Bacteriophages as a potential treatment for *Pseudomonas aeruginosa* mediated chest infections in cystic fibrosis patients

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Abstract

Cystic fibrosis (CF) affects between 1 in 2000 and 1 in 4500 births in caucasians of European descent. It is caused by a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) resulting in abnormal membrane osmolarity. CF is a multi-organ disease, however the cause of death is most often due to respiratory failure caused by infection of the airway epithelia with the bacterium Pseudomonas aeruginosa. Ps. aeruginosa grows in the airways as a biofilm and is recalcitrant to treatment with antibiotics. Therefore alternative therapies are urgently required.

Bacteriophages have been and still are used in ex-Soviet bloc countries as a treatment for many infections. However, to date very few comprehensive studies have been conducted into phage therapy in humans. The aim of this study was to characterise several Ps. aeruginosa phage and to develop an in vitro co-culture model simulating a Ps. aeruginosa lung infection on which bacteriophage could be assessed for their efficacy as a therapeutic agent.

A number of bacteriophage present in the University of Brighton bacteriophage collection were purified, and characterised in a number of ways. Infection process data yielded parameters such as burst size and bacterial kill rate. Genomic characterisation involved analysis of the fragments obtained upon digesting phage genomes with restriction enzymes. Physical parameters of the phage particles were elucidated from transmission electron microscope (TEM) images. Phages were also assessed for their tendency to provoke an inflammatory response in human bronchial epithelial cells (16HBE). This was achieved by measuring the production of proinflammatory cytokines IL-6 and IL-8 by 16HBE cells in response to both pure phage suspensions and cell-free phage lysates.

A phage was selected which had favourable characteristics with regard to its use as a potential therapeutic agent. Criteria included low inflammatory response, low incidence of bacterial resistance and high bacterial kill rate. The selected phage was used to treat in situ Ps. aeruginosa infection on 16HBE cells and the inflammatory response of the cells was assessed. The results suggested that phage therapy does not provoke a strong inflammatory response and is comparable with antibiotics used currently.
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Authors Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

Dated
## Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>16HBE</td>
<td>16-Human Bronchial Epithelial cells</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP Binding Cassette</td>
</tr>
<tr>
<td>aGM1</td>
<td>asialylated Ganglioside 1</td>
</tr>
<tr>
<td>AHL</td>
<td>Acyl Homoserine Lactone</td>
</tr>
<tr>
<td>ALI</td>
<td>Air Liquid Interface</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway Surface Liquid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine TriPhosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>BronchoAlveolar Lavage</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine MonoPhosphate</td>
</tr>
<tr>
<td>CBAVD</td>
<td>Congenital Bilateral Absence of the Vas Deferens</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>Cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CMS</td>
<td>Colistimethate sodium</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disorder</td>
</tr>
<tr>
<td>CSE</td>
<td>Control Standard Endotoxin</td>
</tr>
<tr>
<td>CSS</td>
<td>Colistin Sulphate</td>
</tr>
<tr>
<td>CUAVD</td>
<td>Congenital Unilateral Absence of the Vas Deferens</td>
</tr>
<tr>
<td>DMSO</td>
<td>DiMethyl SulphOxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>EthyleneDiamineTetraAcetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>EPS</td>
<td>ExoPolySaccharide</td>
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<tr>
<td>EU</td>
<td>Endotoxin Units</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>HBD-2</td>
<td>Human Beta Defensin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>IL-#</td>
<td>Interleukin-#</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amoebocyte Lysate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LdH</td>
<td>Lactate deHydrogenase</td>
</tr>
<tr>
<td>LES</td>
<td>Liverpool Epidemic Strain</td>
</tr>
<tr>
<td>LPS</td>
<td>LipoPolySaccharide</td>
</tr>
<tr>
<td>LRW</td>
<td>LAL Reagent Water</td>
</tr>
<tr>
<td>MDRs</td>
<td>Multi Drug Resistance pumps</td>
</tr>
<tr>
<td>MEME</td>
<td>Minimum Essential Media (with Earle's salts)</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity Of Infection</td>
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<tr>
<td>MRSA</td>
<td>Meticillin Resistant Staphylococcus Aureus</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PA</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>PACT</td>
<td>Photodynamic Antimicrobial ChemoTherapy</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCL</td>
<td>Periciliary Compartment Liquid</td>
</tr>
<tr>
<td>PEG</td>
<td>PolyEthylene Glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PMN</td>
<td>PolyMorphoNuclear lymphocytes</td>
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<tr>
<td>sad</td>
<td>surface attachment defective</td>
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<tr>
<td>SARS</td>
<td>Severe Acute Respiratory Syndrome</td>
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<tr>
<td>SCV</td>
<td>Small Colony Variants</td>
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<td>SP-</td>
<td>Surfactant Proteins</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>TSA</td>
<td>Tryptone Soya Agar</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptone Soya Broth</td>
</tr>
<tr>
<td>UTP</td>
<td>Uracil TriPhosphate</td>
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1. Introduction
During the course of their condition cystic fibrosis sufferers will almost inevitably acquire chronic airway infections caused by *Pseudomonas aeruginosa*. Despite intensive antibiotic therapy 80-95% of CF patients mortality is caused by respiratory failure resulting from these pulmonary infections and the accompanying inflammation [Lyczak, Cannon and Pier 2002].

*Ps. aeruginosa* grows in the form of biofilms which exhibit profound resistance to antimicrobial challenges and together the organism’s inherent recalcitrance to antibiotics makes this type of infection extremely difficult to manage. Indeed, once a CF patient has become infected with *Ps. aeruginosa* it is probable that they will never get rid of it. Effective management of these lung infections therefore demands that a new form of treatment needs to be developed. In recent years the increasing antibiotic resistance problem has renewed interest in bacteriophage therapy.

This review will discuss the problems associated with lung infections in CF, the reason why *Ps. aeruginosa* is such a successful pathogen in this disease and the current treatments and their efficacy. The potential for bacteriophages as a therapy for this condition will then be discussed highlighting relevant studies in the literature, together with an appraisal of the general suitability of phages as therapeutic agents.
1.1 Cystic fibrosis

Cystic fibrosis or CF affects over 8000 people in the UK with 30,000 sufferers in the USA and 70,000 worldwide. In the UK 5 babies with CF are born a week, and there are more than 2 million carriers which is approximately 1 in 25 of the population. The median age of survival for CF sufferers is 35-37 years [Cystic Fibrosis Foundation 2009; Cystic Fibrosis Trust 2009]. It is a multi-organ disease and the most prevalent severe autosomal recessive condition amongst the Caucasian population.

1.1.1 Genetic and molecular cause

Although CF has been a well known disease since it was first correctly recognised as a systemic disorder in the 1930s, the gene, in which mutations arise to cause this disorder, was only discovered in 1989. The search for the gene was made difficult due to the lack of gross chromosomal rearrangements in CF that are present in other genetic diseases. Recombination studies had previously placed the gene responsible on the long arm of chromosome 7. A combination of chromosome walking and a related procedure known as chromosome jumping identified a 250 kilo base pair (kbp) gene locus containing 24 exons [Rommens et al. 1989].

By searching cDNA libraries compiled from epithelial tissues with a portion of the putative gene a transcript was found which equated to a protein of 1480 amino acids in total. The protein contained two similar sections linked by a regulatory (R) domain, each containing a transmembrane domain and an ATP-binding domain. The gene product was called the cystic fibrosis transmembrane conductance regulator (CFTR). Comparisons between healthy and CF tissue revealed a 3bp deletion mutation in the gene in CF tissue which caused a loss of phenylalanine in one of the ATP-binding regions of CFTR [Riordan et al. 1989].

This mutation was found to be present in around 70% of CF patients with the remaining 30% having various other mutations in this gene. Different mutations were linked to differences in disease severity, particularly with regards to pancreatic function. The most common mutation termed ΔF508 was seen to be one of the most
severe [Kerem et al. 1989; Dean et al. 1990; Fuchs et al. 1994; Sheppard et al.
1996].

To date 1604 different mutations have been found in the CFTR gene [Cystic Fibrosis
Foundation 2009]. They have been discovered in all domains of the CFTR protein
and fall into 5 different classes which differ in terms of protein maturity, structure
and cellular location of the mutated protein [Thomas et al. 1992; Sheppard et al.
1996; Pasyk et al. 1999; Greger et al. 2001]. Both natural and synthesized mutations
have allowed the structure and function of CFTR to be elucidated further.

Initially CFTR was thought to be just a cyclic adenosine monophosphate (cAMP)
activated chloride ion channel but not all the symptoms of CF could be explained by
a lack of chloride transport [Anderson et al. 1991; Anderson et al. 1991; Berger et al.
1991]. CFTR was also found to be involved in the regulation of other chloride
channels and possibly sodium channels as well [Boucher, Stutts and Knowles 1986;
Gabriel et al. 1993; Schwiebert et al. 1998]. Some trans-membrane ATP transport
and also intra-cellular trafficking was attributed to CFTR [Bradbury 1999;
Vankeerberghen, Cuppens and Cassiman 2002]. It was realised that the CFTR
protein belonged to the superfamily of ABC (ATP-binding cassette) transporters
[Cantiello 2001].

The most common CFTR mutation ΔF508 is thought to have first entered Europe
more than 50,000 years ago [Morral et al. 1994]. The high proportion of carriers that
still remain in the population may be down to a selective advantage against secretory
diarrhoecal conditions such as cholera [Gabriel et al. 1994].

1.1.2 Overview of the disease

The symptoms of CF are derived from the effect that the mutant CFTR has on the
epithelial cell surfaces on which it is expressed. Severity and scope of the symptoms
vary according to the mutation carried, but broadly include; pancreatic insufficiency,
gastrointestinal malfunction, differences in sweat composition, male infertility, and
pulmonary disease, which will be discussed in greater detail in section 1.2 [Quinton 1990; Koch and Hoiby 1993].

1.1.2.1 Pancreatic insufficiency

The healthy pancreas produces various enzymes including lipase, amylase, and trypsin, which are secreted into the duodenum in a bicarbonate rich fluid in response to ingestion of food. Production of the bicarbonate fluid relies on a chloride/bicarbonate dependant water secretion. The CF pancreas, due to its defect in chloride secretion fails to produce the correct volume of fluid to dilute the pancreatic enzymes so the pancreatic duct becomes blocked. This leads to maldigestion of fat and protein which are lost in the faecal matter resulting in eventual malnutrition. CF is the biggest cause of pancreatic insufficiency in infants and the malnutrition it causes has serious consequences for the development of the child [Taylor and Aswani 2002].

1.1.2.2 Gastrointestinal conditions

Frequently, the first indication that a newborn may have CF is a condition called meconium ileus which affects between 10-15% of CF patients. Within the first few hours after birth a healthy infant passes its first bowel movement known as the meconium. This is a tarry substance derived from substances ingested in the uterus. The meconium in a new born with CF becomes thick and sticky and forms an obstruction resulting in the bowel becoming distended and painful [Chaudry et al. 2006].

Bowel obstruction and constipation becomes a common theme throughout the life of the some CF patients. This is a result of both pancreatic dysfunction and poor chloride/water transport across the intestinal epithelia. Other complications that can affect some CF sufferers include appendicitis and gastro-oesophageal reflux [Chaudry et al. 2006].
1.1.2.3 Sweat composition

In healthy individuals sweat is secreted into the first half (secretory coil) of the sweat gland as an isotonic solution. It then passes through the second half (reabsorptive duct) where sodium chloride is reabsorbed across the duct wall with a lesser quantity of water resulting in release of hypotonic sweat on to the skin surface. In CF individuals the reabsorption of sodium chloride is disrupted and hypertonic sweat is the result [Quinton 2007].

This defect provides a useful means of diagnosis that has been used since the disease was first recognised to the present day. Sweat chloride levels of 60 mmol L\(^{-1}\) and above are associated with a positive diagnosis for CF, levels of 40-60 mmol L\(^{-1}\) are classed as borderline [Stern 1997; Dalcin and Abreu e Silva 2008].

1.1.2.4 Congenital bilateral absence of the vas deferens

Congenital bilateral absence of the vas deferens (CBAVD) affects 99% of all male CF patients. Moreover healthy males who suffer from infertility due to obstructive abnormalities such as CBAVD or congenital unilateral absence of the vas deferens (CUAVD) often carry one mutant CFTR allele. It is thought that this condition is a result of CFTR possessing a development function [Radvpou et al. 2008]. The presence of CFTR in healthy 18 week old foetuses further strengthens this theory [Harris et al. 1991].

Female CF patients are also usually less fertile than their healthy counterparts. This is generally due to thickened mucus in the cervix as a result of impaired chloride transport, and malnutrition delaying sexual development [Radvpou et al. 2008].
1.1.2.5 Diagnosis and Treatment

All newborn infants in the UK are tested for CF by measuring levels of immunoreactive trypsinogen, which will be raised in a child with CF. Confirmation of the diagnosis can be achieved by a number of different tests due to the multi-organ manifestation of the disease and often more than one type of test is used which along with supporting factors such as family history can provide a confident diagnosis. Sweat testing is a very good indicator of CF although some CF patients can have relatively normal sweat electrolyte levels, chloride levels of greater than 60 mmol/L are indicative of CF. Genotyping can test for 70 known CF mutations accounting for 90% of the CF population; however those with rare mutations will not be identified. A strong indication of CF, if there are no aggravating factors, is the discovery of obstructive azoospermia on analysis of semen samples, however there are other conditions that can cause this. Abnormal sinuses on X-rays are a sign of pansinusitis a condition which is highly prevalent in CF sufferers. Malabsorption and failure to thrive as an infant is a clear sign of a lack of pancreatic function and this can be confirmed by a good response to enzyme treatment. A rarely used technique, only employed in difficult to diagnose cases due to its technical complexity, is nasal potential difference which measures the sodium movement across the cell membranes. Another technique only used in the diagnosis of atypical CF is bronchoalveolar lavage which measures the concentration of neutrophils as a function of inflammation in the airway fluid [Stern 1997].

The treatment of pulmonary symptoms will be discussed in detail in section 1.2.3, but aside from this treatment the management of CF mainly involves alleviating the malabsorption and digestive symptoms. Sufferers with pancreatic insufficiency take enzymes with each meal to aid the absorption of important nutrients. To assist with this the patients are put on a well planned diet high in fat to boost calorie consumption with nutritional drinks and supplements if required to help maintain weight [Cystic Fibrosis Trust 2009].
1.2  Cystic fibrosis associated pulmonary infections

The dominant symptom of cystic fibrosis is the debilitating pulmonary infections occurring throughout the life of the individual. Several micro-organisms including bacteria, viruses and fungi cause these infections and most, if not all, bacterial infections develop in the form of a cell aggregate surrounded by a glycocalyx, or slime, known as a biofilm. These grow on the surface of the airway epithelia, where the thick mucus characteristic of CF accommodates such growth. Biofilms will be discussed in greater detail in section 1.3.

1.2.1  Conditions in the CF lung

The main cause of death in over 95% of CF sufferers is respiratory failure as a result of chronic respiratory infection [Lyczak, Cannon and Pier 2002]. The infections start early in life and both the bacteria and the sufferers own inflammatory reaction are responsible for the damage to the lungs.

There has been a great deal of research into how different types of CF mutation lead to different clinical prognoses for airway infection, in a similar way to the differences found in pancreatic function. The discovery that normal CFTR may act as an epithelial receptor for the ingestion of Ps. aeruginosa and subsequent removal by apoptosis of the infected cell has strengthened this argument [Pier, Grout and Zaidi 1997]. However another study which investigated pulmonary function and how it correlates to genotype, and immune status found that genotype only modestly influenced clinical parameters such as forced expiratory volume [Parad et al. 1999].

1.2.1.1 Epithelial ion transport and the mucociliary escalator

In healthy lungs the first line of defence against infection is the mucociliary clearance mechanism, known as the mucociliary escalator. The mucous glands and goblet cells produce mucus which traps any microorganisms and particles. This mucus is then propelled out of the lungs by finger-like projections of the airway
epithelial cells called cilia beating in a co-ordinated fashion; coughing and sneezing reactions also assist this process. The volume of airway surface liquid (ASL) found in the periciliar liquid compartment (PCL), which is the area between cilia, is carefully maintained by isotonic absorption across the epithelia. This enables cilia to be fully extended on top of which sits the layer of mucus, which is transported by cilia movement [Widdicombe 2002]. It is still not clear exactly how both the volume and the composition of the ASL are maintained, with the two major hypotheses being passive or active ion transport. Difficulties in taking measurements in vivo combined with unreliable results in vitro have lead to a great deal of confusion and conflicting reports in this field of study [Tarran 2004].

Before the CFTR gene and protein, and hence the true nature of the disease, were discovered studies suggested that, similar to the sweat glands, there were defects in the ion and fluid transport across the airway epithelia. An increased rate of sodium (Na\(^+\)) absorption and a decrease in chloride (Cl\(^-\)) absorption along with the consequent water movement lead to dehydrated airway fluids which in turn hinder the mucus transport [Boucher, Stutts and Knowles 1986; Boucher et al. 1988]. This was further investigated and was thought to be due to a defect in the regulation of a cyclic adenosine monophosphate (cAMP) activated chloride transport mechanism [Frizzell, Rechtemmer and Shoemaker 1986], more specifically in cAMP-dependant phosphorylation by protein kinase A or C [Hwang et al. 1989]. Controversy over the airway fluid differences continued, with one study claiming that Cl\(^-\) conductance was responsible for the differences [Smith, Karp and Welsh 1994] while another stated that there was no difference in the ionic composition of ASL from CF and non-CF individuals [Knowles et al. 1997]. A further study found that in the CF airway there was hyper-absorption of isotonic ASL leading to reduced PCL size and invasion of the PCL by mucus. This effectively traps the cilia making them unable to transport mucus out of the lung [Matsui et al. 1998]. However caution should be exercised when reviewing results relating to the osmolarity of ASL as many groups have reported higher NaCl concentrations in CF airways [Zabner et al. 1998].

In healthy bronchial tissue CFTR is found primarily in the submucosal gland cells, particularly the serous gland cells. This could indicate a mechanism for the abnormal production of mucus in CF patients [Engelhardt et al. 1992]. This was further
supported by a study demonstrating that fluid transport in cultured CF gland cells was different to non-CF cells, with a bias towards absorption and no cAMP-dependant secretion [Jiang et al. 1997]. Mucus of normal epithelial cells appears to protect the cells from bacterial invasion and also reduce numbers of viable bacteria. CF epithelia produces up to 5 times more mucus however this seems to make the cells more susceptible to bacterial invasion. Moreover, when challenged with a bacterial presence normal epithelial cells produce more mucus, whereas CF cells do not respond in this manner [Sajjan, Keshavjee and Forstner 2004]. Thicker mucus with a higher percentage of solids such as that found in the CF airway may restrict the movement of bacteria leading to higher localised levels of bacterial cells. This in turn would lead to the production of quorum sensing molecules which could convert the bacterial cells to a biofilm phenotype leading to chronic infection [Matsui et al. 2006].

1.2.1.2 Innate immune system

CF lung disease is a result of not only the bacteria that cause the infection but also damage caused by the host defence system. Once the bacteria have breached the defences of the lung there is a huge inflammatory reaction, including a mass migration of protease releasing neutrophils, which leads to further bronchial obstruction and destruction of the lung tissue. As this continues it will eventually cause respiratory failure [Bals, Weiner and Wilson 1999].

If the mucociliary escalator does not expel the bacteria, the next line of defence is usually the innate immune system. The epithelium detects micro-organisms via pattern recognition molecules known as toll-like receptors (TLRs) and responds via an inflammatory reaction. This involves the release of a cascade of antimicrobial peptides, chemokines and cytokines that among other things recruit phagocytic cells and lymphocytes and cause an inflammatory cascade [Bals, Weiner and Wilson 1999; Hiemstra 2001; Bals and Hiemstra 2004; Tosi 2005]. The best known family of antimicrobial peptides are the defensins, these include human β-defensin 1 and 2 (HBD-1 and HBD-2) that are expressed in the epithelial cells and the gland cells of the airway. It is thought that the production of HBD-2 is a result of inflammation and
the production of HBD-1 occurs without prior inflammatory signals [Singh et al. 1998; Schutte and McCray 2002].

Bals et al discovered that the decrease in antimicrobial activity in the CF lung was not solely explained by the high salt content of the airway surface fluid. They, and others, suggested that it may be a combination of high salt concentration and the absence of or decrease in an unidentified antibacterial agent. However they were unable to ascertain exactly what it was [Bals et al. 2001; Sajjan, Keshavjee and Forstner 2004]. There has been some evidence to suggest that this unidentified antimicrobial substance may be a defensin due to its low molecular mass and thermodynamic stability. This defensin is ineffective at high salt concentrations but its activity can be recovered once the salt concentration is reduced. This salt dependant variation in activity explains why the CF lung, which may have a higher salt concentration in the mucus, is unable to kill bacteria efficiently [Smith et al. 1996]. Human β-defensin-1 (hBD-1), which exhibits broad spectrum antimicrobial activity, has been found to suffer salt inactivation in the ranges of salt concentration found in the CF lungs [Goldman et al. 1997]. The secretion of lysozyme by the serous cells of the submucosal glands was found to be affected by chloride concentration. However this effect was limited to the membrane polarization of lysozyme secretion and not the total amount secreted [Duszyk 2001]. High levels of chloride have also been found to affect polymorphonuclear leukocytes conferring reduced abilities to kill *Ps. aeruginosa* and increased apoptosis/lysis and IL-8 production [Tager, Wu and Vermeulen 1998].

Surfactant protein A (SP-A) and D (SP-D) are important up-regulators of macrophage killing of bacterial pathogens. The levels of SP-A are found to be decreased in airways that have been injured due to infection and this could provide a mechanism by which successive infections are able to proceed [LeVine et al. 1998; Restrepo et al. 1999]. Another theory is that infected CF lungs contain more of the lactoferrin cleaving proteases, cathepsins. Lactoferrin is an important glycoprotein that has antimicrobial, as well as anti-biofilm, properties and it is found in unusually low quantities in the infected CF lung given the bacterial burden. However cathepsins are found in large quantities and have been shown to cleave lactoferrin rendering it incapable of any anti microbial properties. [Rogan et al. 2004]
As with the levels and composition of the ASL, there has been much speculation
surrounding the levels of pro-inflammatory cytokines produced by CF airways. The
two main hypotheses are thus: (i) The CFTR defect in some way causes
inflammatory mediators to be present on the CF airway before infection, or (ii)
infection occurs first, encouraged in some way by a CFTR defect, and there is a
disproportionate inflammatory response by the CF epithelia.

The discovery that very young CF infants, from 4 weeks old, had already elevated
levels of IL-8, neutrophils, and neutrophil elastase, before any evidence of infection
led to speculation that there was a defect in the inflammatory response of CF airways
[Balough et al. 1995; Khan et al. 1995]. A later study however disagreed with this
and found that before any signs of infection the levels of IL-8 and elastase in BAL
fluid from CF infants was comparable with that of the healthy controls [Armstrong et
al. 1997]. Levels of neutrophil associated defensins from CF patients with chronic
bronchitis were also found to be at levels that are known to be toxic to epithelial cells
[Soong et al. 1997]. BAL fluid from CF mice models infected with *Ps. aeruginosa*
was found to have very different levels of the murine cytokines TNFα, macrophage
inflammatory protein-2 (MIP-2) and KC/N51 (KC) to non-CF mice. In the CF
animals cytokine release was much higher on day 1 post infection but decreased to
much lower levels thereafter [McMorran et al. 2001]. In a cell culture model with
nasal epithelial cells taken from CF individuals and healthy controls the basal levels
of IL-8 and activation of nuclear factor-κB (NF-κB) were similar between non-CF
and healthy cells. Moreover when the level of bacterial adherence to non-CF cells
was increased to match that of CF cells there was still no difference between the two
cell types in either IL-8 production or NF-κB activation. This study favours the idea
that the massive inflammatory response is not due to some inflammatory defect in
CF cells per se but due to the fact that CF cells lend themselves more to bacterial
binding and hence the subsequent immune response than do normal cells [Scheid et
al. 2001]. Further studies reported inconsistent results [Aldallal et al. 2002].

Bronchial sub-mucosal gland cells which, in healthy individuals, express high levels
of CFTR [Engelhardt et al. 1992] were found, in basal conditions, to release higher
levels of IL-8 and IL-6 in CF versus non CF airway cultures. However on
stimulation by *Ps. aeruginosa* LPS the non-CF airway exhibited a much greater
increase in IL-6 and IL-8 release from basal levels than the CF airway [Kammouni et al. 1997]. A similar study confirmed the basal level findings for IL-8 but found no difference between CF and non-CF gland cells in IL-6, IL-1β, or IL-10 levels. These findings could also be reproduced both in vivo and in vitro. Furthermore the levels of IL-8 were dependant on Cl− concentrations with higher concentrations leading to greater production of IL-8 [Tabary et al. 1998]. The mechanism for this increased IL-8 production was elucidated further by the discovery of a lack of the NF-κB inhibitor IκBα. This leads to constitutive upregulation of NF-κB which in turn results in high levels of basal IL-8 secretion [Tabary et al. 2001].

A study which demonstrated release of IL-10 but very little IL-6 or IL-8 by bronchial epithelial cells freshly obtained from healthy individuals showed the precise opposite in cells obtained from individuals with CF [Bonfield, Konstan and Berger 1999]. Further evidence of a higher basal IL-8 production by CF airway epithelia versus non-CF epithelia was produced by using tracheal grafts in immune-deficient mice. Moreover on infection of the mice with Ps. aeruginosa the CF grafts exhibited a faster and more exaggerated inflammatory response with rapid destruction of the epithelial surface compared to non-CF grafts [Tirouvanziam et al. 2000].

Lymphocytes were studied due to their similarity to the pancreatic acinar cells, which are known to be affected by the CF mutation. Lymphocytes secrete cytokines when stimulated in healthy individuals; however in CF individuals they are unable to do so. This could have a detrimental effect on the level of antibodies produced to fight the infection [Bubien 2001].

However other studies have confirmed that apart from a few exceptions IL8 and ICAM-1 (inter-cellular adhesion molecule-1) expression are relatively normal in CF epithelia when compared with wild type. Certainly there could be much interference due to other genetic and environmental factors that affect the outcome of these investigations, thereby making any meaningful conclusions difficult [Aldallal et al. 2002]. This indicates that the reasons behind the lack of antibacterial action in the CF lung is not as simple as first thought, and many factors may combine as a result of the lack of CFTR function. This also supports the theory that the CFTR protein may have a diverse range of functions, rather than just a transporter protein.
1.2.1.3 Adhesion and engulfment

There are two main hypotheses by which bacteria may adhere to CF airway epithelial cells to initiate an infection, and avoid being engulfed and lysed. Firstly the CFTR mutation leads to an increase in asialylated versus sialylated gangliosides on the epithelial cell surface on which bacteria can bind without being internalised. Secondly that CFTR is itself a receptor that binds to bacterial cells and engulfs them for lysozomal lysis. Hence a lack of functional CFTR would prevent this and allow the bacteria to survive.

CF airway epithelial cells exhibit defective sialylation of gangliosides at the cell surface, moreover these asialylated gangliosides (aGM1s) provide a receptor for both *Ps. aeruginosa* and *Staphylococcus aureus* [Imundo et al. 1995]. This indicates that sialylation of surface glycolipids is CFTR dependant [Bryan et al. 1998]. This was confirmed in a subsequent study which indicated that mutant CFTR leads to hyperacidification of the trans-golgi network, and correction of this by treating the cells with a weak base resulted in the correct sialylation of the gangliosides [Poschet et al. 2001]. Piliated strains of *Ps. aeruginosa* exhibit an 8 fold increase in adherence to epithelial cells presenting excess aGM1 compared to wild type cells. Similarly a 5 fold increase was seen in cytotoxicity, which was also found to be due to the cytotoxin ExoU, and a 4 fold increase in internalization of piliated bacteria was seen in those cells with an excess of aGM1. The same effect is not seen in cells presenting an excess of GM1, suggesting sialylation of the ganglioside prevents bacterial binding [Comolli et al. 1999]. The pili act as ligands that are capable of binding to the aGM1s [Bryan et al. 1998]. Furthermore there is a greater quantity of aGM1 receptors found on CF cells compared to non-CF cells and this quantity is even greater when such cells are regenerating after injury. This is obviously of significance in the CF lung which is constantly undergoing injury due to infection and inflammation [De Bentzmann et al. 1996]. This could mean that bacteria are more readily able to adhere to the airway cells, providing the first step towards creation of a biofilm, or that the functional CFTR is actually a receptor of *Ps.*
*Aeruginosa* enabling it to be engulfed and that if CFTR is dysfunctional there will be decreased uptake [Bals, Weiner and Wilson 1999; Chmiel and Davis 2003].

The internalisation and subsequent removal of *Ps. aeruginosa* from the lungs by epithelial cells is dependent on localisation of effective CFTR at the cell membrane. It is the first extracellular domain that is responsible for this activity, a domain of CFTR that is not needed for ion channel function. Moreover intact LPS produced by the bacteria appears to be a ligand for this process. This would explain not only the persistence of *Ps. aeruginosa* in the lungs of CF patients who do not have effective CFTR, but also the emergence of clinical strains of *Ps. aeruginosa* that produce an incomplete LPS structure. This probably isn’t the only mechanism that epithelial cells use to bind *Ps. aeruginosa* as there also appears to be a specific point on the bacterial cell membrane that docks with the CFTR [Pier et al. 1996; Pier, Grout and Zaidi 1997].

This novel function of CFTR may explain the apparent resistance to certain infections that heterozygotes of the CFTR gene possess. *Salmonella typhi*, which causes typhoid fever, establishes infection by translocating across the submucosal layer of the intestine. The receptor it uses is the same first extracellular domain of CFTR that airway epithelial cells use to bind to and engulf *Ps. aeruginosa*. Therefore those with limited or no copies of functional CFTR are likely to be resistant to the infection [Pier et al. 1998].

*Burkholderia cenocepacia* has been shown to be enclosed by normal epithelia into a vacuole which then destroys the bacteria. However CF epithelial cells also appear to engulf *B. cenocepacia*, but this time they are surrounded by intermediate filaments of the cell’s cytoskeleton, and also appear to be dividing. This suggests that similar to other species they are able to utilize the cells cytoskeleton, possibly even to transcytose through the epithelial layer causing bacteraemia, a condition occasionally seen in *B. cepacia* infected CF patients [Burns et al. 1996; Sajjan, Keshavjee and Forstner 2004].
1.2.2 Successive infections

Colonisation of the bronchial airways usually occurs within the first two years of life [Bals, Weiner and Wilson 1999]. Most of the bacteria that cause infections in CF lungs are opportunistic pathogens and some exist in the respiratory tract of healthy individuals without causing disease [Lyczak, Cannon and Pier 2002]. The most commonly found bacteria in cultures from CF-affected lungs are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However in recent years the epidemiology of CF infections has been perceived to be changing and other organisms of note are *Haemophilus influenzae*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Legionella pneumophilia*, *Mycobacterium* spp., viruses and fungi [LiPuma].

In CF affected lungs the primary bacteria to cause infections is usually *S. aureus*, in fact before the advent of antibiotics this was the most common organism associated with CF and was the primary cause of death [Gilligan 1991]. Due to the widespread carriage of *S. aureus* in the nasal passages, the majority of CF associated *S. aureus* infections are caused by the patient’s own flora [Govan 2000]. *S. aureus* can bind to the aGM1 proteins on the epithelial cells surface which is probably the reason for the prevalence of infections by this organism in CF airways [Imundo *et al.* 1995]. The incidence of methicillin resistant *S. aureus* (MRSA) in CF patients was until the last few years relatively low, however it is now on the increase. Risk factors for CF patients acquiring MRSA include hospital admission, intensive antibiotic treatment, and chronic infection with *Aspergillus fumigatus* [Nadesalingam, Conway and Denton 2005].

*Haemophilus influenzae* is one of the most common organisms found in very young CF infants up to 1 year [Saiman 2004]. *H. influenzae* has been demonstrated to form biofilms on airway epithelial cells *in vitro* [Starner 2006]. These pioneering bacteria often predispose the lung to subsequent infection with *Ps. aeruginosa*, by allowing increased attachment of the bacterial cells due to the inflammatory damage. *H. influenzae* may continue to be found in the lungs after onset of infection with *Ps. aeruginosa* but gradually the extent of pseudomonal infection takes over the preceding bacterial colonisation [Anderson *et al.* 1991; Lyczak, Cannon and Pier 2002].
*Burkholderia cepacia* (originally called *Pseudomonas cepacia*) emerged as an important CF pathogen from the early 1970s onwards. Although less common than *Ps. aeruginosa*, the clinical effects of its presence were found to be more serious. It could lead to a rapid decline in pulmonary function, was resistant to many antibiotics, and was easily transmissible between CF patients [Isles et al. 1984]. This acute progression of the infection, which affects around 30% of those infected, is termed cepacia syndrome and is characterised by a necrotising pneumonia and septicaemia [Gilligan 1991; Govan 2000]. Today it is still considered in terms of its unpredictable clinical effects as the CF pathogen which causes the most concern, it is still however far less prevalent than *Ps. aeruginosa* [Govan 2000]. *B. cepacia* is actually a complex (*B. Cepacia* complex: Bcc) of distinct genomic species, termed gemovars, the most common of which are *B. cenocepacia* and *B. multivorans*. Whether a patient is infected with a particular gemovar is thought to influence clinical outcome and prognosis [Jones et al. 2004]. There is evidence that it co-infects the CF lung along with *Ps. aeruginosa*, but usually arrives after pseudomonal infection has set in. It appears that interspecies communication via quorum sensing occurs between these two bacteria. Moreover this occurs in a one way direction with *B. cepacia* responding to quorum sensing signals from *Ps. aeruginosa* but not vice versa [McKenney, Brown and Allison 1995; Riedel et al. 2001].

*Stenotrophomonas* (formerly known as *Xanthomonas*) *maltophilia* was first isolated from the airways of CF patients in 1975. Its incidence has increased since that time, however its prevalence varies widely between geographic locations [Denton and Kerr 1998]. There are limited data suggesting that the isolation of this organism from CF lungs does not correspond to worsening lung function or a poor prognosis [Goss et al. 2004]. Other CF pathogens include *Ralstonia* species, *Achromobacter xylosoxidans*, *Cupriavidus* species, and *Pandoraea* species [LiPuma].

*Aspergillus fumigatus* is the principle fungal pathogen associated with CF. Infection of the airways with *A. fumigatus* leads to allergic bronchopulmonary aspergillosis. The incidence of this is low and tends to occur in adolescence into adulthood [Gilligan 1991].

Viruses are thought to play a role in predisposing the CF lungs to further infection by initiating a damaging inflammatory reaction, particularly in young CF sufferers. The
common respiratory viruses such as influenza, parainfluenza and respiratory syncytial virus are prevalent in CF children, just as they are in non-CF children [Gilligan 1991].

1.2.3 Current and Experimental Treatments

The ultimate treatment choice for CF is gene therapy, with the airway epithelia being the most likely target. This would involve restoring the function of the CFTR in the airway epithelia by inserting a CFTR gene without the CF-causing mutation. However fully effective gene therapy has not yet been performed in humans. Various problems including efficiency of transduction and inflammatory responses to vectors used have compounded to slow progress thus far [Ramsey 1996]. The CF associated therapies in use now manage the symptoms of CF and attempt to prolong the infection free period and hence the life of the CF sufferer. Aside from the physical therapy and the nutritional requirements CF lung treatments can be split into 5 types;

(i) bronchodilators to open the airways,

(ii) medication to break down the viscous mucus,

(iii) steroids to reduce inflammation,

(iv) antibiotics, and finally if appropriate,

(v) lung transplantation.

Bronchodilators act on the bronchial wall muscles causing them to relax and widen the airways. This not only improves the breathing but also facilitates mucus clearance and access to the airways by other inhaled drugs such as antibiotics. Bronchodilators fall into two groups, the beta-2-agonists and the anticholinergenics and both are inhaled either as a dry powder or as an aerosol in a nebulizer. However they are not beneficial to all patients and pre-testing with small quantities of these drugs and assessing the response confirms if the patient is a suitable candidate for this therapy [Ramsey 1996; Halfhide, Evans and Couriel 2005].
The respiratory mucus in CF is notoriously viscoelastic and hence difficult to clear. One of the main factors that causes this viscoelasticity is the extracellular DNA released by numerous autolysing neutrophils, hence this became a target for drug therapy. Purified recombinant human DNase I is an enzyme that breaks down this DNA resulting in a much reduced mucus viscoelasticity, aiding, alongside other medications mentioned, the clearance of purulent sputum [Fuchs et al. 1994].

The nature of the over-zealous inflammatory reaction in CF airways is also a target for drug therapy. Commonly glucocorticoid steroids such as prednisone are administered to CF patients and have been found to be beneficial in modulating the inflammatory reaction. However as with other CF therapies these are not without their drawbacks including growth retardation. For this reason ibuprofen is considered a potentially safer alternative, particularly for children with CF [Ramsey 1996].

Antibiotics are a conspicuous feature in most CF sufferers lives from infancy (the antibiotic treatment used for *Ps. aeruginosa* infection will be discussed in detail in section 1.4.4.1). A broad range of methods of administration of antibiotics is utilised in CF, with oral, nebulised, and intravenous antibiotic delivery being familiar to all CF sufferers. This extensive use of antibiotics, though necessary is not without complications. These range from hypersensitivity and allergic reactions to the risk of resistance and selective advantage to alternative pathogens and toxic effects of antibiotics such as renal failure and hearing loss. Oral and nebulised antibiotics are usually used for prophylaxis and to manage chronic infection (when not suffering an exacerbation) and short term or recently acquired infections.

Pulmonary exacerbations are usually treated in hospital with antibiotics delivered intravenously over the course of 14-21 days. The antibiotics are chosen based on the identification of the causative bacteria from sputum samples [Ramsey 1996]. The isolates are tested for their sensitivity to different antibiotics *in vitro*, however significant differences can be apparent between sensitivity *in vitro* and *in vivo*.

Prophylactic antibiotic treatment is a controversial issue with some studies reporting an advantage in preventing some infections [Weaver et al. 1994] and others reporting no difference, or worse, a selective advantage for other resistant bacteria to cause infection [Beardsmore et al. 1994; Ramsey 1996]. However it may be an
advantage to prevent infections by bacteria such as \textit{S. aureus} to limit early respiratory damage which can encourage \textit{Ps. aeruginosa} infection. Prolonged treatment (from CF diagnosis to three years old) with flucloxacillin can help to prevent \textit{S. aureus} infections particularly when the patient has a viral infection such as a cold. If, even with this treatment, sputum cultures become positive for \textit{S. aureus} then a second oral antibiotic is added e.g. sodium fusidate or rifampicin. If the patient is still unwell after 4 weeks of treatment then intravenous antibiotics are normally administered. Antibiotics are tailored to the sensitivity of the isolates, broad spectrum antibiotics could cause resistance or select for \textit{Ps. aeruginosa} infection. If MRSA is isolated tetracyclines are usually used for “mild” infections with glycopeptides such as nebulised or IV vancomycin used if the infection is more severe [UK Cystic Fibrosis Trust Antibiotic Working Group 2009].

If \textit{Haemophilus influenzae} is isolated from sputum samples antibiotics are started immediately regardless of whether the patient is symptomatic. Antibiotics used are co-amoxiclav or doxycycline as resistance can occur to macrolides and amoxicillin. As with \textit{S. aureus} if cultures are still positive after 4 weeks then IV antibiotics are used [UK Cystic Fibrosis Trust Antibiotic Working Group 2009].

\textit{Burkholderia cepacia} complex is increasing rapidly in prevalence in CF and is associated with particularly extensive resistance to antibiotics including colistin. Hence \textit{B. cepacia} complex infection requires treatment with 2-3 antibiotics working in synergy, the antibiotics used depend on the gemovar that is infecting and its sensitivity profile [UK Cystic Fibrosis Trust Antibiotic Working Group 2009].

Other avenues of CF therapy include compounds that may correct the ion transport defect, either by reducing the sodium ion absorption or activating alternative chloride channels. Amiloride a sodium channel antagonist and nucleotide triphosphates such as UTP and ATP, which activate non-cAMP dependent chloride channels have been considered to this end [Knowles \textit{et al.} 1990; Ramsey 1996]. Additionally a protease inhibitor isolated from the blood has shown potential usefulness in aerosolized form to block the action of neutrophil elastase in the CF airway thereby moderating the inflammatory response [Cantin and Woods 1999].
Potentiators are experimental compounds that increase the ion channel activity of mutant CFTR for those mutations that allow for normal trafficking of the protein such as G551-D. Another class of drugs called correctors improve the trafficking of the CFTR protein for mutations such as ΔF508. Both of these types of compounds have been shown to be effective in vitro, although potentiators have so far been found to achieve a better outcome than correctors [Pedemonte et al.].
1.3  *Pseudomonas aeruginosa*

Chronic infection by *Pseudomonas aeruginosa* is the usual cause of mortality in CF sufferers. Despite infections with other organisms, almost without exception *Ps. aeruginosa* will come to dominate the CF airways. *Ps. aeruginosa* is an opportunistic pathogen, usually infecting those with a disadvantage such as a weak immune system or trauma. *Ps. aeruginosa* infections, aside from CF, are found most commonly in burns patients, those with indwelling medical devices such as catheters and in otitis media, which is an infection in the middle ear. Infections by *Ps. aeruginosa* are characterised by their biofilm mode of growth with the production of a slimy glycocalyx known as alginate and by their recalcitrance to antibiotics, leading to chronic infections in many cases [Drenkard and Ausubel 2002].

1.3.1  Ecology and classification

*Pseudomonas aeruginosa* is a Gram negative aerobic rod-shaped bacterium that produces a distinctive blue/green pigment and an exopolysaccharide known as alginate. It is an opportunistic pathogen of animals and humans. There is little data on the prevalence of *Ps. aeruginosa* in the environment although it is known to exist in soil and various categories of water, and is a well known plant pathogen. The majority of the studies of *Ps. aeruginosa* have concentrated on its implication in human disease as it can cause infections of varying severity affecting many different organs. Another important aspect of *Ps. aeruginosa* as a pathogen is its resistance to many antibiotics making it very hard to treat [Todar 2004]. *Ps. aeruginosa* possesses all of the major bacterial resistance mechanisms, alginate reduces antibiotic diffusion, efflux pumps remove antibiotics from the bacterial cell, enzymes such as beta-lactamases degrade certain antibiotics, and mutations in various genes affect antibiotic targets [Lambert 2002].
A well known characteristic of *Pseudomonas aeruginosa* strains is their ability to produce various pigments, indeed this used to be the method of identifying different strains. The usual blue/green pigment (pyocyanine) and yellow/green fluorescent pigment (pyoverdine) can be replaced by a brown (pyomelanin) or red (pyorubin) pigment or an unpigmented variant. This appears to happen spontaneously but can also be triggered by bacteriophages or antibiotics [Howarth and Dedman 1964; Ogunnariwo and Hamilton-Miller 1975; Meyer 2000].

Before it was discovered that *Ps. aeruginosa* could produce alginate it was considered a non-encapsulated bacterium. There had been incidences of encapsulated strains but these were thought of as rare one-offs. Cetin et al investigated two such encapsulated strains out of 242 incidences of *Ps. aeruginosa*, one from sputum and one from a urine sample. On Gram staining these strains it was found that there was an unstained area around the rods, and when the bacteria were serially passaged, they appeared to lose their capsule. They also discovered that one of the encapsulated strains was more pathogenic for mice than its non-encapsulated counterpart. They also interestingly reported that there was no difference between the two types of strain in susceptibility to antibiotics, however which antibiotics they tested was not disclosed [Cetin, Toreci and Ang 1965]. It was subsequently discovered that the encapsulated mucoid form occurred more frequently in the pulmonary infections of cystic fibrosis patients. The alginate produced by *Pseudomonas* spp. is similar to the alginates found in seaweed, differing in their ratio of manuronic and guluronic acids, and the inclusion of o-acetyl groups in the bacterial alginates [Linker and Jones 1966; Evans and Linker 1973].

*Ps. aeruginosa* is a motile bacterium, moving by use of flagella and type IV pili (twitching motility). These structures also allow the initial stages of biofilm formation to take place [O'Toole and Kolter 1998]. It has two main quorum sensing systems, LasR and LasI, and RhlR and RhlI, and there is also evidence of *Ps. aeruginosa* being involved in interspecies communication with other bacterial species [Gray et al. 1994; McKenney, Brown and Allison 1995; Winson et al. 1995]. *Ps. aeruginosa* has many virulence factors that confer an advantage during infection namely an extracellular lipopolysaccharide, LasR dependant exoenzymes, adhesins and flagella [Tang et al. 1996]. In addition, as mentioned previously *Ps. aeruginosa*
is able to grow as a biofilm on airway epithelium which provides it with many advantages over the planktonic form of growth.

1.3.2 Biofilms

1.3.2.1 Definition and components

The idea of bacteria favouring growth on a surface as opposed to dispersed throughout a liquid media was first proposed in the 1930s and 1940s. Bacteria in sea water samples were found to increase in number more readily on the surface of the sample bottles rather than in the sea water itself [Zobell 1943]. Biofilms were originally described as slime layers found often in streams and aquatic environments [Jones, Roth and Sanders 3rd 1969]. They have since been found to be the standard mode of growth for most bacteria in most situations, including infections [Costerton, Stewart and Greenberg 1999]. In the review by Costerton et. al. the definition of a biofilm is as follows:

→Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces.”

[Costerton et al. 1995]

A second review written several years later by Donlan and Costerton expanded on this simple definition to include the discovery that biofilm bacteria exhibit a characteristic phenotype different to that of their planktonic counterparts, thus the revised description of a biofilm became:

→A microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription”

[Donlan and Costerton 2002]
As can be seen from these definitions, it appears that there are three essential factors needed for a biofilm; microbes, glycocalyx, and a surface (or interface) [Dunne 2002].

The glycocalyx is a “slime”, primarily constructed of various polysaccharides produced by the microbial cells, that protects the cells from damaging influences in the environment, and provides the structural framework of the biofilm [Gilbert, Collier and Brown 1990; Hoyle, Jass and Costerton 1990; Sutherland 2001]. The microbial cells are dispersed throughout the glycocalyx in a heterogeneous manner, forming microcolonies [Costerton et al. 1994].

Advantages for bacteria living in sessile communities include, shelter, homeostasis, nutrient availability, metabolic co-operation and increased gene transfer [Davey and O'Toole 2000]. The elevated levels of gene transfer demonstrated in biofilms may pave the way for the emergence of new pathogens, and greater resistance of existing pathogens to bactericidal substances [Watnick and Kolter 2000]. Resch et al. also raised the possibility that spontaneous release of bacteriophages may occur more frequently in biofilms than in planktonic populations. This activity may confer some advantage on the biofilm cells in that lysed cells could provide nutrients for neighbouring cells in the community [Resch et al. 2005]. In some cases initial colonizing cells enable other species of bacteria to bind whereas they were unable to bind to the uncolonized surface [Leung et al. 1998]. In this manner a bacterial biofilm possesses characteristics similar to multi-celled organisms [Webb, Givskov and Kjelleberg 2003].

1.3.2.2 Prevalence

Biofilms are found throughout the biosphere and are particularly abundant in aquatic conditions. They are responsible for nutrient cycling and degradation of toxic products, and as the bottom of the food chain, particularly in the oceans, ecosystems depend on their presence [Davey and O'Toole 2000].
The importance of biofilms was first understood by those involved in industrial water systems due to the prevalence of biofilms in pipelines and other flow related equipment, including dental water lines. Biofilms can also form in diverse industrial conditions such as in dairy lines, petroleum pipelines and ships hulls. A large body of research has contributed to effective strategies to deal with this potentially very costly problem. Methods used include biocides which are regularly tested to ensure they remain effective against biofilms, enzymes to break down the glycocalyx, and biofilm detection devices installed in pipelines [Johansen, Falholt and Gram 1997; Donlan and Costerton 2002; Dunne 2002]. However biofilms can also be used to advantage in industrial applications such as the treatment of sewage or hydrocarbon based pollution [Davey and O'Toole 2000].

