Lytic bacteriophage as an adjunct to antibiotic therapy for *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients

A thesis submitted to Imperial College London

For the degree of Doctor of Medicine (Research)

July 2021

Dr. Isaac Martin
BSc, MBBS, MRCPCH
National Heart and Lung Institute
Imperial College London

ACKNOWLEDGEMENTS

I wish to thank so many individuals who made this journey possible.

Firstly, to my supervisors Professor Eric Alton and Professor Jane Davis for their wisdom and guidance throughout this process. Jane deserves special thanks as she has been an incredible supervisor and mentor, providing just the right amount of encouragement and critique to get me to the finish line. At the end of this journey, I feel lucky to consider her a friend in addition to the guidance she has provided.

Also for their input into this MD, thanks go to Dr. Sandra Morales, Dr. Dervla Kenna, Zoë Payne, Dr. Susan Lehman, Dr. Rossa Brugha, Dr. Sara Fontanella and Dr. Rishi Pabary. There are many other people in Jane's team that I am fortunate to have worked with and and who managed to keep team morale high, even in stressful times. In particular, Ronan Murphy gave me some much-needed tutorials when I was new to the lab and didn't even know my way around a pipette, and I thank him for his patience.

There are many other friends and colleagues at the Royal Brompton Hospital whose wisdom I have benefitted from and whose humility and enthusiasm I have been inspired by, from the wider paediatric respiratory team to the microbiology department to the electron microscopy team.

I am also thankful for the infrastructure within the world of CF that promotes this kind of research; from the patients and carers of those with the disease, to organisations like the CF Trust that promote and fund it.

Lastly, I am so grateful to my husband, Olugbenga, who has provided me distraction when I needed it, and has supported me with quiet encouragement throughout this very long process.

Declaration of Originality

I declare that all the work within this thesis is my own unless stated otherwise and that all external sources are appropriately acknowledged and referenced.

COPYRIGHT DECLARATION

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

PUBLICATIONS AND PRESENTATIONS

The work in this thesis has contributed to the following journal publications:

Martin I, Kenna DTD, Morales S, Alton E, Davies JC. Variability in Bacteriophage and Antibiotic Sensitivity in Serial *Pseudomonas aeruginosa* Isolates from Cystic Fibrosis Airway Cultures over 12 Months. *Microorganisms*. 2021;9(3).

Martin I, Waters V, Grasemann H. Approaches to Targeting Bacterial Biofilms in Cystic Fibrosis Airways. *Int J Mol Sci*. 2021;22(4).

Davies JC, **Martin I**. New anti-pseudomonal agents for cystic fibrosis- still needed in the era of small molecule CFTR modulators? *Expert Opin Pharmacother*. 2018;19(12):1327-36.

Smith WD, Bardin E, Cameron L, Edmondson CL, Farrant KV, **Martin I**, et al. Current and future therapies for *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. *FEMS Microbiol Lett*. 2017;364(14).

And the following abstracts/presentations in conferences:

Martin I, Morales S, Alton E, & Davies JC. Interactions between an antipseudomonal bacteriophage cocktail and conventional antipseudomonal antibiotics varies with bacterial strain and planktonic/biofilm culture systems. *Pediatric Pulmonology*. 2018 (53):252-252. 111 River St., Hoboken, 07030-5774, NJ USA: Wiley.

Martin I, Davies JC, Morales S, Alton EW. WS07.4 Combined killing effects of lytic bacteriophages and antibiotics in biofilm-grown *Pseudomonas aeruginosa* from CF airway cultures. *Journal of Cystic Fibrosis*. 2017;16:S12-S3.

ABSTRACT

Longer lifespan and improved quality of life for patients with cystic fibrosis (CF) have been attributed to, at least in part, advances in treating pulmonary infection in this disease. The acquisition of ubiquitous environmental organisms and the development of inflammation and airway scarring as a result is a major hurdle to improving the lives of patients with CF. This is especially true for *Pseudomonas aeruginosa* (Pa), the dominant respiratory pathogen in CF patients, due to the development of antibiotic resistance as well as the development of a biofilm mode of growth. The problem posed by this organism has necessitated exploration of novel therapies, including bacteriophage.

As phage therapy in the context of CF is unlikely to be used in isolation, the first part of this thesis explores the efficacy of a cocktail of 4 lytic bacteriophages when used as adjunctive therapy to the commonly used antibiotics ceftazidime and tobramycin in high-throughput models of Pa infection. Reduction in both biofilm biomass and cell viability was demonstrated when combining phage with the two antibiotics in a biofilm model of infection across 16 different clinical Pa strains. To interrogate the effect of combination therapy more thoroughly, checkerboard experiments combining phage and ceftazidime or tobramycin individually in both planktonic and biofilm Pa culture were performed. Although there were individual examples of synergy, there were also examples of antagonism. Efficacy of the phage cocktail varied based on Pa strain, type of culture (biofilm vs. planktonic), as well as the antibiotic used. For 3 of the 16 clinical strains used in these experiments, there was an observed conversion to mucoid phenotype on Congo Red agar. As a small pilot study, transcriptomic analysis on 1 of these strains was undertaken and demonstrated upregulation of genes involved in alginate production with phage in both planktonic and biofilm culture, mirroring the phenotypic findings seen.

Lastly, bacteriophage sensitivity was tracked over time with the objective of answering questions pertaining to the recruitment of participants to prospective clinical trials involving phage. With the objective of knowing whether recruitment of patients could be based on historical sensitivity to phage, I selected 24 patients with chronic Pa infection and looked at serial isolates over the course of 12 months, performing antibiotic sensitivity testing, plaque assays as well as variable number tandem repeat (VNTR) typing of each isolate. Results showed that even when representing a single Pa strain, there was a high degree of variability

in sensitivity to both bacteriophage and antibiotics. There were high rates of sensitivity to bacteriophage – even in multi-drug resistant (MDR) strains -which supports the development of this therapy. However, the observed variability implies that selection of patients in future trials should be on the basis of current, not past, isolate testing.

Broadly speaking, my results with relation to the development of bacteriophage therapy in CF lung disease are promising, showing broad efficacy in a large number of clinical Pa strains in both biofilm and planktonic culture, both as adjunctive therapy with use of commonly used anti-Pa antibiotics, and in MDR strains. The variability of results, both across strains, but also in individual strains at different time points, should be taken into consideration as trials with phage are developed.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 2
DECLARATION OF ORIGINALITY 3
COPYRIGHT DECLARATION 4
PUBLICATIONS AND PRESENTATIONS 5

ABSTRACT 6

LIST OF FIGURES 11
LIST OF TABLES 12
LIST OF ABBREVIATIONS 13

1 CHAPTER 1: INTRODUCTION 16

1.1 CYSTIC FIBROSIS 16
1.2 AIRWAY AND SPUTUM PROPERTIES IN THE NORMAL LUNG 18
1.3 AIRWAY AND SPUTUM PROPERTIES IN CYSTIC FIBROSIS 19
1.4 THE IMMUNE SYSTEM, INFLAMMATION AND THE AIRWAY 22
1.5 INFLAMMATION AS A THERAPEUTIC TARGET IN CF 24
1.6 *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS 25
1.7 ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA* 27
1.8 BIOFILMS IN PA INFECTION 29
Introduction to Biofilms 29
Mucoidy 31
Quorum sensing 33
Intracellular signalling pathways 34
1.9 THE ROLE OF ANTIBIOTICS IN PULMONARY EXACERBATION 34
1.10 OTHER ANTIBIOFILM AGENTS IN CLINICAL USE 36
1.11 BACTERIOPHAGE: STRUCTURE AND CLASSIFICATION 38
1.12 BACTERIOPHAGE: LIFE CYCLE 40
1.13 APPLICATIONS FOR BACTERIOPHAGE THERAPY 44
Proposed Advantages of Phage Therapy 45
Proposed Shortcomings of Phage Therapy 46
Strategies to Enhance the Efficacy of Phage 47

2 CHAPTER 2: METHODS USED IN EXPERIMENTS 49

2.1 ETHICS 49
2.2 STORAGE AND CULTURE OF *PSEUDOMONAS AERUGINOSA* ISOLATES 49
2.3 BACTERIOPHAGE COCKTAIL, SERIAL DILUTIONS AND STORAGE 50
2.4 PREPARATION OF ANTIBIOTIC SOLUTIONS 50
2.5 STANDARD PLAQUE ASSAY 51
2.6 PLANKTONIC GROWTH ASSAYS 53
2.7 STATIC BIOFILM ASSAY 54
The resazurin assay 54
The Crystal Violet assay 55
2.8 DISC DIFFUSION ASSAY 56
2.9 VARIABLE NUMBER TANDEM REPEAT TYPING OF *PSEUDOMONAS AERUGINOSA* 57
2.10 STATISTICAL ANALYSIS 59

3 CHAPTER 3: BACTERIOPHAGE USE ON *PSEUDOMONAS AERUGINOSA* BIOFILMS 60

3.1 INTRODUCTION 60

3.2 HYPOTHESIS 63
3.3 AIMS AND OBJECTIVES 63
3.4 MATERIALS AND METHODS 64
Selection of Pa strains 64
Plaque assay 64
Biofilm assays 64
Statistical analysis 65
Comparison of resazurin and crystal violet biofilm assays 66
Assessment of reproducibility 67
3.5 RESULTS 69
The Plaque Assay 70
Comparing planktonic and biofilm susceptibility to phage 71
Bacteriophage and antibiotics in biofilm 72
3.6 DISCUSSION 75

4 CHAPTER 4: EXPLORING ANTIBIOTIC AND BACTERIOPHAGE INTERACTIONS AT SUB-INHIBITORY CONCENTRATIONS IN PLANKTONIC AND BIOFILM CULTURE 78

4.1 INTRODUCTION 78
4.2 HYPOTHESIS 81
4.3 AIMS AND OBJECTIVES 81
4.4 MATERIALS AND METHODS 81
Selection of Pa strains 81
Planktonic and biofilm assays 81
Antimicrobial checkerboard assay 83
Coefficient of drug interaction (CDI) 83
Statistical analysis 84
Assay optimisation and choice of antimicrobial concentrations 84
4.5 RESULTS 87
4.6 DISCUSSION 92

5 CHAPTER 5: ASSESSING IMPACTS OF PHAGE EXPOSURE ON BACTERIAL BEHAVIOURS: A PILOT STUDY 95

5.1 INTRODUCTION 95
5.2 HYPOTHESIS 97
5.3 AIMS AND OBJECTIVES 97
5.4 METHODS 98
Congo Red agar 98
Selection of strain 98
Selection of phage concentrations and time for harvest 99
RNA extraction and generation of data 99
Statistical analysis 100
Biological pathway analysis 101
5.5 RESULTS 101
Congo Red agar 101
Selection of strain 102
Selection of phage concentrations and time point for harvest 103
RNA extraction 105
Work with statistician 105
Biological pathway analysis 107
5.6 DISCUSSION 107

6 CHAPTER 6: TRACKING VARIABILITY IN SENSITIVITY TO PHAGE AND ANTIBIOTICS OVER TIME 110

6.1 INTRODUCTION 110
6.2 HYPOTHESIS 112
6.3 AIMS AND OBJECTIVES 112
6.4 MATERIALS AND METHODS 112
Database and strain repository 112
Choice of patients and isolates 113
Antibiotic sensitivity testing by disc diffusion 113
Plaque assay 113
VNTR typing 114
Variable Number Tandem Repeat Dendogram 114
Statistical analysis 115
Lung function tests 115
6.5 RESULTS 115
Variable Number Tandem Repeat Analysis 116
Patient and isolate breakdown 120
Antibiogram profiles and phage sensitivity 121
Results from patients with the Liverpool Epidemic Strain 125
6.6 DISCUSSION 128

7 CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS 132

7.1 PHAGE IN BIOFILMS 132
7.2 PHAGE AND ANTIBIOTICS AT SUB-INHIBITORY CONCENTRATIONS 132
7.3 PHAGE AFFECTING PHENOTYPE 133
7.4 PHAGE AND ANTIBIOTIC SENSITIVITY OVER TIME 134
7.5 FINAL THOUGHTS 134

