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Anti-infective strategies in *Pseudomonas aeruginosa* biofilm formation

A thesis submitted to Trinity College, Dublin for the degree of Doctor of Philosophy

Priya Kapoor, BSc

Department of Clinical Microbiology

2014
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Summary

*P. aeruginosa* infection is one of the main causes of lung function decline in patients with cystic fibrosis (CF). *P. aeruginosa* is associated with biofilm formation which can increase the rate of resistance to antibiotic treatment. Biofilms are communities of cells encased within an exopolysaccharide matrix. The overall aim of the project was to explore the activity of novel therapeutic agents against established *P. aeruginosa* biofilms. The study investigated the phenotypic characteristics of clinical CF *P. aeruginosa* strains such as biofilm forming ability and *in vivo* virulence and the resistance to antibiotic single and combination therapy. In addition, the activity of novel synthesised metal-based complexes and the interactions of *P. aeruginosa* with unusual CF pathogens in a mixed bacterial environment were also explored. The static and dynamic *in vitro* models which were employed to study microbial biofilms provided diversity in terms of biofilm growth, development and the response to antimicrobial therapy.

Biofilm formation was common characteristic of CF *P. aeruginosa* strains; however, the levels differed with the different *in vitro* models. *In vivo* virulence of these strains was more pronounced with *P. aeruginosa* strains that produced the phenazine pigment, pyocyanin. The results demonstrated that antibiotic efficacy was less effective against biofilms than planktonic cells and the synergistic interactions of antibiotic combinations were strain specific. Tobramycin-ceftazidime was the only combination that displayed synergistic interactions against the 24 and 72 hour biofilm of one clinical *P. aeruginosa* isolate. Overall, antibiotic combinations were similar to the activity of either antibiotic used as monotherapy.
The flowchamber biofilm system which was used as part of this study allowed direct visualisation of biofilm structures over a specific period of time. The study also showed that *P. aeruginosa* strains were sensitive to treatment with novel silver-coumarin compounds irrespective of their phenotypic traits. The antimicrobial effects of the agents used in the study may be useful for further study as possible treatments of *P. aeruginosa* infection. Furthermore, the interactions of *P. aeruginosa* and other CF pathogens in a mixed species environment implied that *P. aeruginosa* is capable of remaining the dominant pathogen. *Herbaspirillum* sp. and *Paracoccus yeeii* which did not readily form single species biofilms with the static *in vitro* biofilm model did not interfere with the growth and development of *P. aeruginosa* biofilms. In contrast, it was found that *S. maltophilia* was capable of altering the growth of *P. aeruginosa*. *S. maltophilia* which is capable of forming biofilms is frequently isolated from sputum of cystic fibrosis patients. The study also showed that *S. maltophilia* cell-free supernatants inhibit the growth of certain *P. aeruginosa* biofilms. LC/MS analysis was used to identify the different proteins between *S. maltophilia* strains and findings suggest that specific proteins may be influencing virulence and response to antibiotics during co-infection.
Acknowledgements

Completion of this thesis would not have been possible without the selfless support of others and I would like to take this opportunity to express my sincere appreciation. Firstly, I would like to sincerely thank my supervisor Professor Philip Murphy for offering me the opportunity to study a PhD and for all his patience, support and guidance over the past few years.

I would like to thank Dr. Bernie Creaven and Maeve Sullivan for the gift of silver compounds and Dr. Denis O’Shea for the gift of the copper compounds.

Many thanks to Dr. Thomas Bjarnsholt and his group, especially Anne, from the University of Denmark for allowing me to undertake a research placement in the university to further my research skills.

I would like to thank Jonathan Collins for the CF strains and all his advice and Dr. Emma Caraher for all her guidance. I also acknowledge Dr. Siobhan McClean for all her help with the final part of this thesis and Prof. Sean Doyle and Grainne O’Keeffe from NUI Maynooth for carrying out the LC/MS analysis. To all the other CF researchers and microbiologists in Tallaght hospital for their help over the part few years.

Last but not least I would like to thank my parents, who have been so supportive and understanding throughout my four years and for always pushing me to finish.
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Abbreviations

μl  Microlitre
μM  Micromolar
ABC-transporter  ATP-binding cassette transporters
ABPA  Allergic bronchopulmonary aspergillosis
AgNO₃  Silver nitrate
AHL  Acyl-homoserine lactone
AI  Autoinducers
AMNCH  Adelaide and Meath Hospital, Incorporating the National Children’s hospital
AP-1  Activator protein-1
APH  Aminoglycoside phosphotransferase
APM  Ammonium phosphate monobasic
APS  Ammonium persulfate
ATCC  American Type Culture Collection
ATP  Adenosine Tri-phosphate
Bcc  *Burkholderia cepacia* complex
BIC  Biofilm inhibitory concentration
BZT  Benzotriazole
BZTM  1H-benzotriazole-1-methanol
*C. albicans*  *Candida albicans*
CAMP  Cationic antimicrobial peptides
CBAVD  Cogenital bilateral absence or atrophy of the vas deferend
CBD  Calgary Biofilm Device
CCa  Coumarin
CDC  Centres for Disease Control and Prevention
CF  Cystic fibrosis
CFTR  Cystic fibrosis transmembrane conductance regulator
CFU  Colony forming units
cGMP  Cyclic guanosine monophosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Cl</td>
<td>Chloride</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory standards institute</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CFRI</td>
<td>Cystic Fibrosis Registry of Ireland</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>CV</td>
<td>Crystal violet</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonuclease acid</td>
</tr>
<tr>
<td>DSF</td>
<td>Diffusible signal factor</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>E-test</td>
<td>Epsilometer test</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharide</td>
</tr>
<tr>
<td>FAC</td>
<td>Ferric ammonium compounds</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiry volume in 1 second</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug administration</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FIC</td>
<td>Fractional inhibitory concentration</td>
</tr>
<tr>
<td>FICI</td>
<td>Fractional inhibitory concentration index</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>Ga</td>
<td>Gallium</td>
</tr>
<tr>
<td>Ga(NO₃)₃</td>
<td>Gallium nitrate</td>
</tr>
<tr>
<td><em>gfp</em></td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>H. influenzae</td>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
</tr>
<tr>
<td>HSF</td>
<td>Heat shock factor</td>
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</table>
HSL  Homoserine lactone
H₂O  Hydrogen oxide
IL   Interleukin
KH₂PO₄ Monopotassium phosphate
LA   Luria agar
LB   Luria broth
LC   Liquid chromatography
LES  Liverpool epidemic strain
IRT  Immunoreactive trypsinogen
LPS  Lipopolysaccharide
M    Molar
MBC  Minimal bactericidal concentration
MBZT 5-methyl-1H-benzotriazole
MES  Manchester Epidemic strain
MDR  Multidrug resistance
MHA  Mueller-Hinton agar
min  Minutes
MIC  Minimal inhibitory concentration
ml   Millilitres
mmol Millimole
MRD  Modified Robbin’s Device
MRSA Methicillin resistant staphylococcus aureus
mRNA messenger Ribonucleic acid
MS   Mass spectrometry
mw   molecular weight
Na   Sodium
NaCl Sodium chloride
Na₂HPO₄·2H₂O Disodium hydrogen phosphate
(NH₄)₂SO₄ Ammonium sulphate
NB   Nutrient broth
ng   Nanogram
<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OP</td>
<td>Oropharyngeal</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGFE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td><em>P. yeeii</em></td>
<td><em>Paracoccus yeeii</em></td>
</tr>
<tr>
<td>PMA</td>
<td>Polymorphonucleotide activation</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonucleotide</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic acid</td>
</tr>
<tr>
<td>RSCV</td>
<td>Rugose small colony variants</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SCV</td>
<td>Small colony variants</td>
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<tr>
<td><em>S. aureus</em></td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td><em>S. maltophilia</em></td>
<td><em>Stenotrophomonas maltophilia</em></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N;-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptone soy agar</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptone soy broth</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TSP</td>
<td>Transferable solid phase</td>
</tr>
<tr>
<td>TVC</td>
<td>Total Viable Counts</td>
</tr>
<tr>
<td>w/w</td>
<td>weight/weight</td>
</tr>
<tr>
<td>v/w</td>
<td>volume/weight</td>
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<td>v/v</td>
<td>volume/volume</td>
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Chapter 1: Literature Review

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive inherited disorder most common in northern Europeans, affecting approximately 1 in 3000 births (Walters et al., 2007) and 60,000 individuals worldwide (Gibson et al., 2003). The disease persists from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene which encodes a chloride (Cl<sup>-</sup>) channel in secretory epithelia (Riordan et al., 1989). Mutations in the CFTR gene result in a loss or reduction of chloride secretion. This leads to the production of thick, viscous mucus in the lungs. Mucus prevents cilia beating effectively on normal epithelia, allowing the growth of pathogens in the chronic CF lung (Govan et al., 1996). Mucins which are long, highly glycosylated polymers form a mesh with an optimal pore size for trapping inhaled particles such as bacteria and fungi (Boucher et al., 2007). There are two types of mucins in the CF airways, MUC5AC and MUC5B, which are two of the major components that form the mucus matrix (Groneberg et al., 2002).

1.1.1 Cystic Fibrosis Transmembrane Regulator (CFTR) gene

The CFTR gene consists of 180,000 base pairs located on the chromosome 7 and encodes the membrane protein 1480-amino acid (Riordan et al., 1989; Quinton et al., 2008). The CFTR glycoprotein acts as a regulated chloride channel. CFTR is a part of the adenoseine triphosphate (ATP)-binding cassette (ABC) transporter proteins (Riordan et al., 2008). The process by which CFTR functions involves DNA which is transcribed to mRNA. The mRNA from the nucleus and endoplasmic reticulum (ER) from the cytoplasm cause
translation of polypeptide within the ER. Maturation of the ER results in protein folding within the lipid bilayer (Lukacs et al., 1994; Ward et al., 1994).

1.1.2 Mutations in the CFTR gene

Approximately 1,500 CFTR mutations have been identified. Clinicians select a suitable treatment for CF patients according to mutations which are separated into different classes. Some of the most common mutations among the CF population include misfolded CFTR resulting in degradation of the F508del-CFTR gene prematurely (Van Goor et al., 2006), reduced chloride channel ion transport of R117H (Sheppard et al., 1993), and reduced production of normal plasma membrane due to transcriptional dysregulation as a result of the G551D gene (Lewis et al., 1994).
**Figure 1.1:** CFTR mutations which are separated into classes. There are six classes representing six mutations of the CFTR gene (Rowe *et al.*, 2005).

### 1.1.3 Inflammation in the CF airways

Inflammatory responses in the CF airways occur due to increases in bacterial concentrations and polymorphonucleocytes. The inflammatory process prevents the spread of colonising bacteria but also leads to tissue damage (Perez *et al.*, 2007). Many studies have documented increases in cytokines regulated by NFκB such as interleukin IL-8, IL-6 and IL1β, tumour necrosis factor (TNF)α, leukotriene B4 and free neutrophil elastase. Airway inflammation can be detected in bronchoalveolar lavage (BAL) fluid samples as early as infancy (Khan *et al.*, 1995). In response to infection, CF patients have higher levels of IL-8 and neutrophils in BAL samples compared to non-CF infected individuals (Muhlebach *et al.*, 1999). Another signalling pathway IL-10, found in low
quantities in BAL samples can reduce the inflammatory response by neutrophil apoptosis and T-cell stimulation (Lentsch et al., 1997; Schottelius et al., 1999). Other studies have described IL-23 and IL-17 as proinflammatory cytokines involved in cell signalling of TH-17 which is enhanced during CF (Dubin et al., 2007; McAllister et al., 2005). The viscous mucus which traps pathogens can trigger the pathway of IL-23 and IL-17 by recruiting neutrophils to the site of infection (Ferretti et al., 2003). These inflammatory responses have shown to decrease after treatment of the pulmonary exacerbation (Konstan et al., 1993; Konstan et al., 1994; Bonfield et al., 1995; Sagel et al., 2001; Colombo et al., 2005).

1.1.4 Nitric oxide and CF infection

Nitric oxide (NO) acts as an antimicrobial in the lungs of individuals without CF. CF patients with reduced levels of NO may experience hypersusceptibility to infection. Increased metabolism of NO to NO$_2^-$ and NO$_3^-$ results in lower NO levels in exhaled breath of CF patients than produced by normal individuals (Mhanna et al., 2001). Reduced sodium (Na$^+$) has been linked to Na hyperabsorption and Cl$^-$ secretion caused by inhibition interactions between CFTR-ENaC (epithelial sodium channel). Low levels of NO also contribute to a reduction in the activity of guanylate cyclase which is an enzyme that produces cyclic guanosine monophosphate (cGMP) (Jayaraman et al., 1999).

1.1.5 Diagnosis of CF

The diagnosis can be confirmed by a series of tests of abnormal ion concentrations and genotyping of the most common mutations. A series of clinical tests which do not directly
detect defects in the CFTR gene assist in the diagnosis of CF. The procedures involve testing for pancreatic insufficiency or paranasal sinuses (Ratjen and Doring, 2003). Studies have reported that approximately 15% of newborns with CF can be affected with meconium ileus (Evans et al., 2001). Infertility is common among the majority of males caused by cogenital bilateral absence or atrophy of the vas deferend (CBAVD) (Trezise et al., 1993; Dodge et al., 1995). The hypothesis for infertility in CF females is increased viscous production in the cervix which may lead to a reduction in transport of sperm (Hodges et al., 2008).

1.1.6 Screening for CF

Prenatal screening allows identification of CF before birth while newborn screening can detect the condition after birth. Screening protocols generally involve two steps, phenotypic tests that measure immunoreactive trypsinogen (IRT) in dried blood spots followed by a repeat immunoreactive trypsinogen test if there is uncertainty about the result of the sweat test (Ranieri et al., 1994). According to the NCCLS guidelines (2000), a sweat test involves stimulation of sweat by iontophoresis of a cholinergic drug, pilocarpine nitrate to a selected area of the skin. This is followed by a quantitative or qualitative analysis of sweat to determine electrolyte concentration, conductivity or osmolarity for CF diagnosis (LaGrys et al., 1996). Elevated sweat chloride levels are related to abnormal CFTR function. Infants with sweat chloride concentrations of 40-59 mmol/L are likely to be diagnostic of CF and require repeat sweat testing and follow-up tests. A level of > 60 mmol/L sweat chloride by a second sweat test signifies diagnosis of CF (NCCLS, 2000). DNA analysis of a blood sample can also detect ≥ 1 CFTR specific
mutation. Infants without a mutation but a highly elevated IRT value are also classified as being at risk for CF as there may be other mutations that have not been detected (Wilfond et al., 2005). List below shows the common clinical symptoms of CF.

1.1.7 Common symptoms associated with CF diagnosis (adapted from Ratjen and Doring, 2003).

- Chronic cough
- Bacterial colonisation in the airways
- Clubbing
- Airway obstruction
- Sinusitis
- Nasal polyps

1.1.8 Diagnostic microbiology

Sputum samples are the preferred source of airway secretions and an accurate indicator of microorganisms in the lower airways of CF patients (Thomassen et al., 1984; Henig et al., 2001). Other procedures including throat swabs, oropharyngeal cultures (OP) and broncho-alveolar lavage fluids (BAL) are preferred for paediatric patients that cannot readily expectorate sputum (Armstrong et al., 1996; Rosenfeld et al., 1999). BAL samples which are collected from the lower respiratory tract are a more sensitive means of measuring infection. This procedure can be more invasive for the patient and generally requires a general anaesthetic (Henig et al., 2001). While standard culture testing can identify different microorganisms present in samples, molecular techniques allow direct
detection of bacteria, fungi and viruses (Fredricks et al., 2005; Khot et al., 2008; Rabodonirine et al., 1997; Gutierrez et al., 2001; Gilchrist et al., 2011). Willner et al., (2012), concluded reproducibility of these assays varies depending on the DNA extraction method since the volume of BAL obtained from younger patients can be low (Shields et al., 2000).

1.1.9 Colonisation of the CF airways by \textit{P. aeruginosa}

\textit{P. aeruginosa} isolated from the CF lung displays morphological diversity to environmental isolates (Govan et al., 1996). Inflammation in the CF lungs as a result of \textit{P. aeruginosa} colonisation leads to high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are generated by neutrophils as part of the innate immune response. ROS and RNS lead to mutations that enable \textit{P. aeruginosa} to colonise the CF airways (Ciofu et al., 2005). \textit{P. aeruginosa} isolates in the CF lung are often mucoid, biofilm-formers, lack flagella and pili and are highly resistant to antibiotics. Many \textit{P. aeruginosa} strains have been described as hypermutable as they display a higher spontaneous mutation frequency than wild-type bacteria and contribute to increased clonal expansion (Oliver et al., 2000). Dominant mutations such as the conversion of non-mucoid to mucoid colony morphology are caused by an over-production of alginate and this can affect the clinical outcome of the patient (Ramsey et al., 2005). Mutations within the \textit{mucA} gene, encoding a member of the extracytoplasmic function of anti-sigma factors causes a change to the mucoid phenotype. Mutations in \textit{algW}, \textit{mucC} and \textit{mucD} genes can also lead to conversion to the mucoid phenotype (Hasset et al., 2009).
1.2 Treatment strategies of Cystic Fibrosis

1.2.1 Antibiotic Susceptibility testing

Previous studies have suggested pulmonary exacerbations have been associated with new microorganisms or a change in the bacterial density of colonising isolates (Regelmann et al., 1990; Smith et al., 1988; Wat et al., 2003; Van Ewijk et al., 2005). Chronic bacterial infection can be maintained with antibiotics such as tobramycin, ceftazidime, colistin, piperacillin/tazobactam, meropenem and ciprofloxacin, commonly administered for P. aeruginosa and B. cepacia (Banerjee et al., 2000). CF patients infected with P. aeruginosa and suffering from acute exacerbations of chronic multiresistant lung disease are often treated with potential synergistic combinations of anti-pseudomonal antibiotics (Aaron et al., 2002). Cephalosporins, macrolides and tetracycline are often administered for staphylocoecal infection. Due to bacterial resistance, flucloxacillin can be administered as it is more appropriate at minimising this effect (Hodson, 1995).

Beardsmore et al., (1994), suggested that prophylactic therapy can reduce the frequency of S. aureus isolates in sputum samples compared with intermittent therapy.

1.2.2 Antibiotic combination therapy

Bacterial mutations enable resistance to develop in response to the majority of antibiotics. Tolerance is defined as a reduction in the killing effect of an antibiotic against a bacterial population (Tomaz et al., 1985). Many studies have focused on exploring the activity of antibiotic combinations against CF bacteria. Synergistic therapies allow reductions in the quantities of the agents used (Harrison et al., 2008). Zhai et al., (2010), showed lower
concentrations of polymyxin B and fluconazole exhibited fungicidal activity against a range of fluconazole-resistant *Candida albicans* isolates and moulds. *In vitro* studies by Seibert *et al.*, (1992), reported synergistic interactions between cefpirome and vancomycin against several enterococci and the majority of MRSA strains by the checkerboard assay. In addition, Hermann *et al.*, (2010), found colistin and tobramycin inhibited the mature biofilm of *P. aeruginosa* by inhibiting different components of the structure. Additionally, the study by Moskowitz *et al.*, (2005), reported that no specific antibiotic combination was effective against the majority of *P. aeruginosa* isolates and the results vary depending on each individual strain.

### 1.2.3 Aerosolised antibiotic therapy for CF patients

Numerous studies have examined the relationship between aerosolised antibiotics and morbidity of CF patients. Antibiotic inhalation enables the delivery high doses of antibiotics directly to the site of infection (Geller, 2009). Recent studies focusing on aerosolised antibiotics in conjunction with regular oral antibiotic treatment found significant improvements in pulmonary function with reduced bacterial colonisation in CF sputum. Wall *et al.*, (1983), found CF patients receiving 80 mg tobramycin by inhalation and 1 g ticarcillin orally experienced reduced exacerbations. The study by Hoiby *et al.*, (2005), found improved lung function of patients treated with daily use of oral ciprofloxacin and inhaled colistin. Antibiotics such as colistin and tobramycin can be inhaled more effectively due to improvements in particle size, distribution and duration of treatment (Heijerman *et al.*, 2009; Geller, 2009). Many commonly administered antibiotics may be developed as liposomal formulations as research has shown they may
be more effective at penetrating the biofilm matrix. One possible disadvantage of these formulations is bacterial resistance due to prolonged exposure; however, this effect can often be dependent on the concentration present within the CF airways (Geller, 2009).

1.2.4 Macrolide antibiotic therapy

A previous study involving 29 *P. aeruginosa* chronically infected patients found the macrolide antibiotic, roxithromycin resulted in an 80% reduction in sputum viscosity, speculated to be linked to chloride transport being restored (Dupont et al., 1990). Saiman et al., (2003), found a CF patient group receiving 250 mg or 500 mg of oral azithromycin 3 days a week showed improvements in FEV1% and experienced fewer exacerbations than the placebo group. Jaffe et al., (1998), found CF children at end-stage lung disease, colonised with *P. aeruginosa* and treated with long-term azithromycin also had higher median FEV1% values. These studies have shed light on the use of macrolides as a long term therapy for CF patients. In addition, Gillis et al., (2004), found sub-MIC concentrations of azithromycin reduced biofilm development of *P. aeruginosa* cultivated in the flow cell system by delaying initial adherence.

1.2.5 Anti-inflammatory agents for CF patients

Anti-inflammatory agents may be used for CF therapy to deal with lung disease progression due to inflammation in the CF airways. Analysis of BAL is the most common method used by clinicians for assessing inflammation as well as infection in the lungs. Neutrophils are the main inflammatory cells in the CF lungs in which DNA and filamentous actin increase the viscosity of the CF sputum reducing the clearance of mucus (Perks et al., 2000; Tang et al., 2005). Anti-inflammatory treatments that restore
airway surface liquid and interfere with the function of the CFTR proteins have shown more promise. Corticosteroids have the ability reduce mucus and interfere with the inflammatory mediator action. Aspirin and ibuprofen have been shown to have effective anti-inflammatory action against neutrophils by inhibiting neutrophil migration. At higher concentrations the agents can inhibit the action of the main proinflammatory transcription agents, NFkB and AP-1 or HSF (Kopp et al., 1994; Dong et al., 1997; Jurivich et al., 1995).

1.3 Cystic Fibrosis in Ireland

1.3.1 Statistics of CF in Ireland

According to the Cystic Fibrosis Registry of Ireland (CFRI), Ireland has approximately 1 in 1600 infants born with this inherited condition per annum which is an average of 30-40 births each year. The ‘median survival’ is defined as following an entire CF population until half have died. As of 2011, the CFRI has estimated that the median survival of males and females is 24.3 and 29.4 years, respectively. During the same year more deaths have occurred in male patients than in females. A higher decline rate was observed for males born between 1985-1994; however, the results presented in Figure 1.2 show females exhibit similar birth cohort survivorship curves throughout the years. Respiratory or cardiac failure accounts for the largest proportion of all deaths of the Irish CF population.
Figure 1.2: Statistical analysis of the ‘median survival’ of (A) females and (B) males with CF in Ireland who were born between 1985-2004. Image adapted from the CFRI.
1.3.2 Respiratory complications

The CFRI has reported that respiratory failure accounts for 70% of all deaths due to patients contracting deadly infections in the hospital environment. This rate has declined steadily in recent years from approximately 69.5% in 2009 to 69.3% in 2011. Respiratory complications occur more frequently in the adult CF population than among infants. \textit{P. aeruginosa} was the most commonly contracted organism among Irish CF patients in 2011. A recent study by Van deale et al., (2005), found patients were more susceptible to colonisation of more than one \textit{P. aeruginosa} genotype due to patient-to-patient transmission.

Other organisms that persist in the CF lungs include \textit{B cepacia}, accounting for a significantly lower proportion of lung infection (Figure 1.3). Govan et al., (1993), indicated patient-to-patient spread is more problematic with \textit{B. cepacia} strains. Studies by Vandamme et al., (1997), and Clode et al., (2000), indicated \textit{B. cenocepacia} was the most dominant species of the \textit{B. cepacia} complex amongst the CF population, followed by \textit{B. multivorans} and \textit{B. cepacia}. Many CF centres segregate \textit{B. cepacia} infected patients particularly those with transmissibility marker-positive \textit{B. cenocepacia} strains to avoid cross infection. According to LiPuma et al., (2002), segregation does not prevent naturally acquiring the species from the environment.

The CFRI reports \textit{S. aureus} infections have risen steadily in recent years with approximately 32.2% of adults with CF infected in 2011. A study by Elizur et al., (2007), found staphylococcus infection increased from 22% to 27% over three years in one CF centre. Previously, Campana et al., (2007), found 6.7% of sputum samples were
methicillin-resistant *Staphylococcus aureus* (MRSA) infected form one CF centre. Bacterial resistance has been reported as a common occurrence after quinolone treatment which is considered as a high risk factor when treating MRSA infections (LeBlanc *et al.*, 2006). SCV variants of *S. aureus* have demonstrated reduced clinical and microbiological responses even after prolonged antibiotic treatment (Seifert *et al.*, 2003).

![Respiratory Infections vs. Age](image)

**Figure 1.3:** Prevalence of CF infections according to age. *P. aeruginosa, S. aureus* and *H. influenzae* represent the most common pathogens. Image adapted from the Cystic Fibrosis Foundation, 2006.
1.4 Cystic Fibrosis pathogens.

Several of the pathogenic organisms commonly found in the CF lungs are shown in Table 1.1.

Table 1.1: Pathogens associated with CF infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative bacteria</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Burkholderia cepacia complex</em></td>
</tr>
<tr>
<td></td>
<td><em>Stenotrophomonas maltophilia</em></td>
</tr>
<tr>
<td></td>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td></td>
<td>MRSA</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td></td>
<td><em>Candida albicans</em></td>
</tr>
</tbody>
</table>

1.4.1 *Burkholderia cepacia complex* (Bcc)

*Burkholderia cepacia* is a gram-negative bacterium commonly found in the environment, associated with increased morbidity and mortality in CF patients. There are 17 closely related species associated with the *B. cepacia* complex (LiPuma *et al.*, 2010). Overall, the *Burkholderia* genus is comprised of approximately 43 phenotypically different species (Vial *et al.*, 2007). *Burkholderia multivorans* and *Burkholderia cenocepacia* are the more
prevalent groups among the CF population (LiPuma et al., 2001; Speert et al., 2002). The quorum sensing system of Bcc controls biofilm development (Eberl et al., 2001) and the rate of resistance to antimicrobials (Chernish and Aaron, 2003). Numerous studies have indicated that once acquired, Bcc is rarely eradicated from the site of infection. A transmissible, multiresistant epidemic *B. cepacia* strain known as ET-12 has been detected in numerous CF centres in the UK (Pitt et al., 1996). During the end stages of infection with Bcc, lung transplantation remains the most promising therapeutic option for patient survival (Heath et al., 2002).

17 species of the *Burkholderia cepacia* complex (LiPuma et al., 2010).

<table>
<thead>
<tr>
<th><em>Burkholderia cepacia</em> complex species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cepacia</em></td>
</tr>
<tr>
<td><em>B. multivorans</em></td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
</tr>
<tr>
<td><em>B. stabilis</em></td>
</tr>
<tr>
<td><em>B. vietnamiensis</em></td>
</tr>
<tr>
<td><em>B. dolosa</em></td>
</tr>
<tr>
<td><em>B. amblifaria</em></td>
</tr>
<tr>
<td><em>B. anthina</em></td>
</tr>
<tr>
<td><em>B. pyrrocinia</em></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Burkholderia cepacia</em> complex species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. latens</em></td>
</tr>
<tr>
<td><em>B. diffusa</em></td>
</tr>
<tr>
<td><em>B. arbores</em></td>
</tr>
<tr>
<td><em>B. metallica</em></td>
</tr>
<tr>
<td><em>B. seminalis</em></td>
</tr>
<tr>
<td><em>B. contaminans</em></td>
</tr>
<tr>
<td><em>B. lata</em></td>
</tr>
<tr>
<td><em>B. ubonensis</em></td>
</tr>
</tbody>
</table>

1.4.2 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a motile, gram-negative bacterium, found in soil and the aqueous environment. It is an aerobic bacterium which is able to grow under anaerobic
conditions in the presence of nitrate (Govan et al., 1993). Infections with this pathogen are common in immunocompromised individuals and are one the most prevalent species associated with CF respiratory tract infection. Costerton et al., (1999), described the prevalence of infection to vary according to the different age groups. The report commented that approximately 65-80% of cases are age related. Other reports suggest that CF patient groups may contract distinct strains such as Manchester epidemic strain (MES) and Liverpool epidemic strains (LES) (Cheng et al., 1996). A previous survey published in 2004 in the UK of CF clinics in England and Wales revealed LES was the most common CF clone with 11% of CF patients reportedly infected with the strain (Scott et al., 2004).