Due to their nutrient value most bodily fluids are capable of supporting optimal bacterial growth, therefore any metal or plastic surfaces that come into contact with these fluids, such as medical devices, could harbour biofilms. Hence biofilm mediated infections are commonly associated with medical implants e.g. catheters, prosthetic heart valves in fact any foreign body [Costerton, Stewart and Greenberg 1999; Davey and O'Toole 2000]. Inserting a medical implant can result in a "race for the surface", with host tissue often coming second to bacteria after the initial protein conditioning film [Gristina 1987].

*Staphylococcus epidermidis* is a particular problem with regard to infections associated with prosthetic heart valves and intravascular catheters. It has been shown that this organism grows as considerable biofilms on these devices [Christensen *et al.* 1982]. This is due to them being a natural skin dwelling organism with opportunities to contaminate wounds [Resch *et al.* 2005]. Bacteria can also readily adhere to contact lenses and their storage containers providing a source for infection [Donlan and Costerton 2002].

Intraurethral catheters are notorious for biofilm growth, and infection in those patients undergoing long-term catheterisation is seen as inevitable. Biofilms can develop so extensively as to fully occlude the catheter and in many cases treatment is usually considered ineffective until the catheter is removed [Stickler *et al.* 1998]. Biliary stents undergo similar problems with the potential for occlusion of the stent due to biofilm and mineral build-up [Leung *et al.* 1998]. In Costerton's short review
he suggests that the prevalence of biofilm associated infections could be due to the strategy of targeting only planktonic infections with antibiotics and vaccines leading to sessile growth becoming a selective advantage [Costerton 2001].

Endothelial and epithelial surfaces are less likely to play host to such growth due to host defences. However if the surface is weakened or injured in some way, like the respiratory epithelial surface is in cystic fibrosis patients, then biofilms may be able to overcome these difficulties [Costerton et al. 1995]. Native valve endocarditis occurs when bacteria or fungi that are in the bloodstream encounter a damaged heart valve and form a biofilm. Many different organisms can cause this due to the many ways organisms can enter the bloodstream. Otitis media is a biofilm infection of the middle ear which is common in children. Prostatitis (infection of the prostate) if left untreated can rapidly develop into a chronic infection associated with biofilm development. Oral biofilms are well known to occur on the tooth surface in healthy individuals soon after brushing. In protected areas of the mouth such as in the gingival crevice biofilms can lead to varied severities of gum disease [Donlan and Costerton 2002].

Many of the biofilm pathogens that affect patients with a compromised immune system are common place ubiquitous organisms that wouldn't normally affect healthy individuals [Costerton, Stewart and Greenberg 1999]. This is certainly the case with Ps. aeruginosa biofilms on the airway surface of CF patients.

### 1.3.2.3 Formation and structure

Sauer et al. (2002) described five distinct stages of development of Ps. aeruginosa biofilms. Initial attachment was found to require flagella in order for growth to occur successfully. In the second stage motility ceased but the Las quorum sensing systems were activated. The third stage occurred when cell clusters become thicker than 10μm plus the Rhl quorum sensing system was activated. The fourth stage was characterised by the cell clusters reaching 100 μm, the maximum size for the clusters. After 9 days the final stage of development was entered, at this point motile bacteria within the void spaces of the cluster appeared to swim away through
openings leading to a change in structure of cell cluster. [Sauer et al. 2002] This change in cell cluster appeared to be preceded by rapid circular movement of the motile bacteria within these clusters which eventually led to disaggregated microcolonies that were liable to disperse. This was thought to be due to flagella mediated motility [Tolker-Nielsen et al. 2000].

Before bacteria are able to colonise a surface it inevitably becomes pre-conditioned by macromolecules within the aqueous environment. Surface conditioning is a term used to describe the adherence of these macromolecules that alter the surface properties often promoting bacterial adhesion. This is a major factor in the colonisation of medical implants, with bloodstream elements such as albumin, lipids, and fibronectin coating the foreign body very rapidly after insertion of the device [Gristina 1987; Dunne 2002].

Before becoming part of, or starting, a biofilm a bacterium first gets close enough to the surface to allow its motility to slow. This gives the bacterium chance to either adhere to the surface or manoeuvre itself over the surface so as to find already aggregated bacteria [Watnick and Kolter 2000]. Bacteria position themselves on a surface in a number of ways using flagella, pili or other methods of translocation [Davey and O'Toole 2000].

There are thought to be two stages in the attachment of bacteria to a conditioned surface. The first, reversible, stage involves the bacterial cell manoeuvring close to the surface, either by liquid flow or by directed chemotaxis or bacterial motility. Once the cell is very close to the surface attractive or repulsive forces such as hydrophobic, van der waals or electrostatic forces influence the outcome [Dunne 2002]. Usually both the substratum and the bacterial cell surface are negatively charged and so are weakly repulsive. However in some cases such as S. maltophilia the bacterium is positively charged and as a result can bind strongly to most surfaces including Teflon [Jucker, Harms and Zehnder 1996].

The second stage results in a stronger attachment that can only be broken by specific intervention by chemical or physical means. This attachment involves molecular complexes forming between the surface substances and bacterial exopolysaccharides or molecules on the surface of bacterial appendages [Dunne 2002].
Surface Attachment Defective (sad) mutants of *Ps. aeruginosa* that lack either a functional flagellum or pili are unable to form mature biofilms. Those with no functional flagellum show very limited initial attachment to the surface, whereas those with no functional pili are able to attach but not to form microcolonies. This would indicate that microcolonies are formed by aggregation of cells due to twitching motility (mediated by type IV pili) rather than by bacterial growth [O'Toole and Kolter 1998; O'Toole et al. 2000]. The microcolony is the basic unit of biofilm growth and are very dense areas dispersed throughout the biofilm separated by much sparser areas [Costerton et al. 1994; Costerton et al. 1995].

In further work on the regulation of biofilm development, Heydorn *et al.* [Heydorn et al. 2002] found that a *Ps. aeruginosa* strain lacking the pilA gene showed some surprising results. The PilA mutant cells not only exhibited greater initial attachment, but also as the biofilm developed grew unevenly with areas of the strata having no growth. When the biofilms reached maturity the PilA mutants exhibited uneven growth with discrete microcolonies in contrast to the flat uniform growth of the wild-type. The authors proposed that these results may suggest a different reason for twitching motility. Rather than using pili mediated motility for forming microcolonies, *Ps. aeruginosa* may use this mode of transport for spreading out across the surface and ensuring all of the substratum is covered [Heydorn et al. 2002; Klausen et al. 2003].

In some strains of *Ps. aeruginosa* the initiation of biofilm development coincides with the appearance of so called small colony variants (SCVs). Small colony variants have been found in other pathogenic bacterial species and are believed to be auxotrophic mutants with reduced metabolism and slower growth than their wild type counterparts. Also in contrast to the wild type Large colony variant the SCVs formed dense strongly adhered biofilms in a few hours. Microscopic examination revealed that the SCVs were hyperpiliated which would explain not only their adhesiveness but also their hydrophobicity. The isolation of these variants from CF patients has led to speculation that this may be a useful adaption for the bacteria representing a more highly pathogenic status. This is particularly relevant in light of their increased antibiotic resistance and cytotoxicity [Déziel, Comeau and Villemur 2001; Haussler *et al.* 2003; Haussler 2004; von Gotz *et al.* 2004].
The *Ps. aeruginosa* exopolysaccharide also contributes to the adhesion of the bacterial cells to the surface and each other [Ramphal and Pier 1985]. Specific polysaccharide lyases have been found in spent biofilm media from some bacteria indicating that these are induced to promote cell detachment from the surface in times of starvation [Allison *et al.* 1998].

In some cases it is believed that an initial surface colonizer may promote the attachment of a second species that was unable to attach to the uncolonized surface, such as *E.coli* and *Enterococcus* species on biliary stents [Leung *et al.* 1998].

If the environment is no longer able to support the bacteria in the biofilm, the situation is shifted from favouring sessile growth to favouring dissociation of cells from the biofilm [Dunne 2002]. Microcolonies may break off from the biofilm and cause secondary infections when they reach for example a capillary bed [Donlan and Costerton 2002]. This was demonstrated in the investigation of clinical isolates of *Ps. aeruginosa* biofilms from CF patients which showed a tendency towards reduced adherence as the infection continued [Lee *et al.* 2005]. This dispersal also appeared to be facilitated by an organised programme of cell death in the micro-colonies which allowed surviving cells to detach. In one study on *Ps. aeruginosa* the cell death was regulated by the release of a prophage [Webb *et al.* 2003]. Amongst the potential reasons for cell detachment from biofilms are toxic accumulation of metabolic by-products and starvation. Demonstration of detachment due to starvation in continuous flow biofilm cultures has been achieved and followed a similar pattern to that seen in natural biofilm development such as voids appearing in micro-colonies [Hunt *et al.* 2004].

Biofilms generally consist of ~15% cells and ~85% matrix [Donlan and Costerton 2002]. The exopolysaccharide, or matrix, which holds everything together in a biofilm is usually polyanionic, but can occasionally be neutral. It acts to stabilize the biofilm and protect it from high shear environments. It tends to be highly hydrated therefore protecting the biofilm to some extent from desiccation [Sutherland 2001].

Lam *et al* first described the tendency for *Ps. aeruginosa* to form exopolysaccharide-overproducing mucoid microcolonies in the lung. They reported that non mucoid strains still produced exopolysaccharide if grown under certain conditions, and that
the lung environment may favour this mucoid growth. They studied post mortem
lung from CF patients and also infected rat lung, and found that in both cases there
was considerable congestion caused by bacteria surrounded by fibrous matrices
[Lam et al. 1980]. A later study confirmed that exopolysaccharide production by Ps.
aeruginosa cells increased after attachment [Hoyle, Williams and Costerton 1993].
Both mucoid and non-mucoid forms of Ps. aeruginosa are capable of forming
biofilms however mucoid strains tend to produce thicker biofilms with a greater
degree of heterogeneity. This suggests that alginate is important in the architecture of
a biofilm [Hentzer et al. 2001; Nivens et al. 2001].

Dunne suggested that visually the biofilm would resemble a lava lamp [Dunne
2002]. The biofilm has very basic homeostasis, a rudimentary circulatory system and
the ability for the cells within it to interact and work together. Biofilms resemble in
many ways eukaryotic tissue, in their degree of co-operation [Costerton et al. 1995].
The system of channels found throughout biofilms may permit molecules of up to
2,000 kDa to diffuse through the biofilm [Costerton et al. 1994]. Liquid flow
through these channels can be complex and is determined by the shape and width of
the channels. In some cases direction of flow within a biofilm is different to that of
the bulk liquid [Stoodley, DeBeer and Lewandowski 1994].

Biofilms contain many different structures including mushrooms and streamers that
can be up to 3 mm in length. These structures are more likely to form under turbulent
flow and oscillate within the flow [Stoodley et al. 1998].

The speed and heterogeneity with which a biofilm forms can be reliant on the
availability of the carbon source in the nutrients surrounding it [Wolfaardt et al.
1994]. There are also differences in overall biofilm structure according to the species
of bacteria that reside in them. Confocal microscopy studies revealed major
differences in cell density from the surface attached cells through to the outer
reaches of the biofilms between Ps. aeruginosa and Vibrio parahaemolyticus
[Lawrence et al. 1991]. Further studies using freeze substitution transmission
electron microscopy on Ps. aeruginosa biofilms found deeper levels of complexity
within the structure of the biofilm. With cell surface associated polysaccharides,
secreted exopolysaccharides and other matrix materials appearing continuous with
one another. There were also discrete populations of cells in different growth states indicating a complex level of organisation [Hunter and Beveridge 2005].

It is also likely that there is a great deal of difference in the metabolic activity of the cells within a biofilm with a large number of inactive cells [Werner et al. 2004].

Quorum sensing involves the secretion, by the bacteria, of chemical messengers that alter gene expression according to the density of the bacterial cells. It is therefore thought that quorum sensing genes play a role in all stages of the formation of biofilms, from attachment through maturation to dispersal [Parsek and Greenberg 2005]. *Ps. aeruginosa* biofilm infections of indwelling catheters have been found to produce high levels of acylated homoserine lactones (AHLs) a known family of quorum sensing molecules [Stickler et al. 1998]. AHLs have also been found in naturally occurring aquatic biofilms [McLean et al. 1997]. De Kievet et al investigated the two quorum sensing systems in *Ps. aeruginosa* and highlighted not only the differences in static biofilm formation due to quorum sensing mutations but also differences due to the medium in which the biofilm was grown. When static biofilms were grown in media with carbon as the nutrient source the quorum sensing deficient strains did not produce mature biofilms whereas the wild type did. However in media with citrate as the nutrient source the lack of a quorum sensing system had no effect on biofilm development. On checking twitching and flagella motility the team discovered that in the wild-type strain both flagella and pili mediated motility were significantly reduced in the citrate media when compared to the carbon based media. The quorum sensing deficient strains exhibited no twitching motility on either media, and a similar level of flagella mediated motility, as compared to the wild-type strain, on both media. This effect is believed to be due to the piliA gene that encodes for the pili sub-unit being regulated by the carbon metabolism regulator Crc [O'Toole et al. 2000; De Kievit et al. 2001].

Strains of *Ps. aeruginosa* that are deficient in the quorum sensing system LasI form biofilms that are thin with densely packed cells. These biofilms were also very susceptible to sodium dodecyl sulphate, a potent disinfectant, when compared to the wild type biofilms [Davies et al. 1998].
In disagreement with some studies Purevdorj et al claim that quorum sensing systems do not influence biofilm development as much as some reports suggest. In this study the hydrodynamics of the flow cell system appeared to be more important than the presence of cell to cell signalling. In particular they discovered the ability of a biofilm to translocate en mass via the movement of ripples in the biofilm at right angles to the fluid flow [Purevdorj, Costerton and Stoodley 2002]. This was supported by further evidence that knocking out the LasI/LasR quorum sensing system had little effect on Ps. aeruginosa biofilm development [Heydorn et al. 2002].

A further twist in the speculation surrounding quorum sensing was provided by the evidence that nutritional restriction could override quorum sensing controls. In both planktonic and sessile Pseudomonas aeruginosa systems an abundance of iron resulted in the quorum sensing signals being down regulated, whereas if iron was restricted the opposite occurred. As mentioned previously, this is the same for both modes of bacterial growth hinting that the iron response system must be equivalent in each case. This may be due to the close proximity of the iron box” and Lux box promoter regions [Bollinger et al. 2001].

As with attachment and metabolic interactions there is the possibility of cross-species quorum sensing. Ps. aeruginosa and B. cepacia exhibit one way communication with B. cepacia detecting AHL molecules from Ps. aeruginosa [Riedel et al. 2001]. There are many other mixed culture biofilms in nature, in which the individual species or strains are able to interact and cooperate in terms of substrate usage and metabolism [Møller et al. 1998].

As part of the definition of a biofilm sessile cells exhibit an altered phenotype in comparison to planktonic cells. Adhesion of bacteria to a surface stimulates the release of a sigma factor which reactivates a large number of genes. In fact it has been shown that 30% of the proteins that can be separated by 2D electrophoresis were expressed at different levels by the different types of growth [Costerton et al. 1995].

However, a later study comparing Pseudomonas aeruginosa in biofilm and planktonic modes of growth found that only 1% of genes were expressed differently
between the two. Half of which were activated and half repressed in the biofilm mode of growth. Some of these genes were known to be involved in antibiotic resistance and treatment of the biofilm with tobramycin caused altered levels of expression in around 20 genes [Whiteley et al. 2001]

Davies and Geesey investigated the expression of the *Ps. aeruginosa* gene *algC* which encodes for phosphomannomutase, an enzyme that is necessary for the production of alginate. They found that the expression of *algC* was nearly 20 times higher in biofilm cells than in the same strain in suspension. Interestingly they also found that the levels of *algC* found in bacterial cells that had been shed by the biofilm were between that in biofilm cells and that in planktonic cells. This could be explained in one of two ways; either *algC* was down regulated once the cells became detached, or the cells experienced down regulation of the *algC* gene prior to detachment, and the resulting reduction in alginate led to their separation from the biofilm [Davies, Chakrabarty and Geesey 1993; Davies and Geesey 1995].

Phenotypic variation during biofilm growth may not arise solely from the native bacterial genome it may be that bacteriophage elements also have an effect. In a study on strain PAO1 it was found that amongst the genes upregulated in the biofilm mode of growth were those from a temperate bacteriophage Pf1. Moreover this upregulation translated to a 100-1000 fold increase in Pf1 phage in the biofilm. These data could indicate the importance of phage mediated gene transfer amongst the biofilm cells [Whiteley et al. 2001]. It is also thought that a related bacteriophage Pf4 may be responsible for the so called SCV (small colony variant) phenotype often found within *Ps. aeruginosa* biofilms. Infection of the bacteria with this phage can lead to a symbiotic relationship between the two, the phage killing some but not all of the bacteria. The advantage of this phage infection may be conveyed by the filamentous nature of its virion, acting in much the same way as bacterial pili would [Webb, Lau and Kjelleberg 2004].

There is evidence that within mixed biofilms, metabolic interactions between species influence gene expression [Møller et al. 1998].
1.3.2.4 Antibiotic resistance
A well characterised advantage of sessile growth is the resistance of the bacterial cells within the biofilm to various anti-microbials as compared to their planktonic counter-parts. Bacterial cells grown as biofilms were found to suffer only 25% of the killing experienced by planktonic cells of the same strain [Jensen et al. 1990]. These do not just concern antibiotics used in medicine but the biofilm mode of growth affords protection to bacterial cells from all manner of environmental dangers.

There are three main theories that attempt to explain the reason that bacteria within biofilms exhibit such marked antibiotic resistance. One attributes this to the reduction in growth rate that many sessile bacteria experience [Anwar, Strap and Costerton 1992], another takes into account other physiological changes that result from differences in gene expression between planktonic and biofilm bacteria, while a third claims that the antibiotic diffusion is hindered by the extracellular matrix [Hoyle, Jass and Costerton 1990; Gilbert, Das and Foley 1997; Costerton, Stewart and Greenberg 1999; Donlan and Costerton 2002]. Other studies have demonstrated the spread of antibiotic resistance through a biofilm by transfer of a transposon containing an antibiotic resistance gene [Roberts et al. 1999].

A subset of dormant cells known as persister cells are known to occur within biofilms, these cells do not actively grow and are completely resistant to the action of antimicrobials. It is suggested by some that it is these cells that are responsible for the majority of biofilm antibiotic resistance as their presence is usually detected by treatment with antibiotics leaving only the persister cells surviving. The presence of Ps. aeruginosa persister cells has been noted after the use of ofloxacin [Spoering and Lewis 2001; Keren et al. 2004; Lewis 2005]. It has long been known that bacteria at different stages in the growth curve exhibit differences in their susceptibility to antibiotics, with faster growth leading to greater susceptibility [Gilbert and Brown 1978]. Oxygen availability is limited deeper within biofilms [Xu et al. 1998], and Xu et al suggested that in some model biofilms, only the top fifth of the cells in the biofilm were metabolically active [Xu, McFeters and Stewart 2000]. Growth rate and nutrient limitation, particularly deep in the biofilm can confer cell surface differences which may in turn affect the ability of antibiotics to bind to their target receptors [Brown, Allison and Gilbert 1988].
In one study a strain of *Ps. aeruginosa* was used to study biofilms on urinary catheter material, where the planktonic cell MIC for tobramycin was calculated to be less than 1.0 μg/ml. However in a mature biofilm even a tobramycin concentration of 1000 μg/ml didn’t kill all the cells. The study suggested that this may not be due to difference in metabolic rates between biofilm cells and planktonic cells, because in some cases biofilm cells have been shown to have a higher metabolic rate than their planktonic counterparts [Nickel et al. 1985]. This is further backed up by the findings that bacterial cells exhibiting similar growth rates but different modes of growth can have vastly different levels of resistance. With biofilm cells of *B. cepacia* at stationary phase being 15 times more resistant to ciprofloxacin and ceftazidime than planktonic cells at the same phase [Desai et al. 1998]. Another study on *Burkholderia pseudomallei* showed that biofilms were able to survive up to 200 times the planktonic MIC of ceftazimide and co-trimoxazole. However dispersed cells from the biofilm exhibited the same susceptibility as planktonic cells [Vorachit et al. 1993]. *Ps. aeruginosa* biofilms have also been shown to be resistant to imipenem as well as tobramycin [Coquet, Junter and Jouenne 1998]. There are also differences in antibiotic susceptibility between bacteria from a young biofilm and cells from a mature biofilm, with those from the older biofilms benefiting from a higher degree of resistance [Anwar et al. 1989]. Complete eradication of 2 day old *Ps. aeruginosa* biofilms was achieved with a combination of tobramycin and piperacillin however the same concentration of antibiotics applied to an 8 day old biofilm only reduced the bacterial population to 20 % of the original. Moreover after cessation of antibiotic treatment the population increased to 100% once more [Anwar et al. 1992].

Xu et al, taking into account the physiological heterogeneity of the biofilm cells, have suggested an extension of the first theory. The outer cells that are metabolically active and fast growing are killed by antibiotics, whereas the cells below are inactive and are not growing, so will not be affected by the antimicrobials [Xu, McFeters and Stewart 2000]. A possible mechanism to explain how a slowing in growth rate can lead to antimicrobial resistance could involve modification of the bacterial cell envelope in nutrient limitation conditions. If a particular nutrient is depleted the cell surface may be altered to allow more receptors for that substance to be exposed, rendering the bacteria more sensitive to small amounts in the environment. This
modification could result in antimicrobial targets on the cell surface being altered also, hindering the bactericidial action of the antimicrobial substance [Brown, Collier and Gilbert 1990].

Other phenotypic changes brought about by biofilm induced differences in gene expression could have an effect on antibiotic resistance. Quorum sensing, already known to be important in the formation of biofilms may also be important in antibiotic resistance. Biofilms treated with a quorum sensing inhibitory drug (furanone C-30) were found to become more sensitive to treatment with tobramycin [Bjarnsholt et al. 2005]. The production of antibiotic inhibiting enzymes known as β-lactamases can be induced in *Ps. aeruginosa* biofilms as a result of treatment with β-lactams [Giwercman et al. 1991].

Phenotypic differences in adherence between two *S. epidermidis* strains also exhibited differences in antibiotic efficacy. A strain that initially adhered to a surface but did not develop into a multi-layered biofilm was more susceptible to antibiotics than a wild type strain that formed mature biofilms [Schwank et al. 1998].

The idea that the biofilm glycocalyx prevents diffusion of the antibiotics is particularly relevant in the case of mucoid *Ps. aeruginosa* biofilms. Mucoid strains of *Ps. aeruginosa* possess a far greater resistance to antibiotics than non-mucoid strain and this difference can be eliminated by the use of alginase prior to antibiotic treatment [Bayer et al. 1991; Hentzer et al. 2001]. It has been demonstrated that the diffusion of both gentamicin and tobramycin was hindered by a 2% suspension of alginate and that this effect was reversed by treating with alginate lysase [Hatch and Schiller 1998]. However in the same year a separate study found imipenem resistance of *Ps. aeruginosa* may not be due to a diffusion barrier as the antibiotic was not hindered in its movement through alginate [Coquet, Junter and Jouenne 1998]. There have been mathematical models to predict the diffusion of antibiotics through the biofilms to ascertain whether such a diffusion barrier does account for antibiotic resistance. The findings indicated that, unless an additional enzymatic inhibition mechanism (e.g. beta-lactamase) was present, the extent of the biofilm diffusion barrier was not sufficient to explain the lack of antibiotic susceptibility seen. However these models use assumptions including considering biofilm thickness to be uniform throughout, and the biofilm to be homogeneous in its
cellular distribution [Stewart 1996]. As discussed previously this is not the case for the majority of biofilms which exhibit vast differences in thickness throughout and are composed of cell dense microcolonies with spaces between.

An extension of the third theory was proposed for *Ps. aeruginosa*, that antibiotics may bind to alginate produced by the bacteria rendering them incapable of attacking the bacterial cell itself. It was found that the low levels of binding of antibiotic to alginate does occur but does not translate to the high incidence of antibiotic resistance seen in biofilms [Tannenbaum, Hastie and Higgins 1984; Nichols *et al.* 1988].

Antibiotic resistance in many different species of planktonic bacteria has also been attributed to multi drug resistance pumps (MDRs). These appear to have some impact on resistance in biofilms as well but not on all antibiotics despite their reported lack of specificity [Masuda, Sakagawa and Ohya 1995; Poole and Srikumar 2001]. Arguably more important is a sub-population of “super resistant” persister cells, discovered during dose response assays, these were thought to have a greater impact on biofilm resistance in this study [Brooun, Liu and Lewis 2000].

Several different methods have been employed to improve the efficacy of antibiotics in biofilms, although not all of them would be appropriate as clinical treatments. These primarily involve disrupting or breaking down the structure of the biofilm to allow greater access to the cells within. Ultrasound was found to have a moderate improvement in the efficacy of gentamicin treatment of *Ps. aeruginosa* biofilms [Huang *et al.* 1996]. Low strength electric fields applied at the same time as industrial biocides rendered the compounds more effective at killing *Ps. aeruginosa* biofilm cells than planktonic cells treated with just the biocide [Blenkinsopp, Khoury and Costerton 1992]. Slime-dispersants such as EDTA and sodium chloride were used to reduce the viscosity of *Ps. aeruginosa* derived alginate. In doing so they produced an increase in the diffusion of gentamicin and ceftazidime, although the effect was more pronounced for gentamicin [Gordon, Hodges and Marriott 1991].

Although many theories have been extensively studied it is probable that biofilm associated antibiotic resistance is a complex issue that can't necessarily be attributed to one specific cause.
1.4 *Pseudomonas aeruginosa* mediated pulmonary infection

1.4.1 When and why *Pseudomonas aeruginosa*?

Although *Ps. aeruginosa* can cause airway infections in non-CF patients, particularly those that are already unwell, it is the most prevalent CF pathogen and accounts for the majority of CF deaths. Therefore an important area of research is concerned with when and how it dominates the CF airways. *Ps. aeruginosa* seems to arrive in the lungs much earlier in the patient’s life than first thought, with positive cultures appearing in patients less than 3 years old. However these early isolates tend to be non-mucoid and do not exhibit the resistance to antibiotics that later isolates possess [Burns *et al.* 2001]. It is believed that improvements in the medical care of CF patients that led them to live longer may have provided mucoid *Ps. aeruginosa* the chance to colonise CF lungs [Lyczak, Cannon and Pier 2002]. In particular continuous anti-staphylococcal therapy may increase the risk of *Ps. aeruginosa* acquisition [Ratjen *et al.* 2001].

Interestingly it also seems that females acquire *Ps. aeruginosa* infection slightly earlier in their lives than males do and subsequently exhibit a lower life expectancy [Demko, Byard and Davis 1995]. As with most infections in CF patients genotype can affect both the incidence and severity of *Ps. aeruginosa* infection [Kubesch *et al.* 1993].

1.4.1.1 Binding to the epithelial surface

As discussed previously one of the major hypotheses concerning the pathogenesis of CF lung disease involves asialylated gangliosides (aGM1s) found in abundance on CF epithelia, and linked to mutations in the R-domain of the CFTR protein [Bryan *et al.* 1998]. aGM1s are particularly found in regenerating respiratory epithelia after damage or injury suggesting an opportunity for pseudomonas adhesion after other
previous infections have caused epithelial injury [De Bentzmann et al. 1996]. *Ps. aeruginosa* was found to bind strongly to these receptors and this binding was reduced if the bacteria were pre-treated with purified aGM1s or the epithelial cells were treated with an aGM1 antibody. *S. aureus* was also found to bind strongly to aGM1s [Imundo et al. 1995]. Pili aided by LPS bind to aGM1s on epithelial cells [Dasgupta et al. 1994; Comolli et al. 1999]. This can be supported by the fact that *Ps. aeruginosa* isolates from CF patients that have only very recently been colonised are fully motile containing pili and flagella. However isolates from chronically infected patients are very rarely motile, and have lost their pili and flagella. This indicates that these motile structures are necessary for the initial attachment and establishment of infection [Woods et al. 1980; Irvin et al. 1989; Feldman et al. 1998] but are no longer required once the infection has become established [Mahenthiralingam, Campbell and Speert 1994]. This same lack of motility has been shown in vitro biofilm experiments [Sauer et al. 2002]. Experimental infections in mice have shown that piliated strains of *Ps. aeruginosa* cause more severe and disseminated pneumonias, whereas unpiliated strains tend to cause less severe localised infections [Tang, Kays and Prince 1995].

CFTR in lipid rafts on epithelial cell surfaces is also considered to be a receptor for *Ps. aeruginosa* engulfment and killing by apoptosis in epithelial cells. CF affected epithelial cells have limited or no functional CFTR in their membranes hence this defence against *Ps. aeruginosa* is severely restricted [Pier, Grout and Zaidi 1997; Cannon et al. 2003; Kowalski and Pier 2004]. This receptor appears to require pili as a ligand, hence pili deficient mutants of *Ps. aeruginosa* are incapable of triggering apoptosis of the infected cell [Jendrossek et al. 2003]. On the epithelial cell surface there are also CD95/CD95 receptors which again appear to be the focus of ligand interaction between epithelia and *Ps. aeruginosa*. This interaction also triggers apoptosis in the epithelial cell providing another form of defence against infection. It is thought that the expression of CD95/CD95 ligand is upregulated by CFTR after infection and therefore if this system is disrupted by a lack of functional CFTR the bacteria are able to remain resulting in infection [Grassme et al. 2000; Cannon et al. 2003].
Tracheobronchial mucin may also provide a means for adherence of *Ps. aeruginosa* potentially protecting it from opsonophagocytosis [Vishwanath and Ramphal 1984; Vishwanath *et al.* 1988]. A subsequent study confirmed this and found that CF mucin acted as a chemoattractant for most *Ps. aeruginosa* strains studied. The specific mucin components that were most highly chemotactic were strain dependent [Nelson *et al.* 1990; Ramphal and Arora 2001].

Also investigated was the ability of alginate to bind to epithelial cells. Alginate was found to bind strongly particularly to injured epithelial cells [Ramphal and Pier 1985]. Moreover alginate from different strains of *Ps. aeruginosa* was found to bind to both buccal and tracheal epithelial cells but to differing extents, indicating that alginate from different strains had altered structural components [Doig *et al.* 1987]. This difference in alginate from different strains of *Ps. aeruginosa* was confirmed by investigating the cross-reactivity of alginate specific antibodies [Pedersen *et al.* 1989].

As with some other forms of *Ps. aeruginosa* infections, in the CF airway bacterial cells can be internalised or invade the epithelial cells without further consequence to the bacteria. Once inside the epithelial cells they are protected from antibiotics and can go on to develop a biofilm phenotype in safety [Garcia-Medina *et al.* 2005]. This could reflect a difference in receptor i.e. binding to aGM1 as opposed to CFTR, or binding to an incomplete or mutant CFTR that is unable to trigger the apoptotic reaction usually seen in healthy cells. *Ps. aeruginosa* can also invade and disrupt the airway by the production of virulence factors known as rhamnolipids which are thought to disrupt the airway epithelial tight junctions allowing paracellular invasion of the airway by the bacterial cells [Zulianello *et al.* 2006].

### 1.4.1.2 Adaption to the CF lung

*Pseudomonas aeruginosa* cells appear to undergo adaption to the CF lung with proteome analysis revealing markedly altered protein production in cells isolated from CF airway infection versus cells from a burn infection. Amongst those proteins with altered expression were virulence factors such as protease and porins associated
with nutrient and antibiotic uptake [Doring, Goldstein and Roll 1985; Sriramulu, Nimtz and Romling 2005]. Other important virulence determinants that are also upregulated in the CF lung are exoenzyme-S, exoenzyme-U, and exoenzyme-T. These are cytotoxic proteins that cause changes in eukaryotic cell cytoskeletons, epithelial cell damage and induce T-cell proliferation [Tang et al. 1996; Bruno et al. 1998; Allewelt et al. 2000; Krall et al. 2002]. Exotoxin-A is not thought to be as important as proteases and exoenzyme-S, U, and T as it is inactivated by polymorphonuclear leukocyte elastases [Doring, Goldstein and Roll 1985; Apodaca et al. 1995]. Most of these virulence factors are regulated by quorum sensing signals such as homoserine lactone, and Pseudomonas autoinducer (PAI) that act as cell-cell signalling molecules [Jones et al. 1993; Passador et al. 1993; Winson et al. 1995; Storey et al. 1998; Bjarnsholt et al. 2005]. There is also evidence that the virulence factors and quorum sensing signals released by *Ps. aeruginosa* may in turn cause *B. cepacia* to release virulence determinants [McKenney, Brown and Allison 1995].

Respiratory mucin itself may induce changes in gene expression with one study identifying 3 separate genes that were induced after exposure to mucus. One encoded for a virulence factor, pyochelin, one for a transcriptional regulator and the third was suspected to be involved in the production of exopolysaccharide [Wang et al. 1996]. Hence the CF lungs appear to be a unique environment in which *Ps. aeruginosa* undergoes several adaptations. Whilst the expression of many different proteins is significantly changed in CF vs. non-CF isolates [Sriramulu, Nimtz and Romling 2005], by far the most extensively studied adaptation is the conversion to mucoidy.

### 1.4.1.3 Mucoidy

The incidence of mucoid strains of *Ps. aeruginosa* in the CF lung was first reported in 1964, where isolates from non-CF patients infected with *Ps. aeruginosa* were found not to possess the same ethanol-insoluble mucoid substance as those with CF [Doggett, Harrison and Wallis 1964]. This link between CF and mucoid *Ps. aeruginosa* was further investigated by Doggett who found that 70% of sputum samples from CF patients contained mucoid *Ps. aeruginosa*. However samples from
patients known to be infected with *Ps. aeruginosa* but without CF only showed 2.1% mucoidy [Doggett 1969]. Later studies revised these figures suggesting that mucoid *Ps. aeruginosa* accounts for up to 90% of CF associated lung infections. It is believed that the CF *Ps. aeruginosa* infection starts with a non-mucoid form but very quickly transforms to mucoid when in the lung. It appears that the CF lung provides an environment that favours the conversion to mucoidy [Lam *et al.* 1980]. Evidence for this comes from those patients who have undergone a heart-lung transplant, these patients never suffer from infections caused by mucoid *Ps. aeruginosa*, possibly due to the fact that there are no mutant CF genes in the new lung [Anderson *et al.* 1991]. The mucoidy phenotype is notoriously difficult to maintain *in vitro* causing difficulty in mucoidy studies [Govan 1975].

There have been several theories put forward to explain the conversion of *Ps. aeruginosa* to mucoidy.

Antibiotic treatment, with which mucoidy probably offers a selective advantage, has been cited as a potential cause with several studies noting differences in gene expression and conversion to mucoidy on application of antibiotics. CF patients undergo almost constant antibiotic treatment and therefore may provide an ideal environment for mucoid conversion [Hoiby, Vagn and Gunnar 1975; May *et al.* 1991; Whiteley *et al.* 2001; Drenkard and Ausubel 2002].

Berry *et al* have suggested that it may be the osmolarity that triggers the bacteria to become mucoid. They have found that the *Ps. aeruginosa* *algD* gene, which is essential for alginate production, is regulated by the product of another gene known as *algR*. *algR* has significant homology to the *E. coli* gene known as *ompR* which is concerned with the response to changes in osmolarity in the environment [Berry, DeVault and Chakrabarty 1989].

Another set of genes *algU* and *mucA*, *B*, *C*, and *D* are also involved in mucoid conversion, *algU* encodes for a stress σ factor and is a positive regulator for mucoid conversion, *mucA*, *B*, *C*, and *D* negatively regulate *AlgU*. A high proportion of CF mucoid isolates have been found to have mutations in *mucA* [Boucher *et al.* 1997].
The conversion to mucoidy and vice versa also appears to be greatly influenced by culture conditions [Terry, Pina and Mattingly 1991], mucoidy being especially favoured in liquid culture with a high concentration of magnesium [Chan et al. 1984].

Differences in oxygen tension may also have an effect of triggering mucoid conversion, with evidence coming from differing prognoses for endocarditis sufferers dependant on the side of the heart that the infection exists [Bayer et al. 1990].

There is also potential for mucoidy to be triggered by free oxygen radicals such as hydrogen peroxide, which are produced by the phagocytic cells polymorphonuclear leukocytes. Mucoid strains of *Ps. aeruginosa* undergo far less ingestion by macrophages than do their non-mucoid revertant counter parts [Krieg et al. 1988] suggesting an adaptive response to avoid phagocytic killing [Mathee et al. 1999]. It also appears that pseudomonas-produced alginate can scavenge hypochlorite which is produced by activated phagocytes, providing a mechanism whereby bacterial cells can avoid phagocytic killing by oxidative burst. This was demonstrated when addition of alginate to non-mucoid bacteria afforded protection from hypochlorite [Learn, Brestel and Seetharama 1987]. However in some studies the potential advantages of mucoidy, particularly antiphagocytic killing, have been disputed [Blackwood and Pennington 1981]. As well as environmental triggers such as oxygen free radicals, lysogenic conversion by phages could also be responsible for the shift to mucoidy [Martin 1973; Miller and Rubero 1984; Vaca-Pacheco et al. 1999].

Mucoidy appears to confer a number of advantages on the bacterial cells, notably antibiotic resistance and avoidance of non-opsonic polymorphonuclear leukocyte killing [Govan and Fyfe 1978; Pedersen et al. 1990; Bayer et al. 1991]. O-acetylation of the alginate increases the resistance to opsonic killing of *Ps. aeruginosa* and is vital in the avoidance of non-opsonic alginate-antibody detection [Schiller, Alazard and Borowski 1984; Pier et al. 2001]. Another study went further in an attempt to demonstrate that it is indeed alginate which is to blame for the resistance of phagocytosis. Eftekhar and Speert treated 8 mucoid strains of *Ps. aeruginosa* with alginate before testing them for their resistance to phagocytosis.
They showed that all 8 exhibited degraded alginase but only 5 showed an increase in resistance to phagocytosis. They hypothesized that this could be due to differences in the biochemistry of the alginate between the strains and also differences in the amount of alginate each strain produces. Bayer et al investigated the role of alginate in protecting the bacteria from both antibiotic activity and phagocytosis. They discovered that a non mucoid strain of *Ps. aeruginosa* was twice as susceptible to the antibiotic amikacin as its mucoid counterpart, especially during the early antibacterial effect, but pre treatment of the mucoid strain by alginase eliminated the difference. Also that the mucoid strain was better able to resist phagocytosis than the non mucoid strain, however if the mucoid strain was pre treated with alginase then significantly more phagocytosis occurred. Non mucoid cells showed a moderate amount of non opsonic phagocytosis by polymorphonuclear leukocytes which was greatly enhanced by pre treatment with low concentrations of amikacin. Mucoid cells were quite resistant to non opsonic phagocytosis which didn’t change even when the cells were pre treated with amikacin, however if they were treated with alginase instead their susceptibility was substantially raised [Bayer et al. 1991].

1.4.2 **Specific effect *Pseudomonas aeruginosa* has on the lungs and immune system.**

The decline in pulmonary function during infection with *Ps. aeruginosa* is attributed to epithelial inflammation and progressive airway plugging with damaged cell debris [Lyczak, Cannon and Pier 2002]. Initiation of this damage of the lungs by *Ps. aeruginosa* is thought to occur via two stages, firstly bacteria derived proteases cleave immunoglobulins and inactivate lung anti-proteases. In the second stage these proteases are taken up in the formation of immune complexes which stimulate the release of PMN derived proteases. These then become responsible for the greater part of the lung damage [Doring, Goldstein and Roll 1985].
1.4.2.1 Initial reaction to *Ps. aeruginosa* presence

The binding of pili to aGM1s has been found to stimulate the translocation of nuclear factor-κβ (NF-κβ) a transcription factor that in turn stimulates the production of IL-8 by epithelial cells. Interleukins such as IL-8 are chemoattractants for phagocytic polymorphonuclear leukocytes, which release enzymes such as elastase. However CF cells grown *in vitro* were also found to have high levels of constitutively expressed NF-κβ which was hypothesised to be a stress response to the accumulation of mutant CFTR in the endoplasmic reticulum [DiMango *et al*. 1998]. *In vitro* CF cells were shown to express higher levels of IL-6 and IL-8 on exposure to *Ps. aeruginosa* than non-CF cells [Kube *et al*. 2001]. Further investigation revealed that this could not be completely accounted for by the increase in bacterial binding in CF cells [Kube, Fletcher and Davis 2005]. Another study found that the increase in production of IL-8 was due to a prolonged release of the interleukin in CF versus non-CF cells resulting in a protracted inflammatory reaction [Joseph, Look and Ferkol 2005]. Pyocyanin, a *Ps. aeruginosa* virulence factor increased the production of IL-8 by CF and non-CF airway epithelial cells *in vitro* [Denning *et al*. 1998].

Similar results were found *in vivo* in CF mice infected with *Ps. aeruginosa* on agarose beads, with increased production of TNFα, murine macrophage inflammatory protein-2, and KC/N51, another murine inflammatory marker, in CF mice versus non-CF mice [Heeckeren *et al*. 1997].

1.4.2.2 Phagocytosis

The ability of *Ps. aeruginosa*, particularly mucoid strains, to resist killing by phagocytosis is a major factor in the pathogenesis of this infection [Krieg *et al*. 1988]. A possible reason for this resistance is the ability of *Ps. aeruginosa* to bind to respiratory mucin. Both mucoid and non-mucoid strains of *Ps. aeruginosa* were found to be resistant to phagocytosis when incubated with mucin. This was shown to be due to bacterial adherence to mucin rather than mucin affecting the leukocytes.
themselves [Vishwanath et al. 1988]. A further study found that purified alginate from mucoid \textit{Ps. aeruginosa} could prevent neutrophil chemotaxis, the activation of the complement system and also induce oxidative burst by neutrophils. This action could enhance \textit{Ps. aeruginosa} mediated infection by preventing the early clearance of bacteria by neutrophils and inducing them to release toxic oxygen radicals [Pedersen et al. 1990]. The level of O-acetylation of the alginate appears to influence its resistance to opsonic phagocytosis [Pier et al. 2001].

Clinical strains of \textit{Ps. aeruginosa} are capable of cytotoxicity directed at PMNs whereas laboratory strains such as PA01 are not. This aspect of pathogenicity is mediated by the type III secretion system, and is \textit{exoU} dependant. \textit{ExoU} is regulated in co-ordination with \textit{exoT} by \textit{exsA} [Finck-Barbançon et al. 1997]. The PMNs are destroyed by a process known as oncosis which involves the cell swelling and eventually bursting, causing the release of toxic mediators. This is likely to be the cause of the massive inflammatory reaction seen in the lungs of the host [Dacheux et al. 1999; Dacheux et al. 2000].

\subsection*{1.4.2.3 Damaging effects on epithelial layer}

The damaging effects of \textit{Ps. aeruginosa} on the CF epithelial cell layer has been demonstrated \textit{in vitro}. Uninfected cells showed no damage, while in comparison cells infected with \textit{Ps. aeruginosa} showed significant damage including cells extruding from the epithelial layer, loss of cilia and mitochondrial damage. This study also showed that \textit{Ps. aeruginosa} preferentially adhered to damaged epithelium [Tsang et al. 1994]. A later study found that \textit{Ps. aeruginosa} infection adversely affects the transport of particles along the airways and out of the lungs [Cowley et al. 1997]. The reason for this impaired transport is likely to be the loss of cilia demonstrated in the Tsang study [Tsang et al. 1994]. This damage is limited to the CF airway. Six hours after infection by \textit{Ps. aeruginosa} the normal airway epithelia remained intact however destruction of CF epithelia started 3 hours after infection and by 6 hours there was widespread damage [Tirouvanziam et al. 2000].
Ps. aeruginosa strains can be split into two groups, those with an invasive phenotype and those with a cytotoxic phenotype. The latter cause more epithelial damage than the former, and in a typical infection there is an inverse relationship between the two phenotypes. The gene responsible for determining between the ability to invade and cytotoxicity was ExsA a transcriptional regulator of exoenzyme S. Those cells that were cytotoxic had lost the ability to produce Exo-S [Fleiszig et al. 1994; Fleiszig, Zaidi and Pier 1995; Fleiszig et al. 1997]. One of the mechanisms of action of Exo-S is as a Rho-GTPase-activating protein; GTPases are involved in the organisation of the actin cytoskeleton in eukaryotic cells. Many bacterial toxins target these GTPases to allow invasion into epithelial cells [Krall et al. 2002].

1.4.2.4 Antibody production

Although Pseudomonas is not eradicated from the lungs, there is evidence to show that the immune system produces antibodies to mucoid pseudomonas but not its non-mucoid counterpart. This is an indication that non-mucoid strains are less invasive than the alginate producing strains [Doggett and Harrison 1972]. The production of antibodies to Ps. aeruginosa virulence factors continues to increase over several years of chronic infection indicating that antigens are continually being produced and not immediately neutralised [Doring and Hoiby 1983].

Exoenzyme S (Exo-S) is a virulence factor released by many CF clinical isolates of Ps. aeruginosa and is associated with more severe forms of infection. It has been found to be a mitogen, a type of antigen that causes naïve T-cells to proliferate without the requirement of Major Histocompatability Complex (MHC) molecules unlike superantigens. The increased level of T-cell proliferation due to mitogens or superantigens versus a normal antigen-specific immune response could cause a much higher level of inflammatory reaction [Bruno et al. 1998].
1.4.3 Treatment

1.4.3.1 Conventional treatment

Prevention of patients acquiring *Ps. aeruginosa* infection in the first instance should not be overlooked when regarding treatment. Strict hygiene including hand washing by both care personnel and the patient and their family, and care of medical equipment are of the upmost importance. In the same way the segregation of patients colonised by *Ps. aeruginosa* from those not yet infected is an obvious first line in prevention of infection [Giamarellou 2000; Saiman and Siegel 2004].

On first isolation of *Ps. aeruginosa* from sputum sample or swabs, treatment is aggressive and aimed at eradication of the organism to prevent chronic infection developing. This treatment usually occurs in the form of oral ciprofloxacin for up to 3 months combined with a nebulised antibiotic such as colistin or tobramycin [Marchetti *et al.* 2004].

Chronic *Ps. aeruginosa* infection is treated with nebulised antibiotics either colistin or if there is no improvement in pulmonary function tobramycin for inhalation is used, this can also be used for those patients intolerant to colistin. Tobramycin has been found to be highly effective in eradicating early *Ps. aeruginosa* infection [Ramsey *et al.* 1999; Ratjen, Döring and Nikolaizik 2001; Gibson *et al.* 2003] however clinical isolates are showing signs of becoming increasingly resistant to tobramycin and this may pose a serious problem in the future [Barclay *et al.* 1996; Obritsch *et al.* 2004]. Resistance to colistin is rare and it is widely used in CF, however there are concerns as to its potential for neurotoxicity and nephrotoxicity [Conway *et al.* 1997].

Intravenous antibiotics are administered at the first sign of an exacerbation. Often patients with chronic infections will play host to several different strains of *Ps. aeruginosa* and therefore a cocktail of at least 2 antibiotics for which most strains are sensitive, and have different modes of action, should be used [Lang *et al.* 2000]. Specific antibiotics which may be used include, but are not restricted to, colistin, tobramycin, anti-pseudomonal penicillins and β-lactams. Macrolides such as
azithromycin, when used long term appear to have a positive effect on those with chronic *Ps. aeruginosa* infections, particularly if conventional antibiotics are not improving the condition. Macrolides appear to exert an anti-inflammatory effect on the airway, by down-regulating production of inflammatory mediators such as IL-8 as well as interfering with bacterial adherence, motility and alginate production [Jaffé et al. 1998; Wolter et al. 2002].

There are many complications inherent in the extensive use of antibiotics in those with chronic *Ps. aeruginosa* infection. Oral antibiotics such as ciprofloxacin carry the possibility of allergic reactions and also the possible risk of a secondary infection with *Clostridium difficile* due to a selective growth advantage brought about by the antibiotic.