REFERENCES 136

LIST OF FIGURES

Figure 1.1.1 How different CFTR gene mutations impact protein function in health and in CF. 17
Figure 1.3.1: Diagram and EM images of healthy cilia when compared with cilia in dehydrated PCL. 20
Figure 1.4.1. Schematic of the postulated pathophysiologic cascade in CF lung disease. 22
Figure 1.6.1. Prevalence of common respiratory pathogens in CF as patients age. 26
Figure 1.7.1. Innate resistance to classes of commonly used antibiotics in Pa infection. 27
Figure 1.8.1. Diagram of the biofilm lifestyle for *Pseudomonas aeruginosa*. 30
Figure 1.9.1. Fluorescence imaging of *Pseudomonas aeruginosa* biofilms. 36
Figure 1.12.1. The life cycle of lytic bacteriophage. 42
Figure 2.5.1. Visual outcome for the plaque assay. 52
Figure 2.6.1. Basic spectrophotometry (246). 53
Figure 2.7.1. Visual outcomes of both biofilm assays in 96 well plates. 56
Figure 2.9.1. VNTR assay output and summary data. 59
Figure 3.4.1. Comparison of the absolute reads from no treatment wells of both biofilm assays. 66
Figure 3.4.2. Example reproducibility from strain 1 in two subsequent experiments using the same protocol. 68
Figure 3.4.3. Reproducibility in 5 Pa strains at different time points (biological replicates). 69
Figure 3.5.1. Distribution of the three biological replicates of the plaque assay experiments. 70
Figure 3.5.2. Comparison of planktonic and biofilm susceptibility to phage. 71
Figure 3.5.3. Biofilm readout for the 16 strains with focus on the 4 most antibiotic resistant strains. 73
Figure 3.5.4. Percent reduction in biofilm biomass and cell viability with antibiotic combination and phage. .. 74
Figure 4.4.1. Example setup of a synergy checkerboard assay. 83
Figure 4.4.2. Checkerboard output for Strain 11 in biofilm culture with ceftazidime and phage. 86
Figure 4.5.1. Photo of the crystal violet assay from one biological replicate for Strain 15. 87
Figure 4.5.2. Two different clinical strains demonstrating the additive effects of combining individual antibiotics and phage. 88
Figure 4.5.3. Two strains demonstrating different types of inhibition when combining individual antibiotics and phage. 89
Figure 4.5.4. Example of biofilm results for Strain 14 combining phage with ceftazidime or tobramycin. 90
Figure 5.5.1. Congo Red agar on Day 1 and 7 for 3 strains that demonstrated phenotypic change with phage. 101
Figure 5.5.2. Overnight growth curves for Strains 1 (a), 10 (b) and 13 (c). 103
Figure 5.5.3. Analysis of Strain 10 with growth curve and resazurin assay, as well as crystal violet biofilm assay. 104
Figure 5.5.4. RNA integrity numbers (RINs) for different treatment groups. 105
Figure 5.5.5. Heatmap with cluster dendrogram demonstrating top 100 differentially expressed genes for different treatment groups. 106
Figure 6.5.1. Example antibiograms and bacteriophage sensitivity profiles for 4 different patients chosen to illustrate variability in results. 125
Figure 6.5.2. Antibiograms and bacteriophage sensitivity profiles for 4 patients with the Liverpool Epidemic Strain. 127

LIST OF TABLES

Table 1.11.1. Overview of bacteriophage structure, family and viral genome. 39
Table 1.11.2. Bacteriophage families and corresponding genome characteristics. 40
Table 4.5.1. Summary data for checkerboard experiments across 10 clinical strains + PA01. 91
Table 6.5.1. Properties of selected patients and their Pa isolates. 120
Table 6.5.2. Inter-patient antibiogram and plaque assay variability. 121

List of Abbreviations

ACT	Airway clearance therapy
AHLs	N-acyl-L-homoserine lactones
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ASL	Airway surface liquid
AST	Antibiotic sensitivity testing
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
CDI	Coefficient of drug interaction
CF	Cystic fibrosis
CFRD	CF-related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CFU	Colony forming units
CoV	Coefficient of variation
CSLD	Chronic suppurative lung disease
CV	Crystal violet
DNA	Deoxyribonucleic acid
eDNA	extracellular DNA
ds	Double-stranded
EM	Electron microscopy/micrograph
ENaC	Epithelial sodium channel
ESBL	Extended spectrum ß-Lactamase

EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FDA	US Food and Drug Administration
FEV1	Forced expiratory volume in one second
FI	Fluorescence intensity
HRCT	High resolution computerised tomography
IFN	Interferon
IQR	Interquartile range
QS	Quorum-sensing
LES	Liverpool Epidemic Strain
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MCC	Mucociliary clearance
MED	Minimum effective dose
MIC	Minimum inhibitory concentration
MHB	Mueller-Hinton broth
MMR	DNA mismatch repair system
mRNA	Messenger RNA
MS	Mass spectrometry
NBD	Nucleotide-binding domain
NO	Nitric oxide
OD	Optical density
Pa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PCD	Primary ciliary dyskinesia
PCL	Periciliary layer
PCR	Polymerase chain reaction

PFU	Plaque forming units
PHE	Public Health England
RBH	Royal Brompton Hospital
RIN	RNA integrity number
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
SMB	Salt-magnesium buffer
ss	Single-stranded
TEM	Transmission electron microscopy/micrograph
TMM	Trimmed mean of M-values
TNF	Tumour necrosis factor
VNTR	Variable number tandem repeat

1 CHAPTER 1: INTRODUCTION

1.1 CYSTIC FIBROSIS

Cystic fibrosis (CF) is a genetic condition that is estimated to affect more than 70,000 people worldwide (1) and roughly 10,500 people in the UK as of 2017 (2). Despite the fact that it is considered a rare genetic disorder, it is the most frequent life-limiting genetic disease within the Caucasian population, with an approximate incidence of 1 in every 2,500 births (3). CF is inherited in an autosomal recessive fashion, whereby affected individuals inherit two defective copies of a gene that encodes for an ion channel. This protein is called the cystic fibrosis transmembrane conductance regulator (CFTR) and is vital to varied human exocrine functions.

In a healthy human, the CFTR protein sits at epithelial cell surfaces and allows the passage of chloride ions through a c-AMP mediated mechanism (4). These ion channels are spread throughout the body and deranged chloride ion movement in different organs leads to a variety of deficits according to the epithelia where they are expressed. The first scientific description of CF was in 1938 by Dorothy Anderson (5) and describes hallmark pancreatic/gastrointestinal and pulmonary manifestations of disease; however, it is now well understood that CFTR is expressed in the reproductive system, liver and bone, to name but a few other organ systems (6). There is also a developing understanding about expression of the CFTR protein in other organs in the human body and burgeoning research into the innate immune system has led to recognition that impaired function of neutrophils and macrophages leads to further clinical manifestations of the disease (7). Consequently, CF is a truly multisystem disease with complex and varied expression.

In sweat glands on the skin, lack of ability to reabsorb chloride leads to sweat with abnormally high salt content, and this is the basis for the sweat test, first described by Gibson and Cooke in 1959 (8). It is thought that the first reference to sweat as an indicator of disease predates any modern scientific understanding, with stories of medieval folklore making references to children who tasted salty when kissed and were believed to be bewitched and soon die. In 1606, Alonso de los Ruyzes de Fonteca, a professor of medicine in Spain, similarly noted that his fingers tasted salty after wiping the brow of one of these ‘bewitched child’ (9). Salt content in sweat using the pilocarpine-iontophoresis sweat test and the principles described in 1959

(8) is still used to this day to for diagnostic confirmation of the disease (6), and has been used as an outcome measure in many of the new small molecule therapies as a surrogate marker for efficacy in clinical trials (10).

There are currently over 2,000 documented CFTR mutations, many of which are recognised as giving rise to deranged chloride ion function and CF (11). There is, however, a large degree of phenotypic variation amongst patients with this disease, as each genetic variant is associated with different degrees of impairment in the production, expression, function and longevity of the chloride ion channel. These different mutations (from Class I through to Class VI) can be seen in Figure 1.1.1.

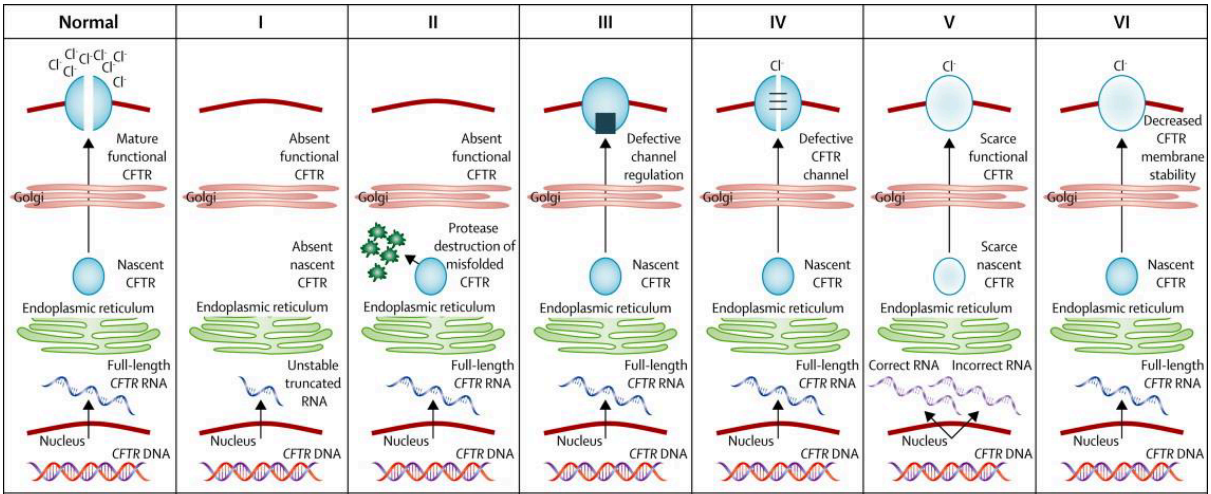


Figure 1.1.1 How different CFTR gene mutations impact protein function in health and in CF. Reproduced from (12).

On one end of the disease spectrum, there is complete lack of CFTR protein production (Class I mutation) and on the other, normal production of protein that has decreased conductance (Class IV) or is less stable than the wild-type protein (Class VI).

The most common disease causing mutation in the USA and in the UK results in a phenylalanine deletion at position 508 (F508del) (1, 2), which is categorised as a class II mutation, but also exhibits properties of other mutation classes. For instance, for the small amount of CFTR that makes it to the cell membrane, it also demonstrates defective channel

regulation (Class III) and decreased stability of the CFTR protein (Class VI) (13). Whilst pancreatic dysfunction correlates well with genotype (and hence, mutation class), extent of lung damage is not so predictable. Even amongst people with the same genetic mutation, there is a high degree of phenotypic variation (14, 15). Studies examining chloride conductance within twins homozygous for the F508del variant showed a higher correlation between monozygotic pairs, concluding that there must be other genes and factors contributing to both chloride ion conductance and manifestation of disease (16). Other modifier genes in CF with a bearing on disease severity are being studied, including non CFTR ion channels, genes involved in host defence and those involved with airway responsiveness (17).

1.2 AIRWAY AND SPUTUM PROPERTIES IN THE NORMAL LUNG

The human respiratory tract is lined by an extensive epithelial surface, from the nose and larynx to the distal alveoli where gas exchange takes place. Pseudo-stratified columnar ciliated cells line the respiratory epithelium in the nose into the lower airways, with mucous secreting goblet cells punctuating this epithelial lining. There are fewer goblet cells and cilia in the very distal airways, in favour of other cells, including those that secrete surfactant (18). This surface is exposed to a high burden of potentially harmful substances, from particulate matter to bacteria that make their way into the airway from the outside environment. Though the first line of mechanical defence of the airways is the cough reflex, the airway mucosa is host to other strategies against substances from the external environment.

The layer formed by cilia, periciliary liquid (PCL) and mucous has many functions, but broadly, foreign substances become stuck in the mucus while the cilia work to remove it. The nasal mucosa traps and filters out the vast majority of particulate matter before it reaches the more distal airways (19) and as such, it is the first line of mucosal defence. The mucus layer itself consists of heavily glycosylated network of mucins that binds and traps inhaled molecules which are then moved proximally and out of the lower airways by the collective action of the cilia (20). This is termed mucociliary clearance (MCC), and is the basis for understanding – at least in part - the mechanism underlying many chronic suppurative lung diseases, including primary ciliary dyskinesia (PCD) and CF (20).

The mucus is separated from the cilia by a PCL layer, which together comprise the airway surface liquid (ASL). Bathed in this low viscosity PCL, the cilia beat at a frequency of about 8-15 Hz, which enables movement of mucus and trapped particulate matter out of the distal airways where they might otherwise cause damage (20, 21). Further studies have expanded the complexity of this model, identifying a "brush layer" with mucins spanning the PCL and cilia (22), but the basic model of MCC remains the same.

1.3 AIRWAY AND SPUTUM PROPERTIES IN CYSTIC FIBROSIS

The predominant clinical problem unifying patients with CF is lung disease and this is the leading cause of mortality and morbidity amongst this group (23). This dual function of ASL in innate defence, the mechanical action of the mucociliary escalator and chemical/immune barrier to infection, has been a large focus of understanding the underlying mechanism of disease in CF and identifying therapies. There is a large body of evidence exploring the ways in which deranged CFTR function leads to an abnormal ASL and the clinical picture seen in CF patients (24). Historically, there have been several hypotheses as to the primary mechanism underlying the pulmonary dysfunction seen in this patient group.

One early theory for the airway dysregulation seen in CF was the high-salt hypothesis, which looks at the chloride ion as a central component of salt regulation and transport. This arose after early studies noted much higher Cl^- and Na^+ concentration in the ASL of CF airway epithelial cultures when compared with similar airway cultures from healthy controls (25). In this theory, the impaired ability for airway epithelial cells to secrete Cl^-, in combination with excessive absorption of Na^+ results in extra salt in the ASL, thought to contribute to the thick secretions seen in the CF airway (26). *In vitro* studies like the one mentioned, however, were not replicated *in vivo*, where it was shown that there was little to no difference in salt content from BAL liquid in patients with CF – at varying stages of disease - compared to healthy controls (27).

In addition to functioning as a chloride ion channel, the CFTR protein is now known to participate in bicarbonate ion transport and exerts and inhibitory effect on epithelial sodium channels (ENaCs) (28). The low volume hypothesis of CF, which, over years has become the favoured theory underlying the mechanistic change in CF airways, asserts that low or absent chloride ion transport, coupled with increased sodium reabsorption via ENaC, results in in a

dehydrated PCL and an increase in viscosity of airway secretions, in turn leading to impaired MCC (29). Early research in humans using a radiolabelled, aerosolised compound showed gross impairment in MCC in patients with CF when compared to controls, supporting this hypothesis (30).