More severe chronic lung infection in CF patients is caused by mucoid P. aeruginosa. This species produces numerous toxins and virulence factors which are known to enhance colonisation and persistence in the respiratory tract forming biofilms (Aaron et al., 2000). Virulence factors of P. aeruginosa include elastase, phospholipase C, exotoxin A, exoenzyme S, rhamnolipids. Phenazine pigment known as pyocyanin and pyoverdine are also secreted by P. aeruginosa. Additionally, flagella, pili and lipopolysaccharide are frequently identified during biofilm development of P. aeruginosa (Wagner et al., 2008; Lyczak et al., 2000).

1.4.3 Staphylococcus aureus

Staphylococcus aureus is a gram-positive facultative anaerobic bacterium associated with the normal flora of the skin and nasal passage. S. aureus has multiple virulence factors
which contribute to severe disease progression. SCV's are often associated with persistent infection and higher antibiotic resistance levels (Beiser et al., 2007). *S. aureus* contains a quorum sensing system known as the *agr* system which regulates cell-surface proteins, exotoxins and hydrolytic enzymes (Chan et al., 2004). Under certain *in vitro* conditions the *agr* system may influence attachment and biofilm formation of *S. aureus* (Yarwood et al., 2004). The production of polysaccharide intracellular adhesion (PIA) is necessary for multi-layered cell cluster formation (Vuong et al., 2000). A recent finding by Boles et al., (2008), suggested the *agr* system regulates the conversion of planktonic to biofilm formation of *S. aureus*. Numerous hyper-mutable strains containing a defective DNA mismatch repair system have been isolated from CF patients. Studies have shown these strains are capable of adapting to certain selective pressures (Oliver et al., 2010; Prunier et al., 2003; Prunier et al., 2005).

**1.4.4 Haemophilus influenzae**

*Haemophilus influenzae* is another common bacterium associated with severe CF lung infection. Studies have shown that CF patients are infected during childhood with this bacterium and any inflammation and damage caused to the airways may enable *P. aeruginosa* to colonise the lungs more effectively (Smith et al., 1997).
1.4.5 Microbiome of the CF airways

The studies of polymicrobial infections have gained much interest among researchers in recent years since numerous microorganisms exist within the respiratory tract of CF patients (Kiska et al., 1996; Shelly et al., 2000; Saiman et al., 2003). Many authors have suggested that these infections can be problematic when selecting suitable treatments. The different species may have different growth rates and nutritional requirements. Recently studies on the CF airway microbiome have been conducted by several research groups to provide a detailed account of the pathogens within the airways. This allows a more detailed identification of CF pathogens that would otherwise not be noticed with standard laboratory culturing. Organisms are identified by 16S rRNA sequencing of sputum samples (Sibley et al., 2011). CF reports on paediatric patients have documented some of the highest levels of different bacterial species. Harris et al., (2007), found a total of 65 pathogens existed within a selected group of CF patients. Recently, Sibley et al., (2011), identified 2,015 strains using partial sequence of the 16S rRNA which was separated into 110 operational taxonomic units. The operational taxonomic units (OTU) were classified into 33 families from five phyla. Tunney et al., (2008), described the detection of high numbers of anaerobic species in sputum samples equal to or greater than _P. aeruginosa_ by molecular methods.

1.4.6 Polymicrobial infection

There are limited studies on the interactions between bacterial species commonly found in the CF lung. Recently, Allison et al., (2000), reported synergistic interactions in mixed species biofilms may form thicker and more stable structures than single species. Other
studies have described bacteriocins which are toxins produced by certain species may inhibit the growth of other species (Kreth et al., 2005). Soong et al., (2006), found that a synergistic relationship existed between P. aeruginosa and the influenza virus. The virus possesses neuroaminidase activity which could increase bacterial adherence post viral infection. Another study by Yang et al., (2011), found co-culturing S. aureus and P. aeruginosa resulted in the formation of colonies with different morphology to each monospecies and that P. aeruginosa was able to influence the growth of S. aureus in vitro.

1.4.7 Fungal infection

Fungal isolates are frequently recovered from respiratory secretions of CF patients (Mortensen et al., 2011; Paugam et al., 2011). Aspergillus fumigatus is the predominant fungal pathogen with a prevalence rate of approximately 60% (LiPuma et al., 2010), followed by Aspergillus terrus as the second most common species (Pihet et al., 2009). Colonisation of A. fumigatus generally results in allergic bronchopulmonary aspergillosis (ABPA) which is characterised by impaired mucociliary clearance, frequent bronchial obstruction and respiratory exacerbations. Generally, 10-15% of patients will develop ABPA which mainly occurs in older children and adults (Stevens et al., 2003). Spore germination and the release of antigens may be caused by excess mucus which could stimulate the T-helper cell type (Th) 2-biased responses leading to ABPA. Inflammation in the CF lungs as a result of A. fumigatus colonisation may be caused by the antibodies IgG and IgE. Patients may become infected with more than one strain, although single strains may become dominant over the course of infection (Cimon et al., 2001; deValk et al., 2009; deValk et al., 2005). Rarely, Aspergillus fumigatus may present as a fungal ball
known as an aspergilloma which can develop within the lung parenchyma and may be an indication of lung transplantation in CF patients (Zmeili et al., 2007; Ryan et al., 1995). Another form of pathology has been recognised as non-allergic fungal bronchitis. Denning et al., (1995), described fungal bronchitis as *Aspergillus* infection confined to the tracheobronchial tree in which there is evidence of bronchial inflammation and excess mucus production with *Aspergillus* as the only pathogen.

Few studies have reported on the frequency of resistance *Aspergillus* sp. to antifungal agents. A CF fungal study by Mortensen et al., (2011), found 4.5% of *A. fumigatus* isolates from CF patients were resistant to azoles, all of which were treated with azoles. Recently, Burgel et al., (2011), found 4.6% of *A. fumigatus* isolates recovered from one CF centre were resistant to itraconazole. Studies have found the TR/L98H resistance mechanism associated with *A. fumigatus*azole resistance is prevalent in CF patients from many countries including the United Kingdom, Denmark and the Netherlands (Snelders et al., 2008).

### 1.5 Physiology of *Pseudomonas aeruginosa* biofilms

Biofilms are communities of bacterial cells growing on a surface encased within an exopolysaccharide matrix. It has been estimated that approximately 65% of bacterial infections are biofilm related (Costerton et al., 2003), such as infections associated with cystic fibrosis patients, dental diseases, wounds and infections associated with indwelling
devices such as catheters, orthopedic devices and heart valves (Singh et al., 2003).

1.5.1 Motility of *P. aeruginosa* isolates

Three types of motility are associated with *P. aeruginosa*: swimming, swarming and twitching. Swarming motility involves the movement of cells across a hydrated, viscous, semi-solid surface while swimming motility involves movement across a low-viscous surface (Deziel et al., 2003; Kohler et al., 2000; Rashid et al., 2000). Twitching motility enables the formation of microcolonies and facilitates bacterial interactions, forming cell clusters and strengthening the attachment of cells to a surface (Sauer et al., 2002). Deligianni et al., (2010), observed isolates capable of twitching motility produce towering structures, while others are associated with the formation of thick lawns. Molecular studies by Caiazza et al., (2007), identified mutations in the *SadB* gene participate in inverse regulation of biofilm formation and motility. Flagellar motility is important for *P. aeruginosa* surface attachment while type-IV pili facilitate microcolony formation (Henrichsen et al., 1972).

1.5.2 Quorum sensing in *P. aeruginosa* biofilm

Quorum sensing (QS) is defined as the communication between bacteria which leads to the development of specific bacterial structures. *P. aeruginosa* contains two QS systems, *las* and *rhl* systems. The *las* system is comprised of the transcriptional activator *lasR* and an AI synthase enzyme *lasI* and the *rhl* system is comprised of the transcriptional activator *rhlR* and the AI synthase enzyme *RhlI* (Latifi et al., 1996; Pesci et al., 1999). Both of the systems are autoinducer synthases which catalyse the formation of
homoserine lactone (HSL) known as autoinducer N-(3-oxododecanoyl)-homoserine lactone (Pearson et al., 1994). Siderophore production and the formation of mature biofilms of \textit{P. aeruginosa} are dependent on the N-3-oxododecanoyl homoserine lactone (3OC\textsubscript{12}-HSL). Recent studies found both QS systems existed within the sputum of CF patients that had been colonised with \textit{P. aeruginosa} (Parsek et al., 2000; Singh et al., 2000; Davies et al., 1998). According to the study by Davies et al., (1998), the \textit{las} quorum sensing system enables biofilms to develop into towering-shaped structures. Whiteley et al., (1999), suggested approximately between 3 to 10\% of the \textit{P. aeruginosa} genome is controlled by the QS system.

The QS regulatory system of \textit{B. cepacia} relies on two different proteins, \textit{Cepl} which controls the synthesis of N-octanoylhomoserine lactone (C8-HSL) and N-hexanoylhomoserine lactone (C6-HSL) and \textit{cepR} which after binding with C8-HSL either activates or represses transcription to certain target genes (Eberl et al., 2001). Studies have documented that \textit{B. cepacia} strains defective in the \textit{cep} QS system develop biofilms that differ from the wild-type. In addition, the ability of such bacteria to swarm across an agar plate has been linked to the activity of this system (Allison & Hughes, 1991). Lewenza et al., (2001), found the \textit{cep} gene was an important regulator of protease production and repressor of the synthesis the siderosphore ornibactin.
1.5.3 *P. aeruginosa* biofilm formation

The biofilm formation process consists of a number of formation steps (O'Toole *et al.*, 2000; Sauer *et al.*, 2002).

These stages are listed below:

- Reversible attachment involves free-floating bacterial cells adhering to a surface.

- Irreversible attachment involves growth and aggregation of cells into microcolonies.

- Maturation involves biofilm growth into the typical mushroom and tower-shaped structures.

- Detachment involves dispersal of surface bacterial cells to new locations.

![Figure 1.4: Biofilm formation process depicting stages of reversible attachment, irreversible attachment, maturation and detachment (Costerton *et al.*, 2002).](image-url)
1.5.4 Reversible attachment

The first step of the biofilm formation process involves initial reversible attachment to a conditioning surface (Marshall et al., 1985; Boland et al., 2000). Attachment of the microorganisms occurs by specific interactions such as van der Waals forces, electrostatic and hydrophilic interactions or a combined effect of the different forces between the surface on which the cells and molecular groups that have adhered (Van Loo dsdrecht et al., 1990; Carpentier et al., 1993; Zottola and Sasahara, 1994; An et al., 2000). A small amount of EPS is produced which surrounds the biofilm (O'Toole et al., 1998). At this point the structure is not stable enough and is capable of dispersion into planktonic cells by external forces. Biofilm architecture is facilitated by the direction of cell growth which has been described as horizontal, vertical or a combination of both directions (Stoodley et al., 1999).

1.5.5 Irreversible attachment

The exopolysaccharide matrix facilitates the conversion from reversible to irreversible attachment by surrounding the individual cells, forming a community with unique physiological characteristics (Allison et al., 2000; Costerton et al., 1994; Costerton et al., 2001). Cells begin to distribute evenly forming multi-layered structures containing void sections through which fluid can flow (Costerton et al., 1994). Glucose, fructose, mannose, galactose, pyruvate and uronic acid residues of D-mannuronic and L-guluronic acid are present within the EPS matrix (Brisou et al., 1995; Franklin et al., 1993). EPS forms the bulk of biofilm architecture at 75 - 90% (Chang et al., 2007). Davies et al., (1993), found the expression of alginate genes may be critical to biofilm formation by *P.*
aeruginosa. Nivens et al., (2001), showed that O-acetylation of alginate was essential for biofilm maturation. The production of EPS may be required for initial structure composition, not just for attachment to abiotic surfaces (Danese et al., 2000; Prigent-Combaret et al., 2000).

1.5.6 Maturation

A recent study by Davey et al., (2003), indicated that the development of mature biofilm structures is mainly facilitated by quorum sensing. Bacterial motility which is caused by type-IV pili and flagella forces cells to become distributed (O’Toole et al., 1999; Klausen et al., 2003). Biofilms increase in thickness forming mushroom or pillar shaped structures containing an array of active and dormant cells with void spaces and channels (O’Toole et al., 2000; Sauer et al., 2002). Any voids that appear may decrease in size as the structures mature (Venugopolon et al., 2005). Quorum sensing influences the concentration of a diffusible autoinducer. This provides a regulatory signal in response to cell density which is essential for the formation of mature P. aeruginosa structures (Davies et al., 1998). The production of biosurfactants, known as rhamnolipids, by P. aeruginosa has been shown to maintain all channels surrounding the microcolonies (Davey et al., 2003). If the nutrient supply is limited, biofilms can expand or migrate in order to search for nutritional or carbon sources (Stoodley et al., 1999).

1.5.7 Detachment

When biofilms reach a particular mass, cells begin to detach from the outer layers of the matrix and re-colonise at new locations (Korber et al., 1989; Sternberg et al., 1999). The
sole purpose of biofilm detachment is to prevent starvation of the cells (Rice et al., 2005).

Cells located within the interior of the biofilm structure have less access to nutrients and studies have suggested a large volume of metabolic waste can accumulate within this area (Sternberg et al., 1999). Boyd et al., (1995), and his group described a less polymerized exopolysaccharide forms a less dense matrix, facilitating the detachment of cells. Additional studies have shown that alginate lysae; a degradative enzyme cleaves the polymer matrix into short oligosaccharides increasing the detachment process (Boyd et al., 1995). Research by Sauer et al., (2002), and Hoiby et al., (2002), reported that cells removed from the biofilm may retain some of the same mechanisms of antimicrobial resistance as attached cells.

1.5.8 Formation of non-surface attached microcolonies

During the biofilm development process bacteria may develop as microcolonies that are not attached to any particular surface. These structures may differ from biofilms that attach to surfaces enclosed within a mucous membrane. Recent clinical studies have found samples recovered from infectious sites in the human body contain microorganisms that do not directly attach to the site of colonisation. Biofilm clusters of chronic wounds that harbour *P. aeruginosa* tend to be larger and have a longer healing process than other strains (Alhede et al., 2011; Gjødsbol et al., 2006). Barclay et al., (1996), described detachment of bacterial cells as an important strategy if species are to survive in a stressful environment.

Previously, Costerton et al., (1987), described the microcolonies of different species may encase within their own unique exopolysaccharide matrix. The larger microcolonies
which are surrounded by a matrix are more protected to a stressful environment (Morris et al., 1997). Limited studies have compared phenotypic characteristics of bacteria that are not attached to the typical biofilm structures that adhere to a surface. Recently, Alhede et al., (2011), reported non-attached bacterial aggregates have been associated with infections in the chronic CF lungs, otitis media and wounds.

1.5.9 Antimicrobial resistance of biofilms.

According to Costerton et al., (2001), there are several mechanisms which are responsible for biofilm resistance to antimicrobial agents listed below:

- Reduced penetration of antimicrobial agents through the EPS matrix.
- Growth rates of biofilm-forming isolates are different for each strain.
- Changes to the physical biofilm structure due to different growth conditions in comparison to planktonic cells.

1.5.10 Reduced penetration of the antimicrobial agent through the EPS matrix.

Previous studies have reported that *P. aeruginosa* exopolysaccharide matrix can slow the diffusion of antibiotics into the biofilm. Many antibiotics are capable of diffusing through the EPS matrix of the biofilm but may lose their efficacy during this process. These processes can enhance resistance to low concentrations of aminoglycoside antibiotics, such as tobramycin (Brooun et al., 2000). Wild-type strains may be able to reduce the efficacy of antibiotics at the outer layers of the biofilms more rapidly than the rate at
which they diffuse into the biofilm matrix (Kumon et al., 1994; Shigeta et al., 1997).

1.5.11 Different growth rates of biofilm-forming isolates.

Cells growing within a biofilm undergo phenotypic changes which are associated with surface attachment and antibiotic resistance. Multidrug resistant pumps (MDR) are thought to play a role in the resistance of *P. aeruginosa* to antimicrobial agents. The four efflux pumps of *P. aeruginosa* are MexAB-oprM, MexXY-oprM, MexCD-oprJ and MexEF-oprNIO (Poole et al., 2001). DeKievit et al., (2001), indicated that two MDR efflux pumps of *P. aeruginosa*, MexAB-OprM and MexCD-OprJ are heterogeneous throughout the biofilm population, with greater expression occurring in cells located at the substratum. According to Allison et al., (2000), *P. aeruginosa* strains that do not have the MexAB-OprM efflux system are more resistant to fluoroquinolones such as ciprofloxacin.

1.5.12 Changes to the biofilm structure due to an altered mode of growth.

Bacteria within biofilms may employ specific mechanisms to resist the activity of antimicrobial agents. *P. aeruginosa* strains that produce rugose small colony variants (RSCV) demonstrated resistance to kanamycin, amikacin, carbenicillin, gentamicin, tobramycin and tetracycline (Drenkard et al., 2002). Zhang et al., (1996), reported that accumulation of acidic waste products might lead to pH differences between the bulk fluid and biofilm interior which could antagonise the activity of antibiotics, particularly against the aminoglycoside agents. Tuomanen et al., (1986), mentioned accumulation of agents by some organisms might cause them to enter a non-growing state in which they
are protected from antimicrobial agents. The study by Tack et al., (1985), revealed that aminoglycosides are less active against the same strain in anaerobic rather than aerobic conditions.

1.5.13 Persister cells

Studies have documented that a group of cells known as ‘persisters’ is responsible for *P. aeruginosa* high tolerance to antimicrobial agents (Lewis and Speoring, 2001). Persisters are phenotypic variants of wild-type cells that produce a similar amount of persister cells upon re-inoculation (Keren et al., 2004). They were first described by Joseph Bigger in 1944 that discovered penicillin could not inhibit a culture of *staphylococcus* (Bigger et al., 1944). Lewis et al., (2001), proposed that persister cells ensure the survival of a cell population in the presence of other inhibitory agents. The gene expression profile of persister cells of *E. coli* showed that their formation was caused by chromosomally encoded toxin-antitoxin molecules. Overexpression of toxins *ReIE* and *HipA* increases the production of multidrug tolerant persister cells to aminoglycosides such as tobramycin and kanamycin (Keren et al., 2004).

1.6 Biofilm formation in different model systems.

Biofilms are dynamic and organised communities of cells and can form in static or flowing systems (Costerton and Stewart, 2001). The literature supports the use of numerous models with different biofilm forming abilities. The flowing system enables the internal structure of biofilm to adapt to the flow velocity at which they grow. This
alters the rate of the internal mass transfer and microbial activity (Singh et al., 2003; Beyenal and Lewandowski, 2002; Goodman and Geesey, 2001). The flow cell system for studying biofilms allows individual cells to be monitored over time (Singh et al., 2002) but does not allow an array of studies to be conducted at any one time. More rapid approaches for the study of biofilms include the microtitre plate assay (Moskowitz et al., 2004) and the ‘colony biofilm assay’. The ‘colony biofilm assay’ involves a high density of bacteria applied as a spot onto an agar plate which allows communities of cells to develop on polymer membranes (Anderl et al., 2000).

1.6.1 Calgary Biofilm Device

The Calgary Biofilm Device (CBD) consists of a 96-well plate and peg lid on which biofilms are grown. The pegs are designed to sit in the reaction vessel and the bottom of the channel which allows medium to flow across the pegs and create a shear force. The device is placed onto a shaker table and after a sufficient amount of time cells adhere to the pegs. Biofilms that are formed can be treated with various antimicrobial agents to determine the susceptibilities of different strains (Ceri et al., 1999).

![Figure 1.5](image-url)  
**Figure 1.5:** Calgary Biofilm Device (CBD) for biofilm susceptibility testing. (A) The tilted table creates shear force for biofilm formation. (B) The lid containing pegs is designed so that it fits into the tray. (C) The polystyrene plate contains 96 pins for biofilms to form (Ceri et al., 1999).
1.6.2 Modified Robbins Device (MRD)

*In vitro* biofilms can be formed under shear force on diverse substrata under controlled flow conditions by the modified Robbins Device. This device operates by pumping inoculums through a channel in which removable studs are set against a lumen surface. The studs where the biofilm develops, as a result of shear force, are able to be removed and studied (Stoodley *et al.*, 1999; Nickel *et al.*, 1985). Unlike other *in vitro* model systems simultaneous biofilm formation of different microbial species can be conducted on different media and substrata. The discs can be removed from the MRD and various antimicrobials can be tested for antibiofilm activity (Coenye *et al.*, 2007).

1.6.3 Colony biofilms

The colony biofilm assay involves growing bacteria on the top of polymer membranes that have been placed onto TSA agar plates. Any colonies that develop are transferred to fresh TSA agar plates and monitored for 24 hours. This method allows penetration of antimicrobial agents to be measured form all sections of the biofilm (Anderl *et al.*, 2000). Transmission electron microscopy (TEM) results by Walters *et al.*, (2002), have shown that bacteria can grow in an evenly distributed manner throughout the colony.
1.6.4 Flow cell system

More advanced bacterial culture systems are emerging for studying the stages of biofilm development in a non-destructive way (Allegrucci et al., 2006). The studies involving flow cells frequently found in the literature allow biofilms to be studied under controlled conditions and enable direct observation using a microscope. Continuous flow provides a steady supply of nutritional requirements, enhancing the expression of various genes for greater biofilm growth (Sauer et al., 2004). The concentration of nutrients supplied has been reported to influence the architecture of developing biofilms (Stoodley et al., 1999). Typically anaerobic species are analysed with this model but studies have extended to aerobic bacterial cultures (O'Sullivan et al., 2009). The system enables biofilms to develop under hydrodynamic conditions. Visual analysis of the biofilms which have developed may be carried out without stopping flow which could disturb biofilm growth (Manz et al., 1999).

Figure 1.6: 1. (a) Schematic diagram of the flow cell system. (b) The upper glass of the flow cell on which biofilms are grown can be viewed using a microscope (Drake et al., 2002). 2. Confocal image of a gfp-tagged P. aeruginosa biofilm grown in this system for 6 days (Banin et al., 2006).
1.7 Novel agents to control *P. aeruginosa* biofilms

1.7.1 Lactoferrin

Lactoferrin is an iron-binding protein of the innate immune system, found on mucosal surfaces (Harbitz *et al*., 1984). Recent findings by Ammons *et al*., (2009), demonstrated the importance of lactoferrin in blocking biofilm formation of *P. aeruginosa*. Other studies have shown that lactoferrin displays bacteriostatic activity through the ability to sequester iron (Bullen *et al*., 1972). Additionally, the agent is able to bind to lipopolysaccharides and disrupt bacterial membranes (Ellison *et al*., 1994; Leitch *et al*., 1999). Synergistic interactions of lactoferrin with other agents such as xylitol, a rare alcohol sugar commonly found in fruit and vegetables, have also been reported against *P. aeruginosa* biofilms. As single agents, lactoferrin and xylitol weakened the biofilm structure of *P. aeruginosa*, however, biofilms were almost completely disrupted with both agents in combination (Ammons *et al*., 2009). Another study by Caraher *et al*., (2007b), found human lactoferrin increased the susceptibility of Bcc biofilms to many antibiotics. Overall, the studies mentioned suggest iron chelators may be useful for disrupting bacterial biofilms in the CF lung.

1.7.2 Gallium nitrate

Singh *et al*., (2007), on studying the effect of transition metal gallium nitrate against *P. aeruginosa* indicated that organisms are unable to distinguish between iron and gallium. The availability of iron enhances virulence, promotes biofilm formation and enhances the cell cluster development and maturation of biofilms into three-dimensional structures. In
addition, Singh et al., (2007), suggested gallium may prevent *P. aeruginosa* biofilm formation when applied to sites of high lactoferrin by enhancing the activity of lactoferrin. Another possible suggestion may involve a direct relationship between iron acquisition genes and pathogenesis indicating low levels of iron in the CF lungs is essential for *P. aeruginosa* virulence. The *PvdS* gene which controls pyoverdine production is regulated in a low iron environment and the *PvdS* protein is synthesised only under conditions of reduced iron availability (Singh et al., 2007). Additionally, Musk et al., (2005), found ferric ammonium compounds (FAC) inhibited *P. aeruginosa* biofilm formation in a dose-dependent manner indicating that *P. aeruginosa* biofilm formation can be influenced by the concentration of iron.

**1.7.3 Ethylenediaminetetraacetic acid (EDTA)**

The most specific mechanism of action of EDTA against gram-negative bacteria involves the disruption of the lipopolysaccharide structure in the outer membrane which leads to a more permeable membrane (Marvin et al., 1989). As a result, many researchers have explored the interactions of EDTA with other antimicrobial agents. The study by Lambert et al., (2004), reported a possible avenue of combined treatment with EDTA and ampicillin and other antipseudomonal antibiotics for the treatment of patients chronically colonised with *P. aeruginosa*. Recent findings by Al-Bakri et al., (2009), described synergistic interactions of aspirin and metal chelator, EDTA involved altering the mechanisms involved in membrane permeability, virulence factor production and a reduction in extracellular polysaccharide and prostaglandin production.
1.7.4 Aspirin

Farber et al., (1992), demonstrated salicylic acid (SA), a major metabolite of acetylsalicylic acid, inhibited adherence, growth and biofilm production of S. epidermidis. Steptanovic et al., (2004), explored the activity of aspirin against Candida species biofilm formation revealing a significant decrease in biofilm growth at concentrations ≥ 1.73 mM. Anti-adhesion properties of aspirin were reported by Demirag et al., (2007), when the agent was used to treat graft infections caused by coagulase-negative staphylococcus. Other studies found salicylic acid was involved in down-regulation of several virulence factors of P. aeruginosa (Prithiviraj et al., 2005).

1.7.5 Heparin

Researchers have explored the ability of anticoagulants to disrupt biofilms since most antibiotics do not possess this mode of action. Gotz et al., (2002), described heparin to alter the processes that involve attachment and adhesion for biofilm formation. In combination with teicoplanin and ciprofloxacin, heparin displayed strong antibiofilm activity against P. aeruginosa and S. aureus. A study by Heilmann et al., (1996), indicated heparin enhances detachment of biofilms that produce biofilm matrix polysaccharide poly-N-acetylglucosamine/polysaccharide intercellular adhesion of S. aureus, S. epidermidis, S. lugdunensis and S. carnosus.

1.7.6 Natural products

Natural extracts derived from plants have provided medicinal properties for treating infectious diseases for thousands of years. A number of recent studies have investigated
the activity of plant extracts on specific pathogens. Rasmussen et al., (2005), demonstrated extracts of garlic inhibit quorum sensing and polymorphonucleotide activation (PMA) in *P. aeruginosa* and reduce antibiotic resistance to tobramycin. The active component of garlic, allicin is effective against many gram-negative pathogens (Ankri et al., 1999). In other studies, ginseng has shown to prevent *P. aeruginosa* biofilm formation (Hoiby et al., 2011), by reducing signalling of quorum sensing systems (Song et al., 2010). Ginseng has shown to stimulate and develop the TH1 immune response allowing clearance of severe infection (Song et al., 1997).
1.8 Project Aims and Objectives:

1) To phenotypically characterise clinical *P. aeruginosa* isolates from the cystic fibrosis lung.

2) To determine the effects of antibiotics singly and in combination against a range of clinical CF and reference strains.

3) To assess the ability of novel metal-based agents to inhibit established *P. aeruginosa* biofilms. To determine the effects of selected synthesised compounds against biofilms grown in the flow cell system.

4) To investigate the growth and development of *P. aeruginosa* biofilms in a mixed culture with other identified CF pathogens and to explore any interactions between these species. To analyse the protein profiles of CF strains that may be interfering with the growth of *P. aeruginosa* with the aim of identifying proteins that may be contributing to virulence during co-infection.
Chapter 2: Characterisation and Antibiotic Susceptibility testing of P. aeruginosa strains from patients with cystic fibrosis.

2.1 Introduction

P. aeruginosa biofilms are difficult to eradicate with standard antibiotic therapy and isolates recovered from CF patients undergo mutations that are likely to become highly antibiotic resistant (Ogle et al., 1988). Antibiotic susceptibility testing allows rapid identification of the susceptibility profile of certain microorganisms using standardised laboratory methods (Saha et al., 2001). The most common way of assessing the activity of antimicrobial agents is to determine the minimum inhibitory concentration (MIC) and synergism between antimicrobial combinations is determined by the fractional inhibitory concentration index (FICI) (Horrevorts et al., 1987).

Biofilm quantification may be analysed using a variety of different techniques. Moskowitz et al., (2004), developed the biofilm inhibitory concentration (BIC) assay based on the assay by Ceri et al., (1999), for a more rapid and reliable means of screening various antimicrobial agents against biofilms. This technique allows biofilms to develop by attaching to the surfaces of polystyrene pegs. Masten et al., (1981), described the importance of this in vitro model in a CF clinical setting as it acts as an initial guide when selecting suitable concentrations of antibiotics for CF patients, since many CF pathogens are able to form biofilms.