Inhaled aminoglycosides such as tobramycin, colistin sulphate, colistimethate and polymyxin B are very useful in the treatment of CF infections in that they allow a high concentration of the antibiotic to be delivered directly to the site of infection. Due to the limited systemic absorption this also reduces the dangers of toxic serum levels building up. Aminoglycosides however, even when inhaled, are not without dangers including bronchoconstriction, for which bronchodilators (see section 1.2.3) can be co-administered, bronchospasm and, in extreme cases, respiratory failure. Intravenous antibiotic administration is usually limited to treatment of pulmonary exacerbations due to the aforementioned potentially toxic levels of antibiotic which can build up in the serum. Complications include renal failure, ototoxicity causing hearing impairment, hypersensitivity and anaphylaxis, and risk to the foetus in pregnancy. Additional issues associated with all antibiotics regardless of methods of administration include the development of bacterial resistance, and secondary infections by other organisms such as *Candida* due to a selective growth advantage [Hoiby and Koch 1990; Banerjee and Stableforth 2000; Doring et al. 2000; Gibson, Burns and Ramsey 2003; UK Cystic Fibrosis Trust Antibiotic Working Group 2009].

Meropenem an antibiotic used intravenously to treat *Ps. aeruginosa* associated pulmonary exacerbations has been shown to release ten times the concentration of endotoxin at twice the MIC when treating a broth culture. This subsequently causes an increased release of the inflammatory mediators TNF-α and NO as shown when
mouse cells were treated with the antibiotic-treated culture filtrates [Yokochi et al. 1996].

1.4.3.2 Experimental treatment of CF related *Ps. aeruginosa* infection

Hatch and Schiller put forward a possible way round the antibiotic resistance problem. If the antibiotic is delivered in conjunction with alginate lyase the growth inhibitory activity of aminoglycosides such as gentamycin and tobramycin can be restored up to ~56% and ~84% respectively [Hatch and Schiller 1998]. Alginase has also been shown to increase the susceptibility of mucoid stains of *Ps. aeruginosa* to phagocytosis [Eftekhari and Speert 1988]. Dunne Jr and Buckmire suggested another possible area for therapy. They discovered a polymannuronic acid depolymerase produced by mucoid *Ps. aeruginosa* that acted on its own alginate and was released in response to certain environmental conditions. If these conditions could be recreated in *Ps. aeruginosa* infection it may result in the enzyme being released and acting on the bacterial alginate thereby making the bacteria more vulnerable to attack by more conventional methods [Dunne and Buckmire 1985].

Also along these lines is the use of other proteolytic enzymes such as serratiopeptidase in order to lower the MIC of antibiotics in the treatment of biofilm mediated infections of prosthetics. [Selan et al. 1993]

Another tactic, this time used by Gordon *et al* was to use slime dispersants to decrease the strength of the alginate gel to aid the diffusion of antibiotics. They showed that chelating agents such as EDTA are more effective at reducing the strength of the gel than slime dispersants containing sodium salts, although both brought about a significant reduction in gel strength. This in turn led to an increase in diffusion for two antibiotics; gentamicin, and ceftazidime [Gordon, Hodges and Marriott 1991].

Huang *et al* investigated the possibility that ultrasound may assist the antibiotics in the control of biofilms. They found that at higher levels of ultrasonication gentamicin efficacy was enhanced in *Ps. aeruginosa* biofilms. This observation could be due to the ultrasonication shearing the surface layer of the cells of the
biofilm thereby enabling the antibiotic to reach more cells than if the biofilm remained intact [Huang et al. 1996].

Another idea is that quorum sensing could be used as a target for the treatment of Gram negative infections. This follows on from the fact that quorum sensing is involved in the production of some virulence factors and that by knocking out this system you could render the organism non-pathogenic. This could be achieved either by inhibition of the synthesis of autoinducers by using synthetic AHL precursor analogues, or by preventing the activation of quorum sensing genes by alternative autoinducers, such as furanone compounds [Hartman and Wise 1998; Hentzer et al. 2002].

Possibly the largest field of research in anti-pseudomonal strategies is in the development of vaccines. Vaccines aimed at developing antibodies to alginate have been found to be safe and effective and may be used to prevent chronic Ps. aeruginosa infection in CF patients either by active or passive immunisation. More specifically these vaccines seem to enable the killing of the mucoid Ps. aeruginosa cells by opsonic phagocytosis [Klinger et al. 1983; Woods and Bryan 1985; Pier, Small and Warren 1990; Pier et al. 1994; Johansen 1996; Cryz et al. 1997; Jones 1998; Lang et al. 2004; Zuercher et al. 2005].

Research into the use of phage therapy is currently enjoying resurgence with many scientists believing that it is the answer to the ever-growing problem of multiple antibiotic resistance [Barrow and Soothill 1997]. However the re-emergence of phage therapy research is not without controversy, and has attracted many critics [Projan 2004].

1.4.4 Experimental models of CF infection

Chronic respiratory infection mediated by Pseudomonas aeruginosa was first investigated in rats. This initial model was developed in 1979 by introducing bacteria enmeshed in agarose beads intra-tracheally [Cash et al. 1979]. This system was then taken up by others to investigate the potential, in rats, for immunisation against
Pseudomonas infection [Klinger et al. 1983; Woods and Bryan 1985; Pier, Small and Warren 1990]. This method of introducing Pseudomonas into the trachea via agar beads was also used to investigate local cellular responses to chronic infection and phagocytosis in cats [Winnie et al. 1982; Thomassen et al. 1984], and the effect of mucoidy in guinea pigs [Blackwood and Pennington 1981]. Later this protocol, when applied to rhesus monkeys, enabled the demonstration of Pseudomonas aeruginosa mediated lung damage similar to that found in CF patients [Cheung et al. 1993]. More recently a similar protocol using alginate beads was used to show the difference in inflammatory reaction and bacterial clearance between infections with mucoid and non-mucoid strains [Claus et al. 2009].

However as with many animal models of disease the mouse became the preferred choice. This is likely to be due to the availability of many different strains of mice, their relative low cost and short gestation period. Strains of mice that were genetically susceptible (e.g. C57BL/6 mice) or resistant (e.g. BALB/c mice) to Pseudomonas aeruginosa were used to demonstrate the differences in inflammatory reaction to Pseudomonas infection and the effect of this on bacterial numbers in the airways [Morissette, Skamene and Gervais 1995; Sapru 1999; Tam, Jackson Snipes and Stevenson 1999; Claus et al. 2009; Peying 2009].

There are no naturally occurring CF-type diseases in animals other than humans so the early work on in vitro airway infections was performed on otherwise healthy (with the exception of genetically susceptible mice strains) animals. This was a disadvantage in CF studies as the lungs of healthy animals were likely to respond differently to those affected by CF.

The first step towards a true CF animal model occurred in 1991 when Koller et al. introduced a mutation, via a gene-targeting construct, into exon10 of the mouse CFTR gene. Exon 10 is the site of the most common mutation found in human CF patients, ΔF508. The protein encoded for by the mouse CFTR gene has greater than 78% homology with the human CFTR protein. This mutation was successfully inserted into embryonic stem cells [Koller et al. 1991]. Very shortly afterwards two further groups achieved the same goal with different mutations also affecting exon 10 of the mouse CFTR gene [Dorin et al. 1992; Ratcliff et al. 1992]. The stem cells were injected into mouse embryos which were used to develop mice homozygous for
the defective CFTR gene [Colledge et al. 1992; Dorin et al. 1992; Snouwaert et al. 1992].

Two of the groups reported that the homozygous mice had both chloride ion transport deficiencies in epithelial membranes [Clarke et al. 1992] and some pathological abnormalities reminiscent of human cystic fibrosis [van Doorninck et al. 1995]. Unfortunately the majority of the mice studied died within days of birth due to severe intestinal obstruction leading to peritonitis [Colledge et al. 1992; Snouwaert et al. 1992]. There were very few pathological abnormalities outside of the GI tract in mice compared to human CF cases, which may be due to differences in CFTR expression. The most obvious difference between mice and human CF cases in these studies was the lack of murine pulmonary symptoms. This could be explained by the fact that the mice did not live long enough to display changes in the airway and were not challenged with pathogens [Snouwaert et al. 1992; Snouwaert et al. 1995].

The remaining group achieved the CF mouse via a slightly different gene targeting method to the others [Ratcliff et al. 1992]. They too showed chloride conductance deficiencies, however the disease pathology was somewhat different. The mice survived for longer than they did in the previous two studies due to the much reduced abnormal pathology in the GI tract, however only one mouse showed any sign of airway damage. It should be noted that the sample size of homozygous mutants was very low (n = 6) [Dorin et al. 1992].

Two years later two groups produced CF mice that carried the most common mutation for CF found in humans ΔF508. These mice showed reduced chloride ion transport in epithelial membranes and abnormal GI tract pathology indicative of CF. However again there were no significant abnormalities in any of the other organs that are usually affected in human CF cases [Colledge et al. 1995; van Doorninck et al. 1995].

Davidson et al. first demonstrated a reduced ability of CF mice to clear bacterial infection, suggesting that they may after all provide a model for human CF related lung disease [Davidson et al. 1995]. Further studies showed that CF mice had higher mortality [Heeckeren et al. 1997; Gosselin et al. 1998] and a more pronounced
inflammatory response to *Pseudomonas aeruginosa* applied using the agar bead method used in previous animal infection models [Cash *et al.* 1979; Heeckeren *et al.* 1997; McMorran *et al.* 2001]. There was no difference in clearance between CF and non-CF mice however the method of infection may have precluded the ability for healthy mice to clear the infection normally [Heeckeren *et al.* 1997; Gosselin *et al.* 1998; McCray *et al.* 1999; Chroneos *et al.* 2000]. In some studies the CF mice did carry a higher bacterial burden after several days of infection than their wild type counterparts [Gosselin *et al.* 1998; McMorran *et al.* 2001; Schroeder *et al.* 2001]. Moreover, in one study *Ps. aeruginosa* appeared to have converted to mucoidy in the murine lung [Coleman *et al.* 2003].

Other methods of creating a respiratory infection in mice were sought to represent more closely the acquisition of infection in nature. These included an aerosol method that was first used in non CF mice [Yu *et al.* 1998; Yu, Nasr and Deretic 2000]. Another strategy involving deposition of bacterial suspension directly into the trachea with a specially shaped needle avoided the invasive nature of traditional techniques [Hoffmann *et al.* 2005]. As did a technique using bacteria embedded beads inserted in a non-invasive fashion [Guilbault *et al.* 2005]. A later study used a method of placing bacterial suspension just above the nasal passages of the mice and allowing them to inhale the bacteria. These techniques more closely mimicked the method of infection in nature and enabled differences in bacterial clearance from murine lungs to be established [van Heeckeren *et al.* 2006]. This was due to the lack of a persistent physical presence of agar bead containing bacterial cells in the lung airways.

The lack of consistency in the results of experiments on these mice raises doubts about the validity of CF mice as a model for CF. Results from one study suggested that the lack of an effective mucociliary escalator mechanism seen in human CF patients was not faithfully replicated in CF mice [Cowley *et al.* 1997]. However another study using a different strain of CF mice with an alternative mutation showed that compared to their wild type littermates CF mice showed a similar deficiency in mucociliary transport to human CF patients [Sapru 1999]. There are also striking differences between different strains of CF mice which may indicate the influence of a second chloride channel in some strains [Kent *et al.* 1997; McCray *et
Moreover the lung pathology was similar in some studies between CF and wild type mice that had been infected [Gosselin et al. 1998].

It is clear that the situation in the CF lung arises from a complex interplay between host and bacteria and differences between the physiology of mice and humans can confuse the situation further.

The differences between species of model animals in terms of their respiratory responses to stimulants such as infections and allergens means that these results are unreliable when applied to humans. An alternative to using animal subjects is tissue culture. Tissue culture as a cell biology technique has existed for many decades. It enables the use of human airway epithelial cells, either primary cultures or cell lines giving a better idea of what effect infections or treatments may have rather than studying cells from a different species which may react differently. It is also cheaper and less time consuming than animal studies [Forbes 2000], meaning more extensive series of experiments can be performed in the early stages of drug development, for example, and negates the need for extensive ethical approvals if cell lines are used.

There are two major cell types in the culture of lung/airway cells; alveolar cells and airway cells which include bronchial and nasal epithelial cells. The experiments within this thesis are only concerned with airway epithelial cells, since these are the cells involved in airway infections in CF.

The morphology of the airway epithelia is complex and extremely important in the correct functioning and defence of these cells in vivo therefore successful in vitro cultures must closely mimic this structure. In order to do this, basic epithelial cells in culture must differentiate into several types of cell, such as ciliated cells and goblet cells which produce mucous. One of the first studies to observe the differentiation of tracheal epithelial cells used hamster cells grown on collagen. These primary cells tended to dedifferentiate when attachment took place but eventually became ciliated and began to produce mucin [Wu, Nolan and Turner 1985]. Further studies revealed that both the growth surface and the culture medium were very important for differentiation of the airway epithelial cells. Most groups found that collagen coated surfaces [Wu, Nolan and Turner 1985; Wu, Sato and Whitcutt 1986; Moller et al. 1987; Wu et al. 1990; Hicks et al. 1997; Tristram, Hicks and Hard 1998] or denuded
trachael grafts [Wu et al. 1985; Wu et al. 1990] were optimal growth surfaces for differentiation. The correct formulation of the cell culture media was also necessary for epithelial morphology, with vitamin A (retinoic acid) [Wu, Sato and Whitcutt 1986; Wu et al. 1990; Gray et al. 1996; Wu, Zhao and Chang 1997; Sachs, Finkbeiner and Widdicombe 2003], growth factors [Masui et al. 1986; Gray et al. 1996], and calcium levels [Martin et al. 1991; Sachs, Finkbeiner and Widdicombe 2003] being particularly vital.

Alternative culture methods have been sought in order to optimise differentiation of the cells. One very successful method produced 3D cultures of “balls” of epithelial cells. These structures formed in agitated culture media seeded with cells. They usually became ciliated with cilia beating demonstrated by the movement of the 3D structures. Mucin secretion and longevity (cultures remained viable for up to 6 months) were also features of these cultures [Jorissen et al. 1989; Bridges et al. 1991; Nicolas et al. 2002; Ulrich and Döring 2004].

Arguably the most significant development in airway epithelial culture has been the ability to grow cells at an air-culture media interface known as ALI (air-liquid interface). This is thought to more closely adhere to the in vivo situation with cells exposed to the air in the airway lumen while being fed from below by the blood supply. The Whitcutt chamber was the first manifestation of this culture method, and the technique has changed little since [Whitcutt, Adler and Wu 1988]. The apparatus consisted of a chamber with a porous membrane at the base on which the cells grow. This could be placed into the well of a multi-well plate with a small space between the membrane of the insert and the base of the well. Culture media was be placed into this space to allow the cells to draw nutrients through the pores in the membrane. This technique along with the correct cell culture media has proved very successful in achieving highly differentiated airway epithelial cell layers [Whitcutt, Adler and Wu 1988; Yamaya et al. 1992; Gray et al. 1996; Hicks et al. 1997; Tristram, Hicks and Hard 1998; Bals et al. 2004]

This method of culture has been further developed, by adding inflammatory mediator cells in order to more closely mimic the in vivo situation. Monocytes and dendritic cells isolated from human blood were added to the apical and basal sides of the inserts respectively. Results indicated that these cells behaved in vitro as they do in
vivo and there was communication and contact between the different types of cells. This form of airway cell culture would be very useful for studying host pathogen interactions [Radyuk et al. 2003; Rothen-Rutishauser, Kiama and Gehr 2005].

Many different host-pathogen interaction studies of the airway epithelia have been performed in vitro. Important information on the adsorption replication and inflammatory response of viruses such as severe acute respiratory syndrome (SARS), influenza and rhinoviruses has been elucidated from such studies [Matsukura et al. 1996; Lopez-Souza et al. 2004; Sims et al. 2005; Sims et al. 2008]. Pathogenic fungi have also been studied using in vitro infection models e.g. Aspergillus fumigatus and Cryptococcus neoformans [Botterel et al. 2008; Guillot et al. 2008].

Bacterial pathogens represent by far the largest number of studies in vitro airway pathogen studies and the vast majority are host-pathogen interaction studies rather than treatment models. These have particularly focussed on Burkholderia cepacia, a notable CF pathogen that can cross the epithelial barrier and lead to systemic infection known as cepacia syndrome. Other groups have studied bacterial translocation through and attachment to the epithelial layer. Bacteria such as Mycobacterium tuberculosis and Mycoplasma pneumoniae both very important respiratory pathogens have been studied in this way [Bermudez et al. 2002; Krunkosky et al. 2007]. The translocation of TB in particular has been studied for periods of up to 5 days at a time [Bermudez et al. 2002].

Haemophilus influenzae has been investigated for its role in promoting cytokine release in airway cells and the role this has in both chronic obstructive pulmonary disorder (COPD) and the early stages of CF [Frick et al. 2000; Chin et al. 2005]. However in 2006 an important step was made by Chin et al who demonstrated that H. influenzae could form biofilms on differentiated airway epithelia grown at ALI. This correlated with the data they collected from airway lavage samples obtained from young CF patients [Starner 2006]. This important discovery showed that it was possible to simulate an infection in vitro that closely resembled the situation in vivo.

Burkholderia cepacia has been extensively studied recently due to its emerging importance in CF. Much of this work has concentrated on the ability of B. cepacia to traverse and breakdown the epithelial cell layer and turn bacterial internalisation by
the epithelial cells to their advantage. Antibiotic protection assays have provided much progress in the understanding of bacterial internalisation and the associated modes of infection [Burns et al. 1996; Cieri et al. 2002]. In these studies bacteria are added to the cell culture system for a defined length of time before an antibiotic (often gentamicin) is added at sufficient concentration to kill any bacteria that remain outside of the epithelial cells. In vitro studies have also successfully shown the extent of damage that *B. cepacia* causes to tight junctions. Moreover *B. cepacia* cells have been found free within epithelial cell cytoplasm having apparently prevented phago-lysosomal fusion [Sajjan, Keshavjee and Forstner 2004; Kim et al. 2005; Sajjan et al. 2006]. Co-culture models have also shown that the bacteria form aggregates within the mucous layers of cells which are likely to be a precursor to biofilms. These were successfully studied for up to 24 hours [Schwab et al. 2002].

Due to its prominence as the most significant CF pathogen there have been many in vitro studies of *Pseudomonas aeruginosa*. Early work in this field concentrated mainly on adherence studies and several groups demonstrated that *Ps. aeruginosa* uses pili to adhere to epithelial cells [Woods et al. 1980; Doig et al. 1988; Saiman, Cacalano and Prince 1990; Saiman and Prince 1993]. This was achieved using epithelial cells in suspension and later in submerged culture. Further studies confirmed that *Ps. aeruginosa* pili attach to asialylated glycolipids (aGM1s) on the epithelial cell surface [De Bentzmann et al. 1996].

In general, after this, studies on *Ps. aeruginosa* formed close parallels with studies on *B. cepacia* with 3 main strands of investigation; (i) invasion studies using antibiotic protection assays, (ii) breakdown of the epithelial barrier, and (iii) cytokine release.

Antibiotic protection assays demonstrated that although some *Ps. aeruginosa* cells were able to invade epithelial cells without being destroyed by lysozymes, the bacterial titre did not increase within the cells. This may indicate that although capable of cellular invasion this is not their preferred mode of infection [Fleiszig et al. 1994; Fleiszig, Zaidi and Pier 1995; Fleiszig et al. 1997; Garcia-Medina et al. 2005]. These assays are usually supported by electron or confocal micrographs.
Investigation into the ability of *Ps. aeruginosa* to significantly injure the epithelial cell barrier is of vital importance as it is this action along with the immune system response that eventually leads to respiratory failure in CF patients. *In vitro* evidence has shown that *Ps. aeruginosa*, when placed on airway epithelial cells at ALI for up to 16 hours severely affects the integrity of the epithelial barrier. *Ps. aeruginosa* infection disrupts tight junctions, triggers apoptosis and causes cells to break away from the insert membrane [Grassme *et al.* 2000; Garcia-Medina *et al.* 2005; Zulianello *et al.* 2006; Chugani and Greenberg 2007; Rejman *et al.* 2007]. This highlights the difficulty in creating an infection model of *Ps. aeruginosa in vitro*. The progression of the infection to destruction of the epithelial layer appears to be much faster *in vitro* than *in vivo*. 
1.5 Bacteriophages

1.5.1 History and classification

Viruses are thought to be the most ancient entities on earth able to infect almost all known organisms with related viruses infecting organisms from different phyla [Bamford 2003; Krisch 2003]. Bacteriophages are ubiquitous throughout the environment, in particular in the aquatic environment where they significantly contribute to the global cycle of nutrients in the oceans and bacterial population dynamics [Bergh et al. 1989; Chibani-Chennoufi et al. 2004; Mann 2005].

Bacteriophages are thought to have been discovered independently 2 years apart in 1915 and 1917 by Twort and d’Herelle respectively, although there has always been much controversy surrounding this issue as to who was the true discoverer [Duckworth 1976]. Shortly after his discovery d’Herelle successfully used phages to treat a number of patients suffering from dysentery [Sulakvelidze, Alavidze and Morris 2001]. Phages became the focus of intensive study to establish their infection process and morphological characteristics, as it was thought that they would be likely candidates for bacterial infection therapy. Early electron microscopy images showed researchers for the first time the distinctive head and tail shape that most phages possess, images also captured adsorption and lysis supporting culture based experiments investigating the infection process [Ellis and Delbruck 1939; Luria and Anderson 1942; Luria, Delbruck and Anderson 1943].

Bacteriophages form one order containing 13 families and 30 genera, these divisions are based mainly on the morphological characteristics of the virion. The vast majority of phage strains are tailed (either contractile or non-contractile). The remaining phage families are either polyhedral, filamentous, or pleomorphic [Bradley 1967; Ackermann 2003].
1.5.2 Bacteriophage biology

1.5.2.1 Lytic lifecycle

The lytic lifestyle was studied and characterised in the first half of the 20th century, just over two decades after the discovery of bacteriophages. It was found that there were distinctive steps in the infection process of a phage; adsorption of the phage to the host cell, multiplication of new phage progeny, and finally lysis of the bacterial cell to release the new phages. This continues through several increments of cell lysis until the infected bacterial culture clears indicating the majority of bacterial cells have been killed by the phages [Ellis and Delbruck 1939]. Elements of this process were shortly afterwards recorded by electron microscopy with images clearly showing phages adsorbed to the surface of bacterial cells and later lysis of the cells releasing the phage progeny [Luria and Anderson 1942; Luria, Delbruck and Anderson 1943]. The lytic lifecycle is very short often only minutes long [Ackermann 1999].

Phages initiate infection by first adsorbing to the outer surface of the host cell, different phage use different bacterial elements as a receptor, appendages such as pili being commonly used. All tailed phage attach via a base plate at the end of their tail, tail-less phages attach to the bacterial cell and usually release their DNA into the bacterial cell wall before it enters the cytoplasm, as they do not have the injection equipment that can deliver the DNA directly [Ackermann 1999].

The channelling of the phage DNA out of the capsid and across the complex cell membrane and wall of the bacteria after adsorption is thought to be achieved through a number of different mechanisms which haven’t been fully elucidated yet. After studies of the E.coli phages T4, T5, and T7, three separate mechanisms have been proposed. The tail of T4 contracts on adsorption to the bacterial cell causing the base-plate of the phage to puncture the bacterial cell using lysozyme to digest the peptidoglycan. The DNA then crosses the cell wall through the phage tail channel at a very fast rate of approximately 4000 bp S⁻¹, the whole phage genome being transferred in 30 seconds. In the non-contractile T5 only 8% of the genome is transferred initially after which it stops whilst two proteins are synthesized from it.
(A1 and A2). After this the remainder of the DNA is transferred taking around 6 minutes in total. If A1 and A2 are not produced the remainder of the DNA is not transferred but remains attached to the first 8% spanning the bacterial cell wall, indicating that these proteins may pull the DNA through. T7, also non-contractile transfers DNA in a similar way, the first 850 bp are transferred possibly due to the pressure inside the capsid, the bacterial polymerase is then activated to pull around 20% of the phage genome through. This contains the gene for the phage RNA polymerase which is synthesized and takes over the transfer of the DNA transcribing as it does so. The whole genome is transferred in 10 minutes [Letellier et al. 2003].

In an early study of the infective process of a *Ps. aeruginosa* RNA phage by Bradley electron micrographs showed areas within the bacterial cell where immature virions were being assembled ready for lysis. Later micrographs showed a large bulge in the cell wall of the bacteria before it ruptured releasing phage progeny [Bradley 1966].

Phage DNA is usually transcribed in three stages, early, middle and late. Early genes are concerned with taking over the host DNA machinery and preventing destruction of the phage DNA. Middle genes are involved with DNA synthesis of copies of the phage genome for the progeny. Late genes encode for the phage structure and lysis proteins [Ackermann 1999].

The phage heads, called proheads until maturation, are formed within the bacterial cell in stages. Often a scaffolding core is the initial stage in head formation, gradually the head becomes larger and the protein lattice forms the shape of the head, finally DNA can be packaged inside before addition of the tail (tailed phages only). Tails are usually assembled in an independent pathway to the heads, the two pathways then converge and the tail is attached [Murialdo and Becker 1978; Kellenberger 1990; Kellenberger and Wunderli-Allenspach 1995].

The packaging of the DNA into the capsid precursors during virion assembly in most cases is thought to start with the production of concatameric DNA. This is a long piece of DNA containing multiple repeated copies of the phage genome. The points at which one genome ends and another starts are recognised and cut by phage-borne enzymes before packaging can begin [Catalano, Cue and Feiss 1995].
Lysis of the bacterial cell after phage maturation occurs by several methods depending on the type of phage involved. Double stranded DNA containing phages produce endolysin an enzyme which digests peptidoglycan in the cell wall; it reaches the cell wall by the action of a second protein which permeabilises the cell membrane called holin. Some phages also contain genes which code for two alternative lysis proteins which are thought to interfere with the cell membrane or wall. Single stranded DNA containing phages possess only one lysis protein which is thought to interrupt peptidoglycan synthesis. Filamentous phages (Inoviridae) can emerge from the infected bacterial cells through phage encoded channels in the bacterial cell wall. This means lysis does not take place and although the bacterium survives this process the growth rate is usually reduced [Young 1992; Young, Wang and Roof 2000].

Latent period (the time taken from initial infection to lysis) duration can be affected by host density with lower concentrations of bacteria leading to a longer period of time before lysis. A compromise has to be achieved because a shorter latent period leads to a smaller burst size. When bacterial hosts are plentiful a smaller burst size is less important as there are more hosts to infect, therefore plenty more progeny can be produced. When hosts are scarce the latent period gradually evolves to become longer enabling many more progeny to be produced increasing the possibility that more bacterial cells will be encountered and infected [Abedon, Herschler and Stopar 2001; Abedon, Hyman and Thomas 2003].

1.5.2.2 Lysogenic lifecycle

As part of a lysogenic cycle a temperate phage will infect a bacterial cell but rather than causing the production of progeny and eventual lysis of the host cell it inserts its genome which then remains as a prophage. This phage DNA is then passed on to daughter cells through bacterial divisions until induced to become lytic. This is the process by which phage can transfer virulence genes [Takeda and Murphy 1978; Waldor and Mekalanos 1996]. In fact this phage mechanism may account for a good
deal of both phage and host evolution [Cheetham and Katz 1995; Hendrix et al. 1999].

There are several different methods by which phages insert their DNA; via a plasmid that doesn’t integrate into the bacterial chromosome, by transposition, or by site-specific recombination using either integrases or invertase-resolvase enzymes [Campbell 2003].

Bacteria can have several prophages within their own genome accounting for up to 20% of the DNA in some cases. Often these prophages increase the fitness of their host, including the ability to adjust to environmental conditions and infect host cells [Canchaya et al. 2003; Casjens 2003]. The highly pathogenic \textit{E. coli} strain O157 Sakai genome contains 24 prophage-like elements and these are believed to be important in the development of its highly virulent status [Ohnishi, Kurokawa and Hayashi 2001]. Prophages can also prevent lytic phages attacking the bacterial host. In \textit{Ps. aeruginosa} PAO1 2 prophages reside in the genome, one is a bacteriocin derived from a phage tail the second is a filamentous phage similar to Pf1 (see section 1.5.4.3) [Canchaya et al. 2003; Casjens 2003]. Several virulence factors produced by \textit{Ps. aeruginosa} have prophage origins including adhesion to host cells, production of cytotoxins and alteration of LPS [Vaca-Pacheco et al. 1999; Newton et al. 2001; Wagner and Waldor 2002].

Growth conditions, such as nutrient availability and temperature, affect the stability of prophages by altering their spontaneous induction frequency [Lunde et al. 2005]. Occasionally prophages will mature and be released from the bacterial cell by lysis so as to continue the lysogenic infection elsewhere [Bertani 1951].

1.5.2.3 Specificity and resistance/defence

Bacteriophage resistance mutants can arise spontaneously as can be observed by regrowth in an infected culture [Luria and Delbruck 1943; Demerec and Fano 1945]. Bacteria employ defences against phage infection such as the phase-variable protein Ag43 found in \textit{E. coli} residing in the intestinal tract. The ability to “turn-off” this
membrane protein results in an avoidance of bacteriophage infection. It may be that the phage uses this protein as an adsorption site [Grzyn and Thomas 2002].

In another study on the effect of bacteriophage on the diversification of *Ps. aeruginosa* cultures two distinct resistant mutants evolved. The phage used was pilus specific (PP7) hence the two types of mutants were believed to be hyperpiliated with non-retractile pili, and unpiliated. The hyperpiliated mutants caused a large reduction in the number of phage particles in the broth culture due to irreversible binding of the phage to the pili which were then unable to continue the infection due to the lack of pilus retraction [Brockhurst, Buckling and Rainey 2005].

Stable populations of bacteria and bacteriophage can be achieved by continuing development of resistance by the bacteria to the phage and mutations in the phage that allow it to overcome that resistance [Balakshina, Kulakov and Boronin 1992].

1.5.2.4 Bacteriophages and biofilms

In the same way that diffusion of antibiotics can be hindered, the exopolysaccharide of biofilms can also prevent phage access to the bacteria, however some phages manage to get over this hurdle. Phage SF153b, like many other phages carries on its base plate a glycanase which is able to degrade the exopolysaccharide of its host *Enterobacter agglomerans*. This leads to a two phase reduction in the number of biofilm bacteria upon infection with the phage. The first drop in cell number is likely due to the sloughing off of cells as a direct result of the glycanase action, the second is likely due to normal lytic phage infection [Hughes, Sutherland and Jones 1998].

Phage release is a normal process in the development of biofilms. Resch *at al.* showed phage release in planktonic cultures of *S. aureus* decreased rapidly after 8 hours; however phage release in biofilms decreased much more slowly and there was still detectable phage after 72 hours. This was believed to be due to proteolytic enzymes released by planktonic cultures which denatured the phage. The advantage of phage release to a biofilm culture is the release of nutrients from lysed cells into a nutrient deprived biofilm. Moreover phage release drives the competence for transformation due to free DNA released from lysed cells and disruption of the cell
membrane [Resch et al. 2005]. As already mentioned previously *Ps. aeruginosa* phage Pf4 may be responsible for driving the emergence of the small colony variant (SCV) from biofilms. This may be involved in the spread of the biofilm, as SCVs were found in large numbers in the biofilm reactor run-off and possessed enhanced adhesion properties [Webb, Lau and Kjelleberg 2004]. This also appears to be the case for bacteriophage PP7 which drives the emergence of small rough (SR) colony variants [Brockhurst, Buckling and Rainey 2005].

1.5.3 *Pseudomonas* bacteriophages

The first bacteriophage to be discovered that was specific for *Ps. aeruginosa* was isolated from the faeces of patients with colitis in 1945 [Fastier 1945]. *Ps. aeruginosa* specific phages can be found throughout the environment due to the prevalence of the host itself. Hence phages have also been found in the sputum of CF patients chronically infected with *Ps. aeruginosa* [Tejedor, Foulds and Zasloff 1982].

Up until recently the main biotechnological use of *Pseudomonas* phages has been for the phage-typing of isolated strains of the bacteria. The varying susceptibility of each strain to a bank of bacteriophages provides a unique epidemiological fingerprint [Farmer and Herman 1969].

Bacteriophages that use *Ps. aeruginosa* as their host fall into five families: Podoviridae, Myoviridae, Inoviridae, Siphoviridae, and Leviviridae. Most *Pseudomonas* phages have genomes in the range of 35 kbp to 75 kbp, with the exception of the large bacteriophages such as SDM-1 and phiKZ, which have genomes in the region of 280kbp [Kwan et al. 2006].

1.5.3.1 Podoviridae

F116 is a well-characterised bacteriophage used in this study; it has been described as a transducing phage in the podoviridae family. Its head is 65 nm in diameter with
an 80 nm tail, it has a genome of 65kbp and requires pili to adsorb and infect the bacterial cell. Although originally regarded as a non-integrating transducer which inserts its genome separately as a plasmid, recent evidence suggests that it does possess an integrase gene which throws this theory into doubt [Slayter 1964; Miller, Pemberton and Richards 1974; Byrne and Kropinski 2005].

Phages LKD16 and φKMV which exhibit a broad host range of *Ps. aeruginosa* strains are members of a subgroup of the T7 supergroup within the podoviridae. Recent genomic analysis has found that they belong in their own T7 subgroup of widespread *Pseudomonas* phages called the φKMV subgroup. This also includes LKA1 which is more distantly related to φKMV and LKD16 than they are to each other, and also has a much narrower host range. However all three phage are less sensitive to restriction enzymes than other phage [Lavigne *et al.* 2003; Ceyssens *et al.* 2006].

Phage φPLS27 is LPS specific has a genome of 42.7 kbp and is very similar to the coliphage T7 [Allan *et al.* 1989].

Other phages specific for *Pseudomonas* in the podoviridae family that have been isolated include 119X, Gh-1, and PaP2.

### 1.5.3.2 Myoviridae

The large φKZ bacteriophage resides in a separate genus of the myoviridae family of viruses along with Lin21, NN, PTB80 and Lin68. It has a large icosahedral head measuring nearly 150 nm in diameter with a 180 nm contractile tail [Fokine *et al.* 2005]. Due to a lack of restriction enzyme sites in its genome these phages are resistant to a number of restriction enzymes produced by *Ps. aeruginosa* as a defence against phage infection. The average G + C content is 36.8% which is significantly lower than its host genome. The vast majority of potential proteins found from φKZs genome have no homology to any other known proteins, however, fifteen gene products show similarities to proteins from other human pathogens, e.g. *Mycobacterium tuberculosis*, *Haemophilus influenzae*, and *Vibrio cholerae*.
[Mesyanzhinov et al. 2002]. This phage also has a wide host range, it is able to infect up to 50% of known clinical isolates of *Ps. aeruginosa* [Krylov et al. 2003]. Another phage SDM-1 shares 99% genome sequence homology with φKZ the only differences being 2 insertions, one in each phage genome. [Kwan et al. 2006]

φCTX is a temperate, non-inducible P2-like phage which carries a cytotoxin gene increasing the virulence of the bacterial host [Hayashi et al. 1990]. It is thought to use LPS as a receptor for binding to the bacterial cell. This group of phages are likely related to R-pyocins which are also produced by *Ps. aeruginosa* [Yokota, Hayashi and Matsumoto 1994; Nakayama et al. 1999].

The phages EL and RU are also found in this family but show significant differences to φ KZ with a third of the predicted EL gene products being similar to those of φKZ in a genome of approximately 211 kbp. This suggests they may belong to a different genus [Krylov et al. 2003; Hertveldt et al. 2005].

1.5.3.3 Inoviridae

Several filamentous phages have been isolated that are specific for *Ps. aeruginosa* among these are Pf1 which is 2000 nm long and 6 nm in diameter, two DNA strands run the length of the virion [Marvin, Wiseman and Wachtel 1974; Thiriot et al. 2004]. Like Pf3 the capsid of this phage contains an adsorption protein similar to that found in the Ff phages of *E.coli*, indicating a possible evolutionary relationship. Pf1 attaches to *Ps. aeruginosa* via the type IV PAK pilus [Bradley 1973; Hill et al. 1991].

Pf3 attaches to its host via the RP4 pilus and progeny phage are released without bacterial cell lysis. It has a circular genome consisting of 5.8 kbp, the virion is 720 nm long and 6.5 nm in diameter [Newman et al. 1982; Allan et al. 1989].
1.5.3.4 Siphoviridae

Bacteriophage B3 is a transposable phage which infects *Ps. aeruginosa*, it has a genome of 38,439 bp and is thought to be related to the *E. coli* phage Mu [Braid et al. 2004]. It was first characterised in 1964 with a head measuring 52 nm in diameter and a 163 nm long tail [Slayter 1964]. Similar to D3112 there are a wide range of *Ps. aeruginosa* strains that are sensitive to it, moreover some *B. cepacia* strains are also sensitive to this phage [Nzula, Vandamme and Govan 2000]. It also uses pili to adsorb to bacterial cells, but pili exclusively are not sufficient, other elements are necessary [Roncero, Darzins and Casadaban 1990]. B33 is very similar to B3, it is a temperate phage with an icosahedral head measuring approximately 50 nm in diameter and a tail of 135 nm in length [Morgan and Stanisich 1976].

First characterised in 1974 phage D3 has a head of 70 nm diameter with a 150 nm flexible tail. It establishes a lysogenic lifestyle in *Ps. aeruginosa* strain PAO1, inserting a genome of 56.4 kbp. In doing so it causes changes to the structure of the LPS altering the serotype and potentially giving the bacteria an advantage over host immune functions [Holloway and Cooper 1962; Miller, Pemberton and Richards 1974; Kropinski 2000; Newton et al. 2001].

D3112 is a transposable phage with a genome of 37.6 kbp, it is related to several other phages with different host species including phage λ, enterobacteriophage mu and the *S. aureus* phage φ12 [Rehmat and Shapiro 1983; Wang, Chu and Guttman 2004].

DMS3 is a temperate phage induced from starved *Ps. aeruginosa* clinical isolate cultures. It is pilus-specific and has an icosahedral head 50 nm in diameter and a tail of 110 nm in length. It was found to be capable of transduction in strains PA14 and PAO1 and could also cause transduction between those strains [Budzik et al. 2004].

G101 was first characterised in 1974, it has an elongated head 75 nm long and 60 nm wide with a 200 nm long non-contractile tail [Miller, Pemberton and Richards 1974]. Some *B. cepacia* strains are sensitive to it as well as *Ps. aeruginosa* [Nzula, Vandamme and Govan 2000].
PO4 was isolated from sewage in 1973, its head is 58 nm in diameter and it has a 186 nm long flexible non-contractile tail. This phage also uses pili as a receptor site for adsorption and it has been shown that in some strains of *Ps. aeruginosa* the pili retracts on phage binding drawing the phage closer to the bacterial cell surface. Moreover the binding of phage appears to stimulate the formation of pili [Bradley 1972; Bradley 1972; Bradley 1973].

1.5.3.5 Leviviridae

PP7 is a small phage first characterised in 1966. It has a capsid of up to 28.4 nm diameter and a genome of 3588 nucleotides. It is believed to adsorb to the pili. Another phage from the leviviridae family that is believed to infect *Ps. aeruginosa* is PRR1[Bradley 1966; Tars *et al.* 2000].

1.5.4 Suitability of bacteriophages as a therapeutic agents

The increasing recalcitrance of many pathogens to antibiotic treatment is becoming a major concern for the future management of bacterial infections. Antibiotic treatment can apply a selective growth advantage to those bacteria that are able to evade killing by antibiotics. Strategies include antibiotic degrading enzymes such as beta-lactamases, the ability to actively remove the antibiotic from the bacterial cell e.g. multi-efflux pumps, changes to the outer surface of the bacteria, and the biofilm mode of growth [Murray 1991; Berkowitz 1995]. This has led to increased interest in phages as a novel therapy for antibiotic resistant bacterial infections. However until relatively recently there have been few well designed controlled studies into the efficacy of phages as treatments and little consideration as to the potential drawbacks of this strategy [Carlton 1999]. However of the limited studies completed so far side-effects to phage therapy appear rare and reversible, with good bioavailability and low immunogenicity [Alisky *et al.* 1998].
Arguably the major concern regarding the therapeutic use of phages is the transfer of virulence determinants [Projan 2004]. There are many examples of phage encoded toxin genes in pathogenic bacteria, indeed phage are thought to be responsible for much of the pathogenic evolution, from host cell attachment strategies to secretion of toxins, and intra-cellular growth [Coleman et al. 1989; Cheetham and Katz 1995; Broudy, Pancholi and Fischetti 2001; Banks, Beres and Musser 2002; Boyd and Brussow 2002; Wagner and Waldor 2002; Brussow, Canchaya and Hardt 2004]. For example there is evidence to suggest that conversion of non-mucoid strains of *Ps. aeruginosa* to mucoidy could be mediated by lysogenic conversion by phages [Martin 1973; Miller and Rubero 1984; Newton et al. 2001]. There is also the possibility that phages could transfer toxin genes to normally non-pathogenic natural flora, or transfer antibiotic resistance genes to previously susceptible strains [Blahova et al. 1998]. The concern is that if phages are not screened for these types of genes before use then they could potentially transfer new virulence factors to the pathogen that is being treated. Phages that are being considered as therapeutic solutions would have to be thoroughly screened and possibly genetically engineered to remove the problem genes [Alisky et al. 1998].

High specificity of phages often down to the strain level means the potential therapeutic phage would have to be closely matched to the target bacteria. Alternatively phage cocktails would be required containing several different phages enabling a broader range of action [Alisky et al. 1998; Projan 2004].

Resistance development is also a potential problem of phage therapy with many bacteria developing resistance mechanisms to phages [Projan 2004]. However in many of the studies completed only one dose of phage has been necessary to achieve eradication of the target pathogen limiting the chances of resistance development. Cocktails of different phages having different mechanisms of infection may also help overcome this problem, however resistance can still develop [Smith, Huggins and Shaw 1987]. Phages could also be engineered to overcome the bacterial resistance mechanisms, or naturally occurring mutant phages could be selected [Alisky et al. 1998]. Due to their specificity and unlike antibiotics phages are very unlikely to cause resistance to develop in normal flora [Barrow and Soothill 1997].
Some researchers have claimed that the wide scale lysis of bacterial cells as a result of phage infection could potentially release large quantities of endotoxin. This could be avoided by genetically modifying phages to not produce holins but remain lethal to bacterial cells whilst keeping them intact, using, for example redonucleases that result in unrepairable bacterial DNA damage [Dixon 2004]. However this disadvantage of phage therapy has been disputed by others, plus it is also a problem with lytic antibiotics which are currently used to treat infections [Soothill et al. 2004].

The method of application of phage treatments could vary according to the infection. Burns associated and wound and skin infections could be treated by direct application [Ahmad 2002]. A study on how orally delivered phages fared revealed that after 10 days of treatment phages were found in 47 out of 56 blood samples and 9 out of 26 urine samples. This indicates that to some extent phages could overcome the harsh conditions of the stomach to pass into general circulation, especially if phage treatment was preceded by medication to neutralise the stomach acid [Weber-Dabrowska, Dabrowski and Slopek 1987]. However once within the circulatory system the phage particles can be quickly removed by the spleen. Long-circulating phage mutants were developed by injecting phage into mice and isolating the remaining phage from blood samples after 7 hours. These phages were propagated in bacterial cultures, purified and re-injected into mice. This was repeated nine times resulting in phage which remained at high levels in the blood up to 18 hrs post injection [Merril et al. 1996].

Due to their relatively inexpensive propagation, phages could be used to great effect in undeveloped countries with poor access to antibiotics [Ahmad 2002].

1.5.5 Experimental bacteriophage therapy strategies

In a series of studies in the 1980s, 550 patients (including 114 children) in total who were suffering from antibiotic resistant infections or sepsis caused by various pathogens were treated with phages specific for the causative organism. The results were summarised by Weber-Dabrowska et al. Positive outcomes were recorded in on
average 92.4% patients with 6.9% showing some temporary improvement and 0.4% showing no improvement. Although these are encouraging results the studies were not as vigorously conducted as might be expected for example they were not randomised double blind studies, which is the usual method for clinical trials [Slopek et al. 1983; Slopek et al. 1983; Slopek et al. 1984; Slopek et al. 1985; Slopek et al. 1985; Slopek et al. 1985; Slopek et al. 1987; Weber-Dabrowska, Mulczyk and Gorski 2000]. A further smaller study treating skin infections caused by various pathogens with phages revealed that half of the treated patients exhibited outstanding results including a suppression of inflammation and negative swabs. However 7 of the 31 patients had their phage treatment stopped due to a lack of improvement or side effects [Cislo et al. 1987].

A study on the efficacy of intra-muscularly injected phage against an experimental *E. coli* infection (either intra-muscularly or intra-cerebrally injected) revealed a lower mortality in phage treated than antibiotic (tetracycline, ampicillin, chloramphenicol, trimethoprim and sulphafurazole, and streptomycin) treated mice. Injection of phage up to 5 days prior to infection had a protective effect. The resistant bacterial mutants that did occur were found to have highly reduced virulence [Smith and Huggins 1982]. The same authors conducted another study on the efficacy of phage therapy in the treatment of *E. coli* mediated diarrhoeal disease in calves piglets and lambs. When orally treated with a mixture of 2 phages (B44/1 and B44/2) calves could be protected from *E. coli* infection however phage B44/1 resistant mutants arose in some calves leading to worsening disease state, the addition of a third phage B44/3 seemed to prevent this. Phage B44/3 in conjunction with B44/1 also appeared more effective at treating the disease after the onset of symptoms than a B44/1 B44/2 mixture. B44/1 was the most virulent phage as much larger concentrations were found in faecal samples compared to the other phages. Treatment of *E. coli* mediated diarrhoea in piglets by two phages (one that was specific for the resistant mutants of the other) at the onset of symptoms was found to be effective with markedly reduced numbers of *E. coli* in faecal samples 8 hours post treatment. A similar effect was seen in lambs. This shows the importance of carefully chosen phages that take into account the development of resistant bacteria, in these studies a highly virulent phage was selected with a secondary phage active against organisms that had become resistant to the virulent phage [Smith and Huggins 1983]. A further
experiment in calves infected with one of 7 strains of \textit{E. coli} found that treatment with the specific phage for that strain was effective at concentrations as low as $10^2$, with the phage suspension sprayed onto the calf litter. Low numbers of resistant mutants occurred but appeared to be of very low virulence. Infections caused by a mixture of 6 strains were controlled by suspensions of 6 different phages but not as effectively as the single strain infections, with a virulent phage resistant mutant occurring. Mutant phages were derived that were effective against this resistant mutant. Treatment of calves with higher doses of phage ($10^5$) 6 hours prior to infection prevented the onset of symptoms, lower doses ($10^2$) did not have this protective effect. This study illustrates the need to monitor the development of resistant mutants even if using a cocktail of phages. In this case a multi-strain infection treated with several phages specific to each \textit{E. coli} strain developed a virulent resistant mutant, possibly due to genetic transfer between strains [Smith, Huggins and Shaw 1987].

Phage treatment in mice when given simultaneously with a lethal bacterial inoculation was found to be protective for \textit{Acinetobacter baumanii} (at low phage concentrations) and \textit{Ps. aeruginosa} infection (at high phage concentrations) but not for \textit{S. aureus} infection. This could have been due to the high levels of \textit{S. aureus} required to achieve a fatal effect [Soothill 1992].

Soothill has also shown that the destruction of skin grafts by \textit{Ps. aeruginosa} infection (a complication often seen in human burns patients) in guinea pigs could be prevented by the direct application of a phage specific for the causative strain [Soothill 1994].

In an experiment testing the efficacy of phage as a prophylactic against \textit{S. aureus} infection it was found that although phage were successful in preventing abscess formation when administered at the same time as the bacteria, they required a higher Multiplicity of Infection (MOI) than similar treatment of Gram negative infections, that is more phage particles per bacterial cell. This indicates that phage treatment of Gram positive infections may not be as efficacious as that of Gram negative infections [Wills, Kerrigan and Soothill 2005].
Another possibility is to genetically modify a phage that doesn’t lyse its host during release of progeny so that it becomes non replicative. A modified version of Pf3 denoted Pf3R was used to treat an experimental *Ps. aeruginosa* infection. It was found that the non replicating non lytic phage achieved similar killing levels to a lytic phage but without causing a large release of endotoxin [Hagens *et al.* 2004].

