involved with efflux. The two site directed knock outs of *tmpA* had accumulation levels of ethidium bromide similar to those of K56-2 before the addition of CCCP. Once CCCP had been added, accumulation in all three strains

Table 7.2 Protein size data for mutants

Mutant	Gene Number	Size (KDa)	Amino acids
tmpA	BCAL 2166	41.4	381
fadD	BCAL 3474	60.8	557
lytB	BCAL 2710	35	326
hslU	BCAL 0500	49	447
gltA	BCAL 3128	49	434



A = K56-2 C = fadD E = hslU 1 = hydrophilic fraction
B = *tmpA* D = *lytB* F = *gltA* 2 = hydrophobic fraction

Figure 7.4 SDS PAGE of outer membrane proteins for K56-2 and mutants

Outer membranes were run on a 12.5% Tris HCl gel at 80 volts. Approximately 3 µl of protein product were run on the gel and then silver stained.

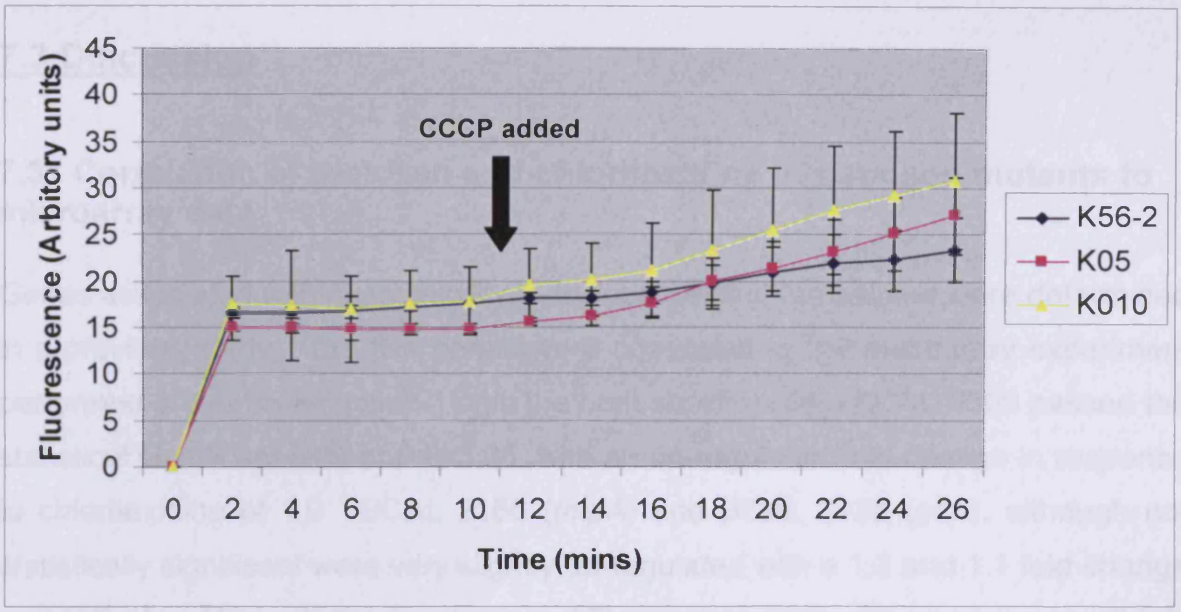


Figure 7.5 Efflux inhibitor assays on wild type K56-2 and *tmpA* mutants (± SE)

tmpA mutants created by site directed mutagenesis. Suspensions were exposed to ethidium bromide and fluorescence measured for 10 minutes. CCCP was then added and fluorescence recorded for a further 16 minutes.

increased, however there was no dramatic increase in accumulation in K56-2 (Figure 7.5).

7.3 Discussion

7.31 Correlation of triclosan and chlorhexidine transposon mutants to microarray data

Genes associated with susceptibility to triclosan and chlorhexidine were determined in a previous study. The five genes were correlated to the microarray experiment performed in this investigation. Only the heat shock protein BCAL 0500 passed the statistical significant filter of $P < 0.05$, with an up-regulated fold change in response to chlorhexidine of 1.9. BCAL 2166 (*tmpA*) and BCAL 3128 (*gltA*), although not statistically significant were very slightly up-regulated with a 1.2 and 1.1 fold change respectively. Although this is very low, efflux pumps may only require a small fold change to make a large difference. BCAL 3474 (*fadD*) and BCAL 2716 (*lytB*) did not pass normalisation and therefore could not be analysed. The chlorhexidine microarray experiment correlates to the heat shock protein mutant which was implicated in chlorhexidine susceptibility, indicating that this gene may be of importance for chlorhexidine resistance. The other genes did not correlate well, although the triclosan associated outer membrane protein (tmpA) could possibly have a role in both triclosan and chlorhexidine resistance, although further investigation is required to confirm this.

7.32 Is triclosan and chlorhexidine resistance associated with membrane changes?

The transposon mutants were found to have roles in phospholipid and peptidoglycan synthesis, stress response, glycosyl transferases and fatty acid synthesis. Interestingly the *tmpA* gene was homologous to a poorly characterised outer membrane protein that may be involved in efflux. All of these genes could contribute to resistance to triclosan and chlorhexidine via modifications of the LPS and outer membrane leaving the bacterium less permeable to the substances (Rees, 2004). This chapter aimed to examine if membrane protein changes and alterations in efflux was associated with the *tmpA* gene.

Changes to the outer membrane and LPS profile have been well documented in the literature as corresponding with changes to resistance. A study in ciprofloxacin resistant *P. aeruginosa* isolated from patients undergoing treatment lost a 31 – 32 KDa outer membrane protein (Daikos *et al.*, 1988). Rajyaguru and Muszynski (Rajyaguru and Muszynski, 1997) found that stepwise exposure to trimethoprim / sulfamethoxazole resulted in a deficiency of a major outer membrane protein at approximately 47 KDa. In addition these mutants expressed outer membrane proteins that were not found in the wildtype strain. LPS differences were also observed in the trimethoprim / sulfamethoxazole and chloramphenicol resistant mutants with the appearance of new high and low molecular weight LPS bands (Rajyaguru and Muszynski, 1997). Tattawasart *et al* (Tattawasart *et al.*, 2000) provided evidence of outer membrane changes in response to biocides. Chlorhexidine and CPC resistant strains of *P. stutzeri* strains had alterations in their outer membranes with the appearance of two new bands. LPS changes were also observed with the loss of the fastest migrating band in chlorhexidine resistant strains (Tattawasart *et al.*, 2000). Many of these strains were also associated with cross resistance to other antimicrobials such ceftazidime and piperacillin (Rajyaguru and Muszynski, 1997).

The *B. cenocepacia* mutants isolated in the study by Rees *et al.* (Rees, 2004) found cross susceptibility to a number of antibiotics such as trimethoprim, chloramphenicol, ciprofloxacin, meropenem, piperacillin and gentamicin which may be due to changes in the outer membrane. It was hypothesised that the triclosan and chlorhexidine sensitive mutants would perhaps show a loss of some major protein bands. Whole cell protein extraction was performed however clear results were not obtained and no differences could be observed between the mutants and the wild type (Figure 7.3). Therefore outer membrane protein extraction was attempted and a silver staining method carried out to clarify results. Nonetheless, a very poor yield of protein was obtained from the kit used to extract membranes, particularly for the hydrophobic fraction. Consequently when ran on a gel, visualisation was very poor (Figure 7.4). No discernible differences could be isolated from the gel obtained, although three mutants appeared to produce a smeared pattern making visualisation more difficult (Figure 7.4). The results of protein analysis were inconclusive as to whether outer membrane changes had

occurred in the mutants as absences of bands were not seen. However, to improve the possibility of seeing outer membrane changes a different extraction approach could be taken. In this investigation an extraction kit was used that did not involve the use of ultra centrifugation. To obtain better yields of protein a method described in Tattawasart *et al.* (Tattawasart *et al.*, 2000) could be used which incorporates ultra centrifugation. To improve the resolution and separation of proteins a 2 – dimensional (2D) protein analysis could be used. 2D analysis could improve the chances of changes in the membrane being seen. Changes in the membrane may not have been seen due to low expression of proteins, as a small change in expression may still have a functional effect.

