CONTROLLED DELIVERY OF BACTERIAL VIRUSES FOR THE ERADICATION OF BACTERIAL INFECTION

Callum J Cooper BSc (Hons), MSc

Welsh School of Pharmacy, Cardiff University

Redwood Building

King Edward VII Avenue

Cardiff

CF10 3NB

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Summary

A rise in antibiotic resistance has prompted renewed interest in the use of phages to treat bacterial infections. This project explored the use of phages to treat *Ps. aeruginosa* infections in the context of potential treatment for Cystic Fibrosis patients.

Initial characterisation of phage activity, by both a conventional plaque based assay and a novel Bioscreen C assay, revealed that 4 *Ps. aeruginosa* phages could infect multiple strains of *Ps. aeruginosa* including some clinically prevalent strains within the UK. This initial activity was increased in some strains following selection with a selective virucide, and a cocktail was designed. The cocktail showed a >2 Log_{10} reduction after 20 h in 10 of 14 strains. When the cocktail was tested against 4 *Ps. aeruginosa* strain biofilms, only a modest level of activity (<1 Log_{10}) was measured. This was increased (>5 Log_{10}) when combined with antibiotics in one bacterial strain in particular; i.e. the strain against which the phage were initially propagated. Assessment of the endotoxin content of the cocktail showed a level far in excess of acceptable levels (approximately 6 Log_{10} EU/mL) that were not reduced sufficiently following purification by affinity chromatography (5.81±0.37 Log_{10} EU/mL). Delivery of the phage cocktail showed acceptable levels of recovery following nebulisation (approximately 99%).

The methods developed here, particularly the Bioscreen C assay for the assessment of phage lytic activity is applicable to a variety of different arenas by the application of standard acceptance criteria on activity levels. The direct quantification of the bacterial content of biofilms following the addition of phage and virucide treatment is novel and allows a cidal activity to be measured.

Results have also highlighted the need for proper quality control within phage preparations for chronic infections. This investigation also highlighted the need for a standardised *in vitro* model that is more representative of the conditions found within a CF lung.

Scientific Publications and Presentations

Parts of this work have been published in scientific abstracts and papers and presented as detailed below.

First Author Publications

COOPER, C.J., DENYER, S.P. and MAILLARD J.Y. (2011) Rapid and quantitative automated measurement of bacteriophage activity against cystic fibrosis isolates of *Pseudomonas aeruginosa*). *Journal of Applied Microbiology* **110**, 631-640

Conference Abstracts

COOPER, C.J., DENYER, S.P. and MAILLARD J.Y (2010). Assessment of the lytic activity of bacteriophage cocktails against *Pseudomonas aeruginosa* strains isolated from Cystic Fibrosis Patients. *110th General Meeting of the American Society for Microbiology. San Diego, California, 2010.*

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ΔF508	Deletion of Phenylalanine at position 508
A_{235nm}	Absorbance at 235nm
A_{530nm}	Absorbance at 530nm
ALG	Alginate lyase
AMB	Ambroxol hydrochloride
ANOVA	Analysis of variance
ATP	Adeonosine Triphosphate
BSAC	British Society for Antimicrobial Chemotherapy
CDFF	Constant depth film fermentor
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
CFU/mL	Colony forming units per millilitre
CI	Confidence interval
CompA	Composition A
EPS	Exopolysaccharide
EU/mL	endotoxin units per millilitre
FDA	Food and Drug Administration
GDPase	Guanine diphosphatase
GM	Genetic modification
HIV	Human Immunodeficiency virus
IFN-γ	Interferon gamma
IL-1	Interlukin-1
IL-8	Interleukin 8
i.p	Intraperitoneal
ISA	Iso-sensitest agar
LAL	Limulus amebocyte lysate
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
Mbp	Mega base pairs
MIC	Minimum inhibitory concentration

MOI	Multiplicity of infection
MPa	Mega Pascals
mRNA	Messenger RNA
MSDM	Membrane spanning domain
NA	Not applicable
NBD	Nucleotide binding domain
NC	Not calculated
NCTC	National collection of type cultures
ND	Not detected
OD_{600nm}	Optical density at 600 nm
$OD_{420-580nm}$	Optical density at 420-580 nm
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PFU/mL	Plaque forming units per millilitre
PPCO	Polypropylene co-polymer
PRE	Pomegranate rind extract
PTFE	Polytetrafluoroethylene
SD	Standard deviation
SDW	Sterile deionised water
SEM	Scanning electron microscopy
SP-A	Surfactant Protein A
TEM	Transmission electron microscopy
TOC	Total organic carbon
TSA	Tryptone soy agar
TSB	Tryptone soy broth
TSC	Tryptone sodium chloride
TVC	Total viable count
v/v	Volume to volume
w/v	Weight to volume
UAC	Uronic acid content

Chapter 1

General Introduction

1.1 The human respiratory system

The lungs are the primary site for gaseous exchange within the body and are divided into lobes. The left lung comprises of two lobes and the right comprising of three lobes. The considerable surface area of the lungs, containing approximately 1500 miles of airways and some 300-500 million alveoli (Ochs *et al.*, 2004), allows for efficient gaseous exchange.

The human lung has multiple mechanisms, both generalised and adaptive, that are designed to protect it from external contaminants and potential pathogens (Table 1.1).

While these innate defence mechanisms usually protect the lung from potential pathogens and maintain a sterile environment, there exist a number of conditions, including cystic fibrosis (CF), infection from the human immunodeficiency virus (HIV) and emphysema in which these mechanisms can be compromised and leave individuals susceptible to secondary infection from opportunistic pathogens.

Mechanism	Function	Innate/ Adaptive
Upper airway filtering	• Speed of air entry and curve of airways causes deposition of particles on airway surfaces based on size	Innate
Coughing and sneezing	• Reflex reaction o Designed to clear airways o Works in conjunction with mucocilliary clearance	Innate
Mucocilliary clearance	• Mucus is pushed up the respiratory tract by the cilia beat • Swallowed and digested	Innate
Phagocytosis	• Macrophages o Non-specific degradation of pathogens by free radicals • Neutrophils o Attracted to infection by release of chemotaxins by macrophages o Similar degradation to macrophages	Innate
Immune function	• Attraction of B and T cells to site of infection	Adaptive

Table 1.1: Defence mechanisms possessed by the lung operating to remove and destroy bacterial pathogens. Adapted from (Sethi and Murphy, 2008).

1.2 Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive condition (Figure 1.1) that is believed to be the most common inherited genetic disease within the Caucasian population (Dodge *et al.*, 2007). Although primarily regarded as a disease of Caucasian populations it has been shown to affect other populations to varying degrees (Boyle, 2007), with sub-Saharan Africa (Poolman and Galvani, 2007) exhibiting a lower incidence of CF and those from an Ashkenazi Jewish population exhibiting a higher incidence rate (van de Vosse *et al.*, 2005).

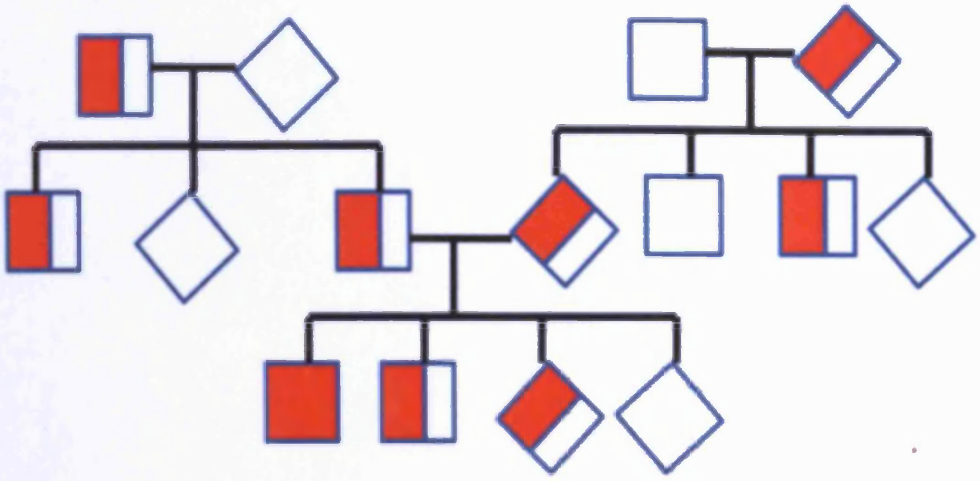


Figure 1.1: Genetic basis of cystic fibrosis. Showing the autosomal recessive trait. Diamonds represent male, squares represent female, half shaded represents carrier phenotype, clear represents normal and filled represents sufferer.

The average life expectancy of current CF patients is just 31 years old (Ratjen and Döring, 2003) with female sufferers believed to have a higher mortality rate (Arrington-Sanders *et al.*, 2006). This is drastically shorter than the average UK life expectancy for non-CF patients at 77 years for males and 81 years for females (Office of National Statistics, 2010). However, due to improved diagnostic and

therapeutic strategies, it has been suggested that the average life expectancy for CF sufferers born within the UK in 2003 will be >50 years old (Dodge *et al.*, 2007).

1.2.1 CF pathophysiology

The CF gene defect is carried on chromosome 7 at locus q31.2 and encodes for the cystic fibrosis transmembrane conductance regulator (CFTR), an adenosine triphosphate (ATP) binding cassette whose structure extends across the cellular membrane (Figure 1.2) where it is responsible for the regulation of chloride ion transport across cellular membranes in multiple organs within the human body including the lungs, liver, gut and pancreas (Saiman and Siegel, 2004).

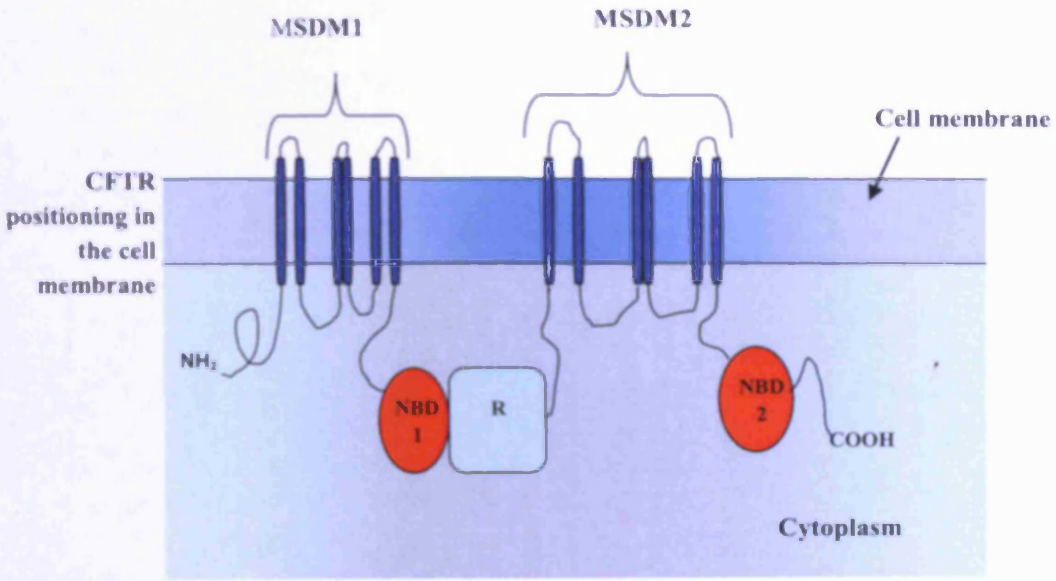


Figure 1.2: The structure of the CFTR protein. The function of the protein is to bind and hydrolyse ATP at the nucleotide binding domains. Adapted from Gibson *et al* (2003). Membrane spanning domain - MSDM, Nucleotide Binding domain - NBD, Regulatory - R domain.

The most common mutation of the chloride channel associated with CF is the result of the deletion of three base pairs encoding for phenylalanine at position 508 (ΔF508), which accounts for approximately 66% of mutations (Accurso, 2007).

The ΔF508 mutation is located within the 1st nucleotide binding domain (NBD 1) and impacts disease severity. In a homozygous state, the mutation results in abnormal protein folding and subsequent degradation that in turn increases the severity of symptoms (McCormick *et al* (2002). However, in a heterozygous state the mutation results in a decrease in the ability to regulate chloride ion transport across the cellular membrane."

However, it should also be noted that other mutations do exist in varying prevalence (Table 1.2) and has been studied in some detail within both UK and international populations (McCormick *et al.*, 2002; Hantash *et al.*, 2006).

Mutation	Gene frequency per 1000 genes	Effect of Mutation
ΔF508	752.0	• Classical form of CF • Deletion of phenylalanine at position 508 • Accounts for approximately 66% of alleles in caucasians
G551D	34.3	• Glycine to aspartic acid substitution at position 551 • Reports of pancreatic insufficiency • Higher prevalence in French Canadians (Gibson *et al.*, 2003)
G542X	18.4	• Premature stop codon mutation (Sermet-Gaudelus *et al.*, 2007) • Halts transcription early
R117H	12.7	• Substitution of arginine for histidine at position 117 (O'Sullivan *et al.*, 2006) • Missense mutation in exon 4 • Reduces level of chloride transport
621+1G→T	12.7	• Guanine to thymine change at position 621 • Resulting from mRNA splicing defect • Patients present with high sweat chloride levels >100 mmol/L (Kerem *et al.*, 1990)

Table 1.2: Breakdown of the 5 most frequent mutations of CFTR within the genotyped caucasian UK CF population. Adapted from McCormick *et al* (2002).

1.2.2 Diagnosis and clinical presentation

Diagnosis of CF patients occurs prenatally or soon after birth (Table 1.3). In older patients diagnosis can occur following the onset of unexpected illness.

Age	Diagnosis
Prenatal screening	Screening of chronic villus fluid for the most common mutations (Ratjen and Döring, 2003)
<2 Weeks	Analysis of dried blood for immune reactive trypsin **AND** screening for common mutations due to the low specificity and sensitivity of the trypsin test (Southern *et al.*, 2007)
2-4 Weeks	Sweat test: salt levels in sweat of >60 mmol/L are indicative of positive results

Table 1.3: Diagnosis of CF in infants

CF typically and primarily presents as the accumulation of viscous mucus within the small airway spaces of the lung (Rubin, 2007) leading to an increased susceptibility to bacterial infection (Gómez and Prince, 2007). The presence of different phenotypes within the gene mutation itself affects the clinical presentation of the disease (Kiesewetter *et al.*, 1993), including the involvement of multiple organ systems including the pancreas (Moskowitz *et* al., 2008) and the intestines (Borowitz *et al.*, 2005). Less significant symptoms include the deterioration of bone density (King *et al.*, 2005) and the clubbing of joints (Ratjen and Döring, 2003).

1.2.3 Important pathogens of cystic fibrosis

The establishment of active bacterial infection within the CF patient is perhaps the most important pathological feature and is believed to be the leading cause of mortality (Rogers *et al.*, 2005).

In the early stages of life numerous bacterial strains, such as *Staphylococcus aureus* and *Haemophilus influenzae* (Saiman and Siegel, 2004) have been shown to establish primary infections within the CF lung. They are ultimately supplanted by *Pseudomonas aeruginosa* which is believed to be resident within 80% of the CF population by the age of 20 (Pritt *et al.*, 2007).

Ps. aeruginosa is arguably the most important pathogen, and will be discussed later in greater detail. The increasing prevalence of *Burkholderia cepacia* complex in both single colonization and in co-colonization within CF patients has resulted in increased mortality (Courtney *et al.*, 2004). Although co-colonization was initially rare, increased co-infection rates could be due to the congregation of patients with mixed respiratory infections in CF centres (Waine *et al.*, 2007) as the *B. cepacia* complex is highly transmissible (McDowell *et al.*, 2004; Festini *et al.*, 2006).

This practice of congregation of patients with multiple infections has given way to the increasing segregation of patients by type of infection (Festini *et al.*, 2006). Although both *Ps. aeruginosa* and *B. cepacia* complex are important pathogens within the CF patient, a number of new bacterial pathogens such as *Stenotrophomonas maltophilia, Alcaligenes xylosoxidans* and *Inquilinus limosus* (Lambiase *et al.*, 2006; Herasimenka *et al.*, 2007) are beginning to emerge. In addition both fungal e.g. *Aspergillus fumigatus* (Lyczak *et al.*, 2002) and viral pathogens such as the influenza virus will present new challenges to physicians.

Currently, and largely due to the voluntary reporting nature of these emerging pathogens, no accurate incidence rates exist at this time.

1.2.4 Current CF therapy

Currently no vaccine or cure exists for the underlying genetic defect of CF; various therapies exist to treat the symptoms and complications from the condition. The accumulation of mucus within the lungs is treated by a combination of a physiotherapeutic regime (Osman *et al.*, 2010) and the use of anti-mucolytic compounds (Fahy *et al.*, 2010). The former approach (i.e. the physiotherapy regime) is often limited in its effectiveness due to poor patient compliance (Osman *et al.*, 2010). CF patients are at increased risk of developing respiratory infections (Rogers *et al.*, 2005). Antibiotics are routinely used to control respiratory pathogens and in the case of *Ps. aeruginosa* infections are used to try to eradicate initial infections before a biofilm can be established (Ho *et al.*, 2009).

1.3 *Pseudomonas aeruginosa*

1.3.1 Structure and morphology

Ps. aeruginosa is a motile Gram-negative bacillus (rod) approximately 0.5-0.8 μm x 1.5-3.0 μm that belongs to the *Pseudomonadaceae* family. While this bacterium is primarily an opportunistic pathogen, it can establish chronic infections in both burn victims (McVay *et al.*, 2007) and those patients with cystic fibrosis (Saiman and Siegel, 2004). It is most prevalent in the over 65's due to immunosenescence (Sansoni *et al.*, 2008).

The organism itself is ubiquitous to the environment and is frequently found in water reservoirs that have been contaminated by human waste and sewage (Pitt, 1986). It has also been shown to cause folliculitis in bathers using hot tubs (Yu *et al.*, 2007) presumably due to the optimum growth temperatures and poor disinfection regimes that are observed in many privately-owned hot tubs.

In reality it is this ability to grow on numerous surfaces and utilise multiple sources of nutrients that allow the organism to present a health risk. When compared to other bacterial agents that are usually associated with CF, namely *H. influenzae* and *Staph. aureus*, it has been observed that the genome of *Pseudomonas* is larger, 6.26 Mega base pairs (Mbp) with approximately 5567 genes, than that of both *H. influenzae* (1.83 Mbp and 1714 genes) and *Staph. aureus* (2.81 Mbp and 2594 genes) (Lambert, 2002). It is also believed that some 1500 of these genes are needed for metabolism and the production of structural proteins, showing a larger than normal capacity for adaptation (Lambert, 2002) when compared to other pathogens.

1.3.2 Virulence factors

The *Pseudomonas* bacterium possesses a number of different mechanisms that allow it to establish and maintain chronic infections within both normal patients and CF sufferers; bacteraemia associated with *Ps. aeruginosa* has increased between 2006 and 2010 (Figure 1.3). This increase in levels of *Pseudomonas* derived bacteraemia could be the result of a changing hospital population in which a greater number of people with deficient immune systems are being treated and could also be in part due to better reporting systems.

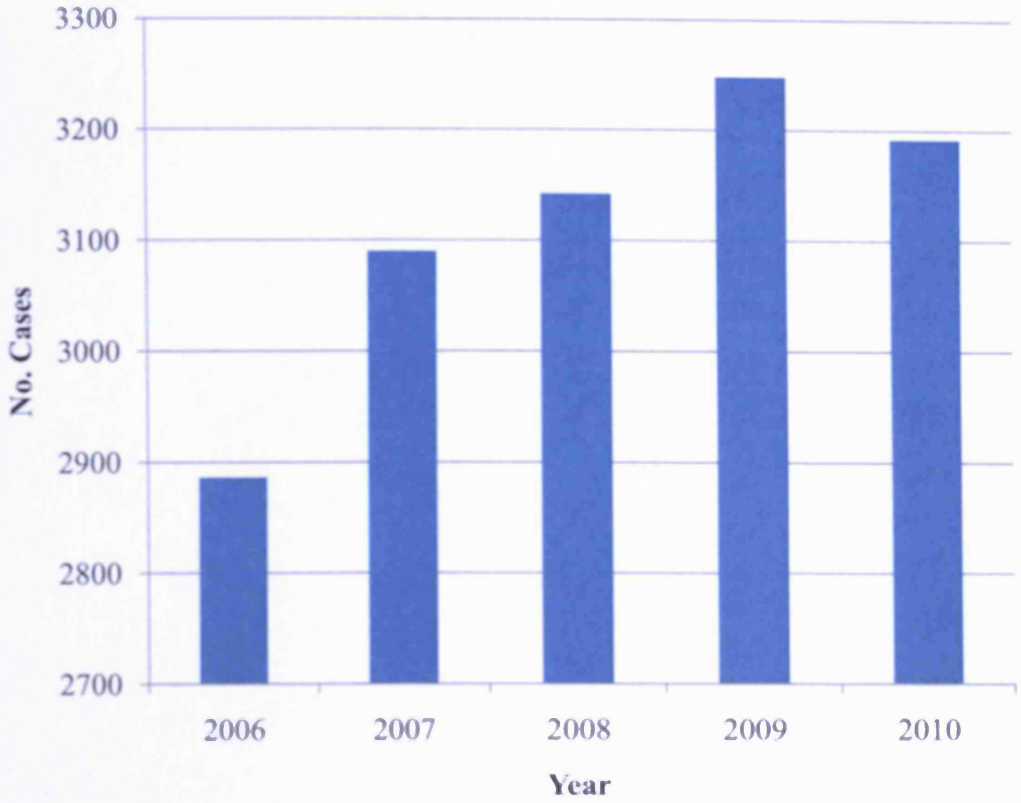


Figure 1.3: Number of bacteraemia cases associated with *Ps. aeruginosa* between 2006 and 2010 within England and Wales. Based upon voluntary laboratory reporting. Adapted from Health Protection Agency (2011).

These factors include the ability to form a biofilm as well as the ability to cause tissue damage through the secretion of proteases and toxins. These various factors will play an important role in the clinical outcome and can affect resistance to antimicrobials (Figure 1.4).

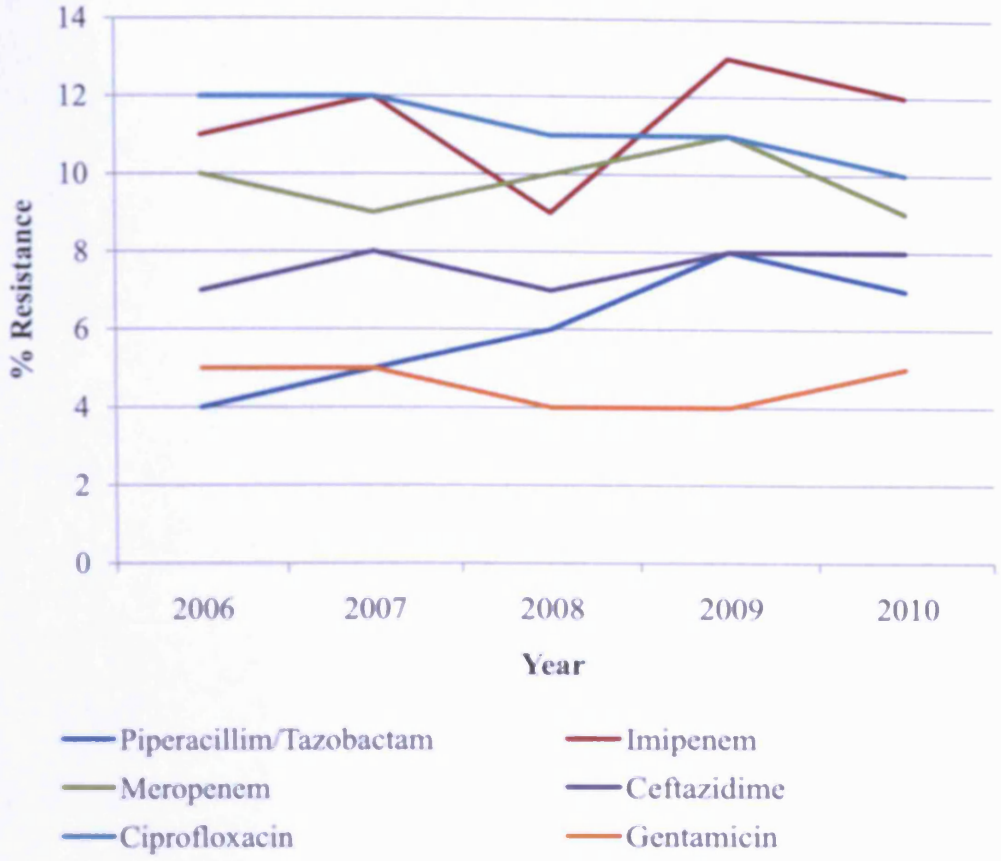


Figure 1.4: Trends in the antibiotic resistance of *Ps. aeruginosa* implicated in bacteraemia to antibiotics between 2006 and 2010 within England and Wales. Based on voluntary laboratory reporting, Health Protection Agency (2011).

1.3.2.1 Alginate production

Alginate is a linear polysaccharide comprised of β-D-mannuronic and α-L-guluronic acid residues that are joined in a 1, 4 linkage (Ertesvåg *et al.*, 1998). The bacterial polysaccharide varies from its algal counterpart through the presence of O-acetyl groups on mannuronate residues (Nivens *et al.*, 2001). In *Ps. aeruginosa* it is synthesised from fructose-6-phosphate in a 7-stage pathway (Figure 1.5).

Alginate overproduction appears to be characteristic of some CF epidemic strains but not all. It has also been shown to fulfil a role in biofilm development (Hentzer *et al.*, 2001) although not as centrally important as previously believed due to the involvement of non-mucoid polysaccharides (Wagner and Iglewski, 2008).

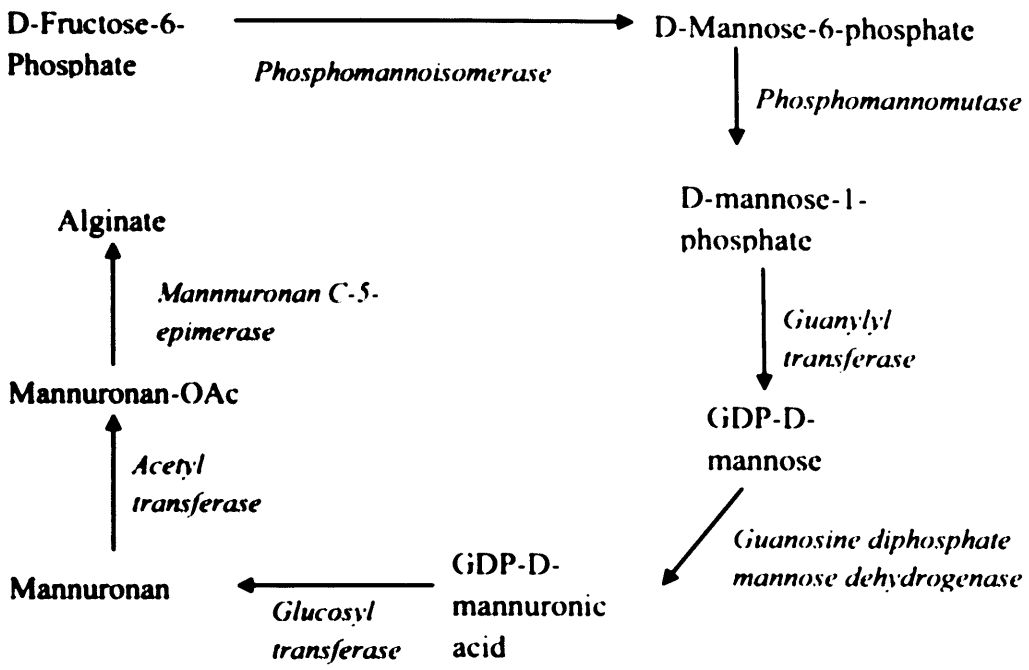


Figure 1.5: Alginate biosynthetic pathway as seen in *Ps. aeruginosa*. Adapted from Ertesvåg (1998). Enzymes catalysing reactions are shown in italics.

The role of alginate within the CF lung is believed to be similar to an adhesin, allowing for successful attachment to cells (Kipnis *et al.*, 2006). It is also believed that alginate has a protective role in which host defence function is compromised, particularly phagocytosis. Also some antibiotics such as the cationic aminoglycosides bind to the alginate (Lambert, 2002). However, Moskowitz and colleagues (2004) presented contradictory evidence that showed mucoid forms of *Ps. aeruginosa* are found to be fully susceptible to cationic aminoglycosides (Moskowitz *et al.*, 2004).

1.3.2.2 Pyocyanin production

Pyocyanin (N-methyl-1-hydroxyphenazine) is a redox-active secondary metabolite that is secreted by *Ps. aeruginosa* to produce a blue pigment and is regulated by the quorum sensing system (Kipnis *et al.*, 2006).

Pyocyanin production has been shown to play an important part in the virulence of *Ps. aeruginosa* and has been shown to be overproduced in some epidemic strains (Fothergill *et al.*, 2007). Pyocyanin can inhibit the beating frequency of cilia on respiratory epithelial cells *in vivo* (Sadikot *et al.*, 2005) and also induce apoptosis in neutrophils in culture (Usher *et al.*, 2002; Allen *et al.*, 2005). This apoptotic feature could result in the release of other compounds into lung spaces resulting in oxidative damage (Kipnis *et al.*, 2006). It may also be responsible for an influx of neutrophils into the lung (Lau *et al.*, 2004) which may account for the acidification of breath exudates that are observed in CF patients (Tate *et al.*, 2002; Di *et al.*, 2006).

The presence of pyocyanin has also been shown to depress the host response to *Ps. aeruginosa* bacteria by increasing the levels of IL-8, an immunosuppressive cytokine (Allen *et al.*, 2005). This suppression of the host's immunity could provide the *Pseudomonas* bacterium with a chance to establish itself within the lung and protect itself from further interventions. Furthermore, the impairment of the host's immune system has been shown to limit the rate at which apoptotic cells are cleared by macrophages (Bianchi *et al.*, 2008).

However, while it has been suggested that pyocyanin may impair CFTR channels in CF by disrupting ATPase (Lau *et al.*, 2004), this action is dependent on the presence of oxygen which has been shown to be lacking from mature biofilms within CF lungs (Worlitzsch *et al.*, 2002).

1.3.2.3 The Type III secretion system and exotoxin production

Ps. aeruginosa possesses the ability to directly damage the respiratory epithelial cells which it binds to, often through the production of exotoxin A. Exotoxin A is a proenzyme of molecular weight 66-71 kDa (Pitt, 1986). It enters target cells through receptor-mediated endocytosis and inhibits protein synthesis leading to cellular death (Sadikot *et al.*, 2005). Death arises because of adenosine diphosphate (ADP) ribosylation (Jenkins *et al.*, 2004), a post-transcriptional modification of protein occurring through the addition of one or more ADP and ribose groups (Sadikot *et al.*, 2005).

Exotoxin has also been shown to be associated with high levels of lethality and also dissemination of infection in animal models (Kurahashi *et al.*, 2004). The toxin can induce apoptosis of human mast cells in culture through capsase-dependant mechanisms (Jenkins *et al.*, 2004), which results in down regulation of anti-apoptotic pathways. Such down regulation of anti-apoptotic pathways could play a significant role in the pathological features that are observed within the CF lung, where the increase of apoptotic cells within the lung, along with other factors, could cause a decrease in lung function (Accurso, 2007) and potentially the influx of non-specific host defences such as phagocytosis.

The type III secretion system possessed by *Ps. aeruginosa* and other Gram-negative bacteria is mediated by pilin and requires contact between the bacterial cell and the epithelia in order to function (Sadikot *et al.*, 2005). This system allows the production, and injection, of at least four effector proteins (ExoS, ExoT, ExoY and ExoU) into host cells. The presence of these effector proteins varies from strain to strain and it has been shown that all strains possess ExoT and around 90% possess ExoY (Vance *et al.*, 2005).

In the case of the most recently discovered protein, ExoY has been shown to be an adenylate cyclase with similar homology to the adenylate cyclase found in

Bacillus anthracis and *Bordetella pertussis* (Yahr *et al.*, 1998), but little is known about its impact on cellular pathology.

Both ExoT and ExoS have been shown to have a high degree of homology and possess both guanine diphosphatase (GDPase) - activating activity and an ADP-ribosyl transferase domain (Cuzick *et al.*, 2006). Although both proteins have been associated with increasing the level of apoptosis (Alaoui-El-Azher *et al.*, 2006; Shafikhani and Engel, 2006) it is unclear if this is independent of one another, or that ExoT inhibits cellular repair of wounded cells (Geiser *et al.*, 2001) and that actual apoptosis is caused by ExoS (Shafikhani and Engel, 2006).

ExoU has been shown to cause high levels of irreversible damage to epithelial cells (Sadikot *et al.*, 2005) and could be responsible for the high levels of IL-8 secretion that are associated with *Pseudomonas* infection (Cuzick *et al.*, 2006).

1.3.2.4 Elastase and protease production

Within the normal human lung alveolar epithelia are lined with surfactant, a complex of lipoproteins that is designed to regulate bacterial clearance and reduces surface tension to increase the level of oxygen absorption (Alcorn and Wright, 2004). Surfactant is comprised of around 90% lipids and 10% protein, predominantly, Surfactant Protein-A (SP-A), SP-B, SP-C and SP-D (Malloy *et al.*, 2005).

The two main components that are specifically affected by *Ps. aeruginosa* proteases are SP-A and SP-D which have been shown to be present in decreased levels in the lungs of CF patients (Beatty *et al.*, 2005); these two proteins are mediators of innate host defences and could be partially responsible for the decrease of host immune function in CF lungs. In addition to this *Pseudomonas* also secretes a number of alkaline proteases which circumvent many functions of

both the adaptive and innate immune systems by promoting tissue invasion (Sadikot *et al.*, 2005).

Ps. aeruginosa also secretes elastase enzymes which have been shown to cleave SP-D (Alcorn and Wright, 2004) resulting in decreased mucocilliary clearance. Furthermore, elastase is responsible for disruption of the epithelial cell lining allowing the invasion of lung tissues by *Ps. aeruginosa* (Sadikot *et al.*, 2005). *In vitro*, it has been shown that inactivation occurs through the disablement of proteinase-activated receptor 2, believed to have a role in the innate immunity of the lung (Dulon *et al.*, 2005) and is presumably responsible for the loss of lung function that is observed in the lungs of CF patients.

1.3.2.5 Other factors

Like many other Gram-negative bacteria *Ps. aeruginosa* possesses a lipopolysaccharide (LPS) layer comprising of a lipid A structure that contains N and O acylated diglucosamine biphosphate backbones. While the same fundamental structure is present in *Pseudomonas* isolated from both CF patients and laboratory adapted strains, there is variation in the number of acyl groups as well as fatty acid types (Pier, 2007). These side chains have often been the basis for serologicical typing due to their antigenic properties (Sadikot *et al.*, 2005).

In the case of CF patients it has been shown that increased inflammation within the lung is due to LPS increasing permeability between epithelial cells (Eutamene *et al.*, 2005). It has also been shown that direct interaction between bacterial LPS and respiratory epithelial cells results in increased uptake and destruction of bacteria (Pier, 2007). This increase in destruction is due to a rapid response from epithelial cells resulting in the production of many proinflammatory cytokines which, in CF lungs, have been shown to be present but in lower levels than those of normal patients.

Ps. aeruginosa expresses a number of pili upon its cell surface. These pili are involved in *Pseudomonas* motility and are partially responsible for "twitching" (Kipnis *et al.*, 2006). This process has been shown to allow the bacterium to travel across hydrated surfaces and aids colonization within the CF lung by binding to epithelial cell membranes (Sadikot *et al.*, 2005). It has been shown that non-pili producing mutants of *Ps. aeruginosa* are less virulent than those which can produce pili, while those which are hyper-pilated mutants (i.e. expressing more) form denser biofilms (Chiang and Burrows, 2003).

The single polar flagellum of *Ps. aeruginosa* has been shown to mediate adhesion to asialoGM1 residues on pulmonary epithelial cells (Kipnis *et al.*, 2006) which in turn allows for initial biofilm formation to occur. Despite playing a role in this key pathogenic stage up to 39% of *Pseudomonas* isolates are non-motile (Zhang *et al.*, 2007) showing that once biofilms are formed flagella are lost. The loss of flagella, following the formation of biofilms, would also account for a lower immune response to the bacteria compared to their flagellated counterparts (Tseng *et al.*, 2006) enabling the infection to persist and develop.

The effect of *Ps. aeruginosa* infection on the presentation of CF symptoms are summarised in Table 1.4.

Cause	Mode of action	Clinical Significance
Pyocyanin production	• Reduces frequency of cilia beat • Induction of neutrophils apoptosis	• Decreased mucus clearance • Acidification of lungs
Biofilm formation	• Attachment to lung surfaces and secretion of exopolysaccharide	• Increased resistance to antibiotics • Frustrated immune system
Elastase production	• Disrupts lung epithelial lining	• Increased tissue invasion • Decreased lung function
LPS release	• Interaction between LPS and cell surface structures	• Increased bacterial attachment to lung surfaces • Inflammation of lung spaces from immune response
Degradation of surfactant proteins	• *Ps. aeruginosa* produces a number of alkaline phosphatases	• Further decrease in surfactant fluid levels, resulting in impaired lung clearance

Table 1.4: Summary of the effects of *Ps. aeruginosa* infection on CF symptoms.

1.4 Bacterial biofilms

1.4.1 Importance of biofilm associated infection

It is believed that biofilm associated infection accounts for 60% of bacterial infections treated within the developed world (Fux *et al.*, 2005). This type of infection presents a challenge to physicians when considering therapeutic strategies (del Pozo and Patel, 2007) and is primarily resident within medical devices (Vickery *et al.*, 2004) and artificial joint replacements (Zimmerli and Ochsner, 2003).

Once established as a biofilm within the lungs of CF patients, *Ps. aeruginosa* proves to be highly resistant to antibiotic therapy, with minimum inhibitory concentrations (MIC) increasing by up to 1000 fold (Li *et al.*, 2008). The resistance of *Pseudomonas* biofilms is in part due to the emergence of persister cells that can survive antibiotic treatment (Mulchay *et al.*, 2010).

1.4.2 Model systems of biofilms

Model systems of bacterial biofilms have been developed which aim to provide similar conditions to those encountered *in vivo*. These systems are often based on approaches similar to those described below.

Although many biofilm models have been developed in order to best mimic the *in vivo* conditions, these model systems can often be divided into two basic categories. Those which are close to *in vivo* conditions but are often difficult to reproduce and those which sacrifice mimicry for reproducibility.

1.4.2.1 Constant depth film fermentor

The constant depth film fermentor (CDFF) (Figure 1.6) functions as a sealed unit in which biofilms of a constant depth can be developed and studied. The unit operates through the addition of liquid media via a sealed inlet that is protected from the outside environment by a bacteria-proof filter (Hope and Wilson, 2006). Inside the sealed unit the biofilms are grown on circular discs of a desired material (usually polytetrafluoroethylene (PTFE; Zanin *et al.*, 2005) which can be recessed to produce biofilms of a chosen depth (Figure 1.6). Once biofilms have grown to the required depth a PTFE scraper removes excess growth which can then be discarded leaving an investigable biofilm which can subsequently be removed for testing.

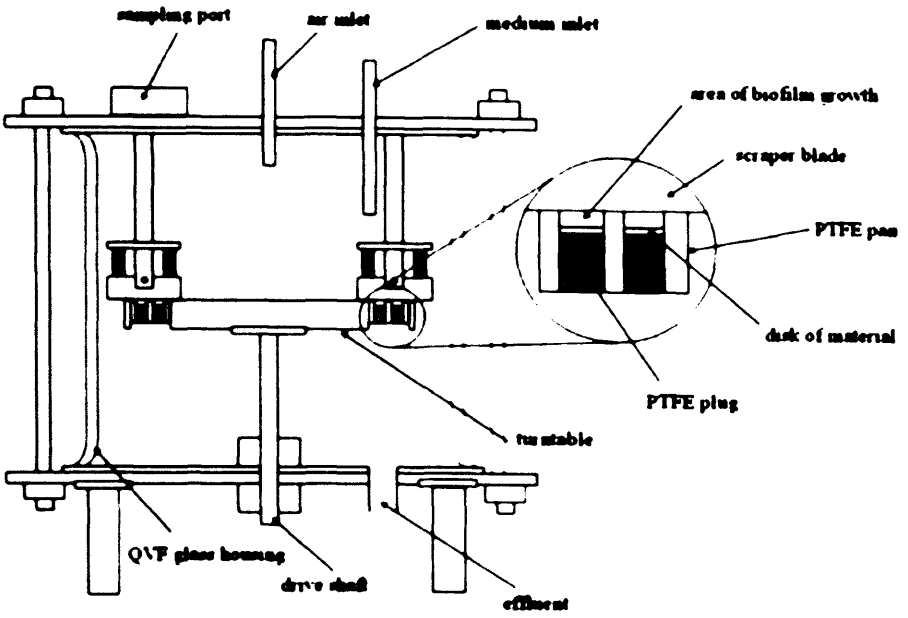


Figure 1.6: Schematic representation of a constant depth film fermentor. (UCL Eastman Dental Institute, 2010)

This method of biofilm growth has been used in a number of investigations to measure the susceptibility profiles of oral biofilms (Hope and Wilson, 2006), *Candida albicans* biofilms (Lamfon *et al.*, 2004), and the effect of photodynamic therapy on *Streptococcus mutans* biofilms (Zanin *et al.*, 2005) primarily within an oral environment.

The CDFF possesses a number of advantages over the other model systems which are discussed later. Firstly, the CDFF is a sealed environment in which growth conditions can be adapted to meet specific requirements (Peters and Wimpenny, 1988), in terms of both media and atmospheric composition. Secondly, the biofilm produced can be altered in terms of depth to possess architecture which is unique to its stage of development. These structures will be conserved, particularly those of the mature biofilm, in which anaerobic conditions within the lower regions have been established.

1.4.2.2 Flow system

The passage of fluid through tubing at a constant rate allows for the establishment of bacterial biofilms while removing unattached bacteria and is the characteristic feature of the flow system of biofilm formation.

This system has been used successfully in conjunction with microscopy techniques (Lee *et al.*, 2005). Using this approach, Lee and colleagues (2005) have been able to directly investigate the microstructure of a biofilm produced by a non-mucoid form of *Ps. aeruginosa* isolated from a CF patient without disturbing the delicate structures contained within, providing an advantage to other systems available. However, the flow system model may not provide the best mimic of *in vivo* conditions. This system also requires the sacrificing of tubes for analysis (Kirisits *et al.*, 2005) thereby limiting the length of time which films can be consistently studied. It has also been reported to be more difficult to use when compared to other systems (Fonseca *et al.*, 2007).

1.4.2.3 Sedimentation

A sedimentation biofilm, (Figure 1.7) unlike the other forms of biofilms discussed above, uses a singular supply of media and there is no media flow over the surface under investigation. Unlike naturally formed biofilms, cells once attached can propagate and form mature biofilms which unless disturbed should not slough off from the attachment surface.

This system has been used as a semi-quantitative method for measuring cell propensity to attach to surfaces (Sriramulu *et al.*, 2005) through the use of a crystal violet assay. This assay measures cell attachment based upon the association of a crystal violet stain to biofilm associated substances (Lee *et al.*, 2005). This method provides semi-qualitative data at best and is subject to easy disruption when adding or removing reagents, thereby limiting its usefulness.

Sedimentation biofilms have also been used to measure the effects of shear stress (Fonseca *et al.*, 2007) on *Ps. aeruginosa* biofilms a simpler manner than with flow cell models.