In a study by Hanlon *et al* there was a reduction in viable counts of a biofilm after treatment with phage that was dependant on cell/phage ratio and length of incubation but not on the age of the biofilm. Also demonstrated was the ability of a *Ps. aeruginosa* phage to diffuse through intermediate concentrations of alginate, and even up to 12% alginate if incubated long enough. Upon rheological assessment of the alginate after incubation with phages, it was discovered that viscosity had decreased by up to 40%. Further analysis of the alginate by chromatography uncovered a shift from larger molecules to smaller ones. This could be indicative of and enzymic reaction. Hanlon *et al* go on to speculate that this could be explained by the up-regulation of the host enzyme alginate depolymerase caused by the bacteriophage infection and the subsequent utilization of the enzyme by the phage [Hanlon *et al.* 2001].

Another strategy that could be employed is to use a phage protein that adversely effects bacterial growth rather than using the whole phage, such as lysins, or bacteriophage tails of which some are closely related to bactericidal pyocins [Shinomiya and Shiga 1979; Projan 2004].
1.6 Aim

Most of the *Ps. aeruginosa* studies in the literature relate to infection models with bacterial cells on either submerged or ALI cultures to study the infection process, or biofilms on inert surfaces to study infection treatments. There are no studies concerning the development of an infection of *Ps. aeruginosa* on airway epithelial cells grown at ALI in order to study potential therapeutic agents. Bacteriophages appear to be a useful tool with which to develop a novel treatment for *Ps. aeruginosa*. In order to confirm that bacteriophages would be appropriate to treat CF related *Ps. aeruginosa* airway infections it must first be established that they cause no undue harm to the airway cells. Initially this could be investigated using an *in vitro* model of *Ps. aeruginosa* infection on airway epithelial cells which could be used to test bacteriophage treatment. To-date no such system has successfully been developed.

Therefore the aim of this study is to investigate the potential of bacteriophages in the treatment of CF associated *Ps. aeruginosa* airway infections. This will be achieved by the following objectives:

- To identify and characterise a selection of bacteriophages specific for *Ps. aeruginosa* from the University of Brighton culture collection.
- To test the effect of these bacteriophages on human airway epithelial cells grown in a cell culture model *in vitro* mimicking conditions found at the human airway epithelia *in vivo*.
- To develop an *in vitro* model of *Ps. aeruginosa* infection of the airway epithelia using a co-culture system.
- To investigate the effects on airway epithelial cells of bacteriophage treatment of the model *Ps. aeruginosa* infection.
2 General Materials and Methods
2.1 Materials and reagents

2.1.1 Materials

- **20NE API strip**: BioMérieux sa. France.
- **Carbenicillin**: Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK.
- **Chloroform (CHCl\(_3\))**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Dimethyl sulfoxide (DMSO)**: Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK.
- **ELISA kits**: BD biosciences ltd. Oxford, UK.
- **Foetal bovine serum (FBS)**: PAA Laboratories GmbH. Pasching Austria.
- **Gelatin**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Glycerol**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Hydrogen peroxide (H\(_2\)O\(_2\))**: Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK.
- **LdH kit (cytotox 96)**: Promega. Southampton, UK.
- **Luria Bertani broth (LB)**: 30g/l in H\(_2\)O – Oxoid Ltd., Basingstoke, Hampshire UK.
- **Magnesium sulphate (MgSO\(_4\)(7H\(_2\)O))**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Minimum essential media with Earles salts (MEME)**: PAA laboratories GmbH., Pasching Austria.
- **Minimum essential media with Earles salts without phenol red**: PAA laboratories GmbH., Pasching Austria.
- **Penicillin/streptomycin solution (100x)**: PAA Laboratories GmbH., Pasching Austria.
- **Phosphate buffered saline (PBS)**: 1 tablet in 100 ml H\(_2\)O – Oxoid Ltd., Basingstoke, Hampshire UK.
- **Polyethylene glycol 8000 (PEG 8000)**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Sodium Chloride (NaCl):** Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Technical agar:** Oxoid Ltd., Basingstoke, Hampshire UK.
- **TNFα:** BD biosciences ltd. Oxford, UK.
- **Tris-base:** Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Trypsin:** PAA Laboratories GmbH., Pasching Austria.
- **Tryptone soya agar (TSA):** 40g/l in H20 – Oxoid Ltd., Basingstoke, Hampshire UK.
- **Tryptone soya broth (TSB):** 30g/l in H20 – Oxoid Ltd., Basingstoke, Hampshire UK.
- **Vanadyl sulphate (VOSO₄):** Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK.

### 2.1.2 Equipment

- **Analytical Balance:** AS200 Analytical standard – Ohaus, Pine Brook, NJ, USA.
- **Autoclave:** 300/75LR – Boxer Laboratory Equipment Ltd, Ware, Hertfordshire, UK.
- **Automated microbiology reader:** Bioscreen C – Thermo Labsystems, Altrincham, Cheshire, UK
- **Centrifuge (Bench-top):** Centaur 2 – Sanyo Electric Co., Ltd, Osaka, Japan
- **Centrifuge (High speed):** RC6 Plus – Sorvall, Thermo Fisher Scientific Ltd, Waltham, MA, USA.
- **Centrifuge (Low speed - for tissue culture):** Multifuge 3s – Heraeus, Thermo Fisher Scientific Ltd. Waltham, MA, USA.
- **Centrifuge tubes:** Fisherbrand – Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK. (part of Thermo Fisher Scientific Ltd, Waltham, MA, USA)
- **Centrifuge tubes (Chloroform resistant):** Iwaki – Sterilin Ltd, Bargoed, Caerphilly, UK.
- **Colony counter:** Stuart – Bibby Scientific Ltd, Stone, Staffordshire, UK
- **Cryo-vials**: Nalgene Labware – Thermo Fisher Scientific Ltd, Waltham, MA, USA.
- **Cuvettes (normal and UV grade)**: Fisherbrand – Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK.
- **ELISA plates**: Iwaki - Sterilin Ltd, Bargoed, Caerphilly, UK.
- **Fluorimeter**: Cary Eclipse – Varian Inc., Palo Alto, CA, USA.
- **Honeycomb multi-well plates**: HC2 – Thermo Labsystems Ltd, Altrincham, Cheshire, UK
- **Incubator (shaking - 37°C)**:
- **Incubator (CO2 controlled - 37°C)**: Hera Cell – Heraeus, Thermo Fisher Scientific Ltd., Waltham, MA, USA.
- **Magnetic stirrer**: HC502 – Bibby Scientific Ltd, Stone, Staffordshire, UK.
- **Micro-pipettors**: Pipetman – Gilson, Inc., Middleton, WI, USA.
- **Oven (for agar)**: MOV-212F Convection Oven – Sanyo Electric Co., Ltd, Osaka, Japan.
- **Petri dishes (90 mm)**: Fisherbrand – Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK.
- **pH meter**: pH Meter 7010 – Electronic Instruments Ltd., Richmond, Surrey, UK.
- **Plate Washer**: Nunc™ Immunowash – Nunc, Thermo Fisher Scientific Ltd., Waltham, MA, USA.
- **Plate reader**: ASYS UVM340 – ASYS GmbH, Dornstadt, Germany.
- **Spectrophotometer**: 6300 Spectrophotometer – Bibby Scientific Ltd T/As Jenway, Dunmow, Essex, UK.
- **Syringe filters**: MiniSart – Sartorius AG, Goettingen, Germany.
- **Tissue culture flasks**: Nunclon Surface – Nunc, Thermo Fisher Scientific Ltd., Waltham, MA, USA.
- **Tissue culture inserts**: Support Transwell-Clear Costar – Corning Inc., Corning, NY, USA.
- **Waterbath (boiling):** SBB6 – Grant Instruments Ltd, Cambridge, UK.

2.1.2.1 Micro-pipettor calibration

Three micro-pipettors were used for accurately dispensing small volumes of liquid, P20 (dispensed 1 μL – 20 μL), P200 (dispensed 20 μL – 200 μL) and the P1000 (dispensed 200 μL – 1000 μL).

To ensure accurate pipetting the micro-pipettors used were verified once a month. This involved pipetting set volumes of water into a weighing boat on a calibrated analytical balance, and verifying that the observed weight was within 1% of the expected weight. Volumes used were 10 μL for the P20, 100 μL and 200 μL for the P200, and 500 μL and 1000μL for the P1000. Volumes were tested in triplicate and the average value was used for verification.

2.1.2.2 Statistical analysis

Unless otherwise stated all statistical analysis was performed using Minitab 15 statistical software package (Minitab Inc., PA, USA).

2.1.3 Cultures

2.1.3.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* strains used were; laboratory strain PA01 (NCIMB 10548), and PAO-GFP. PA01 was purchased from the National Collection of Industrial, Marine and Food Bacteria (NCIMB Ltd, Aberdeen). PAO-GFP was kindly provided by Dr Alice Prince (University of Columbia). This strain contained a
pUCP18-based plasmid with an enhanced GFP gene [Cormack, Valdivia and Falkow 1996] and a beta-lactamase gene conferring carbenicillin resistance as a selection tool (for plasmid map see appendix I).

2.1.3.2 Human bronchial epithelial cells (16HBE)

16HBE cells are a human bronchial epithelial cell line originally isolated from a male one year old heart lung transplant patient [Gruenert, Finkbeiner and Widdicombe 1995].

2.1.3.3 Bacteriophages

A total of seven bacteriophages were used in this study. Three were from the University of Brighton collection, and a further four were isolated from an over-the-counter bacteriophage preparation purchased in the Ukraine. All phage were unidentified with the exception of F116 which has been well characterised in the literature [Slayter 1964; Miller, Pemberton and Richards 1974; Byrne and Kropinski 2005]. Of the remaining phages, those from the University of Brighton were designated #13 and #2 and those from the Ukrainian phage preparation U1-U4.

2.1.4 Preparation of growth media and buffers

2.1.4.1 Growth media

Unless otherwise stated liquid bacterial cultures were grown in tryptone soya broth (TSB). In experiments requiring plating techniques tryptone soya agar was used. Both broth and agar was made according to the manufacturer's instructions and sterilised by autoclaving at 121°C for 15 minutes. The agar was kept molten at 60°C and poured into 90 mm Petri dishes before being allowed to set. Overlay agar was
prepared using TSB and technical agar. Broth powder was mixed with water at the recommended concentration; technical agar powder was then added to a concentration of 0.4% w/v. The preparation was heated to boiling over a Bunsen burner before 5 mL aliquots were placed into bijou bottles and sterilised by autoclaving at 121°C for 15 minutes.

Unless otherwise stated all 16HBE cells were cultured in Minimum essential media (MEME) with Earle’s salts. To this were added 10% v/v foetal bovine serum (FBS) and 1% v/v 100x penicillin/streptomycin solution.

2.1.4.2 Buffers

Phosphate buffered saline (PBS) was prepared using commercial tablets (Oxoid) according to manufacturer’s instructions. All PBS was steam sterilised in an autoclave at 121°C for 15 minutes.

Lambda buffer was prepared with 1.21 g L\(^{-1}\) tris-base, 1.20 g L\(^{-1}\) MgSO\(_4\) (7H\(_2\)O), 0.10 g L\(^{-1}\) Gelatin. Once all the chemicals had been dissolved in water on a magnetic stirrer the solution was adjusted to pH 7.6 using concentrated Hydrochloric acid. All buffers were steam sterilised in an autoclave at 121°C for 15 minutes.
2.2 Bacterial culture

2.2.1 Maintenance of bacterial cultures

Both *Ps. aeruginosa* strains were stored in TSB plus 10% glycerol at -80°C. Each month, or sooner if required, a vial was thawed, transferred to 10 ml sterile TSB and incubated at 37°C with shaking (approx 100 throws per minute) overnight. The resulting culture was streaked out onto a TSA plate which served as a stock plate for culture preparation. Stock plates were replaced every month or sooner if necessary.

2.2.2 Preparation of bacterial broth cultures

Unless otherwise stated all overnight cultures were produced by inoculating 10 mL of TSB with one colony of *Ps. aeruginosa* from a stock plate and incubating overnight at 37°C with shaking (100 throws/min). Overnight cultures of PAO-GFP were prepared in Luria Bertani (LB) broth with 1 mg mL\(^{-1}\) carbenicillin for selection and incubated as described above.

2.2.3 Identification of stock cultures

The identity of the stock cultures was confirmed using an API20NE test kit. Briefly, a colony from an overnight streak plate was suspended in the liquid media provided with the kit. The cupules in the test strip were then filled with the bacterial suspension according to the manufacturer’s instructions. The strip was incubated for 24-48 hours at 30°C.

The results for both PAO1 and PAO-GFP were identical and produced the seven digit code: 1354575 corresponding to a 99.9% excellent identification for *Pseudomonas aeruginosa*. 
2.2.4 Bacterial viable counts

Unless otherwise stated all work requiring bacterial counts was conducted in the same manner. The bacterial sample to be tested was serially diluted in PBS. A 100 μL aliquot of the appropriate dilution was placed on a TSA plate and spread over the agar surface using an alcohol-flamed glass spreader. Each dilution was usually tested in triplicate. The plates were incubated at 37°C overnight. Only plates with between 30-300 colonies were counted.

2.2.5 Repeatability

2.2.5.1 Method

Repeatability experiments were conducted to ensure that enumeration techniques could be relied on to give accurate results. Overnight cultures of PAO1 and PAO-GFP were washed 3 times in PBS by centrifuging the cultures at 3,000 g for 10 minutes, discarding the supernatant and resuspending the bacterial pellets in the same volume of PBS. The suspensions were serially diluted (1:10) down to a dilution factor of $10^{-7}$. This was conducted a further 4 times. A 100 μL aliquot of each of the last 3 dilutions from each set was enumerated by spread plating. The plates were incubated overnight at 37°C.

2.2.5.2 Results

The results, as shown in Table 2.1, and 2.2, were analysed using one way ANOVA to ensure that all the dilution series were statistically indistinguishable from one another.
<table>
<thead>
<tr>
<th>Dilution series</th>
<th>Number of colonies</th>
<th></th>
<th></th>
<th></th>
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<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
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<td>Standard deviation</td>
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<td>41</td>
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<td>34</td>
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<tr>
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<td>35.2</td>
<td>34</td>
<td>34.2</td>
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<td>4.219</td>
<td>3.114</td>
<td>2.000</td>
<td>3.033</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1:** Plate counts of 5 separate $10^{-7}$ dilutions of a washed overnight culture of PAO1.
The Null hypothesis (H\(_0\)) states there is no difference between the results obtained from each set of dilutions. A p-value of less than 0.05 would suggest that H\(_0\) should be rejected. For PAO1 and PAO-GFP the p-values are 0.187 and 0.367 respectively which means the H\(_0\) cannot be rejected and therefore there is no statistical difference between the dilution series for either strain.

<table>
<thead>
<tr>
<th>Dilution series</th>
<th>Number of colonies</th>
<th></th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>Average</td>
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<td>206</td>
<td>224</td>
<td>206</td>
<td>213.6</td>
<td>211.2</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.2:* Plate counts of 5 separate 10\(^{-6}\) dilutions of a washed overnight culture of PAO-GFP.
2.2.6 Relationship between optical density and bacterial titre

2.2.6.1 Method

Three overnight cultures of both PAO1 and PAO-GFP were washed three times each in PBS by centrifuging at 3,000 g for 10 minutes before resuspending the pellet in 10 mL PBS. After the final wash the pellet was resuspended in 25 mL PBS (100% suspension). This was diluted using sterile PBS in 10% increments down to 10% and the absorbance of these suspensions was measured at 420 nm against a PBS blank. Those at approximately 0.5 or above were diluted further and the results extrapolated upwards to ensure a linear relationship. Viable counts were performed on the 100% suspension as described in section 2.2.5. Bacterial titres were calculated and plotted against optical density. The entire experiment was repeated 3 times, and the results were combined to obtain a trend line (Figure 2.1).

2.2.6.2 Results

The results are shown in Figure 2.1.
Both standard curves showed a strong positive correlation with $R^2$ values for PAO1 and PAO-GFP of 0.9975 and 0.9945 respectively.

2.2.7 Characterisation of PAO-GFP fluorescence

The excitation and emission wavelengths of the mutant GFP fluorophore produced by PAO-GFP are 488 nm and 507-511 nm respectively. In order to confirm the published values for the excitation and emission wavelengths excitation and emission scans were performed. During the emission spectrum the fluorimeter
measures intensity of emitted light over a range of wavelengths produced by excitation at a particular wavelength. An excitation spectrum is a reversal of this process, an emission wavelength is input and the intensity of a range of wavelengths of excitation light is measured.

2.2.7.1 Emission spectrum

2.2.7.1.a. Method
An overnight culture of PAO-GFP was centrifuged at 3,000 G for 10 min., the supernatant liquid was discarded and the pellet was resuspended in the same volume of PBS. 3 mL of this suspension was placed in a UV grade macro-cuvette and an emission spectrum was performed on it using 488 nm as the excitation wavelength.

2.2.7.1.b. Results
The spectrum can be seen in Figure 2.2.
The emission spectrum showed a peak at 508.95 nm with an intensity of 582.855 arbitrary units (A. U.), this wavelength could then be used as the emission input for an excitation scan, which is shown in Figure 2.3.

**Figure 2.2:** Emission spectrum of the GFP fluorophore produced by PAO-GFP with an excitation wavelength of 488 nm.
Due to the amount of interference produced by the emission wavelength it was not possible to obtain verification of the excitation wavelength by excitation scan. However it can be assumed from the emission scan that an excitation wavelength of 488 nm enabled the GFP fluorophores to emit light of the expected wavelength.

Figure 2.3: Excitation spectrum of the GFP fluorophore produced by PAO-GFP with an emission wavelength of 509 nm.
2.2.8 Bacterial growth curves

To assess the growth characteristics of both strains of *Ps. aeruginosa* in the different media used growth curves were produced.

2.2.8.1 Growth curves in TSB and LB

2.2.8.1.a. Method
A 2 mL aliquot of an overnight culture of *Ps. aeruginosa* was added to 10 mL of sterile TSB to give an absorbance of 0.2 at 420 nm. Using a sterile 96-well honeycomb micro-titre plate 300 μL of the bacterial suspension was placed in each of 10 wells and 300 μL of sterile un-inoculated TSB was placed in each of 5 wells to serve as a negative control. The plate was placed in a Bioscreen® (automated absorbance reader) and incubated at 37°C. The instrument was set to measure absorbance at 420 nm every 10 minutes for 20 hours with shaking before each reading. The experiment was repeated for both strains in TSB and LB broth.

2.2.8.1.b. Results

The growth curves are shown in Figure 2.4.

*Ps. aeruginosa* strains PAO1 and PAO-GFP had almost identical growth curves for both types of broth. This confirmed that the addition of the GFP expressing plasmid had no effect on the growth characteristics compared to the wild type strain. Growth in TSB achieved a higher optical density compared to that in LB. Stationary phase was reached at approximately the same time for both broths at around 11 hours, hence routine overnight cultures could be assumed to be at stationary phase. Cultures grown in TSB exhibited a great deal of variation in optical density at stationary phase, this could be have been due to clumping of bacterial cells interfering with the readings.
Figure 2.4: Growth curves for both strains of *Ps. aeruginosa* in a) TSB and b) LB broth. 

- **= PAO1,  = PAO-GFP, ▲ = Negative control. Error bars denote standard error, n = 10 for growth curves, n = 5 for control.
2.2.8.2 Growth curves in MEME

2.2.8.2.a. Method
A further growth curve was produced to assess the ability of *Ps. aeruginosa* to grow in minimum essential tissue culture medium, MEME (details in section 2.1.4.1), as this would have consequences in future co-culture work. This was performed as described in section 2.2.8.1.a with MEME used in place of TSB/LB. The negative control was sterile MEME.

2.2.8.2.b. Results
Growth curves are shown in Figure 2.5.

**Figure 2.5:** Growth curves for both strains of *Ps. aeruginosa* in MEME.

- ◆ = PAO1,
- ■ = PAO-GFP,
*Ps. aeruginosa* did not exhibit a classic growth curve when grown in MEME (Figure 2.5), in that there were no clearly defined lag, log and stationary phases. Moreover there were also slight differences (*P* = <0.005, two way ANOVA) between PAO1 and PAO-GFP. PAO1 appeared to show an initial log phase which slowed after approximately 5 hours. PAO-GFP showed a more uniform rate of growth in log phase but neither of the strains appeared to reach a recognised stationary phase in 20 hours. Both strains exhibited a much slower rate of growth in log phase when grown in MEME than when grown in bacteriological media. The differences between the two strains may have been due to interference from green fluorescent protein and the phenol red in the MEME. The slight increase in OD of the negative control early on in the experiment may be due to the instability of the colour of the MEME particularly on incubation. The negative control was observed to have no contamination at the termination of the experiment.

### 2.2.8.3 Growth curves in the presence of carbenicillin

#### 2.2.8.3.a. Method
A final set of growth curves were produced to assess the concentration of carbenicillin required to inhibit growth of PAO1 but not PAO-GFP. In this experiment the two strains were cultured in LB with carbenicillin at 0 mg mL\(^{-1}\), 0.2 mg mL\(^{-1}\), 1 mg mL\(^{-1}\) and 2 mg mL\(^{-1}\). The aim of this experiment was to identify the concentration of carbenicillin required to inhibit the wild type strain but not affect the GFP strain, therefore providing effective selection.

#### 2.2.8.3.b. Results
The results are shown in Figure 2.6.

The sterile MEME control exhibited a slight rise in optical density at the start of the experiment but as there was no change for the remainder of the experiment it can be assumed there was no contamination of this control. MEME contained the pH
indicator phenol red and exhibited colour changes dependent on temperature and length of storage, which may explain the observed increase in OD.

Comparison of the two graphs in Figure 2.6 shows marked differences between PAO1 and PAO-GFP when grown in the presence of carbenicillin. In the absence of carbenicillin PAO1 grew as previously observed however at 0.2 mg mL\(^{-1}\) it exhibited an irregular growth curve. The log phase was reduced in length and the stationary phase was reached at a lower OD value. Approximately 2 hours after stationary phase was reached a rapid increase in OD value was observed before the OD began to decrease steadily. At a carbenicillin concentration of 1 mg mL\(^{-1}\) PAO1 showed a much reduced growth rate with stationary phase at an OD of less than 1 and a gradual decline in OD from that time point onwards. In contrast PAO-GFP, at all concentrations of carbenicillin produced identical growth curves with clearly defined lag, log and stationary phases.

A carbenicillin concentration of 1 mg mL\(^{-1}\) would be an appropriate concentration to use for selection of PAO-GFP as at this concentration the ability of PAO-GFP to multiply was far greater that of PAO1 therefore maintaining the plasmid within the bacterial population.
Figure 2.6: Growth curves for *Ps. aeruginosa* strains a) PAO1 and b) PAO-GFP with varying concentrations of Carbenicillin.

- ▲ = 0 mg ml⁻¹ carbenicillin,
- • = 0.2 mg ml⁻¹ carbenicillin,
- ▲ = 1 mg ml⁻¹ carbenicillin,
- △ = 2 mg ml⁻¹ carbenicillin,
- • = negative control. Error bars denote standard error, n = 5.
2.3   Bacteriophage propagation and storage

2.3.1  Propagation and purification

Bacteriophage propagation and purification was achieved using a PEG precipitation protocol [Yamamoto et al. 1970]. Briefly; single plaques, in the form of agar plugs, were picked from an overlay plate and incubated at room temperature for 2 hours in 2 mL lambda buffer with a few drops of chloroform. Bacterial contamination was removed by filtering the suspension through a syringe filter with a 0.45 μm pore size.

An early-exponential phase *Ps. aeruginosa* culture was prepared by adding 1 mL of an overnight culture to 100 mL sterile TSB in a conical flask and incubating at 37°C with shaking (100 throws/min) for approximately 150 minutes. The culture was infected by adding 1 mL of the phage suspension. This was then incubated at 37°C until visible signs of lysis occurred at approximately 2 hours. Lysis is characterised by a decrease in turbidity and strands of cell debris. Chloroform (10% v/v) was added after incubation to ensure maximal lysis. Sodium chloride was added to give a 1M concentration (5.84 g in 100 mL) and this was dissolved slowly on ice over the course of 1 hour.

Bacterial cell debris was removed by centrifugation at 10,000 g for 10 minutes and the bacteriophages were precipitated with 10% v/v PEG8000 followed by incubation at 4°C overnight. Following centrifugation at 10,000 g for 10 minutes the pelleted bacteriophages were resuspended in 2 mL lambda buffer. 2 mL chloroform was added to the suspension to give a 50% v/v concentration, and it was vortexed for 30 seconds. Centrifugation at 3,000 g for 15 minutes allowed three distinct layers to form. The layers consist of chloroform at the bottom of the tube, the opaque middle layer contains the PEG and the upper aqueous layer contains the phage in lambda buffer. The aqueous layer containing the phage was transferred to a sterile bijou bottle and enumerated via a standard plaque assay.
Phage preparations were stored in bijou bottles at 4°C wrapped in foil to omit light. Phages were shown to be capable of storage in this way for many months with no appreciable reduction in titre (data not shown).

### 2.3.2 Enumeration

Bacteriophage titres were determined using a plaque assay. Bacteriophage suspensions were serially diluted with lambda buffer. A 100 μL aliquot of the appropriate dilution of phage and 100 μL of a 1:2 dilution (in PBS) of an overnight culture of *Ps. aeruginosa* were added to 5 mL of molten overlay agar. The inoculated agar was swirled to mix and was poured onto a TSA plate. This was then swirled to ensure total coverage and allowed to set. The plates were incubated at 37°C overnight.

A pilot experiment was usually conducted first, which utilised a wide range of dilutions of phage which were plated with no replicates. This gave an idea of which phage dilutions produced a number of plaques within the range that could be accurately counted. A second more accurate plaque assay was then performed in which those dilutions that gave an accurately countable number of plaques were plated in triplicate. The resulting average number of plaques was then used to work out the number of plaque forming units per mL (pfu mL⁻¹) in the original suspension.
2.4 Human bronchial epithelial cell culture and storage

2.4.1 Storage

Cells suspended in freezing medium (50% v/v MEME, 40% v/v FBS, 10% v/v DMSO) were stored in cryo-vials in liquid nitrogen.

2.4.2 Culture

When required, cells were thawed rapidly by immersing the cryo-vial to its neck in a 37°C water bath. The contents were then placed in a T-25 flask with 5 mL growth medium pre-warmed to 37°C in a water bath. Cells were incubated at 37°C in a CO₂ controlled incubator. When cells became confluent they were passaged by aspirating the medium and washing the flask with pre-warmed (37°C) PBS. Enough pre-warmed (37°C) trypsin EDTA was added to cover the bottom of the flask which was incubated until the majority of cells had detached. Growth medium was added at the same volume as the trypsin to neutralise and the cells centrifuged at 500 g for 5 minutes. The pellet was resuspended in medium and split between either flasks or inserts at a growth area ratio of 1 to 4, i.e. a 25 cm² flask of confluent cells could be passaged up to a 100 cm² total growth area.

Cell culture inserts (diameter: 23.1 mm or 10.5 mm) were used for all co-culture experiments. The inserts provided a porous membrane on which cells could grow while obtaining nutrients from the cell culture media below the membrane which is illustrated in Figure 2.7.

![Diagram of cell culture insert](image.png)

**Figure 2.7:** Diagram of cell culture insert (not to scale).
2.4.3 Confirmation of confluence

This experiment was performed to establish both the time at which the 16HBE cells formed the most confluent layer on the inserts, with well developed tight junctions, and whether submerged culture (i.e. media on top of the inserts) or air-liquid interface (ALI) (i.e. no media on top of the insert) was the better growth regime for ensuring confluence and tight junction formation.

2.4.3.1 Method

Cells were grown and seeded onto inserts as described in section 2.4.2 with 2.5 mL growth medium below the insert and 1.5 mL above. Prior to the start of the experiment half the inserts were taken to ALI by removal of the apical media. The procedures described below were performed every 2 days for 21 days in total.

On removal from the incubator, medium was aspirated from both above and below the inserts in submerged culture and from below the inserts at ALI. All inserts were washed top and bottom with 1 mL sterile PBS, to remove traces of the discarded media. Following this, 1.5 mL of phenol red free medium was placed above each of the inserts, and 2.5 mL phenol red spiked medium was placed below each of the inserts. The inserts were then returned to the CO₂ controlled 37°C incubator for approximately 5 hours.

Upon removal from the incubator the medium from the top of the inserts was removed and placed into labelled Eppendorf tubes. The remaining medium was aspirated from beneath the inserts, which were subsequently washed with PBS. Normal medium was replaced (2.5 mL below, 1.5 mL above [submerged culture only]) and the inserts were returned to the incubator. For inserts that were being kept at Air-Liquid interface medium was only replaced below the inserts. The optical density (OD) of the medium that had been removed from the top of the inserts was analysed at a wavelength of 482 nm (the isosbestic point of phenol red) in order to determine the extent of phenol red movement, or flux, from the baso-lateral media through to the top of the insert. A lower phenol red flux translates to an epithelial
layer with well developed tight junctions. The phenol red flux assay was performed before the cells had been seeded on to inserts (blank control) and again at intervals until the cells had become over-confluent and started to die. The entire experiment was repeated twice.

2.4.3.2 Results

The data obtained were converted into phenol red flux using equation 1.

The results are presented in Figure 2.8.

\[
J_{PR} = \frac{A_{482} \times mVol}{T \times A \times EC}
\]

**Equation 1. Phenol red flux** Where: \( J_{PR} \) = Phenol red flux (mol cm\(^{-2}\) h\(^{-1}\)), \( A_{482} \) = Absorbance measured at 482 nm, \( mVol \) = Mucosal solution volume (mL), \( T \) = Incubation time, \( A \) = Epithelium surface area, \( EC \) = Molar extinction coefficient (Phenol red = 90061 mol\(^{-1}\) cm\(^{-1}\))
Both types of culture conditions produced an initial sharp drop in phenol red flux, with the submerged culture dropping further. This was taken to be the time when cells were growing rapidly to fill the space on the insert membrane. At 8 days the phenol red flux of those cells grown in submerged culture stopped falling, while the air-liquid interface flux continued to fall but at a decreased rate. The results suggested that the best growth regime to adopt was to grow the cells in submerged culture for 7-8 days before taking them to air/liquid interface 24 hours prior to treatment/experiment. This would allow the cells to produce a highly confluent mono-layer which was important for co-culture experiments. The two methods of culture gave significantly different results (p = 0.01 Scheirer-Ray-Hare test).
2.5   Optimisation of co-culture experiments and cytokine assays

There were two ways in which the epithelial cells in this study were assessed for distress; measuring the level of cytokines released (2.5.1), and measuring the level of LdH released (2.5.4).

2.5.1   Analysis of cytokine release

Throughout this project production of IL-6 and IL-8 by 16HBE cells was assessed by assaying the concentration of each cytokine in samples of baso-lateral media using the ELISA (enzyme linked immunosorbent assay) method. Cytokine ELISA sets (BD Biosciences) were used according to the manufacturer's instructions, the method for IL-6 and IL-8 was identical.

96-well ELISA plates (Iwaki) were coated with 100 µl per well of capture antibody diluted 1:250 in coating buffer (1 M sodium carbonate). The plates were incubated at 4°C overnight. After incubation the coating antibody was discarded and the plate was washed with wash buffer (PBS + 0.5% Tween 20) three times using a plate washer (Nunc-Immuno™ Wash). The plate was blocked using assay diluent (PBS + 10% v/v FBS) at 200µl per well incubated at room temperature for 1 hour. This prevented non-specific binding of the antibodies to the ELISA plate.

Samples to be assayed were thawed and diluted to an appropriate level using sterile MEME in micro-centrifuge tubes. Aliquots of standard cytokine diluted with assay diluent to the highest concentration required for a standard curve (IL-6 = 300 pg mL⁻¹, IL-8 = 200 pg mL⁻¹) were stored at -80°C and thawed when required.

After blocking, the plates were washed three times with wash buffer and blotted on absorbent paper. Standard curves were set up in duplicate in the first two columns of each plate. Serial 1:2 dilutions were performed, using sterile MEME, down each column resulting in 100 µl in each well. The final well in each column contained only 100 µl sterile MEME. The standard curve concentrations are shown in Table 2.3.
100 µl of each diluted sample was added to the remaining wells in duplicate. Following incubation at room temperature for two hours the plates were washed 5 times with wash buffer and blotted onto absorbent paper. Detection antibody/enzyme conjugate was created by adding the detection antibody and the streptavidin-horseradish peroxidise enzyme to assay diluent to achieve a 1:250 dilution of both components. 100 µl of detection antibody solution was added to each well of the plate and incubated at room temperature for 1 hour. After the incubation period the plate was washed seven times allowing wash buffer to remain in the wells for 30 seconds on each wash.

Substrate solution was prepared by combining equal volumes of substrate solution A and B (BD Biosciences) no more than 15 minutes before it was required. 100µl of substrate solution was added to each well and incubated at room temperature in the abscession of light for up to 30 minutes. The plates were checked regularly throughout the incubation period to ensure the colour reaction didn't over-develop; this involved

<table>
<thead>
<tr>
<th>IL-6</th>
<th>IL-8</th>
<th>TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>150</td>
<td>100</td>
<td>125</td>
</tr>
<tr>
<td>75</td>
<td>50</td>
<td>62.5</td>
</tr>
<tr>
<td>37.5</td>
<td>25</td>
<td>31.3</td>
</tr>
<tr>
<td>18.75</td>
<td>12.5</td>
<td>15.6</td>
</tr>
<tr>
<td>9.375</td>
<td>6.25</td>
<td>7.8</td>
</tr>
<tr>
<td>4.6875</td>
<td>3.125</td>
<td>3.9</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.3: Cytokine standard curve concentrations in pg/ml
observing that the yellow reaction colour didn’t become too deep. Once the colour
had developed sufficiently 50µl of stop solution (2 N sulphuric acid) was added to
each well.

The plates were analysed in an optical plate reader (ASYS UVM340) at 450 nm. The
standard curves were used to calculate the concentration of cytokines in the samples
tested.

2.5.2 Concentration range of cytokines produced by 16HBE cells

It was necessary to establish the range of concentration of cytokines produced by
16HBE cells to enable appropriate dilutions of the samples to be made.

2.5.2.1 Method

Cells were grown on cell culture inserts as described in section 2.4.2. Prior to the
start of the experiment the baso-lateral medium was replaced with fresh sterile pre-
warmed (37°C) media. To each insert 50 µL of phage suspension at a concentration
of 5 x 10^{9} pfu ml^{-1} (which corresponds to 1 x 10^{8} pfu in 50 µL) was added. The
phage used were F116, #13, U1, U2, U3, and U4 and each was tested in triplicate.
Three inserts were used for each of the positive control, which was *E. coli* endotoxin
(1mg mL^{-1}), and the negative control which was sterile lambda buffer.

The baso-lateral medium was removed and placed into labelled micro-centrifuge
tubes after 2, 4, 8, and 24 hours. After each sample was taken the medium was
replaced with fresh sterile pre-warmed medium.

Initially only samples from one phage, as well as positive and negative controls were
assayed for their cytokine concentration. This was to get an idea of the concentration
of cytokines produced without unnecessary use of expensive reagents.
Cytokine concentration was found by ELISA using the method described in section 4.2.3.1. IL-6, IL-8 and TNFα were measured; the samples were not diluted for this assay.

### 2.5.2.2 Results

The results for this experiment are shown in Figures 2.9-2.11.

Both sets of results for IL-6 and IL-8 were unreliable due to the fact that the concentrations of the undiluted samples fell outside of the range of the respective standard curves. The highest concentration for the standard curve of IL-6 was 300 pg ml\(^{-1}\) and that of IL-8 was 200 pg ml\(^{-1}\). Samples would have to be diluted in future experiments to ensure accuracy in the calculated results.

The results for TNFα were all very low compared to the other cytokines indicating that 16HBE cells produced very little TNFα in response to a stimulus.
Figure 2.9: Concentration of IL-6 in samples of media from 16HBE cells in response to Bacteriophage #13, Negative control, and Positive control. Error bars denote standard error, n = 3.
Figure 2.10: Concentration of IL-8 in samples of media from 16HBE cells in response to Bacteriophage #13, Negative control, and Positive control. Error bars denote standard error, n = 3.
Figure 2.11: Concentration of TNFα in samples of media from 16HBE cells in response to Bacteriophage #13, Negative control, and Positive control. Error bars denote standard error, n = 3.
2.5.2.3 Method

The next cytokine assay to be set up used the samples for phage F116 obtained in section 4.2.3.2a alongside the positive and negative samples. In this assay they were diluted 1:10 in order to obtain OD values that fell within the range of the standard curve of the assay. This was important as the equation of the standard curve was used to calculate the concentrations of cytokines in the samples.

2.5.2.4 Results

The results are shown in Figures 2.12-2.14. Note that the results for IL-6 and IL-8 (Figures 2.12 and 2.13 respectively) are given in ng ml\(^{-1}\) and those for TNF\(\alpha\) (Figure 2.14) are given in pg ml\(^{-1}\).

TNF\(\alpha\) concentrations were again very low, in this case effectively zero, in subsequent experiments therefore, TNF\(\alpha\) was no longer measured. The concentration of IL-6 in samples was significantly lower than the concentration of IL-8 \(p < 0.0005\) (ANOVA). This indicates that the cells produced more IL-8 in response to stimuli than IL-6. There was no significant difference between treatments i.e controls or phage in either IL-6 or IL-8 concentration \(p = 0.536\) and 0.222 respectively (ANOVA).

The un-extrapolated optical densities for IL-6 and IL-8 were again outside of the range of the standard curve for each cytokine, therefore requiring further dilution of samples in subsequent experiments.
Figure 2.12: Concentration of IL-6 in samples of media from 16HBE cells in response to Bacteriophage F116, Negative control, and Positive control. Error bars denote standard error, n = 3.
Figure 2.13: Concentration of IL-8 in samples of media from 16HBE cells in response to Bacteriophage F116, Negative control, and Positive control. Error bars denote standard error, n = 3.
Figure 2.14: Concentration of TNFα in samples of media from 16HBE cells in response to Bacteriophage F116. Negative control, and Positive control. Error bars denote standard error, n = 3.
2.5.2.5 Method

Samples from the same original experiment were again tested for cytokine content. In this assay F116, Phage 13, U1 and negative and positive control samples at time point 2 hours only were used. The samples were tested for their IL-6 concentration only, to avoid excessive use of expensive reagents. The samples were assayed neat and at 1:10, 1:100, and 1:1000 dilution. This assay protocol was designed to give an idea of the level of dilution required to bring the OD readings within those of the standard curve.

2.5.2.6 Results

Figure 2.15 shows the extrapolated cytokine concentrations for each sample at each dilution. Figure 2.16 shows the standard curve generated in this assay and Table 2.4 shows the raw OD data for each dilution of each sample.
Figure 2.15: IL-6 concentration of several dilutions of samples of baso-lateral media from 16HBE cells in response to 5 treatments. Neat: , 1:10: , 1:100: , 1:1000: . Error bars indicate standard error, n = 3.
Figure 2.16: Standard curve of IL-6 concentration against optical density at 450 nm. Values adjusted for zero. Error bars denote standard deviation, n = 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OD\textsubscript{450} (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat</td>
<td>2.044</td>
</tr>
<tr>
<td>1:10</td>
<td>1.629</td>
</tr>
<tr>
<td>1:100</td>
<td>0.352</td>
</tr>
<tr>
<td>1:1000</td>
<td>0.130</td>
</tr>
<tr>
<td>F116</td>
<td>1.901</td>
</tr>
<tr>
<td>1.503</td>
<td>0.239</td>
</tr>
<tr>
<td>0.003</td>
<td>0.000</td>
</tr>
<tr>
<td>U1</td>
<td>1.869</td>
</tr>
<tr>
<td>1.516</td>
<td>0.238</td>
</tr>
<tr>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Phage 13</td>
<td>1.906</td>
</tr>
<tr>
<td>1.466</td>
<td>0.217</td>
</tr>
<tr>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>1.869</td>
</tr>
<tr>
<td>1.516</td>
<td>0.238</td>
</tr>
<tr>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>1.869</td>
</tr>
<tr>
<td>1.516</td>
<td>0.238</td>
</tr>
<tr>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: Raw optical density (450 nm) data of all dilutions of baso-lateral media samples from 16HBE cells treated with phage and negative and positive controls.
The extrapolated results shown in Figure 2.15 for each treatment differ markedly from one another in terms of dilution levels.

Diluting the samples by 1:1000 was obviously not suitable as the error bars were much larger for these results than any others. To corroborate this, the original OD values seen in Table 2.4 for the 1:1000 dilutions were all either zero or very low. At this level of dilution any cytokine in the sample had been diluted out.

The results for the neat samples were all also very low, and with the exception of the phage 13 samples were all at nearly identical values (464 pg ml\(^{-1}\) ± 1.2). It is likely that these samples exceeded the limits of the cytokine ELISA and that the plate was saturated with IL-6, hence the identical values that fell above the highest concentration in the standard curve.

The results for the 1:10 dilutions were much higher than those for the neat samples, again they were all very similar with the exception of phage 13 (3645 pg ml\(^{-1}\) ± 51). However the OD values all fell above the range of the standard curve (except phage 13). Therefore the results cannot be held to be reliable, and a higher level of dilution is required.

OD values for 1:100 dilutions of the samples fell within the lower end of the standard curve, with the exception of the phage 13 sample which was very low. The extrapolated concentrations for these dilutions were higher than any other dilutions with more variation between samples (average 5543 pg ml\(^{-1}\) ± 318). This level of dilution appeared to be adequate for most of the samples, however it would be optimal to have sample ODs falling within the mid-range of the standard curve. Also it would not be appropriate to dilute samples that contain lower levels of cytokine such as phage 13 to this level. A dilution of between 1:25 and 1:50 was deemed to be appropriate for the majority of future samples.
2.5.3 Assignment of a positive control

2.5.3.1 Method

The positive control in the previous experiment was not adequate as it did not provoke a greater inflammatory response in the 16HBE cells than any other treatments. The purpose of the positive control in co-culture experiments was to show that the cells were able to secrete cytokines in cases where the test conditions did not elicit a cytokine response.

A number of potential positive control candidates were chosen from the literature. They were chosen based on their availability and practicality; concentrations of each were also determined from the literature.

Tumour necrosis factor α (TNFα) has been widely studied as a mediator of interleukin release [Cromwell et al. 1992; Levine et al. 1993; Takizawa et al. 1997; Staiz et al. 1999; Graness et al. 2002; van Wissen et al. 2002; Gilmour et al. 2005; Veranth et al. 2007]. TNFα is produced by phagocytes in response to detection of bacterial invaders, it leads to the production of various cytokines and is implicated in septic shock [Tosi 2005]. Concentration: 10 ng mL\(^{-1}\)

Commercial lipopolysaccharide (LPS) is extensively used throughout the literature as a positive control. [Palmberg et al. 1998; EK et al. 1999; Romberger et al. 2002; van Wissen et al. 2002; Gilmour et al. 2005; Veranth et al. 2007]. However in previous experiments in this study it didn’t appear to provoke a response compared to the negative control. It is included in this experiment for comparison and completeness. LPS is detected by the Toll like receptors (TLRs) on phagocytes and epithelial cells and leads to initiation of the inflammatory cytokine cascade (105). Concentration: 1 mg mL\(^{-1}\)

Vanadyl sulphate (VOSO\(_4\)) was used as a positive control in one study [Veranth et al. 2007]. Vanadium compounds are well known for causing respiratory irritation and disease, particularly amongst those employed as boilermakers. These workers are exposed to fuel oil ash from industrial boilers, which is vanadium rich and many
become ill as a result [Woodin et al. 1998; Mark A. Woodin 2000; Li et al. 2004]. Concentration: 20 µg mL⁻¹

Hydrogen peroxide (H₂O₂) was also used as a positive control in one study [Gilmour et al. 2005]. Reactive oxygen species are known to injure the airway epithelia and cause production of cytokines. However exactly how effective hydrogen peroxide is at stimulating release of interleukins is in doubt [Oslund et al. 2004; Pelaia et al. 2004]. Concentration: 200 µM

*Ps. aeruginosa* and *E. coli* culture supernatants were assessed as inexpensive, easily produced sources of endotoxin that may promote an inflammatory reaction in the cells.

TNFa, LPS, VOSO₄, and H₂O₂ were all adjusted to the stated concentration using MEME. *Ps. aeruginosa* and *E. coli* supernatants were prepared by centrifuging 10 mL overnight cultures of each bacterium at 3,000 G for 10 mins. The supernatant liquid was then syringe filtered (0.2 µm pore size, surfactant-free cellulose acetate) to remove whole bacterial cells.

16HBE cells were grown as before on cell culture inserts. The well and base of the insert were washed by removing the baso-lateral media and replacing it with fresh sterile media, and repeating. A 50 µl aliquot of each of the positive control candidates was added to the appropriate well. 50 µL of PBS was added to separate inserts to serve as a negative control. Each candidate and the control were tested in triplicate.

The baso-lateral media was removed at the same time as the test substances and control were administered and replaced with fresh sterile media. The media that had been removed was placed in labelled eppendorfs and served as the samples for time zero (T = 0). Further samples were taken in the same way every 2 hours for 24 hours.

The samples were tested for their concentration of IL-6 and IL-8 using an ELISA assay (section 2.5.1) before being tested the samples were diluted 1:25 with sterile MEME.
2.5.3.2 Results

Figures 2.17 and 2.18 show the results for IL-6 concentration and Figures 2.19 and 2.20 show the results for IL-8 concentrations.

As is clearly evident from Figures 2.17 – 2.20 vanadium sulphate provoked a much stronger inflammatory response in 16HBE cells than any other positive control candidates. Both IL-6 and IL-8 production was significantly higher for vanadyl sulphate $P = 0.004$, and 0.003 respectively, hence this was chosen as the positive control in subsequent experiments.

TNFα caused very little release of either cytokine throughout the 24 hour experiment. The concentration within the samples hardly varied between each sample time point. However in general the levels of IL-6 and IL-8 produced by cells exposed to TNFα are higher than those for the negative control $P = 0.019$ and $<0.0005$ respectively.

LPS concentrations reached a peak at around 2-4 hours for both cytokines but remained fairly constant for the remainder of the experiment. LPS appeared to provoke a slightly bigger inflammatory response than the negative control for IL-8 ($P = <0.0005$) at most time points but not for IL-6 ($P = 0.051$).

Both bacterial culture supernatants provoked a higher IL-6 release than the negative control up until 12 hours when the concentration decreased. They followed a similar pattern to IL-8 release with results for the *Ps. aeruginosa* supernatant liquid remaining higher throughout the 24 hours.

The smaller sampling intervals also provided a useful profile of cytokine release for the 16HBE cells over 24 hours when faced with a strong inflammatory stimulant such as vanadium sulphate.

IL-6 release peaked at 2-4 hours and gradually decreased before reaching a plateau at 14-16 hours. IL-8 release gradually increased until 12-14 hours where it appeared to plateau before showing signs of decrease towards the end of the experiment.
Figure 2.17: Concentration of IL-6 in baso-lateral media taken from 16HBE cells in response to positive control candidates (T=0 – T=10).
Error bars denote standard error, n = 3.
Figure 2.18: Concentration of IL-6 in baso-lateral media taken from 16HBE cells in response to positive control candidates (T=12 – T=24). Vanadium sulphate: , Hydrogen peroxide: , TNFα: , LPS: , *Ps.aeruginosa* supernatant: , *E. coli* supernatant: , Negative control . Error bars denote standard error, n = 3.
Figure 2.19: Concentration of IL-8 in baso-lateral media taken from 16HBE cells in response to positive control candidates (T=0 – T=10). Vanadium sulphate: ■, Hydrogen peroxide: ■, TNFα: ■, LPS: ■, Ps. aeruginosa supernatant: ■, E. coli supernatant: ■, Negative control ■. Error bars denote standard error, n = 3.
Figure 2.20: Concentration of IL-8 in baso-lateral media taken from 16HBE cells in response to positive control candidates (T=12 – T=24).
Error bars denote standard error, n = 3.
2.5.4 Analysis of LdH release

As well as measuring cytokines released by the 16HBE cells the release of LdH in response to bacteriophages was measured. LdH is released when cells become damaged or “leaky” due to stress.

LdH release by 16HBE cells was assayed using baso-lateral media samples. LdH levels were measured using a Cyto Tox 96 Non-radioactive cell cytotoxicity assay kit following the manufacturer's instructions.