Previous studies have suggested results obtained from in vitro models do not correlate to
the outcome of infection in an *in vivo* environment (Perdreau-Remington et al., 1998). According to the study by Saiman *et al.*, (1999), there has been no record of the most accurate *in vitro* model for antibiotic susceptibility testing of CF species. Aaron *et al.*, (2002), described biofilms formed on polystyrene pegs as less mature than the typical multi-layered biofilm structures. Burton *et al.*, (2007), noted that following crystal violet staining, the dye may interact with negatively charged cells and does not reflect the total amount of cells that are viable and non-viable. Other studies that have quantified viable bacteria by removing cells from the surfaces of the pegs suggest there is a possibility not all cells are dislodged effectively after biofilm disruption (Aaron *et al.*, 2002).

The use of *in vivo* models as a means to investigate virulence of complex bacterial species and their response to treatment has been used by many researchers. Nematodes such as *Galleria mellonella* and mice and insects such as Drosophila have all been employed as alternative models to determine the virulence of bacterial species (Rambaugh *et al.*, 1999). *G. mellonella* has been described as more accurate than other infection models (Seed *et al.*, 2008), and shares similar structural and functional homology to the innate immune systems of mammals (Hoffmann *et al.*, 1995). Phenotypic characteristics of isolates have been shown to play an important role in the rate and severity of disease progression and response to antimicrobial agents within these models (Oliver *et al.*, 2000).

The aim of this chapter was to determine phenotypic characteristics of *P. aeruginosa* clinical CF and reference strains using *in vitro* and *in vivo* models. This section also explored the activity of single and combinations of antibiotics against *P. aeruginosa*.
planktonic and biofilm forming isolates.

2.2 Materials and Methods

2.2.1 Research Ethics.

Ethical approval was not required for the research carried out in this section.

2.2.2 CF sputum samples.

Previously, an Irish CF clinical study was undertaken from 2004 – 2005. Sputum samples were collected from patients who were chronically colonised with \( P. \) aeruginosa from 12 different Irish CF clinics. All strains were stored in glycerol beads at -80°C for further study.

2.2.3 Preparation of Sputasol.

An equal volume of Sputasol (v/v) (Oxoid, Basingstoke UK) was added to the sputum samples. A quantity of 7.5 mls of sterile distilled water was added to 7.5 mls Sputasol and added to 92.5 mls sterile water. All prepared reagents can be used for up to 48 hours.

2.2.4 Preparation of sputum cultures.

Prepared sputum samples were agitated gently for approximately 10 seconds and incubated for 15 minutes at 35°C with gentle agitation to assist homogenisation. A quantity of 10 μl of the homogenised sputum was added to 5 mls sterile distilled water and 1 μl of this dilution was inoculated onto Columbia blood agar (CBA), centrimide agar, \( Burkholderia cepacia \) selective agar (BCSA) and McConkey agar (Fannin, Ireland).
Plates were incubated for 24 - 48 hours at 35°C and total viable counts (TVC) were calculated. Aliquots of homogenised sputum samples were added to glycerol and stored at -80°C. Any bacterial strains that were gram-negative, mucoid or produced a blue/green pigment on Pseudomonas selective agar were identified as \textit{P. aeruginosa}. Any strains that could not be identified were analysed using the Vitek 2 system (bioMerieux) in the microbiology laboratory, AMNCH.

\textbf{2.2.5 Selection of \textit{Pseudomonas aeruginosa} strains.}

All clinical isolates of \textit{P. aeruginosa} used in this study were obtained from 12 different Irish cystic fibrosis clinics and screened in Adelaide and Meath Hospital Incorporating the National Children’s Hospital (AMNCH), Tallaght hospital. All strains were typed using pulsed-field gel electrophoresis (PFGE) in the CF reference laboratory in AMNCH. \textit{P. aeruginosa} strains that represented dominant clones and several clones that were not dominant among patients with cystic fibrosis from the different clinics were used for further study. \textit{P. aeruginosa} ATCC27853 and \textit{P. aeruginosa} PA01 were used as reference strains. All strains were added to glycerol and stored at -80°C. When conducting experimental procedures, strains were grown on Tryptone Soy Agar (TSA) plates and inoculated in nutrient broth for 24 hours at 35°C.
Sources of *P. aeruginosa* strains used in this study.

**P. aeruginosa common clones.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> 1826</td>
<td>CF lung (Irish CF clinic 1)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4276.3</td>
<td>CF lung (Irish CF clinic 2)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 81599</td>
<td>CF lung (Irish CF clinic 4)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1438</td>
<td>CF lung (Irish CF clinic 5)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 5605</td>
<td>CF lung (Irish CF clinic 3)</td>
</tr>
</tbody>
</table>

**P. aeruginosa uncommon clones.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> A5Q5.2</td>
<td>CF lung (Irish CF clinic 2)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4649</td>
<td>CF lung (Irish CF clinic 2)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 5714</td>
<td>CF lung (Irish CF clinic 3)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 88716</td>
<td>CF lung (Irish CF clinic 4)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 6279</td>
<td>CF lung (Irish CF clinic 6)</td>
</tr>
</tbody>
</table>

**Reference strains.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> ATCC27853</td>
<td>Reference strain</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>Reference strain</td>
</tr>
</tbody>
</table>
2.2.6 Antibiotics.

The antibiotics used were tobramycin, ceftazidime, meropenem, ciprofloxacin and colistin and were obtained from the pharmacy at AMNCH. Stock solutions were prepared for tobramycin (40 mg/ml), ceftazidime (50 mg/ml) and meropenem (50 mg/ml) in sterile water and stored at -80°C. A stock solution of colistin (20 mg/ml) was prepared in sterile water and stored at -20°C. Ciprofloxacin solution (2 mg/ml) was stored at room temperature. All reagents were filter sterilised through 0.45 μM pore filters (Fisher Scientific) before use. The final concentration of each antibiotic added to the media was 256 μg/ml. This was the final concentration used for all experimental procedures.

2.2.7 Growth media.

Media was purchased from Oxoid (Basingstoke, UK), or prepared according to the manufacturer’s instructions and sterilised at 121°C for 15 minutes.

2.2.8 Plankonic growth of P. aeruginosa strains.

Overnight cultures of P. aeruginosa clinical and reference strains were prepared in nutrient broth and incubated at 35°C. Bacterial growth was analysed by plating a range of serial dilutions on TSA and determining the CFU/ml at regular intervals of 0, 1, 2, 4, 6, 24, 48, and 72 hours prior to incubation.

2.2.9 Epsilometer test (E-test).

A 0.5 McFarland (1 x 10^8 CFU/ml) standard was prepared for each P. aeruginosa isolate in sterile saline solution. A lawn was created on Muller-Hinton agar (MHA) plates by
swabbing the culture in two directions and left to dry for several minutes. Using a sterile forceps E-test strips (AB BioDisc, Sweden) containing tobramycin (TM), ceftazidime (TZ), meropenem (MP) and ciprofloxacin (CIP) were placed on the surface of the agar with the lowest concentration at the centre of the plate. The strip was pressed gently onto the agar plate so that no air bubbles were formed. Plates were incubated at 35°C for 24 hours. E-test MIC (µg/ml) values were read where the edge of the inhibition ellipse intersects the strip. Antibiotic susceptibility testing of colistin was carried out using the Vitek 2 system, microbiology laboratory at AMNCH.

2.2.10 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination.

MIC values of antibiotics were determined by the microtitre method as described in the Clinical Laboratory Standards Institute (CLSI) guidelines. A quantity of 100 µl of sterile nutrient broth was added to the wells of a 96-well round-bottom microtitre plate (Nunc Inc., Roskilde, Denmark). Stock solutions of antibiotics were prepared in nutrient broth starting at a final concentration of 256 µg/ml. A quantity of 100 µl of this solution was added to first well of the 96-well plate and serially diluted. A quantity of 100 µl of the suspension of the organism was added to each well at a final concentration of 5 x 10⁵ CFU/ml. The microtitre plates were sealed and incubated at 35°C for 24 hours. After 24 hours incubation, the MIC was determined as the lowest concentration of antibiotic showing no visible bacterial growth.
2.2.11 Optimisation of the biofilm quantification assay.

Biofilm quantification was assessed using four different disruption methods to select a suitable assay for testing biofilm biomass following antimicrobial treatment. An overnight culture of *P. aeruginosa* PA01 was suspended in nutrient broth at a final concentration of $5 \times 10^5$ CFU/ml. The wells of a round-bottom polystyrene 96-well plate were inoculated with 100 µl of this inoculum. A transferable solid phase (TSP Screening, Nunc, Inc., Roskilde, Denmark) pin lid containing pegs was added to the plate for biofilms to develop and incubated at 35°C for 24 hours. The first assay involved sonication (UW, Ultrawave) of the plate for 5 minutes to dislodge the cells from the pegs, followed by incubation at 35°C for an additional 6 hours. The second assay involved no sonication and immediate incubation of the plate at 35°C for 6 hours. The third assay involved crystal violet staining of the pegs for 30 minutes followed by 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) exposure for an additional 15 minutes. The final assay involved crystal violet staining of the pegs for 30 minutes followed by exposure to a 1:1 mixture of 1% Triton X-100 (Sigma-Aldrich) and 95% ethanol (Sigma-Aldrich) for 15 minutes. The OD$_{630}$ was measured in a microplate reader (ELx800, Universal Microplate Reader) after each procedure. All assays were carried out in triplicate on three separate occasions.

2.2.12 Biofilm growth determination.

To assess the ability of *P. aeruginosa* isolates used in this study to form biofilms, strains were grown on TSP pin lids as described in Section 2.2.11 and incubated at 35°C for 24 and 72 hours. The TSP pin lids containing pegs were washed three times in sterile water
and allowed to dry at room temperature. The pegs were stained with 0.3% crystal violet, CV (Bener Limited, Ireland) for 30 minutes. Lids containing pegs were washed three times again to remove any dye that did not bind to the biofilm cells. Biofilms that were stained with crystal violet were removed with a (1:1 ratio) solution of 95% ethanol and 1% Triton X-100. The OD<sub>630</sub> was measured in a microplate reader. Biofilm growth was recorded at an OD<sub>630</sub> of ≥ 0.05 (Moskowitz et al., 2004). All experiments were carried out in triplicate on three separate occasions.

2.2.13 Determination of virulence of selected <i>P. aeruginosa</i> isolates in the in vivo <i>G. mellonella</i> model.

Sixth instar larvae of <i>G. mellonella</i> (Greater wax moth) were obtained from Live Foods Direct (Sheffield, England) and stored in wood shavings in the dark at 15°C. Larvae weighing between 0.2 and 0.4g were selected for all experiments. An overnight culture of <i>P. aeruginosa</i> was prepared in nutrient broth at 35°C. <i>P. aeruginosa</i> was grown to late exponential phase, diluted to a final concentration of 1 x 10<sup>8</sup> CFU/ml in nutrient broth and a series of dilutions were prepared Ten larvae were inoculated with 20 µl of each dilution through the last right pro-leg into the haemocoel using a Hamilton syringe (Sigma-Aldrich). Larvae were placed on 9 cm petri dishes containing Whatman filter paper and incubated at 35°C for 48 hours. All experiments included a control which consisted of larvae injected with 20 µl sterile saline. Larval survival was determined at 24 hour intervals by movement in response to touch. All experiments were conducted in triplicate.
2.2.14 Biofilm Inhibitory Concentration (BIC) determination.

Isolates were grown as biofilms as described by Moskowitz et al., (2004), adapted from the Calgary biofilm device to determine the susceptibility of P. aeruginosa strains to antibiotics singly and in combination. An overnight culture of P. aeruginosa was prepared in nutrient broth. The isolate was suspended in 3 mls of nutrient broth to a 0.5 McFarland standard and adjusted to a concentration of 5 x 10^5 CFU/ml. A quantity of 100 μl of this suspension was added to the wells of a 96-well round-bottom plate. A TSP pin lid was placed into the microtitre plate and incubated overnight at 35°C. The TSP pin lid was removed and rinsed three times in sterile water to remove any planktonic cells. Serial two-fold dilutions of antibiotics were prepared and added to a new 96-well plate to which the peg lid was transferred and incubated for 24 hours at 35°C. A quantity of 100 μl of crystal violet was added to each well of a 96-well plate. The lid containing pegs was rinsed three times again, dried at room temperature and added to the crystal violet for a further 30 minutes. To remove the stained biofilm, the lid was placed into a new 96-well round-bottom microtitre plate containing a (1:1 ratio) solution of 95% ethanol and 1% Triton X-100. The OD_{630} was measured in a microplate reader. The biofilm inhibitory concentration was determined as the last well that had an OD_{630} > 0.05. All experiments were conducted in triplicate on three separate occasions.

2.2.15 Fractional Inhibitory Concentration (FIC).

To determine the interactions that occurred between antibiotic combinations the Fractional Inhibitory Concentration (FIC) was calculated: (≤ 0.5 is synergistic, > 0.5 to ≤ 1 is additive, > 1 to ≤ 4 is indifferent and > 4 is antagonistic). This method is frequently
used to determine the activity of interactions between antimicrobial interactions.

2.2.16 Effect of antibiotic combinations on viability of *P. aeruginosa* biofilms.

An overnight culture of each isolate was diluted to an $\text{OD}_{630} = 0.1$ in nutrient broth. A quantity of 100 $\mu$l of this culture was added to each well of a 6-well plate containing 10 mls nutrient broth and incubated at 35°C for 24 hours, 72 hours or 7 days. After each time point the media was removed and changed to media containing concentrations corresponding to the MIC, 5 x MIC, 10 x MIC and 20 x MIC of antibiotics. Calculations of antibiotic concentrations are listed in Appendix 4. Biofilms were treated for a further 24 hours at 35°C. Following antibiotic treatment, all contents of the wells were removed and transferred to centrifuge tubes. Cells were centrifuged (Sigma, SK-15) at 3,000 g for 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 g, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonications and vortexing (Stuart Scientific Autovortex, SA5) until the cells were dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

2.2.17 Statistical analysis.

Statistical analysis was carried out using PASW$^R$ Statistics 18 - SPSS. One-way ANOVA or Student's t-test was used to determine statistical variations for most experiments. A $P \leq 0.05$ value was considered statistically significant.
2.3 Results

2.3.1 Diversity of *P. aeruginosa* isolates grown planktonically.

To compare the growth of CF isolates that were common clones among CF patients, log CFU/ml counts were determined at specific time points. Both reference and clinical isolates showed similar growth curves as seen in Figure 2.1. *P. aeruginosa* ATCC27853 and *P. aeruginosa* PA01 showed a loss of viability at 72 hours compared to the other strains. The other isolates (*P. aeruginosa* 1826 and *P. aeruginosa* 4276.3) had a longer stationary phase and maintaining growth until 72 hours. The level of cell density reached is important when determining the response of these strains to antimicrobial treatment.
Figure 2.1: Differences in planktonic growth curves of selected *P. aeruginosa* isolates. Total viable counts were measured at regular intervals, every 2 hours for 6 hours with final readings at 24, 48 and 72 hours. Graph represents the mean ± standard deviation of two independent experiments.

**2.3.2 Diversity of *P. aeruginosa* isolates grown as biofilms.**

Biofilm formation was quantified by (a) the ability to adhere to polystyrene pegs in 96-well plates quantified by crystal violet straining and (b) viable counts following 24 and 72 hours incubation. The majority of *P. aeruginosa* clinical and reference strains showed a progressive increase in biofilm forming capacity from 24 to 72 hours on the pegs. The growth rate of *P. aeruginosa* 4649 was comparable at 24 and 72 hours. Certain strains (*P. aeruginosa* ATCC27853, *P. aeruginosa* 1826, *P. aeruginosa* 4505.2) consistently formed strong biofilm particularly at the 72 hour time point. Biofilm growth of *P. aeruginosa* 4505.2 on polystyrene pegs was significantly higher than the other strains at 24 hours. In
addition, this strain produced significantly more biofilm than ten out of twelve strains at 72 hours (one-way ANOVA), Figure 2.3 (A). Interestingly, the two models showed variation in the amount of biofilm formed by each strain. A total of eight strains were characterised as strong biofilm formers on polystyrene pegs at 24 hours (OD$_{630} > 0.05$) with an increase in growth at 72 hours. Four clinical CF strains (P. aeruginosa 81599, 1438, 5605 and 6279) which exhibited a low level of adherence to the pegs had high biofilm cell counts at 24 and 72 hours. The results suggest that P. aeruginosa strains are capable of producing biofilms of high cell density at 24 and 72 hours even if the level of adherence to the pegs may be weak. Overall, biofilm formation is a common characteristic of CF P. aeruginosa strains which may be influenced by quorum sensing systems in the biofilm matrix (Davies et al., 1998).

Figure 2.2: 24 hour biofilms of P. aeruginosa clinical CF strains grown on polystyrene pegs. Reference strains (P. aeruginosa PA01 and P. aeruginosa ATCC27853) are in columns 2 and 3, representing positive controls. Columns 4-6 represent clinical CF isolates. (A) Biofilm cells that adhered to the pegs were stained with crystal violet. (B) Crystal violet was removed from the pegs with 95% ethanol and 1% Triton X-100.
Table 2.1: Phenotypic characteristics of *P. aeruginosa* common clones, uncommon clones and reference strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment</th>
<th>Mucoid/ Non-mucoid</th>
<th>Biofilm former</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC27853</td>
<td>pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><strong>P. aeruginosa common clones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1826</td>
<td>pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4276.3</td>
<td>no pigment</td>
<td>mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1438</td>
<td>no pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 81599</td>
<td>no pigment</td>
<td>mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 5605</td>
<td>pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><strong>P. aeruginosa uncommon clones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4505.2</td>
<td>pigment</td>
<td>mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4649</td>
<td>pigment</td>
<td>mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 5714</td>
<td>pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 88716</td>
<td>pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 6279</td>
<td>no pigment</td>
<td>non-mucoid</td>
<td>biofilm former</td>
</tr>
</tbody>
</table>
Figure 2.3: Biofilm formation of *P. aeruginosa* strains. Measurements of biomass were based on (A) OD₆₃₀ of growth on polystyrene pegs and (B) log CFU/ml of viable biofilm cells. At 24 and 72 hours biofilm biomass was quantified for each *P. aeruginosa* strain. Data represent the mean ± standard deviation of three independent experiments.
2.3.3 Characterisation of virulence of *P. aeruginosa* isolates in the *in vivo* *G. mellonella* model.

It was interesting to determine if *P. aeruginosa* strains that form biofilms are more pathogenic in an *in vivo* environment. **Figure 2.4** illustrates *in vivo* virulence of larvae inoculated with the individual *P. aeruginosa* strains used in this study. Larvae were injected with increasing concentrations of *P. aeruginosa* (1 x 10^1 – 1 x 10^5) and assessed for viability at 24 and 48 hours as described in **Section 2.2.13**. Control larvae were injected with 20 μl sterile saline. Results indicate that the degree of virulence was higher when larvae were inoculated with pigment producing isolates *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853 and *P. aeruginosa* 1826 at 24 and 48 hours. Percentage larval survival following inoculation with clinical isolate *P. aeruginosa* 4276.3 was higher than the other strains. This strain does not secrete any redox-active pigments but does form biofilm of high cell density at 24 hours. Larvae inoculated with higher concentrations of 1 x 10^3 – 1 x 10^5 CFU/ml of clinical CF strains *P. aeruginosa* 5605 and 81599 were more viable than the other inoculated larvae even though a pigment was secreted by *P. aeruginosa* 5605. In addition, the degree of virulence appeared to be less severe during infection with clinical isolate *P. aeruginosa* 1438 which forms a weak biofilm within 48 hours and secretes no pigment, 100% larval survival was recorded at 24 and 48 hours post inoculation. Pigments which are virulence factors produced by *P. aeruginosa* may be important virulence factors influencing larval death.
(B) *P. aeruginosa* ATCC27853

% larval survival

\[ \begin{array}{c|c|c|c|c|c|c|c|c|c|c} \hline \text{CFU/larva} & 0 & 10 & 20 & 30 & 40 & 50 & 60 & 70 & 80 & 90 & 100 \\ \hline 10^5 & & & & & & & & & & & \\ 10^4 & & & & & & & & & & & \\ 10^3 & & & & & & & & & & & \\ 10^2 & & & & & & & & & & & \\ 10^1 & & & & & & & & & & & \\ \hline \end{array} \]

(A) *P. aeruginosa* PA01

% larval survival

\[ \begin{array}{c|c|c|c|c|c|c|c|c|c|c} \hline \text{CFU/larva} & 0 & 10 & 20 & 30 & 40 & 50 & 60 & 70 & 80 & 90 & 100 \\ \hline 10^5 & & & & & & & & & & & \\ 10^4 & & & & & & & & & & & \\ 10^3 & & & & & & & & & & & \\ 10^2 & & & & & & & & & & & \\ 10^1 & & & & & & & & & & & \\ \hline \end{array} \]
P. aeruginosa 4276.3

% larval survival

10^1
10^2
10^3
10^4
10^5

CFU/larva

10^1
10^2
10^3
10^4
10^5

CFU/larva

48h
24h

P. aeruginosa 1826

% larval survival

10^1
10^2
10^3
10^4
10^5

CFU/larva

48h
24h
Figure 2.4: Evaluation of virulence of selected reference and CF *P. aeruginosa* strains at different concentrations. Percentage larval survival was recorded at 24 and 48 hours post-infection. Control larvae injected with sterile saline demonstrated 100% survival at 24 and 48 hrs. Larvae were injected with (A) *P. aeruginosa* PA01, (B) *P. aeruginosa* ATCC27853, (C) *P. aeruginosa* 1826, (D) *P. aeruginosa* 4276.3, (E) *P. aeruginosa* 81599, (F) *P. aeruginosa* 5605, (G) *P. aeruginosa* 1438 and incubated at 35°C. Larvae infected with concentrations ≥ 10^3 CFU/ml of *P. aeruginosa* strains (PA01, ATCC27853, 1826) demonstrated significantly reduced viability compared to the other strains (one-way ANOVA). Data represent the mean ± standard deviation of three experiments.
Figure 2.5: *P. aeruginosa* pathogenesis in *G. mellonella* larvae relative to the control. 

(A) Control larvae were injected with PBS which did not affect larval survival. (B) Larvae inoculated with *P. aeruginosa* demonstrated melanisation 24 hours, 35°C post-infection.

2.3.4 Optimisation of biofilm growth quantification.

An initial assay was carried out as demonstrated in Figure 2.6 which illustrates the effect of four different disruption assays against biofilms grown on polystyrene pegs. *P. aeruginosa* PA01 was used as a control organism that forms a biofilm. Findings showed that exposure of the polystyrene pegs to a mixture of 1% Triton X-100 and 95% ethanol at a 1:1 ratio gave the highest absorbance readings indicating that greatest amount of cells were dislodged by this method. This increase in the rate of biomass recovered was found to be significant compared to the other disruption methods (P < 0.001). Average absorbance readings showed that this assay has good reproducible values. Similarly, sonication for 5 minutes was effective in dislodging cells from the pegs. A recent study by Moskowitz *et al.*, (2004), found sonication for a shorter time as more effective than
centrifuging for biofilm cells. A 1:1 ratio of 1% sodium dodecyl sulfate (SDS) and 95% ethanol gave the weakest absorbance range indicating the two agents are not acting effectively as a combination in altering the subsequent attachment of these cells. Absorbance readings of the assay involving no sonication after biofilm growth was higher than that of 1% SDS and 95% ethanol treatment, suggesting this biofilm model has many limitations.

![Figure 2.6: The effect of four different disruption methods against biofilms grown on polystyrene pegs. A solution of 1% Triton X-100 and 95% ethanol was the most effective disruption method against pre-grown biofilms. Fewer biofilm cells were dislodged with the solution of 1% SDS and 95% ethanol. Data represents the mean ± standard deviation of three independent experiments.](image-url)
Table 2.2: E-test (MIC mg/L) results of anti-pseudomonal antibiotics against \( P.\) aeruginosa.

Results of antibiotic susceptibility determined by E-test and Vitek 2 (colistin only) against \( P.\) aeruginosa are summarised below in Table 2.2.

<table>
<thead>
<tr>
<th>( P.) aeruginosa strain</th>
<th>Tobramycin</th>
<th>Ceftazidime</th>
<th>Meropenem</th>
<th>Ciprofloxacin</th>
<th>Colistin</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P.) aeruginosa PA01</td>
<td>2</td>
<td>1.5</td>
<td>0.5</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa ATCC27853</td>
<td>1.5</td>
<td>1.5</td>
<td>0.5</td>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>( P.) aeruginosa 1826</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 4276.3</td>
<td>16</td>
<td>1.5</td>
<td>0.095</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 81599</td>
<td>8</td>
<td>&gt;32</td>
<td>1</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 1438</td>
<td>16</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 5605</td>
<td>2</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 88716</td>
<td>1</td>
<td>12</td>
<td>0.38</td>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>( P.) aeruginosa 5714</td>
<td>1</td>
<td>12</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 4649</td>
<td>1</td>
<td>&gt;32</td>
<td>0.125</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>( P.) aeruginosa 4505.2</td>
<td>4</td>
<td>0.5</td>
<td>0.38</td>
<td>0.38</td>
<td>2</td>
</tr>
<tr>
<td>( P.) aeruginosa 6279</td>
<td>16</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>
2.3.5 Summary of the number of *P. aeruginosa* strains displaying sensitivity to each antibiotic according to the CLSI standards.

E-test results were defined as sensitive or resistant according to the Clinical Laboratory and Standards Institute (CLSI) as shown in Table 2.3 with the exception of colistin in which the susceptibility profiles were analysed with the Vitek 2 system. All *P. aeruginosa* strains displayed sensitivity to colistin. A total of 9/12 strains were sensitive to ciprofloxacin and 1/12 was resistant. The susceptibility profile of meropenem was also high with 9/12 strains displaying sensitivity and 3/12 resistance. Tobramycin was also effective against a large (8/12) number of strains. Finally, an equal number of strains (5/12) displayed susceptibility and resistance to ceftazidime.

**Table 2.3:** Number of antibiotics for which *P. aeruginosa* strains were sensitive (S), intermediate (I) and resistant (R) (*n* = 12).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>CLSI Breakpoints (MIC µg/ml)</th>
<th>Susceptible <em>P. aeruginosa</em> strains</th>
<th>Intermediate <em>P. aeruginosa</em> strains</th>
<th>Resistant <em>P. aeruginosa</em> strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S  I R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>≤ 4</td>
<td>8</td>
<td>16</td>
<td>8/12</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≤ 8</td>
<td>16</td>
<td>≥ 32</td>
<td>5/12</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≤ 4</td>
<td>8</td>
<td>16</td>
<td>9/12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤ 1</td>
<td>2</td>
<td>≥ 4</td>
<td>9/12</td>
</tr>
<tr>
<td>Colistin</td>
<td>≤ 2</td>
<td>4</td>
<td>≥ 8</td>
<td>12/12</td>
</tr>
</tbody>
</table>
Figure 2.7: The susceptibilities of *P. aeruginosa* isolates to antibiotics by E-test on Mueller-Hinton Agar. (A) *P. aeruginosa* 1826, (B) *P. aeruginosa* 4276.3, (C) *P. aeruginosa* PA01 and (D) *P. aeruginosa* ATCC27853. Green agar signifies pigment production by three strains.
2.3.6 Antibiotic susceptibility testing of single antibiotics against *P. aeruginosa*.

Several *P. aeruginosa* isolates representing CF common clones (*P. aeruginosa* 1826, *P. aeruginosa* 4276.3), CF uncommon clones (*P. aeruginosa* 4505.2, *P. aeruginosa* 4649) and reference strains (*P. aeruginosa* PA01, *P. aeruginosa* ATCC27853) were selected for antibiotic susceptibility testing. Table 2.4 illustrates the susceptibilities of antibiotics tested singly against planktonic and biofilm grown isolates. Among the antibiotics selected ciprofloxacin was the most effective single agent against five out of the six *P. aeruginosa* isolates tested. Clinical isolate, *P. aeruginosa* 4276.3 displayed the greatest sensitivity to meropenem indicating antibiotics may be displaying unique effects against the different strains. Polymyxin antibiotic, colistin displayed the weakest activity against all isolates grown planktonically. For the majority of strains, concentrations required to inhibit biofilm-grown isolates were similar to those of planktonic cells. The MIC and BIC values of *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were similar following exposure to ceftazidime. Ceftazidime treated cells exhibited lower MIC values than BIC values against the other *P. aeruginosa* strains indicating this agent can have reduced potency against certain biofilm-forming *P. aeruginosa* isolates. Previous studies have shown that less mature biofilms can have the same antibiotic susceptibility values as planktonic cells (Peeters *et al.*, 2009). Meropenem exhibited weak antibiofilm activity against three isolates, *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853 and *P. aeruginosa* 4505.2 in comparison to planktonic cells. Similarly, tobramycin displayed weaker activity against two isolates, *P. aeruginosa* 4276.3 and *P. aeruginosa* 4505.2, grown as biofilms than planktonic cells.
Table 2.4: Minimum inhibitory concentration (MIC μg/ml) and Biofilm inhibitory concentration (BIC μg/ml) of single antibiotics against *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4276.3</th>
<th><em>P. aeruginosa</em> 4505.2</th>
<th><em>P. aeruginosa</em> 4649</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.25</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>4</td>
<td>128</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>128</td>
</tr>
<tr>
<td>Meropenem</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.0625</td>
<td>0.0625</td>
<td>0.0625</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Colistin</td>
<td>64</td>
<td>64</td>
<td>16</td>
<td>128</td>
<td>32</td>
<td>128</td>
</tr>
</tbody>
</table>
2.3.7 Susceptibility testing of antibiotic combinations.