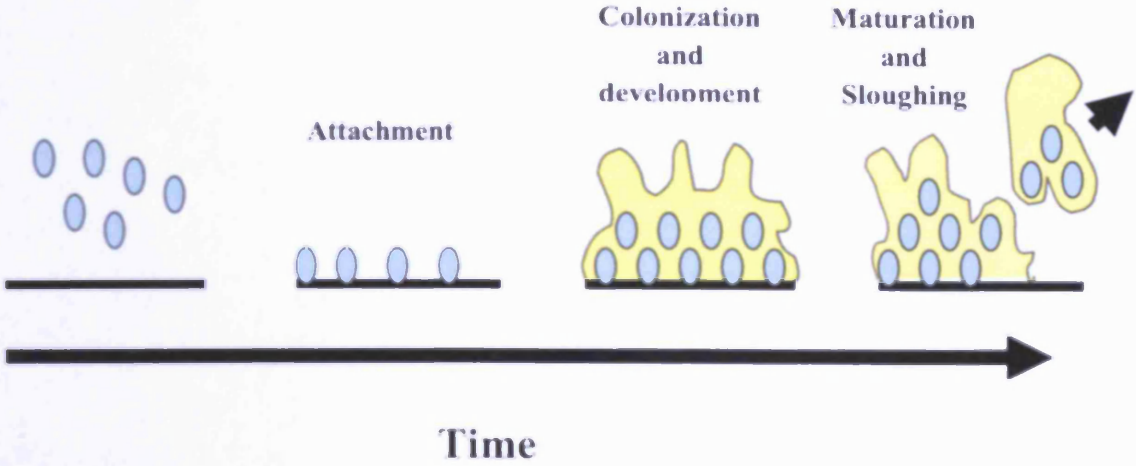


Figure 1.7: Schematic representation of the development of a biofilm.

1.4.2.4 Model systems of cystic fibrosis

As previously discussed CF patients are predisposed to multiple bacterial infections most notably and prevalent is *Ps. aeruginosa*. This increased susceptibility is due to characteristic CF traits which include both decreased mucocilliary clearance and also the production of thick mucus. In order to better understand the mechanisms and pathogenesis that underpin biofilm associated infections, particularly those which are prevalent in CF, in order to increase and develop new therapeutic approaches, the development of model systems is paramount.

The predominant model system that is used in the study of *in vivo* conditions of the CF lung appears to be the flow cell model (Krisits *et al.*, 2005), although murine models, CDFF models (Hentzer *et al.*, 2001) and static growth models (Fonseca *et al.*, 2007) have been used. Each model possesses particular

advantages and disadvantages when compared with one another (Table 1.5). In particular, flow system models of CF lungs are limited in usefulness by the fact that they require a constant flow of medium, while *in vivo* conditions of the CF lung reveal that movement of mucus is frustrated by the defect in the CFTR channel resulting in a decreased ciliary beat (Verkman, 2001). Flow system models often are difficult to produce (Fonseca *et al.*, 2007) and require the sacrifice of portions of biofilm.

In a model system of the CF lung it has previously been shown that *Ps. aeruginosa* exhibits multiple colony morphotypes which could play a role in the establishment and persistence of infection (Kirisits *et al.*, 2005). This is further supported in Lee *et al* (2005) in which non-mucoid *Ps. aeruginosa* isolates from CF patients were tested for production of virulence factors and also quorum sensing molecules. This study showed that non-mucoid strains of *Ps. aeruginosa* were able to form biofilms that, when assessed by a crystal violet assay, were shown to bind less crystal violet than PAO1 implying a less complex biofilm.

Other model systems of CF have explored the addition of an artificial mucus medium on the formation of micro-colonies (Sriramulu *et al.*, 2005). This study found that the addition of artificial mucus supplemented with amino acids promotes the growth of micro-colonies within a biofilm and also alters the phenotypic presentation of *Ps.* aeruginosa grown in this manner. This medium allows colony phenotypes to be more consistent with those found during the initial attachment and invasion phases of *Pseudomonas* colonisation.

System of Growth	Advantages	Disadvantages	Possible applications
Sedimentary	• Simplest to produce	• Highly fragile biofilms created • Poor reproducibility • Difficult quantification of bacterial numbers	• Early screening of drug efficacy
Flow Cell	• Direct visualisation of biofilm structure	• Difficult quantification of bacterial numbers • Specialist apparatus required	• Biofilms within water systems
CDFF	• Biofilm depth reproduction • Structure of biofilm alters over incubation period	• Difficult quantification of bacterial numbers • Specialist apparatus required	• Efficacy of drugs against mature biofilms

Table 1.5: Summary of advantages and disadvantages of different biofilm producing systems and potential applications.

1.5 Bacteriophage

Bacteriophages (phage) are viruses that specifically target bacterial cells and are believed to be amongst the most numerous organisms on Earth (Hanlon, 2007). Phages are also environmentally ubiquitous and reside within all reservoirs that are populated by potential host bacteria. Initially discovered by Twort and d'Hérelle in the early part of the 20th century phages have been used to treat a variety of

bacterial infections (Kutter *et al.*, 2010) and were used in both the First and Second World wars (Sulakvelidze *et al.*, 2001).

With the introduction of antibiotics, namely penicillin, in the early 1940s phage research and therapy have since been confined to the former Soviet Union and Eastern Europe, where phages are currently used in clinical practice. This restricted geographical application has primarily been due to Western scepticism over the benefits claimed (Barrow and Soothill, 1997) and also due to political history (Sulakvelidze *et al.*, 2001).

1.5.1 Structure and replication

The replication of phage centres on one of two cycles, the lytic or the lysogenic cycle (Sulakvelidze *et al.*, 2001). In both these cycles viral attachment to host cells is the initial stage with host cell lysis being the culmination of replication (Figure 1.8). Viral replication is based on the hijacking of the host's metabolic machinery to produce and assemble progeny; this self replication makes the phage an ideal candidate for antimicrobial therapy as this would reduce the number of repeat doses that would be required in order to eliminate a bacterial population.

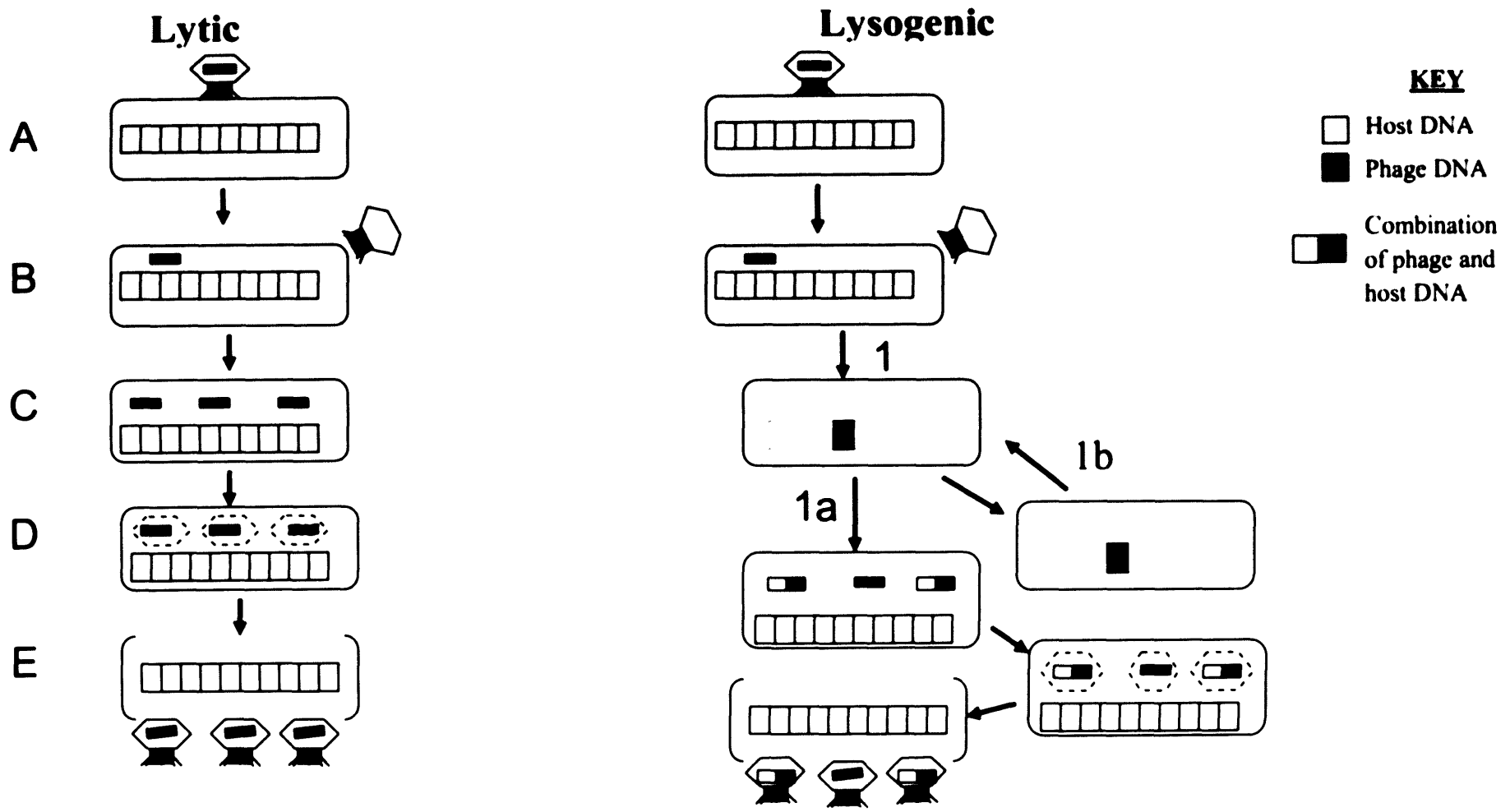


Figure 1.8: Lysis and Lysogeny. The lifecycles of phage. In both lysis and lysogeny bacteriophage follow the same process of attachment (A), injection of genetic material; (B), replication and packaging of viral progeny; (C-D), before the lysis of the cell (E). However, in the lysogenic cycle viral genetic material can be inserted into the host DNA whereby it can lay dormant (1b) until reactivated (1a). Adapted from Sulakvelidze *et al* (2001).

The ability of phage to undergo a lytic lifecycle is the underpinning theory behind phage therapy due to the ability of the phage to destroy the host cell upon replication (Watanabe *et al.*, 2007).

The use of lysogenic phages is best confined to genetic engineering and gene delivery, as genetic material is incorporated into the host genome and is then replicated with bacterial growth (Clark and March, 2006) which would allow for the successful replication of desired genetic sequences.

Phages are usually between 20 and 200 nm in size and while a great deal of morphological variability exists, the majority of phages tend to exhibit some structural similarities. The head (or capsid) of phage is a protein structure that is usually icosahedral in shape (as in the *Pseudomonas* phage ΦKZ; Fokine *et al.*, 2005) and contains the viral genome (Hanlon, 2007). In the case of some *Pseudomonas* phages the capsid structure can vary from the icosahedral structure (Hanlon, 2007) or be absent altogether, as is the case with the Inovirus family, where the primary structure is helical in arrangement and consists of coat proteins surrounding the genome (Holland *et al.*, 2006). The purpose of the capsid is to protect the genetic material from degradation by the extracellular environment. The viral genome can exist as either single or double stranded DNA or RNA; although it is often double stranded DNA and the *Caudovirales* family (tailed phages) of phages account for approximately 95% of published literature.

In the case of *Ps. aeruginosa* specific phages there has been observed a lower ratio of guanine to cytosine (G+C) within their genome when compared to the host genome of PAO1 (54.4:66.6%) and is contrary to the similarities in content that is usually observed in other phage/host comparisons e.g. *Mycobacterium tuberculosis* (63.6:65.6%; (Kwan *et al.*, 2006). This discrepancy could be accounted for in a number of different ways: 1) recent phage invasion from a host with a lower G+C content; 2) genetic conservation throughout the evolutionary history of the phage; and, 3) lateral gene transfer

(Kwan *et al.*, 2006). In the case of *Pseudomonas* phages, particularly those from the *Myoviridae* family (such as ΦKZ and EL) (Hertveldt *et al.*, 2005; Lavigne *et al.*, 2006) the potential for evolutionary divergence to exist is shown by the lack of similar homology to other members of the same family.

In order to mediate attachment to host cells, some phages possess tail fibres with the ability to contract. These have been well characterised in the Enterobacteria phage T4 of *Escherichia coli* which possesses a number of tail fibres which interact with host cell surface receptors (usually LPS molecules or OmpC (surface antigen) proteins) (Rossmann *et al.*, 2004). Once a number of tail fibres have bound to the host cell, the tail sheath shortens to allow gp5 residues on the baseplate (Figure 1.9) to puncture the cell membrane.

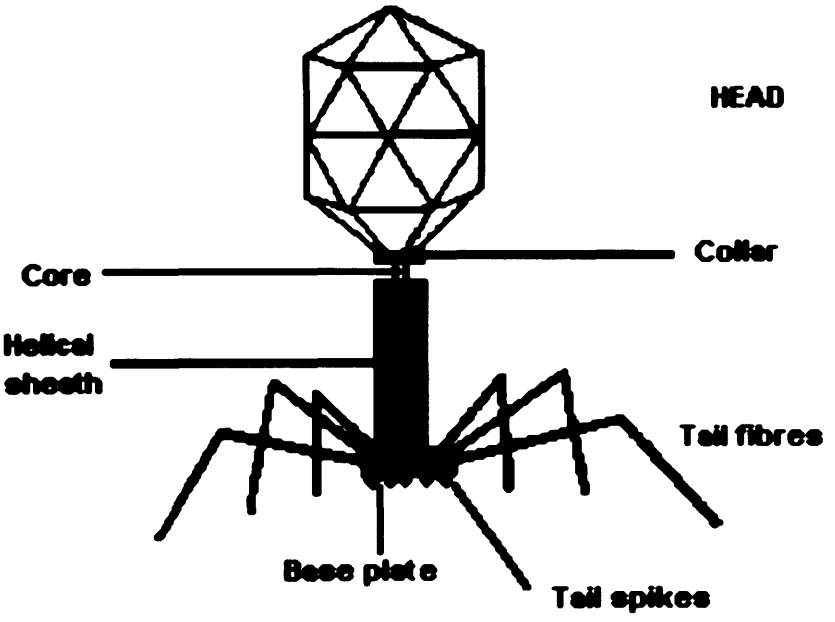


Figure 1.9: Generalised structure of a T4-like bacteriophage.

1.5.2 Bacteriophages as antimicrobial agents

Within Western published literature there seems to be a lack of information surrounding the efficacy of phage therapy in humans, although data for both animal models and *in vitro* studies against a variety of organisms exist.

With the application of phage to the treatment of infection a number of variables have to be borne in mind when considering an administration route (Table 1.6). Although phages are normally assumed to be highly specific to their host, it is possible to develop promiscuous phage under selective conditions (Cooper *et al.*, 2011).

Admin. Route	Advantages	Disadvantages
Oral	• Economical • Can be self-administered • Non-damaging to normal flora • Self-replication of phage	• Neutralisation of gastric acid would be necessary • Endotoxin release from lysed bacteria • Absorption into the body
Ophthalmic e.g. eye drops	• Simple delivery method • Self-replication of phage • Self-administered	• Endotoxin release • Could only be used for eye infections
Topical e.g. cream	• Simple delivery • Self replication of phage • Self administered	• Poor penetration of phage • Limited uses e.g. burns, skin conditions etc
Parenteral including subcutaneous, intravenous, intramuscular and intraperitoneal	• Would require specialist administration although training could be provided to allow self-administration • Rapid spread throughout body • Self-replication of phage	• Clearance by immune system • Time taken to reach site of infection • High levels of purity required • Endotoxin release
Inhalation	• Highly useful for lung conditions e.g. pneumonia • Could be self-administered • Self-replication of phage	• Loss of dosage in airways • Endotoxin release • Viability of bacteriophages following shear stresses • Mucus penetration

Table 1.6: Potential routes of phage administration. Adapted from Clark and March (2006).

In the case of intravenous application, it has previously been shown in a murine model of burns that phage therapy can reduce levels of *Ps. aeruginosa* infection and improve survival rates (McVay *et al.*, 2007). In this particular case survival rates based upon a single phage dose increased from 6% (in non-treated mice) to between 22% and 87% (McVay *et al.*, 2007). This study also showed that the route of administration proved important to clinical outcome with intraperitoneal (i.p.) administration shown to be the most effective. However, despite improvement in outcome, the most logical route of application for phage in this instance would be topical where it has been previously demonstrated in humans that topical application of a phage cocktail was effective (Marza *et al.*, 2006).

Although an actively replicating phage would arguably be most effective in treating bacterial infections, some studies have used non-replicating phage as an alternative (Hagens *et al.*, 2004). The study by Hagens and colleagues (2004) showed that a non-replicating phage was effective at reducing the level of PAO1 infection in mice, although it was less effective than a replicating phage, presumably due to the repeated re-infection of PAO1. Perhaps what is most striking is the survival rates of mice treated with replicating and non-replicating phage, where in the latter case 70% of mice survived the observation period compared to just 20% in the case of a replicating phage. This difference in survival rates is assumed to be due to the release of LPS from lysed bacteria (Hagens *et al.*, 2004) which has been shown to be recognized by TLR4-bearing (Toll like receptor) macrophages, resulting in the release of proinflammatory cytokines such as IL-1 and interferon gamma (IFN-γ) (Gould *et al.*, 2004). However, in the case of the non-replicating phage, host cell integrity was maintained despite cell death, therefore preventing the release of LPS and allowing the bacterial cell to be phagocytised, which in turn will reduce the host response to exopolysaccharide (EPS).

The addition of phage has also shown the ability to reduce the viscoelastic properties of purified EPS of *Ps. aeruginosa* from 2.33 mega Pascal (mPA) to

1.76 mPa after 20 hours while also reducing the bacterial content of these biofilms compared to negative controls (Hanlon *et al.*, 2001).

Although phage therapy in humans is unlikely to supplant antibiotic therapy in the foreseeable future for a variety of reasons including patient compliance and the lack of reliable scientific data surrounding efficacy and side effects, phage therapy does present a number of prospective advantages over antibiotic therapy (Table 1.7).

Bacteriophages	Antibiotics	General Comments
• Highly specific	• Often damages microflora	• High specificity requires pathogen identification for phage therapy
• Low level of resistance	• Increasing levels of resistance to multiple antibiotics	• Phage can be developed against a wide range of hosts • Development of resistance will be confined to particular bacteria for phage
• Actively replicating	• Cleared from body	• Less frequent dosing of phages required
• No serious side effects known	• Allergic response e.g. penicillin • Some antibiotics toxic at high doses	• Phage therapy could result in toxic shock from bacterial lysis
• New phage isolation - easy and relatively simple from environmental sources	• Development of novel antibiotics takes years • New antibiotics tend to be structural modifications	• Believed that every bacterium will possess at least one phage due to natural selection • Would allow for "custom" treatments based on organisms present
• Potentially immunogenic	• Small molecules that do not elicit an immune response except in cases of allergic response	• Encapsulated phage preparations to minimize immune system exposure

Table 1.7: Comparison between antibiotics and bacteriophages for prophylactic and/or therapeutic use. Adapted from Sulakvelidze *et al.*, (2001).

1.5.3 Current use of bacteriophage

Although usage as a therapeutic agent in humans is primarily confined to the former Soviet Union and Eastern Europe, phages have found some applications in the West.

One such recent example is the approval by the US Food and Drug Administration (FDA) in 2006 for the use of a combination of 6 different phages to be added to ready-to-eat meat and poultry products. This was done in order to combat the presence of *Listeria monocytogenes*, the causative agent of listeriosis which accounts for some 2500 cases per year in the USA with 20% of these cases proving fatal (Lang, 2006). Due to the nature of the phage preparation it is believed that some 170 strains of *L. monocytogenes* are susceptible and the risk of resistance developing in this combination product is reduced. While the FDA has authorised the use of this preparation, approval from the US Department of Agriculture which oversees the various meat inspection industries within the US (Lang, 2006) is still being sought. Phage preparations have also been suggested for use in the meat industry against *Salmonella* species (Atterbury *et al.*, 2007) and have recently been approved by the FDA to combat *Escherichia coli* contamination of cheese in 2011(Intralytix Inc., 2011).

Phage preparations have also found a use in aquaculture and have been demonstrated to reduce the appearance of infections in marketable-sized fish in the Far East. The most notable of these infections is caused by *Lactococcus garvieae* in which it was observed that there was no presence of bacteria within the spleen of phage treated fish after 48 h (Nakai and Park, 2002).

Haemorrhagic ascites are a significant disease of fish that can strike at any stage of development and are the result of infection by *Ps. plecoglossicida* and is particularly prevalent in the Asu region of Japan, a primary area for the

freshwater culture of fish. The application of phages to this area was found to reduce the mortality of fish from 65% to just 22.5% (Nakai and Park, 2002).
It should also be noted that various phage preparations are licensed for use as pesticides within the US (Jones *et al.*, 2007). However, there are currently no such preparations available within the UK, although phage based diagnostics are being readily investigated (Kalantri *et al.*, 2005; Pai *et al.*, 2005) and may possess some advantages over traditional diagnostics. In May 2011, the FDA granted clearance for the marketing of the KeyPath MRSA/MSSA Blood Culture Test that is based upon phage amplification technology (FDA, 2011).

Due to the well characterised and publicised rise of antibiotic resistance over the past decade a number of smaller start up companies have begun investigating and developing phage preparations for use on humans. These phage preparations are often for topical use only and are currently in clinical trials (Table 1.8; Wright *et al.*, 2009) or designed for non-human application.

Company	Product Name/Type	Target organism	Licensing stage
Intralytix Inc.	ListShield™ Phage cocktail	*Listeria monocytogenes*	• FDA and USDA approved for use direct on food • EPA approved for surface applications • EU approved
	EcoShield™ Phage cocktail	*Escherichia coli* O157:H7	• No information available on company website • Also actively researching on other bacteria
AmpliPhi Biosciences Corp.	BIOPHAGE-PA	*Pseudomonas aeruginosa*	• Currently undergoing Phase III clinical trials
Gangagen Inc.	StaphTAME: recombinant phage based protein	Methicillin resistant *Staph. aureus*	• Pre-phase I
Novolytics	N/A[1]	Methicillin resistant *Staph. aureus*	• Research only based upon lysogenic phages
Omnilytics	AGRIPHAGE™	Multiple: Including Xanthomonas campestris pv. vesicatoria, or Pseudomonas syringae pv. tomato	• Commercially available

Table 1.8: Commercial companies undertaking phage research including licensed products. [1]: indicates no product name associated with research.

1.6 Aims and objectives

The aim of this study is to investigate the use of bacteriophages to combat *Ps. aeruginosa* infections. This will be done in the following manner:

- Characterise 14 *Ps. aeruginosa* bacterial isolates and 4 phage strains
- Investigate the effect of environmental conditions upon bacterial content and exopolysaccharide content of *Ps. aeruginosa* biofilms
- Develop and validate biofilm models for the growth of *Ps. aeruginosa*
- Develop and validate an alternative method to measure phage activity
- Assess and enhance the lytic activity of *Ps. aeruginosa* phages and design a phage cocktail
- Assess the activity of a phage cocktail against biofilms of *Ps. aeruginosa* alone and in combination with other components
- Investigate the delivery, dosing and quality control related issues surrounding bacteriophage preparations.

At the end of this investigation it is hoped a phage based preparation that possesses activity against both planktonic and biofilm types of *Ps. aeruginosa* will be developed.

Chapter 2

General materials and methods

All materials used in this project were obtained from Fisher Scientific (Loughborough, UK) or Sigma Aldrich (Dorset, UK) unless otherwise stated in the text.

All media and buffers were sterilized by autoclaving at 121°C for 15 min (British Pharmacopeia, 2011a) unless otherwise stated in the text.

All deionised water used in the current investigation was prepared on an ELGA PURELAB Option BP15 (Inorganics at 25°C; between 1-15 MΩ-cm, total organic carbon (TOC) <30 ppb; ELGA Labwater, Marlow, UK).

2.1 Microbial cultures

2.1.1 Bacterial strains

All *Ps. aeruginosa* strains used in this project were kindly provided by Dr E. Mahenthiralingam (School of Biological Sciences, Cardiff University, Cardiff, UK). *Ps. aeruginosa* NCTC 10332 was obtained from the National Collection of Type Cultures (NCTC) (Health Protection Agency, London, UK). All strains are detailed in Table 2.1.

Ps. aeruginosa cultures were stored at -80°C in 10% glycerol and routinely cultured from frozen by streaking to a Tryptone Soy Agar (TSA; Oxoid, Cambridge, UK) slope and incubated at 37°C for 24 h. Working slopes were stored at 4°C for 2 weeks.

2.1.2 Bacteriophage strains

Bacteriophage strains of GL-1, C10176 Small (C10176-S), C10176 Large (C10176-L) and L phage Medium (LP-M) used in this project were kindly provided by Prof. G.W. Hanlon (School of Pharmacy and Biomolecular Sciences, Brighton University, Brighton, UK). Phage cultures were grown from frozen stocks by the agar overlay technique (Adams, 1959) using *Ps. aeruginosa* PAO1 as a host and were stored at -80°C in 10% glycerol.

2.2 Culture preparation and standardisation

2.2.1 Bacterial culture

2.2.1.1 Preparation of bacterial cultures

Bacterial growth was washed from the surface of a single overnight TSA slope using 5 mL of tryptone sodium chloride (TSC; containing 1 g/L tryptone (Oxoid, Cambridge, UK) and 8.5 g/L sodium chloride) and centrifuged at 2600 x *g* for 15 min. The supernatant was then discarded and the bacterial pellet resuspended in 5 mL of fresh TSC. Overnight bacterial tryptone soy broth (TSB; Oxoid, Cambridge, UK) cultures were centrifuged at 2600 x *g* for 15 min and then resuspended in 5 mL of TSC.

2.2.1.2 Standardisation of bacterial cultures by optical density

Washed bacterial suspensions were then standardised by optical density at 600nm (OD_{600nm}) to contain between 1 and 5 x 10^8 colony forming units per millilitre (CFU/mL) (Figure 2.1) on a Helios α spectrophotometer (ThermoFisher Scientific, MA, USA) that had been previously blanked with an appropriate solution (either TSC or Tryptone soy broth (TSB; Oxoid, Cambridge, UK) alone).

2.2.2 Bacteriophage culture

2.2.2.1 Routine culture of bacteriophage suspensions

Five millilitres of phosphate buffered saline (PBS) were pipetted onto the surface of prepared phage plates. The 65% (w/v) "sloppy" agar layer was then scraped into a 50 mL polypropylene co-polymer (PPCO) centrifuge tube (Nalgene, NY, USA). Suspensions were centrifuged at 11000 x *g* for 15 min at 4°C then the supernatant was passed through 0.45 and 0.2 μm membrane filters (Millipore, Cork, Ireland) to remove bacterial debris. Phage suspensions were stored at 4°C for up to 1 month.

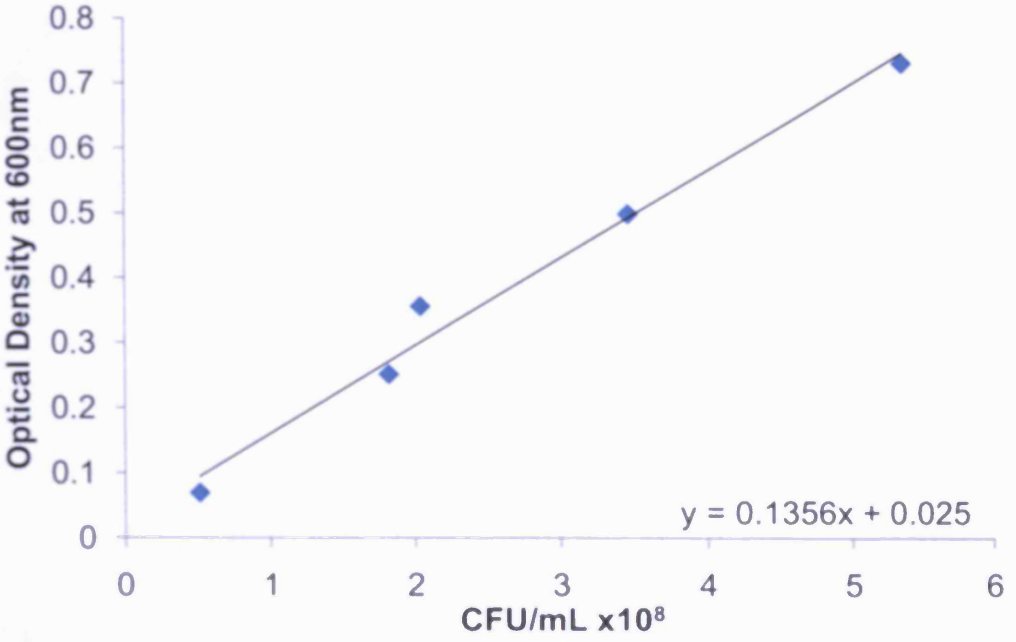


Figure 2.1: Relationship between OD_{600nm} and viable count of *Ps. aeruginosa* strain NCTC 10332. Line represents the best fit by linear regression.

	Strain Name	Source	Comment
	PAO1	Clinical, non-CF	*Ps. aeruginosa* genome sequencing strain (Stover *et al.*, 2000)
Standard	NCTC 10332	NCTC	Also known as ATCC 10145. Quality control strain for API products. Alginate non-producing strain (Edwards and Saunders, 2001)
	C3652	CF	Epidemic Manchester CF strain type (Jones *et al.*, 2001)
	C3719	CF	Epidemic Manchester CF strain type (Jones *et al.*, 2001)
	C3786	CF	Melbourne *Ps. aeruginosa* CF strain - unique (Armstrong *et al.*, 2003)
CF epidemic	LES-400 (LES)	CF	Liverpool epidemic strain - Hypervirulent strain predominant epidemic strain in the UK (Salunkhe *et al.*, 2005; Fothergill *et al.*, 2007)
	Midlands 1-9245 (Mids1)	CF	Midlands 1 epidemic strain (Smart *et al.*, 2006)
	P8959	CF	Liverpool epidemic strain - predominant epidemic strain in the UK (Fothergill *et al.*, 2007)
	C1913	CF	A55 unique - Unique genotype from Vancouver patients (Lewis, 2005)
CF Non-epidemic	C2238	CF	A61 unique - Unique genotype from Vancouver patients (Lewis, 2005)
	C2846	CF	A55 unique -Unique genotype from Vancouver patients (Lewis, 2005)
	C3597	CF	Non-epidemic Manchester CF strain type (Jones *et al.*, 2001)
	C4503	CF	A55 unique - Unique genotype from Vancouver patients (Lewis, 2005)
	PAK-SR (PAK)	CLIN	Streptomycin^{R} parent of Fla-/Pil- mutants of PAK. Sm50. (Ishimoto and Lory, 1989)

Table 2.1: ***Ps. aeruginosa* strains used in the current investigation.**

2.2.2.2 PEG concentration of phage suspensions

For the preparation of concentrated phage suspensions a polyethylene glycol 8000 (PEG) concentration step was performed. The sloppy agar layer from 30 plates of semi-confluent bacterial lysis was removed with 5 mL PBS per plate and transferred to a 50 mL PPCO centrifuge tube. Suspensions were centrifuged at 13000 x *g* for 15 min at 4°C. The supernatant was passed through 0.45 and 0.2 µm membrane filters (Millipore, Cork, Ireland) into a 150 mL conical flask that contained 8% (w/v) PEG 8000 and 2.3% (w/v) sodium chloride and incubated at 4°C overnight with constant mixing. Following incubation the solution was centrifuged at 10000 x *g* for 30 min at 4°C discarding the supernatant upon completion. The resulting pellet was resuspended in 1 mL of fresh PBS and centrifuged at 5000 x *g* for 20 min at 4°C after which the supernatant was passed through 0.45 and 0.2 µm filters (Millipore, Cork, Ireland).

2.3 Enumeration techniques

2.3.1 Bacterial enumeration

The total viable count of bacterial suspensions was performed on all standardised cultures via the drop count method of Miles and Misra (1938). In brief, 100 µL of bacterial suspension was added to 900 µL of TSC buffer and then vortexed. This process was repeated until the desired level of dilution was reached. Three spots of 10 µL of each dilution were then spotted onto duplicate pre-prepared TSA plates. Plates were then incubated at 37°C for 24 h. The concentration of the suspension was then calculated by counting the number of bacterial colonies at a dilution containing 3-30 colonies. This process was validated by one way ANOVA at the 95% confidence interval (CI) on 10 separate dilution series (P=0.486).

2.3.2 Bacteriophage enumeration

Total viable count of phage suspensions were performed according to the agar overlay method (Adams, 1959). In brief, 5 mL of 65% (w/v) TSA (26 g/L) was prepared and supplemented with 100 μL of 50 mM calcium chloride and sterilized. Molten agar was stored at 50°C in a water bath until required. One hundred μL of PAO1 suspension (containing between 1 and 5 x10^8 CFU/mL) was added to the molten agar and gently swirled. To this 100 μL of different dilutions of a bacteriophage suspension was added. The molten agar was then poured over the surface of a pre-prepared TSA plate and spread to ensure even coverage. The "sloppy" agar layer was allowed to set at room temperature before incubation at 37°C for 24 h. Following incubation, plaques (clearing in the bacterial lawn) were counted at a dilution containing between 3 and 30 plaques and the number of plaque forming units per millilitre (PFU/mL) determined. This protocol was validated by one way ANOVA at the 95% CI on 10 separate dilution series of phage (P=0.244).

2.4 Sample preparation for microscopy

2.4.1 Transmission electron microscopy

Concentrated stocks of phage were prepared as previously described (Chapter 2 Section 2.2.2.2). Five microlitres of concentrated phage suspension (approx.10^{14} PFU/mL) was added to 3.05 mm copper 200 mesh grids (Agar Scientific, Stansted, UK) coated in 0.8% (w/v) pioloform powder in chloroform (Agar Scientific, Stansted, UK) and air dried at room temperature for 15 min. Copper grids were then stained in 1% (w/v) methylamine tungstate (Agar scientific, Stansted, UK) for 15 min. Samples were viewed on a Phillips CM12 Transmission electron microscope (TEM; Phillips Healthcare, MA, USA) and imaged on a Megaview III TEM digital camera (Soft Imaging System GmbH, Münster, Germany) using iTEM software (Soft Imaging System GmbH, Münster, Germany). Representative images were taken based upon 15 fields of observation.

2.4.2 Scanning electron microscopy

Bacterial biofilms that had been cultured either as sedimentary biofilms or as CDFF biofilms were prepared for scanning electron microscopy (SEM) imaging as follows. Samples were gently rinsed in 5 mL PBS to remove any loosely adherent cells before fixation in 2 mL of 2% glutaraldehyde (GA) in PBS for 1 h. Samples were rinsed twice in PBS, and then stained with 1% osmium tetroxide (Agar Scientific, Stansted, UK) in PBS for 1 h. Following staining, samples were sequentially dehydrated in ethanol for 5 min at each of the following concentrations, 50% (v/v), 70% (v/v), 90% (v/v) and twice in absolute ethanol.

Samples were dried by critical point drying then affixed to carbon discs and mounted on aluminium studs (Agar Scientific, Stansted, UK). Samples were gold coated and imaged using a Phillips XL20 SEM (Phillips Healthcare, MA, USA) at an accelerating voltage of 20 or 30 kilovolts (kV). Representative images were taken based upon 15 fields of observation.

2.5 Biofilm preparation

2.5.1 Sedimentary biofilms

Ps. aeruginosa strains were prepared and standardised as previously described (Chapter 2; Section 2.2.1). To each well of a 6-well tissue culture plate (Corning Inc., NY, USA) that contained 4 mL of growth medium, either a polytetrafluoroethylene (PTFE) disc (Goodfellow Cambridge Ltd, Huntingdon, UK) or a No. 2 glass coverslip (Menzel-Gläser, Saarbrückener, Germany) was added followed by 1 mL of bacterial suspension (containing between 1 and 5×10^8 CFU/mL). Culture plates were then incubated at 37°C in normal air or 95% air: 5% CO_2 depending on experimental protocol. Prior to use biofilms were gently rinsed in 5 mL PBS.

2.5.2 Constant depth film fermentor (CDFF) biofilms

2.5.2.1 CDFF arrangement

Previously autoclaved CDFF components were removed from the autoclave bags inside of a Herasafe Category 2 biosafety cabinet (Thermo Scientific, MA, USA) and subjected to UV irradiation for 1 h as an additional precaution. PFTE holders were inserted into the rotating platform before the addition of rods and discs. Discs were recessed to the required depth (between 10 and 160 µm) using the tools supplied, subjected to UV irradiation for 1 h and then mounted onto a rotating axle. The glass chamber was then added to the apparatus and the base sealed using silicone grease (Dow Corning, MI, USA). The scrapers, lid, and HEPA filters (Whatman Plc, Maidstone, UK) were then added.

2.5.2.2 Culture conditions

The assembled CDFF apparatus (Figure 2.2) was transferred to a 37°C incubator with a 95% air: 5% CO_2 atmosphere. Bacterial suspensions in TSB (containing between 1 and 5 x10^8 CFU/mL) were perfused in at a rate of 1 mL/min for 15 min using a B. Braun Melsungen Ag perfusor (B. Braun Melsungen AG, Melsungen, Germany). Once dispensed, bacterial suspensions were allowed to attach for 15 min prior to the perfusion of TSB at 0.2 mL/min using a Watson Marlow 502S peristaltic pump (Watson-Marlow Pumps Group, Cornwall, UK). Medium was then continually perfused into the CDFF for 96 h at a rate of 0.2 mL/min.

2.5.2.3 CDFF decontamination

Upon cycle completion biofilm samples were removed and stored in sealed containers for up to 1 h. The base apparatus containing the motor and electrical components was removed and sprayed with 70% v/v ethanol. The CDFF apparatus was dismantled and autoclaved at 121°C for 15 min (British Pharmacopeia, 2011a). The base, lid and glass cylinder of the CDFF were rinsed with water and dried overnight. PTFE discs, rods and holders were

washed in water and then soaked for 1 h in 70% (v/v) ethanol and then dried overnight. All tubing and remaining components were treated by the passage of (0.2 mL/min for media inlet tubing and bacterial tubing), or immersion in (waste tubing), 2.5% Steris HAMO PAA solution (Steris, Biel, Switzerland) for 1 h before rinsing with water and overnight drying. All components were re-autoclaved prior to use.

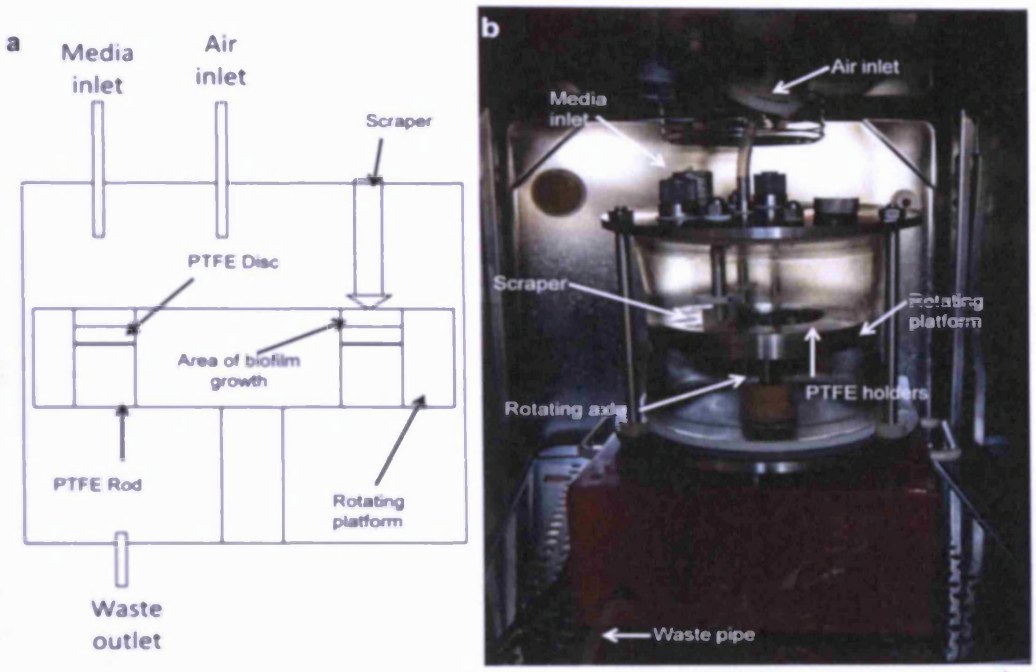


Figure 2.2: Constant depth film fermentor apparatus used in the current investigation. a) shows schematic representation of apparatus and b) shows apparatus *in situ*.

2.5.3 Quality control

In order to ensure purity, samples from biofilms and waste containers were streaked to TSA plates and Kings medium A (Fluka, WI, USA) and then incubated overnight at 37°C. The run was deemed to be successful if only one colony type was present and the Kings medium had turned blue/green.

2.6 Statistical analysis

All data were statistically analysed in the Minitab 15 Statistical Software (Minitab Ltd, UK) by either a one way ANOVA or a Tukey-Kramer A posterior test at the 95% CI unless otherwise stated in the text.

Chapter 3

Characterisation of bacterial and bacteriophage strains and the quantification of exopolysaccharide in biofilms

3.1 Introduction

Ps. aeruginosa is of particular importance to CF patients as it is the primary cause of respiratory infection with around 80% of patients colonized by the age of 20 (Saiman and Siegel, 2004). The organism is able to survive and persist despite intervention in the lung due to the ability to form complex biofilms with characteristic mucus build-up, resulting in an increase in minimum inhibitory concentration (MIC) of numerous antibiotics up to 1000 fold greater than when grown as planktonic cultures (Li *et al.*, 2008). This is in part due to the production of large quantities of exopolysaccharide (EPS) containing alginate, which coats the *Ps. aeruginosa* preventing the uptake of some cationic antibiotics (Haagensen *et al.*, 2007; Platt *et al.*, 2008).

Alginate is a linear polysaccharide consisting of β-D-mannuronic and α-L-guluronic acid residues that are joined in a 1-4 linkage and has been shown to be an important part of *Ps. aeruginosa* biofilms (Hentzer *et al.*, 2001) making it a useful target for quantification of biofilm formation. Currently the main method of biofilm quantification is the crystal violet assay (Gooderham *et al.*, 2008). This method is based on the adherence of bacterial cells to a surface and the interaction of crystal violet stain with the EPS of the established biofilm. This unfortunately is an indirect method of EPS quantification and is highly variable due to the fragile nature of the biofilm produced. The carbazole assay relies on the acidic degradation of the alginate polysaccharide. This forms a 5-formylfuran-2-carboxylic acid intermediate which then reacts with the carbazole to produce the characteristic purple colour (Usov, 1999). Although a direct measurement of the amount of uronic acid within a *Pseudomonas* biofilm is provided by the carbazole assay, it is reliant on EPS production to be consistent, which is known not to be the case (Purevdorj-Gage *et al.*, 2005).

The current literature describes numerous conditions and media that are designed to mimic the *in vivo* conditions of the CF lung (Worlitzsch *et al.*,

2002; Brennan *et al.*, 2007). It is of great importance to create a standard set of conditions that will mimic as closely as possible this environment for testing of the effectiveness of future therapeutics. The synthetic media that are currently available vary greatly in composition and also in their ability to accurately model the *in vivo* CF lung conditions. Perhaps the forerunner of these synthetic media that most closely models the *in vivo* conditions is the artificial sputum medium (Sriramulu *et al.*, 2005). However, this medium is too expensive to scale up to larger applications such as biofilm growth within a CDFF.