Studies examining the appearance and constitution of the PCL have further supported this theory. In healthy adults, for instance, it has been shown that the optimal PCL depth is approximately 7 µL, but reduced in patients with CF (21, 31). This is depicted in Figure 1.3.1.

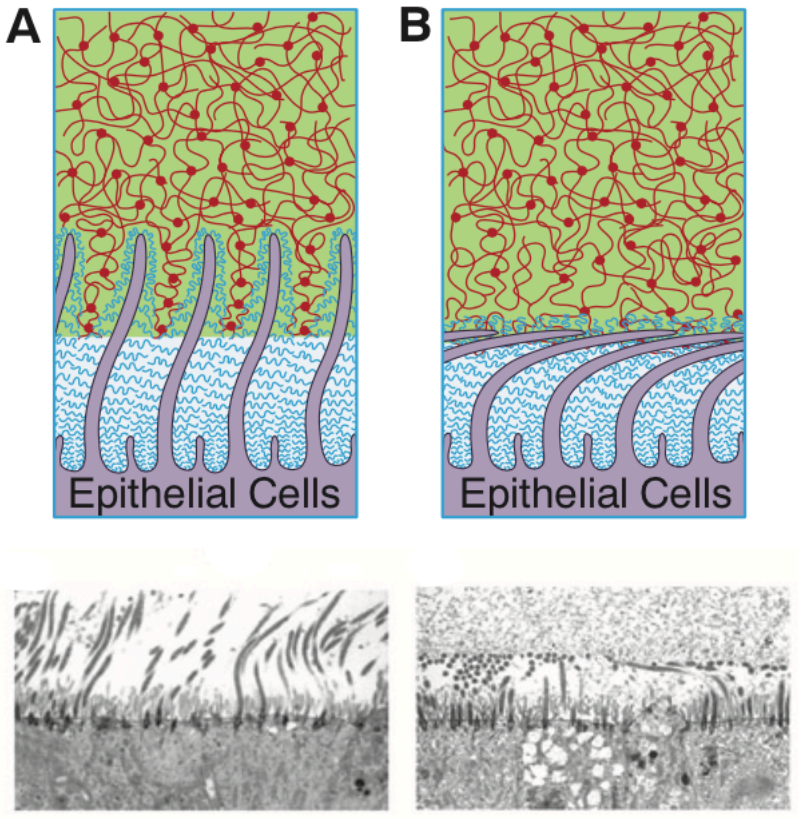


Figure 1.3.1: Diagram and EM images of healthy cilia when compared with cilia in dehydrated PCL.
Upper panel: Diagram of healthy cilia (A) when compared to cilia structurally compressed by a dehydrated PCL (B). Lower panel: Electron microscopy (EM) shows ciliated airway epithelium from a healthy individual (A) after a small volume of ASL added, whereas (B) demonstrates the dehydrated PCL and mechanically distorted cilia with addition of ASL in CF epithelium. Diagram reproduced from (22). Electron microscopy slides below the diagram reproduced from (24).

Mucins tethered on the cilia form a mesh network that attracts water, which can be drawn into the PCL to ensure adequate hydration. In CF, the loss of chloride ion secretion with the

deficient CFTR protein results in sodium and water reabsorption, leading to dehydration of the ASL. Though much research has focused on the role of the CFTR protein as a chloride ion transporter, it also is key in the secretion of bicarbonate in bronchial epithelial cells from the small airways (32). Studies have shown that mucins are released in condensed granules from the crypts of small airways and the presence of bicarbonate allows the mucins to disperse and disaggregate from the epithelial cell surfaces. In CF, lack of bicarbonate leads to a thick and tenacious layer of mucins that adheres to the epithelial surface (33, 34).

Initial studies in both animal models of disease and patients with CF seem to support a low volume, dehydrated ASL as the likely mechanism initiating lung disease in CF (35). Early studies demonstrated that these same properties adversely affected even the first line mechanical defence of cough clearance (36). Far from the complete story, the mechanism being elucidated in studies which look at how the lungs work *in* vivo alludes to something much more complex.

As an example, though *in vitro* work looking at airway epithelial cultures from patients with CF does indicate a depleted PCL, in conditions that mimic the phasic motion of the lung, there is ATP release into the PCL with subsequent restoration of a PCL depth adequate for ciliary function and MCC (37). In this same model, however, infection with respiratory syncytial virus (RSV) disrupted the ATP signalling pathway and reduced PCL depth in CF airway cultures when compared to airway cultures from healthy controls. Thus, though the dehydrated PCL with an overlying viscous mucous layer is indeed a factor seen in CF lung disease, it is by no means the whole picture and factors like viral infection on top of impaired ion transport are likely to be part of the mechanism by which lung damage begins.

The dehydrated PCL with impaired MCC seen in CF is one element contributing to mucus plugging of the small airways, resulting in fixed dilatation and bronchiectasis, hallmark findings on chest imaging. In studies looking at both the histopathology and radiology of end stage CF lungs, there is also marked distention and distortion of the alveoli, denoting emphysema (38). From a clinical perspective, this is represented by more frequent pulmonary exacerbations and admission to hospital, reduced FEV1, worse quality of life scores, and increased mortality.

1.4 THE IMMUNE SYSTEM, INFLAMMATION AND THE AIRWAY

The mucus layer is home to many substances that are bactericidal, including but not limited to IgA, lysozyme and lactoferrin (39). Indeed, it is in the mucus layer where foreign substances first encounter cells from the human immune system like chemokines and cytokines (40), which in turn initiate the recruitment of leukocytes (41). This cascade of inflammatory processes in CF exaggerated and is an intrinsic part of the pathophysiology that is interlinked with the mucus properties described above.

Persistent high level inflammation in response to exogenous pathogens is thought to lead to structural damage of the airway and decline in lung function (42). A schematic of this deterioration can be seen in Figure 1.4.1.

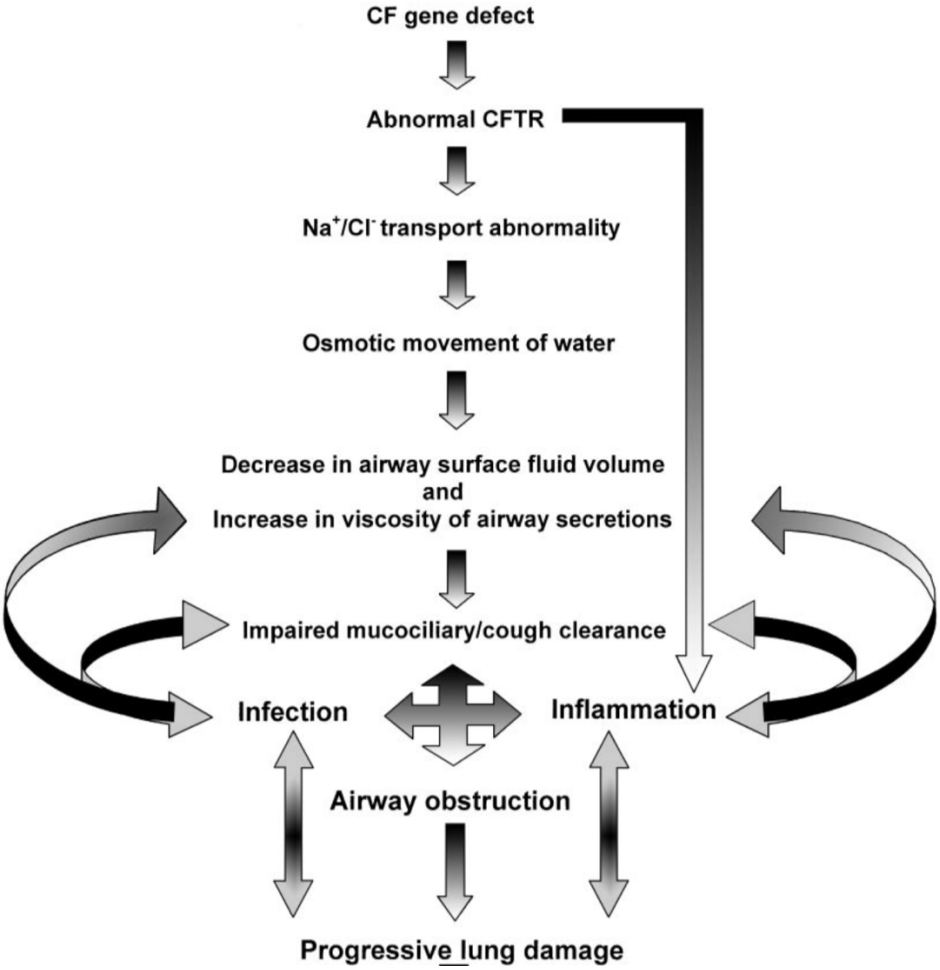


Figure 1.4.1. Schematic of the postulated pathophysiologic cascade in CF lung disease.
It should be noted in this depiction that many processes, including infection and inflammation, are processes that run in parallel. Reproduced from (29).

The cascade of inflammation and infection is still being established. Seminal studies in the 1980s demonstrated structurally normal lungs in newborns with CF (43, 44) and the pathway of microbial infection leading to inflammation was thought to be the explanation of lung disease. Since then, examination of broncho-alveolar lavage fluids of infants in comparison with healthy controls has shown elevated levels of neutrophils and other pro-inflammatory markers (45), even in the absence of any pathogen, suggesting that inflammation could in fact be a precursor to bacterial infection. Indeed, it was noted that even as early as the foetal stage in CF, there were elevated levels of inflammatory proteins when compared with controls (46). These facts suggest that an aberrant inflammatory cascade could exist independently from infection. Flipping the accepted paradigm on its head, it was then hypothesized that elevated levels of inflammation might in fact enable chronic microbial infection, in turn propagating the cycle.

Although inflammation is the body's mechanism for containing and combatting infection, in CF this response is exaggerated, and is responsible for much of the damage of the lung. Defects in the CFTR protein result in increased levels of proinflammatory mediators like IL-8, which acts as a chemoattractant for neutrophils, which in turn lead to damage and scarring of the airways (47). Even when infected with the same organism, BALF from children with CF when compared to controls manifest higher levels of both neutrophils and IL-8, even when accounting for microbial burden (45, 48). Though recruitment of neutrophils should in theory result in less infection, within the context of CF, isolated airway neutrophils have been shown to have impaired bacterial killing potential when compared to those found in healthy controls (49).

In an era of emerging corrective pharmacotherapeutic agents for those with certain CF mutations, the scientific community is again revisiting this topic in more detail to ascertain whether correcting for the basic defect seen in the disease has the ability to reduce levels of inflammation. The small molecule ivacaftor, for instance, which restores functionality to the CFTR protein for those with Type III (gating) mutations, has indeed been seen to reduce both bacterial density and inflammation in the sputum of patients taking this medication (50). As studies begin to explore the relationship of these small molecules that correct CFTR dysfunction, more are beginning to see reduced burden of bacteria (50, 51), though it still

remains unclear whether this is due to inherent antimicrobial properties of these agents, or due to alteration of sputum properties and inflammation that allow for these bacteria to be cleared by innate defences.

1.5 INFLAMMATION AS A THERAPEUTIC TARGET IN CF

A large body of research has looked at interrupting the pathophysiologic cascade of inflammation as a therapeutic target in CF, but results have been varied and denote the complex nature of this disease. Two approaches to tackle inflammation have been used – the early use of broad-spectrum agents and more recently, the study of specific inflammatory mediators or pathways in the hopes of a streamlined therapeutic effect without the side effects seen with the former.

The use of prednisolone was an early approach to tackle inflammation broadly. Though a seminal 4-year study published in 1985 demonstrated improved pulmonary function, weight gain and fewer hospital admissions than placebo (52), further work highlighted higher rates of acquisition of Pa, though exacerbation rates in patients already colonized with Pa remained the same (53). Years later, in participants who had participated in the original trial, there was no significant difference in FEV1 between the placebo and intervention groups; however, the prednisolone treated patients were significantly growth retarded (54). Even when looking at inhaled corticosteroids as an alternative to oral dosing, thereby reducing systemic absorption and improving side effect profiles, there is still no evidence to promote their use in CF (55, 56). Suffice it to say that steroids, though certainly efficacious to some degree at reducing inflammation and improving pulmonary function, have not become a mainstay of CF treatment, outside some specific diagnoses like allergic bronchopulmonary aspergillosis (ABPA).

Ibuprofen is another anti-inflammatory with broad effects that has been studied in the context of CF. In the same year as the seminal study looking at prednisolone, another randomized, double-blind, placebo controlled trial with ibuprofen as an anti-inflammatory agent demonstrated improved preservation of lung function, body weight and fewer hospital admissions with no increase in adverse events (57). A further safety and effectiveness trial revealed similar findings (58), as did analysis of registry data (59). But although a recent

Cochrane Review of multiple studies concludes that high-dose ibuprofen is linked to slowed lung function decline in people with CF (60), it is still not a mainstay of treatment for this disease due to potential side effects like bleeding (61).

Using a more targeted approach to tackle inflammation has been studied, as an alternative to the existing broad spectrum agents with undesirable side effects. Though none of these agents is currently used in clinical practice, there are several approaches that have yielded good laboratory results, but have failed to translate to clinical practice either due to adverse effects of the agent used (62) or due to lack of effect on lung function (63). There are also tales of caution in this domain that tell us that dampening levels of inflammation too comprehensively could have its own risks. One such agent targeted LTB_4, a compound produced by macrophages and PMNs in response to a variety of stimuli, including bacterial pathogens. Inhibiting the action of this compound was theorized to intervene in the development of inflammation (64); however, a clinical trial using LTB_4 inhibitor of was terminated prematurely due to significant increases in the amount of pulmonary exacerbations seen in patients with CF (65). Thus, as with the case for systemic steroid use and higher rates of acquisition of Pa, there is certainly caution to be exercised about reducing inflammation either too comprehensively or not in a way that produces therapeutic benefit.