7.33 Is the *tmpA* gene a triclosan efflux pump?

The poorly characterised *tmpA* gene showed homology to an RND efflux pump and thus may be a previously unknown triclosan efflux pump. Subsequently, this finding could potentially be important as it may explain the high resistance exhibited by this strain and could be a novel target for new therapeutics. Recently, a triclosan specific RND efflux pump has been determined in *P. aeruginosa*, another bacterial species that exhibits high intrinsic resistance to triclosan. This mechanism of resistance was isolated when mutants deficient in five efflux pumps were exposed to triclosan. Resistant mutants were readily obtained and it was found that a triclosan specific efflux pump was being over expressed to compensate for the loss of other pumps (Mima *et al.*, 2007). Similarly, in this investigation the *tmpA* gene could be a RND efflux pump that is specific for triclosan. However, this mutant did exhibit cross susceptibility to other antimicrobials, thus it may be capable of removing other compounds as well as triclosan.

To assess the possibility of *tmpA* being involved in efflux an assay was set up using the efflux inhibitor CCCP. In the *tmpA* mutant CCCP should not increase the accumulation of the effluxes substrate, although a small increase may be seen as this inhibitor will prevent all efflux pumps from working. K56-2 should see an increase in fluorescence when in the presence of CCCP (fluorescence was directly related to accumulation in the cell), due to the inhibition of efflux (Guglierame *et al.*, 2006). However, in this investigation the results were inconclusive. K56-2 did not

exhibit a sharp rise in fluorescence once CCCP was added and was not comparable to the mutants (Figure 7.5). There were some issues that arose during this experiment that may be attributed to discrepancies in results. The substrate used for the assay was ethidium bromide so that fluorescence could easily be monitored. However, this efflux pump could potentially be specific for triclosan or may not transport ethidium bromide as a substrate.

More detailed results may be obtained by growing the wild type in the presence of triclosan to over express the efflux pump. It may also be possible to radiolabel the triclosan and look for biocide accumulation using spectrometry (Nair *et al.*, 2004). The triclosan efflux pump identified in *P. aeruginosa* was over expressed in mutants deficient in other efflux pumps (Mima *et al.*, 2007). To see if the *tmpA* gene was over expressed in K56-2, quantitative real time PCR could be performed. This could be compared to triclosan resistant and sensitive strains to see if it is an important factor. Alternatively a microarray experiment could be performed to look for up regulation of transcription of genes in the whole genome in response to triclosan. This approach would provide a large reservoir of data and would hopefully corroborate with the results obtained in this investigation.

7.4 Conclusions

Triclosan and chlorhexidine susceptible mutants were isolated and characterised previously. To further elucidate the roles these genes play in resistance to these biocides protein extractions were performed to look for alterations in the membranes. No obvious changes were observed, indicating that other factors such as efflux may be more important or that the methods employed were not sufficient to clearly identify changes. Similarly, no differences were observed when outer membrane proteins were extracted. The possibility that *tmpA* was involved in efflux was assessed using efflux inhibitor assays. These results did not indicate that *tmpA* was part of an efflux pump, although problems with reproducibility and issues with the optimisation of the experiment may skew the results. Therefore, to fully elucidate the mechanisms of triclosan resistance in this strain further experiments are required.

Chapter 8.0

Conclusions

Chapter 8.0 Conclusions

8.1 Biocide and antibiotic susceptibility of the Bcc

An up to date survey of the biocide and antibiotic susceptibility of all current members of the Bcc was performed. This provided information on biocide susceptibility in relation to their taxonomy and epidemiology. A panel of *P. aeruginosa* strains was also screened for biocide susceptibility and its relation to CF epidemiology of these bacteria. From this work the following conclusions can be made:

Epidemic strains in CF infections demonstrate elevated biocide resistance

1) Epidemic strains, such as *B. cenocepacia* ET-12 strain J2315 (Govan *et al.*, 1993), the *B. multivorans* Glasgow outbreak strain LMG 16660 (Whiteford *et al.*, 1995) and the *P. aeruginosa* Liverpool epidemic strains (Cheng *et al.*, 1996) were less susceptible to biocides and antibiotics than other non epidemic strains. The Liverpool epidemic strains had a MIC value four times higher than other strains to chlorhexidine. This biocide appears to be effective against other strains of *P. aeruginosa*, thus the lower susceptibility may contribute to these strains persistence in clinical settings and CF patients. There was also evidence that these epidemic strains are less susceptible to a range of unrelated biocides. For example *B. cenocepacia* J2315 was less susceptible to chlorhexidine, CPC, triclosan and povidone compared to other strains, such as *B. anthina*.

Evidence of biocide – antibiotic cross resistance was inconclusive

2) There was conflicting evidence of cross resistance between biocide and antibiotic mechanisms of resistance. Strains such as *B. cenocepacia* J2315 were capable of surviving in a number of unrelated biocides and were classed as resistant using Etest® strips to a number of antibiotics. This indicates there may be some overlaps in mechanisms of resistance.

However, a *B. dolosa* strain was resistant to all 10 antibiotics but sensitive to chlorhexidine and CPC. Further work should be carried out to fully determine the contribution of cross resistance to multi drug resistant organisms

Commercial biocide formulations may be ineffective on Bcc bacteria

3) Two commercial biocides were screened to look for bactericidal effects. Although they were successful at killing the majority of strains tested, including the epidemic *B. multivorans* LMG 16660, two strains a *B. cepacia* and *B. cenocepacia* strain were capable of surviving, in certain strains after an hour exposure to the biocide. This may be a cause for concern and suggests that some of the more resistant strains of the Bcc will not be effectively killed by commercial disinfectants. Therefore, clinics disinfection procedures should be monitored carefully and adjusted to efficiently kill all the Bcc.

Several biocide classes are ineffective against Bcc bacteria

4) From this investigation it can be concluded that QAC biocides such as CPC and benzalkonium chloride, triclosan and povidone are ineffective against the Bcc and therefore should be avoided in situations where these biocides are abundant, such as CF clinics. Although chlorhexidine is more effective against both the Bcc and *P. aeruginosa* it should be noted that some strains, particularly problematic epidemic strains were highly resistant to this biocide, thus caution should be exercised when using this biocide.

Proposal of new biocide challenge testing Bcc reference strains

5) Standard susceptibility tests for biocides currently include *B. cepacia* strain ATCC 25416, they type strain for the Bcc species. However, this strain was one of the more sensitive strains found in this, investigation. Three Bcc biocide resistant strains from this study were proposed as potential reference strains for biocide efficacy testing; *B. cenocepacia* J2315, *B. multivorans*

LMG 16660 and B. *cenocepacia* LMG 18832. This would allow a more accurate decision to be made on the suitability of that biocide for use in clinical situations (Rose *et al.*, 2009).