All *P. aeruginosa* isolates were grown planktonically and as biofilms and treated with a selection of frequently administered antibiotics, alone and in combination. The chosen antibiotics such as ceftazidime, meropenem and colistin were examined in combination with tobramycin as it is one of the main antibiotics of choice for CF patients infected with *P. aeruginosa*. Ciprofloxacin was also tested in combination with these antibiotics as it displayed the greatest anti-pseudomonal activity against the majority of CF isolates as a single agent.

Table 2.6 shows the fractional inhibitory concentration (FIC) indices which determined the interactions that occurred between antibiotic combinations. Results revealed that combinations tobramycin-ceftazidime and tobramycin-meropenem displayed synergistic activity at an FIC ≤ 0.5 against the biofilm of *P. aeruginosa* 1826. Tobramycin-ceftazidime also exhibited synergistic interactions against *P. aeruginosa* 4505.2 at an FIC ≤ 0.25. Interestingly, these antibiotic combinations did not have a similar affect against the same strains grown as planktonic isolates, indicating that these interactions may be specifically targeting the development of biofilms at certain stages. The antibiotic combinations tobramycin-ceftazidime and tobramycin-meropenem did not display synergistic interactions against the reference strains in planktonic or biofilm form. Another interesting finding was observed with ciprofloxacin-colistin which displayed synergistic activity against *P. aeruginosa* 1826 grown planktonically but not against the biofilm. The mechanisms of these findings are currently unknown and warrant further study.
Four antibiotic combinations including tobramycin-meropenem, tobramycin-colistin, ciprofloxacin-ceftazidime and ciprofloxacin-meropenem, displayed antagonistic interactions against the biofilm of clinical isolate *P. aeruginosa* 4505.2 at FIC values of 275, 17, 257, and 8.5, respectively. In addition, two combinations, tobramycin-colistin (FIC ≥ 64.25) and ciprofloxacin-colistin (FIC ≥ 257) were antagonistic against the biofilm of *P. aeruginosa* 4649. These two isolates possess the mucoid phenotype which may be reducing the efficacy of these antibiotics. The remaining results showed that the majority of combinations tested were in the indifferent range. Ciprofloxacin which was an effective single agent displayed the same inhibitory activity in combination with other antipseudomonal antibiotics as ciprofloxacin monotherapy. The combination involving tobramycin-colistin displayed antagonistic interactions (FIC ≥ 4) against biofilm and planktonic cells of two reference strains *P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853. Antagonistic interactions of this combination were also observed against biofilm formation of three clinical strains, *P. aeruginosa* 1826, *P. aeruginosa* 4505.2, *P. aeruginosa* 4649 and planktonic growth of *P. aeruginosa* 4276.3.
Table 2.5: Susceptibility of *P. aeruginosa* planktonic (MIC μg/ml) and 24 hour biofilm-grown isolates (BIC μg/ml) to antibiotic combinations.

<table>
<thead>
<tr>
<th>Antibiotic Combinations</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4276.3</th>
<th><em>P. aeruginosa</em> 4505.2</th>
<th><em>P. aeruginosa</em> 4649</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
</tr>
<tr>
<td>Tobramycin-Ceftazidime</td>
<td>0.5</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tobramycin-Ciprofloxacin</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Tobramycin-Meropenem</td>
<td>1</td>
<td>2</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Tobramycin-Colistin</td>
<td>2</td>
<td>64</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin-Ceftazidime</td>
<td>0.125</td>
<td>0.125</td>
<td>0.0625</td>
<td>0.5</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Ciprofloxacin-Meropenem</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Ciprofloxacin-Colistin</td>
<td>0.125</td>
<td>0.125</td>
<td>0.0625</td>
<td>0.25</td>
<td>0.0625</td>
<td>0.125</td>
</tr>
</tbody>
</table>
Table 2.6: Fractional Inhibitory Concentration’s (FIC) of antibiotic combinations according to FIC Index (FICI).

<table>
<thead>
<tr>
<th>FIC of Antibiotic Combinations</th>
<th>(P.\ aeruginosa) PA01</th>
<th>(P.\ aeruginosa) ATCC27853</th>
<th>(P.\ aeruginosa) 1826</th>
<th>(P.\ aeruginosa) 4276.3</th>
<th>(P.\ aeruginosa) 4505.2</th>
<th>(P.\ aeruginosa) 4649</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
</tr>
<tr>
<td>Tobramycin-Ceftazidime</td>
<td>2</td>
<td>1</td>
<td>0.6</td>
<td>2.5</td>
<td>3</td>
<td>0.5*</td>
</tr>
<tr>
<td>Tobramycin-Ciprofloxacin</td>
<td>5</td>
<td>4</td>
<td>1.5</td>
<td>1.5</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>Tobramycin-Meropenem</td>
<td>1.4</td>
<td>2</td>
<td>0.6</td>
<td>1</td>
<td>0.6</td>
<td>0.5*</td>
</tr>
<tr>
<td>Tobramycin-Colistin</td>
<td>8</td>
<td>65</td>
<td>4</td>
<td>32</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Ciprofloxacin-Ceftazidime</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2.25</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Ciprofloxacin-Meropenem</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.06</td>
<td>1.06</td>
</tr>
<tr>
<td>Ciprofloxacin-Colistin</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.5*</td>
<td>1</td>
</tr>
</tbody>
</table>

* FIC value for synergy ≤ 0.5
2.3.8 Activity of effective antibiotic combinations against mature (72 hour) *P. aeruginosa* biofilms.

Mature *P. aeruginosa* biofilms were grown for 72 hours and exposed to effective antibiotic combinations, tobramycin-ceftazidime and tobramycin-meropenem. These combinations displayed synergistic interactions against particular biofilms grown for 24 hours. Two reference strains (*P. aeruginosa* PA01, *P. aeruginosa* ATCC27853) and two clinical CF strains (*P. aeruginosa* 1826, *P. aeruginosa* 4505.2) were selected for further study. Findings revealed that *P. aeruginosa* 1826 was synergistically inhibited with tobramycin-ceftazidime at an FIC ≤ 0.5. Tobramycin-meropenem showed indifference under the same experimental conditions, indicating antibiotic interactions can be influenced by the age of the biofilm. Similarly, tobramycin-ceftazidime which acted synergistically against clinical isolate *P. aeruginosa* 4505.2 after 24 hours growth did not display the same inhibitory effect against the older biofilm (FIC ≥ 2). Findings suggest that the activity of antibiotic combinations may be reduced against mature *P. aeruginosa* biofilms in comparison to the same strains at 24 hours.
Table 2.7: Susceptibility of mature *P. aeruginosa* biofilms (BIC μg/ml) grown for 72 hours and treated with effective single and double antibiotic combinations.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4505.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin</td>
<td>4</td>
<td>1</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tobramycin-Ceftazidime</td>
<td>4</td>
<td>1</td>
<td>64</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tobramycin-Meropenem</td>
<td>4</td>
<td>2</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

Table 2.8: Fractional Inhibitory Concentration (FIC) of the most effective antibiotic combinations against mature (72 hour) biofilms.

<table>
<thead>
<tr>
<th>Antibiotic Combination</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4505.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin-Ceftazidime</td>
<td>1.03</td>
<td>1</td>
<td>0.5*</td>
<td>2</td>
</tr>
<tr>
<td>Tobramycin-Meropenem</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* FIC value for synergy ≤ 0.5
2.3.9 The effect of tobramycin, ceftazidime, tobramycin-ceftazidime against *P. aeruginosa* biofilms.

Figure 2.8 shows survival of 24 hour *P. aeruginosa* biofilms grown on polystyrene pegs following treatment with effective single (tobramycin, ceftazidime) and combination antibiotics (tobramycin-ceftazidime) at varying concentrations. At concentrations > 64 µg/ml the activities of tobramycin and tobramycin-ceftazidime against *P. aeruginosa* PA01 were similar indicating that tobramycin is the most effective single agent. In contrast, this strain displayed resistance to concentrations > 8 µg/ml of ceftazidime. The activity of tobramycin-ceftazidime matched the activity of tobramycin against *P. aeruginosa* ATCC27853. Clinical isolates *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were also more sensitive to concentrations > 64 µg/ml of tobramycin and tobramycin-ceftazidime than ceftazidime. The activity of single antibiotics showed *P. aeruginosa* 1826 was more resistant to concentrations > 0.5 µg/ml of ceftazidime whereas *P. aeruginosa* 4276.3 was more resistant to concentrations ranging between 8 - 32 µg/ml of tobramycin.
Figure 2.8: Optical density (630nm) of *P. aeruginosa* biofilms (A) *P. aeruginosa* PA01, (B) *P. aeruginosa* ATCC27853, (C) *P. aeruginosa* 1826, (D) *P. aeruginosa* 4276.3 treated with tobramycin, ceftazidime and tobramycin-ceftazidime at varying
concentrations (μg/ml). Results represent the mean ± standard deviation of three independent experiments.

2.3.10 The effect of biofilm maturation on the efficacy of antibiotic treatment.

The activity of combined antibiotic treatment of tobramycin and ceftazidime was evaluated against one reference strain (*P. aeruginosa* PA01) and one clinical strain (*P. aeruginosa* 1826). The concentrations selected were the MIC, 5 x MIC, 10 x MIC, 20 x MIC based on results obtained by the broth microdilution method. The activity of antibiotic combinations was determined based on age of the biofilm and concentration of each agent. The interaction of tobramycin-ceftazidime against *P. aeruginosa* PA01 did not show any significant difference compared to each antibiotic administered alone. On the other hand, at a concentration of 10 x MIC biofilms growth for 72 hours and treated with tobramycin-ceftazidime was significantly reduced (P = 0.008) in comparison to each single agent. The activity of the antibiotic combination against seven day old biofilms of *P. aeruginosa* PA01 showed no significant difference to each single agent as shown in Figure 2.9.

**Figure 2.10** shows greater survival of clinical strain *P. aeruginosa* 1826 at 72 hours in comparison to 24 hours following treatment with the same antibiotics as shown in **Figure 2.9**. *P. aeruginosa* 1826 grown for 24 hours was eradicated with 20 x MIC of tobramycin, indicating high concentrations of particular antibiotics may effectively inhibit less mature biofilm structures. The activity of the tobramycin-ceftazidime revealed a resemblance to the activity of either tobramycin or ceftazidime used singly. The comparisons of each
single and double antibiotic treatment showed no significant difference at any particular time or concentration. The results also revealed that 7 day old biofilm biomass of *P. aeruginosa* PA01 and *P. aeruginosa* 1826 was similar to the less mature biofilm structures suggesting optimum growth may be reached before 7 days with this *in vitro* model.

(A)

![Graph showing antibiotic concentrations and bacterial counts](image)
Figure 2.9: Reduction in biofilm biomass (*P. aeruginosa* PA01) following antibiotic treatment. *P. aeruginosa* PA01 grown for (A) 24 hours, (B) 72 hours and (C) 7 days and challenged with antibiotics at final concentrations of MIC, 5 x MIC, 10 x MIC and 20 x MIC. Viability of the biofilm was recorded following 24 hours treatment.
Figure 2.10: Reduction in biofilm biomass (*P. aeruginosa* 1826) following antibiotic treatment. *P. aeruginosa* 1826 was grown for (A) 24 hours, (B) 72 hours and (C) 7 days and treated with effective antibiotics at final concentrations of MIC, 5 x MIC, 10 x MIC and 20 x MIC. Viability of the biofilm was recorded following 24 hours treatment.
2.4 Discussion

The analysis of clinical isolates described in this chapter was achieved by recovering *P. aeruginosa* from CF sputum samples. A selection of *P. aeruginosa* isolates from the different Irish CF clinics displayed diversity in terms of phenotypic characteristics and biofilm formation. All clinical CF *P. aeruginosa* isolates which included the 5 identified common clones and the 5 uncommon clones had different phenotypic characteristics. Four out of twelve isolates grew as mucoid colonies. The mucoid phenotype identified generally relates to a more rapid impairment in lung function (Parad et al., 1999). Several of the CF strains such as *P. aeruginosa* strains 81599, 5605 and 1438 grew as small white colonies on TSA agar plates. Lam et al., (1980), found *P. aeruginosa* small colony variants (SCV’s) were highly adherent and commonly associated with biofilm formation in the CF lung. SCV’s are often associated with greater biofilm biomass than other strains and increased resistance to antibiotics (Drenkard et al., 2002). The morphological diversity of CF *P. aeruginosa* strains has been documented extensively and may be due to mutations that occur when strains are adapting to a heterogeneous environment (Rainey et al., 1998). Another difference observed was the blue-green pigment produced by eight out of twelve *P. aeruginosa* strains on agar plates. These minor phenotypic differences may be accounting for survival and adaptation by each individual strain.

Biofilm growth of reference and clinical CF strains was examined using two different *in vitro* models. The data demonstrated a progressive increase in growth from 24 to 72
hours for the majority of *P. aeruginosa* isolates. The study also found the biofilm density reached by several *P. aeruginosa* strains differed with each model. Four clinical CF isolates *P. aeruginosa* 81599, *P. aeruginosa* 1438, *P. aeruginosa* 5605 and *P. aeruginosa* 6279 which appeared as weakly adherent on polystyrene pegs grew biofilms of high cell density as determined by viable counts following 72 hours incubation. Results are in accordance with previous studies that have suggested certain species may not colonise the surfaces of pegs but may be depositing on the bottom of the microtitre wells (Smith *et al.*, 2008). Other studies have suggested microcolonies may not be attaching to any surface but may display similar characteristics to adherent bacteria (Alhede *et al.*, 2011). According to Oliver *et al.*, (2000), environmental factors may alter adaptation and survival and influence phenotypic properties of CF isolates. Houry *et al.*, (2009), indicated motility may play a role in biofilm formation through which cells need to find suitable places for biofilms to settle. Heidelberg *et al.*, (2002), suggested the type IV-pili of *P. aeruginosa* may be involved in the attachment of bacteria to various abiotic surfaces. O’Toole and Kohler, (1998), suggested flagella may also promote attachment and that mutant strains may exhibit limited biofilm formation compared to wild-type strains. Findings indicate that several *in vitro* models should be used to assess biofilm development of different *P. aeruginosa* strains.

*P. aeruginosa* mutants that produce a reduced level of pyocyanin may be less virulent in various animal models than wild-type strains (Hendrickson *et al.*, 2001). Rambaugh *et al.*, (1999), linked quorum sensing to the production of virulence factors and increased levels of mortality and morbidity under *in vivo* conditions. The study described the
inactivation of the las QS system caused a reduction in pathogenicity of CF bacteria in the *G. mellonella* model. Results from the present study demonstrated *G. mellonella* infected with *P. aeruginosa* expressed different rates of survival depending on concentration and each individual strain. A number of strains, *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853 and *P. aeruginosa* 1826, which secreted pigments, lead to a greater killing effect than the other strains. Recently, Jander *et al.*, (2000), described pyocyanin as a main virulence factor in *G. mellonella* infected with *P. aeruginosa*. The toxic effects of pyocyanin have been characterised of which contribute to inactivation of vacuolar ATPase of lung epithelial cells and apoptosis of neutrophils (Hasset *et al.*, 2003; Usher *et al.*, 2002). Although *P. aeruginosa* 5605 secretes a pigment, this strain was less virulent in the *in vivo* model suggesting other exoproducts produced by *P. aeruginosa* may be influencing larval mortality. The exact mechanisms that are influencing differences in larval survival with these strains remain inconclusive.

Virulence factor production which is controlled by quorum sensing systems of *P. aeruginosa* strains may also influence the rate at which antimicrobial agents affect the biofilm structure (Pearson *et al.*, 1994). Antibiotic susceptibility testing enables the selection of suitable agents for treatment of CF bacterial infections (Aaron *et al.*, 2002). Findings from the current study indicate the clinical CF isolates develop a unique response to antibiotic treatment. *P. aeruginosa* biofilms were more resistant to antibiotics than planktonic cells. These results correspond to the study by Aaron *et al.*, (2002), which found antibiotics singly or in combination were less active against the strains grown as biofilms than planktonic cells. Recently, Yu *et al.*, (2012), found all *P. aeruginosa*
isolates that were tolerant to antibiotics such as aztreonam were non-mucoid. Mutants defective in the psl gene which is responsible for EPS component synthesis exhibited an increase in resistance to the antibiotic. Findings from the current study indicate that synergistic interactions of antibiotics against *P. aeruginosa* do not rely on whether the isolate is mucoid or non-mucoid.

Ciprofloxacin was identified as a more effective single agent than tobramycin against planktonic and biofilm cells. Previously, susceptibility testing against biofilms found fluoroquinolones such as ofloxacin and ciprofloxacin penetrate biofilm structures more effectively. Aminoglycosides such as tobramycin penetrate the biofilm structure at a slower rate than ciprofloxacin which may be allowing bacteria to acquire adaptive stress responses (Shigeta *et al.*, 1997; Yasuda *et al.*, 1993; Whiteley *et al.*, 2001). The exopolysaccharide matrix can reduce the rate of antibiotic diffusion and aminoglycosides have been reported to stimulate alginate production and increase *P. aeruginosa* biofilm biomass (Hogdes and Gordon, 1991; Bagge *et al.*, 2004). In addition, Stewart *et al.*, (1996), suggested the efficacy of antimicrobial agents may be reduced once adsorbed through the exopolysaccharide matrix. The multi-drug resistance pump MexAB-OprM expressed in *P. aeruginosa* is accountable for much of the intrinsic antimicrobial resistance of this organism (Li *et al.*, 1995).

This section found the MIC and BIC values of colistin were higher in planktonic and biofilm form in comparison to the other anti-pseudomonal antibiotics. Resistance to colistin treatment has been linked to the two signal transduction systems, *phoPQ* and
*pmrAB* which regulate the expression of the OprH gene. It has been suggested that the OprH gene increases membrane permeability of biofilms thereby generating a higher rate of resistance (MacFarlane *et al.*, 1999; Bell *et al.*, 1991). Haagensen *et al.*, (2006), observed *P. aeruginosa* was more resistant to colistin treatment during the early stages of biofilm formation. The study showed the effect of colistin against *P. aeruginosa* biofilms was not influenced by the age or maturation stage of the biofilm and the mechanisms are incompletely understood.

The effect of antibiotic combination therapy was investigated against *P. aeruginosa* planktonic cells and biofilms. Single and combination antibiotic therapy observed higher BIC values for 72 hour *P. aeruginosa* biofilms treated with ceftazidime and meropenem, indicating reduced activity of these agents against mature biofilms. Increased production of exopolysaccharide matrix may slow the penetration of antibiotics through the biofilm structure (Stewart *et al.*, 1996). Another observation involving the antibiotic combinations examined, found tobramycin-ceftazidime and tobramycin-meropenem displayed synergistic interactions against biofilm growth of clinical isolate *P. aeruginosa* 1826 and tobramycin-ceftazidime acted synergistically against clinical isolate *P. aeruginosa* 4505.2. Additionally, tobramycin-ceftazidime displayed synergistic interactions against the 72 hour biofilm of clinical isolate *P. aeruginosa* 1826 but not *P. aeruginosa* 4505.2 while tobramycin-meropenem had reduced activity against all isolates at this time point. Tobramycin belongs to the aminoglycoside group of antibiotics and ceftazidime belongs to the cephalosporin antibiotics. Antibiotics that have different modes of action have been shown to be more effective at eliminating CF pathogens and
synergy of antibiotics is not a novel finding. The exact mechanisms that are causing synergistic interactions warrant further investigation. Recently, the study by Chaudhary et al., (2009), found a patient group with lower respiratory tract infection treated with tobramycin-ceftazidime experienced a better clinical outcome that the group receiving ceftazidime monotherapy. Findings from the present study indicate this combination of antibiotics may be effective for inhibiting *P. aeruginosa* biofilms, however, changes that occur during biofilm maturation may reduce the efficacy of this treatment. Kuman et al., (1995), described certain antibiotics may display synergistic interactions against biofilms during the different stages of the biofilm developmental cycle. Hermann et al., (2010), revealed the combination of colistin and tobramycin was more effective *in vitro* than each single agent in which different areas of the biofilm structure were targeted by each agent. Combined antibiotic treatment of colistin and ciprofloxacin allows biofilm cells to be treated during the different stages of metabolic activity (Pamp et al., 2008). In addition, Skindersoe et al., (2008), found biofilms treated with azithromycin and ceftazidime caused alterations in membrane permeability and signal molecule uptake by interfering with quorum sensing.

Total viable counts were obtained for biofilms treated with tobramycin, ceftazidime and tobramycin-ceftazidime as this combination of antibiotics displayed synergistic activity against biofilms grown on polystyrene pegs. *P. aeruginosa* biofilms were grown for different lengths of time to determine the response of young and mature biofilms to this treatment. Recently, Tre-Hardy et al., (2009), described a biofilm grown for 12 days as more resistant to antibiotic treatment than a biofilm grown for 24 hours. In addition,
Anwar et al., (1989), reported a reduced effect of tobramycin against a 7 day biofilm in comparison to a 2 day biofilm. Consistent with these previous findings, the current study found antibiotic combinations were less effective against 72 hour biofilms than 24 hour biofilms. Synergistic activity was not reported in most cases with tobramycin-ceftazidime as was previously identified against biofilms formed on polystyrene pegs. These differences suggest that therapeutic approaches seem to rely on the age of the biofilm and concentration of antimicrobial agent administered. Inefficacy of antibiotics may be due to slow growth of biofilms rendering the strain more resistant to treatment (Dunne et al., 1993; Evans et al., 1987). Another possible explanation for the difference between the results obtained with different assays may be due to a less developed biofilm structure forming on polystyrene pegs making such strains more susceptible to treatment (Aaron et al., 2002). Recently, Gillis et al., (2009), observed azithromycin displays inhibitory activity against \textit{P. aeruginosa} PA01 during early biofilm development. Cells within the deeper layers of biofilms may be more resistant to certain agents that can effectively inhibit the outer areas (Costerton et al., 1999). Finally, phenotypic and genotypic differences have been identified during the biofilm development process which may be a major factor in the biofilm response to treatment (Ito et al., 2009).
Chapter 3: Antimicrobial activity of metal-based compounds against 

*P. aeruginosa* biofilms.

3.1 Introduction

The phenotypic and genotypic diversity among bacteria of the same species may influence the ability of bacterial communities to withstand environmental stresses (Tilman et al., 1994). Since many bacteria found in the CF lungs are able to form biofilms and develop resistance mechanisms to antibiotics (Cos et al., 2010), many alternative agents have been explored in the literature to suppress biofilm formation.

The use of metals as antimicrobial agents began in ancient times to treat a variety of infectious diseases. Many recent studies have described the use of silver based compounds for many medical applications. Silver is frequently used as a polymer or coating agent for indwelling medical devices such as heart valves and catheters to prevent the formation of biofilms (Dasgupta et al., 1999). Bjamsholt et al., (2007), found *P. aeruginosa* biofilms were inhibited by silver sulfazidime at wound infection sites. The study also found that concentrations required to kill biofilms can be 10-100 times the concentrations required to kill planktonic cells. It is reported that silver uptake by bacteria can cause damage to the cell membrane (Kim et al., 2007). One of the disadvantages of using silver as a treatment is argyia. This is caused by precipitates of silver within the dermis and leads to discolouration of the skin (Bouts et al., 1999).

Other metals such as copper and zinc which are required in trace amounts for bacterial
growth can be toxic to many species at high concentrations (Balsalobre et al., 2003).

Studies have found bacterial species may develop resistance following treatment with certain metals. Parsek et al., (2003), and his group reported that biofilms exhibit resistance to heavy metals such and copper and zinc. The cusA or cusCFBA systems of E. coli enhance the rate of resistance to silver and copper agents (Rensing et al., 2003). Resistance of bacteria to silver has rarely been reported in the literature. The study by Pervical et al., (2008), found wound isolates of P. aeruginosa and S. aureus did not display any resistance to silver.

Recent studies have found coumarin agents possess potent antimicrobial activity. Coumarins are benzopyrene derivatives which are found naturally in many plants and microorganisms and have many uses in pharmaceutical preparations such as antibacterials, antivirals, antioxidant, antiinflammatory and anticoagulants (Hoult et al., 1996; Kostova et al., 2007). The study by Rosselli et al., (2007), showed that coumarins exhibit antimicrobial activity by targeting bacterial nucleic acid synthesis. Coumarin antibiotics such as novobiocin and clorobiocin have potent activity against bacterial infections by inhibiting DNA gyrase (Maxwell et al., 1993). Flavonoids which contain the properties of coumarin agents are able to interact with many drugs through the inhibition of P-glycoprotein (Wang et al., 2007). Warfarin, a derivative of dicoumarol, can prevent tumour formation by reducing the ability of tumor cells to be retained in the pulmonary microvasculature (Mousa et al., 2002).

The aim of this chapter was to determine the effect of metal-based agents against P.
aeruginosa. The antibacterial activity of synthesised copper-benzotriazole and silver-coumarin compounds was explored. This section also studied the effect of gallium nitrate in combination with antibiotics against *P. aeruginosa* planktonic and biofilm cultures.
3.2 Materials and Methods

3.2.1. Bacterial strains and media.
Two reference strains *P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853 were used as controls. Two clinical CF isolates, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were also used for this study. An overnight culture of each isolate was diluted to OD$_{630}$ = 0.1 in nutrient broth. *P. aeruginosa* biofilms were grown for 24 and 72 hours at 35°C.

3.2.2 Media for flow cell biofilm growth.

**BTrace medium**
BTrace media was prepared by adding 10 ml 1M magnesium chloride (MgCl$_2$), 1 ml calcium chloride (CaCl$_2$) and 1 ml Trace metals solution which are required for *P. aeruginosa* biofilm growth in 9 L of sterile Milli-Q-water (Millipore Corporation).

**A-10**
A-10 containing 30g sodium chloride (NaCl), 20g ammonium sulphate, (NH$_4$)$_2$SO$_4$, 60g disodium hydrogen phosphate (Na$_2$HPO$_4$.2H$_2$O) and 30g monopotassium phosphate (KH$_2$PO$_4$) in 1 L sterile water adjusted to a pH 6.4 ± 0.1, acts as a buffer for biofilm cultivation.

BTrace media and A-10 were added together mixed for one minute before supplementing the medium with 10% glucose as a carbon source and mixing again. Media was
autoclaved at 121°C for 15 minutes before use. All chemicals used to prepare this media were purchased from Sigma-Aldrich.

3.2.3 Culture preparation for flow cell experiments.

For flow cell experiments bacterial cultures were streaked on Luria agar (LA) (Sigma-Aldrich) plates and incubated overnight. A single colony was incubated in 5 ml Luria broth (LB) (Sigma-Aldrich) at 35°C on an orbital shaker (200 rpm). The OD$_{630}$ = 0.1 was standardised in sterile 0.9% (w/v) sodium chloride solution.

3.2.4 Synthesis of silver-coumarin compounds.

Silver-coumarin compounds and metal-free coumarin ligands were synthesised by Dr. Bernie Creaven, Department of Chemistry, Institute of Technology, Tallaght, Dublin 24. All compounds and molecular weights are listed in the tables below.
Table 3.1: Molecular weights of synthesised metal-free coumarin ligands.

<table>
<thead>
<tr>
<th>Coumarin compound</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin 2</td>
<td>224.60</td>
</tr>
<tr>
<td>Coumarin 3</td>
<td>220.18</td>
</tr>
<tr>
<td>Coumarin 4</td>
<td>269.05</td>
</tr>
<tr>
<td>Coumarin 5</td>
<td>220.18</td>
</tr>
<tr>
<td>Coumarin 6</td>
<td>254.62</td>
</tr>
<tr>
<td>Coumarin 7</td>
<td>254.62</td>
</tr>
<tr>
<td>Coumarin 8</td>
<td>220.18</td>
</tr>
<tr>
<td>CCa (coumarin-3-carboxylic acid)</td>
<td>190.15</td>
</tr>
</tbody>
</table>
Table 3.2: Molecular weights of synthesised silver-coumarin compounds.

<table>
<thead>
<tr>
<th>Silver-coumarin compound</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag(6-OHCCa)</td>
<td>313.01</td>
</tr>
<tr>
<td>Ag(7-OHCCa)</td>
<td>313.01</td>
</tr>
<tr>
<td>Ag(8-OHCCa)</td>
<td>313.01</td>
</tr>
<tr>
<td>AgCCa</td>
<td>296.01</td>
</tr>
<tr>
<td>Compound 2</td>
<td>331</td>
</tr>
<tr>
<td>Compound 3</td>
<td>327.04</td>
</tr>
<tr>
<td>Compound 4</td>
<td>375.91</td>
</tr>
<tr>
<td>Compound 5</td>
<td>327.04</td>
</tr>
<tr>
<td>Compound 6</td>
<td>361.48</td>
</tr>
<tr>
<td>Compound 7</td>
<td>361.48</td>
</tr>
<tr>
<td>Compound 8</td>
<td>327.04</td>
</tr>
<tr>
<td>Compound 10</td>
<td>366</td>
</tr>
<tr>
<td>Silver nitrate (AgNO₃)</td>
<td>169.87</td>
</tr>
</tbody>
</table>
3.2.5 Synthesis of copper-benzotriazole compounds.