This chapter seeks to characterise 14 strains of *Ps. aeruginosa* including some of the more clinically-relevant strains within the UK upon multiple criteria and to also characterise 4 strains of *Ps. aeruginosa* phage. In addition the effect of varying environmental conditions upon the bacterial content and EPS content of *Ps. aeruginosa* biofilms will also be investigated.

3.2 Materials and methods

3.2.1 Bacterial strains

All *Ps. aeruginosa* strains (Chapter 2; Table 2.1) were used for initial characterisation. For subsequent testing of the effects of media supplementation on the rate of growth and EPS production strains P8959, C2238, NCTC 10332 were used. Bacterial strains were prepared and standardised as previously described (Chapter 2; Section 2.2.1).

3.2.2 Characterisation of bacterial strains

3.2.2.1 Gram stain

Ps. aeruginosa strains were suspended in a drop of deionised water on a glass slide using a flame-sterilised inoculating loop. The bacterial growth was spread, air dried and then heat fixed. Slides were covered in crystal violet for 1 min and washed in deionised water. Gram's iodine was added for 1 min before

decolourization with 70% (v/v) ethanol. Safranin was then added for 45 s to counter-stain before the addition of deionised water.

Slides were blotted dry and viewed under oil immersion at x1000 magnification using an Olympus BX50 Microscope (Olympus, Watford, UK). Images were taken using an Olympus DP10 camera (Olympus, Watford, UK) and visualised using Corel Paint Shop Pro (Corel Corp, Maidenhead, UK).

3.2.2.2 Exopolysaccharide stain

The following methodology was adapted from Harrison-Balestra *et al.* (2003). Glass coverslips 18 x 18 x 0.17 mm (Menzel-Gläser, Saarbrückener, Germany) that had been previously cleaned with absolute ethanol were added to 6 well tissue culture plates (Corning Inc., NY, USA). One hundred microlitres of bacterial suspension (containing between 1 and 5 x 10^8 CFU\mL) and 900 µL fresh TSB were added to each well and then incubated at 37°C for 6 h.

Coverslips were removed with flame sterilised tweezers and one side of the coverslip was then washed with ethanol using a sterile cotton wool swab, attached to a slide and allowed to air dry before being heat fixed.

Once cooled, the slides were stained with a 2:1 mixture of aqueous Congo red (Bios Europe, Lancs, UK) and 10% Tween 80 for 15 min before being washed with deionised water. Slides were then stained with Zeihl carbol fuschin (Pro-Lab Diagnostics, South Wirral, UK) for 6 min before being washed in deionised water and allowed to air dry. Slides were then blotted dry and viewed as above.

3.2.2.3 BBL crystal identification

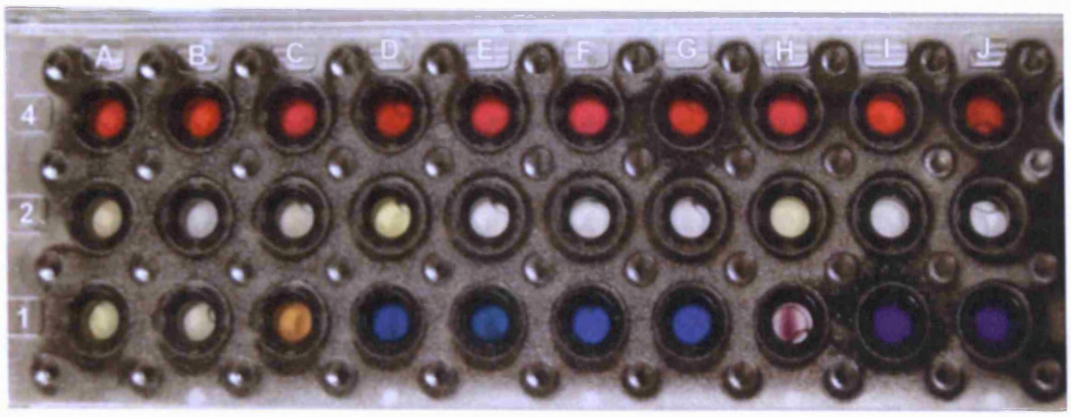
Ps. aeruginosa strains were characterised by the use of the BBL™ Crystal™ Enteric/Nonfermenter ID Kit (BD, NJ, USA). *Ps. aeruginosa* strains were streaked to blood agar plates (containing 10 g of Number 2 Blood agar base

(Oxoid, Cambridge, UK) and 7% (v/v) defibrinated sheep's blood (Oxoid, Cambridge, UK) per 250 mL deionised water) to check culture purity.

Singular *Ps. aeruginosa* colonies were added to the inoculum fluid provided with the BBL crystal kit (BD, NJ, USA) from overnight blood plates. The suspension was then vortexed and turbidity checked against a McFarland standard. The inoculum fluid was then poured into a labelled sample tray and swirled to ensure all wells were filled with fluid before the addition of the test strip. The completed test was then returned to the tray provided, placed inside a plastic bag with a damp piece of tissue and incubated at 37°C overnight.

Following incubation kits were read in a positive/negative manner (Figure 3.1) according to BD specifications that were included with the test kits. The code generated was then inserted into the BBL Crystal MIND Software (BD, NJ, USA) and then identified.

A



B

Score	A	B	C	D	E	F	G	H	I	J
4	-	-	-	-	-	-	-	-	-	-
2	+	-	+	+	-	-	-	+	-	-
1	+	-	+	+	+	+	+	+	+	+
CODE	3	0	3	3	1	1	1	3	1	1

Figure 3.1: Example of BBL crystal. Strain NCTC 10332 was used and shows characterisation as *Ps. aeruginosa* Biotype 1561. A) Shows completed BBL crystal. B) Shows BBL crystal as a positive/negative result and the code generated.

3.2.2.4 Antibiotic susceptibility

The antibiotic susceptibility profile of all 14 *Pseudomonas* strains was evaluated using the disc diffusion method (Andrews, 2009). Iso-Sensitest agar (ISA) plates (Oxoid, Cambridge, UK) were prepared according to manufacturer's instructions.

Bacterial suspensions of all strains were prepared as previously described (Chapter 2; Section 2.2.1) to contain between 1 and 5 x 10^8 CFU/mL, then further diluted by a ratio of 1:100 and enumerated (Chapter 2; Section 2.3.1).

The standardised suspension was spread onto pre-poured ISA plates in three directions using a sterile cotton wool swab. The inoculum was then dried at

room temperature for up to 15 min before the addition of antibiotic discs (Oxoid, Cambridge, UK). The antibiotics used (Table 3.1) were chosen on the basis of current NHS usage against *Ps. aeruginosa* in the Heath Hospital in Cardiff (Hosein, 2007). To ensure compliance with standard methodology (Andrews, 2009) plates were incubated within 15 min of disc addition. ISA plates were incubated at 37°C in air for between 18 and 24 h.

Once checked for semi-confluent growth as described in Andrews (2009), the diameters of the zones of inhibition were measured to the nearest millimetre using a ruler. A mean of triplicate plates was then taken and the results compared to standard diameters (Andrews, 2009) to determine susceptibility.

Antibiotic	Disc Contents (µg)
Colistin (as Colistin sulphate) (CT)	25
Ciprofloxacin (CIP)	1
Ticarcillin (TIC)	75
Ceftazadime (CAZ)	30
Tobramycin (TOB)	10

Table 3.1: Antibiotics used in antibiotic susceptibility testing of *Ps. aeruginosa* strains.

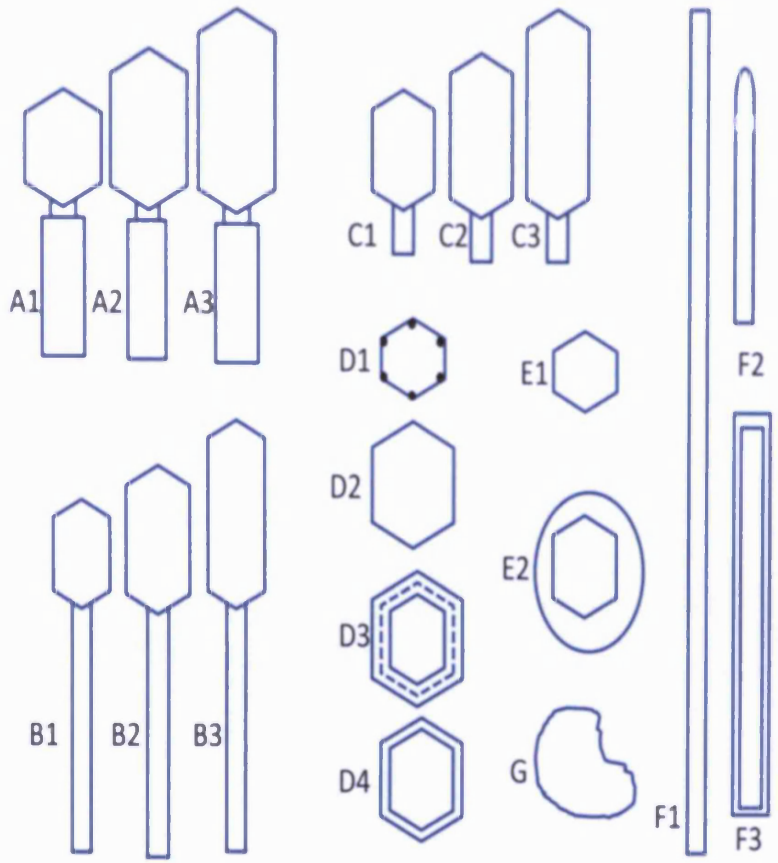
3.2.3 Characterisation of *Ps. aeruginosa* bacteriophages

Structural characterisation and DNA/RNA determination was performed in conjunction with Ms Helen Jones (final year undergraduate student, Welsh School of Pharmacy, Cardiff, UK).

3.2.3.1 Visual characterisation

Concentrated stocks of phages GL-1, C10176-S, C10176-L and LP-M were prepared and concentrated as previously described (Chapter 2; Section 2.2.2) then diluted 1:10 in PBS. Samples were then prepared for TEM imaging as previously described (Chapter 2; Section 2.4.1). Representative images were taken based upon 15 fields of observation. The 4 phage strains used in the

current investigation were classified on the basis of morphology (Figure 3.2; Hull *et al.*, 1989). Phage size was calculated based upon the means of 15 phage particles using iTEM software (Soft Imaging System GmbH, Münster, Germany).



Morphotype	Family	Comment
	Tailed Phage	
A1 to A3	*Myoviridae*	Tails long and contractile
B1 to B3	*Siphoviridae*	Tails long and non-contractile
C1 to C3	Podoviridae	Tails short
	Cubic Phage	
D1	*Microviridae*	Small unenveloped phages containing ssDNA
D2	Unclassified	Large unenveloped phages containing dsDNA
D3	*Corticoviridae*	Large containing dsDNA and internal lipid layer
D4	*Tectiviridae*	Large containing dsDNA, double capsid structure and internal lipid
E1	*Leviviraidae*	Small naked phages containing ssRNA
E2	*Cystoviridae*	Large isometric phages with segmented dsRNA and lipid envelope
	Rod shaped or Filamentous phage	
F1	Inovirus	Long flexible rods containing ssDNA
F2	Plectrovirus	Short straight rods containing dsDNA
F3	Unclassified	Enveloped rigid rods of variable length dsDNA
	Pleomorphic phage	
G	Plasmavirus	Rounded phage with flexible envelope

Figure 3.2: Structural characterisation of *Ps. aeruginosa* phages. Images not to scale. Adapted from Hull *et al.* (1989).

3.2.3.2 DNA/RNA characterisation

The following protocol was adapted from Bradley (1967). Ten microlitres of a concentrated phage suspension (prepared as in Chapter 2; Section 2.2.2) was spotted onto the surface of a glass slide and air dried at room temperature for 1 h.

Phage drops were fixed for 3 h in Carnoy's fluid (containing 60% (v/v) absolute ethanol, 30% (v/v) chloroform and 10% (v/v) glacial acetic acid). Once fixed, coverslips were stained in 1% (w/v) acridine orange (Invitrogen, CA, USA) in McIlvaines buffer at pH 3.8 (containing 35.5% (v/v) 0.2 M Na_2HPO_4 and 64.5% (v/v) 0.1 M citric acid). Slides were then soaked in 0.15 M Na_2HPO_4 for 15 min and examined under ultraviolet light (UV) (Figure 3.3a).

DNA/RNA content was then determined on the basis of emitted fluorescence (Table 3.2). DNA and RNA were distinguished by the addition of 0.1 M molybdic acid (Figure 3.3b).

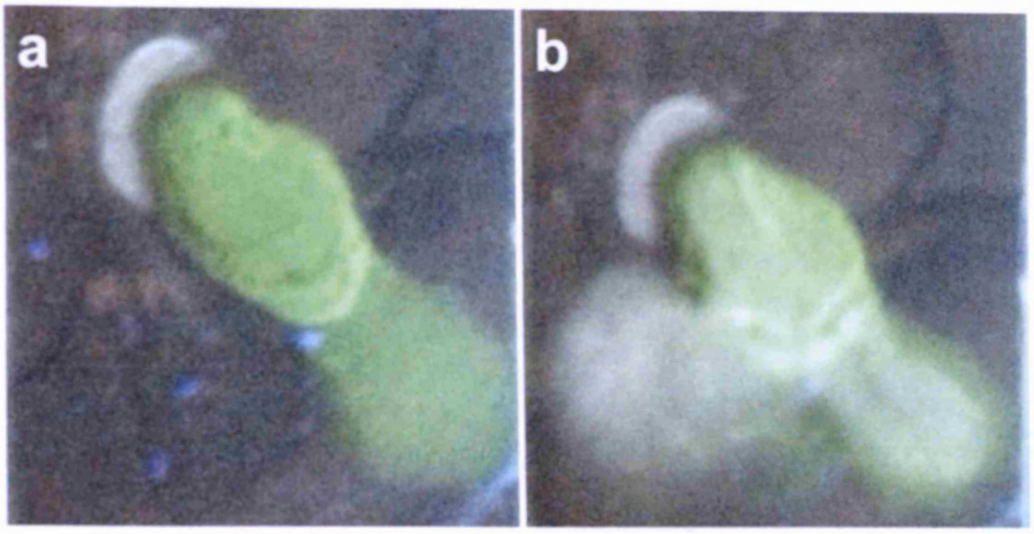


Figure 3.3: Representative image of DNA/RNA characterisation. Phage used was LP-M a) Green fluorescence after staining with acridine orange and b) shows retention of green fluorescence after addition of molybdic acid showing presence of dsDNA. Images not to scale.

	Staining with acridine orange	Addition of molybdic acid
dsDNA	Green	Green
dsRNA	Green	Green fades
ssDNA	Red	Paler green
ssRNA	Red	Paler red

Table 3.2: Determination of DNA/RNA content of bacteriophages using acridine orange staining. dsDNA; double stranded DNA, dsRNA; double stranded RNA, ssDNA; single stranded DNA, ssRNA; single stranded RNA. (Bradley, 1967).

3.2.4 Effect of media supplementation on *Ps. aeruginosa* growth

Ps. aeruginosa strains were prepared and standardised as previously described (Chapter 2; Section 2.2.1).

3.2.4.1 Bioscreen C analysis of growth under aerobic conditions

The Bioscreen C analyzer (Figure 3.4) is an automated spectrophotometer that can directly measure bacterial growth of up to 200 samples at a wide range of temperatures based on sample turbidity over a period of time. Upon completion data are presented in a Microsoft Excel document as optical density per time point sampled.

Figure 3.4: The Bioscreen C analyser.

To each well of a 100 well honeycomb plate (Öy Growth Curves AB Ltd, Helsinki, Finland) 350 µL of growth medium was added. To test wells (5 per strain tested) 50 µL of *Ps. aeruginosa* culture (containing between 1 and 5 x 10^8 CFU/mL) was added. A negative control containing growth medium (TSB or Luria-Bertani broth (LB) broth at pH 7, 6.5, 5 in the presence or absence of 10% (w/v) glycerol) and 50 µL of TSC was also performed.

The assessment of bacterial growth was then undertaken over 20 h using a wideband filter (420-580 nm) with readings taken every 15 min in a Bioscreen C analyzer (Öy Growth Curves AB Ltd, Helsinki, Finland) at 37°C using the

EZExperiment software Version 1.26 (Öy Growth Curves AB Ltd, Helsinki, Finland). Each reading was preceded by shaking cycle of 10 s.

Experiments were performed in triplicate using a fresh bacterial suspension for each run.

3.2.4.2 Analysis of growth in a 95% air 5% CO_2 atmosphere

Upon standardisation 31.25 mL of *Ps. aeruginosa* culture (containing between 1 and 5 x 10^8 CFU/mL) was added to 250 mL of media (TSB, LB or LB Final) and incubated at 37°C in a 95% air: 5% CO_2 atmosphere. Culture flasks were shaken gently by hand prior to sampling in order to adequately mix the sample. OD_{600nm} was determined every hour using un-inoculated media as a negative control on a Heλios α spectrophotometer (ThermoFisher Scientific, MA, USA).

3.2.4.3 Data analysis of growth curves

Data was analysed in Microsoft Excel (Microsoft, MA, USA) whereby optical density at each time point was averaged. Optical density was then adjusted by subtracting the optical density of the blank from optical density of the cultures. Bacterial growth was measured indirectly as the time taken to enter a log growth phase and as an expression of increase in OD ($x10^{-4}$) per min during the log phase of growth (ΔOD/min; Figure 3.5).

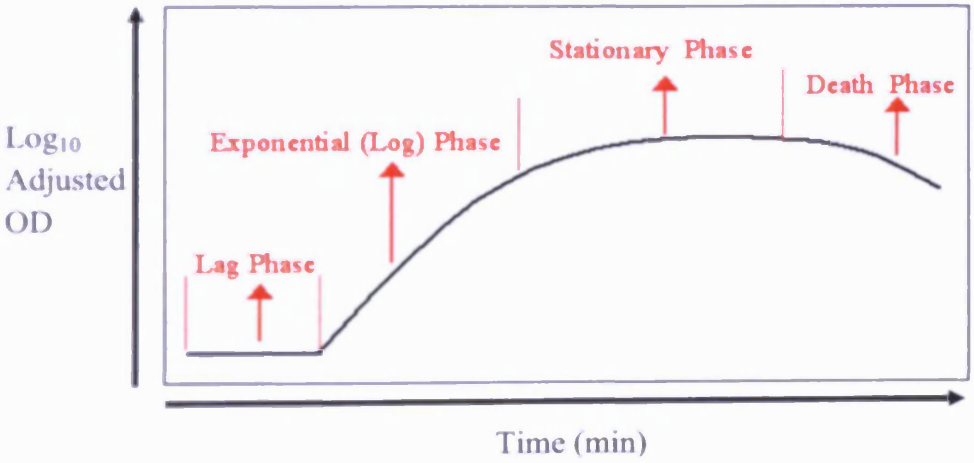


Figure 3.5: The different phases of bacterial growth.

3.2.5 Effect of media supplementation on EPS production of *Ps. aeruginosa* biofilms

3.2.5.1 Biofilm preparation

Sedimentary biofilms of *Ps. aeruginosa* strains NCTC 10332, P8959 and C2238 were prepared on 18 x 18 x 0.17 mm glass coverslips (Menzel-Gläser, Saarbrückener, Germany) as previously described (Chapter 2; Section 2.5.1).

3.2.5.2 Separation of EPS from bacterial biofilms

Coverslips were added face down to a 100 mL glass bottle containing 5 g of 3 mm borosilicate glass beads and 4 mL sterile deionised water (SDW). Glass bottles were then shaken at 200 RPM for 2 min on a Grant Bio POS-300 Orbital shaking platform (Keison UK, Chelmsford, UK). The bacterial suspension was removed, retained and then the process repeated. Coverslips were then removed and rinsed with 1 mL fresh SDW and total viable count (TVC) performed on the combined suspensions. This process was validated on 10 replicates (P=0.066).

Bacterial suspensions were transferred to 50 mL PPCO centrifuge tubes and centrifuged at 13750 x *g* for 30 min at 15°C. The supernatant was poured off and retained while the bacterial pellet was resuspended in 5 mL of fresh SDW. The centrifugation was repeated and the supernatants combined. The combined supernatant was then centrifuged at 13750 x *g* for 15 min at 4°C to remove any further bacterial debris and seeded with 0.1 g sodium acetate and stored on ice. Three volumes of ice cold absolute ethanol (stored at -20°C) were then added to the final solution and then centrifuged as before.

The solution was transferred to a 100 mL glass round bottom flask, attached to a Büchi rotavapor R-114 (Büchi Laboratory-Techniques, Switzerland) and partially immersed into a Büchi waterbath B4-80 (Büchi Laboratory-Techniques, Switzerland) that was preheated to 20°C. Removal of the ethanol

was achieved through the use of a Droi 2-2L vacuum pump and dry ice. Total volume of remaining solution was then recorded for future analysis.

3.2.5.3 Quantification of uronic acid content

A modified carbazole assay was used (Knutson and Jeanes, 1968) to quantify the uronic acid level (alginate) from *Ps. aeruginosa* biofilms cultured under different conditions.

Briefly, 140 µL of the uronic acid sample (Chapter 3; Section 3.2.5.2) was layered on top of 1.2 mL borate working solution (1:40 ratio of borate stock solution (containing 24.7 g of boric acid and 45 mL 4 M KOH per 100 mL): concentrated sulphuric acid) that was prepared fresh daily. The solution was cooled briefly on ice, vortexed for 4 s and then returned to an ice bath. Forty microlitres of a 0.1% (w/v) solution of carbazole in absolute ethanol was then added prior to repetition of the cooling and vortexing procedure. Once processed, samples were placed into a 55°C waterbath for 30 min.

Absorbance at 530nm (A_{530nm}) was determined on a Helios α spectrophotometer (Unicam, Cambridge, UK) that had been previously blanked with deionised water. Uronic acid content was deemed to be positive when the reaction mixture turned purple upon heating. The quantity of uronic acid was calculated based upon the mean absorbance of five replicates compared to standard alginic acid (Acros Organics, Geel, Belgium) solutions ranging from 0.1 mg/mL to 1.0 mg/mL (Figure 3.6). The overall process was validated using a Kruskal-Wallis test on 10 replicates (P=0.437) based upon the quantity of uronic acid /10^6 CFU.

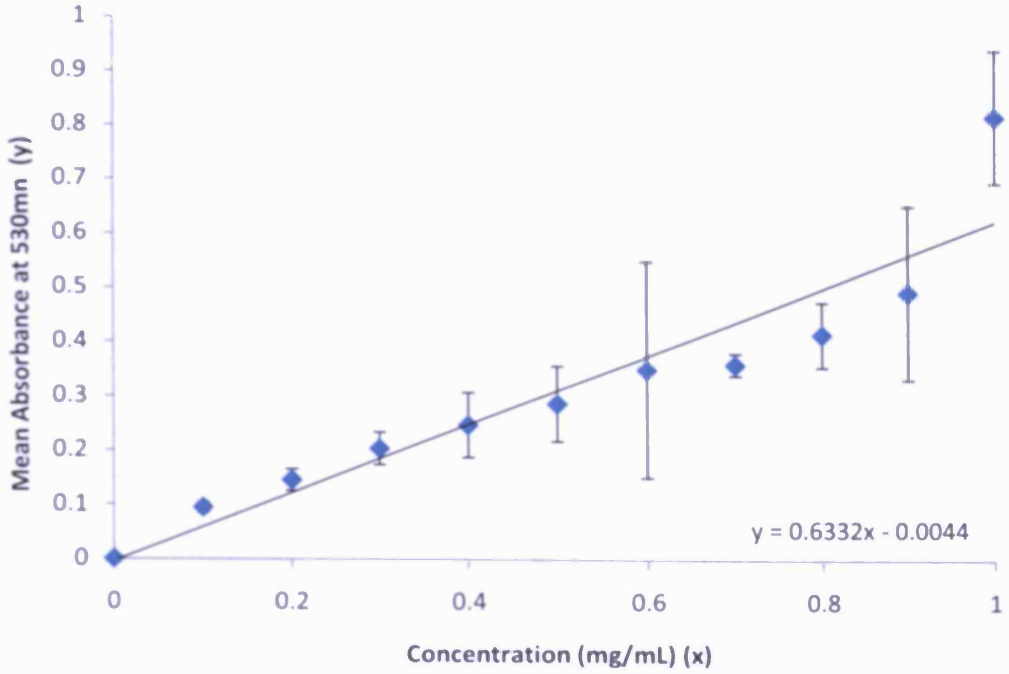


Figure 3.6: Relationship between the uronic acid content of alginic acid standards and absorption at 530 nm. Line represents the best fit by linear regression. Data are the mean of three replicates ±1SD.

3.2.6 Effect of media supplementation on sedimentary biofilm structure

Sedimentary biofilms of *Ps. aeruginosa* strains NCTC 10332, P8959 and C2238 were cultured as previously described (Chapter 2; Section 2.5.1) in TSB for 24 and 96 h and also in LB final broth for 96 h. Samples were then prepared for SEM as previously described (Chapter 2; Section 2.4.2)

3.2.7 Bacterial content of CDFF grown biofilms

PAO1 cultures were prepared and standardised as previously described (Chapter 2; Section 2.2.1). PTFE discs were recessed to depths ranging from 10 to 160 μm. Biofilms were then cultured as previously described (Chapter 2; Section 2.5.2) and bacterial content calculated.

3.3 Results

3.3.1 Characterisation of *Ps. aeruginosa* strains

3.3.1.1 Characterisation of EPS production, biotype and Gram type

All strains were shown to be Gram-negative (Figure 3.7a), with EPS production characterised as either positive or negative using the method of Harrison-Balestra *et al.* (2003) (Figure 3.7b,c). It should be noted that this method is purely qualitative and EPS production was characterised on representative images from 15 fields of vision. Although characterised on a positive/negative basis, varying levels of EPS were seen to be produced between the strains, with P8959 and C2238 appearing to produce the most.

Two biotypes, 7 and 58 dominated the 14 *Pseudomonas* strains (Table 3.3) that were observed when characterised with the BBL crystal system. However, both C4503 and NCTC 10332 were shown to be of a unique biotype (Table 3.3). It was also observed that biotype 7 was predominantly an EPS producer with the exception of PAK-SR, while biotype 58 had no such similarities.

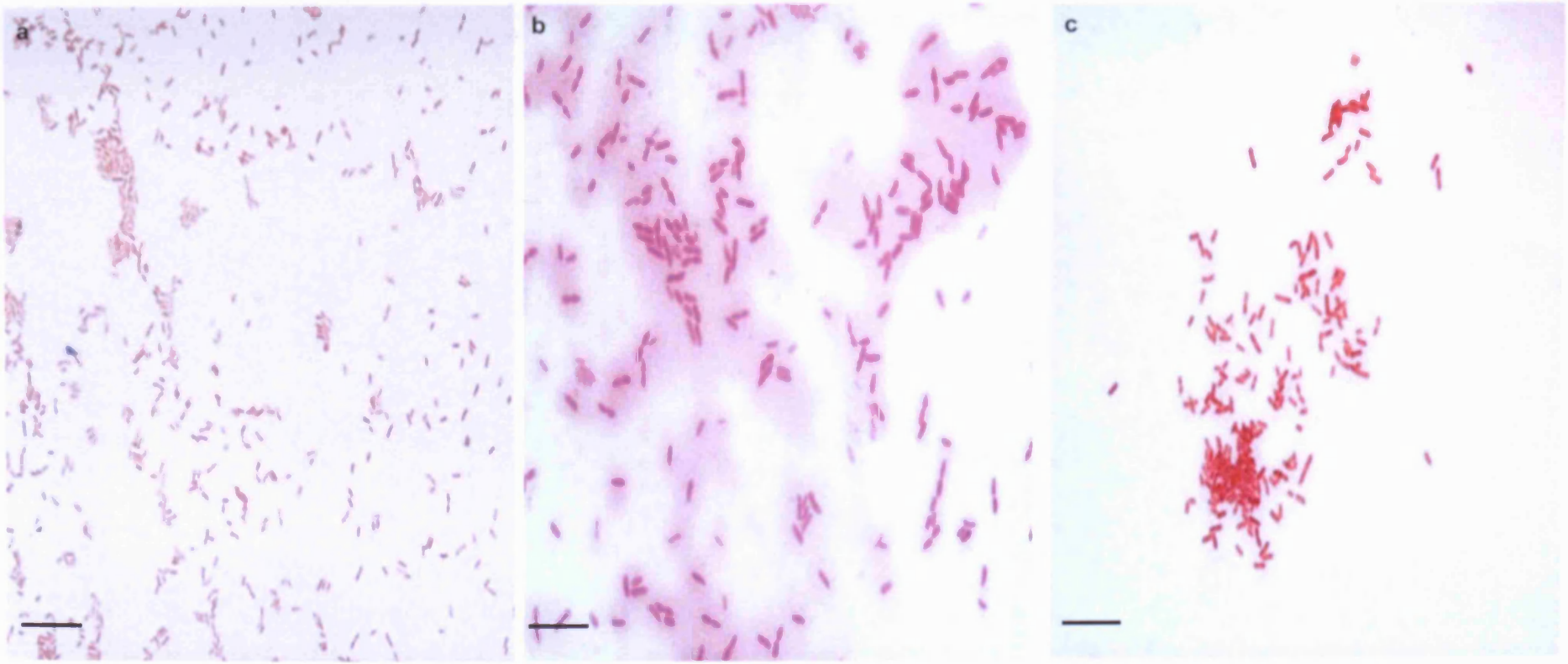


Figure 3.7: Optical microscopy testing used in the initial characterisation of *Ps. aeruginosa* strains. a) Gram stain of C4503, b) EPS positive strain from C2238 and c) EPS negative stain from C3652. Black bar represents 10 μm.

	Bacterial strain	EPS stain[1]	BBL Crystal Results	
			Biotype	Probability[2]
Standard	NCTC 10332	Neg	1561	.9998
	PA01	Pos	7	.9989
CF epidemic	C3652	Neg	58	.9999
	C3719	Neg	58	.9999
	C3786	Pos	58	.9999
	LES	Pos	58	.9999
	Mids1	Pos	7	.9989
	P8959	Pos	7	.9989
CF non-epidemic	C1913	Neg	58	.9999
	C2238	Pos	7	.9989
	C2846	Pos	7	.9989
	C3597	Pos	58	.9999
	C4503	Pos	5495	.9939
	PAK	Neg	7	.9989

Table 3.3: Summary of initial screening of *Ps. aeruginosa* strains. [1]: presence or absence of exopolysaccharide as described in (Chapter 3; Section 3.2.2.2). [2]: Biotype probability determined by BBL Crystal Mind Software as the probability of correct biotype.

3.3.1.2 Antibiotic susceptibility profiles

While each strain has a unique resistance profile it should be noted that strains isolated from CF patients were resistant to multiple antibiotics. Resistance levels were determined based upon the British Society for Antimicrobial Chemotherapy (BSAC) methodology (Andrews, 2009).

All strains with the exception of NCTC 10332 showed resistance or intermediate levels of resistance to at least one antibiotic (Table 3.4), which

varied between strains. Ticarcillin was shown to be the least active against all the *Pseudomonas* strains with 8 strains showing resistance. Colistin was shown to be the most active with only 5 strains showing resistance. With ciprofloxacin, there were only 4 strains with complete resistance but 7 strains with intermediate resistance. This intermediate level of resistance is of clinical relevance as it would suggest that a number of strains are developing resistance to ciprofloxacin.

		Antibiotics				
	Bacterial strain	TIC	CIP	TOB	CT	CAZ
Standard strains	NCTC 10332	S	S	S	S	S
	PAO1	S	S	R	R	S
CF Epidemic	C3652	R	R	S	R	S
	C3719	R	R	R	S	R
	C3786	S	I	R	S	S
	LES	R	I	S	S	R
	Mids1	S	R	R	S	S
	P8959	R	R	R	R	R
CF Non-epidemic	C1913	R	I	R	R	R
	C2238	R	I	S	S	R
	C2846	S	I	S	S	S
	C3597	S	S	S	R	S
	C4503	R	I	S	S	R
	PAK	R	I	S	S	R
	No. of Resistant Strains	8/14	4/14	6/14	5/14	7/14

Table 3.4: Summary of antibiotic susceptibility profiles of *Ps. aeruginosa* strains. TIC-Ticarcillin, CIP-Ciprofloxacin, TOB-Tobramycin, CAZ-Ceftazadime, and CT-Colistin (as Colistin Sulphate). R-Resistant, I-Intermediate, S-Susceptible. Resistance levels determined based upon BSAC methodology (Andrews, 2009).

3.3.2 Growth and EPS production of *Ps. aeruginosa* strains

3.3.2.1 Unsupplemented TSB

When grown in unsupplemented TSB at 37°C and in full aerobic conditions, the rate of bacterial growth varied significantly (P=0.017) between strains (Table 3.5).

In 50% of the strains tested there was no clear log growth phase observed by 20 h, the other 50% exhibited a more consistent linear growth (Figure 3.8). In general it was observed that more non-epidemic CF strains of *Ps. aeruginosa* were able to enter a log phase of growth within 20h compared to non-epidemic strains (Table 3.5).

The change in OD/min was shown to vary between strains with all CF non-epidemic strains exhibiting a higher rate of growth during log phase than both CF epidemic and standard strains (Table 3.5). However, strain C3597 exhibited the slowest growth rate of all the strains that exhibited a log phase of growth.

A 96 h incubation provided the highest total uronic acid content (Figure 3.9), hence this incubation time was chosen to compare different growth conditions on the production of uronic acid by the different strains. It was surprising to observe that the non-mucoid strains, NCTC 10332 and PAO1, also produced some uronic acid (Figure 3.9). However, this was considerably less than the clinical strains.

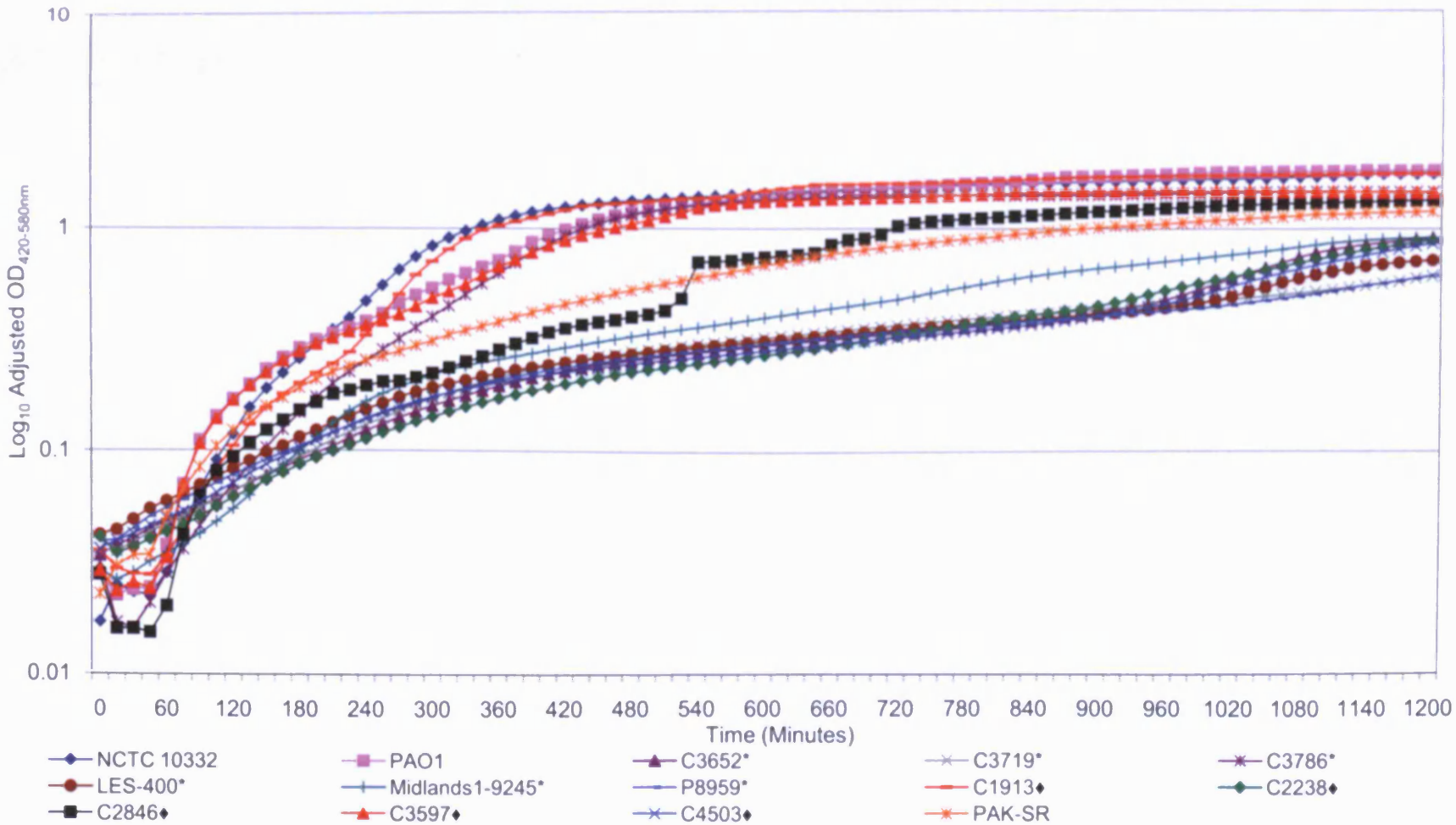


Figure 3.8: Growth of *Ps. aeruginosa* strains under full areobic conditions in TSB at pH 7. Data are the mean of three replicates.

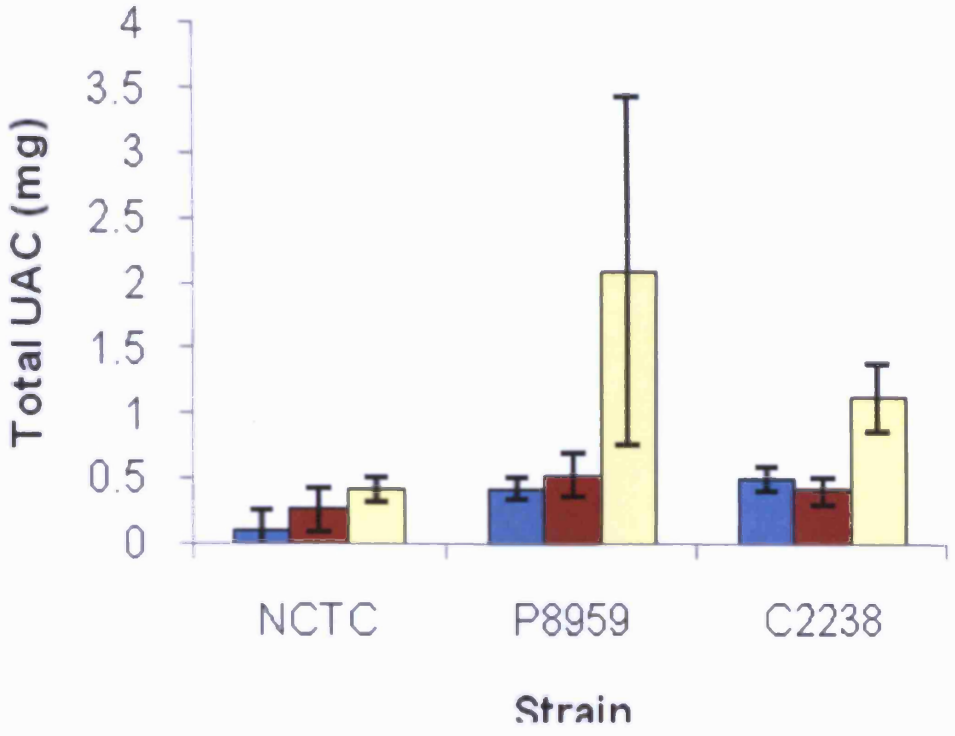


Figure 3.9: Effect of time on total uronic acid content (UAC) of *Ps. aeruginosa* biofilms grown in non-supplemented TSB under full aerobic conditions. Data are the mean of three replicates ±1SD. ■ 24 h, ■ 48 h and ■ 96 h.

	Bacterial strain	Growth slope	
		Time[1] (min)	ΔOD/min[3]
Standard strains	NCTC 10332	45	51.7
	PAO1	45	37.5
CF epidemic strains	C3652	-[2]	-
	C3719	-[2]	-
	C3786	30	41.5
	LES	-[2]	-
	Mids1	-[2]	-
	P8959	-[2]	-
CF non-epidemic strains	C1913	45	62.3
	C2238	-[2]	-
	C2846	45	93.1
	C3597	45	7.72
	C4503	-[2]	-
	PAK	45	11.5

Table 3.5: Summary table of the growth of *Ps. aeruginosa* strains grown under full aerobic conditions. Data are the mean of three replicates. [1]: Time lapse from inoculation to log phase. [2]: no log phase of growth observed. [3]: growth expressed as an increase in $OD_{420\text{-}580nm}$ per min ($x10^{-4}$) during log phase of growth.

3.3.2.2 Effect of media supplementation

The growth of all strains was shown to be lower in unsupplemented LB broth compared to TSB (Table 3.6). Incubation in a 95% air 5% CO_2 atmosphere resulted in a decrease in growth for all strains. However, clinical strains appeared to be more tolerant of diminished oxygen content (Table 3.6).

The clinical strains of *Ps. aeruginosa* (P8959 and C2238) were shown to produce more uronic acids at all conditions (with the exception of C2238 at pH 5) compared to the standard strain (NCTC 10332; Table 3.7). Under full aerobic conditions at pH 7, both TSB and LB were shown to produce among the highest

levels of uronic acid (Table 3.7) with the exception of NCTC 10332 that showed the highest levels of uronic acids at pH 5 in TSB compared to the other conditions tested.

Media supplementation resulted in no significant change ($P \geq 0.05$) of total uronic content for the clinical strains, with the standard strain NCTC 10332, showing a significant increase of total uronic acid content in TSB at pH 5 and a significant decrease in TSB when incubated in a 5% CO_2 atmosphere ($P \leq 0.05$; Table 3.7). It is interesting to observe that NCTC 10332 biofilms contained 50% or less of the total uronic acid content compared to the clinical strains with the exception of TSB at pH 5.

Supplementation with 10% glycerol resulted in a higher bacterial yield for all strains in both TSB and LB ($P \leq 0.05$; Table 3.7) compared to unsupplemented TSB. P8959 showed significantly ($P \leq 0.05$) increased levels of bacteria for all media supplementations with the exception of unsupplemented LB which showed significantly less bacteria per biofilm (Table 3.7).

The rate of growth was shown to vary by strain and also condition tested with both P8959 and C2238 showing few log growth phases (Table 3.6). The overall level of growth did not appear to change between pH 7 (Figure 3.10) and pH5 (Figure 3.11), however some differences between TSB and LB were observed with LB showing lower rates of growth and lower overall growth.