8.2 Molecular basis of chlorhexidine resistance

To further characterise the molecular basis of chlorhexidine resistance a whole genome expression approach using a microarray of *B. cenocepacia* J2315 was performed. The microarray allowed altered gene expression in response to exposure to chlorhexidine to be examined and highlighted genes that may be of importance for survival in chlorhexidine. Genome expression of *P. aeruginosa* in response to triclosan has been researched (Mima *et al.*, 2007), however as far as we are aware this study is the first to examine the transcriptomic response of a bacterium to chlorhexidine. The following conclusions were made:

Novel chlorhexidine up-regulated genes

1) Genes up-regulated in response to chlorhexidine included efflux pumps homologous to the mexAB-OprM efflux pump found in *P. aeruginosa*, outer membrane proteins and transport related proteins such as an ABC transporter. These genes may all be involved in resistance to chlorhexidine as these components have all been described as potential resistant mechanisms and may be potential targets for novel therapeutics.

Knock-out of chlorhexidine up-regulated genes

2) Site directed mutagenesis of two genes, BCAM 0924, a response regulator and BCAL 2353 a transport related gene was carried out. A phenotypic knock down in the MIC was observed with the mutants, compared to the wildtype (40 µg/ml compared to 100 µg/ml). However these mutants could not be confirmed as "true" genetic mutants, since it appears that a single cross over had occurred during the mutation strategy. Thus before the role

of these two genes can be fully established, further mutagenesis should be performed to obtain clean mutations.

B. cenocepacia motility is down-regulated in response to chlorhexidine

3) Genes repressed in response to chlorhexidine were observed. The majority were involved with motility and chemotaxis and raised the question of whether swarming and biofilm induction was effected by chlorhexidine.

The swarming phenotype is highly variable across the Bcc

4) Swarming was screened for 251 strains of the Bcc. Not all strains were capable of swarming with *B. cenocepacia* strains being among the worst swarmers. Sub inhibitory levels of chlorhexidine caused an inhibitory swarming effect in 67% of strains screened, with no differences observed between chlorhexidine resistant or sensitive strains. When other biocides such as CPC and triclosan were screened, the inhibition was not seen, indicating that this swarming down-regulation response may be specific for chlorhexidine.

Sub-inhibitory concentrations of biocides induce biofilm formation

5) Biofilm induction in response to sub inhibitory concentrations of chlorhexidine and CPC was screened. A total of 58.3% and 83% of Bcc strains had an induction of biofilm production in chlorhexidine and CPC. Thus, it appears that sub inhibitory levels of chlorhexidine may induce a biofilm lifestyle in Bcc bacteria leading to increased resistance to antimicrobial agents. This may be a major factor contributing to the ability of Bcc bacteria to contaminate pharmaceuticals and other commercial products (Jimenez, 2007).

Searching for regulation of chlorhexidine dependent swarming down-regulation

6) The inhibition of swarming and induction of biofilms in response to chlorhexidine could be regulated by a two component system regulator. MEME analysis found a potential conserved regulatory binding region up-stream of all gene sets down-regulated by chlorhexidine exposure in *B. cenocepacia*. Transposon mutagenesis was performed to try and uncouple the swarming regulation and determine the regulatory genes involved. This screen found potential interesting genes such as a *cheY* like receiver, which is a response regulator involved in chemotaxis. Several other genes of interest including other regulation and envelope genes were identified. Further work should be carried out to determine the regulatory mechanism of this phenomenon which may be a significant factor in this organism's ability to survive in chlorhexidine. Once identified this regulatory response sensor could provide a potentially useful therapeutic target.

The Bcc are a diverse and versatile group of bacteria that cause many problems. In addition to their antibiotic resistance this study has shown the Bcc posses very high biocide resistance mediated by a number of mechanisms. Health care workers and clinicians should take particular care to ensure that disinfection practices are adequate to kill all members of the Bcc to prevent the acquisition of these infections in CF and vulnerable patients via routine susceptibility testing.

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Chapter 10

Appendices

Chapter 10 Appendices

10.1 Appendix 1; Biocide susceptibility of the *Burkholderia cepacia* complex

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Biocide susceptibility of the *Burkholderia cepacia* complex

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***Objectives*: The *Burkholderia cepacia* complex (Bcc) species are important opportunistic pathogens with intrinsic antibiotic resistance. They are also well known as contaminants of disinfectants, yet their biocide susceptibility has not been studied in detail. We investigated Bcc biocide susceptibility and correlated it to their taxonomy, antibiotic susceptibility and ability to form biofilms.**

***Methods*: Genetically distinct Bcc strains belonging to 12 of the defined species were examined. Biocide susceptibility was assessed by (i) broth dilution MIC assays, (ii) agar growth-based MBC screens and (iii) suspension tests. Antibiotic MIC was determined by Etest® strips, and the ability to form biofilms was examined in a 96-well plate assay.**

***Results*: Biocide susceptibility varied across the Bcc complex with high MIC recorded for chlorhexidine (>100 mg/L), cetylpyridinium chloride (>200 mg/L), triclosan (>500 mg/L), benzalkonium chloride (>400 mg/L) and povidone (>50 000 mg/L). Species-dependent differences were apparent only for cetylpyridinium chloride. There was no correlation between biocide susceptibility and (i) antibiotic susceptibility or (ii) the ability to form biofilms. Biocide MBC was considerably higher than the MIC (chlorhexidine, 6-fold greater; cetylpyridinium chloride, 20-fold greater). Cystic fibrosis outbreak strains (*Burkholderia multivorans* Glasgow strain and *Burkholderia cenocepacia* ET12) possessed elevated chlorhexidine resistance, and Bcc bacteria were also shown to remain viable in current commercial biocide formulations.**

***Conclusions*: Bcc bacteria are resistant to a wide range of biocides and further representatives of this group should be included as reference strains in the development of new anti-infectives and commercial formulations.**

Keywords: antibiotics, resistance and susceptibility, minimal inhibitory concentration

Introduction

The *Burkholderia cepacia* complex (Bcc) species are problematic Gram-negative opportunistic pathogens, and people with cystic fibrosis (CF) are particularly prone to chronic Bcc lung infection.[1] The Bcc consists of multiple closely related species, all of which have been isolated from clinical infections.[1,2] Until very recently, the following 10 species had been formally designated as members of the complex:[3] *B. cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia pyrrocinia* and *Burkholderia ubonensis*. Two recent studies have extended the complex to 17 species by the addition of *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov., *Burkholderia metallica* sp. nov.,[4] *Burkholderia lata* and *Burkholderia contaminans*.[5] Of these 17 species, *B. cenocepacia* and *B. multivorans* are most associated with CF infection, which together account for ~85% to 95% of all Bcc infections in this disease.[1] The epidemiology of Bcc infection outside CF has not been extensively studied; however, the recent studies of Reik *et al.*[6] have shown *B. cenocepacia* (25.6%), *B. cepacia* (18.9%) and *B. multivorans* (15.6%) to be the most common non-CF infections seen in the USA.