1.6 *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS

The pathophysiology of the aberrant CFTR protein in CF translating to a dehydrated and thick mucus layer with impaired mucociliary clearance (30, 66), among other factors that have been touched upon, as diverse as the acidic pH of airway secretions (34, 67) and reduced mucus oxygen concentration (68), to impaired host defences (69), together create an environment in which opportunistic pathogens can thrive. Common pathogens over the lifespan of patients with CF are summarized in Figure 1.6.1.

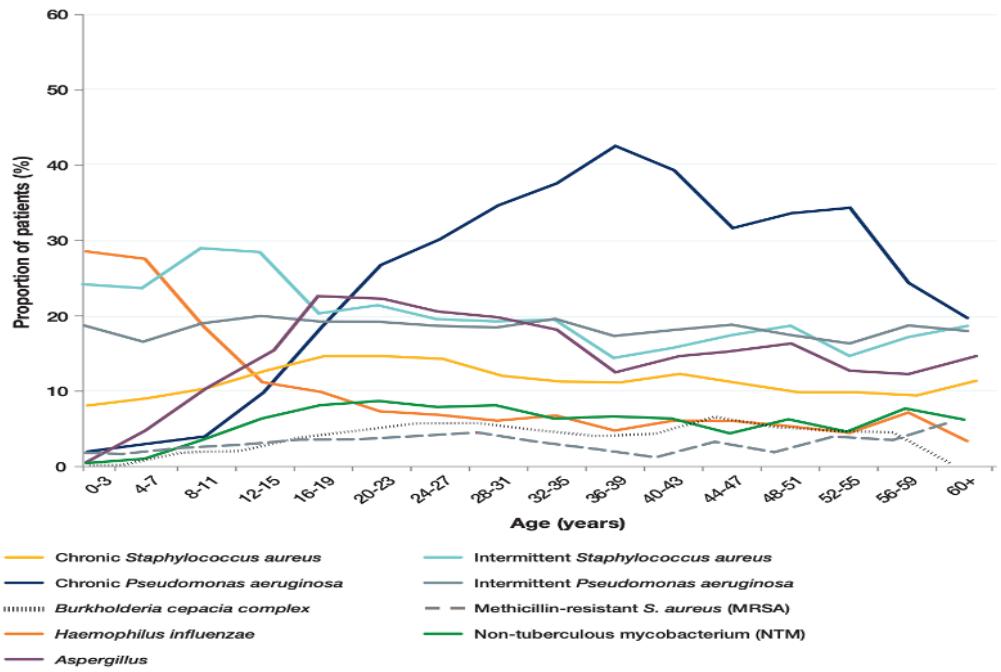


Figure 1.6.1. Prevalence of common respiratory pathogens in CF as patients age. Reproduced from (70).

The most common pathogen in CF is *Pseudomonas aeruginosa* (Pa), a ubiquitous gram-negative bacterium. It is the pathogen with the largest burden in CF, as a high percentage of patients are infected with this Gram-negative bacterium by the time they reach adulthood. Data from the 2020 UK CF registry shows that 4.7% of the paediatric population (<16 years) and 31.9% of adults ≥16 years of age are chronically infected with this organism (70), which has been declining over the past several years (2).

The natural history of infection is typically through initial acquisition of the bacteria, to intermittent infection, and eventually to chronic infection (71, 72). Though nosocomial infection with epidemic strains - often associated with increased rates of AMR - is well documented (73-75), early infection is usually with a 'wild-type' organism that has not yet adapted to the lung environment and is therefore more amenable to standard antibiotic treatment (72) and early eradication (76, 77). If, however, these measures prove unsuccessful, several factors contribute to changes that make the bacteria a chronic, host adapted pathogen.

1.7 ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

The organism has a number of inherent and acquired characteristics which can confer resistance to treatment and allow it to persist in the lungs. Intrinsic characteristics are considered those that decrease the efficacy of a given antibiotic through structural or functional properties of the bacterium on an individual level, as opposed to those acquired through adaptations to the environment and social behaviours, which will be summarised later. Mechanisms of innate resistance are summarised in Figure 1.7.1.

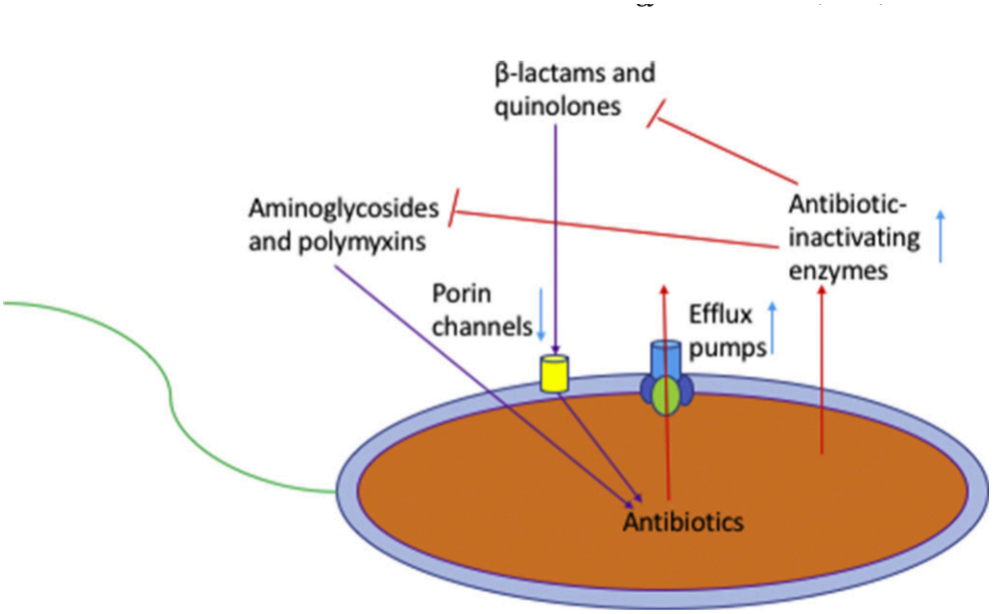


Figure 1.7.1. Innate resistance to classes of commonly used antibiotics in Pa infection. These include reduced outer membrane permeability, efflux pumps and the production of antibiotic inactivating enzymes. Reproduced from (78).

The first intrinsic characteristic in this list is the low outer membrane permeability, making it difficult for antibiotics to make it past the cell membrane and into the cell, where most bactericidal agents take effect (79). Laboratory experiments have demonstrated levels of outer membrane permeability up to 100-fold lower for Pa when compared to *E. coli*, another gram negative rod (80). Generally speaking, it is porins that form protein channels in different configurations that span the phospholipid bilayer of the bacterium's exterior to allow passage and diffusion of different compounds. Though there are many such porins, the OprF porin is known to the be the major non-specific porin in Pa (80), and experimental work has demonstrated that this particular porin is mostly present in a closed state in this bacterial species, accounting for much of the lower outer membrane permeability (81). However, low

outer membrane permeability on its own does not account for the high intrinsic antibiotic resistance of Pa (82).

The presence of bacterial efflux pumps is one such example of proteins with the ability to confer widespread resistance patterns. These are proteins consisting of cytoplasmic membrane transporters, periplasmic linker proteins and outer membrane porins that are instrumental in ejecting noxious substances from within the cell (83). Pa is known to expresses many different classes of efflux pump, each working to decrease intracellular levels of a given antibiotic class. In one class associated with resistance in Pa, the resistance-nodulation-division (RND) family (84), the cytoplasmic and periplasmic components are termed Mex and the outer membrane porin associated with it is termed Opr (83). Perhaps the best known, the MexAB-OprM is responsible for the efflux of ß-lactams and quinolones (85). Notably, the genes encoding for such efflux pumps are seen to be overexpressed in clinical Pa strains, denoting increased survival in the context of medical use of antimicrobial agents (86) and agents are currently being developed that inhibit efflux pumps as a therapeutic target (87).

Pa also has the ability to produce enzymes that degrade and/or deactivate antibiotics. Many antibiotic classes, for instance, have bonds that are susceptible to hydrolysis (88) by enzymes produced by different bacterial species (89). As with many other bacterial species, Pa has an inducible *ampC* gene, which encodes an enzyme (ß-lactamase) that bestows resistance to ß-lactam antibiotics (88). There are four classes of ß-lactamase, each employing a distinct mechanism for inhibiting certain ß-lactam antibiotics (78). It has been noted with some degree of alarm that certain clinical Pa isolates have extended spectrum ß-Lactamases (ESBLs) that confer a high degree of resistance to most penicillins and cephalosporins (90). Expression of genes encoding aminoglycoside-modifying enzymes are similarly responsible for inactivating aminoglycosides like gentamicin, amikacin and tobramycin, typically mainstays of antipseudomonal therapy in CF (91).

Within the microbiology community, great emphasis is made to the changes this organism can undergo that confer new resistance at the level of a single cell. Sequencing of the Pa genome has revealed a comparatively large amount of genetic material with close to 6,000 genes (92), many of which have been shown to both increase virulence and confer resistance

(93, 94), factors which help it thrive in the lung despite host immune defences and antimicrobial treatment. Bacteria can gain antibiotic resistance through acquisition of genes through horizontal gene transfer (78). It is these genes, encoded in the makeup of a given bacterial cell, combined with Pa's ability to acquire them over the course of its evolution, that have been the traditional focus of attention from the wider medical community, necessitating documents like the WHO's 2015 publication on a global action plan for AMR (95).

1.8 BIOFILMS IN PA INFECTION

INTRODUCTION TO BIOFILMS

Bacteria in general exhibit social behaviours and live in communities embedded in a matrix of self-secreted proteins – termed a biofilm. The main constituents of this matrix are extracellular DNA, exopolysaccharides, proteins and metabolites (96). These constituents surround the bacterial community, offering physical protection from antimicrobials, host defences and other environmental stressors. In this context, biofilms are understood to be *tolerant* of antimicrobials due to these social properties that allow communities of cells to adapt to the environment in which they live. In these communities, there is differential access to nutrients, which creates a heterogeneity of bacterial populations with different rates of metabolic activity and cell division (97). This can be contrasted to the planktonic lifestyle, in which free floating bacteria have a more or less standardized access to nutrients as well as antimicrobials in a liquid medium, which forms the basis of much AST. Though the cornerstone of antimicrobial choice in CF rests on conventional AST, this method correlates poorly with clinical outcomes (98).

It is now understood that the predominant mode of growth of bacteria outside of the laboratory is within aggregate communities in contrast to planktonic growth, which may be a transitional phase in the growth and spread of bacteria. Biofilms are a recognised hurdle to the successful treatment in infections of wounds, eye and ear infections, and in prosthetic medical devices (99). Essentially, bacteria require a substratum to bind with and this is often tissue or the surface of a foreign medical device, but in the lungs of CF patients, bacteria aggregate to themselves, forming clusters of communities suspended within the sputum matrix (100). In the CF lung, chronic infection with bacteria grown in biofilms leads to immune-complex mediated chronic inflammation, leading to lung damage and decline (101).

Once in an aggregate or attached to a surface, responding to cues from within the cell and from the environment, Pa then loses its flagella, becoming immotile, and begins secreting proteins that constitute the biofilm matrix (102).

Each stage in the life cycle of a biofilm (summarized in Figure 1.8.1), from attachment, maturation, dispersal and colonization, is a potential target for therapeutic intervention.