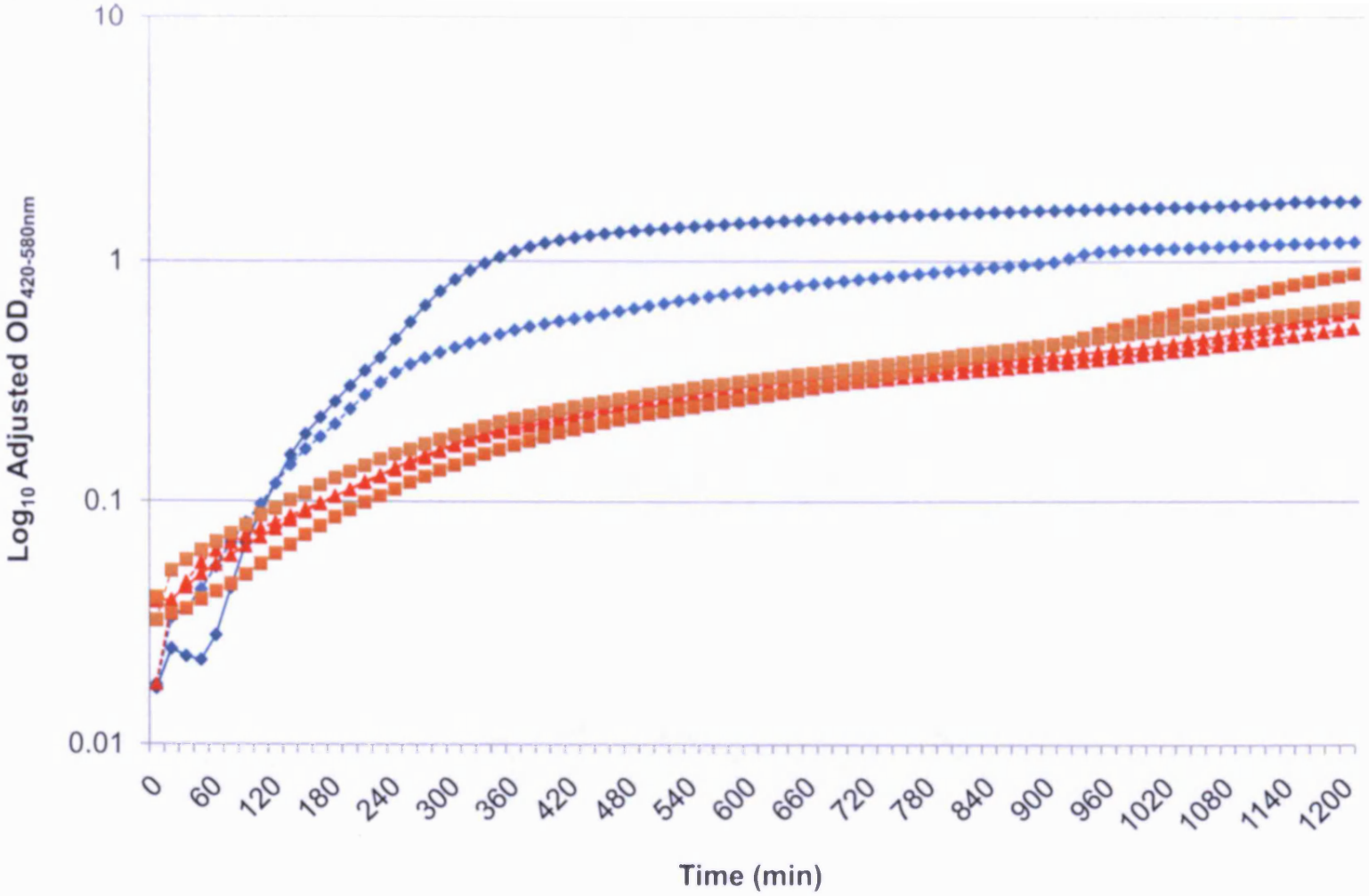


Figure 3.10: Example of Bioscreen C data for the assessment of bacterial growth under full aerobic conditions at pH 7. Data are the mean of three replicates. ♦ NCTC 10332, ▲ P8959 and ■ C2238. Solid lines indicate TSB growth. Dashed lines indicate LB broth growth.

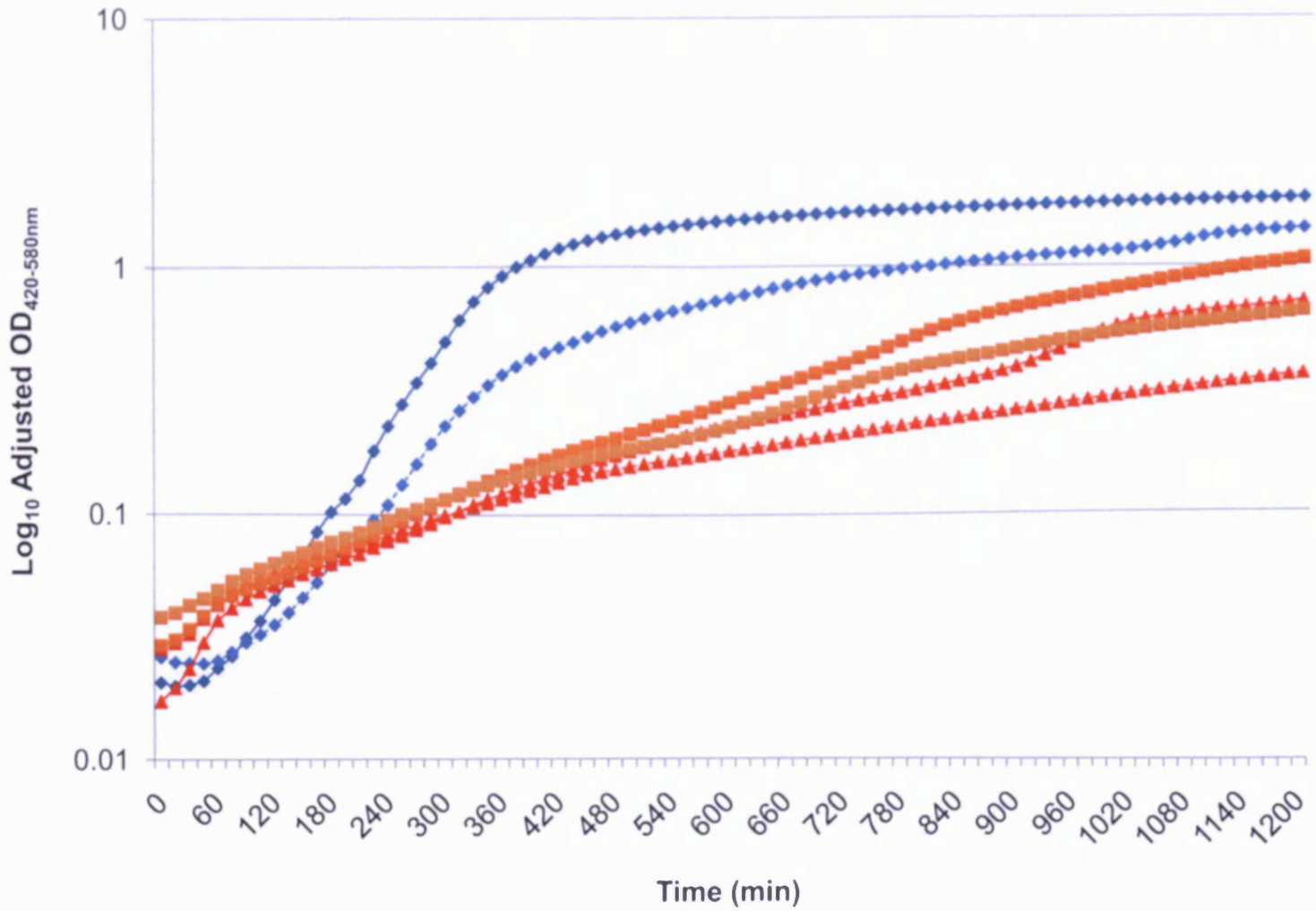


Figure 3.11: Example of Bioscreen C data for the assessment of bacterial growth under full aerobic conditions at pH 5. Data are the mean of three replicates. ♦ NCTC 10332, ▲ P8959 and ■ C2238. Solid lines indicate TSB growth. Dashed lines indicate LB broth growth.

		TSB					LB				
		pH 5	pH 6.5	pH 7			pH 5	pH 7			10% Glycerol, pH 5 and 5% CO_2
					10% Glycerol	5% CO_2			10% Glycerol	5% CO_2	
NCTC 10332	Time[1]	60	60	45	90	-[3]	60	45	15	-	-
	ΔOD/min[2]	94.8[1]	49.8	49.7	33.2	-	13.2	17.8	11.8	-	-
P8959	Time[1]	-	-	-	30	-	-	-	15	-	-
	ΔOD/min	-	-	-	2.81	-	-	-	1.9	-	-
C2238	Time[1]	-	-	-	45	-	-	-	15	-	-
	ΔOD/min	-	-	-	6.36	-	-	-	5.21	-	-

Table 3.6: Effect of media supplementation on *Ps. aeruginosa* growth over 20 h. Data are the mean of three replicates [1]: Time taken to reach log growth, [2]: ΔOD/min is expressed as an increase in $OD_{420\text{-}580nm}$ per min ($x10^{-4}$) during the log phase [3]: no log phase observed.

	TSB					LB				
	pH 5	pH 6.5	pH 7			pH 5	pH 7			10% Glycerol, pH 5 and 5% CO_2
				10% Glycerol	5% CO_2			10% Glycerol	5% CO_2	
					NCTC 10332					
TUC	**1.24 (0.21)**	0.56 (0.08)	0.41 (0.10)	0.45 (0.05)	**0**	0.31 (0.10)	0.42 (0.07)	0.37(0.32)	0.54 (0.08)	0.57 (0.06)
Log_{10} CFU/mL	7.81 (0.04)	7.56 (0.09)	7.36 (0.46)	**8.27 (0.19)**	7.34 (0.14)	7.58 (0.17)	7.49 (0.09)	**8.16 (0.22)**	**8.30 (0.07)**	**8.73 (0.19)**
					P8959					
TUC	1.34 (0.64)	1.17 (0.29)	2.10 (1.35)	1.17 (0.53)	0.75 (0.20)	1.11 (0.30)	0.87 (0.09)	0.74 (0.02)	0.90 (0.06)	1.13 (0.33)
Log_{10} CFU/mL	**7.85 (0.13)**	**7.68 (0.05)**	7.03 (0.30)	**8.28 (0.25)**	**7.64 (0.10)**	**7.57 (0.22)**	**6.20 (0.20)**	**8.03 (0.11)**	**8.16 (0.08)**	**8.63 (0.06)**
					C2238					
TUC	0.54 (0.07)	1.27 (0.16)	1.12 (0.27)	1.19 (0.36)	1.18 (0.32)	0.95 (0.38)	0.80 (0.21)	0.61 (0.03)	1.04 (0.23)	0.94 (0.05)
Log_{10} CFU/mL	7.90 (0.09)	7.87 (0.27)	7.59 (0.46)	**8.31 (0.34)**	7.80 (0.05)	7.41 (0.25)	7.31 (0.19)	**8.58 (0.12)**	8.27 (0.19)	**8.52 (0.15)**

Table 3.7: Effect of media supplementation on total uronic acid content (mg) and bacterial content of 96 h old *Ps. aeruginosa* biofilms. Data are the mean of three replicates (±SD). TUC: total uronic acid content (mg). Bold typeface indicates significant differences compared to unmodified TSB by one way ANOVA at the 95% CI.

3.3.2.3. Effect of media supplementation upon biofilm structure

Investigation by electron microscopy provided clear evidence of the production of EPS in sedimentation biofilms of both clinical isolates and the standard strain grown for 96 h in TSB under full aerobic conditions (Figure 3.12). A 24 h biofilm showed little evidence of EPS production in all strains (Figure 3.12) with the exception of C2238 (Figure 3.12) where clumps of EPS and bacteria were observed to remain on the glass surface. The growth of the sedimentation biofilm for 96 h under 5% CO_2 in LB media (pH 5) supplemented with 10% glycerol showed a more complex and compact structure with a visually increased amount of EPS (Figure 3.12) in all strains when compared to unsupplemented TSB in full aerobic conditions. When grown for 96 h under both full aerobic conditions (Figure 3.12) and in a 5% CO_2 atmosphere the biofilm appears to take on a honeycomb type structure (Figure 3.12). It is also interesting to note that in 96 h incubation bacterial size also appears to decrease.

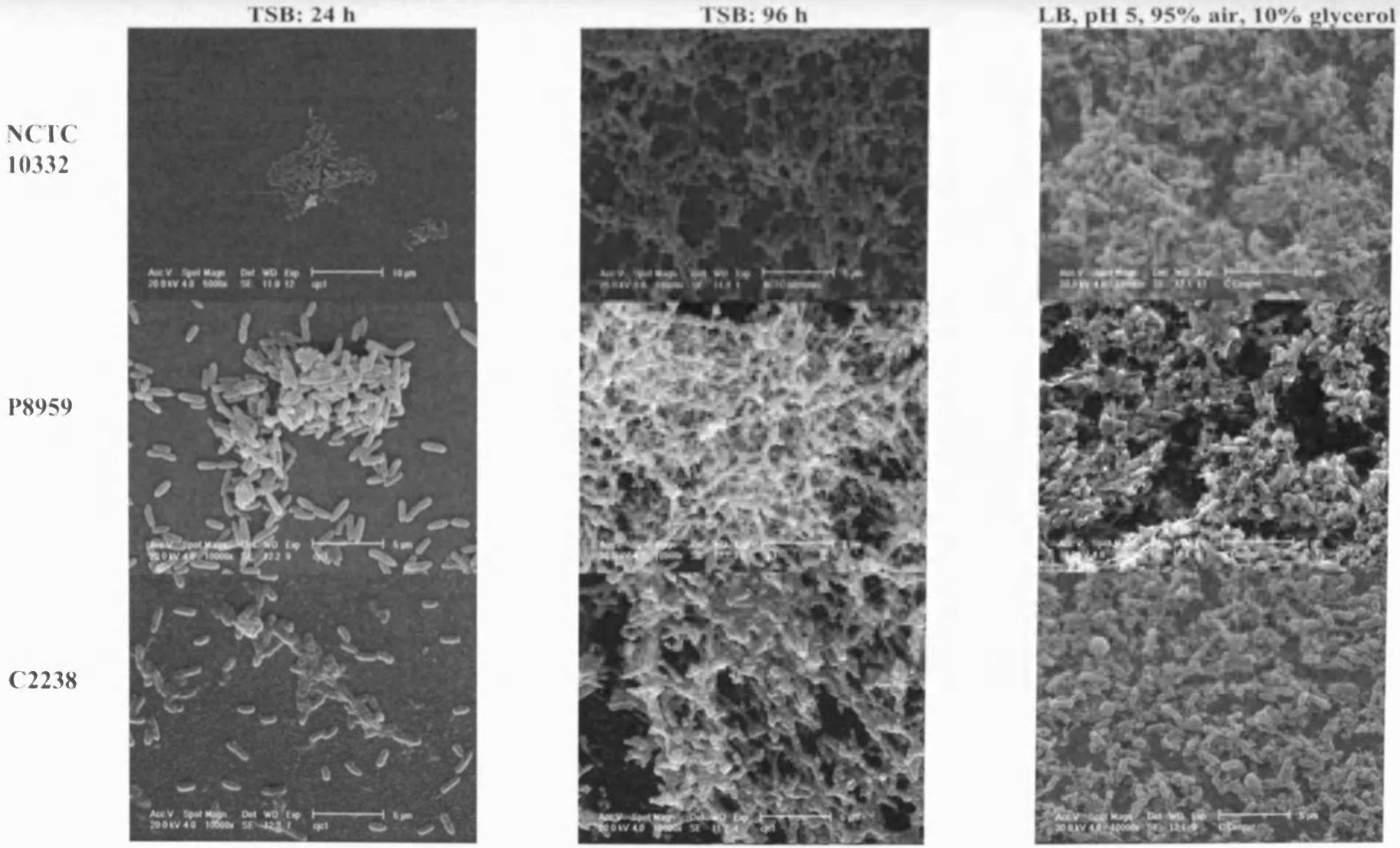


Figure 3.12: Effect of media supplementation on sedimentary biofilms of *Ps. aeruginosa* strains. Images are representative of 15 fields of vision that remained attached to the coverslip. Scale bars are included on each image.

3.3.3 Phage characterisation

All 4 phages were found to contain ds-DNA (Table 3.8). Both phage GL-1 and C10176-S were found to possess A1 structure (Figure 3.13a, d) whereas C10176-L possessed C1 structure (Figure 3.13b) and LP-M possessed B1 structure (Figure 3.13c). All phage possessed capsid diameters of approx. 50 nm while tail domains in GL-1 and C10176-S were approx. 100 nm in length.

An initial morphological characterisation was given to each of the phages using the images shown (Figure 3.13). However, due to the poor image quality and time constraints of an undergraduate project, TEM characterisation should be repeated in order to confirm the initial characterisation.

Phage	**Structure**	**Family**	**Length (nm)**		**DNA/ RNA**
			Capsid	Tail	
GL-1	A1	*Myoviridae*	49.3(5.2)	106.3(9.1)	2-DNA
C10176-L	C1	*Podoviridae*	49.6(6.1)	NC[1]	2-DNA
C10176-S	A1	*Myoviridae*	57.4(6.2)	116.6(15.5)	2-DNA
LP-M	B1	*Siphoviridae*	80.0(5.7)	126.6(13.2)	2-DNA

Table 3.8: Summary table of characterisation data of *Ps. aeruginosa* phages used in the current investigation. Data are the mean of 15 phage particles (±SD) and structural characterisation based upon Figure 3.2. NC indicates not calculated, 2-DNA - double stranded DNA. [1]: Due to breakage caused by preparation not enough tail domains were observed.

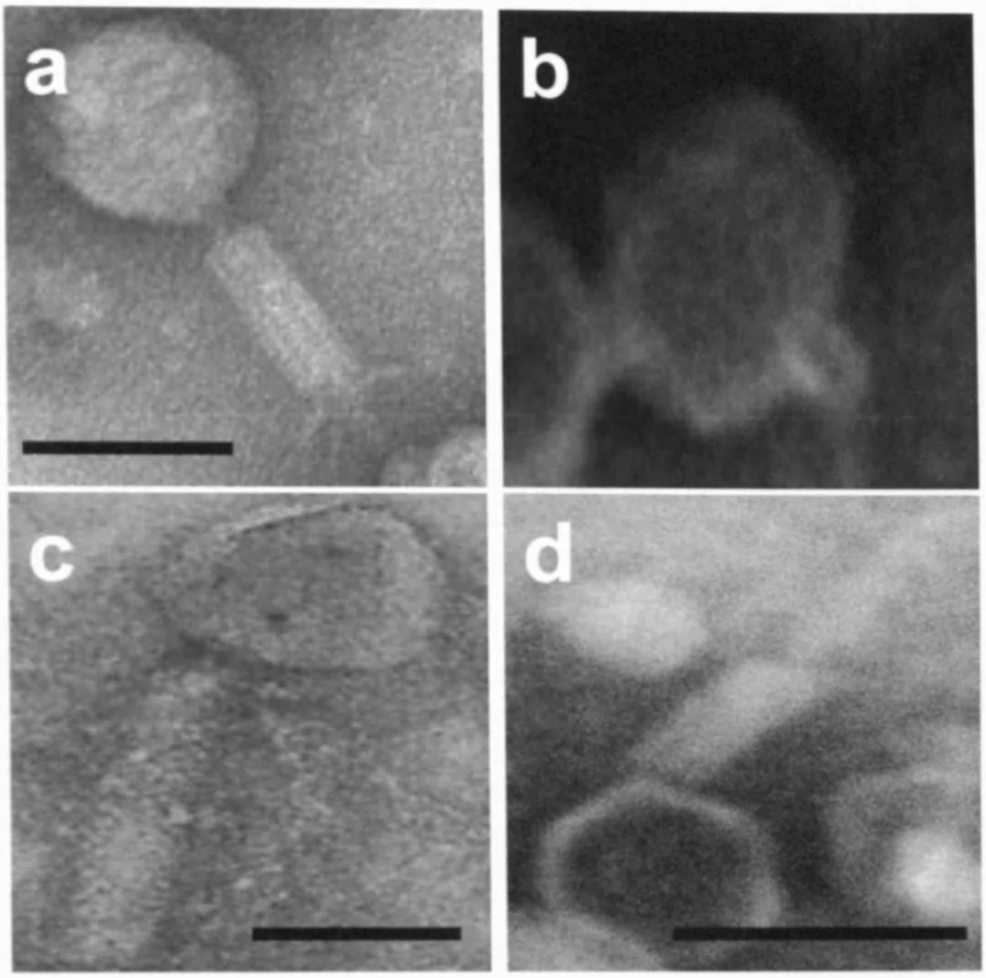


Figure 3.13: Transmission electron microscopy images of the four phages used in the current investigation. a) C10176-S, b) C10176-L, c) LP-M, d) GL-1. Images are representative of 15 fields of vision. Black Bar is representative of 100 nm.

3.3.4 Effect of biofilm depth on the bacterial content of biofilms

As the depth of biofilm was increased the number of bacteria recovered increased significantly (Figure 3.14; P=0.007). The increase in depth of the biofilm to 160 μm produced the largest recovery of bacterial number while 10 μm produced the lowest yield of bacteria. However, this increase was non-linear in nature.

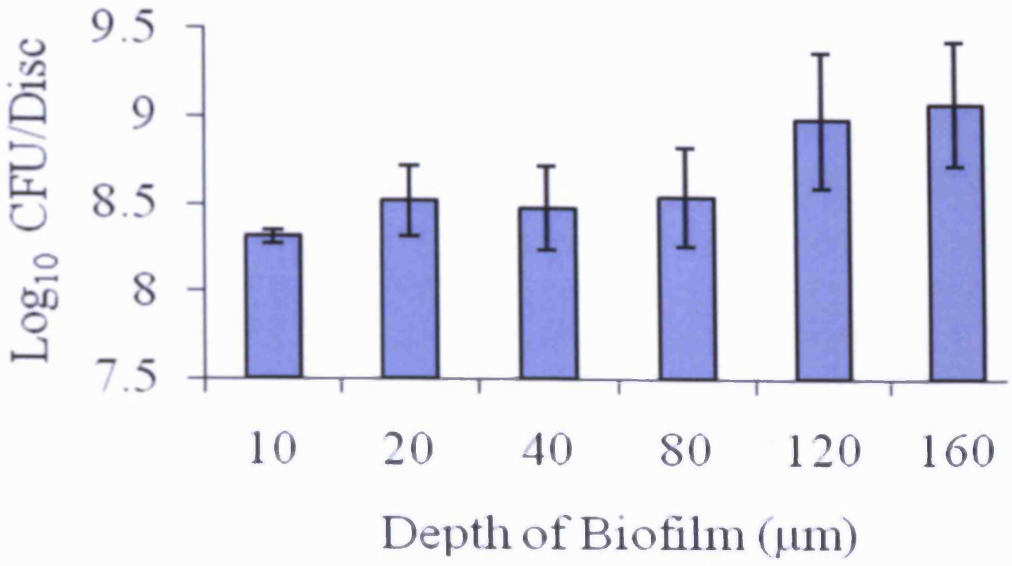


Figure 3.14: Effect of varying depth on the bacterial content of 96 h old CDFF cultured biofilms of *Ps. aeruginosa* strain PAO1. Data are the mean of three replicates ± SD.

3.4 Discussion

3.4.1 Initial characterisation of *Ps. aeruginosa* bacterial strains

The colonization of CF patients with *Ps. aeruginosa* is a major pathological feature of the disease and can lead to increased mortality and morbidity (Pritt *et al.*, 2007). The production of EPS within biofilms has been shown to increase resistance to antibiotic therapy (Li *et al.*, 2008), which is reflected by the therapeutic antibiotic resistance profiles generated here (Table 3.4). Antibiotic therapy in CF patients colonised with *Ps. aeruginosa* varies depending on the stage of infection and often centres on the use of nebulised tobramycin and colistin or oral ciprofloxacin of which approximately 30% or more of the strains tested show some level of resistance to at least one of these antibiotics. Of particular interest is the high level of intermediate resistance that is

observed with ciprofloxacin (Table 3.4) as this would suggest the development of resistance.

On the whole, the epidemic strains possess a greater degree of antibiotic resistance than those isolated from single patients, which would allow strains to persist within individuals. The congregation of CF patients at dedicated CF centres (McDowell *et al.*, 2004) led to the development of epidemic strains such as LES-400 that infect multiple individuals at multiple centres (Jones *et al.*, 2001).

The presence of EPS within 8 of the 11 strains from CF patients shows the importance of biofilm formation to persistence as the most resistant strain (P8959) is one of the largest EPS producers. However, there are a couple of notable exceptions to this (C3719 and C1913) that showed resistance or intermediate resistance to 4 or more antibiotics, although their lack of EPS production here (Table 3.3) does not mean they are incapable of doing so.

3.4.2 Initial characterisation of *Ps. aeruginosa* phage strains

The characterisation of the 4 phages described here revealed that all phages belonged to tailed phage families (Table 3.8) and contain ds-DNA. This is consistent with what is seen in the literature (Miles *et al.*, 1995; Kwan *et al.*, 2006).

When comparing the sizes of previously published *Ps. aeruginosa* phages, the phages described here appear to be slightly smaller in size (Ceyssens *et al.*, 2008). However, although suitable for initial identification, further characterisation work based upon genomic and proteomic analysis would need to be performed (Kwan *et al.*, 2006; Lavigne *et al.*, 2006).

3.4.3 Effects of media supplementation on *Ps. aeruginosa* growth, EPS production and biofilm formation

This investigation has used a simple sedimentation biofilm to assess the production of uronic acids and also bacterial content under differing culture conditions.

Ps. aeruginosa strain NCTC 10332 has previously been described as a non-mucoid strain (Edwards and Saunders, 2001). In our studies it was observed to produce uronic acid levels comparable to clinical strains under certain conditions (Table 3.7). However, a previous investigation by Edwards and Saunders (2001) showed the presence and expression of alginate genes within NCTC 10332.

The production of alginate by *Ps. aeruginosa* is believed to be part of a "coping" mechanism designed to protect against detrimental environmental conditions which could, in part, help to explain increased uronic acid levels at lower pH levels. This has been discussed in greater detail for a variety of stimuli including antibiotics (Wood *et al.*, 2006) and environmental stimuli, including both pH and nitrate concentration (Platt *et al.*, 2008).

The addition of 10% glycerol was chosen as both a secondary source of carbon and also as an aid in the initial attachment of bacteria to a surface (Jesaitis *et al.*, 2003). Although the addition of glucose could have aided in the attachment of bacteria, and hence the increase in the bacterial content of biofilms that has been demonstrated here, (Table 3.7), it is more likely that this was due to the extra nutrients provided by the glycerol.

While no conclusive value exists for the inter-pulmonary pH of CF patients studies have suggested it to be an acidic environment (Tate *et al.*, 2002). When colonized with *Ps. aeruginosa* the natural pH of the lung (approx. 6.15) is altered and decreases to approx. 5.88 in stable CF patients (Tate *et al.*, 2002) and therefore the lower pH values were chosen to reflect both stable and

exacerbated CF patients. This investigation has shown that a decrease in pH had no significant impact on the total content of uronic acids produced by two clinical strains (P8959 and C2238). However, the bacterial content of 96 h old biofilms significantly increased for the CF epidemic strain (P8959) for both pH values when compared to pH 7 TSB, but did not significantly alter for the CF non-epidemic strain (C2238), suggesting that CF epidemic strains are better suited to the acidic environment of the CF lung.

The presence of the characteristic mucus layer and the availability of oxygen within it is a source of much debate, with some groups demonstrating that the mucus layer is anaerobic (Worlitzsch *et al.*, 2002) and that *Ps. aeruginosa* can grow anaerobically in this environment utilizing nitrate as an alternative electron acceptor (O'May *et al.*, 2006). However, more recently it has been suggested that the mucus layer is actually microaerophilic (Alvarez-Ortega and Harwood, 2007). The existence of an environment which possesses a greater accessibility to oxygen is more likely than a true anaerobic environment as any physiotherapy regime that the patient underwent would limit the depth of the mucus layer within the lungs of CF patients.

In this investigation the presence of a 5% CO_2 atmosphere was included in an attempt to mimic a decreased oxygen atmosphere that may be included within the CF lung. Although the bacterial content of both clinical and standard biofilms was shown to increase substantially (Table 3.7), the total uronic acid content did not vary greatly compared to fully aerobic growth. As such it could be suggested that *Ps. aeruginosa* clinical strains are better adapted to a diminished oxygen environment than their standard strain counterpart.

3.4.4 The quantification of uronic acid content as a method of quantifying biofilm formation

In most cases, the static formation of biofilms and the efficacy of treatments are assessed using a crystal violet assay that measures bacterial adhesion to a surface (Hentzer *et al.*, 2001). In this assay the crystal violet is retained by

exopolysaccharides such as alginate and then optical density measured to provide an indication of the level of biofilm. In this investigation, the total uronic acid content of *Ps. aeruginosa* biofilms has been measured along with the bacterial content of biofilms to investigate the effects of media supplementation on *Ps. aeruginosa* biofilms.

The total uronic acid content assay was chosen as alginate is accepted as one of the key components within the EPS matrix of *Ps. aeruginosa* biofilms and is comprised of mannuronic and guluronic acid residues. However, this method of quantification does not take into account some of the other *Ps. aeruginosa* polysaccharides that are encoded for by the *pel* and *psl* gene loci (Ryder *et al.*, 2007). The presence of extracellular DNA has been shown to be present as part of the EPS of *Ps. aeruginosa* and has been suggested to interfere with the uronic acid assay (Whitchurch *et al.*, 2002) although it is still widely used (Killç and Dönmez, 2008). In this investigation the presence of extracellular DNA was tested for against NCTC 10332 under full aerobic conditions in TSB. This showed that the DNA content of extracted EPS accounted for approx. 10% of total uronic acid content, potentially explaining some of the variability observed.

From the work carried out here, the quantification of uronic acids is not sensitive or specific enough to quantify biofilm formation. As such the bacterial content of biofilms will be used as the method of assessment for future work.

3.4.5 Use of sedimentary biofilms

The production of sedimentation biofilms described here, and other biofilms cultured on abiotic surfaces, are believed to differ from those found in the CF lung (Moreau-Marquis *et al.*, 2008) in both complexity and structure.

It is believed that the primary attachment *Ps. aeruginosa* takes place in the presence of lung epithelial cells. The initial attempts to co-culture *Ps.*

aeruginosa in the presence of lung epithelial cells *in vitro* failed within 3-4 hours due to the accumulation of secondary metabolites and lack of oxygen resulting in cellular death. This was overcome in subsequent refinements (Carterson *et al.*, 2005). Despite the ability to co-culture epithelial cells and *Ps. aeruginosa* there is still no consensus on whether *Ps. aeruginosa* binds directly to epithelial cells, with some groups advocating that biofilm formation and growth takes place entirely within the mucus layer in the CF lung (Worlitzsch *et al.*, 2002) and some groups suggesting that direct binding and even cellular internalisation play a role (Garcia-Medina *et al.*, 2005).

Here, we have shown that the supplementation of media does alter both the uronic acid content and the total bacterial content of sedimentary biofilms of *Ps. aeruginosa* (Table 3.7). However, the differences were often insignificant. Here sedimentary biofilms have been shown to be easy to culture and reproducible. As such sedimentation biofilms that show complex structures and EPS production are still valid as an initial method of testing potential therapeutics while more complex and representative models are developed.

The presence of the mucus layer itself is also believed to aid in the initial colonization of CF patients (Landry *et al.*, 2006); the addition of mucin to media (Sriramulu *et al.*, 2005) and the pre-treatment of abiotic surfaces with pig mucin has been shown to increase biofilm complexity and also formation (Landry *et al.*, 2006) as the addition of glycerol demonstrated. The simple media (LB broth, pH 5 10% glycerol and 5% CO_2) described here has also shown to produce biofilms (Figure 3.12) of similar levels of complexity and structure to those produced from more complex media (Sriramulu *et al.*, 2005). This media could therefore be suitable for larger experiments for the culture of *Ps. aeruginosa* biofilms such as those found in CDFF apparatus, as the media is a fraction of the cost of the artificial sputum media.

3.4.6 Summary

This chapter has characterised 14 strains of *Ps. aeruginosa* on the basis of EPS production by light microscopy and antibiotic susceptibility profiles determined using BSAC methodology. From this, 3 strains of *Ps. aeruginosa* were chosen for subsequent testing. A number of *Ps. aeruginosa* phages were structurally characterised using TEM and type of genetic material determined.

The quantification of the total uronic acid content as a measure of *Ps. aeruginosa* biofilms has been shown here to be highly variable, with no significant differences existing between the different conditions tested (with the exception of NCTC 10332 in TSB at pH 5 and 5% CO_2). As such the use of uronic acid content to quantify biofilms is not sensitive or specific enough to determine the effects of environmental and nutritional supplementation.

Chapter 4

Assessment and enhancement of the lytic activity of *Ps. aeruginosa* bacteriophages

4.1 Introduction

With the rise in the level of antibiotic resistance in bacteria there has been an increased interest in alternatives to antibiotic therapy. Although in clinical use in Eastern Europe bacteriophage therapy has only recently begun to attract attention in the West with a number of new products being licensed or currently in clinical trials (Merabishvili *et al.*, 2009).

To produce successful whole-phage based therapeutics a number of criteria must be met. These include the ability to infect a range of strains as well as the ability to reduce the overall level of infection (Hanlon, 2007). Such a phage preparation requires one of two approaches to be undertaken: genetic modification (GM) of the phage genome to broaden specificity and to increase activity or selective breeding for the most infectious phage. The former of these two approaches might be more efficient allowing for the ability to not only contain elements that would enhance penetration of biofilms e.g. such as the production of an alginate lyase (Hanlon *et al.*, 2001), but also to increase the rate and overall level of bacterial kill. In reality, products based on genetically modified phages are probably more difficult to bring to market due to the amount of regulation surrounding GM organisms. The latter approach on the other hand allows for the selection of more virulent phage on the basis of time required to infect the host organism (Jassim *et al.*, 1995), which should lead to an increase in level and rate of bacterial lysis.

This chapter seeks to assess the lytic activity of 4 *Ps. aeruginosa* phages using a qualitative streak test (Merabishvili *et al.*, 2009). In addition, a Bioscreen C based-assay was developed to provide more reliable quantitative data on phage activity and increase the effectiveness of screening. The lytic activity of phage strains was enhanced using a pomegranate rind extract (PRE) (Jassim *et al.*, 1995). This chapter also sought to design and assess the lytic potential of a bacteriophage cocktail.

4.2 Materials and methods

4.2.1 Bacterial and bacteriophage strains

Fourteen bacterial strains and 4 phage strains were routinely cultured as previously described (Chapter 2; Section 2.2). Prior to use bacterial suspensions were standardised as previously described (Chapter 2; Section 2.2.1). Fresh phage cultures were prepared using the agar overlay technique (Adams, 1959) with PAO1 as a host.

4.2.2 Streak test for phage activity and host range

The following method was adapted from Merabishvili *et al.* (2009). Bacterial suspensions were prepared and standardised as previously described (Chapter 2; Section 2.2.1). Ten microlitres of bacterial suspension (containing between 1 and 5 x10^8 CFU/mL) were streaked over the surface of a 100 x 100 mm square petri dish (Sterilin Ltd, Caerphilly, UK) that had previously been divided into a 5 x 5 grid and air dried at room temperature for 15 min.

Ten microlitres of bacteriophage solution (approx. 10^7 PFU/mL) was then spotted at each intersection and air dried at room temperature for 1 h. PBS was used as a negative control. Plates were incubated at 37°C for 3 h and assessed visually for phage activity on the basis of a positive/negative reaction of the bacteria to the phage. Plates were then re-incubated at room temperature in the dark for a further 18-21h after which a secondary reading (Figure 4.1) was scored from 0 to +5 on the basis of the plaque formed by bacteriophage (Table 4.1).

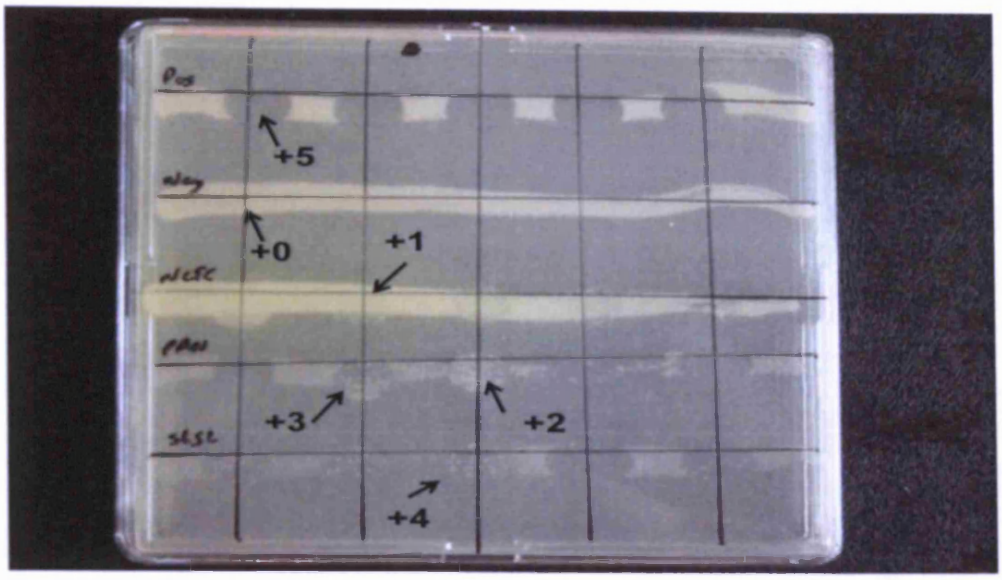


Figure 4.1: Example of a phage streak test plate at secondary reading stage. Labels on the plate correspond to Table 4.1.

Observation	Score
Confluent lysis - bacterial streak completely broken, no bacterial colonies present at spotted intersection	+5
Overgrowth - bacterial streak completely broken, presence of singular bacterial colonies on spot	+4
Semi-confluent lysis - bacterial streak incompletely broken	+3
Multiple small phage plaques	+2
Bacterial streak just affected i.e. little observable disruption to bacterial growth	+1
Negative reaction - no lysis	0

Table 4.1: Scoring system for assessment of bacteriophage activity using a streak test. Based on Merabishvili *et al.* (2009).

4.2.3 Bioscreen C assessment of phage activity

4.2.3.1 Culture preparation

Bacterial and phage suspensions were prepared from working stocks as previously described (Chapter 2 Section 2.2.1 and Chapter 2 Section 2.2.2 respectively); with the exception that bacterial standardisation was performed in TSB. Experiments were performed in triplicate using fresh phage and bacterial suspensions.

4.2.3.2 Plate layout and experimental conditions

Three hundred and fifty microlitres of bacterial suspension (containing between 1 and 5 x10^8 CFU/mL) in TSB was added to each well of a 100 well honeycomb plate (Öy Growth Curves AB Ltd, Helsinki, Finland), followed by the appropriate volume of phage suspension (50 µL unless otherwise stated). A non-phage treated control containing 350 µL of bacterial suspension and 50 µL PBS was performed. A negative control containing TSB alone and 50 µL of PBS was also performed.

The assessment of phage lytic activity was then performed over 20 h using a wideband filter (420-580 nm) with readings taken every 15 min in a Bioscreen C analyzer (Öy Growth Curves AB Ltd, Helsinki, Finland) at 37°C using the EZExperiment software Version 1.26 (Öy Growth Curves AB Ltd, Helsinki, Finland). Each reading was preceded by a 10 s shaking cycle.

4.2.3.3 Data analysis and interpretation

Data were analysed in Microsoft Excel (Microsoft, MA, USA) whereby $OD_{420-580nm}$ at each time point was averaged. $OD_{420-580nm}$ was then adjusted for the contribution of TSB. From this the assessment criteria for phage activity were calculated (Figure 4.2). "Acceptance" criteria were established for two main criteria (Table 4.2): 1) a measurement of bactericidal activity (i.e. activity ≥2 Log_{10} decrease in bacterial number after 8 or 20 h) and 2) a measurement of

bacterial regrowth following phage infection (i.e. time taken to reach an $OD_{420-580nm}$ of 0.1 units above the original $OD_{420-580nm}$) of ≥480 min.

Bactericidal activity was calculated by measuring the bacterial content of test wells and untreated controls, based on $OD_{420-580nm}$. $OD_{420-580nm}$ was converted to Log_{10} CFU from OD/TVC graphs of individual strains and subtracting tests from controls.

Other measurements of phage activity were also calculated but not used for the calculation of phage activity. These included: 1) the initial lytic activity indicated by a decrease in $OD_{420-580nm}$ within 30 min of phage addition (Figure 4.2) and 2) the calculation of the lytic slope as an indication of the rate of bacterial lysis. The set "acceptance" values (Table 4.2) were derived from the literature for phage activity (Hanlon *et al.*, 2001; Atterbury *et al.*, 2007; Fu *et al.*, 2010) and antibiotic dosing times (van Zanten *et al.*, 2007; McCoy *et al.*, 2008).

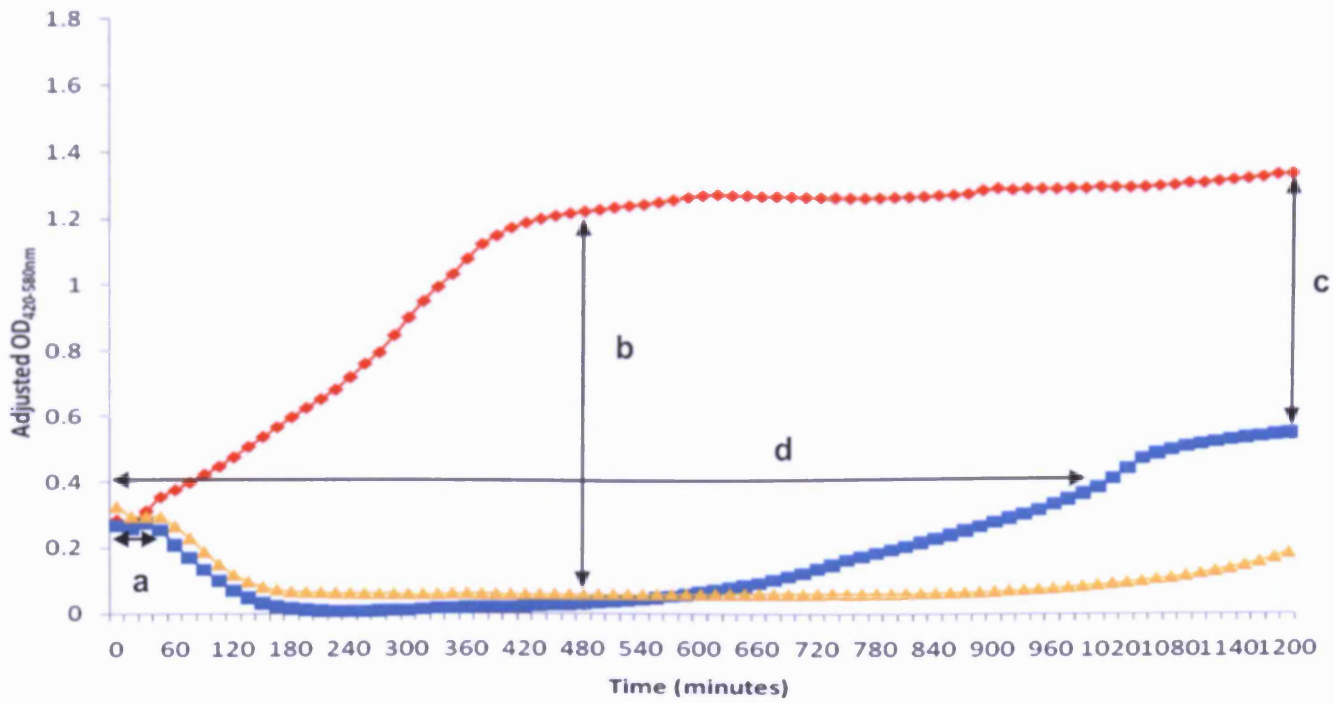


Figure 4.2: Assessment criteria for Bioscreen C analysis. Data used are the treatment of *Ps. aeruginosa* strain PAO1 against PRE and non-PRE treated GL-1. Assessment criteria based on: a) time taken to induce lysis; the level of bacterial reduction at 8 and 20 h (b and c respectively), d) level of bacterial regrowth (time taken to increase $OD_{420\text{-}580nm}$ 0.1 units above the initial $OD_{420\text{-}580nm}$). ●, untreated culture; ■, non-PRE treated phage; ▲, PRE treated phage.