An important feature of Bcc bacteria is their very high innate antimicrobial resistance to both antibiotics and biocides.[1,2] Bcc species are intrinsically resistant to many antibiotics such as aminoglycosides and polymyxin B and often require combination therapy to suppress infection in CF.[7] The antibiotics polymyxin, gentamicin and vancomycin are used at high concentrations in *B. cepacia* Selective Agar, a highly effective

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medium for their isolation from CF sputum.[8] Nzula *et al.*[9] compared the antibiotic susceptibility of six Bcc species and concluded that it was highly variable except for innate polymyxin resistance and not linked to the taxonomic status of the isolates examined. Efflux, the secretion of chromosomal β-lactamases and the impermeability of the outer envelope of Bcc bacteria have been implicated in antibiotic resistance.[1]

In contrast, the molecular basis for biocide resistance in Bcc bacteria has been poorly studied despite these organisms being linked with many instances of contamination in disinfectants and other anti-infective solutions.[10] Pharmaceutical formulations containing benzalkonium chloride,[11] cetylpyridinium chloride[12] and chlorhexidine[13,14] have become contaminated with Bcc bacteria and led to large outbreaks of infection. Even during commercial manufacture, disinfectants such as povidone-iodine may become contaminated with Bcc bacteria.[15] A review of commercial recalls in the USA from 1998 to 2006 demonstrated that Bcc bacteria were the most commonly isolated Gram-negative bacteria in both sterile and non-sterile products.[10] Multiple instances of a single Bcc strain causing either contamination or clinical infection at geographically distinct sites have been identified using multilocus sequence typing (MLST);[16–18] these data suggest that in our burgeoning world economy, the potential for global distribution of contaminating bacteria in anti-infective agents may exist.

The aim of this study was to provide an up-to-date survey of the biocide susceptibility profiles of a representative panel of Bcc species that accounts for taxonomy and genetic diversity. To ensure that a wide genetic diversity of Bcc bacteria were sampled, isolates were also selected on the basis of their MLST type.[19] Biocide susceptibility was investigated using a standardized broth dilution method to determine MICs; this was also correlated to the MBC for resistant and susceptible strains. A possible link between biocide resistance and multidrug resistance has been proposed,[20] and this hypothesis was also explored by correlating biocide and antibiotic resistance profiles of the Bcc isolates tested.

Material and methods

Bacterial collection

A panel of 101 genetically distinct Bcc strains were selected from the Cardiff University collection[3] and the Belgium Coordinated Collection of Microorganisms (BCCM; http://bccm.belspo.be/about/lmg.php). The selected strains encompassed reference strains from the defined Bcc strain panels.[21,22] Additional *B. multivorans* strains from the Glasgow outbreak[23] were kindly provided by Dr Alan Brown and Prof. John R.W. Govan (Edinburgh University, UK). MLST was used to type and select strains spanning the genetic diversity of the Bcc.[19] The strains spanned 11 of the most prevalent Bcc species (Table 1) and also two strains from the Bcc novel Group K (a distinct MLST cluster from the former Group K isolates re-named as the new species *B. lata* and *B. contaminans*); the source of the strains varied and included clinical (56), non-clinical (9) and environmental strains (36). Initially all strains were screened against chlorhexidine (ICN Biomedicals) and cetylpyridinium chloride (ICN Biomedicals) as representatives of agents that could be easily screened in a broth dilution assay. From this screen, a subcollection of 38 strains was selected that spanned the range of

Table 1. Susceptibility of Bcc and other species to chlorhexidine and cetylpyridinium chloride

Species (number of strains tested)	Chlorhexidine MIC (mg/L)		Cetylpyridinium chloride MIC (mg/L)	
	mean minimum	mean maximum	mean minimum	mean maximum
B. cepacia (10)	57	65	170[a]	175[b]
B. multivorans (30)	36.6	46	77.7	101.3
B. cenocepacia (31)	47.4	54.8	175.5[c]	184.5[c]
B. stabilis (3)	40	46.7	146.7	166.7
B. vietnamiensis (5)	52	58	90	106
B. dolosa (3)	26.7	36.7	66.7	86.7
B. ambifaria (6)	33.3	41.7	98.3	116.7
B. anthina (2)	10	20	95	125
B. pyrrocinia (2)	15	25	50	70
B. lata (4)	15	25	183.3[d]	200[d]
B. contaminans (3)	13.3	23.3	200[e]	200[f]
Novel Bcc Group K (2)	50	60	200[g]	200[g]
Mean Bcc (101)	33.03	41.9	129.4	144.3

[a]At the mean minimum cetylpyridinium chloride MIC, *B. cepacia* was significantly more resistant than *B. multivorans*, *B. dolosa*, *B. ambifaria* and *B. pyrrocinia* ($P<0.05$).
[b]At the mean maximum cetylpyridinium chloride MIC, *B. cepacia* was significantly more resistant than *B. multivorans*, *B. dolosa* and *B. pyrrocinia* ($P<0.05$).
[c]At the mean minimum and mean maximum cetylpyridinium chloride MICs, *B. cenocepacia* was significantly more resistant than *B. multivorans*, *B. vietnamensis*, *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia* ($P<0.05$).
[d]At the mean minimum and mean maximum cetylpyridinium chloride MICs, *B. lata* was significantly more resistant than *B. multivorans* ($P<0.05$).
[e]At the mean minimum cetylpyridinium chloride MIC, *B. contaminans* was significantly more resistant than *B. multivorans* and *B. ambifaria* ($P<0.05$).
[f]At the mean maximum cetylpyridinium chloride MIC, *B. contaminans* was significantly more resistant than *B. multivorans* ($P<0.05$).
[g]At the mean minimum and mean maximum cetylpyridinium chloride MICs, Bcc novel Group K was significantly more resistant than *B. multivorans* ($P<0.05$).

chlorhexidine and cetylpyridinium chloride susceptibility observed: 22 strains with low MICs and 16 with high MICs were selected to provide a spectrum of Bcc biocide resistance.

The subcollection was then used (i) to examine the susceptibility to three additional biocides povidone (Sigma Aldrich), benzalkonium chloride (Sigma Aldrich) and triclosan (5-chloro-2-(2,4 dichlorophenoxy)phenol; Fluka, Biochemika) and (ii) to determine the MBC values of chlorhexidine and cetylpyridinium chloride. In addition, 10 non-Bcc species were also screened for chlorhexidine and cetylpyridinium chloride susceptibility as control species (which, except for *Escherichia coli*, can infect the CF lung): *Staphylococcus aureus* [both methicillin-resistant (MRSA, two isolates) and -sensitive (MSSA, four isolates)] and *Pseudomonas aeruginosa* (24 isolates), as well as one control isolate for *Burkholderia gladioli*, *Stenotrophomonas maltophilia*, *Ralstonia mannitolytica*, *Pseudomonas stutzeri*, *Ralstonia picketti*, *Pseudomonas putida*, *Achromobacter xylosoxidans* and *E. coli*. All strains were grown in Tryptone soya broth (TSB) or agar (TSA; Oxoid) and incubated for 24–72 h at 37°C.

Broth dilution MIC method

A broth dilution assay adapted to examine biocide susceptibility of Bcc bacteria was carried out as follows. Aqueous solutions (sterilized by filtration) of chlorhexidine (10 mg/mL) and cetylpyridinium chloride (5 mg/mL) were used to make up stock concentrations of 100 and 1000 mg/L biocides. These solutions were then added to TSB to make up biocide concentrations ranging from 0 to 100 mg/L for chlorhexidine and 0 to 200 mg/L for cetylpyridinium chloride. We found that growth of bacteria in a liquid medium when compared with that on slopes of solid agar produced consistent numbers of viable cells for the Bcc strains studied. Therefore, each Bcc strain was grown overnight (18 h) in 3 mL of TSB at 37°C (in a 14 mL tube, shaken horizontally at 200 rpm); the culture was diluted to an optical density (OD) of 1 (630 nm) corresponding to a viable count of $\sim 1 \times 10^8$ cfu/mL. Approximately 1×10^5 cfu of each test Bcc strain were then added to 1 mL of each biocide concentration, and 200 μL of this was added to three replicate wells within a 96-well plate. The microplates were incubated with shaking (200 rpm) at 37°C for 24 h and the OD was read using a Dynex Technologies MRX® microplate absorbance reader with Revelation application. An endpoint reading at 630 nm was taken with 10 s of shaking beforehand.