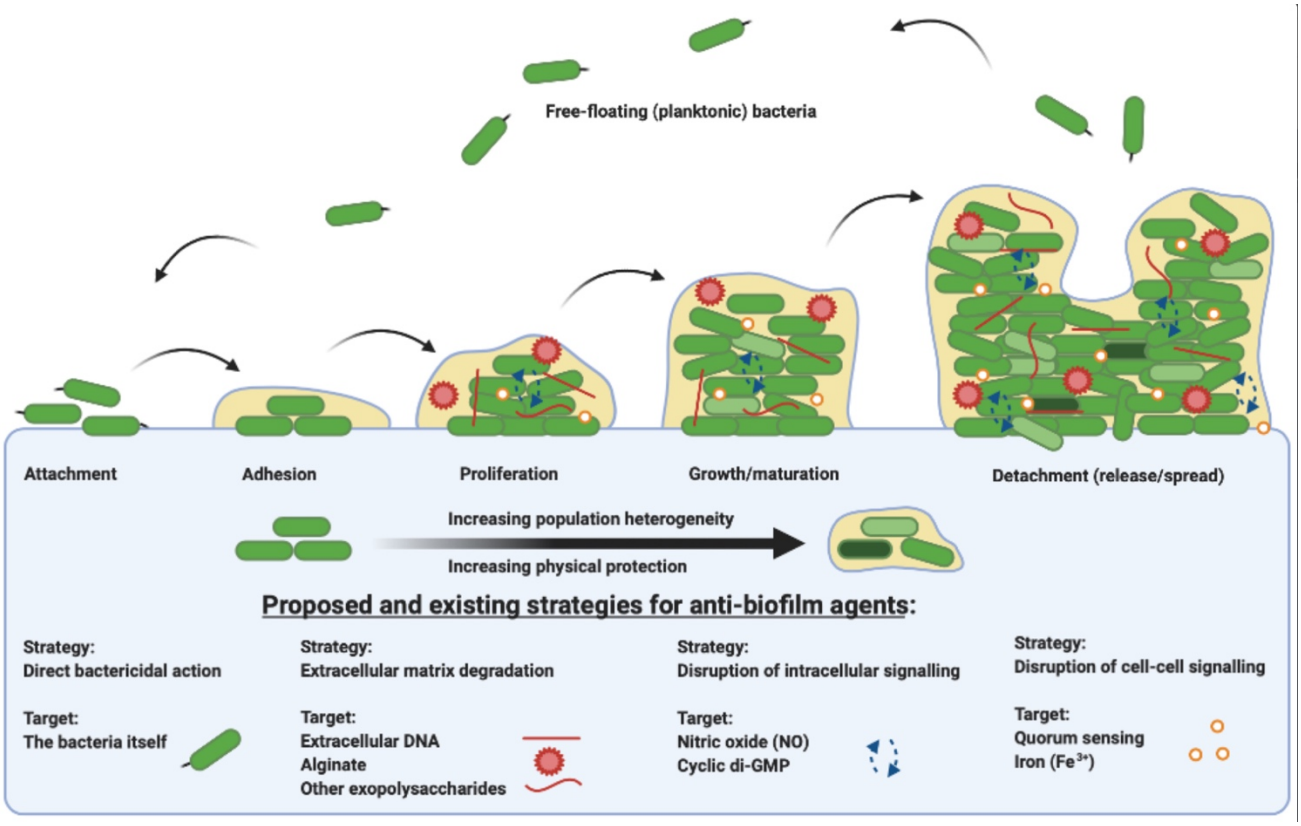


Figure 1.8.1. Diagram of the biofilm lifestyle for *Pseudomonas aeruginosa*.
This is a representation of the life cycle of bacteria, from the point where free-floating planktonic cells adhere to a substrate or surface, through proliferation and maturation/ microcolony formation, to subsequent detachment and dispersal of planktonic cells, propagating and beginning the life cycle, once more. The biofilm structure offers physical protection to bacteria from harmful substances like antibiotics, but also harbours phenotypically distinct subpopulations, some of which have survival advantages over other populations. There are many strategies for targeting biofilms, from targeting the cell, the extracellular matrix, as well as within cell and between cell signalling. Image created with Biorender.com (103).

The high degree of population diversity within a biofilm is thought to be driven in part by nutrient and substrate gradients produced by high concentrations of metabolically active cells, with differential access creating a structure in which phenotypically and genetically distinct populations of cells can survive. Different circumstances – from access of nutrients to

the presence of immune cells or antibiotics in the environment – will favour one mode of growth over another and, in turn, lead to a phenotypic change whereby a constellation of genes are differentially regulated (104). As an example, availability of nutrients has been linked with dispersal of cells from biofilm – phenotypic change from sessile, metabolically dormant cells – to those expressing flagella and can move to colonise other environments (105). Similarly, iron, magnesium and calcium are also involved in Pa biofilm maintenance (106).

The structure of the biofilm, comprised of cells embedded within a matrix of extracellular DNA as well as other exopolymers, is thought to represent a resilient reservoir of heterogeneous organisms that have the ability to disperse and cause pulmonary exacerbations in an opportunistic way, in the context of viral infection or other triggering event (107). This level of population heterogeneity within the biofilm is known to give rise to 'persister cells', a metabolically dormant phenotype that makes up a small fraction of the bacterial population, but is present in higher numbers within slow-growing biofilms. These sub populations of cells have been shown to survive high doses of bactericidal agents (108, 109).

MUCOIDY

One of the other significant phenotypic changes that this particular bacterium can undergo is proliferation of a mucoid phenotype – through copious production of an exopolysaccharide called alginate, which provides a thick and sticky barrier to antimicrobials and host defences (110). Alginate is one exopolysaccharide comprising the extracellular matrix of the biofilm, but is overexpressed in many – but not all – chronic isolates of Pa isolated from patients with CF. Such phenotypic change is a survival mechanism that can be driven by factors inherent in the CF lung (110, 111), but also by external factors aimed at its eradication, including antibiotic administration (112).

The relationship between mucoidy and the biofilm mode of growth is one that has generated much interest. Recent analysis into alginate in Pa infection has demonstrated that expression of alginate alters with oxygen concentration, with more alginate produced in zones of oxygen deprivation (113). Interestingly, a small study of 3 CF patients concluded that mucoid Pa

isolates have more conserved gene expression profiles and maintain their biofilm forming potential longitudinally when compared to their nonmucoid counterparts (114).

Though mucoidy is an adaptive survival response in Pa, it seems that activating the genes that overproduce alginate may come at the expense of disactivating others that increase antibiotic resistance. This hypothesis arose from early studies demonstrating significant differences in antibiotic sensitivity testing (AST) between mucoid and nonmucoid isolates both within the CF community (115, 116) and more broadly (117). A later study looking at matched Pa isolates from CF patients in Denmark showed that genetically similar isolates from the same individual were more antibiotic susceptible if they were mucoid (118). Thus, it would seem that mucoidy, although conferring a survival advantage in the lungs by providing a physical barrier to antimicrobials and host defences, comes at the expense of increased antibiotic susceptibility.

This concept of losing traits that are normally associated with bacterial virulence when adapting to a host environment is not new and has been seen in a variety of studies that have looked at gene expression within the context of Pa infection (119, 120). This relationship was further characterized in a study looking at a mouse model of disease, where late Pa isolates from CF patients were seen to result in less mortality than early isolates, while it was observed that all (early and late) had the ability to establish chronic infection (121). Overproduction of alginate has been seen to be instrumental in conservation of water and nutrients, allowing these to be utilized by Pa within the structure of the biofilm (122). Other work has demonstrated that alginate allows Pa to evade the host immune system (123).

In this manner, biofilms allow for bacteria to survive a harsh environment and then to disperse cells to new areas of the lung when conditions become favourable. Understanding when or how conditions in the lung favour bacterial proliferation, in turn leading to inflammation and clinical signs of a pulmonary exacerbation, is the crux of appropriate treatment in CF. It is therefore also important to understand how Pa can sense what sort of growth should be favoured from cues in the environment, which leads us into the concept of quorum sensing, which is the main extracellular signalling pathway, before I touch upon intracellular signalling.

QUORUM SENSING

Quorum sensing (QS) is a method by which bacteria can communicate with one another by the production, secretion and sensing of small molecules called autoinducers, which allows for regulation of population level phenotypic change (124). At a fundamental level, this allows for a bacterium to modulate gene expression in response to gradients of bacterial density, directing lifestyle choice appropriate to a given environment. Many cellular processes, including but not limited to the transition to a biofilm mode of growth and virulence factor expression, are known to be controlled by the QS system (125). An early study by Singh et al. (126) revealed that the lungs of CF patients are infected by bacterial biofilms by quantitatively analysing two different quorum-sensing signals.

Approximately 6% of the bacterial genome in Pa, comprising genes involved in both biofilm formation and virulence, is activated by two interconnected, N-acyl-homoserine lactone (AHL) signalling systems – the *las* and *rhl* systems (127, 128). These pathways are ubiquitous in bacterial cells and have specific roles in biofilm development and maintenance. Whereas the *las*I system has been seen to be activated in cells undergoing early biofilm formation (129), *Rhl*R/*Rhl*I is activated in the maturation stages of biofilm formation (130). These systems are hierarchically arranged, whereby the *las* system has the power to up-regulate or downregulate *rhl* (131).

The release of extracellular DNA (eDNA), an important component of the biofilm matrix, is controlled by a *third* QS pathway – the Pseudomonas quinolone system (PQS) (132). As the name suggests, this signalling pathway *is* specific to Pa and its role is still being studied, though it has been seen to regulate both the *rhl*- and *las*- QS systems (133). eDNA is known to be important in establishing cation gradients within biofilms and promoting antibiotic resistance (134), and is also a nutrient source during bacterial starvation (135). As the primary method of communication between bacteria, QS in general has been implicated as a key factor in many *intracellular* pathways, including as a negative regulator of Bis-(3'-5')-cyclic dimeric GMP (c-di-GMP), which is a known second messenger involved in the regulation of genes that are expressed in a biofilm lifestyle (136).

INTRACELLULAR SIGNALLING PATHWAYS

There are many signalling pathways with intracellular mediators that have been implicated in biofilm and virulence in Pa, but here I focus on the pathways that have yielded the most promise in pharmaceutical intervention. Nitric oxide (NO), while long recognised as vital signalling compound in eukaryote biology (137), has now been implicated one of the key intracellular signalling molecules involved in bacterial growth – namely, in the transition between planktonic and biofilm lifestyles. Highly water soluble and diffusible in biological systems, NO is ideal for intracellular communication and in this role it has been employed for therapeutic benefit in a number of conditions in humans ranging from cardiovascular disease to erectile dysfunction. Although high levels of NO can be toxic to bacteria, some bacterial species do encounter high levels of this compound in denitrification, a process whereby certain prokaryotes can use nitrate or nitrate for respiration in oxygen-limited conditions (138).

NO has been seen to increase concentration of specific phosphodiesterases (PDEs) which, in turn, reduce the levels of c-di-GMP (139-141). Increased levels of c-di-GMP promote transition from planktonic to a biofilm mode of growth, whereas reduction in levels results in biofilm dispersal (142). Cells are disseminated from Pa biofilms through the modulation of PDEs, which are activated in response to environmental cues. PDEs, in turn, catalyze the degradation of c-di-GMP, and induce expression of features that allow cell motility and dispersal, leading to physiologically distinct features that distinguish biofilm from planktonic cells, (142, 143).

1.9 THE ROLE OF ANTIBIOTICS IN PULMONARY EXACERBATION

The main weapon in our arsenal targeting Pa (and indeed other organisms that lead to increased mortality in CF), is antimicrobial treatment. This cornerstone of care in CF is widely attributed to improvements in quality of life as well as longevity (144), but rates of AMR are high (145), limiting the agents at our disposal. This is worrying because AMR has been associated with worse patient outcomes in Pa infection (146) and has been seen to increase with time using registry data from a number of different countries (147). In addition to this innate resistance, with the infective organisms living predominantly in biofilms in the environment of the human lung, Pa has adaptive behaviours that allow it to modulate gene

expression and cause phenotypic change that further limits the efficacy of our remaining antimicrobial options.

Although aggressive antimicrobial treatment has yielded improved outcomes in patients with CF, precisely what happens to bacterial populations in the context of an exacerbation remains a hot and extensively investigated issue. Sadly, it is known that for patients who experience a pulmonary exacerbation, roughly one quarter will not regain their baseline lung function, even after intravenous antibiotic treatment (148).

Many pulmonary exacerbations do have a viral aetiology, and although this had long been a recognised association, mechanistically some of this is explained by physical damage to airway epithelial cells from viral infection, which in turn makes them more adherent to bacteria and, hence, susceptible to bacterial spread to new areas of the lung (107). It was first noted that respiratory syncytial virus (RSV) shows a strong correlation with Pa acquisition from early investigation of registry data demonstrating Pa acquisition peaks during peaks of RSV infection (149, 150). Later evidence pointed towards increased adherence of Pa to airway epithelial cells infected with RSV (151) and subsequently in a mouse model of CF (152). It has also been shown that rhinovirus infection in a CF airway epithelial cell cultures promoted movement of bacteria from a biofilm to planktonic mode of growth, in turn instigating chemokine responses (153). Importantly, similar models comparing airway epithelial cell cultures from CF patients do not show an exaggerated inflammatory response with viral infection when compared to healthy controls (154), though symptomatically, those with CF are seen to suffer a longer course of illness.

Understanding how current antibiotic therapy interacts with bacterial biofilms has been instrumental in developing optimising current therapies, but also in the development of new antibiotic preparations and defining novel drug targets. Dual antibiotic administration in Pa infection is an approach currently used in the clinical treatment of infection, using the principle of targeting different populations of bacteria for optimal killing. This approach, at least *in vitro*, demonstrates superior penetration into bacterial biofilms and targeting cells with differing levels of metabolic activity (155-157) and is nicely illustrated in a Pa biofilm model in Figure 1.9.1.

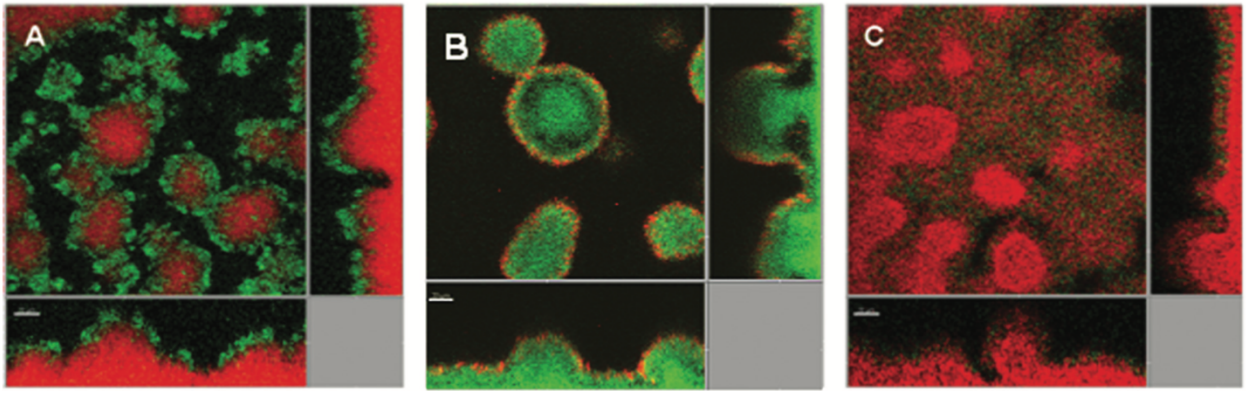


Figure 1.9.1. Fluorescence imaging of *Pseudomonas aeruginosa* biofilms.
Live cells express green fluorescence protein and appear green and dead cells stained with propidium iodide appear red. Biofilms were exposed to colistin sulphate (A), tobramycin (B), and a combination of colistin sulphate and tobramycin (C) for 24 hours. Note that colistin targets cells deeper within the biofilm, while tobramycin targets cells at the surface, while the combination is effective in diffuse killing within the biofilm structure. Reproduced from (155)

The use of liposomal preparations of antibiotics with biofilm penetrating properties is also the focus of several stand-alone reviews and is an approach that shows enormous potential (158, 159). Despite these promising developments, however, it is known that the levels of antibiotics required for eradication of biofilms, in many instances, cannot be achieved in the airway, either through intravenous or nebulized administration (160-163). Though there are many such agents at our disposal, they are insufficient, as evidenced by the high rates of bacterial colonization characteristic of CF, particularly as patients age (1).