Copper-benzotriazole compounds were synthesised by Dr. Denis O’Shea, School of Food Science and Environmental Health, Dublin Institute of Technology (DIT), Cathal Brugha Street, Dublin 1. Copper sulphate (Sigma-Aldrich) and a benzotriazole (Sigma-Aldrich) were added to 50 cm$^3$ ethanol and the resulting clear solutions were refluxed for 3 hours. The reaction mixtures were filtered off, washed in ethanol and air-dried.

Table 3.3: Molecular weights of synthesised copper-benzotriazole compounds and metal-free benzotriazole ligands.

<table>
<thead>
<tr>
<th>Copper-benzotriazole compound</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(C$_7$H$_7$N$_3$)$_2$(SO$_4$)</td>
<td>425.62</td>
</tr>
<tr>
<td>Cu(C$_6$H$_5$N$_3$)$_2$(SO$_4$)</td>
<td>397.62</td>
</tr>
<tr>
<td>Cu(C$_7$H$_7$N$_3$O)$_2$(SO$_4$)</td>
<td>457.62</td>
</tr>
<tr>
<td>Copper sulphate (CuSO$_4$)</td>
<td>159.62</td>
</tr>
<tr>
<td>Benzotriazole</td>
<td>119.12</td>
</tr>
<tr>
<td>1H-Benzotriazole-1-methanol</td>
<td>149.15</td>
</tr>
<tr>
<td>5-Methyl-1H-benzotriazole</td>
<td>133.15</td>
</tr>
</tbody>
</table>

3.2.6 Gallium nitrate.

Gallium hydrate nitrate, Ga(NO$_3$)$_3$.xH$_2$O, was purchased from Sigma-Aldrich. The molecular weight is 255.73 g/mol. All stock solutions were prepared in sterile water.
3.2.7 Solubility testing and preparation of metal-based compounds for experimental procedures.

Stock solutions of 1%, 2.5%, 5% and 10% (v/v) DMSO (99% GC, Sigma-Aldrich) in 1 ml sterile water were prepared. Pre-grown *P. aeruginosa* biofilms were treated with each solution in order to select a concentration to dilute the compounds that would not affect bacterial growth. A 5% (v/v) DMSO solution was selected to dilute the compounds as it was the highest concentration that did not display any inhibitory activity against *P. aeruginosa*.

Stock solutions of silver-coumarin compounds, metal-free coumarin ligands, copper-benzotriazole compounds and metal-free benzotriazole ligands were prepared in a 5% DMSO solution with sterile water to form a uniform solution. Compounds 5 and 10 required heating and vigorous stirring to dissolve the solute. The metal-free coumarin ligands also required heating and vigorous stirring in 5% DMSO to form a uniform solution. Stock solutions of gallium nitrate and silver nitrate were prepared in sterile water as these agents were more readily soluble.

3.2.8 Effect of synthesised compounds against planktonic cultures of *P. aeruginosa*.

Serial dilutions of gallium nitrate and synthesised compounds were prepared and minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were carried out as described in Chapter 1, Section 2.2.12. To determine the MBC, the contents of non-turbid wells were sub-cultured by streaking 10 μl of suspension onto 5% Columbia sheep blood agar plates which were incubated overnight at
35°C and examined for complete inhibition the following day.

3.2.9 Effect of synthesised compounds against biofilms grown on polystyrene pegs.

An overnight culture of *P. aeruginosa* strains was prepared in nutrient broth. The isolates were suspended in 3 mls of nutrient broth to a 0.5 McFarland standard and adjusted to a concentration of $5 \times 10^5$ CFU/ml. A quantity of 100 µl of this suspension was added to the wells of a 96-well round-bottom plate. A transferable solid phase (TSP) pin lid was placed into the microtitre plate and incubated overnight at 35°C. The TSP pin lid was removed and rinsed three times in sterile water to remove planktonic cells. The lid was placed into another plate containing serial dilutions of all compounds starting at a final concentration of 512 µg/ml. After a further 24 hours at 35°C the peg lids were removed and rinsed three times with sterile water and dried at room temperature. The TSP lid was added to a 96-well plate containing 100 µl 0.3% crystal violet for a further 30 minutes. The TSP lid was rinsed again three times in sterile water to remove any crystal violet that did not bind to the biofilm cells. To remove crystal violet stained biofilms attached to the pegs the lid was placed into a new 96-well round-bottom microtitre plate containing a solution (1:1 ratio) of 95% ethanol and 1% Triton X-100. The OD$_{630}$ was measured in a microplate reader. The biofilm inhibitory concentration was determined as the last well that had an OD$_{630} \geq 0.05$ (Moskowitz *et al.*, 2004). All experiments were conducted in triplicate on three separate occasions.

3.2.10 Effect of synthesised compounds on viability of *P. aeruginosa* biofilms.

An overnight culture of *P. aeruginosa* was diluted to OD$_{630} = 0.1$ in LB. A quantity of 10
mls of BTrace minimal medium containing A-10 and 0.5% glucose was added to 6-well microtitre plates (Tissue culture testplate 6, Techno Plastic Products (TPP), Switzerland). A quantity of 100 μl of the culture was added to each well and incubated at 35°C for 72 hours. All of the media was removed from the wells using a 10 ml syringe without destroying clumps at the bottom of the wells. An additional 10 mls of BTrace (A-10 and 0.5% glucose) media containing 100 μl of the appropriate concentrations of silver-coumarin compounds were added to the wells and incubated for a further 24 hours at 35°C. All contents from the wells were transferred to centrifuge tubes. Cells were centrifuged for 3,000 g, 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 g, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexing until the cells were dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

3.2.11 Establishing the flow cell system.

3.2.12 Assembly of the flow cell system channels.

Assembly of the flow cell began by adding a thin layer of silicone (Bluestar Silicones) with a syringe between the flow cell channels, without touching the plexiglass. A glass coverslip was placed over the silicone and pressed firmly in order to cover the whole area of plexiglass. Any coverslips that cracked were removed with a scalpel and replaced again. Flow cells were left for 24 hours to allow the silicone to dry. Tubing (Marpene Tubing, Ole Dich Instrumentmakers Aps.) was connected to each end of the flow channels. Bubble traps were also connected to the flow channels. The 0.5 ml syringes
were mounted on the bubble traps with the inlet and outlet inside the bubble traps and closed with a stopper. Flasks were placed at each end of the system to collect the media. Flasks that were to collect effluent were placed at or above the level of the flow channels so that media will not run out and air will not enter into the system. The flow system was started by removing the stoppers from the bubble traps. The stoppers were replaced when the bubble traps began to overflow.

### 3.2.13 Sterilisation of the flow system.

A 13% sodium hypochloride (AppliChem) solution in 1L Milli-Q-water was prepared and allowed to flow through the system for 3-4 hours. When this had completed, 2L Milli-Q-water was added to the system for 2 hours to get rid of any remaining sodium hypochloride. Sterile tubes were connected to the flask containing BTrace minimal media which was allowed to flow through the system. When the bubble traps began to overflow they were capped so the media was able to flow through the flow cells. Flow rate was set with the 16-channel Watson Marlow pump (Watson-Marlow Flexicon A/S, Denmark) at 0.5 rpm at 35°C.

### 3.2.14 Cultivation of biofilms in the flow cell system.

A-10 and 10% glucose were added to BTrace minimal medium and mixed for 1 minute. Sterile tubes were placed into each flask containing media. Before inoculation, flow was stopped and all tubing between the flow cells and bubble traps was clamped. The flow channels were inoculated with *P. aeruginosa* by injecting 250 µl of the overnight culture diluted to OD$_{630}$ = 0.1 into each flow channel with a 0.5 ml syringe (Thermo Fisher
Scientific, Ireland). The syringe was inserted into the tubing and the hole was sealed with silicone. After inoculation, flow channels were inverted and left without flow for 1 hour, after which medium flow was resumed. Flow velocity was set at 1.75 rpm for all experiments. All bubbles were removed from the flow cell. Biofilms were allowed to develop for 72 hours prior treatment.

3.2.15 Exposure of biofilms to silver nitrate and silver-coumarin compounds.
After 72 hours incubation the flow was stopped and all tubing between the flow cells and bubble traps was clamped. Lids were removed from the bubble traps and flow was resumed at 90 rpm to empty all the bubble traps. The media was changed to medium containing 10% glucose and the appropriate concentrations corresponding to the MIC and
10 x MIC values of silver nitrate and selected silver-coumarin compounds. Concentrations of the agents used are listed in Appendix 5. All clamps were removed and flow was started again at 1.75 rpm. Biofilms were continuously exposed to the compounds for a further 24 hours.

3.2.16 Fluorescent staining.

Baclight Live-Dead stain (Invitrogen, Molecular Probes, 5 mM solution in DMSO) was added to the biofilm after 24 hours. An equal amount of Syto 9 and propidium iodide (PI), (1 µl) was added to 1 litre of sterile Milli-Q-water and covered with foil to avoid contact with light. A quantity of 250 µl was injected the opposite end of the flow cell, covered with foil and left for 15 minutes before viewing under the microscope. The red-fluorescing stain displayed non-viable cells and the green-fluorescing stain displayed cells that were viable.

3.2.17 Microscopy and image processing.

All imaging of P. aeruginosa treated and untreated biofilms was conducted using a Zeiss LSM510 confocal scanning laser microscopy (CSLM; Carl Zeiss, Germany), equipped with an argon-NeHe laser and detectors. The filters were set for simultaneous monitoring of Syto 9 and propidium iodide (PI). Images were obtained using a 63X/1.3 Plan-Neofluar oil immersion objective. Cross sectional analysis through the biofilm was generated by IMARIS (Bitplane AG) software.
3.2.18 Statistical analysis.

Statistical analysis was carried out using PASW® Statistics 18 - SPSS. One-way ANOVA was used to determine statistical variations for most experiments. A $P \leq 0.05$ value was considered statistically significant.

3.3 Results

3.3.1 Effect of copper sulphate/benzotriazole compounds on *P. aeruginosa* planktonic and biofilm formation.

The sensitivity of *P. aeruginosa* to synthesised copper-benzotriazole compounds was assessed by carrying out MIC, MBC and BIC assays. Interestingly, the synthesised compounds did not exhibit any inhibitory effects against *P. aeruginosa* grown planktonically. Copper sulphate displayed antibacterial activity at a high concentration of 3.2 mM against three out of four strains which included two reference strains and one clinical strain. Similarly, compounds displayed weak antimicrobial activity against *P. aeruginosa* biofilm-grown isolates in which results were comparable to the copper sulphate control. The metal-free benzotriazole ligand 5-methyl-1H-benzotriazole exhibited antibiofilm activity against two clinical isolates *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 at a concentration of 1.9 mM. The other ligands benzotriazole and benzotriazole-1H methanol displayed similar biofilm inhibitory activity against *P. aeruginosa* 4276.3 at high concentrations of 2.1 mM and 1.7 mM, respectively. Findings from this study suggest that the metal-free ligands are disrupting *P. aeruginosa* biofilms established on polystyrene pegs and warrant further study.
Table 3.4: Effect of synthesised copper-benzotriazole compounds against planktonic
*P. aeruginosa* strains. Results are expressed as Minimum Inhibitory Concentration (MIC μM).

<table>
<thead>
<tr>
<th>Copper sulphate-benzotriazole compound</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu (BZT)</td>
<td>&gt;1.28</td>
<td>&gt;1.28</td>
<td>&gt;1.28</td>
<td>&gt;1.28</td>
</tr>
<tr>
<td>Cu (MBZT)</td>
<td>&gt;2.4</td>
<td>&gt;2.4</td>
<td>&gt;2.4</td>
<td>&gt;2.4</td>
</tr>
<tr>
<td>Cu (BZTM)</td>
<td>&gt;2.2</td>
<td>&gt;2.2</td>
<td>&gt;2.2</td>
<td>&gt;2.2</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>3.2</td>
<td>3.2</td>
<td>&gt;3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Benzotriazole</td>
<td>&gt;4.3</td>
<td>&gt;4.3</td>
<td>&gt;4.3</td>
<td>&gt;4.3</td>
</tr>
<tr>
<td>5-Methyl-1H Benzotriazole</td>
<td>&gt;3.8</td>
<td>&gt;3.8</td>
<td>&gt;3.8</td>
<td>&gt;3.8</td>
</tr>
<tr>
<td>1H Benzotriazole 1-methanol</td>
<td>1.7</td>
<td>1.7</td>
<td>0.85</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Table 3.5: Effect of synthesised copper-benzotriazole compounds against *P. aeruginosa* biofilms. Results are expressed as Biofilm Inhibitory Concentrations (BIC μM).

<table>
<thead>
<tr>
<th>Copper sulphate-benzotriazole compound</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu (BZT)</td>
<td>&gt;1.2</td>
<td>&gt;1.2</td>
<td>&gt;1.2</td>
<td>&gt;1.2</td>
</tr>
<tr>
<td>Cu (MBZT)</td>
<td>&gt;2.4</td>
<td>&gt;2.4</td>
<td>&gt;2.4</td>
<td>&gt;2.4</td>
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<tr>
<td>Cu (BZTM)</td>
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<td>&gt;2.2</td>
<td>&gt;2.2</td>
<td>&gt;2.2</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>&gt;6.4</td>
<td>&gt;6.4</td>
<td>&gt;6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Benzotriazole</td>
<td>&gt;4.3</td>
<td>&gt;4.3</td>
<td>&gt;4.3</td>
<td>2.1</td>
</tr>
<tr>
<td>5-Methyl-1H Benzotriazole</td>
<td>&gt;7.6</td>
<td>3.8</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>1H Benzotriazole 1-methanol</td>
<td>3.4</td>
<td>6.8</td>
<td>3.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>
3.3.2 Effect of transition metal gallium nitrate (Ga(NO$_3$)$_3$) on growth of *P. aeruginosa* planktonic and biofilm bacteria.

The potential antimicrobial action of gallium nitrate was assessed against *P. aeruginosa* isolates grown planktonically and as biofilms on polystyrene pegs. Gallium nitrate displayed no obvious antibacterial effect as a single agent against planktonic and biofilm formation at concentrations ranging between 1 – 500 µM. Optical density readings of cells treated with gallium nitrate had similar absorbance values to the untreated control. Previous studies have shown gallium to inhibit biofilm bacteria by targeting cells located within the interior of the structure (Singh *et al.*, 2007). The inhibitory activity of gallium in combination with aminoglycoside antibiotic tobramycin was also assessed against biofilms grown on polystyrene pegs. Concentrations of tobramycin ranging between 0.5 - 512 µg/ml were prepared. In contrast to the results obtained from the study by Singh *et al.*, (2007), tobramycin appeared as the only agent inhibiting *P. aeruginosa* biofilm formation in this model. Findings revealed gallium nitrate is not enhancing the anti-pseudomonal activity of tobramycin.
Table 3.6: Effect of gallium nitrate (Ga(NO$_3$)$_3$) on antibiotic efficacy of tobramycin.

Results are expressed as MIC and BIC (µg/ml).

<table>
<thead>
<tr>
<th>Gallium nitrate (µM) + Antibiotic</th>
<th>$P. aeruginosa$ PA01</th>
<th>$P. aeruginosa$ ATCC27853</th>
<th>$P. aeruginosa$ 1826</th>
<th>$P. aeruginosa$ 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
</tr>
<tr>
<td>2 µM Ga + Tobramycin</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>5 µM Ga + Tobramycin</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>20 µM Ga + Tobramycin</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>50 µM Ga + Tobramycin</td>
<td>1</td>
<td>2</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>200 µM Ga + Tobramycin</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>500 µM Ga + Tobramycin</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Tobramycin control</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Gallium nitrate displayed no effect against $P. aeruginosa$. Results are shown in Appendix 3.
3.3.3 Effect of silver-coumarin compounds against planktonic and biofilm formation of *P. aeruginosa*.

The metal-free coumarin ligands did not display any antimicrobial effect against *P. aeruginosa* strains grown planktonically or as biofilms. Results are shown in Appendix 2.

Results presented in Tables 3.7 and 3.8 show the sensitivity of *P. aeruginosa* strains to silver-coumarin compounds in comparison to the silver nitrate control. Silver-coumarin compounds used in this study were designed to enhance this effect by complexing with coumarins. Results revealed compounds 6, 7, 8 and 10 were active against all four strains grown planktonically. Compounds 5 and 6 showed the most potent inhibitory effect against planktonic cultures of two *P. aeruginosa* isolates at 6.1 and 5.5 μM, respectively. Several compounds exhibited a moderate decrease in biofilm formation in comparison to the silver nitrate control when exposed to various concentrations of the different agents. Compounds 3 and 7 resulted in biofilm inhibition greater than silver nitrate against three strains, as quantified by staining with crystal violet. Another three compounds 4, 5 and 6 reduced biofilms in two out of the four strains tested. Overall, the compounds displayed enhanced activity to silver nitrate against biofilm formation in at least one of the isolates examined. In addition, compounds 3 and 4 exhibited greater activity against biofilms than planktonic cells. The interactions of these compounds with *P. aeruginosa* appear to be specific to each strain.
Table 3.7: Effect of silver-coumarin compounds against planktonic *P. aeruginosa* strains. Results are expressed as MIC (µM) and MBC (µM).

<table>
<thead>
<tr>
<th>Silver-coumarin compound</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>Ag60H</td>
<td>12.7</td>
<td>25.5</td>
<td>25.5</td>
<td>12.7</td>
</tr>
<tr>
<td>Ag70H</td>
<td>25.5</td>
<td>25.5</td>
<td>102.2</td>
<td>25.5</td>
</tr>
<tr>
<td>Ag80H</td>
<td>12.7</td>
<td>25.5</td>
<td>102.2</td>
<td>12.7</td>
</tr>
<tr>
<td>AgCCa</td>
<td>13.46</td>
<td>26.9</td>
<td>26.9</td>
<td>107.7</td>
</tr>
<tr>
<td>Compound 2</td>
<td>24.1</td>
<td>193.3</td>
<td>48.3</td>
<td>96.6</td>
</tr>
<tr>
<td>Compound 3</td>
<td>24.4</td>
<td>195.6</td>
<td>48.9</td>
<td>195.6</td>
</tr>
<tr>
<td>Compound 4</td>
<td>21.3</td>
<td>85.1</td>
<td>42.5</td>
<td>170.2</td>
</tr>
<tr>
<td>Compound 5</td>
<td>6.1</td>
<td>12.2</td>
<td>24.46</td>
<td>195.6</td>
</tr>
<tr>
<td>Compound 6</td>
<td>11</td>
<td>44.2</td>
<td>22.1</td>
<td>44.2</td>
</tr>
<tr>
<td>Compound 7</td>
<td>22.1</td>
<td>44.26</td>
<td>22.1</td>
<td>44.26</td>
</tr>
<tr>
<td>Compound 8</td>
<td>12.2</td>
<td>97.8</td>
<td>24.46</td>
<td>48.9</td>
</tr>
<tr>
<td>Compound 10</td>
<td>10.9</td>
<td>21.8</td>
<td>21.8</td>
<td>43.7</td>
</tr>
<tr>
<td><strong>Silver nitrate (AgNO₃)</strong> control</td>
<td><strong>23.5</strong></td>
<td><strong>94.18</strong></td>
<td><strong>23.5</strong></td>
<td><strong>94.18</strong></td>
</tr>
</tbody>
</table>
Table 3.8: Effect of synthesised silver-coumarin compounds against *P. aeruginosa* biofilms. Results are expressed as BIC (µM).

<table>
<thead>
<tr>
<th>Silver-coumarin compound</th>
<th><em>P. aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P. aeruginosa</em> 1826</th>
<th><em>P. aeruginosa</em> 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag60H</td>
<td>215.48</td>
<td>1635.7</td>
<td>107.7</td>
<td>107.7</td>
</tr>
<tr>
<td>Ag70H</td>
<td>408.9</td>
<td>1635.7</td>
<td>51.1</td>
<td>204.46</td>
</tr>
<tr>
<td>Ag80H</td>
<td>817.8</td>
<td>1635.7</td>
<td>51.1</td>
<td>204.46</td>
</tr>
<tr>
<td>AgCCa</td>
<td>204.46</td>
<td>408.9</td>
<td>204.46</td>
<td>51.1</td>
</tr>
<tr>
<td>Compound 2</td>
<td>24.1</td>
<td>3093</td>
<td>3093</td>
<td>24.16</td>
</tr>
<tr>
<td>Compound 3</td>
<td>48.9</td>
<td>782.7</td>
<td>48.92</td>
<td>97.84</td>
</tr>
<tr>
<td>Compound 4</td>
<td>21.3</td>
<td>2724</td>
<td>85.1</td>
<td>340.5</td>
</tr>
<tr>
<td>Compound 5</td>
<td>24.46</td>
<td>1565.5</td>
<td>97.84</td>
<td>48.92</td>
</tr>
<tr>
<td>Compound 6</td>
<td>22.13</td>
<td>2832.7</td>
<td>354.09</td>
<td>22.13</td>
</tr>
<tr>
<td>Compound 7</td>
<td>44.26</td>
<td>1416.39</td>
<td>44.26</td>
<td>177</td>
</tr>
<tr>
<td>Compound 8</td>
<td>24.46</td>
<td>1565.5</td>
<td>195.69</td>
<td>1565.5</td>
</tr>
<tr>
<td>Compound 10</td>
<td>174.8</td>
<td>2797.8</td>
<td>43.71</td>
<td>174.86</td>
</tr>
<tr>
<td><strong>Silver nitrate (AgNO₃) control</strong></td>
<td><strong>94.18</strong></td>
<td><strong>1506.9</strong></td>
<td><strong>188.36</strong></td>
<td><strong>47.09</strong></td>
</tr>
</tbody>
</table>
3.3.4 Summary of selected clinical CF *P. aeruginosa* strains.

A selection of *P. aeruginosa* CF strains were recovered from sputum samples from different Irish CF clinics and typed using pulsed-field gel electrophoresis (PFGE) at the CF reference laboratory, AMNCH. Samples were plated onto selective agar as described in Section 2.2.5 and phenotypic characteristics are listed below in Table 3.9. Four out of the sixteen strains grew as mucoid colonies while twelve were non-mucoid. Approximately half of the isolates produced a green pigment and the remaining grew as small white colonies on MHA after 24 hours.

Table 3.9: Phenotypic characteristics of CF *P. aeruginosa* strains.

<table>
<thead>
<tr>
<th><em>P. aeruginosa</em> strain</th>
<th>pigment/ no pigment</th>
<th>mucoid/ non-mucoid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> 57084</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 57064</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 5714</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 5658</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1828</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1872</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1883</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 398</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 9589</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 69960.2</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 6279</td>
<td>no pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1885</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 8456-1</td>
<td>pigment</td>
<td>mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4649</td>
<td>pigment</td>
<td>mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4505.2</td>
<td>pigment</td>
<td>mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 88716</td>
<td>pigment</td>
<td>mucoid</td>
</tr>
</tbody>
</table>
3.3.5 Investigation of the effect of silver-coumarin agents on planktonic growth of clinical CF *P. aeruginosa* strains.

The antimicrobial activity of effective silver-coumarin compounds was assessed against a range of CF *P. aeruginosa* strains of different phenotypic characteristics. Virulence factors including pyocyanin production secreted by *P. aeruginosa* are known to target epithelial cell function in the CF lung resulting in chronic lung infection (Gardner *et al.*, 1996). The amount of exopolysaccharide has been shown to be an important component in biofilm production and function (Matsukawa *et al.*, 2004). Strains appeared sensitive to these agents irrespective of their phenotypic traits as seen in Figure 3.2. Following treatment with synthesised compounds 6 and 8, a greater reduction in MIC and MBC values in comparison to silver nitrate was not observed for most strains. Differences between MIC and MBC values were generally not more than one or two-fold greater in concentration for each agent. *P. aeruginosa* 57064 and *P. aeruginosa* 4505.2 displayed greater sensitivity to the compounds in comparison to silver nitrate with a one-fold reduction in either the MIC or MBC values. Three additional strains (*P. aeruginosa* 5714, *P. aeruginosa* 69960.2, *P. aeruginosa* 6279) showed greater bactericidal sensitivity to compound 6 in comparison to silver nitrate in which MBC values were reduced by at least 2-fold.
Figure 3.2: MIC and MBC (μM) values of (A) compound 6 (mw 361.48), (B) compound 8 (mw 327.04) and (C) silver nitrate control against clinical CF *P. aeruginosa* strains. Results represent the mean ± standard deviation of three independent experiments.
3.3.6 Inhibition of mature *P. aeruginosa* biofilms by silver-coumarin compounds using two different static models.

To investigate the influence of effective silver-coumarin compounds on mature biofilm structures, analysis was carried out using two different static models. Biofilms were grown for 72 hours and viability was assessed following a further 24 hours exposure to silver nitrate and compound 8. Biofilm biomass was compared to the untreated control. Less mature biofilms have been described as generally more susceptible to antimicrobial treatment than cells in more older structures (Tre-Hardy *et al.*, 2009). Comparative studies of biofilms grown for 72 hours revealed that reference strains (*P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853) were more resistant to treatment since the activity of silver nitrate and compound 8 did not significantly reduce growth in comparison to the untreated control. As shown in Figure 3.3 biomass of clinical isolate *P. aeruginosa* 1826 was significantly reduced with concentrations of MIC of silver nitrate (*P = 0.029*), 10 x MIC of silver nitrate (*P = 0.011*) and MIC of compound 8 (*P = 0.043*) in comparison to the control. In addition, the agents did affect bacterial viability of clinical isolate *P. aeruginosa* 4276.3, suggesting that the concentrations sufficient to suppress mature biofilm formation are strain dependant. Taken together the data indicate that biofilm suppression by these agents may be influenced by the age of the biofilm.
(A) *P. aeruginosa* PA01

(B) *P. aeruginosa* ATCC27853
Figure 3.3: The effect of silver nitrate and compound 8 against 72 hour *P. aeruginosa* biofilms grown in BTrace medium. The activity of all agents was compared to the untreated control. Results are shown as total log counts and represent the average of three experiments.
The results obtained from 72 hour biofilms formed on polystyrene pegs, treated with effective compounds showed that the activity of the silver-coumarin compounds was comparable to silver nitrate as shown in Figure 3.4. Results obtained with this static model are in keeping with the previous model, in which the optical densities of 72 hour biofilms treated with the compounds were similar to the silver nitrate control. Increasing concentrations did not necessarily increase the rate of biofilm inhibition against *P. aeruginosa* PA01. The results indicate that resistance of 72 hour *P. aeruginosa* biofilms may be greater than at 24 hours. The two clinical isolates, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3, were more sensitive strain to all agents at higher concentrations. The efflux system has been described as one of the main resistance mechanisms of most microorganisms exposed to high concentrations of heavy metals for a lengthy period of time (Nies *et al.*, 1999).
(A) *P. aeruginosa* PA01

![Graph showing optical density (630nm) vs. concentration (μg/ml) for *P. aeruginosa* PA01. The graph includes data for Compound 6, Compound 8, and Silver nitrate.]

(B) *P. aeruginosa* 1826

![Graph showing optical density (630nm) vs. concentration (μg/ml) for *P. aeruginosa* 1826. The graph includes data for Compound 6, Compound 8, and Silver nitrate.]

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Figure 3.4: The effect of varying concentrations of compound 6, compound 8 and silver nitrate against mature *P. aeruginosa* biofilms. Biofilms were grown on polystyrene pegs for 72 hours prior treatment. Results represent the mean ± standard deviation of two experiments.
3.3.7 Investigation of *P. aeruginosa* biofilm formation using the flow cell system.

Biofilm forming ability of the reference and CF clinical isolates was investigated in the flow cell system. This model allows the structural development of biofilms to be monitored with confocal laser scanning microscope (CLSM). The results show that clinical CF and reference strains develop different structural biofilms as depicted in Figure 3.5. The microcolonies formed by clinical isolates, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were larger than *P. aeruginosa* PA01. Both of these CF isolates developed large, thick structures while the overall structure of *P. aeruginosa* 1826 was more uniform. In addition, an uneven distribution of microcolonies was observed with the clinical strains. The microcolonies of *P. aeruginosa* PA01 appeared smaller and less defined while *P. aeruginosa* ATCC27853 formed a flat structure on the surface of the flow-cell chamber. It is interesting that *P. aeruginosa* PA01 which produced a strong biofilm with the crystal violet staining biofilm assay showed reduced thickness in the flow cell system. The results suggest that the environmental conditions of the static model may be favouring biofilm growth of *P. aeruginosa* PA01.