B. multivorans LMG 13010 was included in all assays as a control strain as preliminary experiments with this strain exhibited a reproducible effect. Addition of biocides to the growth media resulted in changes in OD. Therefore, the MIC value was designated as the concentration of biocides that resulted in an 80% knockdown of culture OD in comparison with the control wild-type growth with no added biocide. Viable counts performed on the control strain after running the broth dilution MIC assay demonstrated the presence of 9.7×10^5 cfu/mL at a chlorhexidine concentration of 20 mg/L, while no viable cells were detected above this concentration. Using the 80% knockdown in culture OD, the MIC for the control strain was found to be 30–40 mg/L, which corresponded well to the absence of viable cells in concentrations of chlorhexidine above 20 mg/L. The results were subjected to statistical analysis in order to determine levels of susceptibility.

Agar dilution MIC method

To screen the subset of 38 strains against the three additional biocides povidone, benzalkonium chloride and triclosan, an agar dilution method was used. The method allowed for higher concentrations of biocides to be examined when the broth dilution assay was prevented due to the biocides altering the OD reading. Stock solutions of the three biocides were made up as follows: 10000 mg/L of triclosan in DMSO (this solvent did not inhibit the growth of Bcc bacteria at the concentrations used); 200000 mg/L of povidone in water and 10000 mg/L of benzalkonium chloride in water. After autoclaving, TSA was cooled to 50°C and the appropriate amount of each biocide was added to 40 mL of agar for concentration ranges of 0–500 mg/L for triclosan, 0–400 mg/L for benzalkonium chloride and 0–50000 mg/L for povidone. The bacterial strains were cultured and diluted to 1×10^8 cfu/mL as described above and 200 μL of this suspension was then added to a 96-well plate in order to create a master plate containing eight replicates of each test strain. The strain suspensions from the master plate were then replicated onto the biocide-containing agar plates using a 96-well replicator and left to grow for 48 h at 37°C. The MIC was designated as the concentration of biocides of which no visible growth was apparent.

MBC

Bacterial cultures were grown as described above and diluted to $\sim 10^6$ cfu/mL. Chlorhexidine or cetylpyridinium chloride was added to diluted bacteria up to a maximum of 5000 mg/L; 200 μL of this suspension was placed into 96-well plates and incubated at 37°C with shaking for 24 h. After incubation, 20 μL of the bacterial suspension was placed into 100 μL of neutralizer solution (0.75% azolectin and 5% Tween)[24] and left in contact for 5 min. The neutralizer solution was previously screened for efficiency of biocide termination and lack of toxicity towards the test bacteria (data not shown). After neutralization, the culture was replicated onto TSA and incubated for 24–48 h at 37°C; the MBC value was observed as the concentration at which growth ceased. Given the known starting viable count of bacteria, the dilution made in the neutralizer and the volume transferred by the replica plater (1.5 μL), in the absence of bacterial growth, the MBC assay was capable of detecting a 99.995% rate of bacterial kill.

Antibiotic MIC testing: Etest® strips

The susceptibility of Bcc strains to 10 antibiotics was determined using Antibiotic Etest® strips as described by the manufacturer (AB Biodisk). The strains (60 tested) were selected on the basis of their resistance to chlorhexidine and cetylpyridinium chloride. The 10 antibiotics examined were amikacin, azithromycin, ciprofloxacin, ceftazidime, chloramphenicol, imipenem, meropenem, piperacillin, tobramycin and trimethoprim/sulfamethoxazole; all these antibiotics may be used to treat microbial infection in individuals with CF. The selection of antibiotics used to treat Bcc infection in CF is dependent on multiple factors including strain antibiogram, patient tolerance and clinic practice. Double or triple antibiotic combination therapy is recommended with meropenem/high-dose tobramycin combined with ceftazidime, trimethoprim/sulfamethoxazole, chloramphenicol or amikacin.[7] The bacterial strains tested were revived on agar plates, and fresh colony growth resuspended in Iso-Sensitest broth (Oxoid) to an OD of 0.5 at 630 nm. This culture was then swabbed onto Iso-Sensitest agar plates and left to dry for 15 min. Etest® strips were then placed on top of the lawn of bacteria and left at 37°C overnight. The MIC was read from the strips and the resistance/susceptibility determined using the manufacturer's guidelines. Spontaneous resistance of each Bcc strain was measured by counting the number of colonies that grew within the initial zone of

clearing after 48 h of incubation; spontaneous resistance was defined by the appearance of one or more colonies after 48 h of growth.

Suspension Tests

Two commercial skin cleanser biocide formulations were assessed in a standardized suspension test assay:[25] HibiSCRUB™ (containing an alcoholic solution of 4% chlorhexidine gluconate; Regent Medical Ltd) and Cuticura™ Waterless Hand Sanitizer (containing an unspecified amount of triclosan; Cuticura Labs Corp., NY, USA). Bacterial cultures were grown as described above and diluted to $\sim 10^8$ cfu/mL, and 1 mL of this suspension was added to 9 mL of commercial biocide. After exposure for 5, 10, 20 and 60 min, 100 μL of this suspension was removed and placed into 900 μL of a neutralizer solution (0.75% azolectin and 5% Tween 80) for 5 min. A control experiment containing water instead of biocides was also carried out at the same time. To enumerate viable bacteria, serial dilutions and viable counts were conducted.

Biofilm assays

An adapted biofilm assay was used to determine the biofilm production for Bcc strains.[26] Plates (96-well) were coated with 1 mg/well of porcine mucin (Sigma Aldrich, M1778; 200 μL of a 5000 mg/L solution was placed in each well) and left at 37°C overnight. Porcine mucin was used to aid the adherence of bacterial cells to the wells of the plate as mucin is a protein commonly found in the lungs. Plates were washed three times with PBS and 0.1% Tween 20 and then stored at 4°C until use. Bacterial cultures were grown in nutrient broth as described above and diluted to $\sim 10^6$ cfu/mL; 200 μL of each strain was then placed into 96-well plates. Eight replicates of each strain were assessed with the control row containing only broth. The plates were incubated at 37°C statically for 72 h. After incubation, the plates were washed three times with PBS to remove planktonic growth. The remaining biofilm was fixed with methanol for 15 min. Once methanol was removed and plates were dried, biofilms were stained with 1% Crystal Violet for 5 min. The stain was removed by washing with water and plates were dried. Biofilm thickness was measured by adding 33% glacial acetic acid and taking an OD reading at 570 nm using an automated plate reader (see above).

Statistical analysis

Due to the multifactorial mechanisms of biocide action, the MIC values obtained in replicate experiments spanned a range; therefore the results were split into a mean minimum value and a mean maximum value. All the results were analysed using the statistical software Minitab V.14. Kruskal–Wallis and Mann–Whitney statistical tests were carried out on the datasets and significant differences were determined ($P<0.05$ for Kruskal–Wallis and $P<0.05$ for Mann–Whitney). An antibiotic profile score was also calculated as a measure of overall antibiotic resistance within a Bcc species as follows: (i) for a given antibiotic, the mean MIC for all the strains in the species tested was calculated and (ii) the mean for all 10 of the antibiotics tested was then calculated to produce an overall score value for the Bcc species. Mann–Whitney tests were applied to determine the significance of the species' mean antibiotic profile score ($P<0.05$); however, the significance of the score was not determined for Bcc species represented by less than two strains. The antibiotic profile score did not correlate to clinically defined break points for resistance; it just served as a measure of the overall multiple drug resistance observed in each Bcc species.