Samples that had been stored at -80°C were thawed at room temperature. A 50µL aliquot of each sample was added to duplicate wells of a 96-well assay plate (Nunc). Assay buffer, provided in the kit, was allowed to thaw before 12 mL was added to reconstitute one vial of substrate mix. 50μL of substrate mix was added to each well of the assay plate. The plate was incubated at room temperature for up to 30 minutes with regular checks to ensure the reaction didn't proceed too far. Following incubation 50 µL of stop solution, provided with the kit, was added to each well to stop the reaction.

The plate was read in an optical plate reader (ASYS UVM340) at 490 nm. All results were expressed as absorbance units.
2.6 Conclusion

This chapter details all the methods that were used throughout this project. All manufacturers’ details that are not covered in this chapter will be covered in the individual chapter in which the material or equipment is first used.

The analysis of cytokine release by the 16HBE cells is an important part of this chapter that is necessary to complete future experiments. LPS was found to not be a suitable positive control for cytokine release hence several other candidates were considered for this role. A good positive control should provoke the airway epithelial cells to produce high levels of cytokine in order for other treatments to be compared against it. Out of all the candidates vanadyl sulphate was by far the best positive control causing a much higher concentration of both IL-6 and IL-8 from 16HBE cells. Hence vanadyl sulphate was used as a positive control for all experiments in which IL-6 and IL-8 were measured.
3 Bacteriophage Characterisation
3.1 Introduction

Bacteriophages are grouped into a number of families, 3 under the order of Caudovirales and a further 6 which have yet to be assigned to an order [Fauquet et al. 2005]. They are classified according to morphology and genome structure. Therefore, characterising novel or unidentified phage focuses on genome structure and obtaining images of the phage from which to extract morphological data.

Many phages that use Pseudomonas aeruginosa as their host have been characterised and classified in this manner. The first reported isolation of a bacteriophage specific for Ps. aeruginosa was in 1945 [Fastier 1945], and to date over 40 bacteriophages have been named and characterised that use this organism as their host.

Phage genomes vary significantly in size, with one study of 18 phages revealing a range from 35,580 base pairs (bp) (ФCTX) to 281,083 bp (SD1-M). The genomes of a large number of Ps. aeruginosa phages have been sequenced including F116 gh-1, D3, D3112, Ф-CTX, ФKMV and ФKZ [Ackermann 2003]. These studies have revealed a lack of conservation between most phage genomes [Kwan et al. 2006], indicating that many have evolved independently. Aside from total size and sequence phage genomes can also be characterised according to the number and size of DNA fragments produced as a result of digestion by specific restriction enzymes. These can be used to create a restriction map of the genome unique to each species of phage [Allan et al. 1989].

Studies employing transmission electron microscopy (TEM) have provided the most significant advances in understanding bacteriophage and their mode of action from 1941 onwards [Luria, Delbruck and Anderson 1943]. Six basic morphological types were ascertained from this work, and this morphology forms the basis of classification [Luria and Anderson 1942; Bradley 1967]. The study of bacteriophage by TEM usually requires the negative staining of lysates and pure phage preparations [Brenner and Horne 1959].

A third method of phage characterisation involves studying the infection process. All lytic phages follow the same general process upon infection of their host. First of all the phage adsorbs to the bacterial cell wall using specialist adsorption apparatus such
as tail fibres. Following this the phage DNA, usually contained within the phage head, is injected into the bacterial cell where it is taken up by the cell’s translational machinery. The cell in effect becomes a phage factory producing all the separate components for the phage encoded by the phages DNA. The progeny phage are assembled close to the cell membrane and eventually released by lysis of the host cell. These new phage then go on to infect further host cells in the same way [Carlson 2005; Guttman, Raya and Kutter 2005].

Experiments investigating the infection process yield information on the following important characteristics of each phage:

- **Adsorption** can be measured by the decrease in phage titre during the latent period.
- **Eclipse period**: Defined as the time taken after phage infection for the first mature phage to be produced inside the host cells.
- **Latent period**: This is the time from initial phage infection to the first phage progeny being released from the host cells by lysis.
- **Burst size**: This is simply defined as the number of progeny phage released by each infected host cell.
- **Stationary phase**: Although not usually applied to phage it is a useful descriptive factor for the experiments in this section. It is the time taken from initial phage infection to the time at which the rate of phage production levels off, usually due to the lower number of bacterial cells present resulting in less infection events.

All of these parameters can be calculated from measurements of phage production and bacterial survival obtained throughout a phage infection [Carlson 2005]

Figure 3.1A shows a classical graph of a phage infection process with one line representing free phage in the culture. The other line represents both free phages and intracellular phages that have matured but not yet been released by lysis, hence giving a measure of the eclipse period. This measurement can be achieved by lysing the bacterial cells with chloroform. In the initial stages of the infection after
adsorption the number of free and intracellular phages drops to a lower number of that of the free phages only. This is because the chloroform added to these samples kills the bacteria already infected but before mature phage progeny have been produced. In the free phage samples no chloroform is added hence the infected bacteria remain alive and hence produce plaques.

Phage infection process data is a useful tool for comparing the action of different phages and for assessing the suitability of a phage for use as a therapeutic agent, for example larger burst sizes could indicate a phage that would clear an infection rapidly.

In the process of this study several vials of unidentified phages specific for *Pseudomonas aeruginosa* were obtained from different sources, some of them appearing to be highly virulent and therefore good candidates for therapy. The three areas of phage biology discussed above (infection process, physical size and shape, and genome structure) were used in order to characterise the phages and to ensure that they were all different. The experiments were also used to identify the most appropriate phages to take forward for further study.

The phages themselves were given number/letter codes to identify them prior to formal identification, these are as follows: #2L, #2S, #13, and U1-U4 isolated from an over-the-counter phage preparation that can be purchased from pharmacies in the Ukraine. Bacteriophage F116 was also characterised alongside the unidentified phages. It has been fairly well characterised in the literature as a transducing phage in the podoviridae family. Its head is 65 nm in diameter with an 80 nm tail. It has a genome of 65 kbp and requires pili to adsorb and infect the bacterial cell [Slayter 1964; Miller, Pemberton and Richards 1974; Byrne and Kropinski 2005].

The phage genomes were characterised by assessing the different sized DNA fragments arising from a restriction enzyme digest of DNA isolated from phage lysate. Their physical attributes such as tail length and head size were ascertained from TEM pictures of the phages. Finally their infection process was looked at in detail by analysing both bacterial and phage titres at time intervals during the course of a phage infection of a bacterial culture.
Figure 3.1.A: Graph showing relationship between number of phage particles over time during the infection process. - :Free phages, - :Free plus intracellular phages (culture treated with chloroform). B: Diagram of stages of phage infection corresponding to the graph.
3.2 Methods and results

3.2.1 Materials
Those not present in chapter 2 only.

- **Agarose**: Bioline Ltd. London, UK.
- **Boric acid**: Sigma-Aldrich. St Louis, MO, USA.
- **DNA extraction kit (Lambda mini kit)**: Qiagen Ltd. Crawley, West Sussex, UK.
- **EDTA**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Ethanol**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Ethidium bromide**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Glacial acetic acid**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Hyperladder I and IV**: Bioline Ltd. London, UK.
- **Iso-propanol**: Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK.
- **Lithium hydroxide**: Sigma-Aldrich. St Louis, MO, USA.
- **Loading buffer**: Bioline Ltd. London, UK.
- **Restriction enzymes (EcoRI, BamHI)**: New England Biolabs. Ipswich, MA, USA.
- **Uranyl acetate**: TAAB Laboratories Equipment Ltd. Aldermaston, Berkshire, UK.

3.2.2 Equipment
Equipment not present in chapter 2 only.

- **Electrophoresis gel tank/tray**: Bio-Rad Laboratories. Hercules, CA, USA.
- **Filter paper**: Whatman plc. Maidstone, Kent, UK.
- **Formavar coated grids (TEM)**: Agar Scientific Ltd. Stanstead, Essex, UK.
- **Oven**: Memmert Anderson. Memmert GmbH + Co.KG, Schwabach, Germany.
- **Transilluminator and camera (Geneflash)**: Syngene. Cambridge, UK.
- **TEM camera (Ultrascan 1000CCD)**: Gatan. Pleasanton, CA, USA.
3.2.3 Isolation of bacteriophages

3.2.3.1 Method

Three of the bacteriophage used in this study had been isolated previously. F116 was part of the University of Brighton’s culture collection. Phage #13 and #2 were isolated and propagated as part of an undergraduate student research project [Bowyer 2006]. Aliquots of these bacteriophage stock cultures were purified and propagated using the method detailed in section 2.3.1.

The remaining phages were isolated from an over-the-counter Pseudomonas aeruginosa phage preparation-for-injection obtained in the Ukraine. The Ukrainian phage preparation was serially diluted to 10^{-10} in lambda buffer and all dilutions, including the neat preparation, were subjected to a plaque assay using Ps. aeruginosa strain PAO-GFP as a host. Phages were isolated from plaques using agar plugs according to the method described in section 2.3.1. These were repeatedly subjected to plaque assays and isolation from single plaques until the phage isolate appeared to be pure, as determined by plaque characteristics. These include size shape and the presence of a halo around the plaque.

3.2.3.2 Results

F116 and #13 were successfully purified and propagated. Phage #2 appeared to be a mixture of phage due to the presence of very large and very small plaques on plaque assay plates. Contamination during the assay was ruled out due to the inclusion of appropriate controls. Both types of plaque were isolated from agar plugs and
appeared to be pure after three rounds of plaque assays. The two phages now denoted #2L (large plaques) and #2S (small plaques) were propagated.

The Ukrainian phage preparation yielded a number of morphologically distinct plaques at different dilutions on plaque assay plates. Four plaques were chosen for purification on the basis that they had very different characteristics such as size and shape. These were denoted U1-U4. After three rounds of plaque assays the phage appeared to be pure. All four isolates were successfully propagated and enumerated using the method described in section 2.3.2, and stored as previously described.

3.2.4  Bacteriophage infection process

3.2.4.1 Method

This method was adapted from a previously published protocol [Carlson 2005] which generated data for a number of phage parameters, e.g. burst size, adsorption rate and eclipse period, from one experiment.

A 1ml aliquot of an overnight culture of *Ps. aeruginosa* (PAO-GFP) was added to 100 ml sterile TSB in a flask which was incubated at 37°C with shaking until the OD$_{420}$ reached approximately 0.45. The culture was divided equally between two flasks which were prewarmed to 37°C in a water bath. To one flask 1.2 x 10$^{10}$ pfu of bacteriophage was added, to the other the same volume of sterile lambda buffer was added as a control. Samples were taken from both flasks every 10 minutes for the first 60 minutes and then every 30 minutes thereafter.

**Control flask**

Samples (100 µL) denoted C were taken from the control flask, diluted to the appropriate level (pre-determined from a pilot experiment) and 50 µl spread-plates were prepared in triplicate to enumerate viable bacteria.
**Phage-infected flask**

From the infected flask 3 different samples were taken:

*Free phages*

Samples (1 mL) taken to measure free phage were filtered (Minisart syringe filters, 0.45 μm pore size, surfactant-free cellulose acetate) to remove bacteria. This prevented bacteria that were already infected but had not yet produced mature phage progeny from continuing the infection and producing plaques (see Figure 3.1). Samples were subsequently diluted to the appropriate level and 50 μl were plated in triplicate as a plaque assay. Prior to this experiment a control test confirmed that there was no retention of phage in the filter by passing phage suspension of a known concentration through a syringe filter and testing the filtrate by plaque assay, results not shown.

*Free and intracellular phage*

Samples were treated with chloroform to lyse the bacteria and release the intracellular phage. Samples were then diluted and plated as described above. A control test confirmed that chloroform did not denature the phage by adding 10% v/v chloroform to a phage suspension of a known concentration and testing the phage concentration after 1 hour, results not shown.

*Bacterial survivors*

The third type of samples measured bacterial survivors. The sample was first serially diluted to 10^{-5} and 50 μl of each dilution was added to 5 ml of sterile PBS which was then membrane filtered (Cellulose acetate 0.45 μm pore size filter, Sartorius, Aubagne, France). The bacterial survivor samples were filtered to remove phage from the surviving bacteria, if the samples were simply spread-plated the phage remaining in the samples would infect the bacteria on the plates leading to a reduction in the number of bacterial colonies.

Figure 3.1 provides a diagram of the samples taken and how they were processed. Optical density at 420 nm was also measured at the same time at which samples were taken. This showed how the turbidity of a bacterial culture was affected by
phage infection. All plates were incubated overnight at 37°C and enumerated the next day. Each experiment for each phage was performed a total of 3 times, with the exception of F116 which was performed 5 times. The enumeration results from the different samples were then used to calculate various infection parameters.

**Eclipse period**: This is the time taken for the free and intracellular phage titre to begin to rise.

**Latent period**: The time taken for the free phage titre to begin to rise.

**Stationary Phase**: the time point at which the phage titre no longer rises.

**Burst size**: As defined in section 3.1. This is calculated by subtracting phage titre at the start of infection from the phage titre after stationary phase, and dividing this by the number of bacteria killed.

**Percentage of adsorption**: This is the drop in free phage titre during the eclipse period expressed as a percentage of the phage titre at the start of the infection.

**Percentage bacterial death**: This is the drop in bacterial survivors throughout the experiment expressed as a percentage of the bacterial titre at the start of infection.

The results are presented in section 3.2.2.2.
Figure 3.2: The sampling regime of the bacteriophage infection process method (section 3.2.2.1).
3.2.4.2 Results

Figures 3.2-3.7 show the infection profile for each phage isolate. Infection process data was obtained successfully for all phage with the exception of #2L and #2S. Plaque assay plates from these phage persisted in containing both large and small plaques on the same plate, which could indicate contamination by a second phage. It was not possible after several attempts to isolate and purify the phage so further analysis was not attempted. Aside from exhibiting a characteristic pattern of infection the graphs also provide a means of calculating a number of infection parameters as indicated in section 3.2.4.1. Table 3.1 provides a summary of the main infection parameters for the phage isolates investigated.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Eclipse period (min)</th>
<th>Latent period (min)</th>
<th>Stationary phase reached (min)</th>
<th>Burst size (pfu)</th>
<th>Adsorption (%)</th>
<th>Bacteria Killed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F116</td>
<td>10 ± 0</td>
<td>20 ± 0</td>
<td>46.6 ± 2.8</td>
<td>83.2 ± 21.9</td>
<td>98.2 ± 0.8</td>
<td>&gt;99.9 ± 0</td>
</tr>
<tr>
<td>#13</td>
<td>10 ± 0</td>
<td>10 ± 0</td>
<td>70 ± 16.3</td>
<td>293.9 ± 130.4</td>
<td>4.3 ± 0</td>
<td>99.8 ± 0.2</td>
</tr>
<tr>
<td>U1</td>
<td>10 ± 0</td>
<td>10 ± 0</td>
<td>120 ± 0</td>
<td>596.8 ± 241.1</td>
<td>8.3 ± 6.0</td>
<td>&gt;99.9 ± 0</td>
</tr>
<tr>
<td>U2</td>
<td>10 ± 0</td>
<td>10 ± 0</td>
<td>105 ± 12.3</td>
<td>176.8 ± 70.3</td>
<td>33.6 ± 6.2</td>
<td>&gt;99.9 ± 0</td>
</tr>
<tr>
<td>U3</td>
<td>10 ± 0</td>
<td>10 ± 0</td>
<td>80 ± 10</td>
<td>1175.8 ± 148</td>
<td>8.8 ± 5.8</td>
<td>&gt;99.9 ± 0</td>
</tr>
<tr>
<td>U4</td>
<td>10 ± 0</td>
<td>10 ± 0</td>
<td>100 ± 10</td>
<td>525.0 ± 75.4</td>
<td>32.2 ± 6.4</td>
<td>&gt;99.9 ± 0</td>
</tr>
</tbody>
</table>

Table 3.1: Summary of the infection characteristics of 6 bacteriophage (n = 3*; ± standard error).

* n = 5 for F116

All graphs (Figures 3.3-3.8) showed a similar general pattern characteristic of a lytic phage infection. In the first 20 minutes there was a decrease in the titre of both free phage and free plus intracellular phage. This was caused by the adsorption of the phage to the bacterial cell in order to commence infection of that cell. Usually the
free plus intracellular titre rose first as new phage progeny were produced within the bacterial cells. This was quickly followed by a rise in the titre of free phage, which occurred as the infected bacterial cells lysed releasing phage progeny into the culture medium. Not all phages showed this sequential nature of phage titre increases e.g #13 and U3. Also not all phages showed the initial drop in titre, some showed no change in the first 10 minutes and then increased.

In all cases and corresponding with the rise in free phage titre there was a dramatic decrease in bacterial survivors. These numbers, without exception became too low to show on the graphs without obscuring the other results.

The free phage titre and free plus intracellular phage titre eventually reached a plateau, this was the point at which there were few bacterial survivors left hence the probability of a phage contacting and infecting a bacterial cell was reduced so few new phage were produced.

As would be expected the bacterial titre in the control cultures increased steadily over the course of the experiments.

Optical density values for infected and control cultures are shown on the same graphs on a secondary axis for comparison to titres. Although with all phage the increases and decreases in OD values corresponded to increases and decreases in bacterial titre, this relationship was not proportional. For example in Figure 3.2 there was a 5 fold increase in control culture OD but only a corresponding titre rise of approximately 0.5 log10.
Figure 3.3: Infection process data for bacteriophage F116. • = Control culture (cfu mL⁻¹), □ = Free phage (pfu mL⁻¹), ▶ = Free and intracellular phage (pfu mL⁻¹), ◀ = Bacterial survivors (cfu mL⁻¹), ▲ = OD₄₂₀ Control culture, ▲ = OD₄₂₀ Infected culture. n = 5, error bars represent standard error of the mean.
Figure 3.4: Infection process data for bacteriophage #13. = Control culture (cfu mL$^{-1}$), = Free phage (pfu mL$^{-1}$), = Free and intracellular phage (pfu mL$^{-1}$), = Bacterial survivors (cfu mL$^{-1}$), = OD$_{420}$ Control culture, = OD$_{420}$ Infected culture. n = 3, error bars represent standard error of the mean.
**Figure 3.5:** Infection process data for bacteriophage U1. 
- Circles = Control culture (cfu mL$^{-1}$), 
- Squares = Free phage (pfu mL$^{-1}$), 
- Triangles = Free and intracellular phage (pfu mL$^{-1}$), 
- Inverted triangles = Bacterial survivors (cfu mL$^{-1}$), 
- Downward triangles = OD$_{420}$ Control culture, 
- Upward triangles = OD$_{420}$ Infected culture. 
$n = 3$, error bars represent standard error of the mean.
Figure 3.6: Infection process data for bacteriophage U2. 

- = Control culture (cfu mL\(^{-1}\)), □ = Free phage (pfu mL\(^{-1}\)), △ = Free and intracellular phage (pfu mL\(^{-1}\)), ○ = Bacterial survivors (cfu mL\(^{-1}\)), ▽ = OD\(_{420}\) Control culture, ▲ = OD\(_{420}\) Infected culture. \(n = 3\), error bars represent standard error of the mean.
Figure 3.7: Infection process data for bacteriophage U3. Control culture (cfu mL$^{-1}$), Free phage (pfu mL$^{-1}$), Free and intracellular phage (pfu mL$^{-1}$), Bacterial survivors (cfu mL$^{-1}$), OD$_{420}$ Control culture, OD$_{420}$ Infected culture. n = 3, error bars represent standard error of the mean.
Figure 3.8: Infection process data for bacteriophage U4. 

- □ = Control culture (cfu mL$^{-1}$), ■ = Free phage (pfu mL$^{-1}$), ▼ = Free and intracellular phage (pfu mL$^{-1}$), ● = Bacterial survivors (cfu mL$^{-1}$), ▲ = OD$_{420}$ Control culture, ▼ = OD$_{420}$ Infected culture. $n = 3$, error bars represent standard error of the mean.
3.2.5 Bacteriophage genome characterisation

3.2.5.1 Bacteriophage DNA extraction: Method

Restriction enzymes cut DNA strands at sites specific to the restriction enzyme. These sites consist of particular sequences on the DNA strand. Digesting the genome of a bacteriophage with one or more restriction enzymes will lead to a characteristic series of different length DNA strands, known as a restriction pattern. This method exploits the differences in restriction patterns of phage genomes to establish if the phages are all different.

The phage DNA was extracted using a Qiagen lambda mini kit, according to the manufacturer’s instructions (Qiagen GmbH, Hilden, Germany). Briefly, 500 µL of an overnight culture of *Ps. aeruginosa* was added to 50 mL of sterile TSB and incubated with shaking for 4 hours at 37°C, to take the culture to mid-exponential phase. Phages were added to this culture at a multiplicity of infection (MOI) of 2 (i.e. 2 phage for every bacterial cell) and incubated for approximately 40 minutes until lysis was apparent. A few drops of chloroform were added to the flask which was incubated for a further 15 minutes to ensure thorough lysis. 10 mL of the lysed mixture was centrifuged at 10,000 g for 10 minutes to remove bacterial debris. The supernatant liquid was transferred to a fresh container, 30 µl of buffer L1, containing RNase and DNase to remove bacterial RNA and DNA, was added and the mixture was incubated at 37°C for 30 minutes. 2 ml of buffer L2, which precipitates bacteriophage particles using PEG, was then added and the lysate was incubated on ice for 1 hour. The lysate was centrifuged at 10,000 g for 10 minutes, the supernatant was discarded and the tubes were allowed to drain thoroughly before continuing. The pellet was resuspended in 1 mL of buffer L3, which is similar to lambda buffer for resuspension of the bacteriophage, followed by 1 mL of L4, containing sodium dodecyl sulphate (SDS) to denature the phage protein coat to release the phage DNA. It was then incubated at 70°C for 10 minutes before being cooled on ice. 1 mL of buffer L5, containing potassium acetate to precipitate the denature phage proteins, was added and mixed quickly by inverting. The mixture was then centrifuged at 15,000 g at 4°C for 30 minutes to remove the phage proteins. The supernatant was
quickly transferred to a clean container and centrifuged again at the same speed and temperature for 10 minutes to remove any remaining protein. The supernatant was added to a disposable Qiagen anion exchange chromatography column that had already been primed with 1 mL of buffer QBT, which is a low concentration salt solution which equilibrates the column allowing for binding of DNA. The supernatant ran through the column by gravity and was followed by 2 mL of wash buffer QC, a medium concentration salt solution which removes any impurities from the column. The Phage DNA was eluted by adding to the column 1.5 mL of buffer QF, a high concentration salt solution, and allowing it to drain into a clean centrifuge tube. The DNA was then subjected to washing by iso-propanol precipitation to remove the salt. 1 mL of iso-propanol was added to the eluted DNA which was subsequently centrifuged at 15,000 g for 30 minutes at 4°C. The pellet was washed with 70% ethanol. A further centrifugation step, 10 minutes at 15,000 g, was performed before the pellet containing the DNA was resuspended in 20 μL of TE buffer a DNA storage buffer which protects the DNA from degradation.

3.2.5.2 Selection of suitable restriction enzymes

The RESTRICT software programme [Rice, Longden and Bleasby 2000] was used to subject 24 known *Ps. aeruginosa* phage genomes to single virtual digests by all the restriction enzymes in the REBASE database. This was to enable the choice of appropriate restriction enzymes. Two suitable enzymes were chosen based on them giving 3-20 fragments between 500 base pairs (bp) and 25 kbp (kilobase pairs) in most phage genomes. These criteria would provide an easily resolvable gel picture. The two enzymes chosen were BamHI and EcoRI.
3.2.5.3 Assessment of the success of phage DNA extraction

3.2.5.3.a. Method

A 1% agarose gel was first prepared by dissolving 1% v/v agarose in 100 mL TAE buffer (4.84 g L-1 Tris-base, 1.09 g L-1 Glacial acetic acid, 0.29 g L-1 EDTA). To this 0.01% v/v ethidium bromide was added and mixed by swirling. The gel was poured into a small gel tray with a well comb in place and left to set. Once set the comb was removed and the Gel was placed into an electrophoresis tank, TAE buffer was added to the tank until it covered the gel. 100 µL of ethidium bromide was added to the buffer in the tank.

A 20 µL aliquot of a 1:10 dilution in water of the whole-genome extract of each phage was mixed with 10% v/v loading buffer. 10 µL of these were then loaded into separate wells on the gel along with 5 µL of a DNA ladder (hyper ladder I) which contains DNA fragments of known lengths for comparison.

The electrophoresis tank was connected to an electrical power pack and run at 100 volts. The gel was checked regularly on a transilluminator to visualise the DNA. Images of the gel were taken using the image capture system connected to the transilluminator.

3.2.5.3.b. Results

Figure 3.9 shows the gel images obtained following electrophoresis of whole-genome extracts. There were no visible bands for phages #13 and U4 suggesting that very little or no DNA was present. There were very faint bands visible for phages #2L and #2S, again suggesting that very little DNA was present.
Figure 3.9: Gel electrophoresis images of whole-genome DNA bands. Lanes: 1) Hyperladder I (DNA marker), 2) F116, 3) #13, 4) #2L, 5) U1, 6) U2, 7) U3, 8) Hyperladder I, 9) Hyperladder I, 10) #2S, 11) U4, 12) Hyperladder I. Ladder fragment sizes in base pairs.
3.2.5.4 Restriction digest of bacteriophage genome

3.2.5.4.a. Method

Digestion mixtures were calculated according to the concentration of DNA estimated for each phage from the whole phage genome gel. An excess of restriction enzyme was used each time to allow for complete digestion, and 10% v/v enzyme buffer. The digestion mixture was incubated at 37°C for 2 hours.

The digests were immediately subjected to Gel electrophoresis on a 1.2% agarose/Lithium borate gel at 150 volts. The gel and tank preparation was performed as in section 3.2.3.3 but using 1.2% w/v agarose and Lithium borate buffer (0.42 g L\(^{-1}\) Lithium hydroxide, 1.8 g L\(^{-1}\) boric acid) in place of TAE buffer. Lithium borate buffer holds at a lower temperature during electrophoresis allowing a higher voltage to be applied without melting the gel. The wells were loaded with 10 μl of each digest containing 10% v/v loading buffer. Two DNA ladder markers were used; hyperladder I and hyperladder IV which has a range of smaller DNA fragments (Promega, Madison, USA). The gel was checked regularly on the transilluminator and images of the gel were captured at several intervals throughout the electrophoresis process in order to resolve both large and small DNA bands as they migrated through the gel. The fragment size of each band obtained from 5 pictures taken at different times were assessed using the GEL software programme, and compared to the results from the RESTRICT programme. The distance that each band had run was measured including those of the DNA ladders. This information was then analysed using the GEL program to estimate the sizes of each band.

3.2.5.4.b. Results

A representative gel image of the BamHI restriction digest is shown in Figure 3.10. This digest appears to have been unsuccessful as there were no distinct bands for any of the phage genomes. The DNA appears as a smear down the length of the gel indicating possible degradation/denaturation of the DNA.
Figure 3.10: Gel electrophoresis image of phage genomes digested with BamHI. Lanes: 1) Hyperladder I (DNA marker), 2) F116, 3) #2L, 4) #2S, 5) #13, 6) U1, 7) U2, 8) U3, 9) U4, 10) Hyperladder IV (DNA marker). Ladder fragment sizes in base pairs.
A representative image of the gel containing the EcoRI digestions is shown in Figure 3.11. This digest has been more successful than with BamHI as distinct DNA bands can be seen in at least 6 lanes of the gel. The EcoRI digest produced multiple bands on the gel electrophoresis image for F116, #2S, #13, and U1-U3. No DNA bands were visible for #2L or U4. All gel pictures and measurements obtained can be found in appendix II. Table 3.2 contains the average band sizes as calculated by GEL.

Figure 3.11: Gel electrophoresis image of phage genomes digested with EcoRI. Lanes: 1) Hyperladder I (DNA marker), 2) F116, 3) #2L, 4) #2S, 5) #13, 6) U1, 7) U2, 8) U3, 9) U4, 10) Hyperladder IV (DNA marker). Ladder fragment sizes in base pairs.
None of the observed DNA fragments matched any of the fragments from virtual EcoR1 digests of known *Ps. aeruginosa* phage genomes. A comparison of the observed and expected fragments for phage F116 can be seen in Table 3.3. Poor quality DNA preparations or partial digestion of these preparations could be an explanation for the differences between the expected and observed fragments.

DNA extraction was repeated twice more for phages #13, #2S and U4 due to the lack of DNA bands on the gels for these phages. However each time was unsuccessful with no bands (data not shown).

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Observed fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F116</td>
<td>756</td>
</tr>
<tr>
<td>#13</td>
<td>No observed gel bands</td>
</tr>
<tr>
<td>#2L</td>
<td>1101</td>
</tr>
<tr>
<td>#2S</td>
<td>809</td>
</tr>
<tr>
<td>U1</td>
<td>1160</td>
</tr>
<tr>
<td>U2</td>
<td>734</td>
</tr>
<tr>
<td>U3</td>
<td>1189</td>
</tr>
<tr>
<td>U4</td>
<td>No observed gel bands</td>
</tr>
</tbody>
</table>

*Table 3.2*: Average DNA fragment sizes produced by EcoRI digestion of the phage genomes.
<table>
<thead>
<tr>
<th>Expected fragments (bp)</th>
<th>Observed fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2327</td>
<td>756</td>
</tr>
<tr>
<td>2744</td>
<td>1097</td>
</tr>
<tr>
<td>2932</td>
<td>1139</td>
</tr>
<tr>
<td>4930</td>
<td>1296</td>
</tr>
<tr>
<td>7344</td>
<td>1497</td>
</tr>
<tr>
<td>7956</td>
<td>1988</td>
</tr>
<tr>
<td>9127</td>
<td>2081</td>
</tr>
<tr>
<td>9275</td>
<td>2233</td>
</tr>
<tr>
<td>18560</td>
<td>2435</td>
</tr>
<tr>
<td></td>
<td>2929</td>
</tr>
<tr>
<td></td>
<td>3965</td>
</tr>
</tbody>
</table>

**Table 3.3:** Comparison between observed fragments from an EcoRI restriction digest of phage F116 genome and expected fragments.
3.2.6 TEM imaging

3.2.6.1 Method

A drop of a concentrated suspension (~1x10⁹ pfu mL⁻¹) of each bacteriophage was placed on a formavar coated grid using a pasteur pipette. The titre of each phage suspension was approximately 1 x 10⁹ pfu ml⁻¹. Whatman filter paper was used to draw and blot the suspension across the grid. The grid was placed on filter paper to dry for 2 minutes. A drop of saturated uranyl acetate solution was placed in a vial cap and the grid was carefully lowered into it. The grid was allowed to stain for 2 minutes then transferred to filter paper to dry. Bacteriophage were observed in a transmission electron microscope (Hitachi-7100) and images acquired digitally with an axially-mounted (2k x 2k pixel) camera (Gatan Ultrascan 1000 CCD) at magnifications of x10,000 and x50,000.

Photographs were obtained of all 6 phages at higher and lower magnification. Measurements of phages were made by calculating the scale ratio by measuring the length of the scale bar on each photograph. This ratio was then used to calculate the true sizes of phage particles from measurements taken of the photographs. Each measurement was taken 3 times and the average obtained ± standard error.

Figures 3.12 and 3.13 show phage F116 at magnifications of x 10,000 and 50,000, on these images the head and tail have been labelled for information. F116 is a member of the caudoviridae family and is a tailed phage, as previously noted in the literature [Miller, Pemberton and Richards 1974]. The icosahedral head, as measured on the obtained photographs, had a diameter of 76.78 nm (± 1.79) with a tail measuring 161.89 nm (± 1.59) in length and 27.38 nm (± 1.59) in width. Striations, which are the subunits that form the tail, can be clearly seen in Figure 3.12 as can tail fibres attached to what appears to be a small base plate.

Bacteriophage #13 was similar in appearance to F116 (Figures 3.14 and 3.15). It is a tailed phage with a head diameter of 76.18 nm (± 1.59) and a tail length of 192.83 nm (±0) and width of 24.99 nm (± 1.19). The tail was therefore longer and slightly
thinner than that of F116. It also appeared to have at least one tail fibre and possibly a base plate structure at the end of the tail.

Bacteriophage U1 (Figures 3.16 and 3.17) was also a tailed phage but with much shorter tails than F116 or #13. Its head was 67.81 nm (± 1.53) in diameter and its tail was 68.96 nm (± 2.299) in length and 29.86 nm (± 4.59) in width. There did not appear to be any tail fibres or other associated structures. It is not clear whether this was due to the quality of the image making tail fibres difficult to visualise or a genuine lack of tail fibres.

Bacteriophage U2 was another tailed phage, as can be seen in Figures 3.18 and 3.19, very similar in appearance to F116 and #13. Its head was 78.56 nm (± 2.38) in diameter with a tail of 157.12 nm (± 0) in length and 25.59 nm (± 1.98) in width. The irregular shape of the head in the images would suggest an octahedral structure in contrast to the more regular icosahedral heads of F116 and #13. There were also at least two tail fibres extending from the base of the tail.

Bacteriophage U3 was different in that it appeared to be tail-less. Figures 3.20 and 3.21 show icosahedral phage heads of 68.08 nm (± 0) with no tails visible in either image. Moreover in Figure 3.20 there is a collection of what are most likely to be bacterial pili with numerous phage attached to them. This assumption is based on TEM pictures of pili from other studies which correlate to the size and appearance of the structures in this picture [Touhami et al. 2006].

Bacteriophage U4 was another tailed phage with an icosahedral head of 75.85 nm (± 1.15) in diameter. Figure 3.22 shows several of the phage at 10,000x magnification, Figures 3.23 and 3.24 both show an individual phage at 50,000x magnification. In the first image the phage tail is in the normal orientation and was 151.71 nm (± 0) in length, and 20.69 nm (± 0) in width. Striations can be seen along the length of the tail and tail fibres extend from the base of the tail. Some of the phages in this image appeared to be lacking tails, preparation of bacteriophages for TEM often leads to damage to the phage structure which may account for this observation. In the second image (Figure 3.23) the tail consists of two sections, the first extending from the head was 78.52 nm (± 0) long and 28.57 nm (± 0) wide. The second section was of a similar length; 71.42 nm (± 0) but only 8.93 nm (± 0) wide. The phage head remains the same size but appears “empty”, with regards to stain density in comparison to the
first image. These differences indicate that U4 had a contractile tail and the second image represents the phage after it has infected its host [Bradley 1967].
**Figure 3.12**: TEM image of bacteriophage F116 at 10,000x magnification
Figure 3.13: TEM image of bacteriophage F116 at 50,000x magnification, Tail striations (A), tail fibres (B), and possible base plate (C) are indicated by arrows.
Figure 3.14: TEM image of bacteriophage #13 at 10,000x magnification.
Figure 3.15: TEM image of Bacteriophage #13 at 50,000x magnification, with tail fibre (A) and possible base plate structure (B) indicated by arrows.
Figure 3.16: TEM image of bacteriophage U1 at 10,000x magnification.
Figure 3.17: TEM image of bacteriophage U1 at 50,000x magnification.
Figure 3.18: TEM image of bacteriophage U2 at 10,000x magnification
Figure 3.19: TEM image of bacteriophage U2 at 50,000x magnification.
Figure 3.20: TEM image of bacteriophage U3 at 10,000x magnification
Figure 3.21: TEM image of bacteriophage U3 at 50,000x magnification.
Figure 3.22: TEM image of bacteriophage U4 at 10,000x magnification.
Figure 3.23: TEM image of bacteriophage U4 at 50,000x magnification.
Figure 3.24: TEM image of bacteriophage U4 at 50,000x magnification.
3.3 Discussion

The aim of this work was to isolate, purify and characterise a number of bacteriophage from the University of Brighton’s culture collection. The suitability of each phage as a potential candidate for phage therapy can be assessed using the data from this work in combination with other data such as the inflammatory response to the phage. A second possible use for these data is to facilitate identification of the phage and to determine the most suitable phage for further work.

All phage were successfully isolated and purified with the exception of #2. The original stock of #2 appeared to contain two types of phage, one with a large plaque morphology and a second with a very small plaque morphology. Although 2 different phages appeared to be isolated and purified, upon the initiation of infection process experiments, the two different plaque morphologies reappeared in both #2S and #2L samples. This could be the effects of contaminating phage or it is possible that the #2 phage has a sub-population of phages containing a mutation which leads to different plaque morphologies [Weigle 1953]. Hence due to this uncertainty #2 was no longer investigated. A total of four potentially different phages were isolated from the Ukrainian phage preparation.

The infection process experiments yielded a large quantity of data for each phage isolate. The eclipse period, the time taken for new progeny phage to form in the infected bacterial cell, was calculated to be 10 minutes for all phage. The latent period, the time taken for new progeny phage to be released by the bacterial cell, was also 10 minutes for all phage except F116. These Figures are likely to be a function of the sampling regime as opposed to true times. In order to determine these parameters more precisely a higher frequency of sampling would have to be undertaken. However this is unnecessary for the scope of this work. The latent period for F116 was 20 minutes which indicates there is a slight delay between phage progeny being constructed within bacterial cells and lysis of those cells. Very little investigation has been completed on the lytic lifecycle of F116, for comparison, due to its importance as a transducing phage.
The time taken to reach stationary phase was more varied between the different phage. This was the point at which the exponential increase in phage ceases and the phage titre remains constant, as a result of a low number of surviving bacteria being available for further phage infection and release. F116 reached this point at 46.6 minutes, some time before any other phage. Phage U1 took the longest to reach stationary phase with a time of 120 minutes. The remainder of the phage took between 70 and 100 minutes to reach stationary phase.

Burst sizes were also very varied both between phage and within each set of experiments for any particular phage. Burst size is known to be highly variable being affected by culture conditions as well as variations between phage species [Brussow and Kutter 2005]. F116 exhibited the lowest burst size with an average of 83.2 (± 21.9) phage being released upon lysis of the infected bacterial cell. The largest burst size was that of U3 which released an average of 1175.8 (± 148) phage from each lysed bacterial cell. Evidence of this can be seen in the infection process graph of U3 (Figure 3.7). The titre of free phage rose dramatically between 10-60 minutes post infection with a corresponding slow decrease in bacterial survivors. This indicates a large number of phage have been released from a small number of bacteria. After 60 minutes the number of surviving bacteria fell rapidly, almost certainly as a consequence of the large number of phage that had been released.

The remaining burst sizes ranged from 176.8 (± 70.3) for U2 and 596.8 (± 241.1) for U1. A large burst size could be considered favourable for a phage therapy candidate as the bacterial infection could be eradicated rapidly. However if the bacterial lysis releases large quantities of endotoxin, and many bacteria are being lysed simultaneously, such as occurred after 60 minutes with phage U3, the result could be toxic shock for the patient. It would be necessary to determine the levels of endotoxin released as a result of phage-induced bacterial cell lysis and to compare with levels released from antibiotic-treated bacterial cultures.

As the sampling frequency led to difficulties in determining accurate eclipse and latent periods, the apparent percentage of phage that adsorbed to bacterial cells during the eclipse period was somewhat misleading. F116 was the only phage that exhibited a large drop in free phage after initial infection with 98.2% (±0.8) of phage adsorbing in 10 minutes. U2 and U4 showed an adsorption of 33.6% (±6.2) and
32.2% (±6.4) respectively. However #13, U1, and U3 appeared to undergo very little adsorption with only a 4.3% (±0), 8.3% (±6.0), and 8.8% (±5.8) drop in free phage titre respectively. It is possible that the drop in free phage titre resulting from adsorption is being masked by the sampling regime. In this experiment samples were taken initially every 10 minutes, if the adsorption and subsequent release happened within a ten minute window then it could be missed. As with the eclipse and latent period, the picture may become clearer with a higher frequency of sampling.

All but phage #13 exhibited a bacterial kill of >99.9%, usually with no bacteria detectable in the broth after 150 minutes. This is one of the most important traits for a phage therapy candidate, to totally eradicate the bacterial infection. However #13 illustrates one of the potential disadvantages of using phage therapy, that of bacterial resistance. From Figure 3.3 it is clear that although the majority of bacteria were lysed after 60 minutes, by 120 minutes the number of surviving bacteria had risen. This is likely to be a result of phage-resistant bacteria multiplying within the culture. All other phage appeared to kill the bacteria without any subsequent re-growth.

Uninfected control cultures showed a moderate increase in bacterial titre throughout the 150 minute experiments. However the corresponding optical densities of the culture read at 420 nm increased more than would be expected given the culture titres. This could be the result of large amounts of exopolysaccharide that is produced by Ps. aeruginosa cultures which would alter the optical density of the medium.

Throughout these experiments, for practicality, the same strain of Ps. aeruginosa has been used. This may not be the optimum strain for all phages and therefore their infection process data may reflect this. Future investigation should test many different strains to establish a spectrum of activity for each phage.

Characterisation of the bacteriophages genomes generated inconclusive results. Following digestion with EcoR1 none of the DNA fragment sizes represented by bands on the gels corresponded with the expected DNA fragment sizes of known Ps. aeruginosa phages obtained from the database. This included the observed and expected results for the well characterised F116. This meant that it was not possible to identify the other phages based on their genome restriction patterns. Nonetheless, when used in conjunction with data generated from other means of characterisation,
it may be possible to ascertain whether 8 distinct phages have been isolated or whether two or more phage are of the same type. Hence U1 and U3 appear to have the same size bands after digestion with EcoRI, therefore they could either be two preparations of the same phage or two closely related phages. Gel electrophoresis of U4 produced no bands whether digested or undigested. This could be due to degradation of the DNA.

None of the phage genomes were successfully digested with BamHI, it is unclear why this would be the case. Possible reasons are degradation of the DNA, contamination of the phage DNA with bacterial DNA or possibly denaturation of the enzyme. Further investigation would be required to identify the causative factor.

Although the manufacturer’s instructions were closely followed throughout the DNA extractions from phage samples it is possible that degradation could have occurred at some point in the process. This could be investigated by checking samples of the DNA preparation at various stages of the extraction process, or attempting a different method of extraction. Within the method used there was a step to remove bacterial DNA and RNA contamination by enzymatic digestion, however if the RNase/DNase was denatured or there was a lot of bacterial DNA present this step may not have been fully efficient. This could be improved by using fresh RNase/DNase or by incubating the enzyme mixture for longer. Restriction enzymes were bought in especially for this experiment so were fresh, however fluctuations in delivery/storage temperatures, although very unlikely, cannot be entirely ruled out. Restriction enzymes are fragile and could be easily denatured. This could be resolved by using these enzymes to perform a restriction digest on a known control DNA sample, and checking that the correct size fragments occur.

TEM images revealed the morphology of the bacteriophages. All phages except U3 had tails, and all had icosahedral heads with the exception of U2 which appeared to have an octahedral head. All the phages with long tails (F116, #13, U2, and U4) also appeared to have one or more tail fibres extending from the tail base with U4 being the only phage to show evidence of having a contractile tail. This was seen as a shortened thickened tail with longitudinal striations rather than the typical spiral/axial striations this has been noted in other studies[Bradley 1967; Kellenberger and Wunderli-Allenspach 1995]. The other phages may also have had contractile
tails but evidence of this was not seen in any of the pictures. U3 appears to be a tail-less phage which adsorbs to pili, a common phage adsorption site for those that use *Ps. aeruginosa* as their host [Bradley 1966; Bradley 1973; Bradley 1974; Roncero, Darzins and Casadaban 1990]. The head measurements of F116 in this study match those of previous researchers; however the tail lengths are much longer in this study than those found previously. This discrepancy could be either due to an artefact of the TEM such as twisted tail fibres giving the appearance of a longer tail or that the phage preparation in our stock collection was mislabelled as F116.
3.4 Conclusions

Drawing together all the data from the three methods of characterisation, and not taking into account the unresolved phage #2, it would appear that there were 6 distinct phages in this study. Those that appeared similar in one method appeared entirely different in another. For example U1 and U3 shared similar genome restriction patterns however their morphology was very different.

The data gathered from phage characterisation were considered alongside work in the following chapter, which aimed to determine what affect each of these phages has on human airway epithelial cells, in order to make a decision on which phage was used for phage treatment experiments.
4 Bacteriophage co-culture
4.1 Introduction

If bacteriophages are to be used as therapeutic agents it is necessary to ensure that they will not cause any immunological reaction in the patient. When administered, phages would effectively be foreign proteins in the infected host and could therefore provoke an inflammatory response. In CF it is particularly important to assess the level of response to phages as CF-related lung infections are already associated with an inappropriate inflammatory reaction [Khan et al. 1995; Armstrong et al. 1997; Bonfield, Konstan and Berger 1999; Chan, Chmura and Chan 2006].

The epithelial cells of the airway protect themselves from infection initially via an inflammatory reaction. The Toll-like receptors (TLRs) of the epithelial cells recognise certain elements of invading pathogens and respond by producing inflammatory mediators such as cytokines and chemokines. These molecules further promote the inflammatory reaction by initiating production of antimicrobial peptides and recruiting phagocytic neutrophils. Table 4.1 gives details of the more commonly produced defensive and inflammatory molecules. This usually helps eradicate any infection before it has a chance to establish itself. The TLRs recognise a variety of different substances that could be implicated in pathogen invasion especially endotoxins and RNA and DNA. This enables a response to a wide range of pathogens including bacterial, viral and fungal species [Nicod 1993; Hiemstra 2001; Parkin and Cohen 2001; Travis, Singh and Welsh 2001; Yeh and Chen 2003; Bals and Hiemstra 2004; Janeway et al. 2005; Tosi 2005]. Hence it is possible that the epithelial cells could respond in this manner to bacteriophages, especially if an exaggerated inflammatory response is already occurring such as in CF [Hiemstra 2001; Bals and Hiemstra 2004].

Two cytokines that are commonly amongst the first substances to be produced by airway epithelial cells on detection of potential pathogens are interleukin-6 (IL-6) and interleukin-8 (IL-8) [Cromwell et al. 1992; Nicod 1993]. LdH is also an indicator of distressed cells, it being released when the cell membranes become “leaky”.
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Produced by</th>
<th>In response to</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 Interleukin-1</td>
<td>Small polypeptide</td>
<td>PMNs, Monocytes</td>
<td>TLR activation</td>
<td>Induce a variety of inflammatory mediators (inc IL-6 and IL-8), recruit neutrophils</td>
</tr>
<tr>
<td>IL-6 Interleukin-6</td>
<td>Small polypeptide</td>
<td>Epithelium, Monocytes</td>
<td>TLR activation, injury</td>
<td>Pro-inflammatory acute phase response Anti-inflammatory: Inhibits IL-1 and TNFα</td>
</tr>
<tr>
<td>IL-8 Interleukin-8</td>
<td>Small cytokine-like polypeptide</td>
<td>Epithelium, macrophages, neutrophils</td>
<td>TLR activation</td>
<td>Chemoattractant for neutrophils</td>
</tr>
<tr>
<td>TNFα Tumour necrosis factor α</td>
<td>Small polypeptide</td>
<td>PMNs, Monocytes</td>
<td>TLR activation</td>
<td>Induce a variety of inflammatory mediators (inc IL-6 and IL-8), recruit neutrophils</td>
</tr>
<tr>
<td>α defensins</td>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β defensins</td>
<td>Small cationic peptides</td>
<td>Epithelium</td>
<td>TLR activation</td>
<td>Broad-range antimicrobial IL-1, TNFα</td>
</tr>
<tr>
<td>Proteases e.g. elastase</td>
<td>Enzyme polypeptide</td>
<td>Neutrophils</td>
<td>Chemoattractant e.g. IL-1, IL-8 and TNFα</td>
<td>Antimicrobial</td>
</tr>
</tbody>
</table>

**Table 4.1**: Some of the most common inflammatory molecules seen in the innate immune defence of the airway epithelium, their production and action [Nicod 1993; Konstan and Berger 1997; Tosi 2005]. Abbreviations: PMN = Polymorphonuclear leukocytes, TLR = Toll-like receptors.
The release of inflammatory mediators such as cytokines could be due to the nature of the phage particle itself or to substances remaining from the propagation and purification step. In particular, endotoxin contamination arising from residual bacterial debris has been shown to cause a pronounced inflammatory reaction [Dentener et al. 2000]. However, any phage preparation that on application to 16HBE cells causes the release of high concentrations of IL-6, IL-8, and LdH will not be considered for further study.