1.10 OTHER ANTIBIOFILM AGENTS IN CLINICAL USE

Using existing antibiotics in combination with other agents with antibiofilm properties is an approach that has been used clinically for a number of years within the context of CF lung infection. The agents currently in widespread clinical use were developed without the knowledge that they affected biofilms; however, a significant component of their efficacy has since been attributed to their disruption of bacterial biofilms. They also demonstrate that there are therapies beyond standard antibiotics that could yield improved patient outcomes in CF.

Recombinant human deoxyribonuclease I (dornase alfa or DNase) cleaves eDNA, released from neutrophils as well as bacteria, that provide a structure to the biofilm. DNase therapy

has been shown in meta-analyses to be effective both at reducing the rate of lung function decline, as well as reducing the number of pulmonary exacerbations (164). DNase also disrupts pre-formed bacterial biofilms, which may account for some of its effect (165). Although the predominant mechanism has been attributed to the reduction in mucous viscosity and improved airway clearance, DNase also disperses bacteria and increases antimicrobial susceptibility in staphylococcal species as well as in mixed bacterial species models of biofilm infection (166).

One other compound currently being investigated for biofilm degrading properties is a compound called OligoG (CF-5/20). OligoG is a small (mW 2600 Da) alginate oligosaccharide derived from seaweed that has been shown to alter the viscoelastic properties of sputum in CF patients by increasing the pore size in the mucus layer, thereby increasing particle diffusion through sputum and disrupting the extracellular polymeric network of gram-negative biofilms (167, 168). *In vitro* work has shown that this compound can potentiate the activity of antimicrobials on gram-negative models of infection (*Pseudomonas, Acinetobacter, and Burkholderia* spp.) (169), as well as when combined with antifungals in fungal infection (*Aspergillus* and *Candida* spp.) (170). A dry powder formulation of OligoG was investigated in a small randomized, double-blind, placebo-controlled crossover study in CF patients with chronic Pa infection and although it demonstrated safety, there was only a non-statistically significant trend towards an increase in lung function (171). There is still active interest in this compound, with a dose finding study of OligoG underway (NCT03698448) (172).

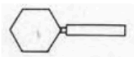
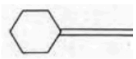
The macrolide azithromycin, although not used as a first line antibiotic against Pa infection, has also been seen to disrupt quorum sensing and block alginate production in a mouse model of infection (173). Additionally, use of this antibiotic has been seen to improve lung function in the subgroup of patients colonized with Pa (174). In a meta-analysis of trials involving patients with CF, the summary data of 4 studies demonstrated improved lung function in those treated with azithromycin, with increased benefit in the subgroup of patients colonized with Pa (174) and it is hypothesized that some of this benefit may be derived from this macrolide's biofilm impairment.

1.11 BACTERIOPHAGE: STRUCTURE AND CLASSIFICATION

Bacteriophages (or phages) are viruses that rely on bacteria to replicate and complete their life cycle. The existence of such viruses was first alluded to in English chemist Ernest Hankin's report in 1896 of the anti-cholera properties in the water of the Ganges (175, 176), and the subsequent description and discovery has been attributed to the work of Canadian Felix d'Herelle in 1917, who described unexplained cell death in Shigella cultures from soldiers in the First World War (177). Phages specifically bind to and infect specific bacterial cells, using surface proteins to recognise specific bacterial hosts. They exist everywhere in the biosphere where bacteria exist and are the most numerous entities on the planet; more numerous than other viruses by several orders of magnitude (178). Bacteriophage have been present for millennia and have been involved in exerting selective pressures on host communities, driving bacterial evolution (179). There is a correspondingly large amount of genotypic diversity in bacteriophage, which is reflected in both their structure and function.

There are many ways of classifying viruses, both by way of looking at the contents of the viral genome (DNA vs RNA, single stranded vs double stranded), and by way of looking at structure (180). Results from electron microscopy (EM) have largely informed the structural classification of bacteriophages. Current classification of bacteriophage by the International Committee on the Taxonomy of Viruses (181) recognises three groups of tailed, and other types of filamentous and isometric phages. This taxonomy, though still evolving, has been largely attributed to a 1967 paper by Bradley (182) and a further evolution of this original classification can be seen in Table 1.11.1. Approximately 96% of phages are tailed (183), and constitute the order of Caudovirales, under which the three families (Myoviridae, Siphoviridae and Podoviridae) fall.

Table 1.11.1. Overview of bacteriophage structure, family and viral genome. Reproduced from (184).

Shape	Order or family	Nucleic acid, particulars, size
	Caudovirales	dsDNA (L), no envelope
	Myoviridae	Tail contractile
	Siphoviridae	Tail long, noncontractile
	Podoviridae	Tail short
	Microviridae	ssDNA (C), 27 nm, 12 knoblike capsomers
	Corticoviridae	dsDNA (C), complex capsid, lipids, 63 nm
	Tectiviridae	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm
	Leviviridae	ssRNA (L), 23 nm, like poliovirus
	Cystoviridae	dsRNA (L), segmented, lipidic envelope, 70–80 nm
	Inoviridae	ssDNA (C), filaments or rods, 85–1950 x 7 nm
	Plasmaviridae	dsDNA (C), lipidic envelope, no capsid, 80 nm

As can be seen from Figure 1.11.1, bacteriophage have both a high degree of variability in terms of structure when viewed by EM, but also in the contents of their genome, which can be double or single-stranded DNA or RNA. All bacteriophage in the order Caudovirales (the three families Myoviridae, Syphoviridae and Podoviridae) contain double stranded DNA.

These families and viral genomes of the different orders of phage are summarised in Table 1.11.2.

Table 1.11.2. Bacteriophage families and corresponding genome characteristics. Reproduced from (183).

Shape	Family	Example	Characteristics[b]
Tailed	Myoviridae	T4	dsDNA, L, tail contractile
	Siphoviridae	λ	dsDNA, L, tail long and noncontractile
	Podoviridae	T7	dsDNA, L, tail short
Polyhedral	Microviridae	ϕX174	ssDNA, C, 12 capsomers, 30 nm
	Corticoviridae	PM2	dsDNA, C, complex capsid, lipids, 60 nm
	Tectiviridae	PRD1	dsDNA, L, inner lipid vesicle, pseudo-tail, 60 nm
	Leviviridae	MS2	ssRNA, L, like poliovirus, 25 nm
	Cystoviridae	ϕ6	dsRNA, L, segmented, envelope, 70 nm
Filamentous	Inoviridae	fd	ssDNA, C, long filaments or short rods 90–1300 nm in length
Pleomorphic	Plasmaviridae	L2	dsDNA, C, envelope, no capsid, ~ 90 nm

Though there is, at present, a developing emphasis on reporting bacteriophage genomes on public databases like GenBank (185), there has been a large amount of information learned from electron microscopy and observation of function and behaviour that has informed decisions about phage therapy and those which have the potential to be harmful. Developments in the area of phage genome research show great promise in understanding these viruses more comprehensively, in a way that may inform the development of therapies involving phage.

1.12 BACTERIOPHAGE: LIFE CYCLE

The first part of the bacteriophage life cycle involves recognition and binding to a receptor expressed on the surface of a host bacterial cell. Viruses in general can use different types of molecules (from glycolipids, carbohydrates and other proteins) to attach to and infect their host (186). These receptor molecules are not simply required for host recognition and binding, but are also integral in the transfer of the viral genome across the cellular membrane

and into the host cell (187). It is widely recognised that bacteriophage have the ability to recognise a variety of receptors, increasing the likelihood of encountering a viable host (188). They also possess the ability to undergo evolution in a dynamic process between virus and bacteria, as the loss and/or acquisition of different host surface proteins as an adaptive mechanism to evade infection necessitates phages to follow suit (189).

The next part of the phage life cycle depends on the type of virus. Broadly speaking, there are two different groups of bacteriophages (outside the traditional classification discussed earlier), both with different roles to play in the lives of bacteria. The lysogenic or temperate phages incorporate themselves into the host genome as prophages while lytic phages are obligate predators and ultimately result in cell death.

Lytic phage of gram-negative organisms (of the order Caudovirales) contain double-stranded DNA, encoding for both the structural proteins required for replication within the cell, but also for at least two types of membrane degrading proteins – holins and lysins (190). Once the phage DNA enters the host cell, it first starts the process of replication using host ribosomes, leading to the production and assembly of virions, whilst simultaneously the phage lysins and holins accumulate within the cell. This infection with bacteriophage DNA also results in the cessation of bacterial DNA, RNA and protein synthesis (191). The activation of the lysins and holins is triggered by an as yet poorly understood mechanism, initiating a timed degradation of the three outer layers of the cell membrane (inner membrane, peptidoglycan and outer membrane) (192). The assembly of viral material within capsids and the accumulation of membrane disrupting proteins is a temporally regulated process whereby hundreds of phage virions have assembled by the time the cell is lysed, leading to the release of viral progeny and initiating the life cycle, once more (192). The ‘burst size’, or amount of viral progeny generated upon cell lysis, has been noted to differ vastly based on the growth media and physiological state of the host bacterium (193). This life cycle of lytic bacteriophage is summarised in Figure 1.12.1.

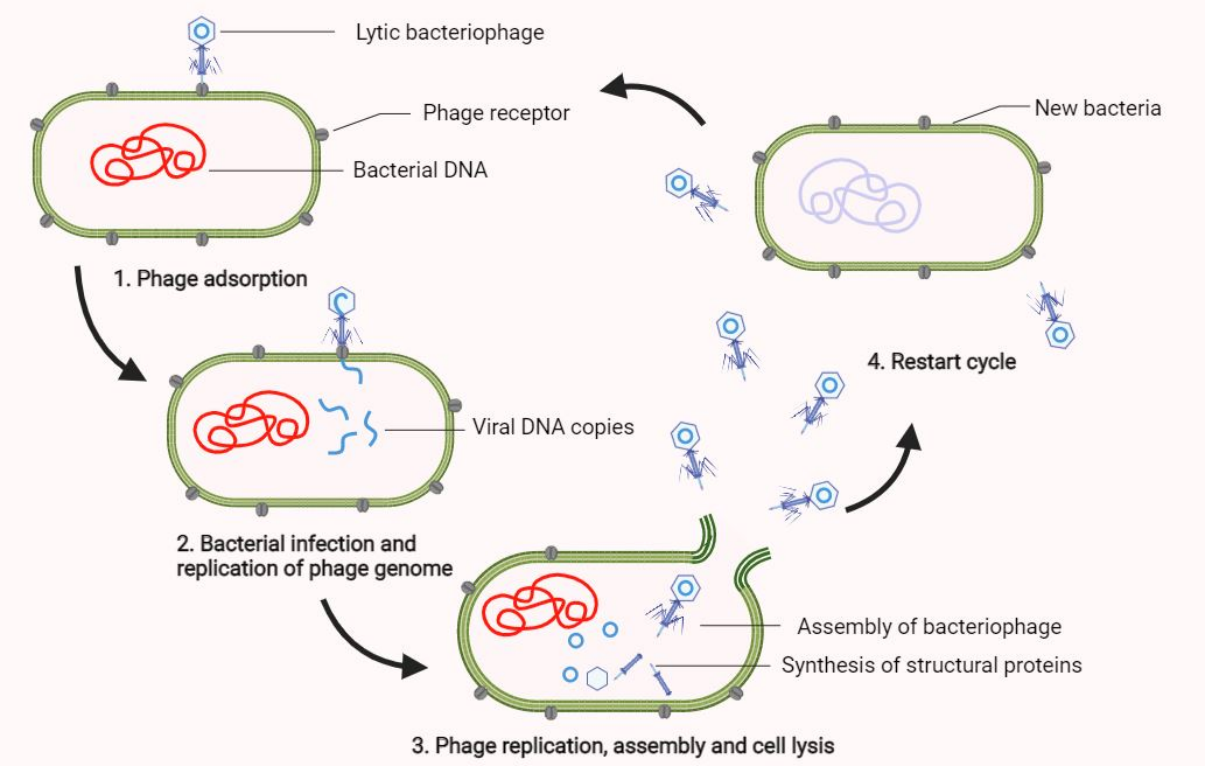


Figure 1.12.1. The life cycle of lytic bacteriophage.
(1) The phage adsorbs to a surface protein on the bacterial cell. (2) Nucleic acid injection. The phage infects the host cell with viral phage DNA, which uses the host cellular machinery to make copies of itself. (3) The phage replicates, using the bacterial cellular machinery to create structural proteins that are used in the assembly of new phage progeny (virions). At the same time, there are productions of lytic proteins (termed endolysins) that accumulate within the cell, degrading the bacterial cell wall, eventually lysing the cell and releasing viral progeny. (4) The phages are released from the lysed bacteria to find another host and repeat the life cycle. Created in BioRender.com.