Results

Chlorhexidine and cetylpyridinium chloride susceptibility

MICs of chlorhexidine and cetylpyridinium chloride were initially determined for 101 strains of the Bcc; after replicate analysis, the mean minimum and mean maximum MIC values for each species were calculated (Table 1). For chlorhexidine, a variable range of growth inhibition was observed around the Bcc species mean (33–42 mg/L), from 10 mg/L (*B. lata*) to 65 mg/L (*B. cepacia*; Table 1). Although there were no significant differences observed between species, strains of *B. cepacia*, *B. vietnamiensis* and *B. cenocepacia* possessed the highest chlorhexidine MIC values observed (Table 1). Approximately 3-fold larger amounts of cetylpyridinium chloride were required to inhibit the growth of Bcc compared with chlorhexidine (mean MIC range, 129–144 mg/L; Table 1). In addition, species differences were apparent, with *B. cepacia*, *B. cenocepacia*, *B. lata*, *B. contaminans* and novel Bcc Group K strains being significantly less susceptible to cetylpyridinium chloride than other members of the Bcc (Table 1; $P<0.05\%$).

The collection of isolates examined contained nine examples of Bcc strains that were involved in outbreaks involving multiple CF patients; interestingly, two of these epidemic strains demonstrated very high levels of chlorhexidine resistance. The *B. cenocepacia* ET12 epidemic strain J2315 and two additional strains that were single-locus variants (part of the same epidemic cable pilus and *B. cepacia* Epidemic Strain Marker-positive lineage) of J2315 had high MIC values for chlorhexidine that were >100 mg/L (one of these, LMG 18827, is shown in Table 2). Of the remaining 28 *B. cenocepacia* strains tested, only one other *recA* Group III-A outbreak strain (MU1; Table 2) and one non-epidemic III-B group strain (LMG 18832; Table 2) possessed a chlorhexidine MIC in excess of 100 mg/L. Other epidemic strains within *B. cenocepacia* III-B such as the Philadelphia–District Columbia[27] and Mid-Western strains[28] were prevented from growing by <60 mg/L chlorhexidine.

The *B. multivorans* isolate LMG 16660, a strain that caused a major outbreak among CF patients in Glasgow,[23] possessed a very high chlorhexidine MIC value (90–100 mg/L; Table 2) compared with the other *B. multivorans* strains. To determine whether this was a feature of the outbreak isolates, 19 additional Glasgow strains (from 17 CF patients involved in the outbreak) were examined and their chlorhexidine MICs were found to range from 44 to 100 mg/L. These minimum and maximum chlorhexidine MICs were significantly higher than the respective mean levels observed for all the other *B. multivorans* strains examined (22–33 mg/L; $P<0.05$). However, in contrast to the elevated chlorhexidine MIC, the Glasgow outbreak strains were significantly more susceptible to cetylpyridinium chloride (76.5–89.5 mg/L; $P<0.05$) than the other *B. multivorans* strains (105–127 mg/L; $P<0.05$).

The Bcc strains were also compared with a control group of non-Bcc species commonly encountered in CF infection. With chlorhexidine, there were no significant differences between the MIC values for Bcc compared with non-Bcc species, although 6 of 10 species tested were prevented from growth by <20 mg/L chlorhexidine. *R. pickettii*, *R. mannitolytica*, *S. aureus* and *P. aeruginosa* required more than 20 mg/L chlorhexidine to inhibit growth. Cetylpyridinium chloride resistance was significantly elevated in several Bcc species compared with

Table 2. Biocide susceptibility and MBCs for selected Bcc strains

Species and strain name	Chlorhexidine		Cetylpyridinium chloride		Triclosan MIC[a] (mg/L)	Benzalkonium chloride MIC[a] (mg/L)
	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)		
B. cepacia (7)						
LMG 17997	100	100	70–100	2000	400	200
ATCC 49709	100	100	>200	500	300	50
IST431	90–100	—	>200	3000	400–450	200
LMG 18821	90–100	300	>200	2000	>500	100
AVC1717	70–80	300	>200	>5000	500	200
ATCC 17759	20–30	100	>200	3000	>500	100
Bcc0176	10–20	50	30–40	50	500	100
B. multivorans (5)						
LMG 16660	90–100	300	>200	>5000	>500	200–250
ATCC 17616	10–20	300	50–150	1000	50	150
C1582	100	300	>200	1000	450–500	150
C1607	100	500	200	>5000	500	250
LMG 18825	20–30	100	50–100	50	50	100
B. cenocepacia (12)						
J2315[b]	>100	100	>200	>5000	>500	350
X100	90–100	500	70–100	>5000	>500	350
LMG 18827[b]	>100	—	>200	—	500	150
MU1	>100	1000	>200	>5000	10	350
WH1	10–20	100	150–180	4000	450–500	200
LMG 16654	80–90	300	>200	>5000	>500	50
DN	100	500	>200	2000	450–500	50
K56-2[b]	90–100	300	>200	>5000	>500	150
PC002	100	300	>200	>5000	>500	>400
ATCC 25609	50–60	300	>200	>5000	400	350
LMG 18832	>100	1000	>200	>5000	>500	350
PC523	10–20	100	150–190	3000	500	200
B. stabilis (2)						
ATCC 35254	10–20	100	200	>5000	500	200
LMG 14294	100	100	180–200	>5000	400	200
B. vietnamiensis (4)						
LMG 16232	100	100	180–200	4000	450–500	100
G4	10–20	300	30–50	500	50	50
J1738	20–30	100	10–30	50	50	50
LMG 10929	100	100	30–50	300	100	50
B. dolosa (2)						
LMG 18943	20–30	300	50–70	2000	450–500	200
AU0090	40–50	100	100–120	5000	450–500	100
B. ambifaria (3)						
MC40-6	20–30	50	100–120	2000	50	50
ATCC 53267	100	—	180–200	5000	>500	50
LMG 19467	20–30	50	70–100	1000	>500	150
B. anthina (2)						
LMG 16670	10–20	50	70–100	500	150–200	50
LMG 20980	10–20	50	120–150	2000	150	50–100
B. pyrrocinia (1)						
LMG 14191	10–20	50	50–70	2000	50	50
Mean maximum value (mg/L)	74	229	155	2998	374	164

[a]The MIC of triclosan and benzalkonium chloride was determined on agar media.
[b]Isolates representative of the *B. cenocepacia* ET12 epidemic CF strain.

other CF colonizers, with *B. cepacia*, *B. cenocepacia* and Bcc novel Group K being significantly less susceptible ($P<0.05$). Overall, *P. aeruginosa* was the only CF pathogen that demonstrated chlorhexidine MICs (33–42 mg/L) and cetylpyridinium chloride MICs (200 mg/L) equivalent to those found in Bcc bacteria.

MBC

After screening 101 strains from the Bcc for growth inhibition, 38 strains spanning the observed MIC ranges for chlorhexidine and cetylpyridinium chloride were selected for MBC analysis (Table 2). The mean Bcc chlorhexidine MBC was 229 mg/L (Table 2), ~6-fold greater than the mean MIC for this biocide. The endpoint difference between the MIC and MBC assays demonstrated that the MIC concentration should not be taken as a measure of biocide efficacy, since for all but two of the strains examined it was below the bactericidal concentration. The majority of strains tested (35 out of 38) required no more than 300 mg/L for a bactericidal outcome. One *B. multivorans* and two *B. cenocepacia* strains had MBCs of 500 mg/L; two *B. cenocepacia* strains needed 1000 mg/L of chlorhexidine to achieve killing, 10-fold above their respective MIC concentration (Table 2). In contrast, 27 of the 38 Bcc strains required over 20-fold more cetylpyridinium chloride than the MIC for a bactericidal outcome, with a mean cetylpyridinium chloride MBC of 2998 mg/L (Table 2). In addition, 13 Bcc strains (eight of which were *B. cenocepacia*) were still viable after a 24 h exposure to 5000 mg/L of cetylpyridinium chloride.