Table 3.10: Phenotypic characteristics of isolates selected for examination and treatment in the flow cell system.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment/ No pigment</th>
<th>Mucoid/ Non-mucoid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC27853</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1826</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 4276.3</td>
<td>no pigment</td>
<td>mucoid</td>
</tr>
</tbody>
</table>
Figure 3.5: CLSM images of 72 hour *P. aeruginosa* biofilms in 10% glucose supplemented minimal media showing different biofilm structures. Images show sections through the biofilm depicting microcolony profiles of the structures.
3.3.8 The effect of silver-coumarin compounds on biofilm development in the flow cell system.

The study progressed to a continuous flow cell model to further determine the mechanisms by which synthesised compounds reduce the viability of established *P. aeruginosa* biofilms in comparison to the silver nitrate control. *P. aeruginosa* 1826 biofilms were grown for 72 hours in separate channels followed by 24 hours treatment with 10 x MIC compound 8 and 10 x MIC of silver nitrate (Figure 3.6). Eradication of the biofilm was observed in comparison to the untreated control in the presence of these agents. The results show no clear differences in the activities of each compound. Findings are consistent with the results obtained of 72 hour *P. aeruginosa* 1826 biofilm formed in the static biofilm models.

(A) *P. aeruginosa* 1826 control
(B) *P. aeruginosa* 1826 + AgNO₃
Figure 3.6: Live-dead stained *P. aeruginosa* 1826 grown for 72 hours in minimal medium and treated with 10 x MIC of silver nitrate and 10 x MIC of compound 8. Images were obtained by CLSM, live cells appear green and dead cells appear red as a result of staining.

The effect of compound 8 and silver nitrate at the same concentration of 10 x MIC was also investigated against *P. aeruginosa* PA01 biofilms in the flow cell system (Figure 3.7). Prior to treatment *P. aeruginosa* PA01 formed small, flat microcolonies. Previously, this strain displayed greater sensitivity to the synthesised silver-coumarin compounds in comparison to the silver-nitrate control in the static biofilm model. This strain was not able to form biofilm of such high surface coverage in the flow cell system. In addition, there was complete inhibition of this strain in the presence of all agents following 24 hours exposure. The exact stages at which the agents affect *P. aeruginosa* biofilm development remain unknown in the present study and warrant further investigation.
Figure 3.7: Live-dead stained images of 72 hour *P. aeruginosa* PA01 biofilm treated with 10 x MIC of silver nitrate and 10 x MIC of compound 8. The results show *P. aeruginosa* PA01 biofilms following 24 hours treatment. Images were obtained by CLSM.
3.4 Discussion

The results so far have demonstrated that CF *P. aeruginosa* strains have different phenotypic characteristics which may influence the response to treatment. This section of the study was aimed at determining the ability of novel metal-based compounds to disrupt *P. aeruginosa* biofilms. Findings were based on crystal violet staining which is a standard method for biofilm quantification. A more advanced study of biofilms using the flow cell system was also used as part of this study.

Results of the antimicrobial action of copper-benzotriazole agents showed that these agents exhibit low antimicrobial activity against *P. aeruginosa*. As mentioned in this Chapter, Section 3.1, reduced concentrations of copper and various other metals may facilitate the growth of certain bacterial species. The current study found *P. aeruginosa* growth was unaffected with all synthesised copper-benzotriazole agents. In addition, copper sulphate exhibited antimicrobial activity against three *P. aeruginosa* strains at a concentration of 3.2 μM indicating that this metal may be toxic to *P. aeruginosa* cells at this concentration. Previously, Visca *et al.*, (1992), described *P. aeruginosa* genes involved in the synthesis of pyochelin were repressed by copper activity. Copper is also known to produce reactive oxygen species which can lead to damage of cellular components (Damerson and Morrison, 1998). Teitzel *et al.*, (2006), described oxidative stress as an important factor for copper toxicity in *P. aeruginosa*. As well as damage to the cell membrane, oxidative stress can increase membrane permeability leading to death of the bacterial species. High amounts of copper may also enhance protein misfolding,
repression of virulence genes, *agr* and *sae* in *S. aureus* (Baker *et al.*, 2010). In other studies, copper has shown to display greater activity through synergistic interactions with other agents. Harrison *et al.*, (2008), found combinations of copper and certain quaternary ammonium compounds enhance toxicity by affecting the nitrate reduction of *P. aeruginosa* biofilms.

Another agent examined against *P. aeruginosa* biofilms grown on polystyrene pegs was gallium nitrate. This agent has been studied by many researchers since it has been FDA approved for intravenous treatment of hypercalcemia of malignancy (Bernstrin *et al.*, 1998). Other studies have shown gallium can enter bacterial cells and act as a substitute for iron in many biological processes including DNA replication (Hubbard *et al.*, 1986; Chitambar *et al.*, 1988). Olakanmi *et al.*, (2000), showed that gallium is effective against *Mycobacterium tuberculosis* under conditions in which the concentration of iron are high. In contrast, Singh *et al.*, (2007), showed gallium was taken up by *P. aeruginosa* biofilms when the concentration of iron was low. Results from the current study found gallium nitrate did not display antibacterial activity against *P. aeruginosa* planktonic and biofilm cultures. This study also found that gallium nitrate did not enhance the activity of the aminoglycoside antibiotic tobramycin. The study by Singh *et al.*, (2007) described biofilms formed under conditions in which concentrations of iron are reduced are more susceptible to antimicrobial treatment. Synergistic interactions were observed with gallium and tobramycin. Tobramycin acts on the cell membrane allowing gallium to inhibit the interior of the biofilm structure. Unlike the study by Singh *et al.*, (2007), *P. aeruginosa* biofilms developed in this study were not grown under conditions in which
the concentration of iron was low.

The synthesised metal-free coumarin compounds used in this study were ineffective against all *P. aeruginosa* strains. In contrast, many of the synthesised silver-coumarin compounds displayed enhanced antibacterial activity in comparison to the silver nitrate control against *P. aeruginosa*. Previous studies have shown coumarins have the potential to enhance the antimicrobial effect of other agents. The study by Thati *et al.*, (2007), showed that these silver-coumarin compounds reduced the rate of respiration of *C. albicans* by disrupting the synthesis of cytochromes in the mitochondrion. Reeves *et al.*, (2004), found coumarin agents increased leakage of the components of the cell membrane. Another study by Smyth *et al.*, (2009), found antibiotic oxicillin displayed greater activity against MRSA in combination with 8-Iodo-5, 7-dihydroxycoumarin. In addition, Zeng *et al.*, (2007), found coumarin agent, esculin enhanced the susceptibility of *P. aeruginosa* to tobramycin by interfering with quorum sensing signalling.

This study adds to the current knowledge about the antimicrobial activity of silver-based agents. The results revealed the effect of silver nitrate and silver-coumarin compounds are effective against *P. aeruginosa* planktonic cells and biofilms. The majority of the compounds showed greater efficacy against planktonic cells than 24 hour biofilms. In addition, biofilms grown for 72 hours on polystyrene pegs were more resistant to treatment with these compounds suggesting the efficacy of these agents may be reduced against mature biofilms. Silver is effective against a broad range of bacteria by acting against the cell membrane by binding to DNA and thiol groups and inactivating enzymes
affecting bacterial replication (Feng et al., 2000; Slawson et al., 1992; Lansdown and Williams, 2007). The antibacterial properties have also been described by Kalishwaralal et al., (2010), which found silver nanoparticles may enhance the detachment of *P. aeruginosa* and *S. aureus* from the wells of a microtitre plate in a concentration dependent manner. Greenfield et al., (1995), described silver sulfadiazine in combination with chlorhexidine inhibited bacterial adherence on vascular catheters. Other *in vivo* studies found silver iontophoretic catheters reduce the rate of *S. aureus* infection (Raad et al., 1996). Youngs et al., (2009), suggested silver-complexes that are water soluble may be suitable for administration via inhalation.

This study also assessed the activity of the silver-coumarin compounds against a broad range of *P. aeruginosa* strains since many clinical CF strains have different phenotypic characteristics as described in this section. The bacteriostatic and bactericidal activity of silver nitrate was recorded against planktonic cultures of 16 *P. aeruginosa* strains. The differences between the MIC and MBC values of silver-coumarin agents in comparison to silver nitrate were generally not more than two-fold, indicating the phenotypic characteristics of *P. aeruginosa* do not appear to influence the efficacy of these agents.

The activity of these compounds was also tested against *P. aeruginosa* biofilms formed in microtitre wells in minimal BTrace media. The concentrations selected were based on the MIC, 10 x MIC and 20 x MIC as determined by the broth microdilution method. Findings revealed that the activity of silver nitrate and the silver-coumarin compounds was not dependant of the concentration. The results revealed that two strains, *P. aeruginosa* PA01
and *P. aeruginosa* 1826 exhibited an increase in biofilm density at the 20 x MIC in comparison to a lower concentration of 10 x MIC. In addition, *P. aeruginosa* 4276.3 was more resistant to the activity of these agents. Previously, Parson *et al.*, (2006), showed that wound dressings that released a larger amount of silver do not lead to greater degree of antimicrobial activity. Bridges *et al.*, (1979), found a selection of *P. aeruginosa* strains collected from burn patients that were resistant to gentamicin were equally resistant to silver, the reasons of which are currently unknown. Gupta *et al.*, (1999), and his group reported that the ability of silver to bind to proteins and efflux pump mechanisms may reduce the toxicity of silver.

*P. aeruginosa* biofilms were grown in the flow cell system to determine if results correlated with those obtained with the static models. Results suggested that silver is able to inhibit and detach established biofilms of certain strains. Assays which enable bacteria to be cultured continuously allow phenotypic characteristics of bacteria to be maintained for lengthy periods of time (Herbert *et al.*, 1956). A continuous nutrient supply is essential for bacteria to grow in any system (Nichols *et al.*, 1999). Results from the current study showed distinct differences in the biofilm structures of selected *P. aeruginosa* strains which may be influencing their response to treatment. Clinical strains, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 developed large microcolonies. Reference strain *P. aeruginosa* PA01 formed small microcolonies and *P. aeruginosa* ATCC27853 developed a flat biofilm structure. Previous studies have suggested the matrix of biofilms under flow conditions may be thicker than those formed in other models, which may be enhancing resistance to antimicrobial agents (Douglas *et al.*, 2006). Davies *et al.*, (1998),
suggested that quorum sensing signalling molecules, known as autoinducers (AI), of *P. aeruginosa* have an important role in differential biofilm morphology which may be giving biofilms their defining structures in a flow environment. The two QS systems, *las* system and *rhl* system, influence biofilm architecture through different mechanisms (DeKievit *et al.*, 2001). It remains unclear if the *las*, *rhl* or both QS systems are involved in attachment and microcolony formation in the present study.

In this study, biofilms grown in the flow cell system that were exposed to effective silver-coumarin compounds showed biofilms were inhibited following 24 hours treatment. These findings were different to the results obtained with the static biofilm models. One possible explanation for the results obtained in the current study could be the continuous nutrient supply containing a suitable concentration of the silver-based agent sloughing off the different cell layers of the biofilm. Recently, Sillankorva *et al.*, (2008), reported that the rate at which some agents reach their host under dynamic conditions can depend on force. The amount of exopolysaccharide secreted by bacteria can be influenced by the different growth conditions. It remains unclear if a greater amount of exopolysaccharide was produced in the static models, indicating that the efficacy silver-based compounds may not be influenced by the matrix of *P. aeruginosa* biofilms.
Chapter 4: Mixed-species biofilm infection.

4.1 Introduction

The pathogens present in the CF lung may change due to environmental conditions and patient-to-patient transmission (LiPuma et al., 1990; Salunkhe et al., 2005). According to Wahab et al., (2004), patients co-colonised with mucoid strains of *P. aeruginosa* and less common bacterial species can experience more severe lung function complications than patients that have contracted just the mucoid phenotype.

Pathogens within the CF airways change with disease progression and antibiotic exposure (Tunney et al., 2013). *P. aeruginosa, S. aureus* and *H. influenzae* have been identified as the primary causes of acute exacerbations and lung deterioration (Saiman et al., 2004). Pathogens less frequently associated with chronic infection include *Streptococcus pneumoniae, Stenotrophomonas maltophilia, Burkholderia cepacia* complex (Bcc), *Achromobacter xylosidans, Acinetobacter* sp., *Herbaspirillum* sp., *Pandoraea* sp. and *Ralstonia* sp. (LiPuma et al., 2010).

As previously described in Chapter 1, Section 1.5, biofilms are bacterial communities encased within an exopolysaccharide matrix. Biofilms may form as single species in which the cell clusters are separate or in a mixed culture consisting of two or more species (Bjarnsholt et al., 2011). Mixed species biofilms may be classified as synergistic
or antagonistic in which the presence of one organism influences the growth of the other organism (Kuramitsu et al., 2007).

Biofilms consisting of one or more species have been described as more thicker and stable than those formed by a single species (Allison et al., 2000). Rao et al., (2004), suggested that microcolonies of certain species may act as protective layers during biofilm formation, enhancing the presence of one species during colonisation. Skillman et al., (1999), found that interactions between the extracellular polysaccharide of two different species resulted in changes to the polymer matrix. Other studies have described rheological interactions between different species may decrease the rate at which antimicrobial agents penetrate the membrane of the biofilm (Allison et al., 1992).

Many standard culturing techniques have been directed at identifying pathogens that are generally more dominant within the lungs. In order for clinicians to select more accurate treatments, a more detailed analysis of the bacteria that exist within these communities of each sputum sample would be necessary (Malic et al., 2009; Gu et al., 2005). The use of molecular methods such as phylogenetic assays has increased the understanding of CF airway pathogens. The 16S rRNA phylochip has detected more than 2,015 bacterial taxa from CF sputum samples (Sibley et al., 2011). Fluorescent in situ hybridization (FISH) is an emerging technique that allows simultaneous identification and visual analysis of bacteria within mixed communities (Moter et al., 2000). With this technique Fazli et al., (2009), noticed P. aeruginosa and S. aureus biofilms colonise the wound surface at
different levels. *P. aeruginosa* tends to reside in the deeper layers and virulence factors that are produced lead to polymorphonucleotide (PMN) destruction and inflammation.

The CF reference laboratory in AMNCH has identified a number of unusual pathogens in CF sputum samples. CF pathogens may develop their own biofilm structures or integrate into biofilms formed by more dominant species (Akiyama *et al.*, 2003; Lembke *et al.*, 2006). In this section the characterisation of unusual CF species was carried out. In parallel studies mixed biofilm interactions of these species with *P. aeruginosa* was conducted with the aim of finding pathogens that may be interfering with the biofilm forming capacity of *P. aeruginosa* and any possible mechanisms of these interactions.
4.2 Materials and Methods

4.2.1 Bacterial strains.

Clinical and reference isolates of *P. aeruginosa* and unusual CF strains are listed below in Table 4.1. The clinical CF strains were identified in the cystic fibrosis reference laboratory in AMNCH by 16S ribosomal RNA (rRNA) gene sequencing. The *Paracoccus yeeii* strains were obtained from the Centre for Disease Control and Prevention (CDC). All isolates were routinely grown on TSA and inoculated in nutrient broth for 24 hours at 35°C when conducting experimental procedures. A culture of all bacterial strains was stored in glycerol at -80°C.

Table 4.1: Cystic fibrosis species used in this study.

<table>
<thead>
<tr>
<th>Species:</th>
<th>Gram-positive/ Gram-negative</th>
<th>Aerobic/Aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>gram-negative</td>
<td>aerobic</td>
</tr>
<tr>
<td><em>Ralstonia sp.</em></td>
<td>gram-negative</td>
<td>aerobic</td>
</tr>
<tr>
<td><em>Acinetobacter sp.</em></td>
<td>gram-negative</td>
<td>aerobic</td>
</tr>
<tr>
<td><em>Herbaspirillum sp.</em></td>
<td>gram-negative</td>
<td>aerobic</td>
</tr>
<tr>
<td><em>Paracoccus yeeii</em></td>
<td>gram-negative</td>
<td>aerobic</td>
</tr>
</tbody>
</table>
4.2.2 Planktonic growth of unusual CF species.

An overnight culture of each isolate was suspended in nutrient broth to a 0.5 McFarland standard and adjusted to a final concentration of $5 \times 10^5$ CFU/ml. A quantity of 100 µl of each culture was added the wells of a 96-well microtitre plate and incubated for 24 hours. After 24 hours, the $\text{OD}_{630}$ was measured in a microplate reader to determine planktonic growth of the unusual CF species.

4.2.3 Single and mixed-species biofilm formation on polystyrene pegs.

The ability of unusual strains isolated from the CF lung to form biofilms as single species and mixed species with *P. aeruginosa* was examined using the microtitre plate assay as described by Moskowitz *et al.*, (2004). An overnight culture of each isolate was suspended in nutrient broth to a 0.5 McFarland standard. A quantity of 5 µl of this inoculum was added to 1 ml nutrient broth to give a final concentration of $5 \times 10^5$ CFU/ml. For single species experiments, 100 µl of the prepared inoculums of each isolate was added to the wells of a 96- well round-bottom plate. A TSP lid containing pegs was placed into the microtitre plate for biofilms to develop and incubated at 35°C for 24, 48 and 72 hours.

For mixed species experiments, a 0.5 McFarland standard of *P. aeruginosa* and the unusual CF isolates was prepared and adjusted to a final concentration of $5 \times 10^5$ CFU/ml as described above. A quantity of 50 µl of two different species was added to the wells of a 96-well plate to give a final volume of 100 µl. A TSP pin lid was added to the microtitre plate and incubated at 35°C for 24, 48 and 72 hours. After each time point the
lids containing pegs were rinsed three times in sterile water, dried at room temperature and added to another plate containing 0.3% crystal violet (CV) for 30 minutes to stain the cells. The plates were rinsed again thoroughly and placed into a new 96-well round-bottom microtitre plate containing an equal volume of 95% ethanol and 1% Triton X-100 to remove the CV stained biofilms. The OD$_{630}$ was measured in a microplate reader. All experimental procedures were carried out in triplicate on three separate occasions.

4.2.4 Cell culture biofilm growth assay of single and mixed species.

To a 6-well microtitre plate, 10 mls of nutrient broth was added. An overnight culture of each isolate was diluted to OD$_{630}$ = 0.1 in nutrient broth. A quantity of 100 µl of the culture was added to each well and incubated at 35°C for 24 and 72 hours to allow biofilms to develop. For mixed species biofilm growth, each mixture was prepared at a 1:1 ratio. Following 24 hours incubation, all contents of the wells were removed and transferred to centrifuge tubes. Cells were centrifuged for 3,000 g, 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 g, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexed until the cells have been dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

For mature biofilm experiments, biofilms were prepared as described above in Section 4.2.4 and incubated for 72 hours. The media was removed from the wells every 24 hours and replaced with fresh media using a 10 ml syringe. This was dispensed gently down the side of the wells to avoid shear force which can destroy any biofilm that has formed on
the bottom of the wells. After 72 hours incubation, all contents from wells were transferred to centrifuge tubes. Cells were centrifuged for 3,000 g, 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 g, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexed until cells are dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

4.2.5 Effect of *S. maltophilia* whole cells and cell-free supernatants on the biofilm forming ability and pre-formed biofilms of *P. aeruginosa*.

Preparation of cell-free supernatants involved inoculating one or two colonies of *S. maltophilia* strains (668, 747) in 50 mls nutrient broth for 96 hours at 35°C. Cell-free supernatants were collected by centrifuging for 3,000 g, 10 minutes and filtering through 0.2 μm pore size filters (Sigma-Aldrich). To assess the interactions between *P. aeruginosa* and *S. maltophilia*, mixed biofilms were prepared by inoculating a cell suspension of 5 x 10^5 CFU/ml of each species at a 1.1 ratio. In addition, a cell suspension of *P. aeruginosa* and an equal volume of cell-free supernatants or concentration of whole cells of *S. maltophilia* were prepared. A quantity of 100 μl of each mixture was added to the microwell plate containing peg lids and incubated for 24 hours at 35°C to allow biofilms to develop.

To determine the effect of *S. maltophilia* on pre-formed *P. aeruginosa* (PA01, 1826, 4505.2) biofilms, strains were grown on polystyrene pegs for 24 hours at 35°C, as previously described. The lids containing pegs were rinsed three times with sterile water
and added to another plate containing 100 μl of *S. maltophilia* supernatants and whole cells. The untreated *P. aeruginosa* strains and cell-free supernatants were added to the wells as controls. Biofilm quantification was carried out after a further 24 hours, 35°C incubation.

4.2.6 Effect of antibiotics on *P. aeruginosa* biofilm formation and pre-formed biofilms with *S. maltophilia* whole cells and cell-free supernatants.

*P. aeruginosa* strains were allowed to form mixed biofilms as previously described in Section 4.2.5. Two-fold serial dilutions of antibiotics were prepared in nutrient broth at a final concentration of 256 μg/ml. Biofilms were exposed to antibiotics for a further 24 hours, stained with 0.3% crystal violet and the optical density measured at 630nm.

4.2.7 Susceptibility of unusual CF isolates to antibiotic treatment.

Minimum inhibitory concentrations (MIC) of unusual species were carried out by the broth microdilution method as previously described in Chapter 2, Section 2.2.10. A quantity of 100 μl sterile nutrient broth was added to the wells of a 96-well round-bottom microtitre plate (Nunc Inc., Roskilde, Denmark). A serial two-fold dilution of tobramycin was prepared in nutrient broth at a final concentration of 256 μg/ml. A 100 μl suspension of each organism was added to the wells at a final concentration of 5 x 10⁵ CFU/ml and the microtitre plates were incubated at 35°C for 24 hours. At 24 hour, the contents of non-turbid wells were sub-cultured by streaking 10 μl of suspension onto 5% Columbia sheep blood agar plates, incubated overnight at 35°C and examined for complete inhibition.
4.2.8 Isolation of proteins from *S. maltophilia* isolates.

*S. maltophilia* isolates were prepared as described in Section 4.2.5, centrifuged at 4,000 g, 10 minutes and filter sterilised to collect the cell-free supernatant. A quantity of 15 µl was added to an ultracentrifuge tube (Amicon, Ultra-15 Centrifugal Filter Devices) and centrifuged at 4,000 g for 30 minutes to collect the protein sample. The concentration of each protein sample was determined using the Nanodrop™ 1000.

4.2.9 10% Ammonium persulfate (APS).

APS (10% w/v) was prepared by adding 0.1g APS to 1 ml distilled water. APS (10% w/v) was prepared fresh on the day for use.

4.2.10 10% Sodium dodecyl sulphate (SDS).

SDS (10% w/v) was prepared by adding 10 g to 100 mls distilled water until all the SDS had been solubilised.

4.2.11 10% Running buffer.

The running buffer (10%) was prepared by dissolving 250 mM Tris base, 1.92 M glycine and 1% SDS in 1 litre distilled water. The mixture was adjusted to a pH 8.3 and stored at room temperature. The buffer was adjusted to a 1X concentration in distilled water before use.
4.2.12 SDS-polyacrylamide gel electrophoresis.

Analysis of proteins produced by \textit{S. maltophilia} was carried out using the SDS-page method. The resolving and stacking gels were prepared as described in Table 4.2. Following the addition of TEMED and 10\% APS the gel was poured immediately as it begins to polymerise rapidly. The prepared resolving gel was poured into the plates, overlaid with water and allowed to set for approximately 30 minutes. Once the gel had polymerised, the water was removed, the stacking gel was added and the combs inserted to allow the wells to form. This was left to set for approximately 45 minutes.

Table 4.2: Materials for preparation of resolving and stacking gel.

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>5.3 ml</td>
<td>3.66 ml</td>
</tr>
<tr>
<td>0.3% Bis/Acrylamide</td>
<td>4.3 ml</td>
<td>780 (\mu)l</td>
</tr>
<tr>
<td>1M Tris/2M Tris</td>
<td>3.25 ml 2M Tris</td>
<td>1.25 ml 1M Tris</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulfate (SDS)</td>
<td>130 (\mu)l</td>
<td>60 (\mu)l</td>
</tr>
<tr>
<td>(N, N', N', N')-Tetramethylethylenediamine (TEMED)</td>
<td>6.5 (\mu)l</td>
<td>6 (\mu)l</td>
</tr>
<tr>
<td>10% (w/v) Ammonium Persulfate (APS)</td>
<td>65 (\mu)l</td>
<td>30 (\mu)l</td>
</tr>
</tbody>
</table>

Protein samples were prepared by adding 5X Laemmli loading dye to each sample, vortexing and centrifuging. The samples were boiled at 95\(^\circ\)C for 5 minutes to denature the proteins and cooled for a further 2 minutes. The electrophoresis rig was prepared and filled with running buffer. The well combs were removed and a quantity of 6 \(\mu\)l of protein...
sample was added to each well and 3 µl of Colorburst™ electrophoresis marker (Fermentas, Thermo Scientific). Samples were electrophoresed at 130 volts, 200 mA for approximately 110 minutes. The gel was removed, rinsed in distilled water and stained overnight with 20 ml PageBlue Protein staining solution (Thermo Scientific) followed by rinsing with distilled water until the bands were visible.

4.2.13 Preparation of protein bands for liquid chromatography/mass spectrometry (LC/MS) analysis.

Protein bands of interest were excised from the sds-page gel. The trypsin buffer was prepared by adding ammonium bicarbonate (100 mM) to a solution of 1 ml acetone nitrile and 9 mls water. A quantity of 1.5 ml modified porcine trypsin (Promega) was added to the mixture to give a final concentration of 15 ng/ml trypsin. A quantity of 5 mls acetone nitrile was added to 5 mls 100 mM ammonium bicarbonate and 200 µl was added to the microfuge tubes containing excised gel pieces to be de-stained. Acetone nitrile (200 µl) was then added for 30 minutes. The acetone was removed and 10 µl trypsin buffer was added. The samples were left on ice for 2 hours with frequent checking so that all gel pieces remained submerged in trypsin. The microfuge tubes were then placed in the incubator overnight at 37°C.

To extract the peptide digestion products the contents of the microfuge tubes were centrifuged and transferred into a fresh tube. The extracts were dried in a vacuum centrifuge overnight. Dried peptides were re-suspended in 15 µl of a 0.1% formic acid solution. Analysis of digested peptides was achieved using an Agilent 6340 Ion Trap
Liquid Chromatography/Mass Spectrometry. Data were analysed using MASCOT search engine. Proteins identified from the database are listed.

4.2.14 Statistical analysis.

Statistical analysis was carried out using PASW® Statistics 18 - SPSS. One-way ANOVA or Student’s t-test was used to determine statistical variations for most experiments. A $P \leq 0.05$ was considered statistically significant.
4.3 Results

4.3.1 Growth of planktonic cultures of unusual CF species.

The growth rates represented by each bacterial species may influence the response to antimicrobial treatment. Few studies have investigated the growth patterns of the species used in this study. Measurements were based on turbidity following 24 hours, 35°C incubation as shown in Figure 4.1. Analysed data showed strong growth capacity of *S. maltophilia* and *Herbaspirillum* species. Growth patterns of seven *Paracoccus* strains were comparable and had the weakest growth rate among the strains tested. Finally, *Ralstonia* sp. and *Acinetobacter* sp. had moderate growth levels. CF strains are showing unique patterns of growth distinguishable from other species.
Figure 4.1: Optical density (630nm) of planktonic growth of unusual CF species measured after 24 hours incubation. Results represent the mean ± standard deviation of three independent experiments.
4.3.2 Adherence of single and mixed species of *Paracoccus yeeii* and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

The sputa of CF patients are frequently assayed for a certain number of bacterial species. There have been reports of unusual bacteria that are rarely isolated from the CF lungs but may be common inhabitants (Rogers et al., 2004). There are limited studies on the virulence factors of *Paracoccus* sp. reported in the literature. The current study found individual species of *P. yeeii* did not form biofilms under the experimental conditions in which OD\textsubscript{630} values were ≤ 0.05. *P. aeruginosa* isolates which form strong biofilms were inoculated with *Paracoccus* sp. at an equal ratio and found to be comparable to biofilm growth of *P. aeruginosa* PA01. In addition, mixed species *P. aeruginosa* ATCC27853 and *P. yeeii* 1968 exhibited a significant reduction in biofilm growth by 72 hours in comparison to the single strain (P = 0.003).

(A)
Figure 4.2: Comparison of growth of single and mixed species biofilms with *Paracoccus yeeii*.