	Evaluation criteria			Additional criterion
	Log_{10} reduction		Time[1]	Lysis[2]
	8 h	20 h	(min)	(min)
"Acceptance" value	≥2	≥2	≥480	≤30

Table 4.2: Standard criteria for the assessment of the lytic activity of *Ps. aeruginosa* bacteriophages in the Bioscreen C analyzer. [1]: Bacterial regrowth (time taken to increase $OD_{420-580nm}$ 0.1 units above the initial $OD_{420-580nm}$) and [2]: time taken to induce lysis.

4.2.4 Enhancement of bacteriophage activity

Pomegranate rind extract stock (PRE) was kindly provided by David Houston (Welsh School of Pharmacy, Cardiff University, Cardiff, UK). The PRE was prepared as described in Jassim *et al.*, (1995). In brief, pomegranate rind (25% w/v) was boiled in distilled water for 30 min then centrifuged at 20000 x *g* for 30 min at 4°C. The supernatant was then autoclaved at 121°C for 15 min and then further purified by membrane ultra filtration (molecular weight cut off 10 kDa) and stored at -20°C.

4.2.4.1 Chemical preparation

A 0.53% (w/v) stock solution of ferrous sulphate in λ buffer (containing 6 mL/L of 1 M Tris-HCl at pH 8.0 (Invitrogen, CA, USA), 5 g/L gelatine and 1.2 g/L $MgSO_4.2H_2O$) was prepared and sterilized by filtration with a 0.45 μm filter (Millipore, Cork, Ireland). A working solution of ferrous sulphate was prepared by adding 4.1 mL stock solution to 14 mL λ buffer. A working solution of PRE was prepared by adding 1.3 mL stock PRE to 8.7 mL λ buffer.

Composition A (CompA) was prepared 1-2 min prior to use by mixing 16.74 mL of ferrous sulphate working solution to 8.3 mL PRE working solution. The solution turned black after approximately 30 s and was protected from light.

4.2.4.2 Phage enhancement

The phage enhancement protocol has been adapted from Jassim *et al.*, (1995). In brief, phage strains were treated with PRE as in Figure 4.3 for 15 min. Subsequent enhancements at 10, 5 and 2.5 min were also performed. By reducing the time allowed for phage infection it is believed that this will increase the infectivity of subsequent phage progeny. The addition of CompA to the phage/host suspension will act as a selective virucide removing phages which have not infected the host.

Controls containing approximately 10^5 PFU/mL and CompA solution were assessed for lytic activity following 20 min at room temperature in the dark. No plaques were detected when regrown using PAO1 as a host. There was no significant difference in plaque numbers between phages treated with PRE containing 2% tween 80 and those treated with PBS ($P \geq 0.05$).

4.2.5 Assessment of lytic activity in a phage cocktail

Phage Cocktails containing up to five *Ps. aeruginosa* phages were tested for lytic activity against all strains of *Ps. aeruginosa* using both the streak test and the Bioscreen C Assay. Phages were chosen on the basis of the streak test assay performed on all enhancements. Phage cocktails containing GL-1, GL-1 *2.5*, LP-M *10*, C10176-S *15* and LP-M *5* (figure in italics represent enhancement time chosen) were prepared by mixing phage suspensions in equal volumes (containing approx. 10^{11} PFU/mL). The streak test and the Bioscreen C assay were then performed as previously described (Chapter 4; Section 4.2.2 and 4.2.3)

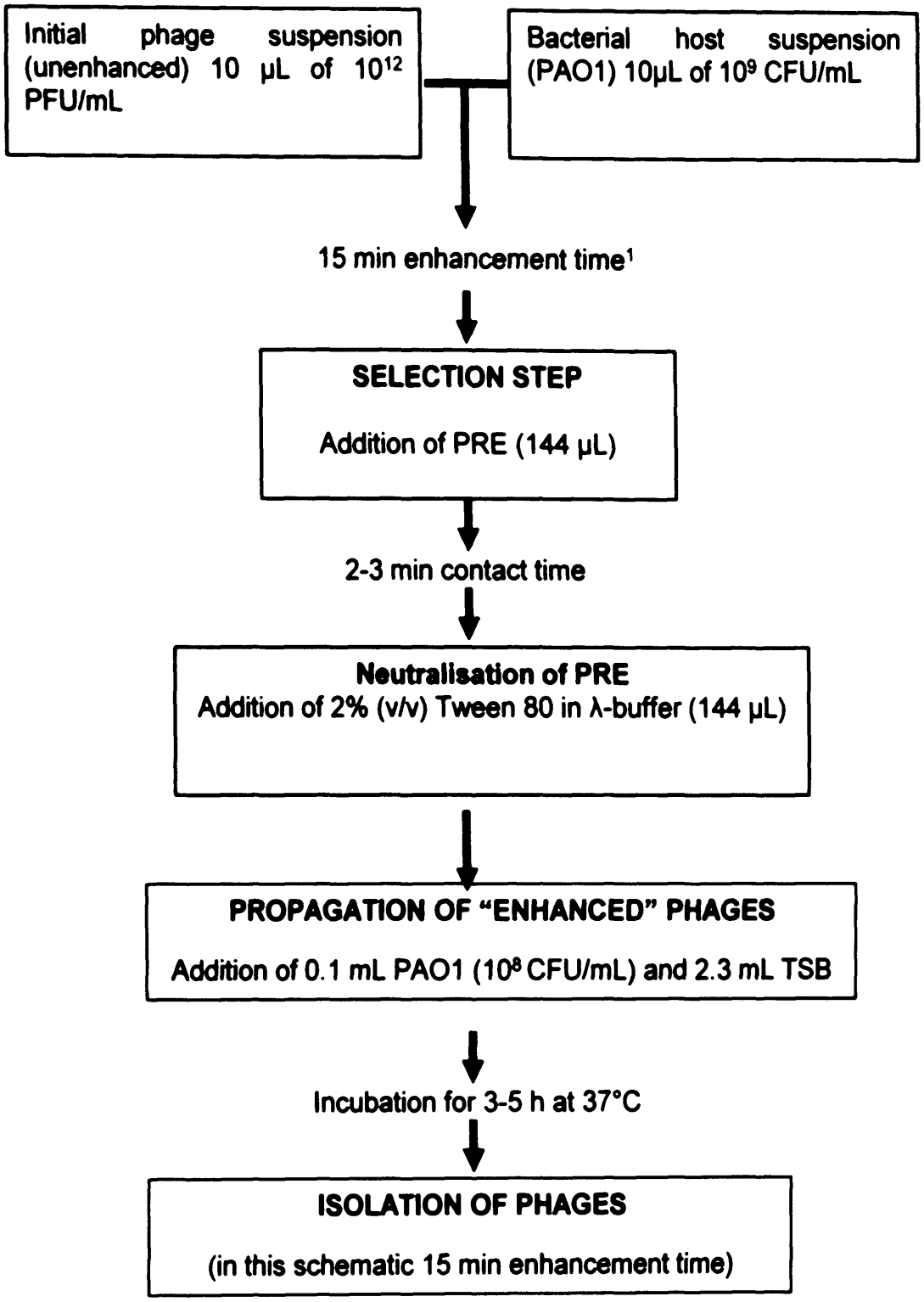


Figure 4.3: Schematic representation of phage enhancement using PRE. [1]:Contact time between phages and bacterial suspension varied (i.e. enhancement time of 15, 10, 5 and 2.5 min were used; see text).

4.3 Results

4.3.1 Assessment of the lytic activity of *Ps. aeruginosa* phages against the host strain

4.3.1.1 Effect of multiplicity of infection on the lytic activity of non-PRE treated phages

Following the addition of phage suspensions at different multiplicities of infection (MOI) to *Ps. aeruginosa* strain PAO1, all concentrations between 10^{12} and 10^{2} PFU/mL (MOI approx. 1400:1 at 10^{12} PFU/mL) caused a decrease in $OD_{420\text{-}580nm}$ (Figure 4.4). However, the time taken to induce this lytic curve varied depending on the phage inoculum size (Table 4.3). No lytic slope was detected in the lowest inoculum size for all phages (10^{1} PFU/mL; Table 4.3).

In terms of the reduction of bacterial number at 8 h, phages GL-1 and C10176-S showed a general trend of decreasing activity as phage inoculum size was decreased (10^{12} to 10^{2} PFU/mL; Table 4.3). However, LP-M and C10176-L showed the highest reductions at 10^{8} or 10^{9} PFU/mL (Table 4.3). The MOI (10^{12} to 10^{3} PFU/mL) was shown to have no significant impact ($P \geq 0.05$) on the level of bacterial lysis (i.e. no difference in bacterial kill). A phage concentration of 10^{1} PFU/mL showed a significantly lower reduction ($P \leq 0.05$; Table 4.3) implying that the MOI only impacts on the overall level of bacterial lysis at extremely low phage levels.

As the MOI was decreased, the amount of time taken for the phages to induce lysis increased (Table 4.3). The rate of lysis (i.e. the slope of the linear decrease in $OD_{420\text{-}580nm}$) was found to vary significantly ($P \leq 0.05$) for all phages with the exception of LP-M (Table 4.3; $P=0.452$).

When using the "acceptance" criteria (Table 4.2) all phages met the required level of bacterial reduction above a concentration of 10^{3} PFU/mL (Table 4.3).

However, all phages failed to meet the acceptable level for time taken to induce lysis.

4.3.1.2 Enhancement of lytic activity following treatment with PRE

The streak test showed an increase in the level of plaque formation in all 4 phages (Table 4.4) following the enhancement process. The largest increase in streak score was between no PRE treatment (i.e. unenhanced) and the 15 min contact time. Subsequent enhancements led to smaller increases to a maximum level in all phages except GL-1 where a maximum activity was achieved following a 5 min enhancement. Both C10176-L and GL-1 showed the largest overall increase in score and LP-M showed the smallest increase (Table 4.4).

When analysed on the Bioscreen C, a phage-treated culture of PAO1 produced lysis within 30 min for all non-PRE treated phages and immediately with PRE-treated phages, with the exception of C10176-L (data not shown). Following enhancement there was a decrease in the gradient of the slope (implying a slower rate of bacterial lysis) for all phages and enhancement times, but this was not statistically significant ($P \geq 0.05$; Table 4.4). Only C10176-L after a 15 min enhancement time showed a non-significant increase in slope gradient ($P \geq 0.05$) when compared to non-PRE treated phage (Table 4.4).

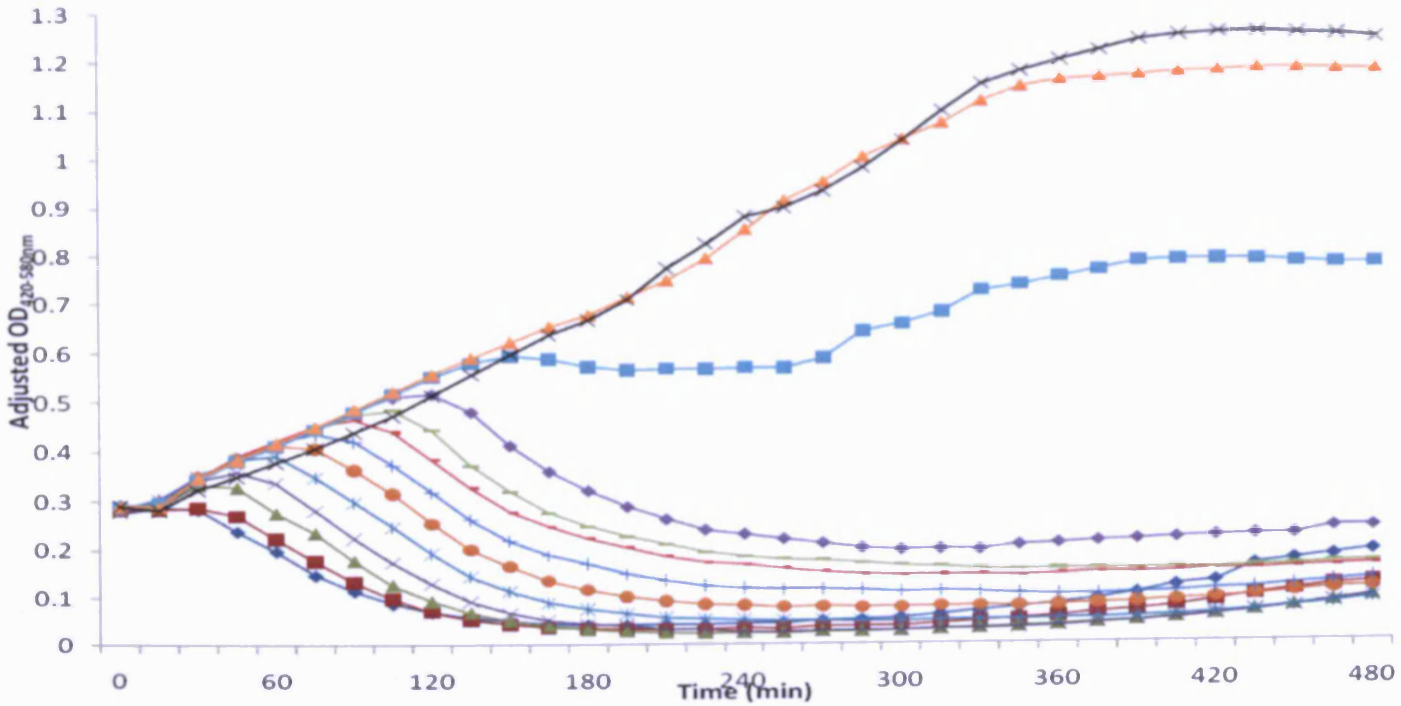


Figure 4.4: Effect of phage multiplicity of infection on the growth of *Ps. aeruginosa* strain PAO1. Phage used was LP-M. Data shown are the mean of 5 replicates (SD not shown as this would over complicate the figure). 10^{12} (♦), 10^{11} (■), 10^{10} (▲), 10^{9} (x), 10^{8} (x), 10^{7} (●), 10^{6} (+), 10^{5} (-), 10^{4} (-), 10^{3} (♦), 10^{2} (■), 10^{1} (▲), Untreated (x) PFU/mL.

Phage concentration ($PFUmL^{-1}$)	Bacteriophage											
	GL-1[4]			C10176-S[4]			C10176-L[5]			LP-M[5]		
	Time[1]	Slope[2]	Log_{10} Reduction[3]	Time[1]	Slope[2]	Log_{10} Reduction[3]	Time[1]	Slope[2]	Log_{10} Reduction[3]	Time[1]	Slope[2]	Log_{10} Reduction[3]
10^{12}	40(10)	20.2	≥2	35(25)	20.0	≥2	45(0)	25.9	≥2	30(15)	20.0	≥2
10^{11}	45(15)	20.6	≥2	55(10)	21.7	≥2	45(0)	24.1	≥2	40(10)	25.2	≥2
10^{10}	60(15)	25.5	≥2	55(10)	26.9	≥2	55(10)	29.9	≥2	55(10)	26.8	≥2
10^{9}	65(10)	22.3	≥2	70(10)	25.6	≥2	65(10)	25.7	≥2	60(0)	30.1	≥2
10^{8}	75(0)	25.2	≥2	85(10)	23.6	≥2	75(0)	27.9	≥2	70(10)	33.1	≥2
10^{7}	85(10)	4.01	≥2	85(15)	24.4	≥2	90(0)	22.9	≥2	75(0)	34.8	≥2
10^{6}	95(10)	20.9	≥2	90(10)	25.3	≥2	95(5)	23.5	≥2	90(0)	27.7	≥2
10^{5}	105(0)	37.2	≥2	110(10)	28.5	≥2	105(0)	25.1	≥2	105(0)	29.4	≥2
10^{4}	115(10)	30.8	≥2	130(15)	36.0	≥2	120(0)	31.7	≥2	120(0)	35.1	≥2
10^{3}	130(10)	32.3	≥2	145(15)	28.5	≥2	130(10)	30.3	≥2	140(10)	30.9	≥2
10^{2}	410(530)	6.38	1.34(0.87)	165(15)	16.8	1.10(0.97)	600(400)	-	1.14(0.71)	390(455)	6.45	1.01(0.58)
10^{1}	-	-	0.38(0.54)	-	-	0.29(0.19)	-	-	0.39(0.50)	-	-	0.15(0.43)

Table 4.3: Summary of the effect of multiplicity of infection on the growth of *Ps. aeruginosa* strain PAO1. [1]: Time (min) taken to induce a lytic slope (±SD). [2]: Slope is expressed as a decrease in $OD_{420-580nm}$ per minute ($x10^{-4}$). [3]: Log_{10} reduction in bacterial number at 8 h. [4]: n=4. [5]: n=5. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

Phage Treatment (min[1])	Streak Test (max.50)	Bioscreen C			
		Slope[2]	Log_{10} Reduction		Time[3]
			8 h	20 h	
Phage GL-1					
0	20	22.0	1.95(0.51)	1.24(1.10)	815±670
15	40	19.9	1.98(0.68)	≥2	>1200
10	45	17.5	≥2	≥2	>1200
5	46	16.0	≥2	≥2	>1200
2.5	43	16.3	≥2	≥2	>1200
Phage C10176-S					
0	29	22.1	≥2	≥2	>1200
15	40	19.6	1.99(0.73)	≥2	>1200
10	44	18.8	≥2	≥2	>1200
5	43	16.0	≥2	≥2	>1200
2.5	47	17.1	≥2	≥2	>1200
Phage C10176-L					
0	20	21.5	≥2	≥2	>1200
15	40	23.1	1.99(0.69)	≥2	>1200
10	44	16.4	≥2	≥2	>1200
5	45	16.9	≥2	≥2	>1200
2.5	48	17.0	≥2	≥2	>1200
Phage LP-M					
0	30	21.5	≥2	≥2	>1200
15	40	19.3	1.99(0.70)	≥2	>1200
10	44	17.3	≥2	≥2	>1200
5	45	16.3	≥2	≥2	>1200
2.5	45	16.2	≥2	≥2	>1200

Table 4.4: Summary of the lytic activity of *Ps. aeruginosa* phages treated with PRE against *Ps. aeruginosa* strain PAO1. Data shown are the mean of 3 replicates (±SD). [1]: Phage-bacteria contact time before exposure to PRE (see text). [2]: Slope is expressed as a decrease in $OD_{420\text{-}580nm}$ per minute ($x10^{-4}$). [3]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. Bold typeface indicates significant differences at the 95% CI by one way ANOVA when compared to non-PRE treated phage.

The level of reduction in bacterial number was calculated from $OD_{420\text{-}580nm}$ as previously described (Chapter 4; Section 4.2.3.3), PRE-treated phages (2.5 min enhancement time) showed a significantly higher level of reduction than non-PRE treated phages ($P \leq 0.05$; Table 4.4) at 8 h. This difference was not significant by 20 h, with the exception of GL-1 (Table 4.4). All PRE-treated phages showed a continuation of the reduction in bacterial number between 8 and 20 h; with the exception of LP-M these increases were not significant ($P \geq 0.05$; Table 4.4).

When comparing the lytic activity of PRE and non-PRE treated GL-1 phage using a streak test, it was observed that the highest level of activity was following a 5 min contact time (Table 4.4). However, the highest level of lytic activity (i.e. Log_{10} reduction in bacterial number) when assessed with the Bioscreen C was observed with a 2.5 min contact time (Table 4.4). The difference in Log_{10} reduction using the Bioscreen C between these two contact times was not significant ($P \geq 0.05$).

In the host strain, PAO1, all PRE treatment times showed the induction of a lytic slope in ≤30 min, thereby meeting the "acceptance" criteria set out in Table 4.2 (data not shown).

Overall GL-1 phage showed the greatest difference between the Log_{10} reduction in bacterial number when comparing untreated and PRE treated samples.

When comparing the time taken for PAO1 to re-grow to an $OD_{420\text{-}580nm}$ value of 0.1 higher than the initial $OD_{420\text{-}580nm}$, it was found that for all phages, except GL-1, both PRE and non-PRE treated phages took >1200 min to reach this value (Table 4.4). When compared against the "acceptance" criteria (Table 4.2) for all phages, both PRE and non-PRE treated exceeded the "acceptance" criteria for regrowth of ≥480 min.

4.3.2 Assessment of the lytic activity of *Ps. aeruginosa* phages against strains isolated from CF patients

Enhanced phages were chosen on the basis of streak test analysis. Where there was no increase or decrease in activity the final stage (2.5 min) of enhancement was chosen in order to provide a comparison between PRE treated and non PRE-treated phages.

The treatment of phages with PRE, when assessed using the streak test method, led to an increase in phage activity with C10176-L and LP-M showing the largest increases and GL-1 showing the smallest (Tables 4.5 a-d). *Ps. aeruginosa* strain C4503 showed increased susceptibility to infection in only the LP-M phage (Table 4.5d). However, some strains, most notably the Mids1 strain (a CF epidemic strain) showed no increase in susceptibility to any of the phages tested (Table 4.5a-d).

Assessment using the Bioscreen C revealed that some of the bacterial strains exhibited no clear lytic slope when challenged with non-PRE treated phages and that following PRE treatment the lytic slope either decreased in gradient (i.e. slower rate of lysis) or ceased to be present (Table 4.5 a-d), and thus failed to meet "acceptance" criteria in almost 50% of strains (Table 4.6). Despite this, the growth pattern of the bacteria varied drastically between PRE and non-PRE treated phages (Figure 4.5).

The activity of the phages based on the "acceptance" criteria described earlier (Table 4.2), the level of bacterial lysis at 8 h (i.e. reduction in bacterial number calculated from $OD_{420\text{-}580nm}$) showed no significant difference between non-PRE and PRE treated phages with the exceptions of C3786 and C1913 treated with C10176-S (Table 4.5b) and C1913 and C2846 treated with GL-1 (Table 4.5a). All bacterial strains showed some level of reduction by 8 h with the exception of Mids1.

Following exposure to phages, all bacterial strains exhibited an eventual increase in $OD_{420\text{-}580nm}$ (Table 4.5a-d) which is indicative of bacterial regrowth, and potentially the presence of phage resistant bacteria. This increase varied between the bacteria/phage combinations. In the vast majority of cases the level of regrowth (time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original) met the required acceptance criteria (i.e. ≥480 min).

Bacterial strain	Non-PRE treated phage				PRE Treated phage			
	Streak test (max.50)	Bioscreen C Log_{10} reduction 8 h	Bioscreen C Log_{10} reduction 20 h	Time[1]	Streak test (max. 50)	Bioscreen C Log_{10} reduction 8 h	Bioscreen C Log_{10} reduction 20 h	Time[1]
NCTC	35	0.99(0.74)	0.72(1.22)	230(35)	-[2]	≥2	1.51(1.68)	180(25)
C3652	34	1.23(0.17)	1.70(0.71)	885(295)	38 *10*[3]	0.90(0.20)	1.21(0.70)	505(140)
C3719	46	0.93(0.17)	0.97(0.06)	585(130)	-[2]	0.79(0.20)	1.39(0.50)	455(160)
C3786	44	0.77(0.67)	0.05(0.95)	110(30)	-[2]	≥2	1.57(1.07)	145(10)
LES	30	1.09(0.13)	≥2	**1185(25)**	34 *2.5*[3]	0.97(0.21)	1.25(0.09)	**940(80)**
Mids1	30	-[4]	1.22(0.76)	**85(20)**	-[2]	-[4]	1.12(0.69)	**140(25)**
P8959	35	1.79(0.34)	≥2	905(175)	-[2]	1.95(0.07)	≥2	915(25)
C1913	6	≥2	0.21(0.63)	310(20)	22 *2.5*[3]	≥2	≥2	345(90)
C2238	23	0.51(0.08)	0.73(0.51)	765(70)	40 *10*[3]	0.57(0.16)	≥2	840(240)
C2846	35	**0.97(0.50)**	0.48(0.71)	240(15)	-[2]	≥2	≥2	345(275)
C3597	50	≥2	0.89(1.32)	560(185)	-[2]	≥2	≥2	495(300)
C4503	34	-[4]	0.10(0.35)	140(20)	-[2]	0.90(1.52)	0.99(2.06)	350(400)
PAK	30	≥2	≥2	**>1200**	46 *2.5*[3]	≥2	≥2	**1020(40)**

Table 4.5a: The lytic activity of PRE and non-PRE treated *Ps. aeruginosa* phage GL-1 when tested against strains isolated from CF patients. Data shown are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420-580nm}$ by 0.1 above the original $OD_{420-580nm}$. [2]: No increase in score [3]: *time*: phage-bacteria contact time (min) before exposure to PRE (See text) [4]: No reduction in bacterial number observed. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

Bacterial strain	Non-PRE treated phage				PRE Treated phage			
	Streak test (max.50)	Bioscreen C			Streak test (max. 50)	Bioscreen C		
		Log_{10} reduction		Time[1]		Log_{10} reduction		Time[1]
		8 h	20 h			8 h	20 h	
NCTC	14	0.47(0.78)	-[4]	220(30)	27 *15*[3]	1.64(1.20)	1.32(1.52)	180(25)
C3652	30	0.97(0.24)	≥2	900(520)	40 *10*[3]	0.95(0.37)	0.93(0.37)	558(200)
C3719	30	0.89(0.24)	1.87(0.71)	890(540)	31 *15*[3]	0.77(0.31)	1.39(0.79)	485(170)
C3786	9	**0.87(1.31)**	0.58(1.04)	**100(25)**	45 *2.5*[3]	≥2	1.90(1.11)	**145(10)**
LES	14	0.91(0.24)	1.46(0.36)	805(280)	30 *2.5*[3]	1.50(1.21)	1.27(0.40)	980(330)
Mids1	18	-[4]	0.68(0.58)	95(30)	-[2]	-[4]	1.55(0.73)	140(25)
P8959	22	1.96(0.20)	≥2	**1100(85)**	-[2]	1.81(0.08)	1.91(0.48)	**825(145)**
C1913	30	**1.50(0.33)**	0.03(0.16)	200(30)	-[2]	≥2	≥2	425(285)
C2238	8	0.56(0.11)	1.02(0.27)	805(50)	40 *10*[3]	0.62(0.13)	≥2	865(340)
C2846	25	0.76(0.67)	0.59(0.77)	215(25)	30 *10*[3]	≥2	≥2	215(50)
C3597	2	≥2	1.78(1.24)	500(280)	46 *5*[3]	≥2	≥2	895(295)
C4503	30	-[4]	0.01(0.12)	140(10)	-[2]	0.81(1.63)	0.83(1.92)	370(435)
PAK	24	≥2	≥2	1095(160)	46 *5*[3]	≥2	≥2	1025(100)

Table 4.5b: The lytic activity of PRE and non-PRE treated *Ps. aeruginosa* phage C10176-S when tested against strains isolated from CF patients. Data shown are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. [2]: No increase in score [3]: *time*: phage-bacteria contact time (min) before exposure to PRE (See text) [4]: No reduction in bacterial number observed. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

Bacterial strain	Streak test (max.50)	Non-PRE treated phage			PRE Treated phage			
		Bioscreen C			Streak test (max. 50)	Bioscreen C		
		Log_{10} reduction		Time[1]		Log_{10} reduction		Time[1]
		8 h	20 h			8 h	20 h	
NCTC	10	0.90(0.78)	0.99(0.99)	205(10)	21 *10*[3]	1.90(0.65)	1.46(0.89)	180(40)
C3652	10	0.94(0.14)	1.79(0.64)	590(85)	36 *10*[3]	1.06(0.15)	1.76(0.88)	1005(340)
C3719	0	0.85(0.05)	1.59(0.46)	565(10)	25 *10*[3]	1.05(0.15)	≥2	>1200
C3786	44	≥2	≥2	130(10)	-[2]	≥2	1.51(0.87)	140(10)
LES	10	1.09(0.18)	≥2	1155(80)	29 *2.5*[3]	0.88(0.27)	1.04(0.43)	860(280)
Mids1	10	-[4]	1.12(0.63)	105(45)	-[2]	-[4]	1.43(0.69)	140(25)
P8959	10	1.99(0.20)	≥2	1180(35)	27 *10*[3]	1.72(0.46)	1.86(0.60)	770(285)
C1913	0	≥2	0.10(0.55)	345(55)	22 *2.5*[3]	≥2	≥2	240(50)
C2238	0	0.59(0.07)	1.48(0.23)	865(135)	49 *5*[3]	0.59(0.14)	≥2	790(325)
C2846	20	≥2	≥2	**305(30)**	24 *10*[3]	≥2	≥2	**205(40)**
C3597	47	≥2	1.34(1.67)	**570(210)**	50 *10*[3]	≥2	≥2	**185(40)**
C4503	20	0.07(0.04)	0.30(0.27)	130(10)	-[2]	0.95(1.70)	0.35(0.99)	380(440)
PAK	16	≥2	≥2	1085(150)	40 *15*[3]	≥2	≥2	1000(165)

Table 4.5c: The lytic activity of PRE and non-PRE treated *Ps. aeruginosa* phage C10176-L when tested against strains isolated from CF patients. Data shown are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. [2]: No increase in score [3]: *time*: phage-bacteria contact time (min) before exposure to PRE (See text) [4]: No reduction in bacterial number observed. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

Bacterial strain	Non-PRE treated phage				PRE Treated phage			
	Streak test (max.50)	Bioscreen C			Streak test (max. 50)	Bioscreen C		
		Log_{10} reduction		Time[1]		Log_{10} reduction		Time[1]
		8 h	20 h			8 h	20 h	
NCTC	32	0.87(0.56)	1.00(0.62)	200(20)	-[2]	≥2	1.76(1.18)	170(30)
C3652	22	1.08(0.02)	≥2	880(280)	35 *10*[3]	0.92(0.32)	1.54(0.79)	505(275)
C3719	20	0.95(0.24)	≥2	**>1200**	31 *10*[3]	0.76(0.29)	1.94(0.08)	**450(250)**
C3786	40	≥2	≥2	130(20)	42 *2.5*[3]	≥2	1.17(1.36)	140(10)
LES	30	1.08(0.19)	≥2	1155(80)	-[2]	0.91(0.23)	1.22(0.42)	940(320)
Mids1	10	-[4]	1.56(0.60)	115(40)	-[2]	-[4]	1.04(1.19)	135(15)
P8959	20	≥2	≥2	>1200	26 *10*[3]	1.76(0.28)	1.99(0.73)	830(285)
C1913	0	≥2	≥2	545(115)	22 *10*[3]	≥2	≥2	350(205)
C2238	20	0.77(0.09)	≥2	>1200	50 *5*[3]	0.51(0.17)	≥2	705(370)
C2846	43	≥2	≥2	**305(10)**	50 *10*[3]	≥2	≥2	**200(40)**
C3597	43	≥2	1.84(0.64)	**605(85)**	50 *10*[3]	≥2	≥2	**220(60)**
C4503	10	-[4]	-[4]	130(10)	21 *10*[3]	0.83(1.54)	0.72(1.89)	335(360)
PAK	4	≥2	≥2	995(75)	42 *2.5*[3]	≥2	≥2	950(55)

Table 4.5d: The lytic activity of PRE and non-PRE treated *Ps. aeruginosa* phage LP-M when tested against strains isolated from CF patients. Data shown are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. [2]: No increase in score [3]: *time*: phage-bacteria contact time (min) before exposure to PRE (See text) [4]: No reduction in bacterial number observed. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

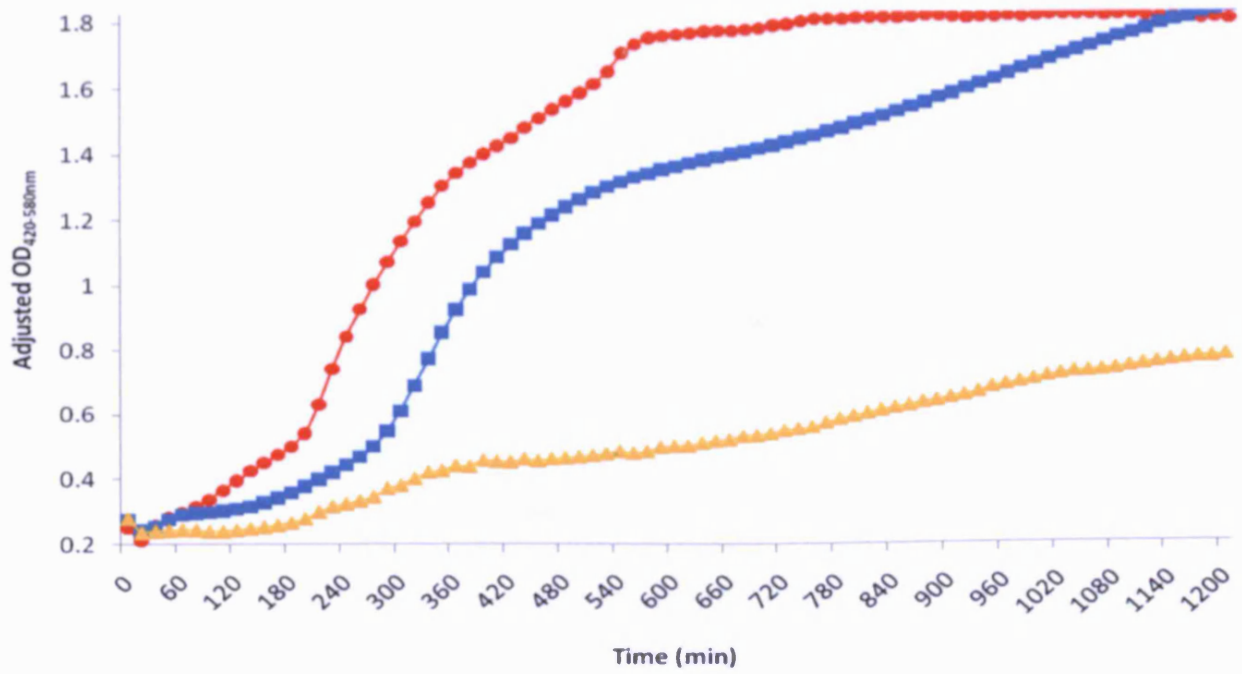


Figure 4.5: Representative change in bacterial growth patterns of *Ps. aeruginosa* strain C1913 following treatment with PRE treated and non-PRE treated C10176-S bacteriophage. Phage treated samples are the mean of 3 replicates; untreated samples are the mean of 6 replicates. ●, Untreated bacteria; ■, non-PRE treated; ▲, PRE treated.

		Evaluation criteria			Additional criterion
		Log_{10} reduction		Time (min)[1]	Lysis[2]
		8 h	20 h		
Phage	**Treatment**	No. of bacterial strains where phage "acceptance" criteria was met (out of 14)			
GL-1	Non-PRE treated	3	3	8	7
C10176-S		3	4	8	7
C10176-L		6	6	8	7
LP-M		7	10	8	7
GL-1	PRE treated	7	7	7	4
C10176-S		6	6	8	4
C10176-L		6	7	6	4
LP-M		7	6	6	4

Table 4.6: Summary of the lytic assessment criteria of single *Ps. aeruginosa* phages from Bioscreen C assessment. [1]: Time taken to increase $OD_{420-580nm}$ by 0.1 about the original $OD_{420-580nm}$. [2]: Time taken to induce a lytic slope.

4.3.3 The lytic activity of phage cocktails against strains of *Ps. aeruginosa*

When the lytic activity of *Ps. aeruginosa* phage cocktails was assessed by the streak test method, all phage cocktails exhibited a score of ≥30 against all strains with the exception of Mids1 and it was not possible to distinguish between the different phage cocktails (Table 4.7a-b).

When analyzed by the Bioscreen C method the lytic activity of phage cocktails (containing between 2-5 phages) varied depending on the bacterial strain that was challenged (Tables 4.7a-b).

Once again, *Ps. aeruginosa* strain C4503 exhibited the least sensitivity to phage infection over all the criteria measured. CF epidemic strains showed less sensitivity to phage infection (i.e. lower "kill") compared to CF non-

epidemic strains (Tables 4.7a-b). In the case of PAO1, all phage cocktails met or exceeded the activity of at least two of the individual phage components.

When assessed using the "acceptance" criteria as previously described (Table 4.2) approximately 50% of strains met the required standard for Log_{10} reduction at 8 h (Table 4.8), although this increased to above 50% for all phage cocktails tested. For the level of bacterial regrowth 50% or above of strains met the required criterion.

Although the 5 phage cocktail (containing GL-1, GL-1 *2.5*, LP-M *10*, C10176-S *15* and LP-M *5*) met the most "acceptance" criteria, both this cocktail and the 3 phage cocktail (containing GL-1, GL-1 *2.5*, and LP-M *10*) showed no significant differences in terms of activity ($P \geq 0.05$; i.e. no greater reduction in bacterial number etc).

Bacterial strain	Phage Cocktail							
	GL-1 and GL-1 *2.5*				GL-1, GL-1 *2.5* and LP-M *10*			
	Streak test	Log_{10} reduction		Time[1]	Streak test	Log_{10} reduction		Time[1]
		8 h	20 h			8 h	20 h	
PAO1	50	≥2	≥2	>1200	50	≥2	≥2	1195(10)
NCTC	40	1.41(0.15)	1.16(0.44)	220(20)	40	≥2	≥2	175(35)
C3652	50	1.06(0.20)	≥2	>1200	50	1.02(0.18)	≥2	>1200
C3719	50	0.92(0.09)	≥2	>1200	50	1.03(0.16)	≥2	>1200
C3786	50	1.91(0.98)	1.03(1.33)	140(10)	50	≥2	1.81(1.39)	140(10)
LES	40	0.95(0.21)	0.99(0.44)	750(225)	40	0.98(0.12)	1.05(0.31)	795(140)
Mids1	4	-[2]	≥2	165(55)	0	-[2]	≥2	150(40)
P8959	50	1.50(0.36)	1.66(0.41)	855(85)	50	1.66(0.30)	1.51(0.82)	785(205)
C1913	44	≥2	≥2	255(65)	44	≥2	≥2	225(60)
C2238	50	0.64(0.22)	3.02(1.11)	860(295)	50	0.70(0.24)	≥2	735(220)
C2846	42	≥2	≥2	510(325)	42	≥2	≥2	465(255)
C3597	50	≥2	1.03(1.96)	535(295)	50	≥2	≥2	365(245)
C4503	30	0.33(0.54)	0.86(0.88)	120(15)	30	0.25(0.55)	0.46(0.87)	125(10)
PAK	50	≥2	≥2	745(320)	50	≥2	≥2	755(320)

Table 4.7a: Summary table of the lytic activity of phage cocktails against *Ps. aeruginosa* assessed using the Bioscreen C. Phage cocktails contained equal parts GL-1, GL-1 *2.5* and LP-M *10*. Data shown are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. [2]: No reduction in bacterial number observed. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

Bacterial strain	Phage Cocktail							
	GL-1, GL-1 *2.5*, LP-M *10* and C10176-S *15*				GL-1, GL-1 *2.5*, LP-M *10*, C10176-S *15* and LP-M *5*			
	Streak test	Log_{10} reduction		Time[1]	Streak test	Log_{10} reduction		Time[1]
		8 h	20 h			8 h	20 h	
PAO1	50	≥2	≥2	>1200	50	≥2	≥2	>1200
NCTC	40	≥2	≥2	200(25)	40	≥2	≥2	210(15)
C3652	50	1.18(0.06)	≥2	>1200	50	1.15(0.08)	≥2	>1200
C3719	50	1.04(0.05)	≥2	>1200	50	1.03(0.04)	≥2	>1200
C3786	50	≥2	1.49(0.87)	135(0)	50	≥2	1.43(0.49)	125(10)
LES	40	0.96(0.16)	0.99(0.32)	755(140)	40	0.95(0.09)	0.96(0.23)	740(75)
Mids1	0	0.57(0.77)	≥2	150(30)	0	-[2]	≥2	160(40)
P8959	50	1.60(0.09)	1.82(0.21)	860(150)	50	1.56(0.08)	1.67(0.19)	755(130)
C1913	44	≥2	≥2	240(95)	44	≥2	≥2	195(65)
C2238	50	0.64(0.30)	≥2	835(355)	50	0.68(0.31)	≥2	840(345)
C2846	42	≥2	≥2	580(415)	42	≥2	≥2	545(395)
C3597	50	≥2	≥2	605(350)	50	≥2	≥2	600(315)
C4503	30	0.19(0.45)	0.79(1.32)	125(10)	30	0.26(0.49)	0.56(0.88)	125(10)
PAK	50	≥2	≥2	785(345)	50	≥2	≥2	785(340)

Table 4.7b: Summary table of the lytic activity of phage cocktails against *Ps. aeruginosa* assessed using the Bioscreen C. Phage cocktails contained equal parts GL-1, GL-1 *2.5* and LP-M *10*. Data shown are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. [2]: No reduction in bacterial number observed. Bold typeface indicates significant differences at the 95% CI by one way ANOVA.

	Evaluation Criteria			Additional Criterion
	Log_{10} reduction		Time	Lysis[2]
	8 h	20 h	(min)[1]	
Phage Cocktail	No. of bacterial strains where phage "acceptance" criteria was met (out of 14)			
GL-1 and GL-1 *2.5*	5	8	9	6
GL-1, GL-1 *2.5* and LP-M *10*	7	10	7	6
GL-1, GL-1 *2.5*, LP-M *10* and C10176-S *15*	7	9	9	6
GL-1, GL-1 *2.5*, LP-M *10*, C10176-S *15* and LP-M *5*	7	10	9	6

Table 4.8: Summary of the lytic assessment criteria of *Ps. aeruginosa* phage cocktails from Bioscreen C assessment. Numbers in brackets represent enhancement time [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 about the original $OD_{420\text{-}580nm}$. [2]: Time taken to induce a lytic slope.

4.3.4 Comparison between the streak test and Bioscreen C assessment

In the host strain (PAO1), there was a good level of correlation between the Bioscreen C and the streak test assessment, where PRE treated phages showed the highest level of bacterial reduction corresponding with the highest scores achieved on the streak test (Table 4.4), with the exception of GL-1. When comparing the level of regrowth, there was no discernable difference between PRE and non-PRE treated phages.

The level of correlation between streak test and Bioscreen C method depended on the phage/bacterial strain. A good correlation (based upon meeting two or more of the "acceptance" criteria) was observed with LP-M, 2 strains; C10176-L, 5 strains; GL-1, 6 strains; C10176-S, 7 strains (Table 4.6). For all other phage/bacteria combinations, the level of reduction in bacterial number at 8 h was not significantly different ($P \geq 0.05$) between PRE and non-PRE treated phages.

The differences exhibited between the Bioscreen C and streak test analysis of phage cocktails further highlights the need for a more discriminatory test for phage activity than the current streak test method, as in this situation it has been shown to be unable to distinguish between different phage cocktails.

4.4 Discussion

4.4.1 Effect of PRE enhancement on lytic activity of *Ps. aeruginosa* phages

In this investigation 4 phages were confirmed, by both an established streak test assay (Merabishvili *et al.*, 2009) and a novel Bioscreen C assay, to possess activity against multiple strains of *Ps. aeruginosa* including some of the more prevalent clinical strains within the UK (Jones *et* al., 2001; Fothergill *et al.*, 2007). All bacterial strains, irrespective of treatment by wild or PRE treated phage, show some regrowth after 20 h, inferring the presence of either uninfected or resistant bacteria.