Triclosan, povidone and benzalkonium chloride susceptibility

The same subgroup of Bcc strains selected for MBC analysis was tested for their growth susceptibility to triclosan, povidone and benzalkonium chloride (Table 2). All Bcc strains tested possessed povidone MICs in excess of 50000 mg/L, except for one *B. pyrrocinia* strain (MIC 2000 mg/L). The mean maximum MIC observed for triclosan was 374 mg/L with 11 Bcc strains having MICs >500 mg/L. The mean maximum MIC of benzalkonium chloride was 164 mg/L, similar to that of the other quaternary ammonium compound tested, cetylpyridinium chloride (155 mg/L for the subgroup of strains tested). Both *B. cenocepacia* J2315 and K56-2 from the ET12 epidemic lineage (which possessed high chlorhexidine and cetylpyridinium chloride MICs; Table 1) had high MICs of triclosan (>500 mg/L), povidone [>50000 mg/L (5%)] and benzalkonium chloride (350 mg/L).

Antibiotic susceptibilities

Antibiotic susceptibility to 10 antibiotics commonly used in CF was determined for 60 Bcc strains (Table 3). Clinically defined resistance or susceptibility to individual antibiotics varied widely for the Bcc strains tested. However, when the total antibiotic MIC score was examined, *B. cepacia*, *B. multivorans*, *B. cenocepacia* and *B. dolosa* possessed the greatest levels of multiple drug resistance (Table 3). *B. dolosa*, in particular, possessed very high levels of antibiotic resistance (Table 3); however, this did not correlate to elevated biocide resistance. For example, the *B. dolosa* strain LMG 18943, a strain associated with an outbreak in a hospital in Boston,[29] had a chlorhexidine MIC value of 20–30 mg/L and a cetylpyridinium chloride MIC value of 50–70 mg/L, but was resistant to all 10 antibiotics tested.

Overall, the Bcc strains examined were most susceptible to meropenem, imipenem, piperacillin and trimethoprim/sulfamethoxazole; *B. cenocepacia* strains such as J2315 were, however, innately resistant to this antibiotic combination. Spontaneous resistant colonies appearing within the zones of clearing on the Etest® strips occurred most frequently with the β-lactam antibiotics (meropenem, imipenem and piperacillin). In addition, *B. multivorans* and *B. cenocepacia* strains (41% and 33%, respectively, of the strains tested) produced the most

Table 3. Mean MIC (mg/L) values for Bcc species to 10 antibiotics

Organism (number of strains tested)	AMK	AZM	CAZ	CHL	CIP	IPM	MEM	PIP	TOB	SXT	Antibiotic profile score
B. cepacia (5)	109.6	125.3	5.98	13.3	5.2	17.6	0.9	13.1	88.23	0.5	37.9[a]
B. multivorans (24)	81.6	110.4	52.9	101.6	1.976	25.8	2.2	21.4	30	0.4	42.8[b]
B. cenocepacia (14)	111.5	81.2	2.6	12.2	1	35.6	3.1	23.4	37.5	9.1	31.7[c]
B. stabilis (2)	8	32.7	1.6	22	16.3	3	1.5	3	4.5	2	9.5[d]
B. vietnamiensis (4)	6.3	13.5	2.5	8	0.2	0.4	0.5	1.25	4.6	0.3	3.8
B. dolosa (4)	224	256	66.7	192.7	10.3	26.2	17.5	82.3	118	12	100.6[e]
B. ambifaria (4)	9.8	23.6	1.2	3.25	0.1	1.1	0.2	1.1	6.6	0.1	4.7
B. anthina (1)	16	32	1.5	4	0.19	2	0.38	1	8	0.19	6.5[d]
B. pyrrocinia (1)	0.094	24	0.75	1.5	0.003	0.19	0.002	0.25	2	0.002	2.9[d]
B. contaminans (1)	1	16	1.5	8	0.25	24	0.75	1	0.38	0.125	5.3[d]

AMK, amikacin; AZM, azithromycin; CIP, ciprofloxacin; CAZ, ceftazidime; CHL, chloramphenicol; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TOB, tobramycin; SXT, trimethoprim/sulfamethoxazole.

[a] *B. cepacia* was significantly more resistant than *B. vietnamensis* and *B. ambifaria* ($P<0.05$).

[b] *B. multivorans* was significantly more resistant than *B. ambifaria* ($P<0.05$).

[c] *B. cenocepacia* was significantly more resistant than *B. vietnamensis* and *B. ambifaria* ($P<0.05$).

[d] *B. stabilis*, *B. anthina*, *B. pyrrocinia* and *B. contaminans* were not statistically significant owing to small sample size.

[e] *B. dolosa* was significantly more resistant than *B. stabilis*, *B. vietnamensis* and *B. ambifaria* ($P<0.05$).

spontaneous resistance to the β-lactams. All other Bcc species tested demonstrated low rates of this phenomenon with *B. vietnamiensis* (at 11% of strains tested) being the next highest observed.

Susceptibility to commercial biocide formulations

To determine whether commercial biocide formulation was bactericidal for Bcc strains that possessed high MIC and MBC to specific disinfectants, suspension tests were performed. Two biocides were evaluated: Hibiscrub, a chlorhexidine-based disinfectant used in clinical settings for pre-operative hand disinfection and skin antiseptisis, and Cuticura, a hand gel sold for both home and clinical use. Four Bcc strains with high resistance to chlorhexidine (90–100 mg/L) were tested: *B. cepacia* LMG 18821, *B. multivorans* LMG 16660, *B. cenocepacia* J2315 and Bcc novel Group K strain 24. *B. multivorans* ATCC 17616 was used as a susceptible control isolate. Five minutes of exposure to the commercial biocide formulation was sufficient to achieve killing of three Bcc strains (strain LMG 16660, Group K strain 24 and ATCC 17616). However, *B. cepacia* LMG 18821 remained viable after 1 h of exposure to Cuticura (only a 1 log reduction in viable count was observed); the strain was killed within 5 min in Hibiscrub. *B. cenocepacia* J2315 remained viable in Hibiscrub after 1 h of exposure; however, it was more susceptible to Cuticura with complete killing observed after 20 min.

Biofilm production

Bacteria within biofilms are less susceptible to killing by antimicrobial agents than planktonic cells.[30] To determine whether there was a correlation between biofilm formation and biocide resistance in a planktonic state, 11 Bcc strains were screened for biofilm production. Six strains were selected with high (>80 mg/L) and five with low (<30 mg/L) resistance to chlorhexidine (Figure 1). No correlation between chlorhexidine MIC and the ability to form a biofilm was observed. For example, *B. multivorans* strain ATCC 17616, which possessed a low chlorhexidine MIC (10–20 mg/L) and low MICs to the other biocide screens (Table 1), produced the most biofilms. In contrast, *B. cenocepacia* J2315, which was representative of a highly transmissible lineage associated with major clinical infections and possessed high biocide (Table 2) and antibiotic resistance (see above), was a poor biofilm former.