(A) *Paracoccus yeeii* controls

(B) *Paracoccus yeeii* + *P. aeruginosa* PA01

(C) *Paracoccus yeeii* + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.
4.3.3 Adherence of single and mixed species of *Acinetobacter* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

*Acinetobacter* sp. has been implicated in a wide variety of nosocomial respiratory infections (Towner *et al.*, 1996). There is evidence to suggest that *Acinetobacter* sp. forms biofilms under static and dynamic conditions and mixed species interactions showed an alteration in the dynamic structure of *P. putida* (Hansen *et al.*, 2007). Quantitative analysis showed that cell attachment of the strain used in the present study was weak with an OD$_{630} \leq 0.003$ following 72 hours incubation. Mixed species biofilms of *P. aeruginosa* and *Acinetobacter* 736 at an equal ratio found the adherence of *P. aeruginosa* PA01 was significantly reduced at 72 hours in combination with this strain (P = 0.002). Similarly, *P. aeruginosa* ATCC27853 experienced a significant reduction in biofilm formation by 72 hours when grown in conjunction with the strain. The values shown in Figure 4.3 are not representative of the quantity of each species in mixed culture.
(A)

Optical density (630nm)

- Actinobacter 736

(B)

Optical density (630nm)

- PA01 + Acinetobacter 736
- PA01 Control
Figure 4.3: Comparison of growth of single and mixed species biofilms with *Acinetobacter* sp.

(A) *Acinetobacter* 736 control

(B) *Acinetobacter* 736 + *P. aeruginosa* PA01

(C) *Acinetobacter* 736 + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.
4.3.4 Adherence of single and mixed species of *Ralstonia* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

*Ralstonia* species have been recovered from a variety of clinical samples including blood and sputum. This species has been described as difficult to differentiate and misidentification can often occur with other species of the same genera as well as *Burkholderia* sp. (Coeyne et al., 1999). Individual species of *Ralstonia* did not form strong biofilms under the experimental conditions, with the exception of one isolate *Ralstonia* 746. This strain showed strong adherence at 48 hours and a decrease in biofilm growth at 72 hours at an OD$_{630}$ ≥ 0.05. There was no difference in biofilm adherence of *P. aeruginosa* PA01 when grown in mixed culture with all four *Ralstonia* species. Similarly, *P. aeruginosa* ATCC27853 showed no difference in the level of adherence at 24 and 72 hours when combined with these strains.

(A)
Figure 4.4: Comparison of growth of single and mixed species biofilms of *Ralstonia* sp.

(A) *Ralstonia* sp. controls

(B) *Ralstonia* sp. + *P. aeruginosa* PA01

(C) *Ralstonia* sp. + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.
4.3.5 Adherence of single and mixed species of *Herbaspirillum* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

*Herbaspirillum* strains were generally not strong biofilm formers as growth did not reach an OD $\text{OD}_{630} \geq 0.05$, under the conditions tested. Selected isolates grown in conjunction with *P. aeruginosa* PA01 did not alter the normal pattern of biofilm growth following 72 hours incubation. The study demonstrated that mixed biofilm growth of *P. aeruginosa* ATCC27853 and *Herbaspirillum* isolates was also comparable to the *P. aeruginosa* ATCC27853 control.

(A)
Figure 4.5: Comparison of growth of single and mixed species biofilms with *Herbaspirillum* sp.

(A) *Herbaspirillum* sp. controls

(B) *Herbaspirillum* sp. + *P. aeruginosa* PA01

(C) *Herbaspirillum* sp. + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.
The \textit{in vitro} model for studying biofilms formed on polystyrene pegs has many disadvantages for mixed cultures. The quantity of each species colonising the surface of the pegs or any structural changes in biofilm morphology could not be identified in the current study. As a result, a comparative analysis was carried out between the optical densities obtained from this assay and total viable counts of several interesting mixed biofilm cultures as described in \textbf{Sections 4.2.4} and \textbf{4.2.5}.

\textbf{4.3.6 Adherence of single and mixed species of \textit{Stenotrophomonas maltophilia} and \textit{P. aeruginosa} (PA01 and ATCC27853) to polystyrene pegs.}

\textit{Stenotrophomonas maltophilia} is an emerging nosocomial bacterial pathogen and has shown to form complex biofilm structures (Waters \textit{et al.}, 2007; Pompilio \textit{et al.}, 2010) although very little is known about the mechanisms of biofilm formation. \textit{S. maltophilia} is frequently cultured from sputum of CF patients. There have been reports of mild lung function decline with this species, however, patients tend to be older and co-infected with \textit{P. aeruginosa} (Goss \textit{et al.}, 2002). The isolates used in the current study had moderate levels of adherence at 24 hours showing evidence of progression to strong biofilm formation at 72 hours on polystyrene pegs with \textit{OD\textsubscript{630}} $\geq$ 0.05. Mixed species biofilms showed a significant increase in \textit{OD\textsubscript{630}} values when \textit{P. aeruginosa} PA01 and \textit{S. maltophilia} 668 were combined in comparison to the single strains (\textit{P} = 0.006). In addition, mixed biofilm cultures of \textit{S. maltophilia} (668, 747) and \textit{P. aeruginosa} ATCC27853 displayed strong adherence to polystyrene pegs with \textit{OD\textsubscript{630}} $\geq$ 0.05, with an increase in \textit{OD\textsubscript{630}} at 48 and 72 hours (\textit{P} = 0.001, \textit{P} = 0.017). Results are consistent with other studies that have identified these species to adhere to abiotic surfaces (deOliverira-
Garcia et al., 2003), however, it remains unclear if *P. aeruginosa* and *S. maltophilia* are mixing or if one isolate is dominant.

(A)
Figure 4.6: Comparison of growth of single and mixed species biofilms with Stenotrophomonas maltophilia.

(A) S. maltophilia controls
(B) S. maltophilia + P. aeruginosa PA01
(C) S. maltophilia + P. aeruginosa ATCC27853

Data represent the mean ± standard deviation of three independent experiments.
4.3.7 Viability of *P. aeruginosa* PA01 biofilms in conjunction with unusual CF strains.

Biofilm growth of selected unusual CF strains was monitored at 24 and 72 hours. No biofilm growth was observed for *Ralstonia* 702 and *Paracoccus* 1968 which is in agreement with the previous assay. *Acinetobacter* 736 established a stable biofilm following 72 hours incubation. In keeping with biofilms formed on polystyrene pegs, *S. maltophilia* exhibited greater biomass than the other species at 24 hours with an increase in growth at 72 hours.

Results presented in Figure 4.7 show biofilms consisting of two species differed to the previous assay for isolates *Ralstonia* 702, *P. yeeii* 1968 and *Acinetobacter* 736 in which growth rates seemed to be identical to the *P. aeruginosa* PA01 control. No observable difference to the growth of *P. aeruginosa* PA01 was detected with *S. maltophilia* 747. Mixed growth of *P. aeruginosa* PA01 and *S. maltophilia* 668 resembled biofilm growth and colony morphology of the *S. maltophilia* 668 control. This affect was seen at 24 hours and maintained until 72 hours. Results suggest that *S. maltophilia* may out-compete certain strains of *P. aeruginosa* for nutritional requirements and dominate mixed cultures in certain *in vitro* models.
(A)

(B)
(C) P. aeruginosa PA01 Paracoccus 1968 P. aeruginosa PA01 + Paracoccus 1968

(D) P. aeruginosa PA01 S. maltophilia 668 P. aeruginosa PA01 + S. maltophilia 668
Figure 4.7: Effect of unusual CF strains on the biofilm growth of *P. aeruginosa* PA01 measured as log CFU/ml. Results are based on different colony morphologies on TSA. The controls represent samples that are not in mixed culture. As a single species, *Paracoccus* 1968 did not show any biofilm formation. Results represent the mean ± standard deviation of three independent experiments.
4.3.8 Viability of clinical isolate, *P. aeruginosa* 1826 biofilms in conjunction with unusual CF strains.

Mixed species biofilm development was assessed with clinical isolate *P. aeruginosa* 1826 as there is a possibility that clinical samples may interact with CF isolates in a different manner to control strains. The results presented in Figure 4.8 differed with previous experiments. *P. aeruginosa* 1826 represented the dominant pathogen in mixed culture with the other species. The study also found that *P. aeruginosa* 1826 was the only viable isolate after 24 and 72 hours incubation with *S. maltophilia*. Findings suggest that clinical *P. aeruginosa* isolates may persist in mixed-culture biofilms with other CF pathogens.

(A)
Figure 4.8: Effect of unusual CF strains on the biofilm growth of *P. aeruginosa* 1826 measured as log CFU/ml. Results are based on different colony morphologies on TSA. The controls represent samples that are not in mixed culture. *Paracoccus* 1968 did not develop any biofilm at 24 or 72 hours. Results represent the mean ± standard deviation of
three independent experiments.

4.3.9 Effect of *S. maltophilia* cell-free supernatants and whole cells on *P. aeruginosa* biofilm development.

To examine the effect of *S. maltophilia* on the ability of *P. aeruginosa* to form biofilms two assays were carried out. *P. aeruginosa* and *S. maltophilia* cell-free supernatants or whole cells were grown together at an equal (1:1) ratio. In addition, *P. aeruginosa* biofilms were established for 24 hours and exposed to *S. maltophilia* culture supernatants or whole cells for an additional 24 hours to investigate any biofilm impairment during early developmental stages. Pre-grown *P. aeruginosa* (PA01 and 4505.2) biofilms treated with *S. maltophilia* supernatants showed a decrease in growth compared to the untreated control. These results did not reach statistical significance, with the exception of two isolates, *P. aeruginosa* PA01 (P = 0.032) and clinical isolate *P. aeruginosa* 4505.2 (P = 0.036). Since *P. aeruginosa* 4505.2 produced substantially more biofilm than the other strains as quantified by crystal violet staining the results suggest that the effect of the *S. maltophilia* supernatant does not depend on the level of biofilm produced.

Upon co-culturing *P. aeruginosa* and *S. maltophilia* at an equal (1:1) ratio, the results revealed *S. maltophilia* cell-free supernatants and whole cells did not promote or decrease *P. aeruginosa* growth in most cases. Virulence factors produced by *P. aeruginosa* such as phenazines have shown to possess toxic effects towards other bacterial species (Kerr *et al.*, 1999). This observation suggests *P. aeruginosa* may be inhibitory towards the other species, however, it remains unclear if changes occur during structural development of *P.*
aeruginosa biofilms with this species. The strains selected for the study secrete pigments which may be enabling P. aeruginosa to persist in this multi-species environment.

Table 4.3: Summary of phenotypic characteristics of selected P. aeruginosa reference and clinical isolates.

<table>
<thead>
<tr>
<th>P. aeruginosa strain</th>
<th>Pigment/No pigment</th>
<th>Mucoid/Non-mucoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa PA01</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td>P. aeruginosa 1826</td>
<td>pigment</td>
<td>non-mucoid</td>
</tr>
<tr>
<td>P. aeruginosa 4505.2</td>
<td>pigment</td>
<td>mucoid</td>
</tr>
</tbody>
</table>

Figure 4.9: The effect of S. maltophilia supernatants or whole cells on established biofilms of P. aeruginosa. After 24 hours of growth, P. aeruginosa biofilms were exposed to S. maltophilia for an additional 24 hours. Values represent the mean ± standard deviation of three independent experiments.
(A) *P. aeruginosa* PA01

(B) *P. aeruginosa* 1826
Figure 4.10: Mixed species biofilms of *P. aeruginosa* with *S. maltophilia* supernatants and whole cells. Bacteria were inoculated at a 1:1 ratio and incubated for 24 hours, 35°C. Control values represents biofilm formation of each single species. Values represent the mean ± standard deviation of three independent experiments.
4.3.10 Effect of antibiotics on the growth of mixed-culture biofilms of \textit{P. aeruginosa} and \textit{S. maltophilia}.

The aim of this study was to determine the antibiotic sensitivity of \textit{P. aeruginosa} and \textit{S. maltophilia} isolates grown singly and in co-culture. To assess antibiotic sensitivity biofilms were grown as described in Section 4.2.5, treated with antibiotics (tobramycin and ceftazidime) for a further 24 hours and biomass was quantified by crystal violet staining. The effect of the antibiotics against \textit{P. aeruginosa} PA01 in mixed culture was comparable to response of each single species at concentrations ranging from 8 to 256 µl. The activity of tobramycin against mixed cultures of clinical isolate \textit{P. aeruginosa} 4505.2 and \textit{S. maltophilia} 668 strains showed that the mixed culture was more sensitive to lower concentrations of tobramycin (one-way ANOVA). The results indicate that \textit{S. maltophilia} may be increasing the sensitivity of \textit{P. aeruginosa} 4505.2 to this antibiotic.

To further monitor if \textit{P. aeruginosa} biofilms treated with \textit{S. maltophilia} cell-free supernatants were more susceptible to antibiotic exposure, biofilms were developed as described in Section 4.2.5. \textit{P. aeruginosa} PA01 treated with the supernatant of \textit{S. maltophilia} 668 responded more effectively to ceftazidime treatment than the \textit{P. aeruginosa} PA01 control. The susceptibility of \textit{P. aeruginosa} PA01 treated with \textit{S. maltophilia} 668 and 747 supernatants to tobramycin was comparable to the \textit{P. aeruginosa} PA01 control. When clinical isolate \textit{P. aeruginosa} 4505.2 was treated with \textit{S. maltophilia} (668, 747) under the same conditions, a slight increase in sensitivity to tobramycin of \textit{P. aeruginosa} 4505.2 treated with \textit{S. maltophilia} 747 supernatant was observed. Similarly, an increase in sensitivity was detected when \textit{P. aeruginosa} 4505.2 exposed to \textit{S. maltophilia}.
*maltophilia* 668 supernatant was treated with ceftazidime (student’s t-test). Findings indicate the supernatants of *S. maltophilia* isolates used in the current study are rendering *P. aeruginosa* 4505.2 more susceptible to antibiotic treatment.

Mixed species biofilms of *P. aeruginosa* (PA01, 4505.2) and *S. maltophilia* 668

Figure 4.11: The effect of antibiotics (tobramycin) against mixed species biofilms. Mixed species biofilms of *P. aeruginosa* and *S. maltophilia* were grown as described in Section 4.2.5. Mixed and single species cultures were exposed to various concentrations of tobramycin for an additional 24 hours. Results represent the average of two independent experiments.
(A) *P. aeruginosa* PA01

![Graph showing optical density (630nm) vs. concentration of antibiotic (µl) for different strains of *P. aeruginosa* and *S. maltophilia*.](image-url)
Figure 4.12: The effect of antibiotics (tobramycin, ceftazidime) biofilms of *P. aeruginosa* (PA01, 4505.2) treated with *S. maltophilia* (668, 747) supernatants as described in Section 4.2.5. Biofilms were exposed to various concentrations of tobramycin and ceftazidime for an additional 24 hours. Results represent the average of two independent experiments.
4.3.11 Susceptibility testing of cystic fibrosis species to Tobramycin.

Table 4.4 illustrates the sensitivity of planktonic cultures of selected unusual species to tobramycin at concentrations corresponding to traditional MIC’s. Paracoccus sp. displayed high sensitivity to tobramycin in which the MIC and MBC values were generally not greater than a concentration of 1 μg/ml. The sensitivity of Ralstonia sp. to tobramycin appeared to be specific to each individual strain. Three strains were weakly sensitive in which MIC and MBC values were > 64 μg/ml. One isolate, Ralstonia 746 displayed moderate sensitivity to aminoglycoside therapy at an MIC value of 4 μg/ml and MBC value of 16 μg/ml. Additionally, weak bactericidal activity was observed against S. maltophilia strains at an MBC > 64 μg/ml. Similarly, the antibiotic exhibited weak bactericidal activity against Acinetobacter 736 and Herbaspirillum isolates at MBC > 64 μg/ml. Results from the present study indicate alternative antibiotics to commonly used aminoglycosides may be required for CF patients infected with these bacterial infections.
Table 4.4: Comparison of susceptibilities of cystic fibrosis species grown planktonically to aminoglycoside antibiotic, Tobramycin (μg/ml).

<table>
<thead>
<tr>
<th>Unusual cystic fibrosis species</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ralstonia</em> 702</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Ralstonia</em> 659</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Ralstonia</em> 735</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Ralstonia</em> 746</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td><em>S. maltophilia</em> 668</td>
<td>64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>S. maltophilia</em> 747</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 767</td>
<td>4</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 762</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 320167</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 320012</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 317533</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 317493</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> 317466</td>
<td>16</td>
<td>&gt;64</td>
</tr>
<tr>
<td><em>Acinetobacter</em> 736</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td><em>Paracoccus</em> 1426</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
</tr>
<tr>
<td><em>Paracoccus</em> 1429</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Paracoccus</em> 1968</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Paracoccus</em> 9205</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Paracoccus</em> 6155</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Paracoccus</em> 4446</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
4.3.12 SDS-page and LC/MS analysis of *S. maltophilia* proteins.

The quantification of protein was determined using the nanodrop\textsuperscript{TM} 1000. The protein concentrations of culture supernatants of *S. maltophilia* 668 and *S. maltophilia* 747 were 2.8 mg/ml and 3.7 mg/ml, respectively. The proteins of these culture supernatants were separated by SDS-page followed by LC/MS analysis of any different protein bands. *S. maltophilia* has previously shown to interfere with the growth of *P. aeruginosa* biofilms.

The results presented in Figure 4.13 show similar protein bands between the *S. maltophilia* strains at 130 kDa, 100 kDa and 70 kDa. Other findings were protein band at approximately 63 kDa which was found to be unique to *S. maltophilia* 668 and 55 kDa protein band was unique to *S. maltophilia* 747. These bands were excised from the gel, trypsin digested and analysed by LC/MS. *S. maltophilia* 668 (70 kDa) was selected as it was one of the common proteins to both strains.

The proteins identified by MASCOT are listed in Table 4.5 with sequence coverages. The three proteins of *S. maltophilia* 668 (band A) were identified as dihydrolipoamide dehydrogenase, cytosol aminopeptidase and s-adenosyl-L-homocysteine. Other proteins that were identified in *S. maltophilia* 747 (band C) were matched to type II citrate synthase, chaperonin GroEL and elongation factor G. The protein at band B was identified as 60 kDa chaperonin which was a common peptide to both *S. maltophilia* strains.
Figure 4.13: Protein analysis of culture supernatants of *S. maltophilia* strains 668 and 747. Proteins were separated by 10% SDS-page and stained with coomassie blue. The migration of molecular weight (kDa) standards is shown on the right of the image.

1. *S. maltophilia* 668
2. *S. maltophilia* 668
3. *S. maltophilia* 747
4. *S. maltophilia* 747
5. Marker
Figure 4.14: Total ion chromatograms from the LC/MS analysis of bands A, B and C of *S. maltophilia* proteins.
Table 4.5: Summary of identified proteins of *S. maltophilia* by LC/MS analysis.

*S. maltophilia* proteins from bands shown in [Figure 4.13](#) were excised and trypsin digested prior to LC/MS identification.

<table>
<thead>
<tr>
<th>Band:</th>
<th>Identification:</th>
<th>Mass (Da)</th>
<th>Sequence coverage (%)</th>
<th>Peptides matched</th>
<th>gi</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Dihydrolipoamide dehydrogenase</td>
<td>50683</td>
<td>20</td>
<td>7</td>
<td>344208053</td>
</tr>
<tr>
<td>A</td>
<td>Cytosol aminopeptidase</td>
<td>51519</td>
<td>9</td>
<td>4</td>
<td>344205968</td>
</tr>
<tr>
<td>A</td>
<td>S-adenosyl-L-homocysteine</td>
<td>53144</td>
<td>6</td>
<td>3</td>
<td>194364404</td>
</tr>
<tr>
<td>B</td>
<td>60 kDa chaperonin</td>
<td>57281</td>
<td>65</td>
<td>34</td>
<td>344209060</td>
</tr>
<tr>
<td>C</td>
<td>Type II citrate synthase</td>
<td>46461</td>
<td>40</td>
<td>11</td>
<td>491553279</td>
</tr>
<tr>
<td>C</td>
<td>Chaperonin GroEl</td>
<td>57312</td>
<td>12</td>
<td>3</td>
<td>194367398</td>
</tr>
<tr>
<td>C</td>
<td>Elongation factor G</td>
<td>77980</td>
<td>7</td>
<td>3</td>
<td>190572941</td>
</tr>
</tbody>
</table>

Band A: Dihydrolipamide dehydrogenase

MAEQFDVVVI GAGPAGYHAA IRAAQGLGLKT ACIDAAALGD GKPALGGTCG
RVGCIPSAL ILSSRQFWNM GHIFGDHGIS FKDARIDVEA MVGREKDIKV
QPTGGIGMLF KANKVAAAYG FGERPGNIV KVTQHDSBIV ELEGTVNIA
AGSDSIELFF AKFGDRTIVD NVGGLDFTTRV PNRNAVIGAV VIGLELGWV
KRLGAEVTL EALPFLAVA DAEVAKTAAK EFKKGGLDIR LGAKVSDKTEI
TGKGKKKVEV VTTDSGSEGK TLTVDKLLVA VGRAATKGI LAEGTVKIN
ERQIEVDAH CHTGVNGWVA VEGCRGPMI ARKGFEESGA VAEILAGFP
HVNFTIPWV IYTEPELAVV GKEAQLRAE GIPYKAGSFY PAMSARAVAM
IPEPGVKIL AHAETDRILG MHLVGAIVSE LVHEGVLTM EFSGADDLAR
ICHAPSLSE VIHDAMAVS KRAINRKHAK
Band A: Cytosol aminopeptidase

**MALLETLNHV** APAAATVCLVL VVGAYADHTLT PAAQALDAA SGRRLAALAQ
**RGDLGKTGTA** TLLTLDLPGV TAPPVLVQGL GEAARFGVPQQ YLKAVGAVVR
**ALKAGAARSA** LFTLSEVAIK DRDAAAWAIQ AVIAADHAY RTYATLGGK
**ADDAGLAQLA** VGAADQAQLA GQAIAAGVE FARELGNLFP NYCTFAYLAE
**VGGKFAEGHD** GAAEIILSET QMEALGMLSL LAVARGSAHR PRLVVLKWTG
**AGDAGKYVLV** KGKSTDGG VNLKTQQGGL EMKMDGCGGA NVIGTFVAVAV
**KAKLPLNLVV** VVPPVANEAID GNAAYRPSDV TSMGSKTIEV GMTDAEGRIL
**LCDALTYAQR** TLTAGCMIVAL GHQTAGMLSS HDDLANELLA
**AGEHVFDRARL** PLPLNDFADYY MLSTFDAYV NIGRRAAGAI TACGFLSRFRA
**EGQRWAHLDI** AGVADDEGKR GMATRGPMVL LSQMLLQVQA RA

Band A: S-adenosyl-L-homocysteine

**MMNAVAKFST** EDDYKIRDIT LADWGGKEDG IAEGHEMPGLM SIRKKHALSL
**PLKGVRVTGS** LMMTHIQAVTL ILTLDKIDAG VRWASCNHIS TQDRAAAIAA
**ATGGTFQAVMK** GESLEEEYWSC TDLALTILTA DGTQLFELPV VDDGGVTLLL
**IHGKYELEN** STWVNEPAAH HEEQVIKNLL KRVATERPGY WGRVWKG

Band A: 60 kDa chaperonin

**MAAKDIRFGE** DARSMMVRGV NVLANAVKAT LGPKGRNVVL EKSFGAPTIT
**KDVGVSVEKE** ELADKFNEMG AQMVKEVASR TNYADGQTT TAVLQALI
**REGAKAVAAG** MNMDLKKRG DIKAUVVAAVE LNTKSKPTAD DMAIAQVTGI
**SANSDISGQ** IIAADMKERG KEGVITVEG SGLDNELDV VGMQFDRGYL
**SPIFENQQPS** QTADLDDDPFI LHDLKKISNV RDLLEVFLEGV AKAGKPLLIV
**AEEETVGAVLS** TLTVAQTIRG FKVVAVAFAG FDYRKAMLE DMVLTGTCV
**ISEEEVLISLE** KAITKLGDA KKQVSKENT TIDVQGDKA AVDARVQGQK
**TQIOQTSSDY** DREKIQERVA KLAVGAVVK VGASTEINMK EKKRVDVADAL
**HATRAAVEEG** VVPPGQGVTAL RKVATLGLKL GANEDQHNGI QIALAMEAPE
**LREIVANAGE** EPSVINKVK EGTFSGYNA ATGEFQDMLO FGILDPKVVT

Band B: 60 kDa chaperonin

**MALLETLNHV** APAAATVCLVL VVGAYADHTLT PAAQALDAA SGRRLAALAQ
**RGDLGKTGTA** TLLTLDLPGV TAPPVLVQGL GEAARFGVPQQ YLKAVGAVVR
**ALKAGAARSA** LFTLSEVAIK DRDAAAWAIQ AVIAADHAY RTYATLGGK
**ADDAGLAQLA** VGAADQAQLA GQAIAAGVE FARELGNLFP NYCTFAYLAE
**VGGKFAEGHD** GAAEIILSET QMEALGMLSL LAVARGSAHR PRLVVLKWTG
**AGDAGKYVLV** KGKSTDGG VNLKTQQGGL EMKMDGCGGA NVIGTFVAVAV
**KAKLPLNLVV** VVPPVANEAID GNAAYRPSDV TSMGSKTIEV GMTDAEGRIL
**LCDALTYAQR** TLTAGCMIVAL GHQTAGMLSS HDDLANELLA
**AGEHVFDRARL** PLPLNDFADYY MLSTFDAYV NIGRRAAGAI TACGFLSRFRA
**EGQRWAHLDI** AGVADDEGKR GMATRGPMVL LSQMLLQVQA RA

Band B: 60 kDa chaperonin

**MNNAVAKFST** EDDYKIRDIT LADWGGKEDG IAEGHEMPGLM SIRKKHALSL
**PLKGVRVTGS** LMMTHIQAVTL ILTLDKIDAG VRWASCNHIS TQDRAAAIAA
**ATGGTFQAVMK** GESLEEEYWSC TDLALTILTA DGTQLFELPV VDDGGVTLLL
**IHGKYELEN** STWVNEPAAH HEEQVIKNLL KRVATERPGY WGRVWKG

Band B: 60 kDa chaperonin

**MAAKDIRFGE** DARSMMVRGV NVLANAVKAT LGPKGRNVVL EKSFGAPTIT
**KDVGVSVEKE** ELADKFNEMG AQMVKEVASR TNYADGQTT TAVLQALI
**REGAKAVAAG** MNMDLKKRG DIKAUVVAAVE LNTKSKPTAD DMAIAQVTGI
**SANSDISGQ** IIAADMKERG KEGVITVEG SGLDNELDV VGMQFDRGYL
**SPIFENQQPS** QTADLDDDPFI LHDLKKISNV RDLLEVFLEGV AKAGKPLLIV
**AEEETVGAVLS** TLTVAQTIRG FKVVAVAFAG FDYRKAMLE DMVLTGTCV
**ISEEEVLISLE** KAITKLGDA KKQVSKENT TIDVQGDKA AVDARVQGQK
**TQIOQTSSDY** DREKIQERVA KLAVGAVVK VGASTEINMK EKKRVDVADAL
**HATRAAVEEG** VVPPGQGVTAL RKVATLGLKL GANEDQHNGI QIALAMEAPE
**LREIVANAGE** EPSVINKVK EGTFSGYNA ATGEFQDMLO FGILDPKVVT

Band C: Type II citrate synthase

**MSDLLQVTIN** AGDKSVVVLV IKPTLGDNCV DIAKLTKEGA FFTYDGGFTA
**TASCKSAITY** IGDQGVLGLY RGYPIEQGLSE KSSYVEVAYL LINGERPSAE
**QLKAPREDIET** AENAVDESIN TLIGSFAKDA HEMAILAAAI AQLSAIYHDS
**LDSLDASQQR** QAARLIAKF PTLSAIYRH GKKFLPMKPD TLSSLYFSRL
**KQFTFQSDQQ** YLDINQPDVKA LDDPLFILHAD NEQNPAYSTV RLVSGTGANP
**YASVAAGVITL** LWPGHAGGN EAVLKMLEEIS GSAADVESAV VKAKDTSFG
**RIMGFRVRV** KNDFDFRAKVI GEMTSKVLQF LGVQPDLLDV AVKLEQAALQ
**DEIFVPARKLY** PNVFYSGIIK YKALQIPTEM EVTMSFLRGT SGWVSHWLEQ
**QVDPEMKIRG** PRQVYTGSDV RGYQG
Band C: Chaperonin GroEL

MAAKDIRFGE DARSMVRGVR NVLANAVKAT LGPKGRNVVL EKSGFGAPTIT
KGVSVMVEAI ELAKFENMG AQMVKEAVSR TNDDAGGTT TATVLAQALI
REGAKAQAAG MNPHDLKRGRI DKAVVAANVE LKSISKPTAD DFIAAQVGTI
SANSDEIYIQ IIAIDAKKVEY KEGVITYVEG SGLDNLQDVAL KGMQFGIRGL
SPYFINIQEQS QTADDDFPFI LLHDKKISNV RDLPVLEGV AKAGFKLLIV
ABEEVEGEALAI TLVWVITIRGI KVVAKVAKPG FDQRRKAMLE DMAVLITGGTV
IIEEVGSLSELE KATIKDLGRO KKVQVSKENT TIIQGVGDFA AVDSRVQAQIK
TQIQDTSYDD DREKIQERVVA KLAGGAVAKV VGASTEEMK EKKDRTDDDAL
HATRAAEEEG VVPEGGVALV RAVSAALAGLQ GANEDQHGI QIALRAMEAP
LREIVANAGER EPSIVNKVK EGTGSGFYNA ATGEFGDLMQ FGILDFTKVFT
RSALQNAASIST NGLMITEAM VAEAPKDEEP MGAGGGMGFLGGMGMDF

Band C: Elongation factor G

MARSTPIERY RNFGMAMID AGKTTSERI LFTYGKSHKI GEVHDOGAATM
DWMEQEQERQG IITQSAATTA FWKGMDEKSLP EHRFNIIDTP GHVDTIEVE
RSRLVLOGAV FVLCAVGGVQ PQGSETVWSQA HNYHVPRIAIF VNRMDRTGAN
FQKVGVQLKGA KLGAVAVQMQ LPQAGEDNFK GVDLLKNGA IHWDEASQGM
KFYESDPAD LQAQAEARQF LMVTTAEASA EELMELKLYGQ EELAKAEILN
ALRTRATELLI VVYMCGSAF KNFGQAMLD GVQILLPSPV DVPDVKVGVDL
DDTVEQMTIK SDDKAFQSSL ALFIITDDPV GALTFFRVSQ GTLNGDDTQL
NCSVKKERI GRILQMSSSH RREEKEVLAG DIAAVGLKLD TTTGDTLCAY
DAPLIERMTT FPEPVISMAV EPFTKSDQEF MGLALGRLAQ EDPFSRVKTD
EEGQTIISG MGELHDIIIV DLKRFKNFEV ANVGAQVSAY RETITADVK
SDYKHAVQSG GKGQYGHVVI ELSPITAADR ADFKLAPAID DDLFLINDIT
GGVIFPEEFP SVEKGLRSTR TSGPLAGFPV UVDVVKLVFG SYHVDSDSEM
APKLAASSMAF KQGAKAEKFV LLEFIMKVEI VTFDYQGQDV MGDSVRRRVG
LQGSDTTDGGQ SASINAMIF LGEMFYAYA LRSQITGGRAT FTMFDHYFP
APTNAEAVMK KGK

Figure 4.15: The seven peptides sequenced of *S. maltophilia* proteins identified from bands A, B and C. All peptides that have been matched by LC/MS are highlighted in red.
4.4 Discussion

Mixed species biofilm interactions have been the focus of many literature studies since the airways of CF patients have been known to harbour many diverse bacterial communities (Zemanick et al., 2011). Although *P. aeruginosa* accounts for approximately 80% of CF related infection, other strains are frequently identified within the same environment (LiPuma et al., 2010). Many reports indicate unusual species can often be misidentified as those that are more common (Coeyne et al., 1999). This study provides an indication of the characteristics of unusual CF species that have been isolated from the CF lung and their ability to form biofilms.