Although the treatment of phages with PRE was shown to increase lytic activity in the host strain (PAO1) and in some of the clinical strains, this increase in activity (either by increase in Log_{10} reduction or bacterial regrowth) was often not significant.

Result variability in the Bioscreen C assessment of lytic activity is most probably caused by the inherent variability that is associated with both bacterial and bacteriophage growth, despite initial standardization of bacterial concentration. The differences in susceptibility of the bacteria may be a result in part to the binding efficacy of the phage to the target cell (Comolli et al, 1999) and also the growth rates of the strains tested.

The PRE treatment was originally conceived as a selective virucide and has been demonstrated to have no effect on bacterial or fungal cells (Jassim *et al.*, 1995), and was designed as a mechanism for bacterial detection (Stewart *et al.*, 1998). Here we have adapted this process to remove phages that have been unable to infect a bacterial host within a specified amount of time. The exact

mechanism for the action of the PRE/ferrous sulphate complex still needs to be elucidated. It is possible that the function would be around the interaction of a phenolic compound with the binding sites of phage. Phenol itself has previously been shown to damage the tail fibres of the *Ps. aeruginosa* phage F116 (Maillard *et al.*, 1995; Maillard *et al.*, 1996). Damage to tail fibres would be consistent with the phages used in this investigation which have all been shown to be tailed (Chapter 3; Section 3.3.3). Although PRE treatment could potentially damage phage DNA preventing successful transcription and replication, phenol has been shown to not significantly impair *Ps. aeruginosa* phage F116 replication (Maillard *et al.*, 1995).

4.4.2 Bioscreen C *vs.* streak test for the measurement of phage activity

With a renewed increase in bacteriophage therapy (Merabishvili *et al.*, 2009) there is a need for a high throughput method that is both sensitive and quantitative. The successful selection of a phage does not hinge on the initial activity screening; the selection process can be enhanced by efficient and quantitative screening methods that will ensure the widest host range and maximum activity.

Here it has been shown that both the Bioscreen C and the streak test can be used to assess the lytic activity of phages and that the Bioscreen C method possesses a number of advantages in terms of both the quantity and type of data generated.

The set "acceptance" values used in this investigation were based upon the literature for other phage based products. The reduction of bacterial number (i.e. Log_{10} reduction) at 8 h and 20 h was based upon reductions that were reported from the literature for other *Ps. aeruginosa* phages (Hanlon *et al.*, 2001; Fu *et al.*, 2010). Regrowth criteria were based upon antibiotic dosing times for CF patients i.e. 2 or 3 times daily dosing (van Zanten *et al.*, 2007; McCoy *et al.*, 2008). This reflects a minimum amount of time (i.e. 480 min)

between dosings that any potential treatment developed would have to maintain activity over.

The streak test provides adequate data for initial screening in terms of yes/no activity; the Bioscreen C method allows for a quantitative assessment of the lytic activity of phages to be made based upon different criteria (i.e. reduction in bacterial number at set time points and bacterial regrowth) that would be useful in phage based products, such as the reduction in bacterial number and also the development of resistance. These criteria, however, should not be used individually but assessed as an amalgamation to reflect the real lytic ability of phages.

The key difference between the two methods is one of throughput. With the streak test a maximum of 4 phage combinations plus one set of controls (assuming 5 replicates per line) can be tested per experiment against a single bacterial strain. In the case of the Bioscreen C method, however, up to 18 combinations can be tested per run assuming the same conditions as the streak test.

4.4.2.1 Limitations of the current Bioscreen C assay

Although the Bioscreen C assay described in the current investigation represents a step towards a standardised, rapid and quantitative method for the assessment of phage activity, a number of drawbacks still need to be resolved.

Perhaps the biggest limitation of the Bioscreen C assay is the conversion of $OD_{420\text{-}580nm}$ to a CFU value from an OD/TVC graph for each bacterial strain, which in turn limits the sensitivity of the assay. At OD values above ~0.8 it is well established that a non-linear relationship between CFU and OD exists, therefore at OD values >0.8 the bacterial content may have been underestimated. This underestimation of bacterial content would also be evident at OD values <0.1 whereby turbidity caused by bacteria would be difficult to distinguish from the background OD. Therefore, a narrow range of

approx. 2 Log_{10} exists in which lytic activity can be calculated accurately using the current method. The use of an OD based system is further complicated by the contribution of bacterial debris to the total OD, which may also further reduce any detection limits.

In order to confirm the lytic activity of phages following initial assessment by Bioscreen C Assay, aliquots should be removed at required time points (i.e. 8 and 20 h as in the current investigation) and assessed for both bacterial and viral content.

Although the Bioscreen method currently possesses a number of flaws which limit its usefulness, these flaws could be addressed by the incorporation of metabolic assays that would allow for the assessment of the level of bacterial viability (Kuda and Yano, 2003; Cerca *et al.*, 2005) or potentially bioluminescent mutants (Marques *et al.*, 2005; Jassim *et al.*, 2007; Thorn *et al.*, 2007) providing direct measurement of bacterial viability, ultimately increasing the sensitivity of this OD-based assay.

4.4.3 Single bacteriophage preparations *vs.* phage cocktails

This chapter has demonstrated the effectiveness of both individual phage and also phage cocktails against *Ps. aeruginosa* strains. The choice of a phage cocktail over a single phage was based upon the current literature where phage preparations currently undergoing clinical trials (Rhoads *et al.*, 2009; Wright *et al.*, 2009) are cocktails targeting multiple species.

Here, the phage cocktails described have shown some increased lytic activity (i.e. increased Log_{10} reduction and reduced bacterial regrowth) as well as a broader host range compared to their individual counterparts, however most increases observed were not significant.

The choice of a 3 phage cocktail (containing equal parts of GL-1, GL-1 *2.5* and LP-M *10*) for future testing over the other cocktails described here was based

upon Bioscreen C assessment of lytic activity. Although more complex phage cocktails met more of the "acceptance" criteria, the 3 phage cocktail exhibited similar levels of activity.

4.4.4 Summary

This investigation has established the host range and overall level of lytic activity for 4 *Ps. aeruginosa* phages against 14 strains of *Ps. aeruginosa* including some clinically relevant strains from CF patients within the UK (Salunkhe *et al.*, 2005; Fothergill *et al.*, 2007).

An alternative assay for the investigation of the lytic activity of *Ps. aeruginosa* phages, based upon optical density reading, was also developed to address some of the deficiencies of current protocols and to further characterise the lytic activity of *Ps aeruginosa* phages.

The optical density assay compared to a currently adopted traditional plaque based assay has shown that the lytic activity of phages can be increased through selective breeding of phages with PRE (Chapter 4.3.1). It is interesting to note that not only did the lytic activity of phages increase against the chosen strain (in this case PAO1) but activity was also increased against a number of other strains. The enhancement of the PRE treated (2.5 min) phages was repeated, this time using *Ps. aeruginosa* strain C4503 as the host. It was observed that where activity had been decreased during the first cycle of enhancement (using PAO1 as a host), the activity against C4503 could be increased again using the enhancement procedure described above (Bhogal, 2010).

The cocktail of 3 phages created (containing GL-1, GL-1 *2.5* and LP-M *10*) will be used for subsequent testing based upon Bioscreen C analysis of activity as it was shown to have a broad range of activity that exceeded the individual components.

Chapter 5

The efficacy of a bacteriophage cocktail against biofilms of *Ps. aeruginosa*

5.1 Introduction

Within the clinical setting, bacterial biofilms present a considerable challenge to physicians (Hall-Stoodley *et al.*, 2004) with approx. 60% of infections treated believed to involve biofilms (Fux *et al.*, 2005). In the clinical setting this type of infection predominates around catheter use (Williams and Stickler, 2008), other medical devices such as endoscopes (Vickery *et al.*, 2004) and artificial joint replacements (Zimmerli and Ochsner, 2003). Once established within biofilms *Ps. aeruginosa* infections prove more difficult to clear with antibiotic therapy resulting from MIC levels that increase by up to 1000 fold when compared to planktonic cultures (Li *et al.*, 2008). In order to successfully treat biofilm associated infections both drug penetration and biofilm disruption need to take place (Smith, 2005). Both mucolytics and nebulised antibiotics are used to treat pulmonary infections in CF patients and enjoy some success in treating early stage *Ps. aeruginosa* colonisation (Ho *et al.*, 2009). In chronic CF patients where biofilms are well established the success rates of these therapies declines dramatically (Boe *et al.*, 2004).

Alginate lyase (ALG) enzymes have been isolated from a variety of sources including marine molluscs (Iwamoto *et al.*, 2001). The majority fit into one of two main groupings based upon substrate specificity. The first is poly (M) lyase (E.C.4.2.2.3) and the second poly (G) lyase (E.C. 4.2.2.11) (Shimizu *et al.*, 2003). A notable exception is the commercially available alginate lyase used in the current investigation which has been previously noted to possess bi-functional activity (Iwamoto *et al.*, 2001). The enzyme depolymerises the polysaccharide via a 3 stage β elimination reaction (Figure 5.1) in which the 1,4 linkage binding the monomer units is severed (Gacesa, 1992) forming optically-active isomers which can then be spectrophotometrically detected.

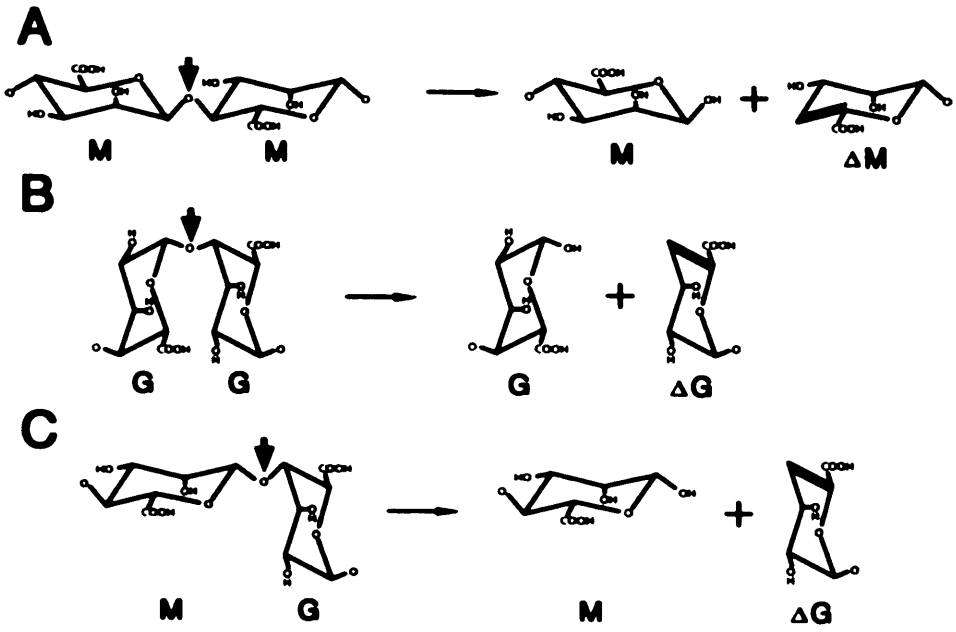


Figure 5.1: Mechanism of alginate degradation by alginate lyase. Thick arrow indicates site of cleavage, thin arrow indicates progression of reaction. Taken from (Yamasaki *et al.*, 2004). A, B and C indicate cleavage of different disaccharide blocks.

Ambroxol hydrochloride (AMB) is a mucolytic compound (Seifart *et al.*, 2005) and also sodium channel blocker (Gaida *et al.*, 2005) that is currently unlicensed for use in the UK (British National Formulary, 2011) and has been shown to affect the structure of *Ps. aeruginosa* biofilms (Li *et al*, 2008). Although the exact mechanism behind the disruption of biofilms is unknown, it is thought to be through the reduction of viscosity (Malerba and Ragnoli, 2008).

Since the mid 20th Century the treatment of bacterial infections has relied upon the use of antibiotics. The widespread use and misuse of these drugs, since their introduction, has led to the development of phenotypes that are resistant to multiple classes, most notably in *Staphylococcus aureus, Clostridium difficile* (Taori *et al.*, 2010) and *Mycobacterium tuberculosis* (O'Sullivan *et al.*, 2010).

Increased susceptibility to bacterial infection is one of the characteristic traits of CF in which sufferers are colonized in early childhood by organisms such as *H. influenzae* or *Staph. aureus* (Valenza *et al.*, 2008). However, these organisms are eventually supplanted by *Ps. aeruginosa* by the age of 20 (Pritt *et al.*, 2007).

The treatment of *Ps. aeruginosa* infection in CF patients varies depending on the stage of infection and these different strategies are summarised (Table 5.1).

Stage of Infection	**Treatment**	**Typical Duration**
Initial colonisation	Oral ciprofloxacin 30-40 mg/kg/day + Inhaled tobramycin 80-300 mg/12 h **Or** Inhaled colistin 1-3 million U/12 h	2-3 weeks or until negative sputum results
Chronic colonisation	Inhaled tobramycin 80-300 mg/kg/12 h **Or** Inhaled colistin 1-3 million U/12 h + Oral ciprofloxacin 30-40 mg/kg/day	Inhaled antibiotics permanently, Ciprofloxacin 2-3 weeks
Exacerbation (or antibiotic resistance)	IV ceftazidime,50–70 mg/kg/8 h + IV tobramycin, 5–10 mg/kg/24 h (NB, This is an example of a typical regime, but alternatives can be used)	

Table 5.1: Treatment regimes and doses for the various stages of *Ps. aeruginosa* infection in CF patients. Adapted from Canton *et al.*, (2005).

This chapter evaluates the effectiveness of a previously described cocktail (Chapter 4; Section 4.3.3) against biofilms of *Ps. aeruginosa*. The addition of other components to the cocktail will be investigated to see if the activity of the cocktail can be increased.

5.2 Materials and methods

5.2.1 Bacterial and bacteriophage strains

Ps. aeruginosa strains PAO1, NCTC, P8959 and C2238 were prepared and standardised as before (Chapter 2; Section 2.2.1). The phage cocktail containing equal parts GL-1, GL-1 *2.5* and LP-M *10* was prepared as previously described (Chapter 4; Section 4.2.5).

Phage cocktails containing either alginate lyase (ALG; 200 mg/L) or ambroxol hydrochloride (AMB; 200 mg/L) and ciprofloxacin (CIP) or tobramycin (TOB) at MIC concentrations (1 mg/L and 4 mg/L respectively; based upon Andrews, 2009) were prepared fresh prior to use. Antibiotics were chosen on the basis of NHS usage for *Ps. aeruginosa* infection at the University Hospital of Wales (Cardiff, UK; Hosein, 2007).

5.2.2 Assessment of the activity of a phage cocktail alone and in combination with other components

5.2.2.1 Bioscreen C assessment

The activity of the phage cocktail alone or in combination with other components was assessed using the Bioscreen C analyser as previously described (Chapter 4; Section 4.2.3).

5.2.2.2 Biofilm assessment

Sedimentary and CDFF biofilms were prepared as before (Chapter 2; Sections 2.5.1 and 2.5.2 respectively). Samples were then treated with 5 mL of phage cocktail alone or in combination with ALG or AMB at final concentrations of 200 mg/L and incubated at 37°C in a 95% air: 5% CO_2 atmosphere.

For the assessment of activity in combination with CIP or TOB biofilms were exposed to phage cocktails, TOB (4 mg/L) and CIP (1 mg/L) alone or in combination for 20 h before the bacterial content of biofilms was calculated.

The bacterial content of biofilms was then quantified as follows. PTFE discs were removed and rinsed gently with TSC buffer. Discs were then immersed in 1.2 mL of CompA that had been prepared as previously described (Chapter 4 Section 4.2.4) for 20 min in the dark. Samples were then sonicated on a MSE Soniprep 150 (MSE, Lower Sydenham, UK) for 30 s with a wavelength amplitude of 2 μm. Bacterial numbers were then quantified (Chapter 2 Section 2.3.1). This process was validated on 10 replicates using a one way ANOVA at the 95% CI (P=0.083).

5.3 Results

5.3.1 Assessment of the lytic activity of a phage cocktail alone or in combination with other components

5.3.1.1 Bioscreen C assessment

The mixture of phage cocktails with either CIP or TOB at MIC concentrations of (1 mg/L and 4 mg/L respectively) were shown to be the most effective treatment for the 4 strains tested by showing the highest bacterial reduction (Table 5.2) at 8 h. There were no significant differences in reduction over the period 8 to 20 h for any cocktail ($P \geq 0.05$; Table 5.2).

The addition of ALG or AMB at a final concentration of 200 mg/L to cocktails of phage also had no significant impact upon the time taken for bacterial regrowth ($P \geq 0.05$; Table 5.2). At MIC concentrations the cocktail of phage did not result in a significant difference in bacterial reduction at 8 h for all the strains tested ($P \geq 0.05$; Table 5.2). At 20 h, phage showed no significant difference in reduction ($P \geq 0.05$) in both standard strains (PAO1 and NCTC; Table 5.2). With the clinical strains (P8959 and C2238), CIP showed

significant differences ($P<0.05$) in reduction compared to the phage alone that was not consistent between 8 and 20 h. TOB only showed significant differences in C2238 (Table 5.2). Analysis also showed no significant differences ($P\geq0.05$) in reduction between the CIP and TOB at either 8 or 20 h.

When comparing the cocktail of phage and either CIP or TOB to antibiotics alone, no strain showed a significant difference in reduction at 8 and 20 h ($P\geq0.05$; Table 5.2) compared to antibiotic treatment alone.

However, when comparing the cocktails on the basis of the assessment criteria previously described (Chapter 4; Section 4.2.3), it was observed that phage cocktails in conjunction with CIP and TOB at MIC concentrations (1 mg/L and 4 mg/L respectively) proved to be the most effective (Table 5.3).

Treatment	Log_{10} reduction (± SD)		Time[1]
	8 h	20 h	
PAO1			
Cocktail only	≥2	≥2	1195 (10)
Cocktail + ALG	1.78 (0.35)	≥2	1150 (90)
Cocktail + AMB	1.82 (0.34)	≥2	1180 (35)
Cocktail + CIP	≥2	≥2	≥1200
Cocktail + TOB	≥2	≥2	≥1200
CIP	≥2	≥2	**70 (25)**
TOB	1.80 (0.39)	≥2	**455 (645)**
NCTC 10332			
Cocktail only	≥2	≥2	175 (35)
Cocktail + ALG	1.10 (0.95)	0.81 (0.97)	160 (40)
Cocktail + AMB	0.34 (0.44)	0.10 (0.60)	160 (40)
Cocktail + CIP	≥2	≥2	565 (550)
Cocktail + TOB	≥2	≥2	755 (465)
CIP	≥2	≥2	180 (25)
TOB	≥2	≥2	**1075 (220)**
P8959			
Cocktail only	1.66 (0.30)	1.51 (0.82)	785 (205)
Cocktail + ALG	1.21 (0.22)	0.60 (0.97)	**545 (35)**
Cocktail + AMB	1.24 (0.25)	0.74 (0.58)	560 (75)
Cocktail + CIP	≥2	≥2	**≥1200**
Cocktail + TOB	≥2	≥2	**≥1200**
CIP	**0.73 (0.36)**	≥2	**155 (10)**
TOB	1.77 (0.20)	≥2	**≥1200**
C2238			
Cocktail only	0.70 (0.24)	≥2	735 (220)
Cocktail + ALG	0.70 (0.17)	≥2	745 (180)
Cocktail + AMB	0.66 (0.21)	≥2	740 (220)
Cocktail + CIP	0.97 (0.16)	≥2	**≥1200**
Cocktail + TOB	1.09 (0.06)	≥2	**≥1200**
CIP	0.39 (0.10)	≥2	**230 (25)**
TOB	0.87 (0.07)	≥2	**≥1200**

Table 5.2: Bioscreen C assessment of a phage cocktail in conjunction with other components. Phage cocktails contained equal parts GL-1, GL-1 *2.5* and LP-M *10*. Data for Bioscreen analysis are the mean of three replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. Bold typeface indicates significant difference at the 95% CI by one way ANOVA to cocktail only.

Treatment	Evaluation Criteria		
	Log_{10} Reduction		Time[1]
	8 h	20 h	
	No. Strains meeting acceptance criteria (max. 4)		
Cocktail only	2	3	3
Cocktail + ALG	-[2]	2	3
Cocktail + AMB	-[2]	2	3
Cocktail + CIP	3	4	4
Cocktail + TOB	3	4	3
CIP	2	4	0
TOB	1	4	2

Table 5.3: Summary of the lytic assessment criteria of a phage cocktail in conjunction with other components from Bioscreen C assessment. Phage cocktails contained equal parts GL-1, GL-1 *2.5* and LP-M *10*. [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$. [2]: No strains met the required level.

5.3.1.2 CDFF grown biofilms

Following exposure of CDFF cultured biofilms to a phage cocktail (containing equal parts GL-1, GL-1 *2.5* and LP-M *10*) or mixtures of phage cocktail with ALG or AMB, all strains showed a significant reduction in bacterial content at 8 h ($P<0.05$) compared to a PBS treated control (Table 5.4). However, at 20 h only PAO1 and P8959 ($P<0.05$; Table 5.4) showed a significant reduction, while NCTC showed a small but statistically insignificant change in bacterial content ($P\geq0.05$; Table 5.4) compared to PBS controls.

In both PAO1 and P8959 there was no significant difference between biofilms treated with the phage cocktail or the phage ALG/AMB combination at 8 h and 20 h ($P\geq0.05$; Table 5.4).

At 8 h NCTC 10332 showed no significant differences between the cocktail only treated biofilm and the phage cocktail in combination with ALG ($P\geq0.05$;

Table 5.4), a significant difference was observed between the phage cocktail only and phage cocktail in combination with AMB ($P<0.05$; Table 5.4). At 20 h, there were no significant differences between each treatment ($P\geq0.05$; Table 5.4). There was also a significant level of bacterial regrowth between 8 and 20 h for each of the treated biofilms ($P<0.05$; Table 5.4).

Treatment	Bacterial strain (Log_{10} CFU ± SD)					
	PAO1		NCTC		P8959	
	8 h	20 h	8 h	20 h	8 h	20 h
Untreated	8.86(0.27)	8.87(0.46)	8.81(0.07)	8.93(0.23)	8.11(0.11)	8.39(0.14)
Phage cocktail only	**8.16(0.19)**	**8.17(0.13)**	8.30(0.14)	8.87(0.07)	**7.38(0.07)**	**7.71(0.11)**
Phage cocktail +ALG	**8.13(0.18)**	**8.10(0.11)**	**7.86(0.30)**	8.83(0.05)	**7.42(0.05)**	**7.39(0.23)**
Phage cocktail +AMB	**8.31(0.14)**	8.24(0.11)	**7.24(0.23)**	8.64(0.14)	**7.47(0.03)**	**7.18(0.23)**

Table 5.4: Activity of bacteriophage cocktail on 96 h CDFF cultured biofilms of *Ps. aeruginosa* at 20 h post phage cocktail addition. Data are the mean of three replicates (±SD). Phage cocktails contained equal parts GL-1, GL-1 *2.5* and LP-M *10*. Bold typeface indicates significant difference at 95% CI by one way ANOVA compared to untreated control.

5.3.1.3 Sedimentary biofilms

The exposure of sedimentary biofilms to a phage cocktail (containing equal parts of GL-1, *GL-1 2.5* and LP-M *10*) resulted in a significant reduction ($P \leq 0.05$) in the bacterial content of PAO1 biofilms. However, the reduction in bacterial number was not statistically significant ($P \geq 0.05$) in the other strains (Table 5.5).

When the reduction in bacterial number of *Ps. aeruginosa* sedimentary biofilms was assayed using phage in combination with antibiotics both standard (PAO1 and NCTC 10332) and clinical (P8959 and C2238) strains showed a significant reduction in biofilm bacterial content compared to an untreated control ($P \leq 0.05$; Table 5.5). In combination with ALG and AMB only PAO1 showed a significant reduction ($P \leq 0.05$) in biofilm bacterial content and NCTC 10332 and P8959 showed an increase in bacterial content that was not statistically significant ($P \geq 0.05$).

Phage cocktails alone, and in conjunction with other components showed significant differences with both ALG/AMB addition in PAO1 biofilms as well as antibiotics in all strains ($P \leq 0.05$; Table 5.5).

Both CIP and TOB at MIC concentrations proved to be the most effective of the strategies employed in this investigation following 20 h of exposure against all strains with the exception of PAO1 (Table 5.5). A significant reduction ($P \leq 0.05$) in bacterial content compared to untreated controls was observed, whereas there was no significant reduction in bacterial content for those strains tested with ALG or AMB alone ($P \geq 0.05$ with the exception of PAO1 Table 5.5).

Treatment	Bacterial strain (Log_{10} CFU ± SD)			
	PAO1	NCTC	P8959	C2238
Untreated	7.47 (0.21)	7.35 (0.27)	7.47 (0.15)	7.35 (0.29)
Cocktail only	**3.99 (1.29)**	6.84 (1.69)	7.33 (0.16)	7.19 (0.29)
Cocktail +ALG	**5.22 (0.39)**	8.24 (0.17)	7.86 (0.06)	6.97 (0.10)
Cocktail +AMB	**5.61 (0.27)**	7.96 (0.21)	7.93 (0.21)	6.96 (0.01)
Cocktail+ CIP	**<2**	**4.66 (0.15)**	**6.23 (0.20)**	**6.33 (0.28)**
Cocktail +TOB	**<2**	**4.65 (0.31)**	**6.08 (0.07)**	**5.35 (0.05)**
ALG only	**7.55 (0.16)**	7.31 (0.21)	7.12 (0.27)	7.58 (0.08)
AMB only	**6.70 (0.06)**	6.96 (0.24)	**6.53 (0.40)**	7.13 (0.13)
CIP only	**3.36 (0.05)**	**4.65 (0.59)**	**4.19 (0.31)**	**4.81 (0.26)**
TOB only	**3.62 (0.48)**	**4.47 (0.35)**	**3.49 (0.30)**	**5.45 (0.16)**

Table 5.5: Activity of phage cocktail with other components on 96 h sedimentary biofilms of *Ps. aeruginosa* at 20 h post cocktail addition. Data are the mean of three replicates (±SD). Phage cocktails contained equal parts GL-1, GL-1 *2.5* and LP-M *10*. Untreated and cocktail only are means of six replicates. Bold typeface indicates significant difference at 95% CI by one way ANOVA compared to untreated control.

5.3.1.4 Effects of extra components on the activity of a phage cocktail

When evaluating the overall effect of extra components on the activity of the phage cocktail used here it was found that the combinations of phage and antibiotics led to the highest activity (i.e. Log_{10} reduction at 20 h) in both Bioscreen and biofilm assessment (Figure 5.2). However, this was often a marginal increase over the other tests.

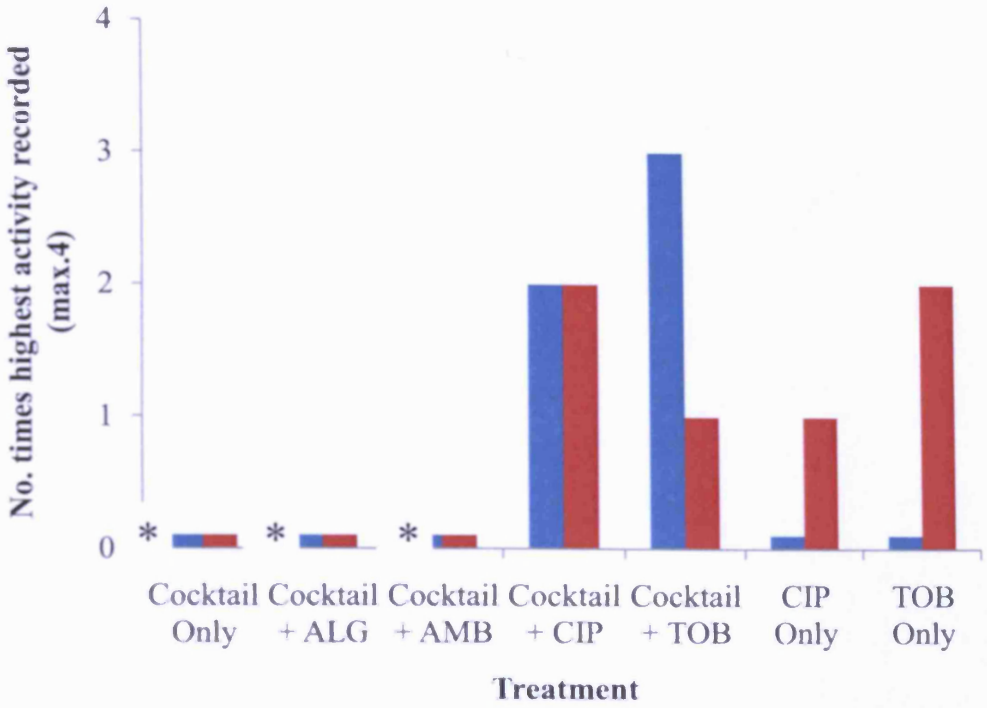


Figure 5.2: Representation of the relative activities of testing in this chapter. Ranking is based upon Log_{10} Reduction at 20 h. ■ Bioscreen C assessment and ■ Sedimentary biofilm assessment. *: no strains.

5.4 Discussion

5.4.1 The activity of a phage cocktail against biofilms of *Ps. aeruginosa*

Bacterial biofilms present an existing and developing challenge to clinicians trying to use successful therapeutic regimes. In the case of CF patients, the formation of *Ps. aeruginosa* biofilms is one of the key pathological features of disease progression, with the biofilm of *Ps. aeruginosa* surviving aggressive antibiotic therapy (Høiby *et al.*, 2010). The cocktail of multiple antibiotics (colistin and tobramycin; Herrmann *et al.*, 2010) increased the survival of mice and reduced *Ps. aeruginosa* content within sputum samples taken from CF patients.

In this investigation a previously described cocktail of phages, containing equal parts GL-1, GL-1 *2.5* and *LP-M 10* (Chapter 4; Section 4.3.3) has been shown

to possess activity against both sedimentary and CDFF cultured biofilms derived from multiple strains of *Ps. aeruginosa* including clinical strains isolated from CF patients. This activity extends to cocktails containing antibiotics (ciprofloxacin and tobramycin), alginate lyase and ambroxol hydrochloride. However, the observed reduction in bacterial content was modest when compared to phage cocktails reported in the literature (Wright *et al.*, 2009; Fu *et al.*, 2010).

The two different models of *Ps. aeruginosa* described here (CDFF and sedimentary biofilms) have shown sizable differences in their susceptibility to phage infection. In comparison to the *Ps. aeruginosa* biofilm layers within the CF lung, the biofilm layers employed here are simplistic and lack components such as an existing layer of mucin (similar to the thick sticky mucus that is found in CF patients; Sriramulu *et al.*, 2005). This has been shown in a previous chapter (Chapter 3; Section 3.3.2.3) to possess similar levels of structural complexity to images from the literature. There is some variation between the bacterial content of biofilms between sedimentary and CDFF cultured models. This is due to the denser packed nature and near consistent nutrient flow that the CDFF method provides, whereas in the sedimentation model, access to nutrients was limited. In the CDFF model in particular biofilms were often more densely packed and visible on the PTFE discs (unpublished observation) compared to their sedimentation counterparts, raising the bioburden, reducing any potential phage penetration and limiting reduction in bacterial content.

The combination of alginate lyase and ambroxol hydrochloride to phage preparations derives from the need to disrupt the biofilm layer and increase penetration not only by phage cocktails but potentially by other therapeutic compounds. The case of alginate lyase reveals an interesting situation in which both enzyme specificity, and also conditions of use, can play a role in activity. The enzyme used here, while suggesting bifunctional activity (Iwamoto *et al.*, 2001), may lack in activity compared to other alginate lyases isolated from

both *Ps. aeruginosa* phages (Glonti *et al.*, 2010) and bacterial spp. (Ma *et al.*, 2008; Uchimura *et al.*, 2010).

Here we have described a method for the direct quantification of the bacterial content of biofilms following the addition of phage. This method contests with the crystal violet method which is often used for biofilm quantification (Knezevic and Petrovic, 2008) and was designed to measure the ability of bacterial cells to bind to a surface, rather than as a quantification of bacterial numbers. The direct quantification method not only neutralises any surviving phage, following transfer to the activated PRE allowing for the "surviving" bacterial content of biofilms to be enumerated, but also does not rely upon the uptake of crystal violet into the exopolysachharide matrix and contains fewer stages, reducing potential disruption caused to the biofilm.

5.4.2 The use of antibiotics in combination with a phage cocktail

For the current investigation both Ciprofloxacin and Tobramycin have been used alone and in combination with a phage cocktail and assessed against both biofilms and planktonic *Ps. aeruginosa*. These antibiotics are currently primarily used to combat *Ps. aeruginosa* infection within CF patients via nebulisation.

Both Ciprofloxacin and Tobramycin are non-lytic antibiotics, acting against DNA gyrase and the bacterial 30S and 50S ribosome respectively. Although this will have little impact on the level of activity exhibited in biofilms, due to the direct quantification of activity by TVC.

However, in the Bioscreen C assay, due to the nature of activity calculation from OD, the level of reduction calculated may under estimate the level of activity of both the cocktail alone and also the activity of the cocktail in combination with antibiotics, due to the contribution of unlysed bacterial cells to the overall OD.

5.4.3 Summary

This investigation has established the activity of a cocktail of phages against biofilms of 4 *Ps. aeruginosa* strains in cocktail with a number of different components designed to increase phage penetration of a biofilm and to increase the level of bacterial kill. Although a modest level of activity against the bacterial strains was detected any potential therapeutic agent would need to increase this activity substantially to remain commercially viable.

Chapter 6

Delivery of a bacteriophage cocktail and quality control issues

6.1 Introduction

The nebulisation of antibiotics for the treatment of respiratory infections has been in clinical use since the introduction of penicillin to treat pneumonia. Today the nebulisation of antibiotics is the primary method of treating *Ps. aeruginosa* infections of CF patients (Ho *et al.*, 2009). Although many companies produce nebulisers they fall into one of two broad categories based upon the method of aerosol production; jet or ultrasonic nebulisers.

The production of an aerosol in the jet nebuliser relies upon the passage of a compressed air stream to create a negative pressure state which draws liquid from a reservoir. Depending on the flow rate of compressed air, particle size can vary and is controlled by the addition of baffles which result in the condensation of larger particles (Boe *et al.*, 2004).

Ultrasonic nebulisers however, produce aerosols of a uniform particle size (Boe *et al.*, 2004) via the vibration of a mesh with specific sized holes with piezoelectric actuators.

The different delivery vectors for potential phage therapeutics have been discussed in greater detail elsewhere (Chapter 1; Section 1.5.2). The advantages and disadvantages of the different type of nebulisers are summarised below (Table 6.1).

Nebuliser type	Advantages	Disadvantages
Jet	• Widely used for the delivery of antibiotics • Low operational cost	• Shear stress could destroy biological components • Requires a compressor pump to create aerosols • Loss of nebulised product in the airways
Ultrasonic	• More portable than jet nebulisers • Lower shear stress to nebulised product could increase availability to target site	• Higher initial cost of manufacture • Loss of nebulised product in the airways

Table 6.1: Summary table of the advantages and disadvantages of different forms of nebulisation.

With renewed interest in the use of phage to treat bacterial infection, attention will have to be drawn to the production of phage preparations in order to ensure they meet regulatory requirements. These requirements will vary depending on the type of application and route of administration, with topical applications requiring little in the way of microbial compliance and intravenous injections requiring strict control (British Pharmacopeia, 2011f). As such, many of the commercially produced phage preparations currently undergoing trials are of a topical nature (Wright *et al.*, 2009).

This chapter seeks to investigate the use of a jet stream nebuliser for the delivery of a cocktail of phages via nebulisation and the stability of the individual phage components. Pharmacopeial requirements, including bacterial content for non-sterile products and endotoxin content will also be investigated.

6.2 Materials and methods

Ps. aeruginosa strains PAO1, NCTC 10332, C2238 and P8959 were routinely cultured for 24 h prior to use in 10 mL of TSB at 37°C and standardised as previously described (Chapter 2; Section 2.2.2).

The phage cocktail, containing equal parts GL-1, GL-1 *2.5* and LP-M *10* was routinely cultured and enumerated as previously described (Chapter 2; Section 2.3.2).

6.2.1 Calculating the efficacy of phage cocktail delivery via a nebuliser into a simulated lung

A cocktail of *Ps. aeruginosa* phages (containing equal parts of GL-1, GL-1 *2.5* and LP-M *10*) was prepared in 0.9% (w/v) saline as previously described (Chapter 2; Section 2.2.2.1) and quantified.

Ten mL of the phage cocktail was transferred to the Ventstream drug chamber of a Porta-neb nebuliser (Respironics Resipiratory Drug Delivery Ltd, Chichester, UK). The nebuliser was connected to a Anderson cascade impactor (Figure 6.1) and air was drawn through the impactor at a rate of approx. 28 L/min. The phage suspension was then nebulised for 10 min after which the remaining phage suspension was recovered and quantified by the agar overlay method (Chapter 2; Section 2.3.2).

Steel sampling plates were removed from the cascade impactor using flame sterilized tweezers and transferred to sterile petri dishes. To each petri dish 10 mL of sterile PBS was added and the plates shaken at 150 RPM for 15 min. PBS was then pipetted into sterile centrifuge tubes and vortexed. The phage content of each impactor stage was then determined by the agar overlay method (Chapter 2; Section 2.3.2).

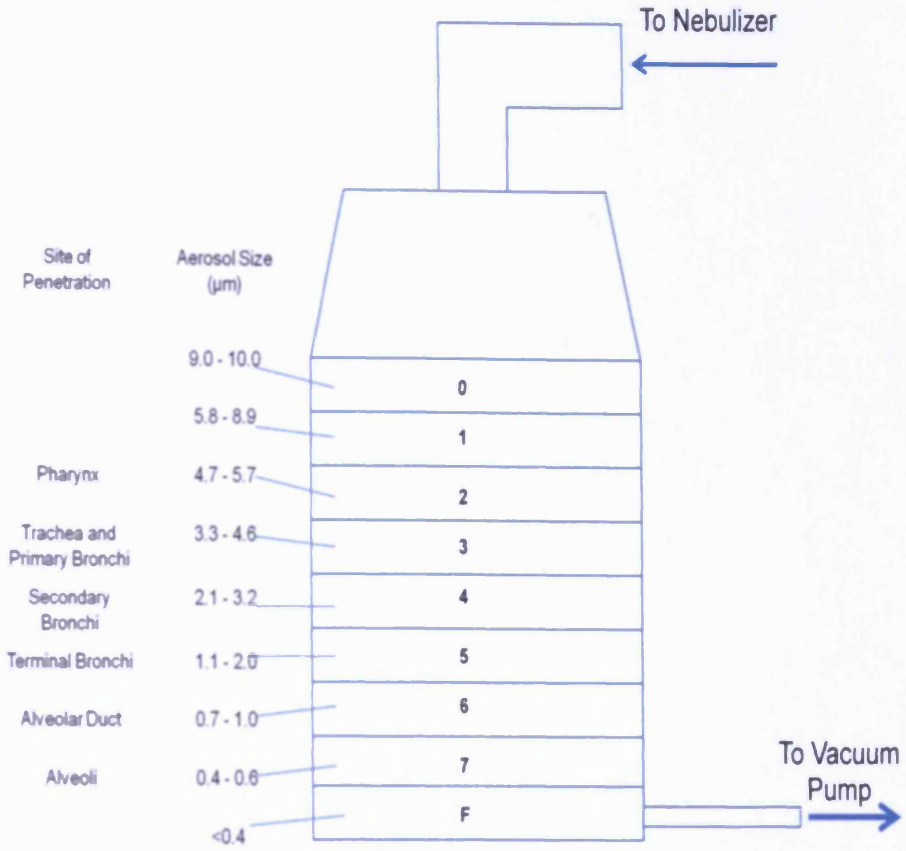


Figure 6.1: Schematic representation of the cascade impactor used to simulate depth of lung penetration.

6.2.2 Zeta potential characterisation of *Ps. aeruginosa* phages

6.2.2.1 Phage preparation

Phage suspensions (GL-1, GL-1 *2.5* LPM, LPM *10*, C10176-S and C10176-L) were prepared as previously described using sterile deionised water to wash the overlay agar from the surface of the plate (Chapter 2; Section 2.2.2).

6.2.2.2 Calibration of the Zetasizer 2000

The Zetasizer 2000 (Malvern Instruments Ltd, Malvern, UK) was calibrated daily by the passage of zeta potential transfer standard solution (Malvern Instruments Ltd, Malvern, UK). In order to be successfully calibrated the zeta potential had to fall within the range of 68 ± 6.8 mV.

6.2.2.3 Determination of the Zeta potential of *Ps. aeruginosa* phages

The zeta potential of phage suspensions was determined by passing 2.5 mL of phage suspension into the flow cell of a Zetasizer 2000 (Malvern Instruments Ltd, Malvern, UK) and five readings were taken. Between each sample the flow cell was flushed with 30 mL of sterile deionised water before the injection of the next sample.

6.2.3 Effect of purification on activity and endotoxin levels in a phage cocktail

All glassware and containers where possible were depyrogenated by dry heat sterilization at 200°C for 1 h (British Pharmacopeia, 2011a). Buffers and reagents were made in cell culture grade water.

6.2.3.1 Cocktail preparation

The phage cocktail containing equal parts of GL-1, GL-1 *2.5* and LP-M *10* (Chapter 4; Section 4.2.5) were prepared in 0.9% (w/v) saline and 50 μM $CaCl_2$ using PAO1 as a host.

6.2.3.2 Purification of a phage cocktail

Endotoxin was removed from the crude phage cocktail preparation via the Endotrap Blue Chromatography system (Hyglos, Germany) as follows. The prepacked column was drained and regenerated using the regeneration buffer supplied. Following regeneration the column was washed twice in 0.9% (w/v) saline with 50 μM $CaCl_2$ (equilibration buffer). The sample was added and flushed through with equilibration buffer. The column was then washed and regenerated prior to the addition of the next sample. Once finished the column was stored in 0.2% (w/v) sodium azide at 4°C. Samples of purified phage cocktail were assessed within 1 h of generation.

6.2.3.3 Endotoxin determination of *Ps. aeruginosa* phage cocktails

Endotoxin levels in suspensions of *Ps. aeruginosa* phage cocktails were determined via a Limulus amebocyte lysate (LAL) kinetic chromogenic

method on an Endosafe®-PTS (Figure 6.2; PTS; Charles River Laboratories International Inc., MA, USA) according to manufacturer's instructions (Charles River Laboratories International Inc., MA, USA) on a PTS testing cartridge (0.05 endotoxin units per millilitre (EU/mL); Charles River Laboratories International Inc., MA, USA). Experiments were performed in triplicate. Controls were also performed on 0.9% (w/v) saline. Where appropriate samples were diluted in LAL reagent grade water (Charles River Laboratories International Inc., MA, USA).