This portion of work tested the ability of each of the phage to elicit an inflammatory response in human airway cells. The production of the cytokines interleukin-6 and interleukin-8 (IL-6 and IL-8), and LdH by 16HBE cells was monitored at various times after the application of the 6 different phages used in this study (F116, #13, U1-U4). After this, endotoxin levels in each of the phage preparations were monitored by the limulus amoebocyte lysate test to establish what influence any contaminating endotoxin may have had on the cytokine results.
4.2 Methods and results

4.2.1 Treatment of 16HBE cells with bacteriophage

Before choosing a bacteriophage for further investigation it was important to investigate the inflammatory response they provoke in the 16HBE cells. A phage that leads to low concentrations of cytokines produced by 16HBE cells and hence a reduced inflammatory response would be the most optimal for use in subsequent experiments. This experiment was performed to address this issue.

4.2.1.1 Method

16HBE cells were grown on inserts in the usual way (section 2.4.2). The wells and baso-lateral surfaces of the inserts were washed with fresh sterile growth medium which was then removed and replaced. Suspensions of 6 bacteriophage (F116, #13, U1-U4) were produced at concentrations of $2 \times 10^9$ pfu mL$^{-1}$ in lambda buffer. A 50 µL aliquot (containing $1 \times 10^8$ pfu) of each phage was placed on the apical surface of an insert. Each phage was tested in triplicate as were the controls. The positive control was vanadyl sulphate 20 µg mL$^{-1}$ and the negative control was lambda buffer.

The baso-lateral medium was removed and replaced with fresh sterile medium, immediately the phage suspensions and controls were added. The medium that had been removed was placed in labelled micro-centrifuge tubes and served as the samples for time zero ($T = 0$). Further samples were taken in the same way every 2 hours for 24 hours. For practical reasons the experiment was split into two parts staggered over 12 hours to allow sampling for 12 hours on consecutive days rather than for 24 hours in one go. The first inserts were set up at time zero and sampled every 2 hours for 12 hours, after this time a second set of inserts were set up and incubated overnight for 12 hours. At this time the baso-lateral medium was removed and the inserts were washed with fresh pre-warmed medium 3 times. At 14 hours post set up sampling began from the second set of inserts as $T14$ and continued every 2 hours until $T24$ was reached.
The samples were tested for their concentration of IL-6 and IL-8 using an ELISA assay (section 2.5.1). Before being tested the samples were diluted 1:25 with sterile MEME. The samples were also tested for comparative levels of LdH (section 2.5.4). It was not necessary to dilute the samples for LdH assays.

4.2.1.2 Results

Figures 4.1 and 4.2 show the results of the IL-6 assay. Over the course of the experiment there were no significant differences in IL-6 production between 16HBE cells that had been exposed to the 6 different phages, \( P = 0.905 \) (one-way ANOVA). Moreover in general the results did not differ significantly from those where cells had been exposed to lambda buffer only, as a negative control \( P = 0.916 \) (ANOVA). The exception to this was during the peak production of IL-6 at between 2-6 hours after application of phage where the response to all phages apart from #13 and U4 was higher than the response to the negative control \( P = <0.0005 \). This seems to indicate that the cells reacted to the phage shortly after application but this response eased. As would be expected the positive control resulted in a significantly higher IL-6 concentration than any other tests \( P =<0.0005 \) (one way ANOVA).

IL-8 results (Figures 4.3 and 4.4) show a similar pattern to IL-6 results with no significant differences in concentration from cells exposed to different phages over the course of the experiment \( P=0.804 \) (one way ANOVA). Again in general the results for the negative controls did not differ from those for the phages \( P = 0.890 \). The only exceptions in this case were the results for F116 and #13 at 6-8 hours which were higher than the negative control \( P=0.021 \). Again as expected the positive control results were much higher than all other tests \( P = <0.0005 \) with a peak at 4-6 hours and another later at 18 hours.

The high concentrations of both IL-6 and IL-8 for the positive controls demonstrated the ability of the 16HBE cells used to respond fully to inflammatory stimuli. Hence the lower concentration observed in the phage co-culture tests can be assumed to be a true low response as opposed to a lack of ability to respond.
The results for LdH (Figures 4.5 and 4.6) were relatively unremarkable with no differences between any of the phage or the negative control $P = 0.314$ and 0.323 respectively (ANOVA). Moreover the results did not increase from time zero, indicating little LdH release. With the exception of some LdH peaks, with large standard errors for phage samples, from 4-6 hours onwards the positive control was higher than the other tests $P = <0.005$ but this difference was less pronounced than with interleukin production.
**Figure 4.1:** IL-6 concentration of baso-lateral media samples taken from 16HBE cells exposed to 6 different bacteriophage (T=0 – T=10). F116: Phage 13; U1: U1; U2: U2; U3: U3; U4: U4. Negative control: , Positive control: . Error bars denote standard error, n = 3.
Figure 4.2: IL-6 concentration of baso-lateral media samples taken from 16HBE cells exposed to 6 different bacteriophage (T=12 – T=24).

Figure 4.3: IL-8 concentration of baso-lateral media samples taken from 16HBE cells exposed to 6 different bacteriophage (T=0 – T=10).

Figure 4.5: LdH release by 16HBE cells exposed to 6 different bacteriophage (T=0 – T=10).
Figure 4.6: LdH release by 16HBE cells exposed to 6 different bacteriophage (T=12 – T=24).
4.2.2 Levels of endotoxin in bacteriophage preparations

In order to confirm whether the response investigated in section 4.2.1 was due solely to bacteriophage particles it was necessary to measure the levels of endotoxin in the phage stocks. The nature of the preparation of phage stocks ensured that live bacteria and bacterial debris were removed however it was possible that some endotoxin remained as there was no specific depyrogenation of the stocks. Endotoxin is likely to provoke an inflammatory response by the 16HBE cells.

4.2.2.1 Method

Endotoxin levels were measured using a Limulus amoebocyte lysate (LAL) system. This system uses Pyrochrome® which contains blood cells from the horseshoe crab which coagulate on contact with endotoxin as a result of an enzymatic reaction. In this assay the enzymatic reaction was utilized to cleave chromogenic peptides which caused a colour change. In this case the test turned from colourless to yellow. By the use of a standard curve produced by diluting a control standard endotoxin (CSE) the levels of endotoxin in samples could be calculated. There are two methods of calculating endotoxin with this assay; the kinetic method which measures the time taken for samples to reach a specified absorbance level, and the endpoint method which measures the absorbance after a certain amount of time. Endotoxin concentrations are usually measured in Endotoxin units per mL (EU mL⁻¹) and the equivalence of this to standard concentration units can be obtained from the certificates of analysis that accompany these tests.

The manufacturer's instructions were modified slightly to allow the use of alternative equipment to that specified. A vial of control standard endotoxin (CSE) from *E. coli* O113:H10 was reconstituted with 5 mL LAL reagent water (LRW). It was diluted to the highest concentration required for the standard curve which was 2.5 ng mL⁻¹ or 25 EU mL⁻¹. 1 ng mL⁻¹ is equivalent to 10 EU mL⁻¹ for this endotoxin as reported in the manufacturer's certificate of analysis. Each phage preparation,
already diluted to the levels used in section 4.2.3 with lambda buffer, was further
diluted in LRW at 1:10, and 1:100, and 1:1000.

Honeycomb well plates that were compatible for the Bioscreen® automated
absorbance reader were used for this assay. The standard curves were created by
diluting the CSE solution serially 1:2 with LRW in the plate wells with a final
volume in each well of 100 μL. The range of the standard curve was 25 EU mL⁻¹-
0.75 EU mL⁻¹. A 100 μL aliquot of each of the dilutions of the phage preparations
was placed into plate wells. Each dilution of each phage preparation was tested in
duplicate as were samples of lambda buffer, PBS and LRW as negative controls.

A vial of Pyrochrome® which contained the LAL reagent was reconstituted with 3.2
mL of pyrochrome buffer provided as a kit. 100 μL of this solution was swiftly
added to each occupied well in the honeycomb plate. The plate was immediately
placed into the prewarmed (37°C) Bioscreen®. The Bioscreen® was set to take
readings every 2 minutes for 2 hours which was the minimum interval possible with
the number of samples to be read, with incubation at 37°C. Due to the type of data
collection employed in this assay, i.e. absorbance readings taken at time intervals
rather than continuously, the endpoint method was used to calculate endotoxin
levels. The endpoint method calculates levels of endotoxin based on the OD value
after a set duration of incubation of the sample with LAL. In this experiment the time
point for taking measurements after the addition of LAL will be chosen based on
how linear the standard curve is. In order to work this out a standard curve of a series
of known endotoxin concentrations was prepared, Pyrochrome® was added and the
OD value for each concentration was measured every two minutes. The set of values
for each time point were analysed to assess at which time the standard curve
demonstrated a straight line.

4.2.2.2 Results

The time point chosen for this method was the point at which the standard curve fit
the linear relationship most faithfully. This point was 10 minutes after the addition of
Pyrochrome®. The phage preparation dilution whose absorbance fell within that of
the standard curve was used to calculate the level of endotoxin for that phage. In all cases this was the 1:1000 dilution. Figure 4.7 shows the levels of endotoxin for each phage preparation, extrapolated upwards to account for the dilution, and also for LAL reagent water, PBS and lambda buffer which are shown on a separate graph due to the differences in endotoxin concentration between these and the phage preparations.

The endotoxin results for all 6 phages were obviously much higher than those for the diluents and buffers $P = \leq 0.0005$. However there were no significant differences in endotoxin levels between the phage preparations $P = 0.086$. 
Figure 4.7: Levels of endotoxin in diluents (A) and phage preparations (B). n = 3, error bars denote standard error.
4.3 Discussion

Although some animal studies have demonstrated recognition of bacteriophages as foreign bodies/proteins and removal from the blood by the spleen [Merril et al. 1996], there has been very little investigation into the innate response to phage, in isolation, at the epithelial cell level. Treatment of a pulmonary infection by phages would almost certainly involve delivering the phage directly to the airway epithelia by means of inhalation. Taking into account the heightened inflammatory response already taking place in the infected airways of CF patients it is important to ensure that this situation is not exacerbated by the treatment regime. Hence it is necessary to establish the inflammatory response of epithelial cells to direct contact with phages.

In this study the release of interleukin-6 and IL-8 was monitored after the application of 6 different phages to the apical side of an in vitro epithelial cell layer at ALI. These cytokines are released as part of an inflammatory cascade in response to pathogens and form an important part of the innate immune system. There was no difference in release of IL-6 or IL-8 between cells treated with the different phages indicating they were all equal in their ability to invoke an inflammatory response. In general this response was low with little difference between the phages and the negative controls. However the release of both cytokines reached a peak shortly after the application of the phages (2-6 hours for IL-6, 4-8 hours for IL-8). At these points the cytokine release was significantly higher for cells treated with phages than those with the negative control. Interestingly this peak subsided and for the rest of the experiment the cytokine release remained low for those cells treated with phage. Moreover the cytokine release for cells treated with the positive control was up to three times higher than for phage treated cells. This seems to suggest that phages do not provoke a large scale inflammatory response in 16HBE cells. The initial peaks (2-6 hours for IL-6, 4-8 hours for IL-8) may have been due to endotoxin in the phage preparations, as a by-product of bacterial lysis. All the phage preparations contained approximately the same concentration of endotoxin and this could potentially mask any differences in inflammatory response seen between the phages. However it has been shown previously in this study (section 2.5.3) that these particular cells do not respond very strongly to commercial endotoxin either. Further studies using different
cell lines and primary cells would need to be completed to get a full picture of inflammatory response in airway epithelia.

LdH release by the 16HBE cells in response to the application of bacteriophages was also studied. LdH is released by epithelial cells that have become “leaky” due to distress most often caused by pathogens. Again the levels of LdH release by cells exposed to the different phages were all equal indicating no one phage was worse than the others at causing a response. The levels also did not increase from the time zero point which was the time at which phages were first placed on the cells, effectively providing a baseline level. This means that there was very little LdH released at all by 16HBE cells in response to phage. This correlates with the low levels of cytokine production and it is likely that the phage do not distress the cells sufficiently to cause the release of LdH through leaky membranes.

Only one cell line was used in this work, meaning that it would have to be repeated using other cell lines before any firm conclusions could be drawn regarding the use of phages as therapeutic agents. However this work provides an initial assessment of the viability of this approach.

As already discussed the initial response of the epithelial cells to the phage suspension may be due to residual endotoxin present. Although there are no defined endotoxin limits for nebulised solutions in the British Pharmacopeia it can be assumed that if the endotoxin present in such a solution is <0.25 EU mL\(^{-1}\), the limit for solutions for injection, then it should be safe. The level of endotoxin in phage diluted to the level used in co-culture with lambda buffer was investigated. The endotoxin levels for all 6 phages were high, ranging from 3500 EU mL\(^{-1}\) to more than 4000 EU mL\(^{-1}\), but are effectively equal to one another. It should be stressed that these results provide an indication only of the endotoxin levels in phage preparations compared to that in the sterile diluents used in co-culture experiments. Endotoxin testing is vulnerable to contamination. Endotoxin testing laboratories in the pharmaceutical industry are subject to rigorous validation of all equipment to ensure that the results are accurate and not influenced by contamination. This was impractical in a research laboratory that had not been used for endotoxin testing before and which formed only a small fraction of the work that took place in it. Therefore it is possible that contamination may affect equipment such as the
honeycomb well plate, however the LRW control shows a very low (not significantly
different from 0 EU mL\(^{-1}\)) endotoxin level indicating that contamination of the
routine equipment was not a significant problem. The manufacturer's protocol was
altered to allow the use of the Bioscreen® automated absorbance reader which
appeared to work well as results with acceptable error levels were obtained, with
minimal contamination.

The endotoxin present was likely to have been carried over from the phage
propagation and purification methods. In these methods there were specific steps to
remove bacterial debris and whole bacteria by centrifugation and chloroform lysis
respectively. However there were no specific steps to remove endotoxin, such steps
must be included in future work to bring the endotoxin level in the phage
preparations closer to 0.25 EU mL\(^{-1}\). Conventional methods of endotoxin removal
such as heating were not suitable due to denaturation of phage proteins. There have
been other methods of endotoxin removal described including those specifically
designed for phage suspensions. One method employed sequential ultrafiltration
followed by chromatography which resulted in an endotoxin level of 0.4-7 EU mL\(^{-1}\)
[Boratynski et al. 2004]. Other methods include various types of chromatography,
gr gradient centrifugation, and extraction/adsorption using solvents and polymers
[Petsch and Anspach 2000]. Future work would involve investigating the most
appropriate endotoxin removal method for the bacteriophages used. Also important
is how endotoxin-free bacteriophage suspensions differ in the epithelial cell response
compared to bacteriophage suspensions containing residual endotoxin. For further
endotoxin work it would be useful to collaborate with a laboratory already equipped
to measure endotoxin levels.
4.4 Conclusions

This work forms an important part of the project; it completed the bacteriophage characterisation work and answered some important questions regarding the use of bacteriophages as potential therapeutic agents for airway epithelia infections.

Other than a brief spike soon after application, phage preparations did not cause a significantly increased inflammatory reaction in comparison with the negative control. Hence they can still be deemed appropriate as potential therapeutics, and warrant further investigation to this end.

The early increased levels of cytokine could be due to residual endotoxin in the phage preparations as a result of the propagation process; this was shown in the endotoxin assays. Future work should concentrate on removing this endotoxin from the phage preparations.
5 Development of a co-culture model with *Ps. aeruginosa* and human airway epithelial cells
5.1 Introduction

One of the major goals of this work was to establish a controlled *Pseudomonas aeruginosa*-mediated infection on human bronchial epithelial cells. This could eventually be developed into a model that more closely mimics conditions at the infected CF airway. A model such as this could be used to investigate the infection process of CF lung disease and also as a testing unit for potential therapeutics. This could partially negate the need for the use of, often inappropriate, animal models. The majority of animal models use mice which are obviously anatomically different to humans and the method of initiating infection usually involves inserting bacteria embedded in alginate beads, which again is an entirely different scenario to acquiring infection in CF lungs [Heeckeren et al. 1997; McMorran et al. 2001]. Mice do not suffer from CF naturally however genetically modified CF mice have been created which show some similarity to the human disease but with some differences particularly in terms of lung disease [Clarke et al. 1992; Colledge et al. 1992; Ratcliff et al. 1992; Snouwaert et al. 1992].

Previous *in vitro* studies that have placed *Ps. aeruginosa* on airway epithelial cells at ALI (air-liquid interface) for up to 16 hours have demonstrated that it severely affects the integrity of the epithelial barrier. *Ps. aeruginosa* infection disrupts tight junctions, triggers apoptosis and causes cells to break away from the insert membrane [Grassme et al. 2000; Garcia-Medina et al. 2005; Zulianello et al. 2006; Chugani and Greenberg 2007; Rejman et al. 2007]. This highlights the difficulty in creating an infection model of *Ps. aeruginosa in vitro*. The progression of the infection to destruction of the epithelial layer appears to be much faster *in vitro* than *in vivo*. The aim therefore of this set of experiments was to initiate and maintain a *Ps. aeruginosa* biofilm infection on airway epithelial cells. Ideally, this should be maintained for a sufficient length of time to allow the assessment of bacteriophages as therapeutic agents, in this case up to 24 hours. In this endeavour, due to the aggressive nature of *Ps. aeruginosa* in culture, it may be necessary to confer a disadvantage or restrictions on the bacteria such that it is unable to grow at its normally rapid rate.
There are several ways in which this could be approached:

- The bacterial culture could be starved of nutrients prior to the experiment in order to slow the growth rate and metabolism.
- The bacteria could be encouraged to form a biofilm by suspending the cells in an artificial glycocalyx such as commercial alginate suspensions.
- The bacteria could be pre-grown on a substrate such as glass or stainless steel to encourage natural biofilm development.

The cells could then be transferred to the surface of the HBE cells growing in the culture model. A methodical approach was taken towards this challenge by attempting various co-culture strategies such as the one mentioned above, with the first attempt being simply using planktonic bacterial cells suspended in liquid buffer.
5.2 Methods and results

5.2.1 Treatment of 16HBE cells with planktonic *Pseudomonas aeruginosa*

5.2.1.1 Method

16HBE cells were grown on inserts as detailed in section 2.4.2. 24 hours prior to the start of the experiment all medium was removed from the inserts and the apical and basolateral surfaces were washed with 500 µL, and 1 mL prewarmed (37°C) PBS respectively. 1 mL of growth medium without penicillin/streptomycin was placed underneath each insert thereby taking the inserts to ALI.

An overnight culture of *Ps. aeruginosa* PAO-GFP was washed by centrifuging the culture at 3,000 g for 10 minutes before resuspending the pellet in 10 mL PBS. This was repeated twice further. A 50 µL aliquot of the bacterial suspension was placed onto each insert; this volume when placed onto the insert would gradually spread over the surface. A set of 3 inserts was each exposed to 50 µL of sterile TSB which served as a negative control.

Visual observations were made at intervals of two hours to assess the suitability of this method for allowing controlled co-culture to proceed over the course of several hours. Suitability was assessed on whether the bacterial infection had overwhelmed the insert which would be evidenced by sections of epithelial membrane detaching from the insert or by the cell culture media becoming green due to *Ps. aeruginosa* pigment.

5.2.1.2 Results

Within 6 hours of the start of the experiment the *Ps. aeruginosa* growth had overwhelmed the apical surface of those inserts that it was placed on and within 8 hours the baso-lateral media had become green due to the pigments produced by *Ps.*
aeruginosa. It was clear that the 16HBE cells were no longer viable as an epithelial layer, as cells could be seen sloughing off the insert membrane, therefore a different approach to co-culture was considered.

5.2.2 Treatment of 16HBE cells with *Ps. aeruginosa* suspended in alginate

In order to allow sufficient time to investigate phage treatment of the *Ps. aeruginosa* infection it was necessary to slow the progress of the infectious process. This was attempted by suspending the bacterial cells in alginate as it was proposed that encasing the bacteria in a synthetic glycocalyx would prevent it from overwhelming the entire insert system. This could be due to physical restraint of the bacteria within a discrete pocket of alginate as opposed to a liquid buffer spread over the surface of the insert. Encasement in alginate may also have the effect of causing the bacteria to express the biofilm phenotype rather than the planktonic phenotype.

5.2.2.1 Method

16HBE cells were taken to ALI as described in section 5.2.1.1, 24 hours prior to the start of the experiment to allow the cells to become accustomed to the new culture conditions.

Sodium alginate (Fisher Scientific UK Ltd. Loughborough, Leicestershire, UK) was prepared at concentrations of 4%, 6%, 8%, and 10% w/v in reverse osmosis water. 100 µL of a *Ps. aeruginosa* suspension containing $2.5 \times 10^6$ cfu mL$^{-1}$ was added to 400 µL of each of the sodium alginate concentrations and mixed well at the same time trying to prevent the introduction of air bubbles. This resulted in a $5 \times 10^5$ cfu mL$^{-1}$ bacterial suspension in sodium alginate, this bacterial titre is relatively low compared to the numbers that can be reached in the CF lung, higher titres may result in the model becoming rapidly overwhelmed.

A 50 µL aliquot of each of the alginate/bacterial cell suspensions was placed on the apical surface of 3 inserts. The controls, also applied at 50 µL volumes, were alginate only (6%) and no treatment as a negative control.
Visual observations were made and samples were taken every two hours to test for LdH content (section 2.5.4) and bacterial contamination of the baso-lateral media. Sampling involved removing the entire volume of baso-lateral media and placing in a labelled micro-centrifuge tube before replacing with fresh pre-warmed (37°C) growth medium. Bacterial contamination was tested by spread plating 100 µL aliquots of the sample neat, and as a 1:10 dilution in PBS, this was performed immediately after the sample was taken. The remaining volume of the samples was stored at -80°C until they could be assayed for LdH concentration.

5.2.2.2 Results

The results of the LdH assay are shown in Figure 5.1. As in the previous experiment the *Ps. aeruginosa* infection overwhelmed the insert system by 8 hours. Bacterial contamination of the baso-lateral media was first detected at 6 hours post experiment start. The experiment was terminated after 16 hours as the 16HBE cells were likely to be non-viable by this time due to the extensive degradation of the epithelial cell layer. LdH release peaked for all alginate concentrations between 4-8 hours which coincided with the detection of bacterial contamination in the baso-lateral media. This method of co-culture still does not provide a sufficient length of time of controlled infection with which to conduct further experiments.
Figure 5.1: LdH release (expressed as Optical density) by 16HBE cells in response to *Ps. aeruginosa* suspended in alginate of varying concentrations. 4% alginate: •, 6% alginate: ■, 8% alginate: □, 10% alginate: ◇, alginate control: △, Negative control: ▼. Results represent the average OD (n=3) ± standard error.
5.2.3 Treatment of 16HBE cells with nutrient-starved *Pseudomonas aeruginosa* suspended in alginate.

As suspending *Ps. aeruginosa* in alginate was not sufficient to prevent overwhelming infection in a short space of time another method of slowing bacterial growth was required. Nutrient starvation has been shown to affect the growth of bacteria, with marine and soil isolates showing reduced metabolism and slow recovery [Nelson and Parkinson 1978; Amy, Pauling and Morita 1983]. Starvation studies on *Pseudomonas* species have also shown changes in protein expression and metabolism and biofilm associated changes such as dispersal and increased exopolysaccharide production [MacKelvie, Campbell and Gronlund 1968; Madhusudhan *et al.* 2003; Hunt *et al.* 2004; Schleheck *et al.* 2009].

5.2.3.1 The effect of starvation on *Pseudomonas aeruginosa* recovery

The ability of *Ps. aeruginosa* to recover after nutrient starvation for different time periods was investigated. This would provide an idea of how long *Ps. aeruginosa* would need to be starved of nutrients to adversely affect its growth, thereby providing an advantage in co-culture.

5.2.3.1.a. Method

A 250 ml overnight culture of *Ps. aeruginosa* in TSB was washed 3 times by centrifuging at 3,000 g for 10 minutes before resuspending in 250 mL PBS. The bacterial suspension was split between 14 centrifuge tubes finishing with each containing 10 mL. Six different starvation routines were followed, with letter codes identifying them. Code A denoted a 24 hour starvation period followed by recovery by resuspension in TSB, with B-G corresponding to an additional 24 hours of starvation for each letter up to 168 hours of starvation. Two tubes were used for each starvation routine.
Each bacterial/PBS suspension was incubated at 37°C with shaking (100 throws per min) for the allotted starvation time. At the end of the starvation period the two suspensions were centrifuged at 3,000 g for 10 minutes and the pellets were re-suspended, one in 10 mL fresh sterile TSB and one in 10 mL fresh sterile MEME-based growth medium, they were incubated at 37°C with shaking (100 throws per minute). Recovery in the two media was monitored by taking 100 µL samples every hour for 12 hours and diluting and spread plating them.

A further tube containing 10 mL bacterial cell/PBS suspension was sampled every 6 hours throughout the experiment. This enabled monitoring of the viable count during starvation.

A control experiment was set up to allow comparison between those cell suspensions that had been starved and a cell suspension that had not been nutrient starved. A further two tubes containing 10 mL of washed and resuspended overnight culture were centrifuged at 3,000 g for 10 minutes. The pellets were immediately resuspended, one in 10 mL TSB and one in 10 mL MEME-based growth medium. The resuspended cultures were incubated with shaking (100 throws per min) at 37°C for 12 hours. Viable count was assessed using the spread plate technique with samples taken every 3 hours.

5.2.3.1.b. Results

The results are shown in Figures 5.2 – 5.6.

Figure 5.2 shows the viable count of a bacterial/PBS suspension over the course of the experiment allowing the effect of nutrient starvation on bacterial viability to be observed.

After nutrient starvation commenced the viable count of the starved culture continued to rise until around 42 hours when a plateau was reached. The first six hours post starvation showed a steep rise in viable count which became less pronounced. After 100 hours of nutrient starvation there appeared to be a gradual
decrease in viable count with the exception of two peaks. The viable count at 168 hours was slightly less than that at 0 hours.

Figures 5.3 to 5.6 show the viable counts of cultures that had been nutrient starved for varying lengths of time and then placed into TSB or MEME-based growth medium to recover. The counts were monitored every hour for 12 hours after placing the cultures into recovery. The viable counts of 2 control cultures are also on each graph for comparison. These were overnight cultures that were not starved but were placed straight into TSB and MEME-based growth medium. The titre of the control cultures were very similar whether the culture had been placed into TSB or MEME-based growth medium. The starting titre was \( \sim 2 \times 10^9 \) cfu mL\(^{-1}\) and over the course of 12 hours increased to \( \sim 3 \times 10^{10} \) cfu mL\(^{-1}\).

After 24 hours of nutrient starvation (Figure 5.3.a) the cultures did not increase in viable count during the 12 hour recovery period. Viable counts at times 0 and 12 hours were almost identical. The titres for recovery in MEME-based growth medium however were significantly lower than for TSB, \( P < 0.0005 \) (one way ANOVA).
For the first 7 hours of recovery following 48 hours of nutrient starvation (Figure 5.3b) the viable count of the recovering cultures remained unchanged. However after this time the viable count for recovery in both media increased to approximately $2 \times 10^9$ cfu mL$^{-1}$ at 9 hours. The viable count of the culture in MEME-based growth medium then decreased again over the next 3 hours, whilst in TSB it remained higher and appeared to be continuing to increase slowly. However these differences were not statistically significant over the 12 hour measurement period $P = 0.5$ (one-way ANOVA).

The viable count of cultures recovering after 72 hours of nutrient starvation changed very little over the course of 12 hours (Figure 5.4a). There was also no significant difference in viable count between those cultures recovering in MEME-based growth medium and those recovering in TSB $P = 0.012$ (one-way ANOVA).

After 96 hours of starvation the viable count of recovery cultures increased for both media, reaching a peak between 6-7 hours (Figure 5.4b). The count then decreased slightly resulting in very similar start and end viable counts, it is unclear as to why this should be the case. The difference between counts from cultures recovering in the two different media was significant $P = 0.004$ (one way ANOVA).

The final three starvation periods of 120, 144, and 168 hours showed very similar responses in terms of culture recovery (Figures 5.5a, 5.5b, and 5.6 respectively). The viable counts of the cultures in both media changed very little over the 12 hour recovery period. There was also no significant difference in count values between the two recovery media for these three starvation periods with the exception of 144 hours. $P$ values were as follows: Starvation period 120 hours $P = 0.554$, 144 hours $P < 0.0005$, and 168 hours $P = 0.935$ respectively (one-way ANOVA). The counts throughout the recovery period after 168 hours of starvation were slightly lower than the other starvation periods. This could be due to a lower number of cells added to the recovery media, possibly due to cells ceasing to be viable after this length of starvation. The aim of this experiment was to ascertain the shortest starvation period required to prevent rapid regrowth of *Pseudomonas aeruginosa* in the culture media without adversely affecting their viability. From the results it is clear that a period of just 24 hours nutrient starvation was sufficient to alter recovery and this information was used to attempt further co-culture experiments.
Figure 5.3: Ability of *Ps. aeruginosa* to recover from nutrient starvation lasting over a period of 24 hours (graph a) and 48 hours (graph b) as shown by viable counts taken during a 12 hour recovery period.

Results represent average viable count (n=3) ± standard error.
Figure 5.4: Ability of *Ps. aeruginosa* to recover from nutrient starvation lasting over a period of 72 hours (graph a) and 96 hours (graph b) as shown by viable counts taken during a 12 hour recovery period.


Results represent average viable count (n=3) ± standard error.
Figure 5.5: Ability of *Ps. aeruginosa* to recover from nutrient starvation lasting over a period of 120 hours (graph a) and 144 hours (graph b) as shown by viable counts taken during a 12 hour recovery period. Recovery in TSB: ●, Recovery in MEME: ■, Non-starvation control in TSB: ▲, Non-starvation control in MEME: ×. Results represent average viable count (n=3) ± standard error.
Figure 5.6: Ability of *Ps. aeruginosa* to recover from nutrient starvation lasting over a period of 168 hours as shown by viable counts taken during a 12 hour recovery period. Recovery in TSB: ●, Recovery in MEME: ■, Non-starvation control in TSB: ▲, Non-starvation control in MEME: ×. Results represent average viable count (n=3) ± standard error.
5.2.3.2 Treatment of 16HBE cells with nutrient-starved *Pseudomonas aeruginosa* suspended in alginate

5.2.3.2.a. Method

The experimental procedure followed in this experiment was similar to that used in section 5.2.2.1. However nutrient-starved *Ps. aeruginosa* suspension was used instead of a fresh overnight culture.

The nutrient starved suspension was prepared in the following way. An overnight TSB culture of *Ps. aeruginosa* was washed by centrifuging the culture at 3,000 g for 10 minutes before resuspending in 10 mL PBS this was repeated twice more. The culture suspended in PBS was incubated for 24 hours at 37°C with shaking (100 throws per min). After the incubation period the suspension was centrifuged at 3,000 g for 10 minutes before being resuspended in 10 mL PBS. The suspension was diluted with PBS to achieve a viable count of $2.5 \times 10^6$ cfu mL$^{-1}$ this suspension was then ready to use in the experiment.

The bacterial suspension was added to solutions of sodium alginate of four different concentrations as detailed in section 5.2.5.1, with a final bacterial concentration in alginate of $2.5 \times 10^5$ cfu mL$^{-1}$. The alginate bacterial cell suspension was then used to treat the cells. The positive control was vanadyl sulphate ($20\mu$g mL$^{-1}$) and the negative control was 6% alginate. 50 µL of each alginate/bacterial cell suspension and control was added to each of three inserts.

Samples were taken every 2 hours for 24 hours by transferring the baso-lateral media into labelled micro-centrifuge tubes and replacing it with fresh pre-warmed (37°C) growth medium.

The concentrations of IL-6 and IL-8 were assayed from the samples using the protocols described in section 2.4.1. The levels of LdH present in the samples were also assessed (section 2.4.2).
5.2.3.2.b. Results

The inserts were observed throughout the experiment as in previous co-culture experiments and it was deemed that the *Ps. aeruginosa* was not overwhelming the insert systems as rapidly as it had before. This was based on the fact that at 8 hours there were no fragments of detached epithelial membrane and the cell culture media had not turned green due to *Ps. aeruginosa* pigment as had happened by this time point in the previous experiments (sections 5.2.1.2 and 5.2.2.2). However by 20 hours the infection was badly affecting the cells, as exhibited by 16HBE cells detaching from the insert membrane and green pigmentation appearing in the cell culture media hence the experiment was terminated.

The concentration of IL-6 (Figures 5.7 and 5.8) was highest in the samples from the positive control treated cells, indicating that the positive control had a strong adverse effect on the cells. This effect peaked at 6-8 hours after treatment and gradually declined throughout the remainder of the experiment. The negative control elicited a lesser response as demonstrated by the lower IL-6 concentrations. These values maintained a fairly constant level throughout the experiment.

At the 0 time point, the IL-6 concentrations for all conditions were effectively zero. This was expected as these samples were taken immediately after the inserts had been washed and fresh media had been added, and before the treatments were added.

The IL-6 concentrations of the samples taken from cells treated with alginate/bacterial cell suspensions peaked at 2-4 hours after treatment. The concentration of IL-6 at this time point ranged between 1300 pg mL\(^{-1}\) and 1700 pg mL\(^{-1}\). There was no statistical difference between the 4 different concentrations of alginate, \(P = 0.543\) (one way ANOVA).

After this initial peak the concentrations of IL-6 decreased for all four concentrations of alginate, until 10-12 hours after treatment at which point they reached zero. This may be due to the stressed cells dying and therefore being unable to produce anymore cytokine, hence the concentration of IL-6 from the negative control was higher at this point.
As expected the highest concentrations of IL-8 (Figure 5.9 and 5.10) were found in those samples from cells treated with the positive control. This peaked at 6-8 hours after treatment, and decreased gradually in a similar manner to IL-6 concentration. As with IL-6 the concentration of IL-8 in the negative control samples remained fairly steady throughout with a small peak at 6-8 hours post treatment and another at 16-18 hours. The concentration of IL-8 in samples from cells treated with alginate/bacterial cell suspension reached a peak at 4-6 hours after treatment, 2 hours later than IL-6. Moreover there was still no significant difference in IL-8 concentration between samples from cells that had been treated with different alginate concentrations P=0.467 (one way ANOVA).

The concentration of IL-8 from the cells treated with alginate/bacterial cell suspension gradually decreased from this point. By 16-18 hours after treatment there was no longer any IL-8 detected in the samples. This demonstrates the IL-8 response lasted for up to 6 hours longer than the IL-6 response. There was a slight rise in the concentration of IL-8 from negative control cells at 16-20 hours. One possible reason for this is that the cells were becoming stressed due to the ongoing experimental procedure.

LdH production by the cells is shown in Figures 5.11 and 5.12. OD values, which are directly proportional to LdH concentration, remained fairly constant at around 0.4 A.U. throughout the greatest proportion of the experiment. However between 6-10 hours after treatment there was a peak in LdH in samples from cells exposed to all treatments. This was most apparent for those cells treated with 4% and 6% alginate/bacterial cell suspension, with these samples reaching an OD value of 0.8 and 0.7 A.U. respectively which were significantly higher than the other treatments P = 0.002 (one-way ANOVA).

From 12 hours after treatment there was also a rise in LdH from those cells treated with the positive control. This was maintained for the remainder of the experiment reaching an OD value of 0.8 at 16-18 hours after treatment.
Figure 5.7: Concentration of IL-6 in samples of baso-lateral media taken from 16HBE cells treated with nutrient-starved *P. aeruginosa* embedded in alginate of varying concentrations (T=0 – T=8).

4% alginate: ■, 6% alginate: ■, 8% alginate: ■, 10% alginate: ■, Negative control: ■, Positive control: ■.

Results represent average IL-6 concentration (n=3) ± standard error.
Figure 5.8: Concentration of IL-6 in samples of baso-lateral media taken from 16HBE cells treated with nutrient-starved *Ps. aeruginosa* embedded in alginate of varying concentrations (T=10 – T=20).

4% alginate: ■, 6% alginate: ■, 8% alginate: ■, 10% alginate: ■, Negative control: ■, Positive control: ■.

Results represent average IL-6 concentration (n=3) ± standard error.
Figure 5.9: Concentration of IL-8 in samples of baso-lateral media taken from 16HBE cells treated with nutrient-starved *Ps. aeruginosa* embedded in alginate of varying concentrations (T=0 – T=8).

4% alginate: ■, 6% alginate: ▲, 8% alginate: ▼, 10% alginate: ▼, Negative control: ■, Positive control: ▲.

Results represent average IL-8 concentration (n=3) ± standard error.
Figure 5.10: Concentration of IL-8 in samples of baso-lateral media taken from 16HBE cells treated with nutrient-starved *Ps. aeruginosa* embedded in alginate of varying concentrations (T=10 – T=20).

4% alginate: ■, 6% alginate: ■, 8% alginate: ■, 10% alginate: ■, Negative control: ■, Positive control: ■.

Results represent average IL-8 concentration (n=3) ± standard error.
Figure 5.11: Levels of LdH (represented by optical density) in samples of baso-lateral media taken from 16HBE cells treated with nutrient-starved *Ps. aeruginosa* embedded in alginate of varying concentrations (T=0 – T=8).

4% alginate: ■, 6% alginate: ■, 8% alginate: ■, 10% alginate: ■, Negative control: ■, Positive control: ■.

Results represent average OD (n=3) ± standard error.
Figure 5.12: Levels of LDH (represented by optical density) in samples of baso-lateral media taken from 16HBE cells treated with nutrient-starved *P. aeruginosa* embedded in alginate of varying concentrations (T=10 – T=20).

4% alginate: ■, 6% alginate: ■, 8% alginate: ■, 10% alginate: ■, Negative control: ■, Positive control: ■.

Results represent average OD (n=3) ± standard error.
5.3 Discussion

Successfully initiating and maintaining an infection in vitro to mimic an in vivo situation comes with inherent difficulties. The in vitro systems developed to date do not yet have the full complement of host defences to help moderate the bacterial invasion. Moreover the necessary use of nutrient rich cell culture media can encourage the growth of bacteria further. These conditions can lead to the bacteria overwhelming the in vitro system and making further studies impossible [Zulianello et al. 2006], in contrast to the in vivo situation in which chronic infections often continue for many years [Costerton 2001; Lyczak, Cannon and Pier 2002; Chmiel and Davis 2003].

The aim of this work was to initiate a Pseudomonas aeruginosa infection on airway epithelial cells and maintain it for up to 24 hours. The majority of in vitro infection studies in the literature involve placing planktonic bacterial cells on epithelial cells for up to 6 hours. Part of the scope of this work was also to attempt to establish a biofilm on the cells, this being the natural mode of infection in vivo [Costerton et al. 1995; Costerton, Stewart and Greenberg 1999].

The first step in this approach was to apply planktonic bacterial cells suspended in PBS to the human bronchial epithelial cells. This, possibly due to the reasons mentioned above, was unsuccessful as the bacterial infection overwhelmed the insert system within 6 hours rendering the airway cells non-viable and impossible to study. The second step was to encase the bacterial cells in alginate as a restrictive measure. This would also place the Pseudomonas cells in an artificial biofilm, alginate being the substance naturally produced by Ps. aeruginosa as a glycocalyx. However this was also unsuccessful as again the bacterial infection overwhelmed the in vitro system. Alginate alone was obviously insufficient in preventing the bacteria from growing in a sustainable manner. In order to produce a more controlled infection the bacterial cells needed to be hindered further.

Nutrient starvation has been shown to adversely affect bacterial growth on recovery into nutrient rich media [Amy, Pauling and Morita 1983]. In this study recovery of Pseudomonas aeruginosa was found to be affected after just 24 hours of nutrient
starvation by incubation in sterile PBS. This effect was largely unchanged with a starvation period of up to 168 hours. On resuspension into nutrient rich TSB or MEME-based growth medium the starved cultures failed to exhibit a rise in bacterial cell numbers over 12 hours, indicating the starvation had altered their ability to reproduce over this time. In some cases there was some variability in the viable counts of the recovery cultures. Recently it has been shown that starvation affects the adhesiveness of *Ps. aeruginosa* cells causing differences in exopolysaccharide release and altering its tendency to aggregate [Myszka and Czaczyk 2009; Schleheck *et al.* 2009]. This could explain the unusual viable count variability at times.

During this experiment the initial count at the start of any recovery period was always slightly lower than would be expected compared to the count results from the starved culture at the time point immediately prior to initiation of recovery. This is likely to be due to the loss of a small number of cells during the centrifugation step. It was considered that the lack of increase in cell numbers after resuspension in the recovery media could be due to the large number of cells being resuspended and naturally limiting the scope for proliferation. However the control experiment showed an increase of just over 1 log\(_{10}\) when resuspended and incubated in the same manner. This suggests that the tendency for cells not to proliferate in recovery media is due to starvation effects, possibly including low ATP levels which will take time to recover to normal levels before cell proliferation takes place.

This treatment was then applied to the cells employed in the bacterial co-culture providing the possibility of controlling the bacterial infection once on the 16HBE cells. The final approach to co-culture was thus using *Ps. aeruginosa* that had been nutrient starved for 24 hours and embedded in alginate to produce an artificial biofilm. This approach was much more successful in that the bacterial infection did not overwhelm the *in vitro* system early on in the experiment as it had in the previous trials. However after 12 hours the bacterial cells were severely affecting the 16HBE cells which could be directly observed by sloughing of the cells. These timings are confirmed in the literature [Zulianello *et al.* 2006].

For all concentrations of alginate the application of the bacterial/alginate suspension led to a peak in IL-6 production by the epithelial cells at 2-4 hours and in IL-8 production at 4-6 hours. The cytokine concentrations then fell to zero at 10-12 hours
for IL-6 and 16-18 hours for IL-8. This followed the same pattern seen in the experiments in section 2.5.2 with IL-8 production peaking later than IL-6 production. The reduction in cytokine production from 10-12 hours onwards leading to a complete absence of either cytokines after 18 hours supported the observations of cells sloughing from the membrane after 12 hours. This indicates that the reduction in cytokine production was probably due to the epithelial cells becoming non-viable. This is further supported by the peak in LdH production prior to the drop in cytokine concentration indicating leaky disrupted cell membranes. This conclusion will need to be tested via a live/dead cell stain.

The vanadyl sulphate positive control caused a peak in both IL-6 and IL-8 production at 6-8 hours which then reduced to a plateau but not to zero. LdH production for the positive control increased and reached a plateau from 12 hours onwards. Evidently the positive control causes the cells to become stressed but doesn’t kill them within the time frame of this experiment. The difference in the time taken for the cytokine concentrations to reach peak values for the positive control compared to the bacteria/alginate suspension may indicate the activation of different inflammatory pathways.

Interestingly growth studies performed with *Ps. aeruginosa* in MEME-based growth medium (section 2.2.9.2) showed that it was not a very suitable growth medium with a markedly different growth curve than with TSB. However when *Ps. aeruginosa* was placed on 16HBE cells with MEME-based growth medium as the nutrient source, it grew very well, as evidenced by the uncontrolled infection on the inserts. It is unclear as to why this highly nutritious growth media may not be suitable for growth of *Ps. aeruginosa* hence it is difficult to explain why it is the reverse when 16HBE cells are present. The 16HBE cells may alter the growth medium in such a way as to make the nutrients more available or the *Ps. aeruginosa* cells may gain their nutrients from the epithelial cells themselves. The 16HBE cells do not produce mucin which could be a nutrient source but other substances may be produced by the cells which the bacterial cells can utilise. Alternatively *Ps. aeruginosa* well characterised as favouring the biofilm mode of growth may proliferate more successfully on the insert surface than in the planktonic mode of growth with which Bioscreen® growth curves are associated.
This work achieved a simulated infection for up to 12 hours which does fall short of the original aim of 24 hours. It may be possible to prolong the length of time with which an infection can be maintained by using 3D cell cultures. These use inflammatory cells such as phagocytes, or another option would be to use antibiotics. However this would fall outside of the scope of the main study which is to investigate the ability of bacteriophages to treat the infection not to the detriment of the epithelial cells. Both phagocytes and antibiotics could reduce the bacterial cell numbers applied therefore disguising the bactericidal effects of the bacteriophages. The 12 hour period provided by this approach was deemed to be sufficient to allow such investigations to take place.

In future work, it may be necessary to develop the infection model further by, for example, including other types of cells such as endothelial or immune system components. Further development would also include the use of primary cells and clinical isolates of *Ps. aeruginosa*. The PAO-GFP strain used in this work could also be visualised by confocal microscopy to assess the progression of infection. The infection model developed in this work will be applied to bacteriophage treatment models in the remainder of the project.
5.4 Conclusions

The establishment of a *Ps. aeruginosa* infection on airway epithelial cells *in vitro* and its maintenance for sufficient time as to perform experiments cannot be achieved without disadvantaging the bacterial cells. Nutrient starvation and suspension of the bacterial cells in alginate as a simulated biofilm controlled the bacterial growth on airway epithelial cells sufficiently to allow further experimentation.
6 Bacteriophage treatment model
6.1 Introduction

This final set of experiments draws together the different themes in this project. The previous sections have involved characterising the potential bacteriophage therapy candidates, using data from infection process experiments to assess the suitability of the phages. The phages have also been assessed for their potential to provoke an inflammatory response in airway epithelial cells. A method of establishing \emph{Ps. aeruginosa} infection on airway cells for long enough to perform further experiments has also been established. From these initial experiments airway epithelial cells, \emph{Ps. aeruginosa} cells and bacteriophages are brought together into one system and the effect on the airway cells can be considered.

The first step towards this objective was to treat a \emph{Ps. aeruginosa} culture with bacteriophages whilst not in contact with 16HBE cells. The culture lysate devoid of whole bacterial cells was then applied to the airway cells. This is a simple first step that can avoid the complications that arise from placing live bacteria onto cell culture systems. This experiment also provided the final set of results for choosing one of the six phages to be used in the next experiments. Results from chapters 3 and 5 were also be considered in making this choice.

The next step was bacteriophage treatment of a \emph{Ps. aeruginosa} infection \emph{in situ} in the \emph{in vitro} system. This involved establishing an infection on the epithelial cells and applying the phage to this at different times after infection. This assessed the limit at which phage treatment was useful after initial infection and how the cells reacted to the phage treatment. Ideally the phage should destroy the infection without an adverse effect on the cells themselves.

In parallel with these two experiments, two similar experiments that use antibiotics currently employed in the clinical treatment of CF airway infections instead of bacteriophages were performed for comparison. It is important to provide a comparison to bacteriophage treatment with the use of antibiotics that are already used in the clinical setting for this condition. These experiments assessed what difference, if any, there was in the reaction of cells treated with phage compared to those treated with antibiotic. If the phage experiment results compared favourably to
The antibiotic results then phage as a potential infection treatment can be considered further.

The antibiotics that were used were the two forms of colistin available; colistimethate sodium (CMS) and colistin sulphate (CSS). Colistin sulphate is cationic and stable and clinically used for topical and oral administration. Colistimethate sodium is a methanesulfonated colistin prodrug converted to reduce toxicity it is usually administered intra-venously and by inhalation. However it is anionic and unstable in aqueous solution and in vivo is easily converted to colistin and a range of methanesulfonated and hydroxymethyl derivatives [Freeman and Ian 1969; Gupta et al. 2009]. Colistin antibiotics interact with the lipopolysaccharide (LPS) in the bacterial cell membrane causing an increase in permeability resulting in cell death, whilst binding to and neutralising endotoxin. In the treatment of CF associated airway infection colistin is usually given in nebulised form at a dosage of 80 mg in 4 mL of saline [Gupta et al. 2009].

The application of phage as a treatment in this chapter, by a suspension applied directly to the airway epithelial cells can be considered to be equivalent to the nebulisation of phage i.e. phage suspensions deposited onto the airway surface in small droplets. Phage therapy of CF associated airway infections is envisaged to be performed in the same way that antibiotic therapy is often performed now. All phage therapy studies to date have used animal models with the phages usually being delivered orally, intravenously, or topically to wounds. In this way any inflammatory markers produced as a consequence of phage treatment are measured in the blood. In this study the inflammatory mediators released from the cells in direct contact with the phage and bacteria will be measured, resulting in a clearer idea of the situation at a cellular level.
6.2 Methods and results

6.2.1 Treatment of 16HBE cells with cell free bacteriophage lysate

To investigate the effects that lysed *Ps. aeruginosa* supernatant, produced as a result of treating infection with phage, would have on 16HBE cells the application of cell-free bacteriophage lysate to the epithelial cells was conducted.