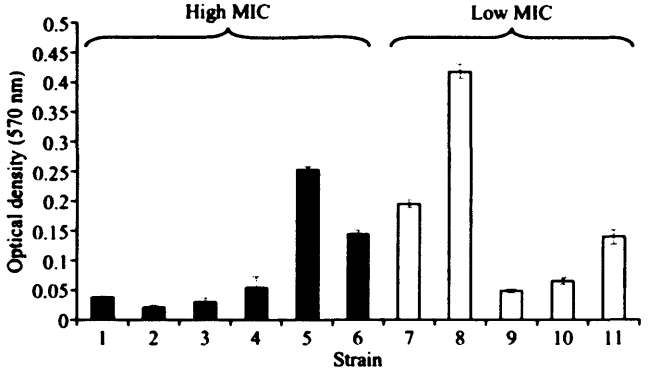

Figure 1. Biofilm production by selected Bcc strains. Six strains with high (black bars) and five with low (open bars) chlorhexidine MICs were tested for biofilm formation. The biofilm biomass readout is shown on the vertical axis. Strains are as follows: 1, *B. cepacia* LMG 18821; 2, *B. multivorans* LMG 16660; 3, *B. cenocepacia* LMG 18832; 4, *B. cenocepacia* J2315; 5, *B. cenocepacia* C4455; 6, *B. cepacia* novel Group K 24; 7, *B. multivorans* LMG 13010; 8, *B. multivorans* ATCC 17616; 9, *B. dolosa* LMG 18943; 10, *B. ambifaria* AMMD; 11, *Burkholderia vietnamensis* G4.

Discussion

Bcc bacteria are known to possess high levels of antimicrobial resistance; however, a survey of their biocide resistance that accounts for the recent changes in their taxonomy has not been performed. Our analysis of the biocides chlorhexidine, cetylpyridinium chloride, triclosan and benzalkonium chloride has demonstrated that susceptibility to these agents varies considerably across a panel of genetically distinct Bcc strains. Species-dependent differences were apparent only for cetylpyridinium chloride. No specific correlations between biocide susceptibility, antibiotic susceptibility and the ability to form biofilms were seen; however, certain CF outbreak strains possessed very high chlorhexidine resistance. The ability of biocide-resistant Bcc strains to survive exposure to two widely used commercial skin washing biocides was also demonstrated, suggesting that there is still room to improve disinfectant formulations to ensure they kill this group of bacteria.

While the correlation of antibiotic susceptibility to six genomovars has been reported,[9] the influence of Bcc taxonomy on biocide susceptibility has not been examined. The MIC for Bcc strains was investigated in great depth for chlorhexidine and cetylpyridinium chloride, with *B. cepacia*, *B. cenocepacia*, *B. lata*, *B. contaminans* and Bcc novel Group K being significantly less sensitive to cetylpyridinium chloride than other species. Previous work correlating clinical antibiotic resistance to Bcc taxonomy did not find any significant trends,[9] however, we found that if an overall antibiotic resistance score was calculated based on the MIC value, then *B. cepacia*, *B. multivorans*, *B. cenocepacia* and *B. dolosa* were significantly more resistant. Interestingly, *B. cenocepacia*, which overall possessed both high biocide (Tables 1 and 2) and antibiotic resistance (Table 3), was also the most prevalent Bcc species encountered in a recent survey of both CF and other clinical infections.[6]

Correlations between antibiotic resistance and biocide resistance were not obvious among the Bcc bacteria. Certain epidemic Bcc strains such as those of the ET12 group (e.g. J2315, Table 2) possessed the highest levels of resistance to both classes of antimicrobial agents. The Glasgow outbreak *B. multivorans* strain[23] possessed chlorhexidine MICs that were nearly double that of other *B. multivorans* strains (Table 2), yet had similar levels of antibiotic resistance within this species group. Other strains, such as the epidemic *B. dolosa* LMG 18943,[29] possessed low chlorhexidine and cetylpyridinium chloride MICs (Table 2) but were resistant to all 10 antibiotics tested. There was also a lack of correlation between planktonic biocide resistance and the ability to form biofilms (Figure 1) for Bcc bacteria. This suggests that despite the advantages biofilm growth confers in terms of survival under antimicrobial stress,[30] not all Bcc bacteria have adopted this phenotype, but instead have other enhanced resistance mechanisms that function during planktonic growth. However, strains such as *B. cenocepacia* C4455 and Bcc novel Group K 24 (Figure 1), which have both high intrinsic biocide

and a good ability to form biofilms, have the potential to be particularly problematic in contamination or outbreak scenarios. The lipopolysaccharide (LPS) structure of Bcc bacteria is very unusual and also varies between species.[31] Given the important role this part of the Bcc cell envelope plays in its resistance to cationic antimicrobial agents, it would be interesting in future studies to compare the LPS structures of strains that are resistant or susceptible to membrane-active biocides such as chlorhexidine.

Maintaining product sterility is a problem faced by many manufacturing industries and a recent survey of the pharmaceutical industry has shown that Bcc bacteria are one of the leading causes of pharmaceutical product recalls.[10] Our analysis of MBC data showed that Bcc bacteria may need more than 25 times more biocides over the MIC value to achieve killing. The concentrations of biocides used in commercial products are dependent on the biocide and type of product application. Chlorhexidine, cetylpyridinium chloride and triclosan are used in a variety of commercial products in concentrations ranging from 0.1% to 4% (1000–40000 mg/L).[32] At the lowest level, this is insufficient to kill Bcc bacteria. For example, two *B. cenocepacia* strains had chlorhexidine MBCs of 1000 mg/L and 31 Bcc strains had cetylpyridinium chloride MBCs in excess of 1000 mg/L (Table 2). Dilute solutions of chlorhexidine appear particularly prone to contamination with Bcc bacteria, and outbreaks associated with mouthwash (0.2%)[33] or blood-product disinfectant solutions (0.5% and lower concentrations)[13] have been reported. The MIC of five disinfectants used for organ irrigation in CF lung-transplant procedures was evaluated by Perry *et al.*[34] Taurolin and Noxyflex were found to be more inhibitory towards the 19 Bcc isolates tested than chlorhexidine, triclosan and povidone, and were specifically recommended for use where these bacteria may be present.[34]

The efficacy of skin disinfection products and procedures may be tested according to standard European guidelines using bacterial species such as *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus hirae*.[25] Preservative challenge test guidelines include the *B. cepacia* Type strain ATCC 25416, among the recommended test strains.[35] However, although this strain possessed the maximal cetylpyridinium chloride MIC (>200 mg/L), its chlorhexidine MIC was 20–30 mg/L and was not among the most resistant encountered in our survey. Three well-defined strains stand out in our analysis as being resistant to multiple biocides (Table 2): (i) *B. cenocepacia* LMG 18832 (ATCC 17765), a urinary tract infection isolate;[22] (ii) *B. cenocepacia* J2315 (LMG 16656), an epidemic CF isolate,[22] and (iii) *B. multivorans* LMG 16660 (C1576), a CF isolate from the Glasgow outbreak.[23] All the latter strains were included in a historical reference panel of Bcc strains[22] and are available from recognized strain collections. Given the major role Bcc organisms may play in the contamination of such products,[10] future testing should also include these bacteria.

In summary, the characterization of Bcc as a group of problematic opportunistic pathogens has advanced considerably in the last decade.[3] The ability to genetically define strains[17,19] and systematically identify new species within the group[4,5] has improved considerably. We have shown that as a closely related group of Gram-negative pathogens, Bcc bacteria possess very high resistance to biocides and commercial formulations of these agents. This understanding, together with the potential use of the biocide-resistant reference strains we have defined, can be used to develop new anti-infectives capable of killing Bcc bacteria and preventing their occurrence in disease[2] and contamination of commercial products.[10]

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Transparency declarations

None to declare.

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10.2 Appendix 2; raw microarray and sequence data

CD-ROM of raw microarray data, including quality control reports and raw microarray imagines produced by the Agilent feature extractor software. Raw sequences generated from the transposon mutagenesis screen are included on the CD-ROM.