Findings from the current study indicate that while the biofilm forming capacity of clinical CF *P. aeruginosa* strains remains strong, adherence of other clinical CF species to polystyrene pegs remains much weaker. *Paracoccus* sp. and *Herbaspirillum* sp. did not develop biofilms under the experimental conditions suggesting biofilm formation is not a specific virulence factor for colonisation in the CF lung. In addition, *Acinetobacter* 736 strain used in this study was viable at 72 hours but did not form biofilm on polystyrene pegs at any time point. Recent studies have shown that the amount of exopolysaccharide produced is involved in biofilm formation of this species (Tomaras et al., 2003). Often bacteria that do not form biofilms require different adaptive mechanisms for colonisation which have not been identified in this study. Recently, Caraher et al., (2007a), found *Pandoraea* sp., which have been associated with deterioration of CF lung function was not attributed to biofilm formation. *Pandoraea* sp. most likely acts as an inflammatory
stimulant for other pathogens that cause infection.

Among the Ralstonia sp., tested, strain 746 adhered to polystyrene pegs with an OD value $\geq 0.05$ within 72 hours indicating biofilm formation may be a specific virulence trait to each individual strain. Previously, Anderson et al., (1990), identified biofilm formation of this genus with Ralstonia picketti isolates. Recovery of Ralstonia sp., in CF sputum samples in generally low compared to other species (Coeyne et al., 2002) and occasionally misidentification can occur (Henry et al., 2001). Nosocomial outbreaks of the species are rare and have been mainly linked to contamination by hospital equipment (Roberts et al., 1990; Gardner et al., 1984). A clinical study by Burns et al., (1998), found a total of 2 out of 559 CF patients were infected with the bacterium. Previous studies have described Ralstonia solanacearum as motile when transferred into water or a suitable medium (Kelman et al., 1973). Overall, limited information is available about the virulence factors of this species.

Although S. maltophilia is a prominent CF pathogen (Fitzsimmons et al., 1993) there have been limited studies of an increased rate in lung function decline. The CFRI reports approximately 2.7% of CF patients tested positive for S. maltophilia in 2011. Other studies have reported that S. maltophilia has been isolated from the CF lungs in approximately 30% of CF patients (Steinkamp et al., 2005). S. maltophilia used in this study showed strong adherence to polystyrene pegs with high OD$_{630}$ $\geq 0.05$ at 72 hours suggesting biofilm formation may be an important virulence trait of this species. This was consistent with the study by Pompilio et al., (2011), by which CF patients' established S.
*maltophilia* infection by biofilm formation and diversity in phenotypic properties during the course of infection. Studies have reported that flagella may allow bacterial adhesion of this species to various abiotic surfaces (Sampaio *et al.*, 2009). Exoproductions produced by *P. aeruginosa* that cause damage to the epithelial mucosa may enhance the ability of *S. maltophilia* colonisation (DeAbreu *et al.*, 2001; Karpati *et al.*, 1994).

The present study focused on mixed biofilm interactions between *P. aeruginosa* and the unusual CF species. According to Spilker *et al.*, (2008), species that are less commonly found in the CF lungs are less frequently identified as the main cause of chronic lung disease but may be contributing to lung deterioration and tolerance to antibiotic treatment. *Paracoccus* sp. and *Herbaspirillum* sp. which did not readily form single species biofilms did not appear to influence *P. aeruginosa* biofilm formation on polystyrene pegs. Similarly, Spilker *et al.*, (2008), found a reduced frequency of *Herbaspirillum* isolates in the CF lungs likely to be caused by other more common strains overtaking *Herbaspirillum*. A clinical study by Filkins *et al.*, (2012), found *P. aeruginosa* was the main organism in the majority of CF patients. Tomlin *et al.*, (2001), described co-colonisation of the lungs with *P. aeruginosa* and other strains such as *B. cepacia* can lead to more serious pulmonary decline than *P. aeruginosa* alone. Interestingly, *Acinetobacter* 736 which did not develop biofilm on the pegs reduced the biomass of both *P. aeruginosa* strains (PA01 and ATCC27853) at 72 hours. When viable counts were obtained of the same strains to quantify the biofilm produced by each species, no difference in the growth of *P. aeruginosa* was observed suggesting that *Acinetobacter* 736 may be influencing the attachment of *P. aeruginosa*. 205
Many patients who acquire *S. maltophilia* have a higher risk of contracting *P. aeruginosa* and *B. cepacia* (Goss et al., 2002). A recent clinical CF study by Saminos *et al.*, (2012), found 33.8% of *S. maltophilia* patients were co-infected with other pathogens. An increase in biofilm growth was observed with *P. aeruginosa* PA01 and *S. maltophilia* 668 on polystyrene pegs at 72 hours which may indicate one species is enhancing biofilm formation of the other. Mixed biofilm cultures of *P. aeruginosa* ATCC27853 and *S. maltophilia* 668 and 747 showed an increase in biofilm growth at 72 hours in the same model. Additionally, the cell-free supernatants showed a significant reduction in biofilm growth of *P. aeruginosa* PA01 and clinical isolate *P. aeruginosa* 4505.2. It was interesting that *P. aeruginosa* 4505.2 was mucoid and formed a biofilm of greater cell density than any other strain used in this part of the study. There is a possibility that extracellular products secreted by *S. maltophilia* are able to disrupt established *P. aeruginosa* biofilms. Previous reports have suggested that extracellular products such as polysaccharides produced by some bacteria are able to control biofilm development during infection (Pihl *et al.*, 2010). Recently, Ryan *et al.*, (2008), indicated the cell-cell signalling system of *S. maltophilia*, known as diffusible signal factor (DSF) is not restricted to xanthomonads. The study found *S. maltophilia* alters the biofilm structure of *P. aeruginosa* through DSF signalling. Resistance of *P. aeruginosa* to cationic antimicrobial peptides (CAMP) may be allowing growth and survival of CF strains in the presence of species that produce DSF since CAMP is a component of the innate immune system. The other *P. aeruginosa* strains (PA01 and 1826) remained unaffected in co-culture at 24 hours. Since pigment production was a virulence factor of the selected *P. aeruginosa* isolates used in the current study, this may have allowed *P. aeruginosa* to
gain a competitive advantage during dual-species biofilm formation with *S. maltophilia* supernatants and whole cells.

Recent studies have demonstrated quorum sensing influences signalling between bacterial communities (Reidel *et al.*, 2001; Eberl *et al.*, 2004). An *et al.*, (2006), reported flagella and type-IV pili influence interactions between *P. aeruginosa* PA01 and *Agrobacterium*. Reidel *et al.*, (2001), found the N-acyl-homoserine lactone (HSL) quorum sensing system enhances synergistic interactions between *B. cepacia* and *P. aeruginosa* during biofilm formation. The study by Yang *et al.*, (2011), showed type-IV pili facilitated mixed species aggregate formation of *P. aeruginosa* and *S. aureus* under flow conditions. Harriott *et al.*, (2009), suggested that the up-regulation of matrix production by *S. aureus* may occur during co-culture with *C. albicans* since differences between single and mixed structural components were observed.

The bacterial interactions that were found in the current study highlight the importance of examining the response of these species to antibiotics. Carmeli *et al.*, (1997), suggested the use of broad spectrum antibiotics in CF patients could lead to acquisition of *S. maltophilia*, in particular the use of carbapenem antibiotics. Since biofilm formation of *P. aeruginosa* and *Burkholderia* sp., is associated with an increase in antibiotic resistance (Cos *et al.*, 2010), susceptibility of tobramycin was assessed for planktonic cultures of this collection of species. Aminoglycosides such as tobramycin are one of the main antibiotic classes for treating CF infections. Interestingly, strains that did not form biofilms were not sensitive to antibiotic treatment such as *Ralstonia, Acinetobacter* and
Herbaspirillum species. Paracoccus sp. displayed the most sensitivity at MIC and MBC ranging 0.25 – 1 μg/ml. S. maltophilia strains resistant to aminoglycosides are known to be substrates of aminoglycoside phosphotransferase (APH) enzymes (Okazaki et al., 2007). Otkun et al., (2005), found antimicrobial resistance of S. maltophilia significantly higher than other species, mainly mediated by intrinsic factors such as multidrug efflux pumps. Further testing of the antimicrobial susceptibility of mixed species biofilms found P. aeruginosa 4505.2 showed greater sensitivity to tobramycin indicating S. maltophilia can reduce the tolerance of mucoid P. aeruginosa isolates. The study by Skillman et al., (1999), described interactions between the matrixes of two species may result in a thicker and more protective barrier to foreign agents, particularly between Enterobacter agglomerans and Klebsiella pneumonia.

Previous studies have suggested that expression of proteins secreted by CF bacteria may vary during initial and chronic infection. The results presented in this section of the study found S. maltophilia strains may interfere with the growth of P. aeruginosa biofilms. SDS-page was employed to detect any differences between the CF S. maltophilia strains. A protein at approximately 63 kDa was detected in S. maltophilia 668 and a protein at approximately 50 kDa in S. maltophilia 747. According to Hanna et al., (2000), these differences among specific strains may be influencing their virulence during CF lung infection. Further information of these protein bands was carried out by LC/MS analysis. A peptide at 70 kDa which was common to both S. maltophilia strains was recognised as a 60 kDa chaperonin. Hartl et al., (1996), described the chaperones to assist in the folding of newly synthesised polypeptides and folding of proteins that have denatured. The S.
*maltophilia* 668 proteins identified at a unique band A were dihydrolipoamide dehydrogenase, cytosol aminopeptidase and s-adenosyl-l-homoserine. Dihydrolipoamide dehydrogenases are homodimeric flavoproteins that are known to enhance virulence and multiplication of infection of pneumococcal disease (Smith *et al.*, 2002). The aminopeptidases have been shown to act as repressors or activators in the operon regulation of virulence genes in many bacterial species (Woolwine *et al.*, 1999). In addition, s-adenosyl-l-homoserine has an important role in cellular homeostasis (Loenen *et al.*, 2006). This identification provides an insight into the proteins that may be allowing certain *S. maltophilia* strains to gain a competitive advantage with other CF bacteria. Other proteins identified in *S. maltophilia* 747 were elongation factor G and type II citrate synthase. Kunert *et al.*, (2007), showed that elongation factor G is a surface protein of *P. aeruginosa* that enhances attachment and immune evasion and limited information was available on the role of type II citrate synthase. A previous study by Kelly *et al.*, (1990), found *P. aeruginosa* strains that have the mucoid phenotype expressed different proteins to non-mucoid strains. In addition, Karaba *et al.*, (2013), found that protein secretion by *S. maltophilia* which caused the supernatants to have protease activity was dependent on the type 2 secretion system. Another study by deOliveira-Garcia *et al.*, (2002), found flagellin protein at 38 kDa was common to *S. maltophilia* strains but the molecular mass differed among strains. From the data presented it is evident that there are differences between CF *S. maltophilia* isolates. The peptides that have been characterised will allow further studies to detect the proteins that may be acting as virulence factors during co-infection.
Chapter 5: General Discussion and Conclusions

Biofilms are communities of bacterial cells growing on a surface encased within an exopolysaccharide matrix. It has been estimated that approximately 65% of bacterial infections are related to biofilm formation. Antibiotic resistance of bacteria growing in biofilms is dependent on a number of factors such as the exopolysaccharide polymers composed of alginate which reduces the ability of antimicrobials to penetrate the cells. \textit{P. aeruginosa} isolates from cystic fibrosis patients can be highly antibiotic resistant due to biofilm formation (Costerton \textit{et al.}, 2003). The main aim of this study was to explore the activity of potential anti-biofilm treatments such as antibiotic combination therapy and synthesised metal-based complexes with the aim of identifying novel agents that may be inhibiting \textit{P. aeruginosa} reference and clinical strains. Mixed species biofilm interactions were also explored to identify any species that may be altering the growth of \textit{P. aeruginosa}. The clinical isolates recovered from sputum samples from different Irish CF centres were useful in understanding the responses of \textit{P. aeruginosa} in a clinical context.

The results showed that clinical isolates produced different phenotypic characteristics which may be influencing specific adaptive behaviour and response to treatment. The results in Chapter 2 demonstrated that phenotypic characteristics are important components for biofilm formation, \textit{in vivo} pathogenesis and antibiotic susceptibility of \textit{P. aeruginosa}. Biofilm density differed with each isolate and each model. Many strains that adhered weakly to polystyrene pegs showed greater biomass determined by total viable counts, indicating that biofilms formed on pegs are not representing fully developed
biofilm structures. Houry et al., (2009), described motility as a major factor influencing the ability of cells to find suitable places to settle. It was evident from the in vivo characterisation of *P. aeruginosa* that phenazines are major virulence factors of CF isolates influencing *G. mellonella* mortality.

The results also suggested that strains may have different susceptibilities to antibiotic single and combination therapy. Resistance to antimicrobial agents is generally higher in *P. aeruginosa* biofilms than in planktonic cells (Nickel et al., 1985). Antibiotics such as aminoglycosides have been shown to diffuse into the biofilm at a slower rate due to binding with the exopolysaccharide matrix (Hogdes and Gordon, 1991). In the current study most of the antibiotic combinations against the biofilms were similar to the activity of the antibiotics used as single agents, however, this study evaluated the importance of choosing the most suitable treatment for a particular patient. E-test results showed all *P. aeruginosa* isolates were susceptible to colistin, however, this effect did not translate against the biofilms. *P. aeruginosa* also displayed resistance to antibiotic combinations involving colistin. The OprH gene increases membrane permeability of biofilms maintaining a higher rate of resistance to colistin therapy (MacFarlane et al., 1999; Bell et al., 1991).

Tobramycin and ciprofloxacin were examined in combination with other antipseudomonal agents as they displayed the greatest inhibitory activity as single antibiotics. Synergistic interactions of tobramycin-ceftazidime and tobramycin-meropenem (FIC ≤ 0.5) were observed against clinical CF isolate *P. aeruginosa* 1826 and
tobramycin-ceftazidime against clinical CF isolate \textit{P. aeruginosa} 4505.2. In addition, tobramycin-ceftazidime was also synergistic against \textit{P. aeruginosa} 1826 but had a reduced effect against \textit{P. aeruginosa} 4505.2. Although synergistic antibiotic interactions were limited in this study, findings suggest that two or more antibiotics that have different mechanisms of action may be used as alternative treatments to single antibiotics. The results showed the synergistic activities of antibiotic combinations seen in 24 hour biofilms may be reduced against 72 hour biofilms. In addition, the efficacy of tobramycin-ceftazidime may not be influenced by the phenotypic characteristics of \textit{P. aeruginosa} strains since these stains differed in exopolysaccharide production. Based on the study by Ciofu \textit{et al.}, (2001), the mucoid phenotype may ensure survival of the biofilm, while the non-mucoid cells act as protective barriers against antimicrobial agents. Overall, findings reveal antibiotic combination therapy is specific to each individual \textit{P. aeruginosa} isolate and antibiotics administered during the early stages of CF infection may be more effective to control infection. The mechanisms that are influencing synergistic interactions warrant further investigation.

A similar response was seen in Chapter 3 following treatment of \textit{P. aeruginosa} biofilms with metal-based agents, in particular silver-coumarin compounds. The activity of silver against \textit{P. aeruginosa} has been studied by many researchers with promising results (Kalishwaralal \textit{et al.}, 2010; Bjamsholt \textit{et al.}, 2007). Synthesised silver-coumarin compounds reduced the rate of respiration by disrupting the synthesis of cytochromes in the mitochondrion of \textit{C. albicans} (Thati \textit{et al.}, 2007). \textit{P. aeruginosa} planktonic cells and biofilms formed under static conditions displayed sensitivity to these agents, however,
results were dependent on the response of each strain to each individual compound. MIC and MBC values of the agents against an array of phenotypically diverse *P. aeruginosa* planktonic isolates revealed that silver exhibits a broad range of activity irrespective of these differences. In addition, mature biofilms of certain *P. aeruginosa* strains formed in static models were more resistant following exposure to the novel compounds. Exopolysaccharide has been shown to play a major role in the absorption of heavy metals (Kocberber and Donmez, 2008). Exopolysaccharide production and its binding with silver may be reducing the activity of the silver-coumarin compounds against mature biofilms. Other metals including copper-benzotriazole compounds and gallium nitrate had a reduced effect against planktonic cells and biofilms suggesting *P. aeruginosa* is resistant to certain metals-based agents.

The study of bacterial biofilm formation is of major significance in understanding the pathogenesis of *P. aeruginosa* during CF lung infection. Arrays of different *in vitro* models have been designed to study biofilm formation in great detail. As described previously, biofilm formation was assessed using a variety of different *in vitro* models. Many authors have suggested that biofilms formed on polystyrene pegs are suitable for rapid screening of antimicrobial agents but the assay has a number of limitations. Smith *et al.*, (2008), described gravitational forces may be depositing cells on the bottom of the wells leading to structures formed on polystyrene pegs that may not be representative of a genuine biofilm. Other studies have suggested that *in vitro* biofilms may not be an accurate representation of biofilms formed in an *in vivo* environment (Aaron *et al.*, 2002). The flowchamber biofilm system which was used as part of this study allowed direct
visualisation of biofilm structures over a specific period of time. The replenishment of nutrients and elimination of metabolic waste from bacterial cultures is a continuous process in order to develop phenotypic characteristics of each individual strain (Nichols et al., 1999). This model enables the structural development of biofilms to be observed over time, however, a large volume of samples cannot be analysed simultaneously. The effect of silver-coumarin compounds against *P. aeruginosa* biofilms examined in this model showed that all cells were inhibited following 24 hours exposure. Results suggest that shear force from this model may be rendering the biofilms more susceptible to the antimicrobial properties of the silver-coumarin compounds.

The unusual CF bacteria from the CF reference laboratory at AMNCH expanded this study to explore the possible biofilm forming ability of these species and any potential interactions with *P. aeruginosa* as seen in Chapter 4. Sibley et al., (2011), described the airways of CF patients that consist of many bacterial species and the contribution of some less commonly isolated species may lead to a rapid decline in lung function. It was demonstrated that *P. aeruginosa* remained the dominant pathogen when grown in mixed culture with several unusual CF species including *Paracoccus* sp., *Herbaspirillum* sp. and *Ralstonia* sp. Since the *P. aeruginosa* isolates used for this study produced virulence factors such as phenazines, that may be exerting toxic effects towards other bacterial species as previously demonstrated by Kerr et al., (1999). It was reported that *S. maltophilia* and *Acinetobacter* sp. influenced the attachment of *P. aeruginosa* to polystyrene pegs. Viability testing also showed that *S. maltophilia* 668 was capable of out-competing *P. aeruginosa* PA01 when grown as mixed species biofilms. Additionally,
exposure to cell-free supernatants of *S. maltophilia* 668 and 747 showed a significant reduction in biofilm formation of *P. aeruginosa* strains. Alternatively, not all *P. aeruginosa* biofilms were sensitive to treatment with *S. maltophilia* whole cells and supernatants. *Stenotrophomonas maltophilia* is gaining much attention as an emerging nosocomial pathogen in CF patients but the clinical outcome of infected individuals has not been studied extensively (Waters *et al.*, 2007; Steinkamp *et al.*, 2005).

Proteomic studies have previously been used to detect a number of virulence associated proteins of *P. aeruginosa* (Hanna *et al.*, 2000). To assess the protein profiles of the CF *S. maltophilia* strains that were virulent in the co-culture model, LC/MS analysis on tryptic digests of excised SDS-page bands was conducted. The selected bands corresponded to proteins that were common and different to each strain. Chaperonins were a common peptide group in *S. maltophilia*. This group in involved in the folding of newly generated polypeptides in the bacterial cytosol by an ATP-dependent reaction (Hartl *et al.*, 1996). The proteins of *S. maltophilia* 668 that were identified at a unique band (63 kDa) included dihydrolipoamide dehydrogenase, cytosol aminopeptidase and s-adenosyl-l-homocysteine. This strain was of interest as it was able to out-compete *P. aeruginosa* PA01 and the cell-free supernatants reduced the biofilm growth of the same strain. As described in Chapter 4, Section 4.4 studies have shown that these proteins regulate virulence factor production in other bacterial species. Dihydrolipoamide dehydrogenases are known to enhance virulence of pneumococcal disease (Smith *et al.*, 2002). Aminopeptidases found in *E. coli* and *P. aeruginosa* may act as repressors or activators in the operon regulation of virulence genes (Woolwine *et al.*, 1999). Finally, s-adenosyl-l-
homoserine is involved in cellular homeostasis (Loenen et al., 2006).

As mentioned previously by Sibley et al., (2011), CF lung infection can often involve numerous bacterial species. The current study indicated that some species may be dominant pathogens in a multi-species environment. The results suggest that extracellular proteins of *S. maltophilia* may be related to persistent, chronic infection and antibiotic resistance. Langford *et al.*, (1989), described the outer membrane proteins of *P. aeruginosa* increased resistance to aminoglycosides. Ciofu *et al.*, (2001), described the outer membrane proteins may differ among mucoid and non-mucoid *P. aeruginosa* isolates. Examining mixed species interactions under *in vivo* conditions and the response to antibiotics would be required for a more accurate analysis. Finally, a larger amount of diverse strains would be useful for examining the interactions of *P. aeruginosa* and *S. maltophilia* during lung infection in CF patients.


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## Appendix 1: Molecular weights and solubility of metal-free coumarin compounds.

<table>
<thead>
<tr>
<th>Metal-free coumarin ligands</th>
<th>Molecular Weight</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin 2</td>
<td>224.60</td>
<td>Hot water, alcohols, Ethyl acetate, dichloromethane, DMSO</td>
</tr>
<tr>
<td>Coumarin 3</td>
<td>220.18</td>
<td>Hot water, alcohols, Ethyl acetate, dichloromethane, DMSO</td>
</tr>
<tr>
<td>Coumarin 4</td>
<td>269.05</td>
<td>Hot water, alcohols, Ethyl acetate, DMSO</td>
</tr>
<tr>
<td>Coumarin 5</td>
<td>220.18</td>
<td>Hot water, alcohols, Ethyl acetate, DMSO</td>
</tr>
<tr>
<td>Coumarin 6</td>
<td>254.62</td>
<td>Hot water, Hot alcohols, Ethyl acetate, DMSO</td>
</tr>
<tr>
<td>Coumarin 7</td>
<td>254.62</td>
<td>Hot water, Hot alcohols, Hot Ethyl acetate, DMSO</td>
</tr>
<tr>
<td>Coumarin 8</td>
<td>220.18</td>
<td>Hot water, alcohols, Ethyl acetate, DMSO</td>
</tr>
<tr>
<td>CCa (coumarin-3-carboxylic acid)</td>
<td>190.15</td>
<td>Hot water, alcohols, Ethyl acetate, DMSO</td>
</tr>
</tbody>
</table>

256
Appendix 2: Effect of metal-free coumarin compounds against *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Metal-free coumarin ligands (μM)</th>
<th><em>P.aeruginosa</em> PA01</th>
<th><em>P. aeruginosa</em> ATCC27853</th>
<th><em>P.aeruginosa</em> 1826</th>
<th><em>P.aeruginosa</em> 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin 2</td>
<td>&gt;569.9</td>
<td>&gt;569.9</td>
<td>&gt;569.9</td>
<td>&gt;569.9</td>
</tr>
<tr>
<td>Coumarin 3</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
</tr>
<tr>
<td>Coumarin 4</td>
<td>&gt;475.74</td>
<td>&gt;475.74</td>
<td>&gt;475.74</td>
<td>&gt;475.74</td>
</tr>
<tr>
<td>Coumarin 5</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
</tr>
<tr>
<td>Coumarin 6</td>
<td>&gt;502.7</td>
<td>&gt;502.7</td>
<td>&gt;502.7</td>
<td>&gt;502.7</td>
</tr>
<tr>
<td>Coumarin 7</td>
<td>&gt;502.7</td>
<td>&gt;502.7</td>
<td>&gt;502.7</td>
<td>&gt;502.7</td>
</tr>
<tr>
<td>Coumarin 8</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
<td>&gt;581.34</td>
</tr>
<tr>
<td>CCa (coumarin-3-carboxylic acid)</td>
<td>&gt;637</td>
<td>&gt;637</td>
<td>&gt;637</td>
<td>&gt;637</td>
</tr>
</tbody>
</table>

Appendix 3: The effect of different concentrations of gallium nitrate against *P. aeruginosa* planktonic cells and biofilms.

<table>
<thead>
<tr>
<th>Gallium nitrate (μM)</th>
<th><em>P.aeruginosa</em> PA01</th>
<th><em>P.aeruginosa</em> ATCC27853</th>
<th><em>P.aeruginosa</em> 1826</th>
<th><em>P.aeruginosa</em> 4276.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>BIC</td>
<td>MIC</td>
<td>BIC</td>
</tr>
<tr>
<td>2 μM</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>5 μM</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>20 μM</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>50 μM</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>200 μM</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>500 μM</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

257
Appendix 4: Calculations of increasing concentrations of antibiotics based on the MIC (µg/ml) values determined by the broth microdilution method.

(A) *P. aeruginosa* PA01

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC</th>
<th>5xMIC</th>
<th>10xMIC</th>
<th>20xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin</td>
<td>0.25</td>
<td>1.25</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>4</td>
<td>20</td>
<td>40</td>
<td>80</td>
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</tbody>
</table>

(B) *P. aeruginosa* 1826

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC</th>
<th>5xMIC</th>
<th>10xMIC</th>
<th>20xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin</td>
<td>0.5</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

Appendix 5: Calculations of increasing concentrations of silver nitrate and compound 8 based on the MIC (µM) values determined by the broth microdilution method.

(A) *P. aeruginosa* PA01

<table>
<thead>
<tr>
<th>Antibiotic (µg/ml)</th>
<th>MIC</th>
<th>10xMIC</th>
<th>20xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nitrate</td>
<td>23.5</td>
<td>235</td>
<td>470</td>
</tr>
<tr>
<td>Compound 8</td>
<td>12.2</td>
<td>122</td>
<td>244</td>
</tr>
</tbody>
</table>
(B) *P. aeruginosa* 1826

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC</th>
<th>10xMIC</th>
<th>20xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nitrate</td>
<td>23.5</td>
<td>235</td>
<td>470</td>
</tr>
<tr>
<td>Compound 8</td>
<td>12.2</td>
<td>122</td>
<td>244</td>
</tr>
</tbody>
</table>