6.2.1.1 Preparation of *Ps. aeruginosa* cell lysate

6.2.1.1.a. Method

A 1 mL aliquot of an overnight culture of *Ps. aeruginosa* was added to 100 mL sterile TSB in a conical flask. The culture was incubated at 37°C with shaking (100 throws per minute) for approximately 3 hours. After this time 1 mL of a suspension of bacteriophage containing $1 \times 10^{10}$ pfu mL$^{-1}$ was added to the flask, swirled to mix and incubated statically at 37°C for 15 minutes. The flask was then incubated at the same temperature but with shaking, as before, overnight. 10 mL of the lysed culture was transferred to a centrifuge tube and centrifuged at 3,000 g for 15 minutes to remove bacterial cell debris. The supernatant was transferred to a fresh centrifuge tube. This process was repeated twice more before the resultant supernatant, containing only bacterial products and bacteriophage, was transferred to a fresh sterile centrifuge tube for use in the co-culture experiment. Membrane filtration was not used in the preparation of the lysate as it would remove all large pieces of bacterial debris. The lysate preparation was repeated for each of the six phages, plus an uninfected control to which 1 mL of sterile lambda buffer was added in place of phage suspension.
6.2.1.2 Co-culture

6.2.1.2.a. Method
16HBE cells were grown on inserts as described previously (section 2.4.2). Prior to the start of the experiment the plate wells and the baso-lateral surfaces of the inserts were washed by removing the media and replacing with fresh sterile growth medium, this was performed twice. A 50 µL aliquot of each of the phage lysates was placed on the apical surface of the inserts, with each phage lysate being tested in triplicate. The positive and negative controls, which were also tested in triplicate, were vanadium sulphate (20 µg mL$^{-1}$), and sterile TSB respectively. The baso-lateral medium was removed and replaced, with the removed media being placed into micro-centrifuge tubes, which were stored at -80°C until analysis. This served as sample T = 0 (zero hour time point). Further samples were taken in this manner every 2 hours for 24 hours.

For practicality the experiment was split into two parts staggered over 12 hours to allow sampling for 12 hours on consecutive days rather than for 24 hours in one go. The first inserts were set up at time zero and sampled every 2 hours for 12 hours, at this point a second set of inserts were set up and incubated overnight for 12 hours. At this time the baso-lateral medium was removed and the inserts were washed with fresh pre-warmed medium 3 times. At 14 hours post set up sampling began from the second set of inserts as T14 and continued every 2 hours until T24 was reached.

All samples were analysed for their concentration of IL-6, IL-8, and LdH (section 2.5.1, and 2.5.4).

6.2.1.2.b. Results
The concentration of IL-6 (Figures 6.1 and 6.2) reached a peak for cells treated with phage lysate between 2-8 hours. Between 2 and 4 hours after treatment there were significant differences in the concentration of IL-6 between samples from cells treated with the 6 different phage lysates. F116 provoked the greatest response whereas U4 caused a lower concentration of IL6 to be produced, $P = 0.026$ (one-way ANOVA). There were significant differences between phage lysates again at 4-6
hours post-treatment with F116 highest and U4 lowest P = 0.001 (one-way ANOVA), and again at 6-8 hours, this time F116 and #13 were significantly lower than the rest P < 0.0005 (one-way ANOVA). After this initial peak from 8 hours post-treatment onwards there were no significant differences in IL-6 concentration between the phage lysate treatments P = 0.367 (one-way ANOVA). As would be expected, after 2 hours post-treatment, the positive control caused a significantly higher concentration of IL-6 than any other treatments P = 0.009 (one-way ANOVA). During the initial 2-8 hour peak in IL-6 concentrations the negative control, as expected, was significantly lower than the phage lysate treatments. However after the IL-6 concentration plateaued for the phage lysate treatments the negative control was not significantly different from these P < 0.0005 (one-way ANOVA).

IL-8 concentrations followed a similar pattern to IL-6. The peak IL-8 concentrations occurred at 6-8 hours post-treatment and at this time there were no significant differences between different phage lysates P = 0.720 (one-way ANOVA). However in the previous 2 hour sample periods the IL-8 concentrations increased towards the peak and there were significant differences between phage lysates. At both 2-4 hours and 4-6 hours post-treatment F116 caused the highest levels of IL-8 production, and U4 the lowest P = 0.002, and <0.0005 respectively. As with IL-6 production after the initial peak (from 8 hours post-treatment) there were no significant differences in IL-8 production between the phage lysates P = 0.341 (one-way ANOVA). Again as expected the positive control provoked a significantly higher response in the 16HBE cells than any other treatments P = <0.0005 (one-way ANOVA). The negative control results for IL-8 followed a similar pattern to the IL-6 results with the concentrations being significantly lower than that for the phage lysate treatments in the initial peak phase. During the plateau phase there was no significant difference between the negative control results and the phage lysate results P < 0.0005 (one-way ANOVA).

There were no real peaks in LdH production in the first 10 hours post-treatment indicating that cells were not overly damaged or stressed during this time. Throughout the experiment the uninfected control caused higher LdH results than the other treatments. This was more pronounced at the end of the experiment when the uninfected control results were significantly higher (P = <0.0005) than the other
treatments but not significantly different to the positive control results $P = 0.201$ (ANOVA). The results for 22-24 hours post-treatment were much lower than the previous results, going against the general trend, but it is unclear as to why this should be the case.

6.2.1.3 Choosing a phage for further work

Due to the fact that the culture systems were expensive and labour intensive only one phage will be used and investigated in detail for the subsequent phage treatment experiments in this project (section 6.2.3). Future experiments should investigate other phages but that is outside of the scope of this project. From the previous chapters and from this experiment phage U4 was chosen to be used in the next set of experiments. U4 has caused a lower level of cytokine production than other phages both in the form of purified phage and culture lysate. U4 also showed a high level of rapid bacterial lysis and little sign of resistant bacterial cells.
Figure 6.1: IL-6 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free bacteriophage lysate (T0-T10).


n = 3, error bars denote standard error.
Figure 6.3: IL-8 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free bacteriophage lysate (T0-T10). F116: ■, #13: ■, U1: ■, U2: ■, U3: ■, U4: ■, Uninfected control: ■, Negative control: ■, Positive control: ■. n = 3, error bars denote standard error.
Figure 6.4: IL-8 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free bacteriophage lysate (T12-T24).

n = 3, error bars denote standard error.
Figure 6.5: LDH release (expressed as optical density) from 16HBE cells exposed to cell free bacteriophage lysate (T0-T10).
n = 3, error bars denote standard error.
Figure 6.6: LdH release (expressed as optical density) from 16HBE cells exposed to cell free bacteriophage lysate (T12-T24).

n = 3, error bars denote standard error.
6.2.2 Treatment of 16HBE cells with colistin treated culture supernatant

In order to establish the advantages of using bacteriophage therapy over the use of antibiotics it is necessary to have a comparable experiment to that in section 6.2.1 where bacterial cell lysates have been produced using antibiotics in the place of bacteriophages. The antibiotic chosen was colistin as this is often used in severe \textit{Ps. aeruginosa} infections which are resistant to other antibiotics. Two forms of colistin were used in this experiment, colistimethate sodium (CMS) and colistin sulphate (CSS). First it was necessary to standardise the antibiotic experiment to the bacteriophage experiment by assessing the concentration of colistin required to produce the same level of bacterial reduction as the bacteriophages.

6.2.2.1 Assessing optimal colistin concentration

6.2.2.1.a. Method
An initial experiment was performed to assess the approximate concentration of colistin required to inhibit a \textit{Pseudomonas aeruginosa} culture. This was performed using a Bioscreen® automated absorbance reader. An overnight culture of \textit{Ps. aeruginosa} was diluted with sterile TSB until its optical density reached 0.5. The suspension was split between 16 test tubes. CMS and CSS (both: Sigma Aldrich UK Ltd, Gillingham, Dorset) were diluted in PBS and added to the cultures to achieve a tube containing each of the concentrations in Table 6.1 for each antibiotic. A 300 µL aliquot of each suspension was immediately added to each of 5 wells in a honeycomb well plate. This was repeated for the negative control which was sterile TSB. The Bioscreen was programmed to take \textit{OD}_{420} readings every 10 mins for 24 hours at 37°C.
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<table>
<thead>
<tr>
<th>Concentration (µg mL(^{-1}))</th>
<th>Molar concentration (µM L(^{-1}))</th>
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<th>CSS</th>
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*Table 6.1:* Equivalent molar concentrations for CMS and CSS.

6.2.2.1.b. Results

Figures 6.7 and 6.8 show the growth curves for *Ps. aeruginosa* cultures with various concentrations of CMS and CSS respectively.

It required lower concentrations of CSS to inhibit *Ps. aeruginosa* growth than CMS. At a concentration of 3.57 µM L\(^{-1}\) CMS there was still growth albeit with a longer lag phase than lower concentrations, however at a concentration of 7.14 µM L\(^{-1}\) and above there was no growth in 24 hours. Concentrations as low as 0.56 µM L\(^{-1}\) of CSS adversely affected growth with exponential phase entered at 800 minutes compared to less than 200 minutes for no CSS. At a concentration of 1.16 µM L\(^{-1}\) CSS and above there was no bacterial growth in 24 hours. These results gave a good indication of the concentration of antibiotic required for the second stage of this experiment, and are in agreement with published Figures [Li et al. 2001].

Both sets of growth curves showed considerable variation in the OD value during stationary phase of cultures with low concentrations of antibiotics. *Ps. aeruginosa*
cultures produce large quantities of exopolysaccharide which accumulates in broth cultures and can be seen as inclusions that are stringy in appearance.

6.2.2.1.c. **Method**

The second stage of the experiment involved calculating the exact concentration of both CMS and CSS required to achieve the same bacterial survival rate in the presence of bacteriophage U4 at a concentration of \(1 \times 10^8\) pfu mL\(^{-1}\) in 150 minutes.

1 mL of an overnight culture of *Ps. aeruginosa* was added to 100 mL of sterile TSB, this was repeated 13 more times giving a total of 14 flasks. The cultures were incubated with shaking (100 throws per minute) at 37 °C for approximately 3 hours. A 100 µL aliquot of CMS diluted to give final concentrations of 80, 68.5, 57, 45.7, 34.3, 22.9 and 0 µM L\(^{-1}\) was added to each of seven flasks and swirled to mix. In the same way aliquots of CSS to give final concentrations of 8.6, 7.1, 5.7, 4.3, 2.9, 1.4, and 0 µM L\(^{-1}\) were added to the remaining seven flasks. The dilutions were chosen based on the previous experiment but adjusted to make them higher as in this experiment the antibiotics were being added to exponential phase cultures rather than at inoculation. All flasks were incubated with shaking at 37°C for 150 minutes.

After this time 3x100 µL samples were taken from each flask and serially diluted 1:10 in PBS down to 10\(^{-6}\). The dilutions were spread plated on TSA in triplicate and incubated overnight at 37°C to ascertain the viable count for each antibiotic concentration.

6.2.2.1.d. **Results**

The viable counts against concentration of CMS and CSS are in Figure 6.9 a and b respectively. Both antibiotic/viable count relationships showed good correlation with \(R^2\) values of 0.9299 for CMS and 0.9525 for CSS. The concentration of each antibiotic required to produce the same reduction in viable count as bacteriophage U4 was calculated from the equation of the line. A final phage titre of \(1 \times 10^8\) pfu mL\(^{-1}\) resulted in approximately 100 cfu mL\(^{-1}\) viable count after a 150 minute infection (section 3.2.4.2). Placing this viable count into the equations for each antibiotic resulted in a concentration of 119.61 µM L\(^{-1}\) for CMS and 6.24 µM L\(^{-1}\) for
CSS. These concentrations were used in future experiments where comparisons are required between bacteriophage and colistin antibiotics.
Figure 6.7: Growth curves of *Pseudomonas aeruginosa* with varying concentrations of Colistimethate sodium.
Concentrations: 57 µM L⁻¹, 28.6 µM L⁻¹, 14.3 µM L⁻¹, 7.14 µM L⁻¹, 3.57 µM L⁻¹, 1.79 µM L⁻¹, 0.89 µM L⁻¹, 0 µM L⁻¹, Sterile TSB (negative control): ×. n = 5, error bars denote standard error.
**Figure 6.8:** Growth curves of *Pseudomonas aeruginosa* with varying concentrations of Colistin sulphate. Concentrations: 35.7 µM L⁻¹, 17.8 µM L⁻¹, 8.93 µM L⁻¹, 4.46 µM L⁻¹, 2.23 µM L⁻¹, 1.16 µM L⁻¹, 0.56 µM L⁻¹; 0 µM L⁻¹; Sterile TSB (negative control): ×. n = 5, error bars denote standard error.
Figure 6.9: Bacterial viable count from a \textit{Ps. aeruginosa} culture containing varying concentrations of colistimethate sodium (a) and colistin sulphate (b). Results represent average viable counts from 3 samples ± standard error.
6.2.2.2 Preparation of colistin-treated *Ps. aeruginosa* supernatant

In the same way that bacterial-cell-free phage lysate was used in section 6.2.1, bacterial cell free colistin treated culture supernatant was used in this experiment.

A 1 mL aliquot of an overnight culture of *Ps. aeruginosa* was added to each of four flasks containing 100 mL of sterile TSB. The flasks were incubated at 37°C with shaking (100 throws per minute) for approximately 3 hours. To one flask 1 mL of a 12 mM L\(^{-1}\) solution of CMS, diluted in PBS, was added giving a final concentration of approximately 120 µM L\(^{-1}\) and swirled to mix. To the second flask 1 mL of a 0.6 mM L\(^{-1}\) solution of CSS was added giving a final concentration of approximately 6.25 µM L\(^{-1}\) and swirled to mix. To the third flask 1 mL of a 1 x10\(^{10}\) pfu mL\(^{-1}\) suspension of phage U4 was added and swirled to mix. To the final flask 1 mL of sterile PBS was added, this flask provided the untreated control.

The flasks were incubated at 37°C with shaking (100 throws per minute) for 150 minutes. From each flask 10 mL was transferred to a sterile centrifuge tube and centrifuged at 3000 G for 15 minutes. The supernatant was transferred to a new sterile centrifuge tube and the process was repeated twice more. The final supernatant was transferred to a fresh sterile tube ready to be used immediately for the co-culture experiment. Three 100 µL samples were removed and spread plated without dilution to confirm that viable bacterial cells capable of growth were not present in the supernatant.

6.2.2.3 Treatment of 16HBE cells with antibiotic treated cell free *Ps. aeruginosa* culture supernatant liquid

6.2.2.3.a. Method

16HBE cells were grown on inserts as described previously. Prior to the start of the experiment the plate wells and the baso-lateral surfaces of the inserts were washed by removing the media and replacing with fresh sterile growth medium, this was performed twice. A 50 µl aliquot of each of the supernatant liquids was placed on the apical surface of the inserts and each supernatant was tested in triplicate. The positive and negative controls, which were also tested in triplicate, were vanadium sulphate (20 µg mL\(^{-1}\)), and sterile TSB respectively. Two further controls consisted of sterile TSB containing 120 µM L\(^{-1}\) CMS in one and 6.25 µM L\(^{-1}\) CSS in the
second. The baso-lateral media was removed and replaced with the removed media being placed into micro-centrifuge tubes, which served as sample $T = 0$. Further samples were taken in this manner every 2 hours for 24 hours.

All samples were analysed for their concentration of IL-6, IL-8, and LdH (method in section 2.4.1).

6.2.2.3.b. Results

In general the concentration of IL-6 (Figure 6.10 and 6.11) from samples taken from cells in this experiment was lower than that in the previous experiment (section 6.2.1.2.b). However, some variation between separate experiments could be expected and of more interest are the relative responses from the different challenges.

The concentration of IL-6 in baso-lateral media taken from 16HBE cells exposed to colistin-treated culture supernatant and various controls reached a peak at between 2-6 hours, with the exception of the positive control which occurred between 6-8 hours. At these peaks there was no significant difference between cells treated with either of the colistin supernatant liquids or U4 lysate $P = 0.057$ and $0.055$ respectively (one-way ANOVA). By 12-14 hours post-treatment all results, apart from the positive control and the cells exposed to the untreated control supernatant had reached a plateau. The uninfected control became significantly lower than the other results $P = <0.0005$ (one-way ANOVA) reaching zero by 18-20 hours. The positive control was significantly higher than the other results from the positive control peak at 6-8 hour post-treatment onwards $P = <0.0005$ (General linear model using Tukey's test).

As with IL-6 the IL-8 concentration levels as shown in Figures 6.12 and 6.13 were lower than with the previous experiment. The IL-8 concentrations of baso-lateral media samples taken from the 16HBE cells increased between 2 and 6 hours post-treatment. At 2-4 hours post treatment there was no significant difference in the IL-8 concentration of media from cells exposed to different treatments $P = 0.07$. At 4-6 hours post-treatment CMS treated culture supernatant caused a significantly higher concentration of IL-8 in the media samples than any other treatment apart from the positive control $P = 0.045$ (one-way ANOVA). At the peak (6-8 hours post-
treatment) there was no significant difference in IL-8 concentration for samples from
cells treated with either CMS, CSS, or U4 treated culture supernatants $P = 0.291$
(one-way ANOVA). However the untreated culture supernatant treated cells
produced more IL-8 than any other treatments apart from the positive control $P =
0.011$. As with IL-6 the IL-8 concentrations for this treatment reduced drastically
from 12-14 hours indicating that the cells were either no longer being stimulated by
this treatment or were incapable of producing cytokines. From this point the positive
control caused the highest concentration of IL-8 in samples compared to other
treatments and this continued throughout the experiment $P <=0.0005$ (general linear
model using Tukey’s test).

In general the levels of LdH produced (expressed as optical density) were fairly
uniform, rising slightly towards the end of the sampling period (Figures 6.14 and
6.15). Between 2-4 and 4-6 hours post-treatment the level of LdH produced by cells
exposed to untreated culture supernatant was higher than for those exposed to either
CMS, CSS, or U4 treated culture supernatant $P = 0.037$, and 0.001 respectively. Later
in the experiment, between 16-22 hours post treatment the results for untreated
culture supernatant were higher than other treatments ($P = <0.0005$) but not
significantly different from the positive control ($P = 0.274$). This increase in LdH
production coincided with the severe reduction in cytokine production by the 16HBE
cells. As with the previous experiment there was a noticeable reduction in LdH
levels for all treatments at 22-24 hours post-treatment.
Figure 6.10: IL-6 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free colistin lysate $T = 0$-10 hours. Colistimethate sodium lysate: ■, Colistin sulphate: ■, Bacteriophage U4 lysate: ■, Untreated *Ps. aeruginosa* supernatant: ■, *colistimethate* control: ■, Colistin sulphate: ■, Sterile TSB control: ■, Positive control: ■. n = 3, error bars denote standard error.
Figure 6.11: IL-6 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free colistin lysate T = 10-24 hours. Colistimethate sodium lysate: , Colistin sulphate: , Bacteriophage U4 lysate: , Untreated *P. aeruginosa* supernatant: , *P. aeruginosa* colistimethate control: , Colistin sulphate: , Sterile TSB control: , Positive control: . n = 3, error bars denote standard error.
Figure 6.12: IL-8 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free colistin lysate T = 0-10 hours. Colistimethate sodium lysate: ■, Colistin sulphate: □, Bacteriophage U4 lysate: ☐, Untreated *Ps. aeruginosa* supernatant: ◇, colistimethate control: ■, Colistin sulphate: □, Sterile TSB control: ◇, Positive control: ◇. n = 3, error bars denote standard error.
Figure 6.13: IL-8 concentration of baso-lateral media samples taken from 16HBE cells exposed to cell free colistin lysate T = 10-24 hours. Colistimethate sodium lysate: ■, Colistin sulphate: ■, Bacteriophage U4 lysate: ■, Untreated *Ps. aeruginosa* supernatant: ■, colistimethate control: ■, Colistin sulphate: ■, Sterile TSB control: ■, Positive control: ■. n = 3, error bars denote standard error.
Figure 6.14: LdH release expressed as optical density from 16HBE cells exposed to cell free colistin lysate T = 0-10 hours.

Figure 6.15: LdH release expressed as optical density from 16HBE cells exposed to cell free colistin lysate T = 10-24 hours.
6.2.3 Bacteriophage treatment of a co-culture infection of *Ps. aeruginosa*

This experiment involved creating an *in vitro* phage treatment model. Starved *Ps. aeruginosa* cells suspended in alginate were placed on 16HBE cells and bacteriophage U4 was used to treat the infection at time increments after the initial inoculation.

6.2.3.1 Method

16HBE cells were grown on inserts as described in section 2.4.2. Prior to the start of the experiment the plate wells and the baso-lateral surfaces of the inserts were washed by removing the media and replacing with fresh sterile growth medium, this was performed twice.

To each of 24 inserts 50 µL of 4% alginate containing $1 \times 10^5$ cfu mL$^{-1}$ *Ps. aeruginosa* was added. To a further 3 inserts 50 µL of a $5 \times 10^5$ pfu mL$^{-1}$ suspension of bacteriophage U4 was added. Further inserts were also treated with 50 µL aliquots of lambda buffer (x 3) and sterile 4% alginate (x 3). 50 µL of 20mg mL$^{-1}$ vanadyl sulphate as a positive control was added to the final three inserts.

All baso-lateral media were removed from the inserts and placed into labelled microcentrifuge tubes which formed the time zero samples. 50 µL of the $5 \times 10^5$ pfu mL$^{-1}$ phage U4 suspension was immediately added to 3 of the 24 inserts containing *Ps. aeruginosa* in alginate. All inserts were incubated at 37°C with 5% CO$_2$. After 2 hours a further set of baso-lateral samples were taken, and 50 µL of U4 suspension was added to the next three inserts containing bacteria in alginate. Further bacteriophage treatments were added to consecutive triplicate inserts containing bacteria in alginate for up to 12 hours after the initial bacterial infection. One set of inserts remained untreated serving as a control. Baso-lateral samples were taken every two hours up to 24 hours after the initial bacterial infection. All samples were placed in labelled microcentrifuge tubes and immediately stored at -80°C.
6.2.3.2 Results

The collective results for this experiment for all three measured parameters (IL-6, IL-8 and LdH) are shown in Figures 6.16-6.21. These results will be discussed here briefly, however to fully understand the difference between phage treatment times after infection the results are presented in further graphs which start 2 hours prior to the phage being added for each set of inserts (Figure 6.22).

Figures 6.16 and 6.17 show all the results for the concentration of IL-6 in samples of baso-lateral media taken from 16HBE cells infected with *Ps. aeruginosa* and treated with phage U4 at different times after infection. The samples from cells that were treated earlier with phage (treatment at 0-6 hours post-infection) reached a peak between 4-8 hours post-infection and then reduced to a plateau at 10 hours onwards. In samples from cells that had been treated with phage 8-12 hours post-infection, and the untreated control, concentrations of IL-6 reached a peak at 4-6 hours post infection and by 8-10 hours had become almost zero, significantly lower than the earlier phage treatment results *P* = <0.0005 (one-way ANOVA). As expected from 4-6 hours after the experiment started the IL-6 concentration of media from cells treated with the positive control was much higher than for any other treatments *P* =<0.0005 (general linear model using Tukey's test).

IL-8 concentrations (Figures 6.18 and 6.19) followed a similar pattern to IL-6. Samples from cells that had been treated with phage up to 6 hours post infection reached an IL-8 concentration peak at 6-8 hours, at this point they were at a similar concentration to the alginate and lambda buffer results *P* = 0.033 (one-way ANOVA). However samples from cells that were treated with phage from 8 hours post infection reached a concentration peak at 4-6 hours then decreased. By 8-10 hours post infection values were effectively zero compared to the earlier treatment times *P* = <0.0005 (one-way ANOVA). Again as expected the positive control results are significantly higher than any other treatments *P* =<0.0005 (general linear model using Tukey’s test).

LdH release (Figures 6.20 and 6.21) from the cells treated with phage 8-12 hours post-infection and the untreated control reached a peak at 6-8 hours post infection. At this point these results were significantly higher than any other results including
the positive control $P = 0.0005$ (general linear model using tukey's test). At 8-10 hours post infection the LdH release from cells that had been treated with phage 0-6 hours post infection was no different from that of cells treated only with alginate or lambda buffer, $P = 0.299$. The $T=0$ phage treatment regime (i.e. phages applied immediately after bacterial infection) remained at a low plateau for the remainder of the experiment. The 2 hour post infection phage treatment and the 4 hour post infection phage treatment peaked for LdH from 18-20 hours remaining high for the remainder of the experiment. The 6 hour post infection phage treatment shows high LdH release throughout the second half of the experiment reaching a peak at 18-20 hours. The 8 and 10 hours post infection phage treatment are also high throughout the second half of the experiment with peaks at 14-16 hours and 12-14 hours respectively. The 12 hour post infection phage treatment regime exhibited a low LdH release throughout the 12-24 hour period.
Figure 6.16: IL-6 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with Ps. aeruginosa and treated with bacteriophage U4 at different times after infection $T = 0$-10 hours.
Phage treatment time post-infection: 0 hours:    , 2 hours:    , 4 hours:    , 6 hours:    , 8 hours:    , 10 hours:    , 12 hours:    , Untreated:    , U4 control:    , lambda buffer control:    , Alginate control:    , Positive control:    . $n = 3$, error bars denote standard error.
Figure 6.17: IL-6 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with bacteriophage U4 at different times after infection T = 10-24 hours.

Phage treatment time post-infection: 0 hours: ■, 2 hours: ■, 4 hours: ■, 6 hours: ■, 8 hours: ■, 10 hours: ■, 12 hours: ■, Untreated: ■, U4 control: ■, lambda buffer control: ■, Alginate control: ■, Positive control: ■. n = 3, error bars denote standard error.
**Figure 6.18:** IL-8 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with bacteriophage U4 at different times after infection T = 0-10 hours.

Phage treatment time post-infection: 0 hours: , 2 hours: , 4 hours: , 6 hours: , 8 hours: , 10 hours: , 12 hours: , Untreated: , U4 control: , lambda buffer control: , Alginate control: , Positive control: , n = 3, error bars denote standard error.
Figure 6.19: IL-8 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with bacteriophage U4 at different times after infection $T = 10-24$ hours.

Phage treatment time post-infection: 0 hours, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, Untreated, U4 control, lambda buffer control, Alginate control, Positive control. $n = 3$, error bars denote standard error.
Figure 6.20: LdH release expressed as optical density from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with bacteriophage U4 at different times after infection $T = 0-10$ hours.

Phage treatment time post-infection: 0 hours: ■, 2 hours: ■, 4 hours: ■, 6 hours: ■, 8 hours: ■, 10 hours: ■, 12 hours: ■, Untreated: ■, U4 control: ■, lambda buffer control: ■, Alginate control: ■, Positive control: ■. $n = 3$, error bars denote standard error.
Figure 6.21: LdH release expressed as optical density from 16HBE cells co-cultured with *P. aeruginosa* and treated with bacteriophage U4 at different times after infection $T = 10-24$ hours.

Phage treatment time post-infection: 0 hours: ■, 2 hours: ■, 4 hours: ■, 6 hours: ■, 8 hours: ■, 10 hours: ■, 12 hours: ■, Untreated: ■, U4 control: ■, lambda buffer control: ■, Alginate control: ■, Positive control: ■. $n = 3$, error bars denote standard error.
Figure 6.22 shows results for each phage treatment schedule from 2 hours prior to the addition of phage to 12 hours after treatment. The results are displayed as line graphs for clarity and to appreciate the trends more fully. The y-axis crosses the point on the x-axis at which the phage treatment is added. Each line represents a different time period between initial *Ps. aeruginosa* infection and addition of phage treatment.

As was suggested in the previous experiments it was clear from Figure 6.22 that the results were divided into two groups. In general the early phage treatment (0 hour – 6 hours) regimes caused both IL-6 and IL-8 levels to be higher than the late phage treatment regimes, *P* =<0.0005.

Samples from cells that were treated with phage U4 immediately following infection (0 hour) started with a low IL-6 and IL-8 concentration as this was the experiment start and corresponded to the baseline interleukin concentration. The concentration of both cytokines for this treatment regime reached a peak at 4-6 hours post-treatment reaching concentrations of approximately 900 pg mL\(^{-1}\) IL-6, and just over 1000 pg mL\(^{-1}\) IL-8. The concentrations decreased and reached a plateau at around 500 pg mL\(^{-1}\) for IL-6 and 700 pg mL\(^{-1}\) for IL-8.

Samples from cells that were treated with U4 2 hours after the initial infection started with a higher IL-6 and IL-8 concentration than the baseline. The IL-6 concentration of these samples remained fairly steady rising slightly between 0-4 hours post treatment before experiencing a peak to around 1000 pg mL\(^{-1}\) at 6-8 hours post-treatment. After this point it decreased to a plateau of just below 600 pg mL\(^{-1}\) at 8-10 hours post treatment. The IL-8 concentration of these samples steadily rose to a peak of 1400 pg mL\(^{-1}\) at 6-8 hours post treatment.

There was a large difference between starting IL-6 and IL-8 concentrations in samples from cells that were treated with U4 4 hours post infection. IL-6 started at approximately 1000 pg mL\(^{-1}\) and IL-8 at 700 pg mL\(^{-1}\). Both cytokine concentrations reached a peak at 2-4 hours post treatment (approximately 1100 pg mL\(^{-1}\) IL-6 and 1400 pg mL\(^{-1}\) IL-8) and decreased to a plateau 6-8 hours after treatment.

With the exception of the IL-6 concentration for the 4 hour treatment results the starting concentration for the 6 hour treatment was the highest of any others. For
both cytokines the concentrations reached a peak at 0-2 hours post treatment reaching more than 1000 pg mL\(^{-1}\) for IL-6 and over 1400 pg mL\(^{-1}\) for IL-8. The IL-6 concentration then slowly decreased and reached a low plateau of approximately 400 pg mL\(^{-1}\) from 8-10 hours post-treatment. The IL-8 concentration dropped and reached a plateau of approximately 700 pg mL\(^{-1}\) from 2-4 hours post treatment with a small rise to 900 pg mL\(^{-1}\) at 6-8 hours post treatment.

The remaining 3 treatment regimes (8, 10, and 12 hours post infection) all followed very similar patterns in both cytokines. The starting concentrations were all very low compared to the early treatment regimes (with the exception of 0 hour). They then all dropped further being approximately zero for the majority of the experiment. Small rises were seen in IL-6 concentration between 4-6 hours post-treatment for the 10 hour treatment regime. Small rises were also seen in IL-8 concentration at 4-8 hours post-treatment for the 10 hour treatment regime and 6-8 hours post-treatment for the 8 hour treatment regime.

In very general terms LdH production appeared to be a reversal of cytokine production. The early treatment regimes appeared to experience low release of LdH throughout the experiment whereas the later treatment regimes experienced higher release of LdH. This was not true of all the results however. The 6 hour treatment regime showed LdH production similar to that of the 8 and 10 hour treatment regimes. By contrast the 12 hour treatment regime showed LdH results similar to those of the 0, 2 and 4 hour regimes.
Figure 6.22: IL-6 (a), IL-8 (b), and LdH (c) release by 16HBE cells infected with *Ps. aeruginosa* and treated with bacteriophage U4 at different times after infection. Treatment times (post-infection): 0 hours: ●, 2 hours: ●, 4 hours: ●, 6 hours: ●, 8 hours: ●, 10 hours: ●, 12 hours: ●. n = 3, error bars denote standard error.
6.2.4  **Colistin treatment of a co-culture infection of *Ps. aeruginosa***

This experiment was identical to experiment 6.2.3 but using colistimethate sodium (CMS) as a treatment instead of bacteriophage. This was to provide a comparison between a treatment already in use for *Ps. aeruginosa* airway infections and bacteriophage therapy. CMS only, was used in this experiment as opposed to both CMS and CSS as CMS is indicated for nebulisation in the treatment of CF associated airway infection whereas CSS is not.

**6.2.4.1 Method**

16HBE cells were grown on inserts as described in section 2.4.2. Prior to the start of the experiment the plate wells and the baso-lateral surfaces of the inserts were washed by removing the media and replacing with fresh sterile growth medium, this was performed twice.

To each of 24 inserts 50 µL of 4% alginate containing $1 \times 10^8$ cfu mL$^{-1}$ *Ps. aeruginosa* was added. To a further 3 inserts 50 µL of a 120 µM L$^{-1}$ solution of CMS was added (diluted in PBS). This is lower than the normal clinical concentration of colistin for nebulisation (20 mg mL$^{-1}$) as this Figure was chosen to represent the equivalent, in terms of killing, of the phage titre used. 50 µL aliquots of PBS and sterile 4% alginate were added to a further 3 inserts for each. 50 µL of 20 µg mL$^{-1}$ vanadyl sulphate as a positive control was added to the final three inserts.

All baso-lateral media were removed from the inserts and placed into labelled microcentrifuge tubes which formed the time zero samples. 50 µL of the 120 µM L$^{-1}$ CMS solution was immediately added to 3 of the 24 inserts containing *Ps. aeruginosa* in alginate. All inserts were incubated at 37°C with 5% CO$_2$. After 2 hours a further set of baso-lateral samples were taken, and 50 µL of CMS solution was added to the next three inserts containing bacteria in alginate. Further antibiotic treatments were added to consecutive triplicate inserts containing bacteria in alginate for up to 12 hours after the initial bacterial infection. One set of inserts remained as an untreated (without antibiotic) control. Baso-lateral samples were also taken every two hours up to 24 hours after the initial bacterial infection.
6.2.4.2 Results

As with the previous experiment the collective results are shown in Figures 6.23 to 6.28, however the results for the different treatment regimes are shown as line graphs in Figure 6.29 in order to explain the differences between the regimes better.

In general all cytokine concentrations in this experiment were slightly higher than those in the previous experiment reflecting the variation that occurs between different experiments.

Between 2-6 hours after the experiment start there was a peak in IL-6 concentration (Figure 6.23) in samples from cells exposed to CMS only (control) and the positive control, with no significant difference between them P = 0.053. As with the phage treatment experiment the different colistin treatment regimes appeared to be split, however in this experiment there were three groups. The early treatments (0 hour, 2 hour, and 4 hour post-infection) reached a peak IL-6 concentration at 8-10 hours post infection and then decreased to a plateau from 14-16 hours onwards (Figure 6.24). IL-6 concentrations for the 6 and 8 hour treatment regimes were lower in the plateau (P= <0.0005) and very variable with large errors prior to this. The late treatment regimes and the untreated infection caused very low concentrations of IL-6 production (effectively 0 pg mL\(^{-1}\)) from 10-12 hours post infection onwards. The differences between the treatment regimes will be explained more fully in Figure 6.28. After the initial CMS control peak the highest IL-6 concentration was from cells exposed to the positive control (P = <0.0005) as would be expected.

The concentrations of IL-8 (Figures 6.25 and 6.26) in the samples followed a similar pattern to that of IL-6. The treatment regimes were again split into three groups. The early treatments (0 hour, 2 hour, and 4 hour post-infection) exhibited a peak in IL-8 concentration between 8 and 12 hours post infection and then decreased to a plateau from 16 hours onwards. The 6 and 8 hour post-infection treatments initially exhibited lower concentrations of IL-8. However from 14 hours onward the 8 hour treatment was the same as the earlier treatments and the 6 hour treatment was lower in terms of IL-8 concentration. From 10 hours onwards the later treatment regimes (10 and 12 hours post-infection) and the untreated control were all effectively zero compared to the other treatments (P = <0.0005). As would be expected the positive
control caused a significantly higher IL-8 concentration than any other treatments after 8 hours (P = <0.0005).

LdH release (Figures 6.27 and 6.28) from the cells followed a very similar pattern to that of the phage treatment experiment, with a large peak in LdH production from cells subjected to late treatment regimes (8 hours, 10 hours, and 12 hours post infection) and the untreated control at 6-8 hours. The early treatment regimes did not exhibit a peak at this point (P = <0.0005). The LdH release for the various colistin treatment regimes during the second half of the experiment showed a similar pattern to that of the phage treatment regimes. However the LdH release for the 12 hours post infection CMS treatment remains high through the latter time points.
Figure 6.23: IL-6 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with colistimethate sodium (CMS) at different times after infection *T = 0-10 hours*.

CMS treatment time post-infection: 0 hours: , 2 hours: , 4 hours: , 6 hours: , 8 hours: , 10 hours: , 12 hours: , Untreated: , CMS control: , Alginate control: , negative control: , Positive control: . *n* = 3, error bars denote standard error.
Figure 6.24: IL-6 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with colistimethate sodium at different times after infection T = 10-24 hours.

CMS treatment time post-infection: 0 hours: ■, 2 hours: ■, 4 hours: ■, 6 hours: ■, 8 hours: ■, 10 hours: ■, 12 hours: ■, Untreated: ■, CMS control: ▲, Alginate control: ■, negative control: ■, Positive control: ■, n = 3, error bars denote standard error.
Figure 6.25: IL-8 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with colistimethate sodium at different times after infection T = 0-10 hours.

CMS treatment time post-infection: 0 hours: ■, 2 hours: ■, 4 hours: ■, 6 hours: ■, 8 hours: ■, 10 hours: ■, 12 hours: ■, Untreated: ■.

**Figure 6.26**: IL-8 concentration of baso-lateral media samples taken from 16HBE cells co-cultured with *P. aeruginosa* and treated with colistimethate sodium at different times after infection $T = 10-24$ hours.

CMS treatment time post-infection: 0 hours: , 2 hours: , 4 hours: , 6 hours: , 8 hours: , 10 hours: , 12 hours: , Untreated: , CMS control: , Alginate control: , negative control: , Positive control: . $n = 3$, error bars denote standard error.
Figure 6.27: LdH release (expressed as optical density) from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with colistimethate sodium at different times after infection $T = 10-24$ hours.

CMS treatment time post-infection: 0 hours, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, Untreated, Untreated, CMS control, Alginate control, negative control, Positive control. $n = 3$, error bars denote standard error.
Figure 6.28: LdH release (expressed as optical density) from 16HBE cells co-cultured with *Ps. aeruginosa* and treated with colistimethate sodium at different times after infection \( T = 10-24 \) hours.

CMS treatment time post-infection: 0 hours: ■, 2 hours: ■, 4 hours: ■, 6 hours: ■, 8 hours: ■, 10 hours: ■, 12 hours: ■, Untreated: ■
CMS control: ■, Alginate control: ■, negative control: ■, Positive control: ■. \( n = 3 \), error bars denote standard error.
The results for the different treatment regimes are shown as line graphs in Figure 6.29. The results are from 2 hours prior to colistin treatment to 12 hours post treatment. The y-axis crosses the x-axis at the point at which the CMS treatment is added. Each line represents a different time period from initial *Ps. aeruginosa* infection to CMS treatment.

The concentrations of both IL-6 and IL-8 in samples from cells treated immediately after infection (0 hours) started low, as this was the baseline cytokine production for the cells. The IL-6 concentration then peaked at over 1000 pg mL\(^{-1}\) at 2-4 hours post-treatment and formed a plateau at this level for the remainder of the 12 hours. The IL-8 concentration also peaked at over 1000 pg mL\(^{-1}\) but 2 hours later at 4-6 hours post treatment, it then slowly decreased to approximately 600 pg mL\(^{-1}\).

The concentration of IL-6 in samples taken from cells that were treated 2 hours post-infection started high at over 1000 pg mL\(^{-1}\) and increased to a peak of approximately 1500 pg mL\(^{-1}\) at 8-10 hours post-treatment. The concentration of IL-8 started at over 500 pg mL\(^{-1}\) and increased to a peak of 1000 pg mL\(^{-1}\) at 4-6 hours post infection. It then decreased towards the end of the 12 hours.

The concentrations of both IL-6 and IL-8 followed similar patterns for cells treated with colistin at 4 hours post-infection. Both started at 1000 pg mL\(^{-1}\) and the IL-6 concentration reached a peak of approximately 1750 pg mL\(^{-1}\) at 4-6 hours post-treatment. The IL-8 concentration reached a peak of 1600 pg mL\(^{-1}\) 2 hours later. Both then decreased in concentration and at the end of the experiment they were at similar values to the start.

Samples from cells treated with CMS at 6 and 8 hours post-infection contained very similar concentrations of both IL-6 and IL-8 throughout the sampling period. The IL-6 concentration for both dropped dramatically during the 2 hours post-treatment, the concentrations then reached a peak at similar levels to the start at 4-6 hours post-treatment. Both then decreased throughout the remainder of the experiment reaching approximately 200 pg mL\(^{-1}\) at the end of the 12 hours. The IL-8 concentrations followed the same pattern with very similar values with the exception of the last sample which contained a higher concentration of IL-8 than IL-6.
The results for the 10 hour and 12 hour post infection treatment regimes were very similar to the phage treatment experiment. Both cytokine concentrations started very low, at a similar level to the baseline despite having had an untreated bacterial infection for up to 12 hours. Both cytokine concentrations then dropped to approximately 0 pg mL\(^{-1}\) and remained at that level for the remainder of the experiment.

The LdH levels for the 0, 2, 4 and 12 hour treatment regimes remained fairly low and uniform throughout this period. In samples from cells treated 6 and 10 hours post-infec-tion the LdH levels increased until 4-6 hours post-treatment when they reached a plateau. The LdH levels for the 8 hour treatment regime started significantly higher than for any other regimes (\(P = <0.0005\)) then dropped dramatically between 0 and 4 hours post-treatment. The levels then rose to a plateau at close to the same level as at the start from 4-6 hours post treatment onwards.
Figure 6.29: IL-6 (a), IL-8 (b), and LdH (c) release by 16HBE cells infected with *Ps. aeruginosa* and treated with CMS at different times after infection. Treatment times (post-infection): 0 hours: ●, 2 hours: ○, 4 hours: ●, 6 hours: ●, 8 hours: ●, 10 hours: ●, 12 hours: ●. n = 3, error bars denote standard error.
6.2.5  Assessing the status of 16HBE cells

When considering the cytokine and LdH results in the previous two experiments it is necessary to establish whether the epithelial cells were still viable under certain experimental conditions.

6.2.5.1 Method

16HBE cells were grown on inserts as described in section 2.4.2. 24 hours prior to the experiment they were taken to ALI (air-liquid interface) as usual. Immediately prior to the experiment the baso-lateral medium was removed and replaced with fresh sterile medium. Nine different conditions were set up with each one tested in triplicate. These were as follows:

1. *Ps. aeruginosa* (PA) at a titre of $1 \times 10^8$ cfu mL$^{-1}$, nutrient starved and suspended in 4% w/v alginate.
2. *Ps. aeruginosa* as (1) but with Phage U4 at a titre of $1 \times 10^8$ pfu mL$^{-1}$.
3. *Ps. aeruginosa* as (1) but with CMS at a concentration of 120 µM L$^{-1}$.
4. *Ps. aeruginosa* as (1) but with CSS at a concentration of 6.25 µM L$^{-1}$.
5. Phage U4 only, at a titre of $1 \times 10^8$ pfu mL$^{-1}$.
6. CMS only, at a concentration of 120 µM L$^{-1}$.
7. CSS only, at a concentration of 6.25 µM L$^{-1}$.
8. Negative control for which nothing was added to the 16HBE cells on inserts.
9. Positive control which was vanadyl sulphate at a concentration of 20 µg mL$^{-1}$.

Each treatment was added to the inserts at a volume of 50 µL. After the treatments were added the inserts were incubated at 37°C at 5% CO$_2$ for 16 hours. This period of time was chosen as this is the point at which 16HBE cells that undergo no phage/antibiotic treatment or late treatment cease to produce cytokines, and therefore may no longer be viable. Therefore it was important to see whether the cells are alive or dead at this stage.

At the end of the incubation time all inserts were apically washed by adding 200 µL PBS and aspirating it off a total of three times. The Cellstain Double Staining Kit
(Fluka: Sigma-Aldrich, St Louis, MO, USA) was used according to the manufacturer's instructions. The staining solution was prepared by adding 10 µL of solution A (Calcein-AM) and 5 µL of solution B (Propidium Iodide) to 5 mL of PBS. 100 µL of this solution was added apically to each insert and incubated at 37ºC for 15 minutes. After this time the inserts were visualised using a laser scanning confocal microscope (Leica) using an excitation wavelength of 490 nm and the 20x objective. Cells fluorescing green are viable and those that are red are non-viable.

Photographs were taken of each insert and these were digitally processed using ImageJ software (Rasband, 2009) to calculate the percentage of live cells for each field of view (800 µm x 800 µm). Averages were taken for each triplicate set of inserts.

6.2.5.2 Results

Figure 6.30 shows the average percentage of live cells within the microscope field of view at the end of the incubation period for each treatment. The first point to note is that after 16 hours of infection with *Ps. aeruginosa* there were no 16HBE cells left on the inserts. All cells had died and lifted off the inserts and were probably washed away during the wash step prior to staining.

There was no significant difference in the percentage of live cells for each of the dual treatment conditions (PA + U4, PA + CMS and PA + CSS), (P = 0.952). There was a significant difference (P = 0.012) in the percentage of live cells between the phage-only control (U4) and the CMS-only control, possibly reflecting the toxicity of colistin (Freeman 1969, Gupta *et. al.* 2009). The percentage of live cells resulting from CMS treatment, and U4 treatment of *Ps. aeruginosa* infection were not significantly different from the percentage of live cells remaining in the negative control (P = 0.908, and 0.920 respectively). A 16 hour incubation with vanadyl sulphate, which had been used as a positive control for the cytokine assays, resulted in less than 50% of live cells remaining.

Confocal images can be found in appendix III.
Figure 6.30: Average percentage of live 16HBE cells within the microscope field of view after exposure to various treatments. PA: Untreated *P. aeruginosa*. CMS: CMS only. CSS: CSS only. PA + CMS: *P. aeruginosa* treated with CMS. PA + CSS: *P. aeruginosa* treated with CSS. PA + U4: *P. aeruginosa* treated with phage U4. U4: Phage U4 only. Negative control: No treatment. V$_{2}$SO$_{4}$: Vanadyl Sulphate (positive control). n = 3, error bars denote standard error.
6.3 Discussion

The first experiment in this chapter investigated the ability of bacterial-cell free phage lysates to provoke the production of cytokines by 16HBE cells. All 6 phage were used to infect cultures of *Ps. aeruginosa* which were allowed to lyse and were then centrifuged to remove any remaining whole bacterial cells. The cultures were not syringe filtered as this may have removed cell debris from the lysate. The idea of this experiment was to test the effect that the products of phage mediated bacterial lysis, such as bacterial cell debris and contents, would have on the cells.

F116 lysate caused the highest concentrations of both IL-6 and IL-8 in the samples taken from the cells in the first half of the experiment, with U4 causing the lowest. This indicated that the F116 lysate caused a more pronounced inflammatory response than the U4 lysate. If phages were applied directly to the airways to treat bacterial infection then the airways would be directly exposed to lysate as the phages infected and killed the bacteria. Therefore it is very important that a phage was chosen whose lysate provoked the smallest response. After this initial peak in the first 10 hours after lysate application the IL-6 and IL-8 concentrations of samples from cells treated with all phage lysates decreased to a plateau. These levels were not significantly different from one another or the negative controls indicating that after the initial reaction the cells no longer reacted strongly to the phage lysates. This was a promising outcome with respect to the potential use of phage as a therapy.

LdH levels in this and many of the other experiments were somewhat unreliable with patterns being difficult to distinguish and large standard errors. It could be that the assay was subject to interference, possibly from bacterial LdH in the lysates. There was a very low level of LdH in the final sample period, this was very different from the level in the previous sample period and the trend was similar for samples from all treatments. Therefore it seems likely that this was not a genuine finding and that this was an error with the LdH assay or with the sample storage.

The cells treated with supernatant liquid from bacterial cultures that had not been infected by phage produced more LdH than any others. It is not clear why this should be the case, however although the supernatant liquids were spread plated it is
possible that there were a very low number of bacterial cells remaining in the supernatant. The supernatants were only subjected to centrifugation, not syringe filtering to ensure large pieces of bacterial debris remained in the supernatant therefore it is possible for whole bacterial cells to remain. If these were to divide and increase in number then they could have the effect of increasing LdH production.