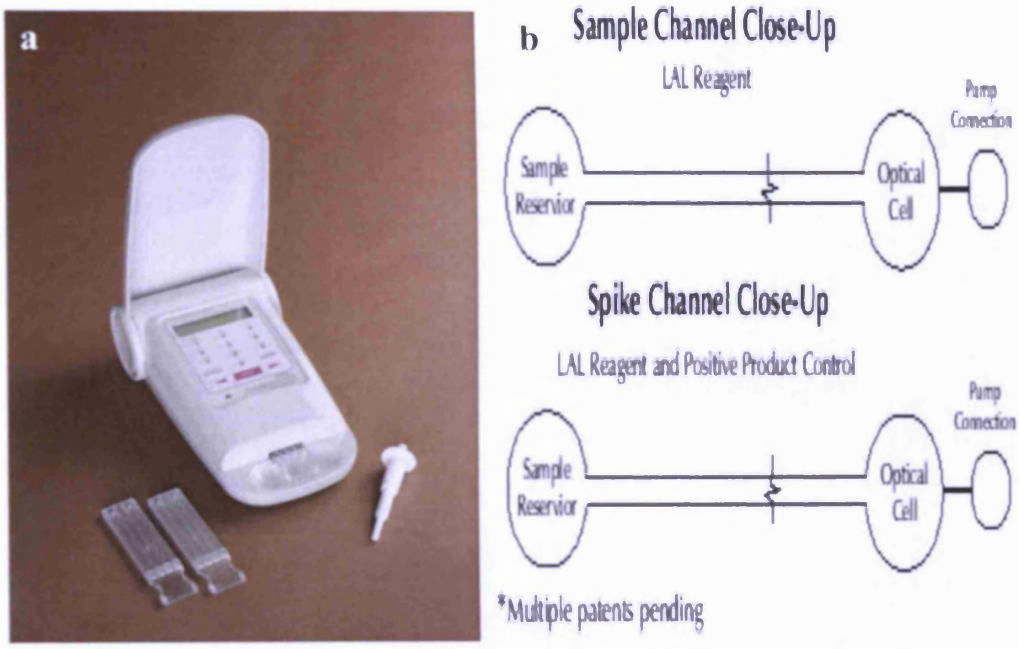


Figure 6.2: Endosafe PTS LAL kinetic assay for the detection of endotoxin and schematic representation of the test cartridge. a) Endosafe PTS machine and b) schematic representation of a PTS testing cartridge.

6.2.3.4 Activity determination of phage cocktails against *Ps. aeruginosa*

The lytic activity of both purified and non-purified *Ps. aeruginosa* phage cocktails was assessed using the Bioscreen C method against PAO1 as previously described (Chapter 4; Section 4.2.5).

6.2.4 Stability of *Ps. aeruginosa* phages

Crude *Ps. aeruginosa* phage suspensions of GL-1, GL-1 *2.5* and LP-M *10* were prepared in PBS as previously described (Chapter 4; Section 4.2.5). Following initial quantification of phage content, triplicate samples were stored at 2-8°C or room temperature (approx. 22°C). Samples were then quantified at 7, 30, 60, 90 and 180 days for phage content by the agar overlay method (Chapter 2; Section 2.2.2) using PAO1 as a host.

6.2.5 Testing phage cultures for microbial contamination

The level of microbial contamination in both purified and crude phage cocktails was assessed in accordance with Pharmacopoeial requirements for non-sterile respiratory products as follows (British Pharmacopoeia, 2011d and 2011e).

6.2.5.1 Total aerobic viable count

One millilitre of *Ps. aeruginosa* phage cocktail was used to inoculate 20 mL of TSA or 20 mL of Sabouraud Dextrose agar (SAB; Oxoid, UK). This was repeated for triplicate plates. TSA plates were incubated at 37°C for 5 days and SAB plates were incubated at 30°C for 5 days. Plates were checked for growth every 24 h.

6.2.5.2 Absence of *Ps. aeruginosa*

One millilitre of *Ps. aeruginosa* phage cocktail was incubated into 100 mL TSB and incubated at 37°C for 48 h. If no microbial growth was detected after 48 h the product was streaked to Cetrimide agar (Oxoid, UK) and incubated at 41-43°C for 24 h.

6.2.5.3 Absence of *Staphylococcus aureus*

One millilitre of *Ps. aeruginosa* phage cocktail was incubated into 100 mL TSB and incubated at 37°C for 48 h. If no microbial growth was detected after 48 h the product was sub-cultured to Baird-Parker Agar (Oxoid, UK) and incubated at 37°C for 72 h.

6.3 Results

6.3.1 Characterisation of *Ps. aeruginosa* bacteriophage charge

When the zeta potential (i.e. surface charge) of phages was assessed both enhanced and unenhanced phages were shown to be negatively charged (Figure 6.3 and Table 6.2). There was no significant difference ($P \geq 0.05$) in the surface charge of the non-PRE treated phages (Table 6.2) with C10176-L showing the least negative charge. Following treatment with PRE the surface charge of phages (GL-1 and LP-M) was increased (Table 6.2). However, this increase was not significant ($P \geq 0.05$) when compared to the non-PRE treated counterparts. It should also be noted that the conductivity (Cond.) value for GL-1 was increased following treatment with PRE (Table 6.2), however C10176-L possessed the highest conductance value.

Bacteriophage	**Charge Detail**		
	Mean (mV)	**Width (mV)**	**Cond. (mS/cm)**
	Non-PRE treated		
GL-1	-16.35(3.36)	4.98(4.73)	6.96(0.14)
C10176-S	-17.11(3.39)	4.05(2.48)	8.34(2.42)
C10176-L	-14.35(2.35)	3.47(1.00)	11.58(3.68)
LP-M	-17.07(2.78)	2.43(1.36)	8.73(2.80)
	PRE-treated		
GL-1 *2.5*	-15.20(3.39)	2.85(1.63)	10.18(3.82)
LP-M *10*	-16.76(2.32)	2.40(1.39)	8.87(3.43)

Table 6.2: Zeta potential characterisation of PRE and non-PRE treated *Ps. aeruginosa* bacteriophages.

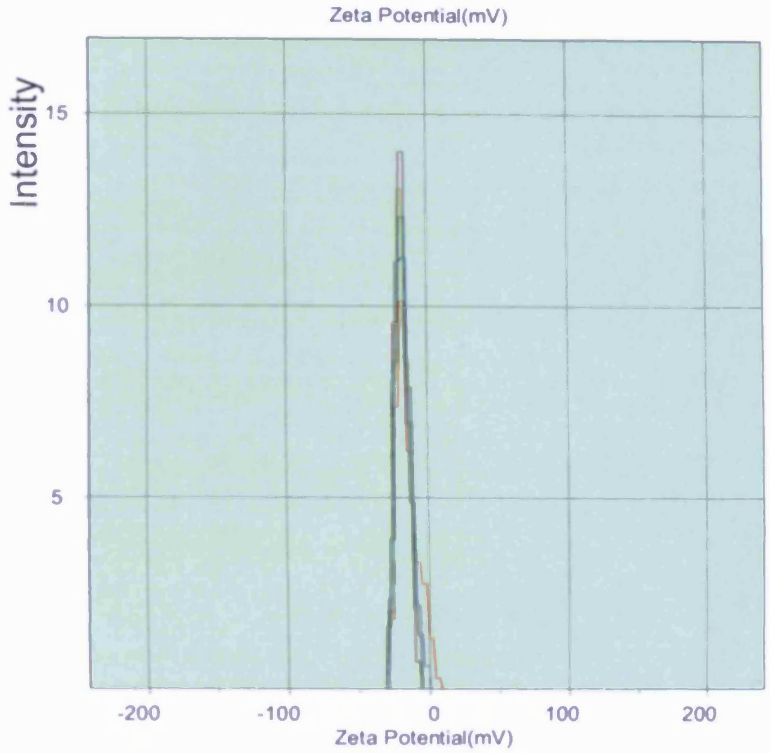


Figure 6.3: Example of the data output from the Zetasizer 2000 showing the negative surface charge of phage LP-M.

6.3.2 Nebulisation of *Ps. aeruginosa* phage cocktails

When quantified there was no significant difference (P=0.848) between the total number of nebulised phage and the total number of phage recovered. By stage 7 of the impactor 99.2% (±4.2%) of all phage had been recovered (Figure 6.4). As the nebulised droplets penetrate the cascade impactor they are sorted by size, with larger particles (between 10 and 3.3 μm) being retained by the earlier levels (stages 0-3). Stages 0-3 represent impact with the throat and upper airways and show approximately 75% of the total deposited phage. The remainder of the deposited phage occurs within the later stages (stages 4-7), representing the secondary bronchi to the alveoli (droplet sizes approximately 3.2 to 0.4 μm).

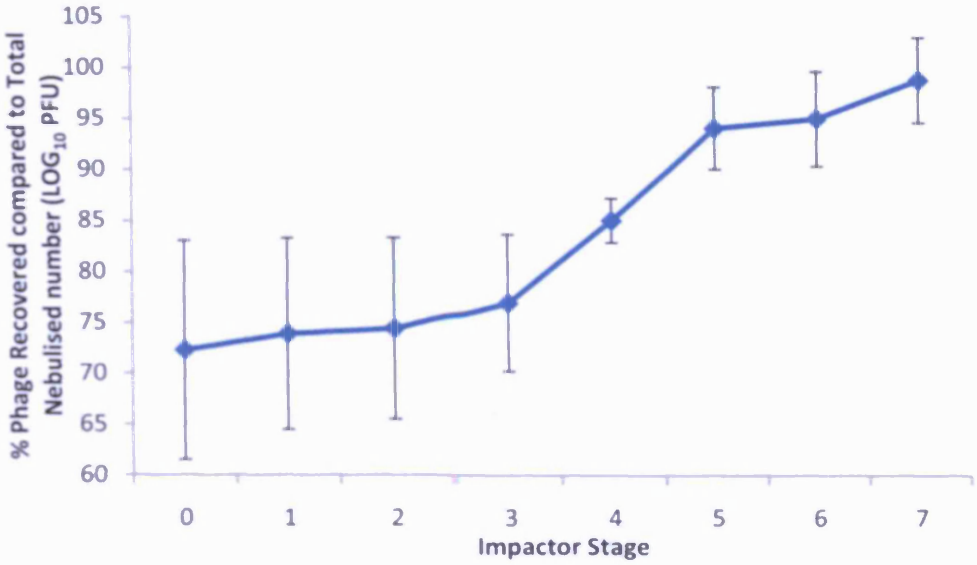


Figure 6.4: Nebulisation of a bacteriophage cocktail. Data are the mean of 3 replicates ±SD. Each impactor stage corresponds to a particular particle size (Figure 6.1).

6.3.3 Stability of *Ps. aeruginosa* phage

There was no significant difference in viable phage at both room temperature and 4°C ($P \geq 0.05$) for all 3 phages over a period of 180 days. Also there was no significant difference in loss of phage viability due to storage conditions at either 4°C or room temperature (Figure 6.5; $P \geq 0.05$) with the exception of LP-M *10* at 180 days storage ($P \leq 0.003$). In general phage LP-M *10* was most susceptible to loss of viability following storage (Figure 6.5c) and GL-1 least susceptible to loss following storage (Figure 6.5a).

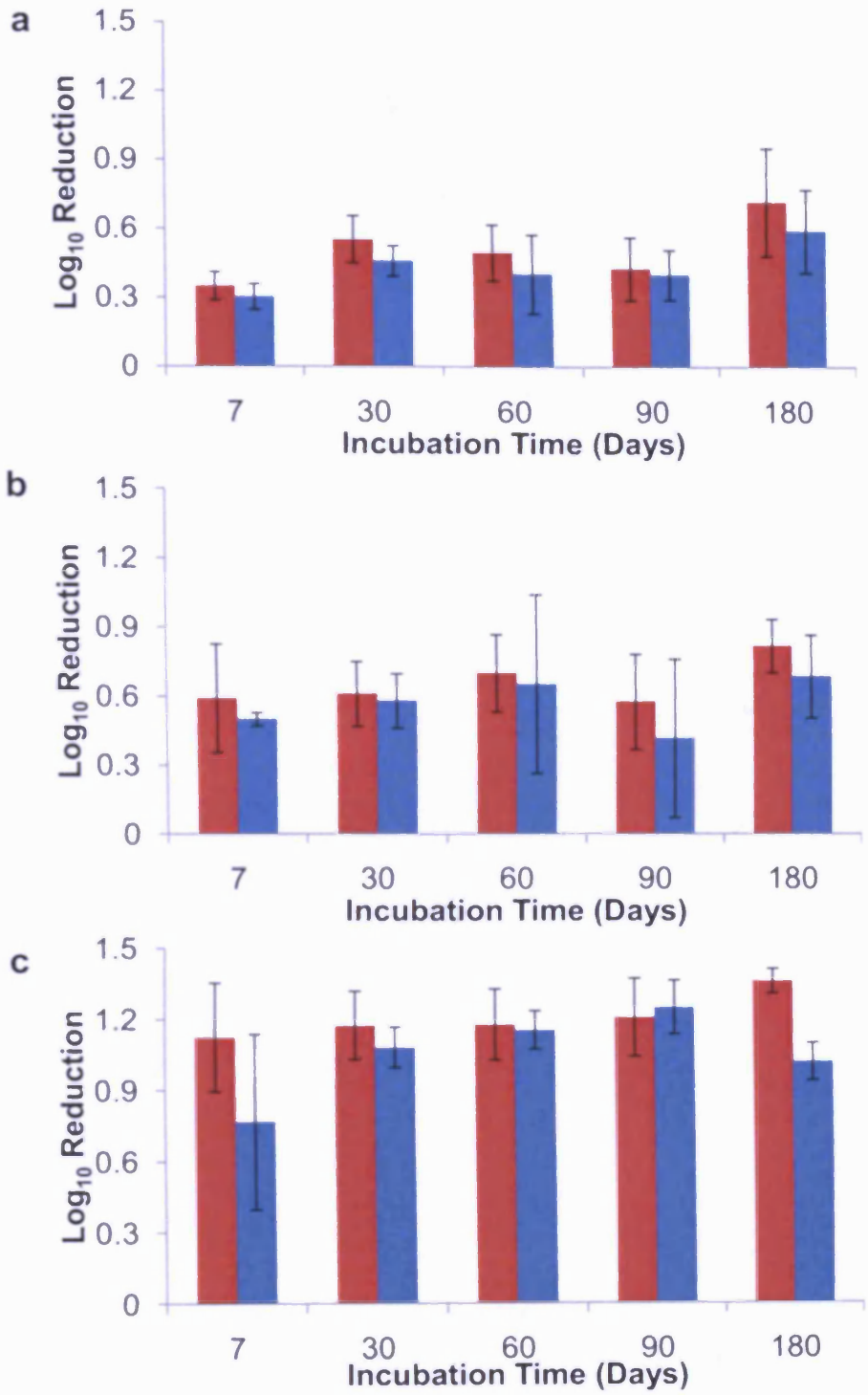


Figure 6.5: Stability of phage cocktail components over a period of 180 days. a, GL-1; b, GL-1 *2.5* and c, LP-M *10*. ■, Room temperature, ■, 4°C. Data are the mean of 3 replicates ± SD.

6.3.4 Effect of Endotrap purification on activity, endotoxin content and microbial content of *Ps. aeruginosa* phage cocktails

All phage cocktails showed no microbial contamination according to pharmacopeial standards (data not shown).

When endotoxin levels were assessed using the Endosafe PTS LAL kinetic assay, crude phage cocktails showed large amounts of endotoxin to be present (>5 Log_{10} EU/mL; Table 6.3). Following purification via passage through the Endotrap system, there was a small drop in endotoxin levels (approximately 0.2 Log_{10} EU/mL) that was non-significant when compared to crude phage cocktails (P=0.327). This level of endotoxin far exceeds the pharmacopeia required levels (<0.2 EU/mL) and as such would fail quality assurance.

The assessment of both purified and non-purified phage preparations on the Bioscreen C analyser shows a small decrease but non-significant ($P \geq 0.05$) in the level of activity of the phages in terms of Log_{10} reductions at 8 and 20 h (Table 6.3). This is potentially due to the small reduction (P=0.124) in the phage content of preparations following passage through the Endotrap system. It should also be noted that the phage activity presented here is slightly lower than previously described (Chapters 4 and 5) but is not significantly different to previously reported activity ($P \geq 0.05$).

Recovery (Log_{10} PFU/mL)	Bioscreen C Assay			Endotoxin Levels (Log_{10} EU/mL)
	Log_{10} Reduction		Time[1] (min)	
	8 h	20 h		
Crude Preparation				
11.02(0.07)	1.85 (0.11)	1.84 (0.19)	>1200	6.05(0.06)
"Purified" Preparation				
10.81(0.18)	1.78 (0.12)	1.77 (0.29)	>1200	5.81(0.37)

Table 6.3: Activity of *Ps. aeruginosa* cocktails following purification on the Endotrap Blue system against *Ps. aeruginosa* strain PAO1. Data are the mean of 3 replicates (±SD). [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$.

6.4 Discussion

6.4.1 Preparation of phage cocktails for delivery

The use of nebulised antibiotics for the treatment of *Ps. aeruginosa* infections in CF patients is well established and has been shown to have some impact on bacterial levels within sputum samples of CF patients (Herrmann *et al.*, 2010). In the current investigation we have shown that the delivery of a bacteriophage based preparation can be readily achieved, and that viable phages can be recovered following delivery. The deposition of phage observed here (Figure 6.4) shows that although phage will be retained by upper airways, as is the case with antibiotics and other nebulised products (Sermet-Gaudelus *et al.*, 2002). Sufficient phage will reach the targeted areas (i.e. the secondary and terminal bronchi) to potentially establish a self replicating phage infection.

The deposition and subsequent recovery of viable phage from a simulated lung model has demonstrated that shear stress and nebulisation of phage particles via a jet nebuliser does not have a severe effect on the ability of phages to replicate for the phages used in this investigation. However, different phages could potentially be more susceptible to damage and loss of viability. In order

to mitigate this risk a number of strategies could be introduced to encapsulate phage particles that have successfully been used for nebulisation including the use of chitosan-alginate microbeads (Ma *et al.*, 2008) and PEGylation (Mok *et al.*, 2007).

The surface of *Ps aeruginosa* biofilms has been shown to demonstrate negatively charged residues within the exopolysaccharide layer of biofilms (i.e. alginate), that have been shown to bind cationic antibiotics e.g. aminoglycosides (Slack and Nichols, 1981). In this chapter we have demonstrated that wild type phages (i.e. non- PRE treated phages) also have a negative surface charge, which may limit the binding potential of phages to host bacterial cells and as a consequence diminish their activity. The small, although statistically insignificant decrease in the negative charges of phage, following treatment with PRE to select for more active phages, may in part explain this enhanced activity as it would reduce repulsion between phage and host cells. However, due to the relatively small change in charge, further experimentation should be carried out in order to determine if this effect is real or merely an artefact.

6.4.2 Quality control of phage preparations

Although the described phage cocktail reaches compliance levels in terms of bacterial numbers for BP (British Pharmacopeia, 2011d) this is for non-sterile manufactured products only. Due to the nature of pharmacopeia compliance, many of the phage preparations currently undergoing clinical trials (Wright *et al.*, 2009) or currently licensed for use (e.g. ListShield™, Intralytix, USA) are for topical use or within the food industry only. For approval for the use in the food industry, the Listshield™ product has demonstrated that the phage cocktail used is at little or no risk of causing adverse side effects (a single protein showed some homology to wheat protein but not enough to induce an allergic response) and that no potentially pathogenic sequences for *Listeria* reside within the phage genome (Food and Drug Administration, 2006).

Although the information is unavailable within the public domain, stability studies would have been carried out to ascertain the "shelf-life" of the product.

However, for topical applications, in order to pass British Pharmacopeia standards (British Pharmacopeia, 2011d), products only need to demonstrate bacterial levels of less than 100 CFU/mL and with all products a stability/preservative efficacy test of the product would be performed. For preparations that are to be used intravenously, microbiological quality assurance will be based around sterility testing (British Pharmacopeia, 2011b) rather than the methods used in the current investigation. In this chapter we have demonstrated the ease at which phage preparations could meet the requirements for non-sterile topical applications. However, for use as a nebulised product, stricter controls including endotoxin levels would have to be met.

As demonstrated here, the production of endotoxin from the lysis of the *Pseudomonas* represents a major stumbling block for the further development of phage products beyond topical applications. For determination of endotoxin levels within phage preparations only animal models have been used to provide an indication (Merabishvili *et al.*, 2009). Here, the use of the pharmacopeia "gold standard" LAL assay (British Pharmacopeia, 2011c and 2011g) has been used to quantify endotoxin levels in both crude and "purified" phage cocktails resulting in unacceptable endotoxin levels (Table 6.3). The LAL assay not only provides quantitative data with regards to the total endotoxin levels within samples, but also is more sensitive than animal models. However, the LAL assay used here required the dilution of samples that were above the detection threshold of the cartridge (1 EU/mL), requiring the extrapolation of endotoxin levels from a positive result.

The purification of phage preparations with the Endotrap Blue system has previously been demonstrated to be effective at reducing endotoxin levels in phage preparations (Merabishvili *et al.*, 2009). In the current investigation

endotoxin levels were not significantly altered by passage through this system. The low reduction in endotoxin content observed here may be in part due to saturation of the column with endotoxin, in which case the column would need to be increased in size or samples passed through multiple columns to reduce endotoxin levels to the required level. However, this would increase any final cost for industrial applications. For laboratory scale preparation, caesium chloride gradients and PEG precipitations have been demonstrated to purify phages (Gill and Hyman, 2010) although this would be impractical for industrial applications.

6.4.3 Summary

This chapter illustrates the range of quality control issues that need to be addressed in the formulation of phage based products in particular endotoxin levels within phage preparations. There will be a need to ensure consistency in performance and stability; the data obtained for the phage cocktail demonstrates wide limits (up to a 6 month shelf life at both room temperature and 4°C storage with no significant loss of activity; a high endotoxin content that far exceeds acceptable standards etc). This investigation has also demonstrated that a non-encapsulated phage cocktail can be deposited throughout a simulated lung and that to enhance deposition in the target areas further, alterations to the size of aerosolized particles produced by nebulisers would have to be made.

Chapter 7

General discussion

7.1 General discussion

7.1.1 Phage as antimicrobial agents

Since their initial discovery phage preparations have been used as a mechanism for the treatment of bacterial infection (Kutter *et al.*, 2010). Although in clinical use within the former Soviet Union for over half a century, a lack of comprehensive information on the efficacy and safety of bacteriophage products, coupled with the wide availability of antibiotics, limited their impact and dispersal within the Western world (Hanlon, 2007). Today, with a well publicised and documented rise in bacterial resistance to antibiotics, alternative strategies are being sought to treat bacterial infection including the use of phage preparations.

It is currently believed that phage represent the most abundant organism on Earth with approx. 10^{31} PFU existing (Hanlon, 2007). This abundance of phage means that lytic phages against most bacterial species are relatively easy to isolate, with a few notable exceptions (e.g. *C. difficile*). Within the current investigation, phage therapy has been described as the use of a replicating lytic phage that can infect and destroy bacteria. These types of preparations are amongst the simplest to produce in a crude form via a simple centrifugation and filtration step, and have been established as safe for use within the food industry (e.g. Listshield, Intralytix Inc.). The use of lytic phage allows for a single dose to set up a self-replicating infection that, in theory, should continue until all the target bacteria are destroyed, while leaving the non-targeted bacteria intact.

Another approach to phage therapy is the induction of lysogenic phages. Traditionally this is accomplished through the use of mitomycin C. However, it has been demonstrated in *Ps. aeruginosa* that many antibiotics can induce lysogenic phages including those more commonly used for the treatment of *Ps. aeruginosa* infections in CF patients (Fothergill *et al.*, 2011). Induction of lysogenic phages with mitomycin C from the *Ps. aeruginosa* strains used in the

current investigation revealed the presence of inducible lysogenic phages in 10 of the 14 strains (Jones, 2009). Currently this approach is only used by one commercial company, Novolytics, to induce lysogenic phages then modifies them to remove both lysogenic properties and potentially hazardous sequences using a proprietary process called ABSEPT. Although the induction of lysogenic phages would allow for synergy between antibiotic and phage therapy it is not without its risks (Table 7.1), as the induction of lysogenic phages may carry virulence factors for the bacterial hosts with it.

	Advantages	Disadvantages
Lytic Phage	• Self-Replicating • Highly abundant for most organisms • Highly specific • Easy to produce in most cases	• Limited Efficacy • Quality control issues particularly in gram-negative organisms • Highly specific • Requires time to lyse cells • Development of resistance by the host • Difficult to commercialise
Lysins	• Short time to lysis • Existing GM technology can be adapted to produce lysins • Low development of resistance • Can be isolated from lytic and lysogenic phages	• Only effective against Gram positive and non-intracellular organisms • Potentially highly specific • High initial investment • Protein based therefore cleared from the body easily • Need multiple doses
Induction of lysogenic phage	• Induction with common antibiotics can provide synergy between antibiotics and phage • Antibiotic efficacy and safety well established • Currently possible with right antibiotics • Broad spectrum	• Induction and transfer of pathogenic regions e.g. toxin production • Potential toxicity following use of high antibiotic concentrations

Table 7.1: Summary of different approaches to phage therapy

Alternatives to the use of phages involving the synthesis of phage lysins are actively being pursued (Daniel *et al.*, 2010). Although lysins remove potential hurdles involving the use of actively replicating lytic phages, they have only been shown to be effective against Gram-positive organisms such as *Staph. aureus* (Daniel *et al.*, 2010). The use of lysins within the clinical setting would require multiple molecular techniques in order to produce an engineered bacterium that could express the lysin in high quantities (Pastagia *et al.*, 2011). This would subsequently then require extraction and purification before efficacy and safety testing could be undertaken.

Currently a number of companies are undertaking research into phage preparations (Table 7.2). Despite the focus of this investigation on the use of phage to treat a systemic infection, phage preparations can be applied to a variety of different fields, from water and industrial decontamination (Table 7.2) to the oil industry.

Company	Product Name/Type	Target organism	Industrial area
Intralytix Inc.	SalmShield™	*Salmonella* serotypes	Food
	SAP-100TM	*Staphylococcus aureus*	Human Health
	ABPP-100TM	*Acinetobacter baumannii*	Human Health
Novolytics	Unknown: No product name available	*Staphylococcus aureus*	Human Health
Omnilytics	Unknown: No product name available	*Bacillus anthracis*	Human Health
		Ps. aeruginosa	Food and Water
		Enterobacter aerogenes	Paper
Ecolyse Inc.	Unknown: No product name available	Sulphate reducing bacteria	Oil and Water

Table 7.2: Summary table of phage based products currently under development by commercial companies.

Preparations based upon whole lytic phage (including phage cocktail), although patentable for a particular application, are relatively easy to circumvent due to the high abundance of other lytic phages. In order to successfully patent a phage preparation containing lytic phages, the patent should be targeted at extra components (e.g. cocktail + antibiotics) or a specific process in the manufacture (e.g. reduction of endotoxin content) or delivery (e.g. nebulisation). Bacterial lysins and also potentially genetically modified phage are highly patentable, as patents could target specific gene sequences and also expression vectors.

7.1.2 Treatment of *Ps. aeruginosa* infections in CF patients

Currently the treatment of *Ps. aeruginosa* infections in CF patients is reliant upon the use of antibiotics (Chapter 5 ;Table 5.1) that is often frustrated if not resulting in complete failure by the formation of biofilms within the lung and the reduction of bacterial metabolic activity reducing sensitivity to antibiotics (Abdi-Ali *et al.*, 2006). The potential advantages of phage preparations over traditional antibiotic therapy have been discussed elsewhere in some detail (Chapter 1; Table 1.7).

This investigation has shown that with the addition of antibiotics to a phage cocktail the level of bacterial reduction in certain *Ps. aeruginosa* biofilms can be increased. The addition of a phage cocktail to individual antibiotics (i.e. CIP and TOB) did improve the overall bactericidal activity against PAO1 biofilm, but not against other bacterial strains (Table 7.3). This may be in part due to the relative activity of phages against biofilms and a lack of penetration into the biofilm layer and the presence of persistor cells within the biofilm matrix (Donlan, 2009). The high level of activity exhibited by the use of antibiotics only is due to the high concentration used based upon MIC levels for *Ps. aeruginosa* (Andrews, 2009). The activity of the cocktail in combination with antibiotics and antibiotics alone could be better distinguished by using concentrations that reflect the in use concentrations for the treatment of *Ps. aeruginosa* in CF patients (Chapter 5; Table 5.1).

Treatment	Bacterial strain (Log $_{10}$ CFU)			
	PAO1	NCTC	P8959	C2238
Untreated	7.47 (0.21)	7.35 (0.27)	7.47 (0.15)	7.35 (0.29)
Cocktail + CIP	<2	4.66 (0.15)	6.23 (0.20)	6.33 (0.28)
Cocktail + TOB	<2	4.65 (0.31)	6.08 (0.07)	5.35 (0.05)
CIP only	3.36 (0.05)	4.65 (0.59)	4.19 (0.31)	4.81 (0.26)
TOB only	3.62 (0.48)	4.47 (0.35)	3.49 (0.30)	5.45 (0.16)

Table 7.3: Summary of the bacterial content of *Ps. aeruginosa* biofilms following 20 h exposure to the described phage cocktail in combination with antibiotics. CIP- ciprofloxacin (1 mg/L), TOB- tobramycin (4 mg/L).

7.1.3 Assessment of phage activity

Currently the assessment of phage activity relies on assays such as the streak test or a spot assay that rely upon the formation of plaques within a lawn or streak of bacteria (Merabishvili *et al.*, 2009). These methods are simple to perform but provide only a yes/no assessment of activity with any indication of the relative resistance of the organisms being tested against, or taking into account, the relative replication rates of the target bacteria.

The current investigation has shown the development of an alternative assay based upon optical density for the initial screening of phages using the Bioscreen C analyser. This assay is not only quantitative and able to look at the reduction in bacterial content compared to an uninoculated control, but also can investigate the relative resistance and regrowth of a phage resistant subpopulation of bacteria (Cooper *et al.*, 2011).

This method has been shown to be particularly useful in distinguishing between subtle differences in activity that are found in phage cocktails (Chapter 4; Tables 7a and 7b) that cannot be distinguished between with plaque based assays.

The addition of "acceptance" criteria to the Bioscreen C assay has allowed for minimum levels of activity to be set based upon the literature (for Log_{10} reductions; Hanlon *et al.*, 2001; Atterbury *et al.*, 2007; Fu *et al.*, 2010) and antibiotic dosing times (for the calculation of bacterial regrowth; (Van Zanten *et al.*, 2007; McCoy *et al.*, 2008). For the criteria used approx. 50% of strains failed to meet the acceptable level of activity (Table 7.4). However, by reducing the acceptance criteria to require only a 1 Log_{10} reduction in bacterial content at 8 h and 20 h approximately 20% more strains showed the required level of reduction. What is interesting to note however is the small increase in the number of strains meeting required levels of activity in the cocktail containing components (Table 7.4; antibiotics, alginate lyase or ambroxol hydrochloride). By increasing the level of activity that each phage is required to possess, the assay is required to discriminate between different phages ensuring that only highly active phage will be accepted.

	Treatment type	Acceptance criteria		
		Log_{10} reduction		Time[1]
		8 h	20 h	
		Established criteria		
Acceptance levels		≥2	≥2	≥480
% Strains meeting required levels	Single phage	42.3	49.2	58.1
	Cocktail	46.4	66.1	60.7
	Cocktail + Components	40.0	75.0	85.0
		Modified criteria		
Acceptance levels		≥1	≥1	≥240
% Strains meeting required levels	Single phage	61.3	81.5	71.8
	Cocktail	69.6	87.5	66.1
	Cocktail + Components	75.0	80.0	85.0

Table 7.4: Established and modified acceptance criteria for Bioscreen C assessment of phage activity. Table is based on both single phages and phage combinations against the 14 strains tested in this investigation. [1]: Time taken to increase $OD_{420\text{-}580nm}$ by 0.1 above the original $OD_{420\text{-}580nm}$.

This investigation has also shown the ability to directly quantify bacterial survivors following the addition of a phage cocktail through the addition of a selective virucide. Although the cocktail described here (Table 7.5) has shown a limited activity against biofilms of *Ps. aeruginosa,* including clinical isolates from CF patients, initial preliminary work involving single phages showed damage to the biofilm layer after 20 h exposure to phage (Figure 7.1). The potential number of phages in the environment (10^{31} PFU; Hanlon, 2007) would allow for a large scale screening process to be undertaken in order to successfully determine the most effective phage candidates for future cocktails.

Biofilm type	Bacterial strain (Log_{10} reduction)			
	PAO1	NCTC 10332	P8959	C2238
CDFF	0.70 (0.13)	0.06 (0.07)	0.68 (0.11)	-
Sedimentary	3.48 (1.29)	0.51 (1.69)	0.14 (0.17)	0.16 (0.29)

Table 7.5: Summary of reduction in bacterial content of the described phage cocktail against biofilms of *Ps. aeruginosa* following 20h exposure. Cocktail contained equal parts GL-1, GL-1 *2.5* and LP-M *10*.

7.1.3.1 Limitations of the assessment of lytic activity of bacteriophages

As previously discussed (Chapters 4 and 5), the current assessment of lytic activity using OD represents a step toward a rapid, standardised, quantitative method for phage activity; however, there are a number of limitations which first must be addressed.

Chief amongst these is the use of OD as a mechanism for determining bacterial content as numerous factors can interfere with this, including bacterial debris and non-lysed bacterial cells. These factors in turn affect the sensitivity and the range at which lytic activity can be accurately calculated resulting in an underestimation of activity.

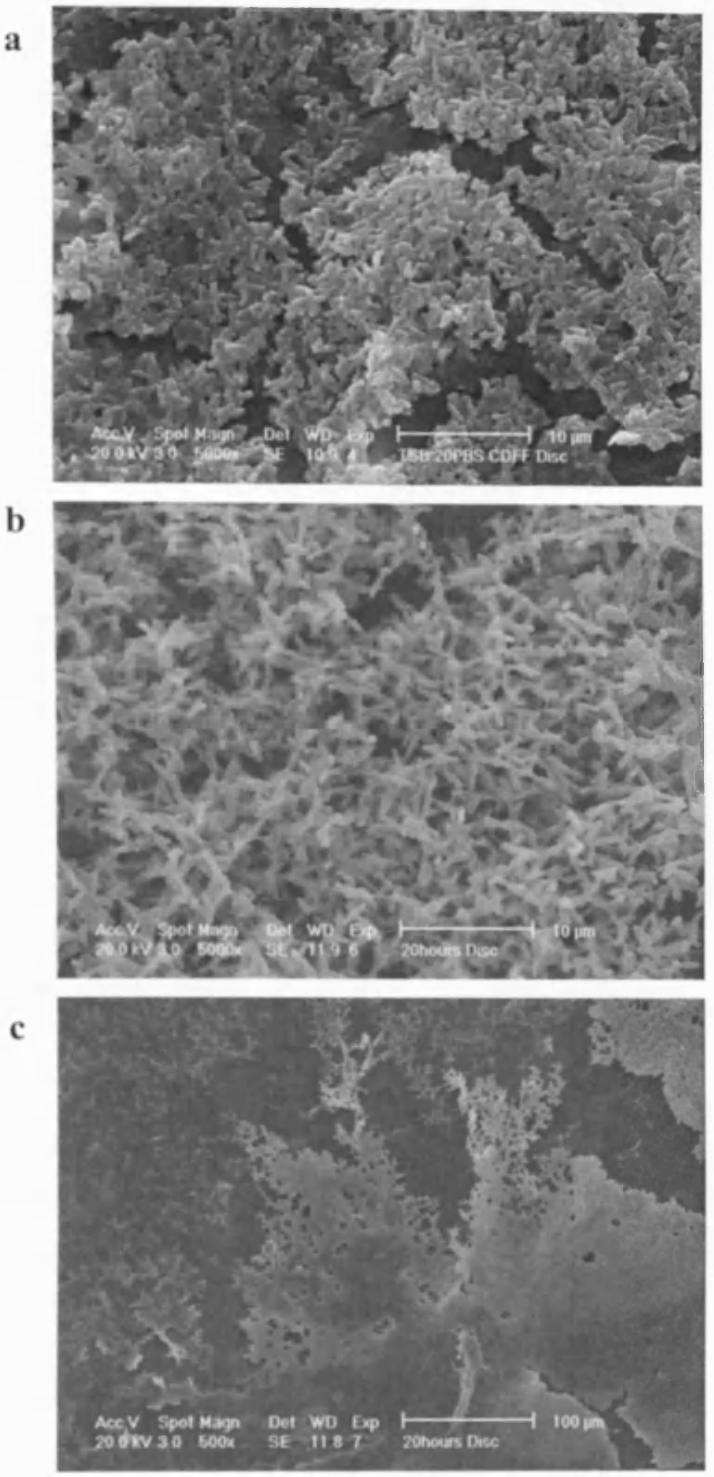


Figure 7.1: Effect of phage addition to 96 h PAO1 CDFF biofilms following 20 h of exposure to LP-M *15* phage. a) PBS treated, b) and c) LP-M *15* phage treated. Scale bars are included on each image.

7.1.4 Delivery and formulation of phage preparations

The use of nebulised antibiotic solutions for the treatment of *Ps. aeruginosa* infections of CF is well established (Tramper-Stranders *et al.*, 2010) and previously discussed. Nebulised or dry powdered formulations would possess numerous advantages over other delivery vectors (Chapter 1; Table 1.4).

In the current investigation it has been shown that bacteriophages can be successfully delivered and recovered following nebulisation with no significant loss of phage viability (approx. 0.1 Log_{10} PFU). The use of nebulised phages has been described within the literature (Huff *et al.*, 2003; Golshahi *et al.*, 2008) and shown to be effective *in vitro* and in animal models. In the study by Huff and colleagues (2003), it was shown that nebulised delivery of a lytic *E. coli* phage could reduce mortality in broiler chickens compared to controls.

The encapsulation of phage (Mok *et al.*, 2007; Ma *et al.*, 2008) possesses a number of advantages and disadvantages (Table 7.6). Encapsulation would allow for greater product stability and a reduction of shear stress resulting from the nebulisation process, potentially increasing the number of viable phage to the target site. In the current investigation it was shown that encapsulation of the phage cocktail used would not be necessary as the individual phage components were shown to be stable at both room temperature and 4°C (< 1.5 Log_{10} reduction following 180 days storage). In addition, following nebulisation, nearly all phage were recovered and their viability maintained (Chapter 6; Section 6.3.2). It should be noted that the stability of any phage preparations should be evaluated as standard practice and as such may require encapsulation to preserve activity.

However, any encapsulating material would need to account for the charge of the phage particle as this would potentially have a serious impact on the ability of the phage to attach to target cells. While alginate microspheres (Ma *et al.*, 2008) would be of little use for delivery of *Ps. aeruginosa* phages in CF patients, particularly if an alginate lyase was used to disrupt the biofilm layer,

as the alginate lyase could potentially degrade the microspheres before delivery. The use of PEG could potentially provide the answer (Mok *et al.*, 2007), by hiding the phage particles from immune cells and increasing stability.

Advantages	Disadvantages
• Encapsulation can allow for more specific targeting of the phage cocktail	• Increased particle size could lead to deposition in the wrong parts of the lung
• Increased product stability	• Encapsulation material could lead to decreased availability of phage
• Could reduce immune response to phage particularly for use in IV preparations	• Would increase the time taken to establish a self replicating infection
	• Poor encapsulation material choice could reduce phage binding

Table 7.6: Summary of the advantages and disadvantages of phage encapsulation.

7.1.5 General conclusions

This thesis sought to characterise, assess and enhance the lytic activity of four *Ps. aeruginosa* phages (i), assess the activity of a cocktail of phages against biofilms of *Ps. aeruginosa* including clinically relevant strains (ii) introducing other components into the phage cocktail including antibiotics, alginate lyase and ambroxol hydrochloride (iii) and to investigate quality control issues surrounding the delivery of a cocktail of phage (iv).

The initial investigation into the host range and activity of the *Ps. aeruginosa* phages used here showed immense potential and high levels of activity when assessed by both broth and by plaque based assays. This activity was then

improved upon for a number of strains following selection based upon time, with some strains showing a reduction in activity.

Although the current investigation has highlighted the potential for phage preparations to be used against biofilms of *Ps. aeruginosa* in CF patients the level of activity would need to be substantially increased in order to be a viable alternative to antibiotics. This substantial increase could be achieved by the screening for more active phage using both the streak test and the Bioscreen C assay (Table 7.7). This would not only allow for the selection of more active phages but also aid in the refinement of the assessment criteria. Phages could also be enhanced further using selective virucides previously discussed (Chapter 4). However, further investigation into the mechanism behind the enhancement should be undertaken.

Method	Advantages	Disadvantages
PRE Treatment	Enhancement against a single strain can increase activity against multiple strains	Mechanism behind the enhancement is unknown
	"Natural" selection on the basis of infection time	Can reduce activity against other strains
Genetic modification	Can be used to turn lysogenic phage into lytic phage	Increases difficulty to pass regulation
	Can remove pathogenic sequences from potential candidates	Expensive and requires genome sequencing
		Requires propriety technology e.g. ABSEPT process (Novolytics, UK)
Increased screening	Easiest to perform	Labour and time intensive
	Potentially increase the number of phages available	Lytic phage may not exist for certain organisms

Table 7.7: Summary table of potential improvements for increasing phage activity.

The current investigation has shown the need for further research into aspects of quality assurance that would surround phage preparations. Here it has been shown that despite the ability to meet the Pharmacopeia based requirements for a number of types of product (Table 7.8), the level of endotoxins present far exceeds acceptable limits and therefore in its current form the preparation would be limited to arenas in which endotoxin levels were not monitored.

	Criteria			
	Microbiology content[a] (CFU/mL or CFU/g)		Endotoxin content[b]	
Application	**TAMC[c]**	**TYMC[d]**	**(EU/mL)**	**Other**
This investigation	ND[e]	ND	>5 Log10	Absence of *Staph. aureus and Ps. aeruginosa*
Aqueous oral preparations	10^2	10^1		Absence of *E. coli*
Transdermal patches	10^2	10^1		Absence of *Staph. aureus* and *Ps. aeruginosa*
Inhalation use[f]	10^2	10^1		Absence of *Staph. aureus*, *Ps. aeruginosa* and bile tolerant gram negative bacteria
Parenteral Preparations	0	0	0.2	See British Pharmacopeia (2011)[g]

Table 7.8: Summary of the different regulatory requirements by application compared to the current investigation. [a]: Based on British Pharmacopeia (2011d) [b]: Based on British Pharmacopeia (2011c and 2011g) [c]: Total aerobic microbe count, [d]: Total Yeast and Moulds count, [e]: Not detected, [d]: Not for nebulised delivery. [g]: Based on British Pharmacopeia (2011f).

Currently the majority of phage research being undertaken centres around topical applications (e.g. for use in infected burns, acne treatment etc) and around the food industry applications. However, some research into the prevention of biocorrosion and water treatment is also being undertaken (Table 7.2). This is in part to meet less stringent criteria that are required within the topical application (Table 7.8), whereas for use for systemic applications much stricter requirements are required to be met (British Pharmacopeia, 2011b).

7.2 Future work

7.2.1 Creation of a co-culture model of *Ps. aeruginosa* and CF cell lines

In order to provide a more representative system which better mimics the *in vivo* conditions of a CF lung, a co-culture between a mucus producing CF cell line and *Ps. aeruginosa* could be used. This system has been used in the modelling of pulpal infection by *Streptococcus anginosus* (Roberts *et al.*, 2008). Although this system would not account for the three dimensional structure usually seen in the lung, it would produce a characteristic thick mucus layer and provide a more representative picture of the CF lung.

7.2.2 Screening a larger range of *Ps. aeruginosa* phages and *Ps. aeruginosa* strains and optimisation of the Bioscreen C assay

The current investigation has established the host range and lytic potential of four *Ps. aeruginosa* phages and has demonstrated how this host range and activity can be changed and altered by the application of a selective virucide.