Though lysogenic phages are not the primary focus of the therapy being developed in this thesis, they should be mentioned to provide an insight into the complexity of the world of viral infection in bacteria. Once the genetic material of lysogenic phages have been injected into the cell, they incorporate themselves into the host genome and exist as prophages. Unlike lytic phages, infection of a bacterial host with lysogenic phage DNA does not stop synthesis of bacterial proteins or cellular function. The step of bacterial lysis mentioned above will only be triggered if there are pressures exerted on the cell that cause sufficient strain on the bacterial host, in which case the phage DNA is excised from the bacterial genome and undergoes similar steps to those mentioned previously for lytic phage. In integrating reversibly into the host genome, a long-term relationship of genetic symbiosis is formed, one

in which the survival of the host bacterial population could be viewed as being advantageous to this particular type of phage.

Lysogenic phages have long been seen to encode for bacterial virulence factors and this fascinating subject is the focus of several early reviews (194, 195). It is now widely understood that phage drive the horizontal transfer of virulence genes between bacteria via transduction; in a separate process from conjugation, driven by other mobile genetic elements, like plasmids. Though they lacked a mechanistic explanation for this phenomenon, Frobisher and Brown first described this process of phage driven conjugation in 1927 in an experiment where non-toxic Streptococcal species that had been exposed to the supernatants of more pathogenic bacteria were able to acquire the ability to produce scarlatinal toxin (196). A later description of the same phenomenon was the discovery that the toxin produced by *Vibrio cholera* is actually encoded by prophage DNA, without which the bacteria is actually rendered non-pathogenic to its host (197).

Contrasting these cautionary tales of lysogenic phage, they have also been proposed as therapeutic agents in certain instances, including in the battle against Pa biofilms. Phage ΦPan70, thought to be an obligate lytic phage, was used in a mouse burn model of Pa infection and demonstrated survival of between 80-100% of treated compared to 0% of non-treated mice. However, on DNA analysis, this phage was discovered to share most of its genome with another known temperate phage (198). Thus, our understanding of bacteriophage is far from complete and phage-bacterial interactions cannot simply be predicted by looking at type of phage and type of host. There must be rigorous scientific foundations for setting up both *in vitro* and *in vivo* work with a view to bringing this therapy to clinical trial.

Within the context of CF and, more specifically, Pa lung infection, phages are also recognised as one of the driving forces behind horizontal gene transfer and the acquisition of genes conferring bacterial resistance (199). The presence of prophage DNA in many clinical Pa strains has been well documented and indicate a complex interplay between virus and bacteria in the CF lung (199-201). A simple example of how bacteria derive survival benefit from this type of infection is with filamentous phages. Filamentous phage (of the family Inoviridae) are elongated nucleoprotein filaments with a unique structure and function (202).

This type of lysogenic phage has been shown to play a key role in the assembly and maintenance of bacterial biofilms, including in the context of Pa infection (200, 203, 204).

1.13 APPLICATIONS FOR BACTERIOPHAGE THERAPY

In response to the threat of AMR, there has been a resurgence of interest in phage and other therapies that are being developed to tackle infections refractory to antibiotic treatment for Pa (205), and with other organisms in the context of CF (206). d'Herelle was the first to attempt to exploit the bactericidal potential of lytic phage in dysentery amongst soldiers in the First World War with an oral phage preparation (177). This concept was exported successfully to Russia and other parts of Eastern Europe, where it persists as accepted antibacterial therapy to this day (207). In fact, there was a huge amount of international support from the scientific community for phage therapy (208), with reports of phage being given in cases of dysentery globally (209-211). Although it is estimated that there were over 800 papers published in scientific journals about phage therapy in humans between 1917 and 1948 (208), it was largely abandoned by Western Europe and North America with Fleming's discovery of penicillin, thought to be the cure-all for bacterial infection.

One other reason for phage having been abandoned by the scientific community was a lack of rigorous scientific evidence supporting it. It is believed, for instance, that an inability to distinguish lysogenic and lytic phages in early use contributed to variability of results and failure in early clinical trials (212). Additional factors including but not limited to a lack of understanding of bacteriophage biology and structure, probably led many to focus their research interests elsewhere. Many eminent scientists at the time were able to point out alack of rigor in many of the early experiments, which rendered any results or positive findings subject to speculation (213-215). But the scepticism garnered by these critiques, even if true, were insufficient justification in turning away from this therapy, especially as it was already known that penicillin was not the panacea that had been promised; both because it is only weakly effective in Gram-negative infection and also due to the development of penicillin resistance (208).

Now, over a century after the discovery of phage, the scientific community is revisiting viruses as a potential treatment for bacterial infection, largely due to the impending threat of antibiotic resistance. When considering any compound for therapeutic use, in addition to the

intended direct effects of the compound in question, an ideal drug should be specific, stable, and with few side effects. Looking at these criteria specifically, here I examine the proposed advantages of phage and barriers to its widespread use.

Proposed Advantages of Phage Therapy

Bacteriophage meets several of the essential criteria for a successful antimicrobial; indeed, in certain respects, it has advantages above and beyond current antibiotic therapy. Firstly, phages are specific (to the extent that this has the potential to also be seen as a drawback and will be expanded upon later). They are specific not only to bacterial species but, furthermore, are only able to target bacteria within a species that express the surface proteins to which they have the ability to adsorb. As an example, bacteriophage T4 which infects *E. coli* and is one of the most widely studied lytic phages, binds *only* to the glucosyl-α-1,3 terminus of rough lipopolysaccharide (LPS) on the bacterial cell surface of some strains of this bacterium (191). This level of specificity has the potential of leaving the rest of the host microbial community intact, in contrast to antibiotics, which usually target broad groups of bacteria (eg. Gram-negative +/- Gram-positive organisms). In the context of CF, antibiotic killing of non-pathogenic bacteria is a topic under intense debate, as it is known that one of the hallmarks of disease progression is a loss of microbial diversity (216-218).

Phage therapy has additional pharmacokinetic properties that distinguish it from other medications that could be advantageous. Unlike antibiotics, which reach a peak level (differing in serum vs gut vs lungs, depending on route of administration), and then decrease over time as they are filtered by the kidneys or metabolised by liver enzymes, bacteriophages will multiply in the presence of a bacterial target. This process of amplification has the theoretical benefit of increasing concentrations of the therapy locally where it is most needed (219). Bacteriophage therapy has also not been associated with many side effects in both Western experiments where it has used in laboratory animals and in small trials involving human subjects (220), but also in Georgia and places where it is a readily available and accepted therapy (207, 221). This is thought to be at least partly attributed to the fact that phages are ubiquitous in the environment, leading the immune system to be accustomed to encountering phage.

PROPOSED SHORTCOMINGS OF PHAGE THERAPY

There are many obstacles to the widespread development of clinical trials involving phage and the potential success of this therapy. In addition to some innate properties of bacteriophage that will be discussed, several of the aforementioned points can also be disadvantageous, depending on the clinical setting in question. The specificity for bacteriophage for only certain types of cell receptors can render the window of viable host cells extremely narrow (222). Additionally, there have been many reports of rapid evolution of phage resistance in bacterial strains when exposed to this therapy *in vitro* (222, 223).

The pharmacokinetics and pharmacodynamic properties of bacteriophage, while also hypothesized to allow for increase in titres at areas where it is most needed, are also the subject of some concern for the development of this therapy. Although phage therapy has never been associated with life-threatening immune reactions like anaphylaxis (220), studies have shown that antibodies against phage can accumulate in serum from subjects exposed to this therapy (224, 225) and it is uncertain what impact this will have on efficacy of treatment. Semler et al. (226) devised experiments in mice that demonstrated efficacy of bacteriophages against *Burkholderia* lung infection when administered via inhalation, but not when administered by intraperitoneal injection. Though the underlying reason for this finding is not known, one hypothesis is the exposure to phage antibodies rendering the therapy inactive. Route of administration is therefore a topic of paramount importance in the development of effective phage therapy.

The storage and stability of bacteriophage is yet another topic that has garnered some attention. It was as early as 1959 when the hydrodynamic shear forces of passing through the head of a hypodermic needle were observed to disrupt the integrity of DNA from T2 and T4 bacteriophages (227). Currently, phage products in development have a shelf-life of roughly a year before the phage titre meaningfully decreases when in solution (228). This easy degradation of phage is something that needs to be considered in both manufacture and storage, but also in the mode of delivery to ensure that the virus is delivered intact to the host bacteria. In addition, though few side effects of bacteriophage therapy have been seen, there are concerns that endotoxins formed during the bacteriophage production process might accumulate and these are further factors that need to be taken into account as this therapy approaches clinical trial (229, 230).

STRATEGIES TO ENHANCE THE EFFICACY OF PHAGE

In order to overcome the natural shortcomings of phage therapy, there are different strategies that have been employed to maximise the efficacy of viral killing. One promising approach that will be explored extensively in this thesis is the use of phage alongside standard antibiotic therapy. This approach was used as early as 1946 with staphylococcal species resistant to penicillin rendered sensitive with the co-administration of phage (231). As bacteriophage therapy within the context of CF lung disease is unlikely to be trialled as a stand-alone therapy (owing largely to the increases in life expectancy that has come from aggressive antibiotic therapies), I have focused on the use of phage as an adjunctive therapy in both biofilm and planktonic models of infection and this will be further explored in the relevant chapters.

Two other widely practiced strategies are the genetic engineering of phages and the administration of multiple phages in 'cocktails'. The topic of genetically engineering phages is one that has been the focus of several well written reviews that explore this approach mechanistically in good detail (232, 233). The reasons for genetically engineering phage seek to address the shortcomings mentioned earlier – from broadening the range of potential hosts, to enhancing lytic activity, to encoding lytic proteins that enhance their activity (232). In an entirely different approach, the use of engineered temperate phages has been successfully used *in vitro* to encode antibiotic susceptibility into *E. coli* strains that were then successfully eradicated by antibiotic treatment (234). The engineering of temperate phages has also been suggested to broaden the availability of acceptable therapeutic agents by rendering them obligately lytic (235).

The latter strategy of making a mixture of different phages seeks to address the reality of the evolution of bacterial resistance to one phage (through, for example, loss of a phage receptor). In this instance, careful selection of other phages in a given cocktail should ensure that the mixture retains the ability to adsorb to target host cells and continue lysing the pathogenic bacteria. To demonstrate proof of principle, one study looked at planktonic and biofilm grown Pa with two different lytic phages, MAG1 and MAG4, and saw that when used alone, there was propagation and lysis in 50% of the strains tested, whereas combining both in a cocktail resulted in 72.9% coverage (236). This approach to therapy has been gaining traction in the West, where widespread and streamlined production of fixed bacteriophage

cocktails targeting one bacterial species is being developed by the pharmaceutical industry in the hopes of marketing a patented product (237, 238).

There are anecdotal reports of clinical successes within clinical contexts relevant to this thesis that have employed the strategies mentioned here. For Pa infection, a randomized, double-blinded, placebo-controlled trial of a topical phage preparation in patients with otitis media refractory to antibiotic treatment demonstrated both safety and efficacy (239). *In vitro* work has similarly demonstrated the efficacy of bacteriophages in reducing the Pa colony count in CF sputum samples (240). In Pa lung infection models in animals, the administration of lytic phage preparations lowered both bacterial load and inflammatory markers in the airway (241, 242). In 2019, a cocktail of genome engineered bacteriophages (to remove repressor genes), delivered intravenously and topically to a 15 year old CF patient with disseminated *Mycobacterium abscessus* infection, was successfully employed on a compassionate basis (206). Bacteriophage cocktails have also been used compassionately in a small number of other CF patients with advanced lung disease to good clinical safety and effect. One small study looked at 3 patients, 2 of whom were treated with anti-Pa phage and one with phage against *Burkholderia dolorosa*; the two treated with Pa infection had good clinical outcomes, while the patient with *Burkholderia* passed away (243). Despite the promise from these studies, there are still no published clinical trials in humans demonstrating the efficacy of this therapy within the context of CF lung infection.

2 CHAPTER 2: METHODS USED IN EXPERIMENTS

2.1 ETHICS

Ethical approval was not required for experimental techniques performed on subcultures of isolates provided by the clinical laboratory for research purposes. Some of these analyses included the use of clinical information; all subjects had consented to data entry into the CF patient registry. The Strategic Research Centre received approval from the Registry Steering Committee for analysis of these data for research purposes (Huntingdon Research Ethics Committee (07/Q0104/2).

2.2 STORAGE AND CULTURE OF *PSEUDOMONAS AERUGINOSA* ISOLATES

Previous members of our laboratory designed a large database and repository for the storage and selection of clinical isolates from patients at the Royal Brompton Hospital (RBH). Prior to the inception of this research project, the database was designed using Microsoft Access and continues growing to this day. This database includes details of CF patients receiving care at RBH as well as corresponding microbiological data, which can (but does not always) include antibiotic sensitivities, Variable Number Tandem Repeat (VNTR) analysis, and details of other co-colonising organisms (bacterial and fungal).