The positive control vanadyl sulphate caused cells to release high concentrations of both IL-6 and IL-8, this was also true of LdH production however the effect was not as pronounced in LdH as it was in IL-6 and 8. This could mean that although vanadyl sulphate provoked an inflammatory response, it didn't cause the cells to become less viable or “leaky”. This was confirmed by the live cell experiment. At the end of 16 hours of exposure to vanadyl sulphate a large proportion of 16HBE cells were viable.

To establish how the airway cells reacted to phage compared to a conventional antibiotic treatment, the experiment was repeated using colistimethate sodium and colistin sulphate instead of bacteriophage.

First the concentrations of both antibiotics that were to be used in the experiment needed to be calculated. To be able to directly compare the phage experiment with the colistin experiment the concentration of CMS and CSS that produced the same bacterial kill rate as the phage over the same length of time was established.

The first step was to investigate the approximate concentration of CMS and CSS that inhibited growth of *Ps. aeruginosa*. The Figure obtained from this step was increased for the second step as the antibiotics were to be added to an early exponential culture as opposed to the newly inoculated culture that was used in the first step. The final concentrations were 120 µM L⁻¹ for CMS and 6.25 µM L⁻¹ for CSS, both of which are lower than clinical doses for nebulisation, which are 20 mg mL⁻¹ (11.43 mM L⁻¹) for CMS (the actual dosage achieved *in vivo* is likely to be lower) and CSS is not indicated for nebulisation due to its toxicity [Katz, Ho and Coates 2001; Gupta *et al.* 2009].

These concentrations were used to produce culture supernatant liquids of *Ps. aeruginosa* that had been treated with antibiotics in the same way as the bacteriophage lysates in the previous experiments.
When this antibiotic-treated culture supernatant was applied to the airway epithelial cells there were the same peaks in IL-6 and IL-8 concentrations in the first half of the experiment as were seen in the phage lysate study. Importantly there was no difference in IL-6 release by cells that had been exposed to phage U4 lysate or CMS or CSS supernatant. However at its peak the IL-8 concentration from cells exposed to CMS treated supernatant was higher than any other treatments. This was unexpected as CMS is less toxic than CSS, however CMS does undergo conversion to more toxic derivatives in aqueous solution and it may be these derivatives which are provoking an epithelial cell response [Freeman and Ian 1969; Gupta et al. 2009]. This means that the response of airway cells to phage lysate was lower than that to CMS treated culture supernatant. As with the phage lysate experiment the concentration of IL-6 and 8 produced by cells exposed to antibiotic-treated culture supernatant maintained a plateau during the second half of the experiment. This indicates that as with the phage lysate experiment the cells underwent an initial response which then dampened after several hours.

The untreated culture supernatant again caused a high level of LdH release by the 16HBE cells. Moreover the increase in LdH corresponded to a drop in cytokine concentration. This was likely to be due to residual bacterial cells in the supernatant. A sudden high release of LdH could be due to cells becoming leaky and non-viable as the bacteria disrupted the tight junctions, these cells would no longer be capable of the production of cytokines.

A phage treatment model was further developed by infecting airway epithelial cells with *Ps. aeruginosa* and treating the infection with bacteriophage at different times after the infection started. It became clear from these experiments that there was a threshold time post-infection when phage treatment was still effective in preventing airway cell damage from the bacterial cells. The early phage treatments which corresponded to 6 hours post-infection or less caused an increase in IL-6 and 8 production before settling into a plateau. However cells that were treated with phage after 8 hours post-infection produced effectively no detectable IL-6 or 8. The late treatment cells in general produced a higher level of LdH than the early treatment cells. This suggests that the late treatment cells had already been damaged by the bacterial infection, leading to the elevated levels of LdH. These non-viable cells were no longer capable of producing cytokines. The exception to this pattern was...
the 12 hour treatment which from 2 hours prior to treatment onwards resulted in very low levels of IL-6 and 8 but also low levels of LdH production. There was a peak in LdH production for this treatment at 6-8 hours hence the majority of the cells may have been damaged at this time leaving little LdH left.

As with the cell-free lysate experiments, this experiment was repeated using colistimethate sodium as a treatment instead of phage for a comparison between the two treatments. The results for this experiment were similar to the phage experiment. The cells that were treated in the first three time points (0-4 hours post infection) displayed an increase in IL-6 and IL-8 production however it took longer to decrease down to a plateau. The response to colistin treatment at 6 hours post-infection was similar to that at 8 hours with cytokine levels remaining lower than with the early treatments. This was different from the phage experiment in which the 6 hour treatment cells responded in a similar way to the early treatment cells, and the 8 hour treatment cells showed similar results to the late treatment cells. The 10 and 12 hour treatments resulted in effectively no cytokine production as before.

This suggests that at early treatments the airway cells react to the infection/treatment for a longer period of time for CMS than with U4. Although the concentration of CMS used in this experiment is “sub-clinical” it was chosen based on its ability to kill the same number of bacterial cells as the phage titre used. Hence this is unlikely to be a reason for the differences seen between CMS and U4. This could mean that U4 destroys the infection more quickly allowing the cells to recover more quickly, or that the CMS itself causes a greater cytokine reaction than U4. This is supported by the results from the cells treated with CMS only which caused a large peak in IL-6 and IL-8 production between 2 and 6 hours after application. If this also occurred in cells that had been infected with Ps. aeruginosa and treated with CMS then that could explain the prolonged increase in interleukin concentration. The U4 control in the previous experiment didn’t cause the same peak in IL-6 and IL-8 concentration. CMS is considered to be a less toxic derivative of colistin designed to be used more safely for nebulisation or intra-venous administration, however it quickly converts to more toxic forms in aqueous solutions. The peaks seen with CMS treatment therefore could be a result of these conversions [Freeman and Ian 1969; Gupta et al. 2009].
The lower cytokine levels from cells treated at 6 and 8 hours suggest that some of these cells had already been damaged and could no longer produce IL-6 and IL-8. After 6 or 8 hours of bacterial infection the cytokine levels were high however shortly after treatment they dropped low indicating that the infection wasn’t resolved in time to allow all the cells to recover. However the cytokine results were higher in this experiment for the 8 hour treatment than with phage indicating that some cells had recovered due to CMS treatment. In the cell viability assay there were a slightly larger number of live cells for the CMS treatment than the phage treatment but this was not significant. This didn’t support the idea that CMS works more slowly than the phage, which was hypothesised in response to cells treated with CMS taking longer to recover from the infection than those treated with phage.

The very low levels of IL-6 and IL-8 production by the 10 and 12 hour treatment cells from 2 hours prior to treatment onwards could be explained by the fact that the treatment was too late and the bacterial infection had already damaged the cells. This was supported in the LdH production for the 10 hour treatment cells which had the highest LdH production 2 hours prior to treatment. This suggests that 8 hours post infection is the optimal time for release of LdH. Hence the LdH production was low for the 12 hour treatment cells from 2 hours prior to treatment onward which was probably due to the cells releasing LdH before this time point.

The hypothesis that the low cytokine levels and high LdH levels were due to the cells becoming damaged and detached from the insert and therefore unable to produce cytokine was substantiated by the live/dead cell experiment. After 16 hours of incubation with *Ps. aeruginosa* only there were no cells left on any of the inserts tested, indicating that all had become detached.

There was a large amount of variability in IL-6 and IL-8 production between the sets of experiments. This was probably due to natural variation between the initial vials of cells or in the incubation, although every care was taken to ensure the cells were treated identically for each experiment. The experiments were all self-contained with positive and negative controls for comparison so this variation should not have adversely affect the conclusions drawn from the results. However more investigation into this anomaly should be conducted to ensure there are no causes within the experimenter’s control for this
The differences in cytokine production between the times of treatment after infection were due to the bacterial infection damaging the cells after around 8 hours. This period of viability will need to be lengthened for longer term studies into phage treatment. The method of bacterial co-culture will need to be refined further to allow a more slowly developing bacterial infection to take place. Strategies for this could be to reduce the initial bacterial titre, to suspend the bacteria in a higher concentration of alginate or possibly to grow a bacterial biofilm separately from the airway cells and then transfer it to the cell culture insert when established.

Another possible limitation with this work is that in the treatment models the phage or antibiotic treatment was added to nutrient starved bacterial cells. As has already been discussed, bacterial cells that are slow growing due to lack of nutrients may not respond to treatment in the same manner as exponential phase cells such as those used in preparation of the cell free lysates. However this may not be too far removed from the in-vivo situation in which a chronic biofilm will consist of populations of cells with different growth rates.

Once the bacterial co-culture method is improved, further work would need to be employed to investigate the phage treatment model. Different concentrations of phage should be tested to establish how the airway cells react to high phage titres and also the larger number of bacteria lysed at one time. This could be compared as before to different concentrations of CMS. Different airway epithelial cells should also be tested including primary cells and those from CF patients. Different cytokines could be monitored to get a more complete picture of the inflammatory response. Finally immune system cells could be co-cultured with the epithelial cells to establish what effect the infection and subsequent treatment has on these and also what affect these cells have on the infection itself. The use of phagocytic cells may help to prolong the bacterial infection without causing excessive damage to the cells.
6.4 Conclusions

Phage treatment of *Ps. aeruginosa* infection was successful *in vitro* with no lasting inflammatory reaction, however with this model the phage treatment should be added within 6 hours of infection.

Phage treatment compares favourably to an already established antibiotic treatment, CMS with regards to cytokine production by the cells. However this will need to be investigated further before bacteriophages can be seriously considered as a treatment for CF-related airway infections.
7 Discussion
The aim of this study was to investigate whether bacteriophages could be considered as appropriate therapeutic agents in the treatment of *Ps. aeruginosa* mediated airway infections in CF patients. Several stages were involved in order to do this. The first was to attempt to identify and characterise the bacteriophages that were to become potential therapies, and to assess them for their ability to provoke an inflammatory response in human airway epithelial cells. Next it was investigated if any of the by-products of phage lysis of bacterial cells provoked an inflammatory response and how this compared to the by-products of antibiotic treatment. A model was then developed of *Ps. aeruginosa* infection on airway epithelial cells *in vitro* and used to test the ability of phage to control the infection and allow the epithelial cells to recover. This was also compared to the antibiotic to ascertain whether phage could be considered as an equal, or arguably better treatment than currently used antibiotics.
7.1 Isolation and characterisation of phages (Chapter 3)

Bacteriophages for this study were isolated from samples in the University of Brighton’s culture collection. Some of these; F116, #13 and #2 had already been partially purified and stored as phage stock suspensions. The remainder U1-U4 were isolated from a *Ps. aeruginosa* specific bacteriophage preparation obtained from a clinical source in the Ukraine. All bacteriophages were checked for purity and with the exception of #2 were assumed to be pure after several rounds of plaque assay. Three commonly used methods of characterisation were employed to assess the properties of the phages. It was hoped that this would lead to identification of those phages that were unknown, and to ensure that there were no duplicate phages amongst the samples. The second aim was achieved and it was thought that there were 6 different phages in the cohort. However it proved more difficult to fully identify the phages from these characterisation methods only.

Infection process data provided details of the various parameters of the phages lytic lifestyles. Knowledge of the phage infection process is vital for estimating the therapeutic potential of the phage, however it must be noted that the situation *in vivo* will be much more complex [Skurnik and Strauch 2006; Uchiyama et al. 2009].

The TEM images allowed for identification of the family to which each phage belonged, however due to the lack of clear results from the genome characterisation further elucidation of the phage identities was not possible. The TEM images did however cast doubt as to the actual identity of the phage that was believed to be F116. The tail in this study appearing much longer than that found in previous work [Slayter 1964; Miller, Pemberton and Richards 1974], hence it cannot be said with any certainty that the phage designated F116 in the University of Brighton culture collection was in fact F116. In future work the identity of F116 could be confirmed or rejected by the use of polymerase chain reaction (PCR) for a known F116 gene.

Further investigation should be carried out to allow formal identification of the phages within the University of Brighton’s culture collection, such as experiments to establish host range and further physical and genome characterisation. In order to be used as therapeutic agents specific phages need to be well characterised and their
bacterial surface receptor known in order to assess the possibility of resistance developing [Skurnik and Strauch 2006]. TEM pictures of the phages adsorbing to their hosts would be helpful in this, as would screening a collection of different Ps. aeruginosa strains for susceptibility to each phage. Different methods for phage DNA extraction and purification should be sought before more detailed genome characterisation such as sequencing takes place. Once a satisfactory method has been found it is of importance to establish whether any of the phage possess virulence genes which could be transferred to other bacteria. In order to do this the phage genome should be sequenced and annotated using gene databases, this would identify any putative or known virulence genes.

It would be advantageous to increase the number of Ps. aeruginosa specific phages present in the culture collection by isolating phages from various environmental samples. Indeed phages that use Ps. aeruginosa as their host are still being isolated and characterised [Ceyssens et al. 2006; Holland, Sanz and Perham 2006; Kwan et al. 2006; Elke et al. 2009; Knezevic et al. 2009; Pieter-Jan et al. 2009; Pieter-Jan et al. 2009; Elke et al. 2009; Knezevic and Petrovic 2008]. Colorimetric techniques that use crystal violet to stain biofilms allow for rapid assessment of the most virulent phages for Ps. aeruginosa biofilm eradication [Knezevic and Petrovic 2008]. Phages that exhibit either a lytic or lysogenic lifestyle were recovered from the sputa of CF patients at levels of on average $10^4$ pfu ml$^{-1}$ [Tejedor, Foulds and Zasloff 1982]. These lower concentrations of phages have been found to aid the development of a biofilm by releasing nutrients and increasing species diversity by the establishment of a phage/bacterial equilibrium [GaidelytÄ—, Vaara and Bamford 2007; Nechaev and Severinov 2008; Letarov and Kulikov 2009; Rice et al. 2009]. Phages isolated from sputa however could be useful as therapeutic agents as they would be specific to the strains of Ps. aeruginosa that the CF sufferer was infected with and at higher concentrations may serve to eradicate the bacteria.
7.2 Phage co-culture (Chapter 4)

There are no in vitro studies published that consider the effect of applying phages in isolation without any bacterial infection.

In this study it was shown that on first application to the epithelial cells the phages did appear to provoke an inflammatory response but this quickly subsided and may have been due to endotoxin in the phage preparations (section 4.2.1.2). For the remainder of the experiment the inflammatory response of the airway epithelial cells to phages was not different to their response to the negative control and significantly less than the positive control.

The endotoxin present in the phage preparations was measured using the LAL chromogenic method (section 4.2.1.3). This was to provide an indication only of the approximate levels of endotoxin as the lab and equipment used was not specifically designed for endotoxin measurement, but was tested via controls. The results indicated that there was significantly more endotoxin in the phage preparations than the negative controls (section 4.2.1.4). There are no definitive endotoxin guidelines in the British Pharmacopoeia for solutions for nebulisation. However they could be assumed to be safe if the endotoxin level fell below the limit required for solutions for injection which is 0.25 EU mL\(^{-1}\). The phage preparations had endotoxin levels of up to 4000 EU mL\(^{-1}\).

These results suggested that provided the endotoxin within the phage preparations could be reduced to a satisfactory level then phages do appear to be an appropriate candidate for investigation as novel antibacterial therapies.

Other trials on human subjects have shown that phage do not cause any strong inflammatory reactions and in fact may help to reduce inappropriately vigorous inflammatory reactions to bacterial infections [Miedzybrodzki et al. 2009]. It is also thought that phage therapy would be safe and beneficial for immune-compromised patients [Borysowski and Górski 2008; Zimecki et al. 2009].

In summary in order for phages to be considered as therapeutic agents they must be free of bacterial components such as endotoxin. They should also be tested in trials on animals followed by humans using a variety of methods of administration to
ensure that no adverse reactions take place and their safety is established [Skurnik and Strauch 2006; Donlan 2009]. This *in vitro* work is the first step towards achieving that end.
7.3 Bacterial co-culture (Chapter 5)

The first step in investigating the usefulness of phages in the treatment of \textit{Ps. aeruginosa} infections of airway epithelial in CF was to create an \textit{in vitro} model mimicking the situation \textit{in vivo}. This could then be used to test the effect of phage treatment on the epithelial cells by way of measuring inflammatory markers released by the cells. The co-culture of bacteria with human cells is not without significant complications as was experienced in this work. \textit{Ps. aeruginosa} in particular multiplies vigorously in optimum conditions; early growth curve work demonstrated slower growth kinetics for this organism in cell culture media compared to bacteria-specific media. Despite this the application of planktonic \textit{Ps. aeruginosa} cells to human bronchial epithelial cells on cell culture inserts resulted in overwhelming bacterial growth, and rapid destruction of the epithelial layer, inappropriate for our requirements.

The ultimate aim for this model was to develop a bacterial biofilm on the epithelial cells as this is the mode of growth in CF \textit{in vivo}. Clearly the first approach (section 5.2.1), placing planktonic bacterial cells suspended in PBS, didn’t allow for enough time to observe a biofilm before the epithelial cells were destroyed. The next approach (section 5.2.2) was therefore to apply an artificial biofilm by suspending planktonic bacterial cells in commercial alginate. It is well-know that chronic \textit{Ps. aeruginosa} infection in CF involves a mucoid form of the bacteria which produces large quantities of alginate [Doggett 1969; Deretic \textit{et al.} 1994]. It was hoped that this would also contain the \textit{Ps. aeruginosa} cells allowing them to be present for a longer duration before overwhelming the model. Unfortunately this approach was as unsuccessful as the first with rapid destruction of the epithelial layer and overwhelming bacterial growth.

The next approach was to impart further growth impairment on the \textit{Ps. aeruginosa} cells by starving them of nutrients for 24 hours prior to deposition onto the epithelial cell. The length of the starvation period was investigated beforehand (section 5.2.3.1). It has been shown that \textit{Ps. aeruginosa} produced more exopolysaccharide under starvation conditions and this resulted in better adhesion and hence biofilm formation [Myszka and Czaczyk 2009]. This tactic, along with suspending the
bacterial cells in alginate, proved to be more successful with a longer period of time during which the bacterial cells were on the epithelial cells before entering the phase of rapid growth (section 5.2.3.2). This length of time was sufficient to begin investigating the effects of phage treatment of the infection.

As anticipated the epithelial cells reacted rapidly to the application of bacterial cells with maximal inflammatory mediator concentrations seen between 2 and 6 hours after infection. Between 10 and 12 hours after infection the cells appeared to stop responding as interleukin concentrations in the cell culture media dropped to zero. This was most probably due to the epithelial cells becoming non-viable due to the damage caused by the infection, which was later corroborated by the live/dead cell staining.

The breakdown of the epithelial cell layer seen eventually in all experiments and confirmed by other studies [Rejman et al. 2007] was thought to be partly due to the action of several virulence factors produced by *Ps. aeruginosa*. Rhamnolipids are believed to be incorporated into the epithelial cell membrane and result in the disruption of tight junctions, thus permeabilising the membrane [Zulianello et al. 2006; Sanchez et al. 2009]. They are also thought to surround biofilms and are implicated in the destruction of polymorphonuclear leukocytes [Alhede et al. 2009]. Pyocyanin, an exotoxin produced by *Ps. aeruginosa* triggers an extensive inflammatory response in epithelial cells which also contributes to the damage [Caldwell et al. 2009]. DNA released from *Ps. aeruginosa* cells is also believed to cause IL-8 release by airway epithelial cells in an unusual pathway not requiring the usual inflammatory mediators [Delgado, Poschet and Deretic 2006]. LPS and flagella activate the toll-like receptors promoting a damaging inflammatory cascade [Raoust et al. 2009].

Starner *et al.* successfully grew *Haemophilus influenza* biofilms on airway epithelial cells in culture at ALI over the course of four days, and measured the cytokine response. This was thought to be the first such *in vitro* model of biofilm formation on airway epithelial cells [Starner 2006]. Various other cell lines or primary cells either differentiated or undifferentiated have been used to study other pathogens such as *B. cepacia* complex and severe acute respiratory syndrome (SARS) virus and [Sajjan et al. 2006; Krunkosky et al. 2007; Botterel et al. 2008; Guillot et al. 2008;
Sims et al. 2008]. Another in vitro model grew a Ps. aeruginosa biofilm on viable well-differentiated primary airway epithelia for up to 20 hours with the classic biofilm architecture being apparent [Woodworth et al. 2008]. This approach should be pursued as the next logical step in developing the phage treatment model.

Further mouse models of Ps. aeruginosa biofilm infection have also been developed using alginate beads and insufflations of bacterial suspension, as well as chronic wound infections in rats [van Heeckeren et al. 2006; Claus et al. 2009; Kanno et al. 2009; Moser et al. 2009; Peying 2009]. It has been shown that there is a good degree of correlation between in vitro studies and in vivo animal models lending support to this work in developing an in vitro model [Rejman et al. 2007]. Drosophila melanogaster known as the fruitfly has been used as a model animal for many varied genetic studies. Drosophila models of Ps. aeruginosa infection have been developed as drosophila have similar tissue physiology and cell-cell signalling to mammals [Apidianakis and Rahme 2009].

Several groups have been working on in vitro skin wound models to investigate the biofilm infections found in chronic wounds and to test potential therapies. Although somewhat different to the airway epithelial infection some of the experiences of growing biofilms in these systems could be utilised in this model [Charles et al. 2009; Steinstraesser et al. 2009].

A successful 3D airway epithelial cell culture model was developed using a rotating-wall vessel bioreactor which enabled well-differentiated cells to form. This was used to model a Ps. aeruginosa infection, and may be a useful model for the further testing of phages as therapeutic agents [Carterson et al. 2005].

In this study a GFP producing strain of Ps. aeruginosa was used as it was anticipated that confocal microscopy investigations of the model would be performed in future work. Comparisons in terms of growth curves and phage infection process were made between the GFP and non-GFP producing strains, and no differences were found. However, recently others have noted that there can be differences between GFP and non-GFP bacteria in terms of their susceptibility to antibacterial agents [Allison and Sattenstall 2007]. Hence the GFP strains should be used for initial investigations but the experiments should be repeated with non-GFP clinical strains for confirmation before the phage therapeutic agent is taken to the next stage.
Another possible tool for monitoring the bacterial cells is to use a lux reporter gene which has the advantage over GFP in that it is only visible in live bacteria, whereas GFP will still be visible for some time after bacterial death.
7.4 The response of 16HBE cells to supernatant liquids of *Ps. aeruginosa* cultures treated with colistin or phages (chapter 6)

Prior to experiments to investigate *in situ* phage treatment of *Ps. aeruginosa* infection on epithelial cells a preliminary experiment was performed. This involved a two step process whereby cultures of *Ps. aeruginosa* were treated with phages or colistin and the resultant supernatant liquid, following centrifugation to remove bacterial cells, was applied to the human bronchial epithelial cells. This would allow establishment of the level of inflammatory reaction of the epithelial cells to the products of antibiotic or phage-treated bacteria without the added complication of an *in situ* infection. Testing both phage and antibiotic-treated culture supernatant liquids would also enable assessment of any differences between an established treatment and the novel phage treatment.

Initially lysed culture supernatant liquids from all 6 phages were tested for their ability to provoke an inflammatory reaction in the epithelial cells (section 6.2.1.2). From these data and previous phage characterisation and co-culture work a single phage was chosen to be used in the more complex treatment models. Phage U4 was chosen as the candidate since it provoked the smallest inflammatory response when applied to epithelial cells in isolation and as a culture lysate and had the best infection profile in terms of bacterial eradication and resistance. This was then used in an experiment to compare colistin-treated and U4-treated culture supernatant (section 6.2.2.3).

The results showed that there was an initial inflammatory spike by the epithelial cells in response to the phage lysate but this subsided. Again this could be due to endotoxin present in the supernatant that had been released during the phage infection. A similar pattern was seen with antibiotic treated culture supernatant. Moreover there was no significant difference between phage and antibiotic treated culture supernatant in terms of the inflammatory reaction it provoked over the majority of the duration of the experiment. However colistimethate sodium did provoke a higher release of IL-8 at the peak of the inflammatory cascade than did U4.
In a comment piece Dixon expressed concern that phage therapy may potentially induce circulatory shock as a result of massive bacterial lysis releasing large quantities of endotoxin [Dixon 2004]. An in vivo study on canine ear infections showed that there were no adverse reaction that may have been a result of endotoxin release due to bacterial lysis [Soothill et al. 2004]. Lysis deficient phages that are either naturally occurring such as filamentous phages or that have been engineered to kill bacteria without lysing them could be a useful way around this issue [Matsuda et al. 2005]. This issue is not exclusive to phages, antibiotics such as the beta-lactams also induce lysis of bacterial cells. Colistin, by contrast, binds to and neutralises endotoxin. The effective treatment of chronic multi-resistant infections could be considered as a compromise between eradication of the bacteria and potential hazards of the treatment such as endotoxin release.
7.5 **Treatment of *Ps. aeruginosa* infection on 16HBE cells with colistin or phages (Chapter 6)**

The treatment of *in vitro* *Ps. aeruginosa* infections on airway epithelial cells at various time points after initiation of the infection revealed a time threshold for treatment. Neither colistin (section 6.2.4) nor phage (section 6.2.3) treatment applied from 8 hours post-infection onwards resulted in complete recovery of the epithelial cells as revealed by cytokine production. Phage treatment resulted in an increased inflammatory reaction after treatment which then quickly subsided, however the reaction resulting from antibiotic treatment took longer to subside. It also appeared that in general phage treatment was more successful at 8 hours than antibiotic treatment. This may reflect a more rapid killing of the bacterial cells by phage than by colistin. Another important element to these experiments was that the phage treatment on its own without any infection present caused a lesser degree of inflammatory reaction than colistin treatment without prior infection. This suggests that in this model phage treatment is safer and more effective than colistin treatment, a therapy already established for use in CF related airway infection.

In this study colistin was used in a method of delivery *in vitro* that arguably mimics nebulisation *in vivo*, i.e. small droplets of colistin solution are delivered directly to the airway surfaces. Colistin is a commonly used antibiotic in CF due to the rarity of resistance in *Ps. aeruginosa*. However it is not ideal; complete *Ps. aeruginosa* eradication is unusual, increasing resistance is being seen, and further development of dosing regimens and methods of administration is needed. Until recently the exact mechanism of action of colistin was unclear, however recent work has suggested that it displaces calcium and magnesium in the LPS causing alterations in the cell membrane of the bacteria leading to permeabilisation and subsequent leakage and cell death. Colistin may also interfere with bacterial cell division and appears to increase the rigidity of the cell wall [Johansen *et al.* 2008; Sabuda *et al.* 2008; El Solh and Alhajhusain 2009; Gupta *et al.* 2009; Molina, Cordero and Pachon 2009; Montero *et al.* 2009; Mortensen *et al.* 2009].

The resistance of many bacteria to multiple antibiotics has been one of the main reasons for the resurgence of interest in bacteriophage therapy research. *Ps.*
*Pseudomonas aeruginosa* in particular is resistant to various categories of antibiotics, utilising most of the known mechanisms of resistance. It can rapidly develop resistance after only a short exposure to an antibiotic [Papaioannidou, Nitsas and Mirtsou-Fidani 2009; Strateva and Yordanov 2009].

Development of resistance by bacteria to phages has been cited as a possible complication associated with phage therapy [Donlan 2009]. In the infection process experiments there was very little resistant re-growth in the presence of any of the phages. However these experiments were relatively short and should therefore be extended in duration to ascertain whether resistance develops after longer periods of time such as 24 hours. Resistance is often associated with changes in the bacterial receptors to which the phage adsorbs [Nechaev and Severinov 2008]. Highly virulent phages avoid resistance by adsorbing to essential bacterial structures. Many *Ps. aeruginosa* phages adsorb to the flagella or pili, both of which are important in the development of a biofilm, the growth mode which is associated with greater bacterial virulence. Hence pili or flagella-deficient mutants may be able to evade some phage infections but may not be as effective at causing the chronic biofilm associated infection that is the source of so many problems [Holland, Sanz and Perham 2006; Andrew *et al.* 2009]. Further studies on other bacterial infections have also shown that bacterial resistant mutants are far less virulent and phages can overcome the resistance mechanism by mutation [Kysela and Turner 2007; Capparelli *et al.* 2009]. Others have also speculated that delivering a large enough dose of a combination of phage would avoid ongoing phage replication and hence the opportunity for the bacteria to develop resistance [Cairns and Payne 2008].

A problem unique to phages is the possible transference of virulence factors across different strains of bacteria via lysogeny [Cheetham and Katz 1995; Broudy, Pancholi and Fischetti 2001; Newton *et al.* 2001; Ohnishi, Kurokawa and Hayashi 2001; Banks, Beres and Musser 2002; Boyd and Brussow 2002; Wagner and Waldor 2002; Parisien *et al.* 2008; Donlan 2009]. This has been demonstrated in *Ps. aeruginosa* with the transference of antibiotic resistance genes and genes that confer mucoidy [Miller and Rubero 1984; Blahova *et al.* 1998]. This lysogenic conversion can even appear to extend to interspecies transfer e.g. between *Staphylococcus aureus* and *Listeria monocytogenes* [Chen and Novick 2009]. Phages to be used as therapeutic agents could be engineered to avoid the carriage of virulence genes and
the development of resistance [Donlan 2009]. They should also only follow a lytic lifecycle with no tendency toward lysogeny.

A further potential issue of phage therapy is the possibility that antibodies could be raised against them and they could subsequently be removed from the body. In a mouse model of Salmonella enteric infection phage therapy did not result in antibodies being raised against the phages, and they remained active for up to 2 weeks [Capparelli et al. 2009]. However in a similar study four different phages were found to be removed from the murine bloodstream within 2-3 days [Uchiyama et al. 2009]. Long-circulating phage mutants were developed by injecting phage into mice and isolating the remaining phage from blood samples after 7 hours. These phages were propagated in bacterial cultures, purified and re-injected into mice nine times, resulting in phage which remained at high levels in the blood up to 18 hrs post injection [Merril et al. 1996]. Phages can also apparently reduce reactive oxygen species produced by polymorphonuclear leukocytes in response to the presence of bacteria, this in turn may prevent some of the damage to the airway surface caused by these reactive oxygen species [Kurzepa et al. 2009].

Mutator strains of Ps. aeruginosa have been isolated from CF airway infections; these are associated with higher rates of antibiotic resistance, increasing virulence and poor lung function [Ferroni et al. 2009; Hoboth et al. 2009]. These mutator populations appear to be focused within the micro-colonies of Ps. aeruginosa biofilms and are probably important in the adaptation to the biofilm phenotype [Conibear, Collins and Webb 2009]. These strains with higher mutation rates may therefore acquire phage resistance more quickly, hence phage therapy candidates would have to be tested against these isolates.

Successful in vitro models of bacteriophage therapy have been tested for indwelling medical devices e.g. catheters. These are without the human airway epithelial cells in this study but do show phages to be promising in prevention or treatment of biofilm infections of these devices [Curtin and Donlan 2006; Del Pozo et al. 2007; Fu et al. 2009].

There have been several animal models of phage therapy developed since the start of this project. A study on mice with experimental imipenem-resistant Ps. aeruginosa bacteraemia found that administering intra-peritoneal phage isolated from sewage,
60 minutes after injecting *Ps. aeruginosa* allowed survival of all mice, of which 100% died if left untreated. However survival fell significantly if mice were treated up to 360 minutes after injection with bacteria [Wang et al. 2006]. Further studies have found a similar affect in mice and drosophila [Vinodkumar, Kalsurmath and Neelagund 2008; Heo et al. 2009]. Phage therapy as a prophylaxis prevented full scale *S. aureus* infection in immunosuppressed mice and also improved the adaptive immune response [Zimecki et al. 2009]. In a burn wound mouse model infected with *Ps. aeruginosa* survival increased significantly in those mice treated with a three phage cocktail. Moreover the route of administration of the phage cocktail had significant bearing on the speed and efficacy of treatment with intra-peritoneal administration conferring the greater advantage (versus intra-muscular or subcutaneous administration) [McVay, Velasquez and Fralick 2007]. There have been animal experiments that have shown phage therapy to be unsuccessful. In an experiment on phage therapy of *Yersinia* enterocolitica in mice no difference was seen between treated and untreated mice [Skurnik and Strauch 2006].

In two case studies of bacteriophage therapy *Ps. aeruginosa* infection of human burns and canine chronic bilateral otitis externa were treated with an appropriate phage as tested for susceptibility *in vitro*. Both conditions improved and both subjects recovered although the burns case was also receiving antibiotics which couldn’t be ruled out as a cause of the improvement. In both cases extensive multiplication of the phage was seen a short time after treatment, with no apparent ill effects. The burns patient had a febrile episode shortly after phage application which could be attributed to phage mediated bacterial lysis, however these episodes had occurred several times prior to phage treatment as well [Marza et al. 2006].

In a phase I trial of a phage cocktail specifically designed to target bacterial populations within venous leg ulcers no differences were seen between phage treatment and control (saline) with respect to either adverse effects or healing [Rhoads et al. 2009]. However in a phase I/II clinical trial of a bacteriophage preparation targeted at *Ps. aeruginosa* in chronic antibiotic resistant otitis, improvements were seen in the treatment group with no side effects but not the control group [Wright et al. 2009].
7.6 Future directions

This work was intended to provide the initial steps in creating an *in vitro* model of *Ps. aeruginosa* infection on airway epithelial cells in order to test bacteriophages as a means of treatment. An *in vitro* model has been created and has shown that phage treatment was more efficacious and provoked less of an inflammatory reaction in airway epithelial cells than the commonly used conventional treatment colistin. However although these results are very promising more work needs to be done to improve the model and to further test the efficacy of different phages as treatments.

A library of phages and a library of various laboratory and clinical strains of *Ps. aeruginosa* should be built up, and screened to assess the phage specificity, infection process and adsorption receptors. Phage preparations should be processed to avoid excess endotoxin and tested in co-culture with epithelial cells to check for an inflammatory reaction to them.

The bacterial/epithelial cell co-culture model although sufficient to produce these results would be improved by lengthening the time after infection and prior to destruction of the epithelial cell layer. In this model commercial alginate was used to encourage biofilm growth. Other groups have used mucus infected with *Ps. aeruginosa* and shown that biofilms form in the thickened dehydrated mucus, which prevented bacterial motility [Matsui *et al.* 2006]. This could be a useful method to establish biofilms on the airway epithelial cells, and would also enable establishment of the rate of phage diffusion through the thick mucus that is found in CF cells. Other biofilm culture methods have been developed which could be manipulated to suit this model. A low shear drip-flow reactor developed in one study was described as mimicking various *in vivo* situations including the CF airway [Goeres *et al.* 2009]. An alternative flat-bed perfusion biofilm model used a continuously perfused cellulose matrix which can be used to test topically applied treatments [Thorn and Greenman 2009]. A polyaxamer hydrogel gel was successfully used in another study to test the antibiotic susceptibility of biofilms grown on it [Clutterbuck *et al.* 2007].

With regard to the airway epithelial section of the model it could be improved by using primary cells and allowing them to fully differentiate. A further step would be
to develop a 3D culture model with a more fully developed innate immune system using e.g. macrophages. This type of *in vitro* system has yet to be developed for airway epithelia, although work is progressing in this field, and its development would be a valuable tool for the study of potential therapeutic agents [Roggen, Soni and Verheyen 2006; Deslee *et al.* 2007; Moreau-Marquis, Stanton and O'Toole 2008].
7.7 The future of bacteriophage therapy

Thanks to the resurgence in interest in bacteriophage therapy due to the problems with increasing antibiotic resistance the use of bacteriophages in the future is likely to take several different directions.

Phages used in conjunction with antibiotics have been found to be more efficacious than phages or antibiotics alone indicating that this type of multi-therapy could be the answer to chronic infections such as those in CF [Hagens, Habel and Blasi 2006; Dzulishvili et al. 2007].

Phages have been recognised as particularly useful in the preservation of food with the approval of the use of phages as a food additive. Phage products are in use for the control of plant pathogens in crops and Listeria in meat and cheese products. This field is rapidly expanding with studies on the control of pathogens using phages at different stages throughout the agriculture and food production chain. This should pave the way for future legislation governing phage therapy [Bren 2007; Garcia et al. 2008].

Phages could also be engineered as delivery systems for other antimicrobial drugs with targeted specificity [Deresinski 2009]. Filamentous phages have been engineered to carry cargos of drugs, genes for gene therapy and imaging agents for investigation, and these can be targeted to specific areas [Yacoby, Bar and Benhar 2007; Yacoby and Benhar 2008].

The idea that using phage-derived antimicrobial peptides as treatments as opposed to whole phages has been suggested as a way to avoid immunogenicity and distribution problems [Projan 2004; Fischetti, Nelson and Schuch 2006]. Alginase enzymes have been found associated with Ps. aeruginosa phages which depolymerise the alginate and may represent a useful form of treatment [Glonti, Chanishvili and Taylor 2009]. Lysins may also prove to be useful antimicrobials, with a low reported incidence of resistance [Fischetti 2008; O’Flaherty, Ross and Coffey 2009; Sarah, Ross and Aidan 2009]. Endolysins have been found to successfully break down the peptidoglycan in Gram positive bacterial cell walls in animal models [Hermoso, Garcia and Garcia 2007], however this strategy would not be applicable to Gram negative bacteria such
as *Ps. aeruginosa*. Also in the case of CF airway infections the phage could be delivered directly to the airway surface and, although further investigation is necessary, it appears that inflammatory reactions to the phages would be small and fleeting.

The method of delivery of phages in CF is likely to be via nebulisation. A study has already been performed that shows that the nebulisation of *B. cepacia* phages can be successfully achieved and that the phages are likely to be well distributed throughout the lungs [Golshahi et al. 2008].
7.8 Alternative novel treatments

Aside from the promising idea of phage therapy there has been a great deal of research into other compounds that may help treat *Ps. aeruginosa* infections. Despite the levels of research being undertaken to attempt to improve the treatment of *Ps. aeruginosa* infections the likelihood of a new anti-pseudomonal drug being released in the near future is low [Page and Heim 2009]. As an indication of the difficulty in finding a new therapeutic agent for this bacterium the investigations have taken many different routes from novel synthetic antibiotics to naturally occurring compounds from a variety of sources.

Several antibiotics are being developed, in particular monobactams such as aztreonam lysinate or new generations of other antibiotic classes [Gibson *et al.* 2006; Coates and Hu 2007]. A randomised double-blind placebo-controlled trial of aztreonam improved symptoms and lung function in CF patients [Retsch-Bogart *et al.* 2009]. A new generation cephalosporin (an anti-pseudomonal β-lactam) known as CXA-101 has shown particular promise as it acts on even multi-drug resistant *Ps. aeruginosa* that produce class-C β-lactamases, and few resistant isolates appeared after exposure [Bulik, Christensen and Nicolau 2009; Juan *et al.* 2009; Page and Heim 2009]. Doripenem and biapenem, new carbapenems have shown promise in treating various Gram negative bacterial infections including *Ps. aeruginosa*, however resistance to previous carbapenems can preclude their use [El Solh and Alhajhusain 2009; Rahal 2009]. Other antibiotics that appear to be useful in the treatment of *Ps. aeruginosa* include liposomal amikacin (an aminoglycoside) and levofloxacin (a fluoroquinolone) [Meers *et al.* 2008; Chachanidze *et al.* 2009; King *et al.* 2009; Okusanya *et al.* 2009]. Species specific antibiotics are being investigated as their high specificity will avoid some of the classic problems with antibiotic chemotherapy [Then and Sahl 2009].

Macrolide antibiotics have been used for some time however recently there has been research into the use of these in modulating the inflammatory reaction of the host rather than a classic antimicrobial action. In several studies azithromycin was shown to alter the gene expression of airway epithelial cells resulting in a reduced reaction to *Ps. aeruginosa* presence protecting the epithelial layer from the extensive damage
usually seen. It does this without reduction in the number of bacterial cells present. Hence macrolides such as azithromycin could be used concurrently with classic antibacterial antibiotics and in the early stages of CF infection to prevent airway injury which predisposes to further infection [Giamarellos-Bourboulis 2008; Halldorsson et al. 2009; Ribeiro et al. 2009; Tsai et al. 2009].

Different combinations of existing antibiotics may also be more effective, such as aztreonam, ceftazidime, and colistin used in one study, and tobramycin and clarithromycin in another. Antibiotics that have different mechanisms of action can act in synergy, that is a combination of antibiotics can be vastly more effective than the individual antibiotic, even in some cases when the organism is resistant to the individual antibiotic. Antibiotic classes that can exhibit synergy in the treatment of *Ps. aeruginosa* include beta-lactams/aminoglycosides, beta-lactams/fluoroquinolones, and fluoroquinolones/aminoglycosides. [Cernohorska and Votava 2008; Cai et al. 2009; Chachanidze et al. 2009; Dales et al. 2009; MacLeod et al. 2009; Oie et al. 2009; Tre-Hardy et al. 2009].

A number of compounds that have antibacterial action but are also harmful to eukaryotic cells could be rendered useful by specific targeting using a specialised delivery system. Filamentous phages specific for *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Escherichia coli* have been engineered to carry a large antibacterial drug cargo which they then deliver directly to the site of infection. This specific delivery system was found to substantially increase the efficacy of the drug [Yacoby and Benhar 2007].

Silver-carbene complexes are known to have good antibacterial action. Silver has been coupled to a methylated caffeine carrier to form a complex which was aerosolised to allow direct application to the airways thus reducing the risk of systemic toxicity. This system also provided a sustained release of the antimicrobial effect resulting in fewer doses. This form of treatment was found to be effective against *Ps. aeruginosa* and did not cause any adverse reactions in mice or in airway epithelial cells grown *in vitro*. [Cannon et al. 2009; Hindi et al. 2009].

Other methods of dealing with the *Ps. aeruginosa* threat include vaccines targeted against alginate or flagella and these systems have undergone trials. They appear to
be of limited success and do not prevent all episodes of infection [Zuercher et al. 2005; Zuercher et al. 2006; Döring, Meisner and Stern 2007].

CFTR (cystic fibrosis transmembrane conductance regulator) correctors that appear to reduce the over-production of inflammatory mediators in airway epithelia also show promise. Amitriptyline, usually administered as a tricyclic anti-depressant, reduces Ps. aeruginosa infection in mice and appears efficacious in human trials. It blocks the production of sphingomyelinase and acid ceramidase. These enzymes belong to a pathway that has numerous cellular effects including inflammation and apoptosis [Dechecchi et al. 2007; Jenkins, Canals and Hannun 2009; Riethmuller et al. 2009].

Compounds are also being developed which will ameliorate the effect of antibiotics such as the liposomal formulation in a recent study which improved the efficacy of tobramycin on Ps. aeruginosa and B. cenocepacia [Halwani et al. 2008]. Enzymes such as DNase and alginate lyase are also thought to be useful. They reduce the viscosity of the mucus and the biofilm glycocalyx to allow diffusion of co-administered antibiotics [Alkawash, Soothill and Schiller 2006; Alipour, Suntres and Omri 2009; Yang et al. 2009]. In addition, there are agents such as arginine and nitrate that increase the growth rate of bacteria in anaerobic biofilms hence enhancing the action of antibiotics such as tobramycin and ciprofloxacin. [Borriello et al. 2006]. Inhibitors of the multi-efflux pumps which contribute to antibiotic resistance in Ps. aeruginosa improve the action of antibiotics usually removed by these pumps [Tohidpour et al. 2009].

Another treatment that is targeted at preventing antibiotic resistance is the use of iron chelators. Iron is released by CF airway epithelia and contributes to antibiotic resistance. FDA approved iron chelators used in conjunction with tobramycin prevented and reduced Ps. aeruginosa biofilm formation on CF airway epithelia in vitro. Compounds such as ferric picolinate can be nebulised for ease of treatment [Musk and Hergenrother 2008; Moreau-Marquis, O'Toole and Stanton 2009]. EDTA another metal chelator is also effective at destroying biofilms particularly when used in conjunction with an antibiotic [Banin, Brady and Greenberg 2006]. EDTA scavenges divalent cations from LPS in the bacterial membranes leading to the loss of LPS from the membranes. This causes dispersal of cells from biofilms and
increased membrane permeability, hence other antibacterial compounds become more effective in the presence of EDTA

Novel therapy options are being explored from natural sources. Recently interest has arisen surrounding the potential benefits of by-products of *Lactobacillus plantarum* a lactic acid producing bacteria which is found in some fermented foods. Supernatant liquids of *L. plantarum* have been shown to inhibit the damaging effects on epithelial cells of *Ps. aeruginosa* virulence factors [Ramos *et al.* 2009]. Maggots are already used successfully in chronic wound infections. Maggot excretions and secretions have now been shown to prevent and break down *Ps. aeruginosa* biofilms on biomaterials [Cazander *et al.* 2009]. Garlic has been found to block quorum sensing in *Ps. aeruginosa* infections in mice and hence may be an effective prophylactic treatment. However it is still unclear what compound in garlic extract is responsible for this action [Harjai, Kumar and Singh 2009]. In a further study a computer programme screened traditional Chinese medicines for quorum sensing inhibitors and discovered baikalein. Baicalein inhibits biofilm formation and works in synergy with ampicillin [Zeng *et al.* 2008]. However a study suggesting that *Ps. aeruginosa* can form biofilms without the use of quorum sensing signals means that quorum sensing blockers may not be as effective as originally thought [Schaber *et al.* 2007].

A more unusual idea for the control of *Ps. aeruginosa* is photodynamic antimicrobial chemotherapy (PACT). This system uses photosensitisers, such as toludine blue which can diffuse through the viscous CF mucus, which are activated by red light (635 nm). This combination can kill *Ps. aeruginosa* depending on the concentration of the photosensitiser and the mode of growth of the bacteria [Donnelly *et al.* 2007]. Nano-particles have been shown to be useful in the implementation of PACT treatment. A dynamic-nanoplatform constructed from a polyacrylamide hydrogel matrix can carry photo-sensitisers such as methylene blue, which would otherwise be degraded *in vivo*, in a targeted manner to the site of infection [Wu *et al.* 2009]. In another study free radical scavenging nano-particles reduced the effects of certain virulence factors [Elswaifi *et al.* 2009].

New and more virulent strains of *Ps. aeruginosa* are appearing such as the Liverpool epidemic strain (LES) which has spread throughout the UK. Moreover it is believed that this strain has evolved in part due to the action of lysogenic conversion, as
several prophage elements have been found in its genome [Winstanley et al. 2009].
A new strain of multi resistant *S. aureus* has also been spreading amongst some of
the CF population. As with the LES it is thought that the antibiotic resistance may be
spreading between strains by the way of an antibiotic-inducible bacteriophage
[Rolain et al. 2009]. This highlights some of the potential complications associated
not only with developing phages as therapeutic agents but in convincing sceptics of
their merit.

However bacteriophages are likely to be utilized more extensively in the future,
particularly when faced with ever increasing antibiotic resistance. In fact some
groups believe that phage treatment should not just be used after antibiotics have
failed and may be an alternative to be considered as an initial treatment choice
[Miedzybrodzki et al. 2007]. They are also very useful at treating biofilms due to the
carriage by many phages of an exopolysaccharide depolymerise [Azeredo and
Sutherland 2008]. However more research needs to be done to solve some of the
challenges that arise with phage therapy and a separate regulatory category for
licensing of phage products is also needed with protocols for characterisation and
safety testing in place [Hanlon 2007; Skurnik, Pajunen and Kiljunen 2007; Verbeken
et al. 2007; Mattey and Spencer 2008]. This work along with the many other studies
into the different aspects of phage therapy will hopefully help to forward the
prospect of phage therapy and make it a realistic choice for the treatment of chronic
infections in the near future.
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Appendix I
Map of the plasmid containing the GFP construct within the PAO-GFP strain of *Ps. aeruginosa*. 

![Diagram of the plasmid containing the GFP construct](image)
Appendix II
Remainder of the bacteriophage genome restriction digest gel electrophoresis images (chapter 3).
Appendix III
A representative image for each condition in the live/dead cell staining assay (chapter 6)

Colistimethate sodium control

Colistin sulphate control
Bacteriophage U4 Control

*Pseudomonas aeruginosa* treated with colistimethate sodium
*Pseudomonas aeruginosa* treated with Colistin sulphate

*Pseudomonas aeruginosa* treated with bacteriophage U4

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Positive control (vanadyl sulphate)

Negative control