The screening of a larger range of phages will allow for the selection of a more active cocktail with an enhanced host range. The Bioscreen C method described here can rapidly and quantitatively assess phage based on multiple criteria, including the reduction of bacterial content and the regrowth of resistant sub-populations compared to the qualitative assessment provided by plaque based assays. This would be done in order to minimise the potential for

a subpopulation to develop and also increase the activity of cocktail selected phage, which could then transfer to increased activity against biofilms.

The Bioscreen C assay as described in the current investigation relies upon the conversion of optical density to bacterial number from an OD/TVC graph. This conversion has its limitations as OD values above approx. 0.8 are non linear in nature and as such could lead to an underestimation of phage activity (Cooper *et al.*, 2011). In order to increase the accuracy and sensitivity of the Bioscreen assay a metabolic assay for measuring bacterial viability (Kuda and Yano, 2003; Cerca *et al.*, 2005) or potentially bioluminescent mutants (Marques *et al. 2005*; Jassim and Griffiths, 2007; Thorn *et al.*, 2007) could be incorporated. However, particularly with the use of mutants, these may not be wholly representative of clinical isolates.

The application of "acceptance" criteria to the data generated by the Bioscreen C assessment of phage activity is currently based upon *Pseudomonas* phages only and, in a number of cases, the phages tested here failed to meet the required acceptance criteria for further testing. In order to refine these criteria, other bacterial species such as staphlococci etc should be tested against, as should an increased number of *Pseudomonas* phages.

7.2.3 Alternative formulations and efficacy of nebulised phage preparations

In this investigation we have shown that the individual components of a phage cocktail are stable in suspension and remain viable for a period of 6 months. The production of a dry powder formulation of phage has been shown to be possible (Puapermpoonsiri *et al.*, 2009; Golshahi *et al.*, 2010), which possess a number of advantages over liquid formulations (Table 7.9).

	Advantages	Disadvantages
Liquid formulation	Less complicated to produce than dry powder Less processing required to produce final product	Endotoxin content Potentially more susceptible to storage conditions
Dry powdered formulation	Increased stability of final product Can increase phage concentration within powders	Endotoxin content More complicated to produce Loss of activity during lyophilisation Will require stabilising agents in order to protect the phage

Table 7.9: Summary of liquid formulation versus dry powdered formulation.

Although *Ps. aeruginosa* is the main infectious agent within CF patients, colonizing up to 80% of sufferers by the age of 20 (Hassett *et al.*, 2009), the presence of other infectious agents such a *B. cepacia* complex increases both mortality and morbidity (Waine *et al.*, 2007). Cocktail of phages to target multiple potential pathogens are currently well researched, particularly for topical applications such as infected burns. However, cocktails of phages that target *Ps. aeruginosa* and also include phages that target emerging pathogens such as *Inquilinus limosus* (Herasimenka *et al.*, 2007), could potentially reduce the bacterial load of CF patients prior to the establishment of a persistent infection.

This investigation has demonstrated how a cocktail of *Ps. aeruginosa* phages can be successfully delivered into a simulated lung using a jet nebuliser and viability of recovered phage assessed. In order to further develop a successful phage preparation, different types of nebuliser should be assessed to determine

their usefulness in delivering phage preparations. Although this would determine the efficacy of the delivery options, it would not determine the activity of those preparations following delivery against biofilms of *Ps. aeruginosa*. As such it is recommended that biofilms of *Ps. aeruginosa* clinical isolates are evaluated for their susceptibility to nebulised phage.

7.2.4 Development of new depyrogenation strategies

The presence of endotoxin within phage cocktails remains one of the main stumbling blocks for the successful implementation of phage products beyond topical applications and use within the food industry. Endotoxin removal via the Endotrap affinity column has been shown to be effective in reducing the endotoxin content of a phage preparation containing both staphylococci and *Pseudomonas* phages (Merabishvili *et* al., 2009), although endotoxin levels were assessed using a rabbit pyrogenicity test. The current investigation has shown passage of a *Pseudomonas* phage cocktail through the Endotrap system did not reduce the endotoxin content of the cocktail sufficiently to be in line with pharmacopeia standards when assessed using the gold standard LAL assay.

Current depyrogenation methods, such as dry heat treatment (200°C for approx. 1 h) and repeated acid/base washes are incompatible for biologically active products such as phages. As such alternative methods would need to be developed which would preserve the biological activity of phage while reducing the endotoxin content to acceptable levels.

Chapter 8

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Chapter 9

Appendix

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ORIGINAL ARTICLE

Rapid and quantitative automated measurement of bacteriophage activity against cystic fibrosis isolates of *Pseudomonas aeruginosa*

C.J. Cooper, S.P. Denyer and J.-Y. Maillard

Welsh School of Pharmacy, Cardiff University, Cardiff, Wales, UK

Keywords
bacteriophage, lytic activity, *Pseudomonas aeruginosa*.

Correspondence
Jean-Yves Maillard, Welsh School of Pharmacy, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3NB, UK.
E-mail: maillardj@cardiff.ac.uk

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Abstract

Introduction: *Pseudomonas aeruginosa* is an opportunistic pathogen and is the main cause of respiratory infection in cystic fibrosis patients. Most strains prevalent within the UK are resistant to two or more antibiotics leading to the search for new therapeutic strategies including the use of bacteriophages.
Methods and Results: The infectivity of four bacteriophages was increased using an enhancement protocol based on the use of pomegranate rind extract. Their efficacy against 14 *Ps. aeruginosa* strains was measured using a qualitative streak test and a novel quantitative assay based on the Bioscreen C microbial growth analyzer. Streak test analysis illustrated an increase in the lytic activity of enhanced bacteriophages, whereas Bioscreen analysis showed that both enhanced and unenhanced bacteriophages failed to meet acceptable levels of activity in *c.* 50% of strains tested.
Conclusions: The quantitative Bioscreen C analyzer showed comparable but not identical results in phage activity and identified significant bacterial regrowth by 20 h postinfection.
Significance and Impact of the Study: With the resurgence of interest in bacteriophage therapy against infectious bacterial diseases, a rapid high throughput quantitative method for screening phage activity and bacterial resistance is required. The use of the Bioscreen C analyzer meets these criteria and was shown to be more stringent than the traditional streak test.

Introduction

Pseudomonas aeruginosa, an environmentally ubiquitous organism, is an opportunistic human pathogen that plays a key role in Cystic Fibrosis (CF) patients with around 80% of patients colonized by the age of 20 years (Saiman and Siegel 2004). With an increase in the level of antibiotic resistance over the past decade, there has been a renewed interest in alternatives to antibiotic therapy. Although in clinical use in Eastern Europe, bacteriophage therapy has only recently begun to attract attention in the West with a number of new products being licenced or currently in clinical trials (Merabishvili *et al.* 2009).

To successfully produce whole phage based therapeutics, a number of criteria must be met. These include the ability to infect a range of bacterial strains as well as the ability to reduce the overall level of infection (Hanlon 2007). One of two distinct approaches may be undertaken to create such a phage preparation: genetic modification (GM) of the phage genome to broaden specificity and to increase activity, or the selective breeding of phage, isolating the most infectious. The former of these two approaches is undoubtedly attractive allowing the ability to not only increase the rate and overall level of bacterial kill but also to provide genes that when expressed would enhance phage virulence, e.g., biofilm penetration through the production of an alginate lyase (Hanlon *et al.* 2001). However, products based on genetically modified (GM) phages might be more difficult to bring to market due to the level of regulation surrounding GM organisms. The latter approach on the other hand allows for the selection of more virulent phages on the basis of infectivity (Jassim *et al.* 1995).

The activity of phages is generally measured using traditional plating on agar of target bacteria where infection

will be identified by plaque production (Adams 1959). Plaque morphologies and counts, however, vary yielding a subjective end point. The traditional streak assay, which relies on such a protocol is limited in its capacity and does not provide evidence for the emergence of bacterial resistance and re-growth (Merabishvili *et al.* 2009). Both phage induced lysis and bacterial re-growth can be followed by turbidometric measurements, offering an alternative and potentially high throughput method (Maillard *et al.* 1996).

In the present study, the efficacy of a novel high throughput method for measuring phage activity was tested against four *Ps. aeruginosa* phages and 14 strains of *Ps. aeruginosa* including isolates from CF patients. Phage activity was concurrently tested with a traditional streak test.

Materials and Methods

Host cells and bacteriophage cultures

Bacterial strains were kindly provided by Dr E Mahenthiralingam (School of Biosciences, Cardiff University, Cardiff, UK) (Table 1). *Ps. aeruginosa* PAO1 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK). *Ps. aeruginosa* NCTC 10332 (reference strain) was obtained from the Health Protection Agency (London, UK). Four bacteriophages, C10176-Large (C10176-L), C10176-Small (C10176-S), GL-1 and L-Phage-Medium (LP-M), were kindly provided by Prof. GW Hanlon (School of Pharmacy, University of Brighton, Brighton, UK). Bacterial strains and bacteriophages were stored at −80°C in 10% glycerol. Bacterial cultures were routinely prepared in tryptone soy broth (TSB; Oxoid Ltd, Cambridge, UK) and incubated at 37°C for 24 h. Cultures were centrifuged at 2600 *g*, resuspended in tryptone sodium chloride (TSC; 1 g l^{-1} tryptone, 9·25 g l^{-1} NaCl) to a standardized optical density at 600 nm (OD_{600}) corresponding to 1–3 × 10^8 CFU ml^{-1}. Bacteriophage suspensions were routinely prepared using the soft overlay agar method (Adams 1959) with *Ps. aeruginosa* PAO1 used as the host. Five ml of phosphate buffered saline (PBS; Sigma, Dorset, UK) was used to remove the sloppy agar layer containing the phage. The phage suspension was then centrifuged at 11 000 *g* for 15 min at 4°C and passed through 0·45 and 0·2 μm membrane filters (Millipore, Cork, Ireland).

Preparation of pomegranate rind extract

Pomegranate rind extract (PRE) was kindly provided by David Houston (Welsh School of Pharmacy, Cardiff University, Cardiff, UK) and prepared as described in Stewart *et al.* (1998). Briefly, PRE was diluted by adding 1·3 ml of PRE in 8·7 ml of λ buffer immediately prior to use and 8·3 ml added to 16·7 ml of a freshly prepared ferrous sulfate solution (4·8 mmol l^{-1} $FeSO_4.7H_2O$). The solution was protected from light.

Enhancement of *Ps. aeruginosa* bacteriophage activity

The phage enhancement principle used in this study is based on the selection of the more virulent phage on the basis of invasion time. The enhancement protocol was adapted from Jassim *et al.* (1995) and is shown in Fig. 1.

Table 1 *Pseudomonas aeruginosa* strains used in this investigation

Strain name	Source	Comment
NCTC 10332	NCTC	Also known as ATCC 10145. Quality control strain for API products. Not producing alginate (Edwards and Saunders 2001)
PAO1	Clinical, nonCF	*Ps. aeruginosa* genome sequencing strain (Stover *et al.* 2000)
C3652*	CF	Epidemic Manchester CF strain type (Jones *et al.* 2001)
C3719*	CF	Epidemic Manchester CF strain type (Jones *et al.* 2001)
C3786*	CF	Melbourne *Ps. aeruginosa* CF strain – unique (Armstrong *et al.* 2003)
LES 400*	CF	Liverpool epidemic strain- Hypervirulent Strain predominant epidemic strain in the UK (Salunkhe *et al.* 2005; Fothergill *et al.* 2007)
Midlands-1-9245 (Mids-1)	CF	Midland 1 epidemic strain
P8959*	CF	Liverpool epidemic strain- predominant epidemic strain in the UK (Fothergill *et al.* 2007)
C1913*	CF	A55 unique- Unique genotype from Vancouver patients (Lewis *et al.* 2005)
C2238♦	CF	A61 unique- Unique genotype from Vancouver patients (Lewis *et al.* 2005)
C2846♦	CF	A55 unique-Unique genotype from Vancouver patients (Lewis *et al.* 2005)
C3597♦	CF	Nonepidemic Manchester CF strain type (Jones *et al.* 2001)
C4503♦	CF	A55 unique- Unique genotype from Vancouver patients (Lewis *et al.* 2005)
PAK-SR	Research Strain	Streptomycin^R parent of Fla-/Pil- mutants of PAK. Sm50

Strains indicated by * are epidemic CF strains. ♦ nonepidemic CF strains.

The initial exposure time (referred to as enhancement time) was 15 min and subsequently shortened to 10, 5 and 2·5 min.

Streak test assay

The following methodology was adapted from (Merabishvili *et al.* 2009). Ten microlitres of bacterial suspension (*c.* 1×10^8 CFU ml^{-1}) was streaked over the surface of a 100 × 100 mm square petri dish (Sterilin Ltd, Caerphilly, UK) that was previously divided into a 5 × 5 grid and air dried at room temperature for 15 min. Ten microlitres of bacteriophage suspension (*c.* 1×10^7 PFU ml^{-1}) was then spotted onto each intersection and air dried at room temperature for 1 h. PBS was used as a negative control. Plates were incubated at 37°C for 3 h and a primary assessment for activity was made on the basis of the extent of phage lysis. A secondary reading was performed after a further incubation for 18 h at room temperature and a score of '0' (no plaque) to '+5' (confluent lysis) was attributed (Merabishvili *et al.* 2009) (Fig. 2). Ten separate experiments were conducted and a final score out of a maximum value of 50 was calculated.

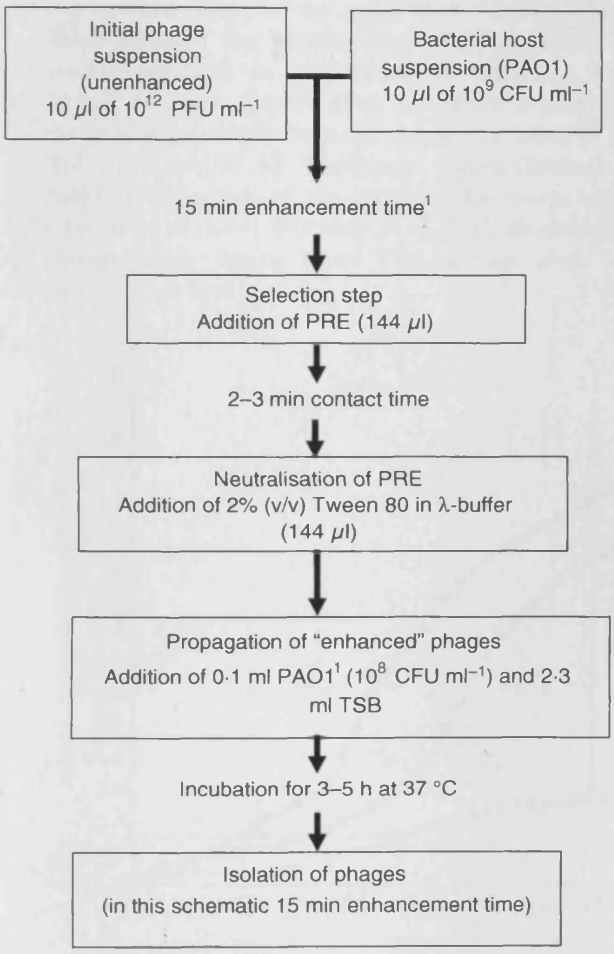

Figure 1 Schematic representation of bacteriophage enhancement using pomegranate rind extract. [1]:Contact time between phages and bacterial suspension varied (i.e. enhancement time of 2·5, 5, 10 and 15 min were used; see text).

Bioscreen assay

Three hundred and fifty microlitres of a bacterial suspension in TSB (*c.* 1×10^8 CFU ml^{-1}) was added to each well of a 100 well honeycomb plate (Oy Growth Curves AB Ltd, Helsinki, Finland) with TSB used as a negative control. To each well, 50 μl of standardized bacteriophage suspension (10^{11} PFU ml^{-1}) was added. This phage/bacterial cell ratio was determined in preliminary experiments to give the most appropriate phage activity, without directly lysing the bacterial cell on initial contact, thus allowing phage replication inside the host. To increase the reproducibility of the reading, a total of 10 wells for each phage were inoculated for each honeycomb plate. The plates were then incubated at 37°C for 20 h and turbidity recorded using a wideband filter (420–580 nm) with readings taken every 15 min in a Bioscreen C analyzer (Oy Growth Curves AB Ltd, Helsinki, Finland). Each reading was preceded by a 10 s shaking cycle. A total of three independent experiments were performed using fresh bacterial cultures and phage cultures

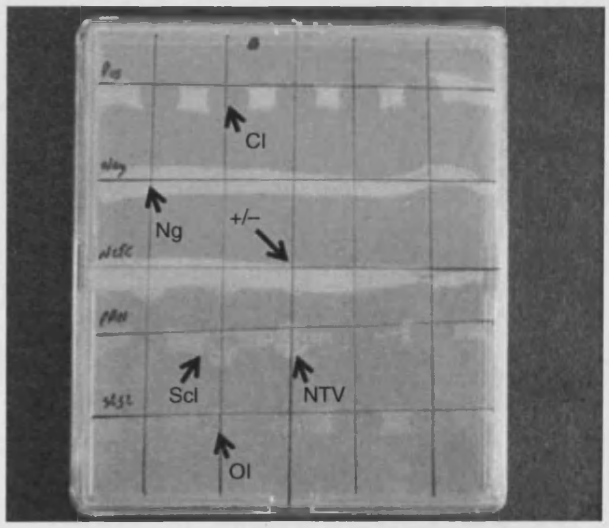

Figure 2 Example of a phage streak test plate at secondary reading stage. Cl – confluent lysis (+5), Ol – overgrowth, bacterial line completely broken, presence of singular bacterial colonies on spot (+4), Scl – Semi-confluent lysis- bacterial line not completely broken (+3), NTV – Multiple small phage plaques (+2), +/– Bacterial line just affected i.e. little observable disruption to bacterial line (+1) and Ng-negative (0).

for each repeat. Once completed, the mean optical density (out of 10 wells) for each sample at each sampling time was calculated. Bacterial number at 8 and 20 h incubation time was calculated using a standard graph plotting optical density reading *vs* CFU ml^{-1}. Reduction in bacterial number (expressed as Log_{10} reduction) following phage exposure was calculated by comparing bacterial number recovered after 8 and 20 h incubation from wells exposed to phage and those not exposed to phages (control) (Fig. 3).

For this study, phage activity with the Bioscreen C analyser was measured against set 'acceptance' values attributed to two main parameters: (i) a ≥ 2 log_{10} decrease in bacterial number after 8 and 20 h (i.e. measurement of bactericidal efficacy) and (ii) a time of ≥480 min to reach a $OD_{420-580}$ of 0·1 above the original $OD_{420-580}$ (measurement of bacterial re-growth postphage infection) (Fig. 3 and Table 2). Other parameters were also calculated but did not contribute to the assessment of phage efficacy. These included the measurement of phage initial lytic activity indicated by a decrease in $OD_{420-580}$ within 30 min of phage addition (Fig. 3) and the calculation of the lytic slope, which indicates the rate of bacterial lysis. The attribution of set 'acceptance' values (Table 2) was based on a review of the literature for phage activity (Hanlon *et al.* 2001; Atterbury *et al.* 2007; Fu *et al.* 2010) and antibiotic dosing times (Van Zanten *et al.* 2007; McCoy *et al.* 2008).

Statistical analysis

Data were analysed for significant differences using a one-way ANOVA in Minitab 15 (Minitab Ltd, Coventry, UK).

Results

Assessment of phage activity using the streak test method

Phage activity was enhanced against *Ps. aeruginosa* PAO1. Phages showing an increase in activity were then tested against the different clinical isolates.

The streak test showed an increase in the level of lytic activity of the four phages against *Ps. aeruginosa* PAO1 (Table 3). The largest level of increased lytic activity against PAO1 was after the first enhancement step of 15 min (data not shown). The further enhancement steps following 10, 5 and 2·5 min contact time lead to smaller increases in activity (data not shown). Ultimately, the maximum level of lytic activity against PAO1 was reached after the final enhancement time of 2·5 min for all phages, except for GL-1, for which a maximum activity was reached after the enhancement time of 5 min (Table 3). When used against all bacterial isolates, the highest increase in activity was observed for C10176-L and LP-M and the smallest for GL-1. For some bacterial strains, notably for the CF

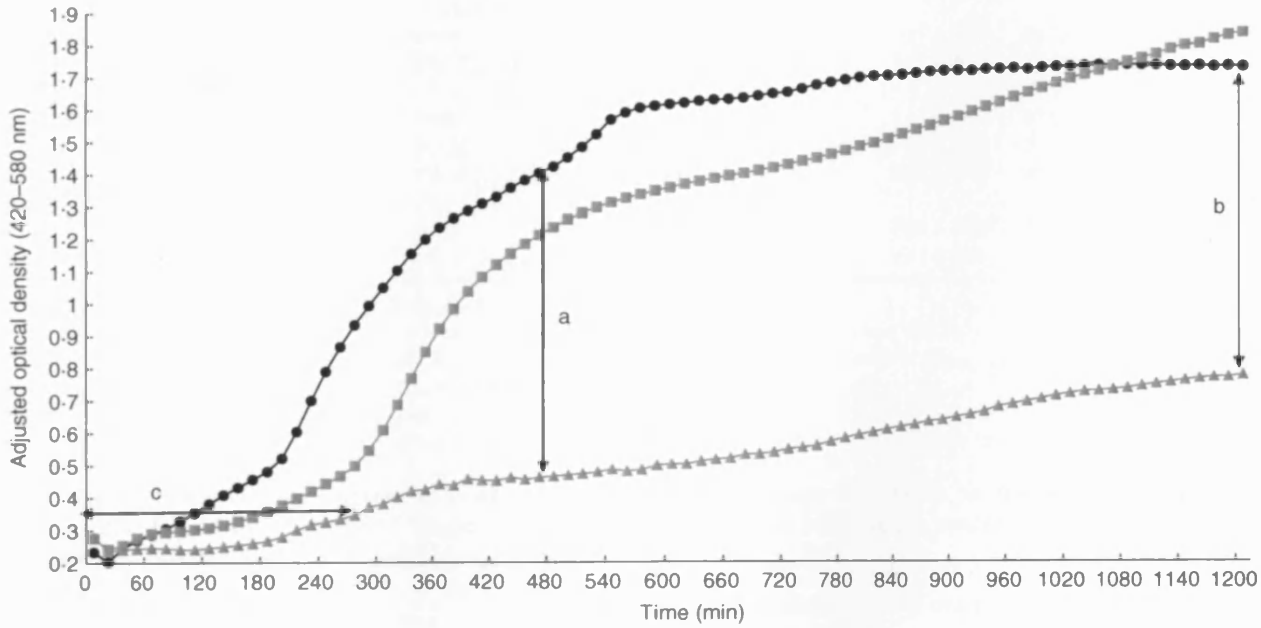

Figure 3 Representative change in bacterial growth patterns of *Pseudomonas aeruginosa* strain C1913 following treatment with original and enhanced C10176-S bacteriophage. (a and b) bacterial reduction at 8 and 20 h respectively. (c) time taken for the $OD_{420-580}$ to increase by 0·1 above the initial $OD_{420-580}$. Phage treated samples are the mean of three replicates; untreated samples are the mean of six replicates. (●) Untreated Cultures; (■) nonPRE treated phage and (▲) PRE treated phage.

Table 2 Summary of the lytic assessment criteria of *Pseudomonas aeruginosa* bacteriophages from Bioscreen C assessment

		Evaluation criteria			Additional criterion
		Log_{10} reduction		Time* (min)	
		8 hours	20 hours		Lysis† (min)
'Acceptance' value‡		≥2	≥2	≥480	≤30
Phage	Treatment	No. of bacterial strains meeting the acceptance value out of 14			
GL-1	Non-PRE	3	3	8	7
C10176-S	treated	3	4	8	7
C10176-L		6	6	8	7
LP-M		7	10	8	7
GL-1	PRE	7	7	7	4
C10176-S	treated	6	6	8	4
C10176-L		6	7	6	4
LP-M		7	6	6	4

*Time taken to increase $OD_{420-580}$ by 0·1 above the original $OD_{420-580}$
†Time taken to induce a lytic slope.
‡Set 'acceptance' value (see text).

Table 3 Comparison of lytic activity of phages against *Pseudomonas aeruginosa* PAO1 between the streak test method and the Bioscreen C analyzer

		Bioscreen C analyser			
		Log_{10} reduction ($CFU\ ml^{-1}$)			
Phage treatment	Streak test (score out of 50)	8 hours	20 hours	*P*-values*	Time† (minutes)
C10176-L					
None‡	20	2·06 ± 0·50	2·28 ± 0·89	0·724	>1200
PRE§ (2·5)¶	48	3·14 ± 0·10	3·20 ± 0·46	0·834	>1200
C10176-S					
None	29	2·04 ± 0·52	2·31 ± 0·83	0·662	>1200
PRE (2·5)	47	3·14 ± 0·10	3·23 ± 0·52	0·668	>1200
GL-1					
None	20	1·95 ± 0·51	1·24 ± 1·10	0·367	815 ± 670
PRE (5)	46	2·66 ± 0·25	2·72 ± 0·13	0·712	>1200
PRE (2·5)	43	3·15 ± 0·11	3·18 ± 0·40	0·905	>1200
LP-M					
None	30	2·01 ± 0·45	2·28 ± 0·89	0·667	>1200
PRE (2·5)	45	3·01 ± 0·30	2·97 ± 0·55	0·917	>1200

Data are based upon the means of three replicates.
**P*-values showing differences in log_{10} reduction at 8 and 20 h.
†Time taken to increase $OD_{420-580}$ by 0·1 above the original $OD_{420-580}$
‡None: phage not treated with PRE (i.e. original phage).
§PRE: phage exposed to PRE.
¶(time): phage-bacteria contact time (min) before exposure to PRE (see text).

epidemic strain Midlands-1, no increase in phage activity was observed, whereas only LP-M phage showed some increase in activity against CF nonepidemic strain C4503 (Tables 4–7). Overall, C10176-L and LP-M showed increased levels of activity against 11 strains, C10176-S against 10 strains and GL-1 against six strains (Tables 4–7). Surprisingly, all enhanced phages showed decreased levels of activity against Midlands-1 (data not shown). Finally, the nonepidemic CF bacterial strains were overall more susceptible to the enhanced phages when compared to epidemic strains.

Assessment of phage activity using the Bioscreen C method

When PAO1 was challenged with phages, a lytic slope was observed within 30 min of the initial inoculation for original phages and immediately upon exposure for

Table 4 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to C10176-L phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (score/50)	Log_{10} reduction*	Time† (minutes)
10332	10	0·90 ± 0·78	205 ± 10	21 (10)‡	1·90 ± 0·65	180 ± 40
C3652	10	0·94 ± 0·14	590 ± 85	36 (10)	1·06 ± 0·15	1005 ± 340
C3719	0	0·85 ± 0·05	565 ± 10	25 (10)	1·05 ± 0·15	>1200
C3786	44	2·58 ± 1·05	130 ± 10	–§	2·74 ± 0·60	140 ± 10
LES-400	10	1·09 ± 0·18	1155 ± 80	29 (2·5)	0·88 ± 0·27	860 ± 280
Mids-1	10	ND¶	105 ± 45	–	ND	140 ± 25
P8959	10	1·99 ± 0·20	1180 ± 35	27 (10)	1·72 ± 0·46	770 ± 285
C1913	0	4·36 ± 1·79	345 ± 55	22 (2·5)	3·57 ± 2·24	240 ± 50
C2238	0	0·59 ± 0·07	865 ± 135	49 (5)	0·59 ± 0·14	790 ± 325
C2846	20	2·39 ± 0·95	**305 ± 30**	24 (10)	3·04 ± 0·83	**205 ± 40**
C3597	47	3·78 ± 0·82	**570 ± 210**	50 (10)	2·47 ± 0·09	**185 ± 40**
C4503	20	0·07 ± 0·04	130 ± 10	–	0·95 ± 1·70	380 ± 440
PAK-SR	16	2·90 ± 0·22	1085 ± 150	40 (15)	3·05 ± 0·26	1000 ± 165

Data shown are the mean of three replicates. Bold typeface indicates significant differences.
*Log_{10} reduction in bacterial number at 8 h.
†Time taken to increase $OD_{420-580}$ by 0·1 above the original $OD_{420-580}$
‡(time): phage-bacteria contact time (min) before exposure to PRE (see text).
§Dashes indicate no increase or decrease in lytic activity.
¶ND: no reduction in bacterial content determined.

Table 5 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to C10176-S phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (max. 50)	Log_{10} reduction*	Time† (minutes)
10332	14	0·47 ± 0·78	220 ± 30	27 (15)‡	1·64 ± 1·20	180 ± 25
C3652	30	0·97 ± 0·24	900 ± 520	40 (10)	0·95 ± 0·37	558 ± 200
C3719	30	0·89 ± 0·24	890 ± 540	31 (15)	0·77 ± 0·31	485 ± 170
C3786	9	**0·87 ± 1·31**	**100 ± 25**	45 (2·5)	**3·32 ± 0·80**	**145 ± 10**
LES-400	14	0·91 ± 0·24	805 ± 280	30 (2·5)	1·50 ± .121	980 ± 330
Mids-1	18	ND§	95 ± 30	–¶	ND	140 ± 25
P8959	22	1·96 ± 0·2	**1100 ± 85**	–	1·81 ± 0·08	**825 ± 145**
C1913	30	**1·50 ± 0·33**	200 ± 30	–	**4·49 ± 1·05**	425 ± 285
C2238	8	0·56 ± 0·11	805 ± 50	40 (10)	0·62 ± 0·13	865 ± 340
C2846	25	0·76 ± 0·67	215 ± 25	30 (10)	2·73 ± 1·52	215 ± 50
C3597	2	3·73 ± 0·78	500 ± 280	46 (5)	3·39 ± 0·59	895 ± 295
C4503	30	ND	140 ± 10	–	0·81 ± 1·63	370 ± 435
PAK-SR	24	2·84 ± 0·26	1095 ± 160	46 (5)	3·04 ± 0·35	1025 ± 100

Data shown are the mean of three replicates. Bold typeface indicates significant differences.
*Log_{10} reduction in bacterial number at 8 h.
†Time taken to increase $OD_{420-580}$ by 0·1 above the original $OD_{420-580}$
‡(time): phage-bacteria contact time (min) before exposure to PRE (see text).
§ND: no reduction in bacterial content determined.
¶Dashes indicate no increase or decrease in lytic activity.

enhanced phages. However, there was no significant ($P \geq 0·05$) change in the rate of $OD_{420-580}$ decrease per minute between original and enhanced phages (data not shown). A lytic slope was not necessarily observed with all CF isolates exposed to the parent or enhanced phages (data not shown). Where a decrease $OD_{420-580}$ was noted (against *Ps. aeruginosa* LES400, P8959 and PAK-SR), there was no statistically significantly

Table 6 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to GL-1 phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (max. 50)	Log_{10} reduction*	Time† (minutes)
10332	35	0·99 ± 0·74	230 ± 35	-‡	2·09 ± 0·98	180 ± 25
C3652	34	1·23 ± 0·17	885 ± 295	38 (10)§	0·90 ± 0·20	505 ± 140
C3719	46	0·93 ± 0·17	585 ± 130	-	0·79 ± 0·20	455 ± 160
C3786	44	0·77 ± 0·67	110 ± 30	-	2·72 ± 1·43	145 ± 10
LES-400	30	1·09 ± 0·13	**1185 ± 25**	34 (2·5)	0·97 ± 0·21	**940 ± 80**
Mids-1	30	ND¶	**85 ± 20**	-	ND	**140 ± 25**
P8959	35	1·79 ± 0·34	905 ± 175	-	1·95 ± 0·07	915 ± 25
C1913	6	**3·30 ± 0·85**	310 ± 20	22 (2·5)	**5·06 ± 0·33**	345 ± 90
C2238	23	0·51 ± 0·08	765 ± 70	40 (10)	0·57 ± 0·16	840 ± 240
C2846	35	**0·97 ± 0·50**	240 ± 15	-	**2·90 ± 0·08**	345 ± 275
C3597	50	3·80 ± 0·78	560 ± 185	-	2·98 ± 0·19	495 ± 300
C4503	34	ND	140 ± 20	-	0·90 ± 1·52	350 ± 400
PAK-SR	30	2·78 ± 0·23	**>1200**	46 (2·5)	2·98 ± 0·31	**1020 ± 40**

Data shown are the mean of three replicates. Bold typeface indicates significant differences.
*Log_{10} reduction in bacterial number at 8 h.
†Time taken to increase $OD_{420-580}$ by 0·1 above the original $OD_{420-580}$
‡Dashes indicate no increase or decrease in lytic activity.
§ (time): phage-bacteria contact time (min) before exposure to PRE (see text).
¶ND: no reduction in bacterial content determined.

difference ($P \geq 0{\cdot}05$) between original and enhanced phages (data not shown). In the strains where no lytic slope was observed (e.g. C1913), the growth pattern of the bacterial isolates was dramatically altered by the phage (example in Fig. 3) and the final $OD_{420-580}$ at 20 h was lower following exposure to enhanced phages. It has to be noted that as our protocol is based on OD reading, bacterial cell debris following lysis might have contributed somewhat to the OD values recorded and as such might have masked initial cell lysis following initial phage interaction/replication in their host. However, the presence of cell debris cannot explain solely the dramatic change in bacterial growth pattern recorded following phage treatment.

When the phage bactericidal activity to PAO1 was evaluated, enhanced phages showed a significantly higher ($P \leq 0{\cdot}05$) level of bacterial reduction compared to their original counterparts at both 8 and 20 h (Table 3). There was no significant difference ($P \geq 0{\cdot}05$) in the reduction of bacterial number after 8 and 20 h incubation (Table 3). There was, however, a significant difference ($P = 0{\cdot}034$) between the efficacy of the original and enhanced phages at 8 h and these results were in accordance to those obtained from the streak test (Table 3). In the case of GL-1 where a 5 min PRE treatment yielded the highest score by streak test, there was no significant difference ($P \geq 0{\cdot}05$) in phage activity between the 5 and 2·5 min enhancement time (Table 3) when measured with the Bioscreen. Furthermore, in PAO1, there was no significant difference in bacterial re-growth following original and enhanced phage exposure (Table 3).

There was generally no significant difference ($P \geq 0{\cdot}05$) in activity between original and enhanced phages at 8 h exposure against CF isolates (Tables 4–7), with the exception of *Ps. aeruginosa* C3786 and C1913 exposed to C10176-S (Table 4) and C1913 and C2846 challenged with GL-1 (Table 6). After 8 h incubation at 37°C, all bacterial strains showed a reduction in bacterial number when exposed to original or enhanced phages with the exception of Midlands-1.

Following exposure to phages, all bacterial strains showed an eventual increase in $OD_{420-580}$ (Tables 4–7). This $OD_{420-580}$ increase is indicative of bacterial re-growth and potentially indicates the presence of resistant bacteria to the phage. However, the extent of $OD_{420-580}$ increase varied between bacteria/phage combinations. In the majority of cases, the time taken to increase the $OD_{420-580}$ by 0·1 above the initial $OD_{420-580}$ was reduced when bacteria were exposed to enhanced phage (Tables 4–7).

Comparison of activity between the streak test and the Bioscreen C

Overall, there was some correlation between the streak test assay and the Bioscreen C assay against PAO1, where

Table 7 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to LP-M phage

	Original phage			Enhanced phage		
	Streak test	Bioscreen C analyser		Streak test	Bioscreen C analyser	
Bacterial strain	(score out of 50)	Log_{10} reduction*	Time† (minutes)	(max. 50)	Log_{10} reduction*	Time† (minutes)
10332	32	0·87 ± 0·56	200 ± 20	–‡	2·01 ± 0·80	170 ± 30
C3652	22	1·08 ± 0·02	880 ± 280	35 (10)§	0·92 ± 0·32	505 ± 275
C3719	20	0·95 ± 0·24	**>1200**	31 (10)	0·76 ± 0·29	**450 ± 250**
C3786	40	3·03 ± 1·02	130 ± 20	42 (2·5)	2·70 ± 0·99	140 ± 10
LES-400	30	1·08 ± 0·19	1155 ± 80	–	0·91 ± 0·23	940 ± 320
Mids-1	10	ND¶	115 ± 40	–	ND	135 ± 15
P8959	20	2·03 ± 0·17	>1200	26 (10)	1·76 ± 0·28	830 ± 285
C1913	0	5·57 ± 0·45	545 ± 115	22 (10)	4·84 ± 0·86	350 ± 205
C2238	20	0·77 ± 0·09	>1200	50 (5)	0·51 ± 0·17	705 ± 370
C2846	43	3·00 ± 0·36	**305 ± 10**	50 (10)	3·01 ± 0·31	**200 ± 40**
C3597	43	3·90 ± 0·82	**605 ± 85**	50 (10)	2·66 ± 0·26	**220 ± 60**
C4503	10	ND	130 ± 10	21 (10)	0·83 ± 1·54	335 ± 360
PAK-SR	4	2·95 ± 0·26	995 ± 75	42 (2·5)	2·85 ± 0·33	950 ± 55

Data shown are the mean of three replicates. Bold typeface indicates significant differences.
*Log_{10} reduction in bacterial number at 8 h.
†Time taken to increase $OD_{420-580}$ by 0·1 above the original $OD_{420-580}$
‡Dashes indicate no increase or decrease in lytic activity.
§ (time): phage-bacteria contact time (min) before exposure to PRE (see text).
¶ND: no reduction in bacterial content determined.

all enhanced phages with the exception of GL-1 showed the highest reduction of bacterial number with the Bioscreen C (Table 3). This fact corresponded to the highest scores achieved with the streak test (Table 3). However, with enhanced GL-1, the largest reduction in bacterial number was obtained after an enhancement time of 2·5 min, compared to the highest score on the streak test that was obtained after a 5 min enhancement (Table 3). When comparing the time taken for PAO1 to re-grow to an $OD_{420-580}$ value of 0·1 higher than the initial $OD_{420-580}$, it was found that for all phages, except GL-1, bacteria treated with both original and enhanced phages took >1200 min to reach this value (Table 3).

When the activity of phages was assessed against CF isolates, the level of correlation between the streak test and the reduction of bacterial number from the Bioscreen C method depended upon phage/bacterial strain. A good correlation was observed with LP-M and two strains, C10176-L and five strains, GL-1 and six strains and C10176-S and seven strains (Tables 4–7). For all the other phage/bacteria combinations, however, the level of reduction in bacterial number at 8 h was not significantly different ($P \geq 0·05$) when exposed to the original or the enhanced phages.

When phage performance was compared to the set 'acceptance' values, it was observed that an 'acceptable' status was achieved against 50% or less strains (Table 2). However, enhanced phages appeared to have a better activity than the original phage although bacterial re-growth was faster in many bacteria.

Discussion

With the renewed interest in bacteriophage therapy for a variety of conditions ranging from *Ps. aeruginosa* infections in cystic fibrosis patients to the use of bacteriophages to reduce the level of *Salmonella* colonization of broiler chickens (Atterbury *et al.* 2007), there is a need for a high throughput method, which is both sensitive and quantitative.

Although a successful phage product does not hinge on the initial screening of activity, the selection of phages may be enhanced by efficient and quantitative screening methods to ensure maximum activity against the widest host range. Here, we have shown that both the streak plate method and the Bioscreen method produced similar results, at least with phages showing a large increase in activity. Although the streak test provides adequate qualitative data for initial screening in the form of a single number, it does not provide the quantitative data that would be needed to select phages for practical applications. Conversely, the data generated by the Bioscreen C analyser allows for the assessment of multiple criteria. These criteria, however, should not be taken individually, but should instead be combined, and phages selected on the basis of increased characteristics between all criteria combined. Our assay relied on the calculation of bacterial

number from a pre-established OD/TVC graph. Such extrapolation of bacterial number has its limitation as the OD/TVC graph is not linear above an OD value of *c.* 0·8. At best, this limitation means that our bacterial number calculated from OD value >0·8 might have been underestimated. However, the conversion of OD value in bacterial number, and the subsequent calculation of Log_{10} reduction, enables the direct comparison of phage activity between strains that show different growth kinetic to be established. Our method aimed to provide criterion to compare phage activity against multiple bacterial strains that might show different growth characteristic. Although, the conversion of OD values to bacterial number has its drawbacks, our results proved to be reproducible and enabled phage activity to be effectively compared.

In this manuscript, two methods were compared and some similarities were observed. It has to be noted that the parameters used in the two methodologies were different, notably the different phage: bacteria ratio, the incubation temperature following exposure and the media. These parameters might have contributed to the differences in results observed. The parameters used with the Bioscreen were developed in preliminary experiments and chosen as they provided the best results. The parameters used in the traditional streak test are those described in the literature and used routinely. The main objective of this paper was to report an automated method to improve on the traditional streak test. The main practical difference between the two methods remains with the number of bacteria/phage combination that can be tested. With the streak test, a maximum of four phage combinations and one set of controls (five replicates per bacterial line) against a single bacterial species could be tested in any one experiment. However, with the Bioscreen assuming the same number of replicates, a maximum of 18 phage combinations could be investigated showing that the Bioscreen has a higher throughput. In addition, the Bioscreen method provided important quantitative information on bacterial re-growth following phage exposure over a 20 h period.

In this study, four *Ps. aeruginosa* bacteriophages were confirmed to possess activity against multiple strains of *Ps. aeruginosa* including some of the more prevalent strains within the UK (Salunkhe *et al.* 2005; Fothergill *et al.* 2007). Result variability is most probably caused by the inherent variability that is associated with both bacterial and bacteriophage growth despite initial standardization of bacterial content. The differences in susceptibility of the phages may be a result in part to the binding efficacy of the phage to the target cell (Hart *et al.* 1994) and also the growth rates of the strains tested. The nature of the PRE treatment also accounts for a lack of increase in both rate and level of kill as it was originally designed to select bacteriophages on the basis of invasion time rather than other factors (Jassim *et al.* 1995).

The use of the Bioscreen C analyzer produced a quick real-time assessment of bacteriophage activity. It allows the rapid visualization of bacterial lysis demonstrating phage activity although measurement of the rate of lysis was shown not to be a reliable criterion for the comparison of lytic activity. Measurement of OD in the Bioscreen assay is automated and does not rely on human qualitative assessment on plaque formation as in the streak assay (Merabishvili *et al.* 2009) and was shown to be more stringent compared to the streak test and provided quantitative results within 8 h. In comparison, the streak test method can take up to 24 h (Merabishvili *et al.* 2009). The Bioscreen method could be further refined with the use of metabolic assays to assess the level of bacterial viability (Kuda and Yano 2003; Cerca *et al.* 2005) or potentially bioluminescent mutants (Marques *et al.* 2005; Jassim and Griffiths 2007; Thorn *et al.* 2007), which provide direct measurements of bacterial viability, ultimately increasing the sensitivity of this OD-based quantitative assay.

The set 'acceptance' values used in this study aim to ensure that selected phages possess acceptable properties to be used for therapy. The phages used in this study meet these criteria in 50% or less of strains tested, implying that their activity is not yet sufficient to be applied to therapeutics. These 'acceptance' values were set after a review of the literature and taken into account antibiotic dosing to prevent bacterial re-growth. It is conceivable that our values are too stringent and might need to be revised. To this aim the testing of phages that are currently undergoing licencing or already licenced for human applications would be of benefit.

The Bioscreen method is an *in vitro* set up and phage behaviour *in vitro* might be different from their behaviour *in situ*. The chemical and physical parameters used in the Bioscreen set up (e.g. growth media, temperature; % CO_2) can be modified to represent better conditions found in practice.

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