Clinical Pa strains were provided by the RBH microbiology laboratory and form a large part of this database and repository. All isolates had been identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis in the microbiology department at the hospital as being Pa growth. When MALDI-TOF analysis yielded an equivocal result, isolates were grown on cetrimide agar at 43° C to determine whether the growth was, in fact, Pa. Samples from CF patients were taken directly from sweeps of bacterial colonies grown on agar with a plastic inoculation loop. Strains were stored on glass Microbank™ beads in a -80° C freezer.

When a particular isolate was chosen for use in an experiment through the database (see later chapters for selection criteria), the corresponding vial was briefly removed from the

freezer and placed on dry ice, a bead was selected with a sterile 22 G needle and colonies were seeded on nutrient agar for overnight growth in the incubator at 37° C.

Many of the isolates chosen for use in these experiments had been selected at random by a previous Masters student in the laboratory, Yaqi Betty Hu, and used for continuity.

2.3 BACTERIOPHAGE COCKTAIL, SERIAL DILUTIONS AND STORAGE

Our long time commercial collaborator, Ampliphi Biosciences Corporation, provided our team with the bacteriophage cocktail used in all of the experiments. Between the years 2006 and 2008, they had identified 52 anti Pa phage by methodology described previously (212). They then performed plaque assays on clinical strains from a number of different hospital sources to assess lytic activity and narrowed down selection to ten phage, which were found to lyse 96% (77/80) of the strains tested. From these ten phage, further work selected 4 (Pa193, Pa204, Pa222, Pa223) which, when used in combination, demonstrated lysis in plaque assay on 78% of clinical strains from CF patients at RBH.

Each component of this 4 phage cocktail was provided at a concentration of $1x10^9$ PFU/ml. When mixed together in equal volumes, each individual phage was therefore at a concentration of $2.5x10^8$ PFU/ml. Previous work by Ampliphi Biosciences Corporation demonstrated a non-statistically significant 0.8 Log reduction in viable phage at 4° C over the course of 12 months. A new batch of this phage cocktail was therefore used every 12 months through the course of these experiments.

The phage cocktail was prepared and stored in salt magnesium buffer (SMB). For individual experiments, the 4 bacteriophage mix was serially diluted in PBS by a factor of 10 consecutively six times (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) for use in the plaque assay and for 96-well plate experiments, stored in sterile Eppendorf tubes.

2.4 PREPARATION OF ANTIBIOTIC SOLUTIONS

Antibiotics used for planktonic and biofilm experiments were ceftazidime and tobramycin, selected due to their differing mechanisms of action (ß-lactams act on the cell wall while aminoglycosides work at the level of the bacterial ribosomal 30S subunit) as well as their use

in clinical practice. Both were prepared from dry powder (ThermoFisher Scientific) and dissolved into sterile water to make stock solutions to 10 g/L. Stock solutions were further diluted with sterile water to reach a series of concentrations to reach the specified final concentrations required depending on treatment protocol. Antibiotic solutions were labelled and kept in the -20°C freezer for use in further experiments. Solutions were thawed for use in experiments a maximum of three times before new solutions were made based on the size of aliquots that had been prepared.

2.5 STANDARD PLAQUE ASSAY

Laboratory strain PA01 was used as a control for plaque assay experiments as the phage preparation demonstrated consistent lytic activity to a high (10^{-6}) dilution. An overnight culture of selected Pa isolates was grown in nutrient broth following overnight culture on nutrient agar. Overnight culture was diluted in fresh nutrient broth to an optical density (OD) of 0.1 (WPA Biowave CO8000 Cell Density Meter) and 100 µL of this bacterial suspension was pipetted into a 5mL sterile plastic tube. 3mL of semi-solid nutrient agar (13 g/L Oxoid Nutrient Broth + 4 g/L Oxoid Agar) was added to each 5mL tube with bacterial suspension, gently inverted to mix, poured over sterile nutrient agar plates (13 g/L Oxoid Nutrient Broth + 15 g/L Oxoid Agar) and allowed to dry in the safety cabinet for 20-30 minutes.

5µL aliquots of each phage cocktail dilution (neat phage to 10^{-6} dilution) were spotted onto the surface of the Pa inoculated plates in rows with multichannel pipettes. Each assay was performed in triplicate on a single agar plate using sterile pipette tips. 5 µL of SMB was used as a negative control for each technical replicate. Plates were then kept in the safety cabinet until the spots had dried before being inverted and incubated overnight (16-24 hours) at 37°C.

Agar plates were examined over a dark background. If there were no discernible plaques at any dilution, the phage cocktail was said to have no lytic effect on the Pa strain being examined. If, however, plaques were observed, the maximum dilution at which they were seen was used to calculate the phage titre in plaque forming units/ml (PFU/mL) using the following calculation:

((PFU in spot)/(spot volume in mL))* 1/(dilution factor of tube from which spot was taken)

If lytic activity was seen in the negative control spot, then results for that replicate were discounted on the basis of contamination. Visual of the output from this assay is shown in Figure 2.5.1.

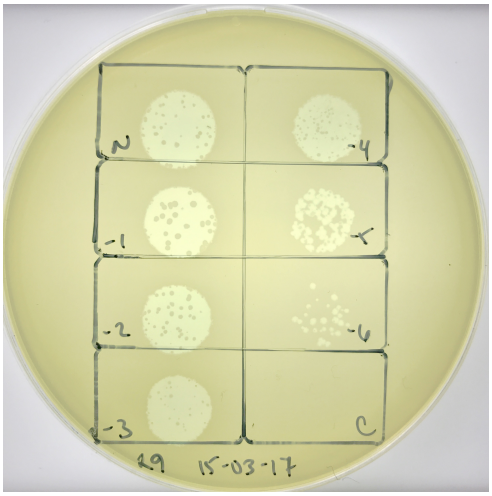


Figure 2.5.1. Visual outcome for the plaque assay.
Plaque assay outcome for strain PA01, which reliably generates discrete plaques up to 10^{-6} serial dilutions. This was performed using 10 µL spots of the bacteriophage cocktail at ten-fold serial dilutions on a lawn of Pa grown on a Nutrient agar plate. Agar plates were then examined over a dark background, but for the purposes of demonstration by photo, this is demonstrated over a light background.

If there were no discernible plaques at any dilution, the phage cocktail was said to have no lytic effect on the Pa strain being examined. If lytic activity was seen in the negative control spot, then results for that replicate were discounted due to contamination. It should be noted that output of this assay is expressed in the apparent phage titre in PFU/ml, as described above, and output is on a logarithmic scale whereby increasing PFU/ml indicates more active viral particles per unit volume and therefore higher levels of sensitivity.

In many instances, individual plaques were not easily distinguished, though there was plaque formation. Thus, a simplification of the above formula was proposed, in which PFU in spot was either 0 or 1. This modification was made for several reasons: it standardised an approach to all bacteria and removed the subjectivity in counting on the majority of occasions where it was difficult to define a number of well-defined plaques, but also accounting for the fact that the logarithmic scale used in the calculation was most heavily influenced by the dilution factor from which the spot was taken. Thus, for most strains that were being analysed, the number

of individual plaques seen (usually from 1-6), at the greatest dilution did not make a sizable difference in terms of PFU/ml on the logarithmic scale being used.

2.6 PLANKTONIC GROWTH ASSAYS

Sterile plastic 96 well plates (ThermoFisher Scientific) were used for both planktonic and biofilm assays. Overnight cultures of Pa strains were diluted in Mueller Hinton broth (MHB) to an OD of 0.05 with a portable spectrophotometer as described previously.

For planktonic culture, 180 μL of overnight culture (diluted to OD 0.05) was pipetted into each well of a 96 well plate. 20 μl of SMB was then pipetted into negative control wells, 20 μl of each dilution of phage, antibiotic preparation, or combination was added to individual wells according to individual experimental protocol so that the final volume in each well was 200 μl. Plates were then sealed with a breathable, oxygen permeable seal and placed overnight (16-20 hours) in the incubator at 37°C at 200 rpm.

Outcomes for planktonic growth were optical density and fluorescence reading with resazurin to approximate bacterial activity. Both were read with the plate reader (BMG Labtech FLUOstar Omega). Negative controls were wells with no bacteriophage or antibiotics added.

For the first reading, optical density was measured spectophotometrically as an approximation of cell growth as described elsewhere (244, 245). A diagram showing the basic principle of spectrophotometric measurement is described in Figure 2.5.1.

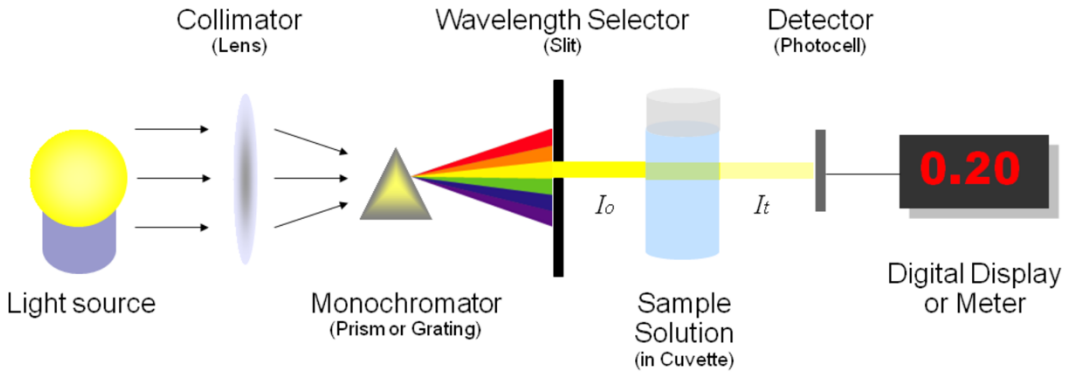


Figure 2.6.1. Basic spectrophotometry (246).

The multi plate reader was calibrated to OD_{600}. It should be noted that the linear range of absorption to colony forming units (CFU in bacteria per ml) has been described for

Pseudomonas aeruginosa by other colleagues in my laboratory and elsewhere in scientific literature (245).

2.7 STATIC BIOFILM ASSAY

The static biofilm assay in microtiter plates has been used in a number of permutations for some years due the fact that many experimental protocols can be tested at once, and for the fact that it is high throughput (247-249). Biofilms were assessed for both cell viability and total biomass to give a robust measurement of adherent bacterial colonies. Outcomes for biofilm growth were measured with resazurin for an approximation of cell viability and with crystal violet for an approximation of biomass.

To grow biofilms, 200 μL of overnight culture (diluted to OD 0.05 in Mueller Hinton broth – Sigma Aldrich) was pipetted into each well of a 96 well plate. Plates were incubated at 37°C for 24 hours static to allow cells to adhere to the sides and bottom of the wells. With a multi-channel pipette, wells were emptied and washed with a phosphate buffered saline (PBS) solution before 180 L of Mueller Hinton broth was added to all wells. 20 μl of SMB was then pipetted into negative control wells, 20 μl of each dilution of phage, antibiotic preparation, or combination was added to individual wells according to individual experimental protocol so that the final volume in each well was 200 μl. Plates were then sealed with a breathable, oxygen permeable seal and placed overnight (16-20 hours) in the incubator at 37°C at 200 rpm.

THE RESAZURIN ASSAY

Resazurin salt (Alamar Blue, IUPAC name: 7-hydroxy-10-oxiphenoxazin-10-ium-3-one) is a chemical that has been widely used to approximate cell viability (250). Resazurin is a non-fluorescent blue dye that is reduced to a pink, highly fluorescent chemical called resorufin by metabolically active cells. A 1% w/v stock solution of resazurin was prepared by dissolving powder in 100 ml of distilled water. From the stock solution, 50 ml of a 0.02% w/v working solution of resazurin was made and stored in the fridge at 4° C in a sterile tube. The volume of resazurin added was 10% of the total volume of each well (e.g. 20 μL of 0.02% w/v resazurin working solution in a total well volume of 200 μL).

For evaluating biofilm cell viability, all wells were emptied of free-floating cells by microchannel pipette and washed with PBS before introduction of 180 µL of MHB, then 20 µL of resazurin 0.02 % w/v working solution according to the manufacturer's instructions (ThermoFischer Scientific). Plates were covered with aluminium foil and incubated at 37°C for 3-4 hours before being transferred to the plate reader for analysis. Plates were analysed using a plate reader using emission and excitation wavelengths of 540 and 590 nm, respectively ($FI_{540/590}$). The gain was set to 95% (251) based on a positive control of resorufin that had been prepared beforehand.

The Crystal Violet assay

Crystal violet is a dye that stains cells and extracellular proteins, and after a simple washing step, remains on all matter adherent to the plastic wells (biofilms) before solubilisation for spectrophotometric quantification. This is a common high-throughput method for biomass quantification of adherent biofilms (247, 248). For this assay, a 0.1% crystal violet solution was created with sterile water from a liquid 1% aqueous stock (Sigma Aldrich). Plates containing viable cells and resazurin were emptied in waste containers for safe disposal. They were then washed twice by submersion and inversion in water before tapping out the remaining liquid content onto paper towels. 180 µL of 0.1% crystal violet solution was added to each well of the plate and gently rocked for 15 minutes at room temperature. Emptying and washing steps were then repeated and plates allowed to dry in the microbiological safety cabinet. 200 µL of 95% ethanol was added to each well to solubilise the biofilms stained with crystal violet and then left alone for 30 minutes before absorption readings with the spectrophotometer (OD_{550}).

Outcomes for both assays are depicted visually in Figure 2.6.1.

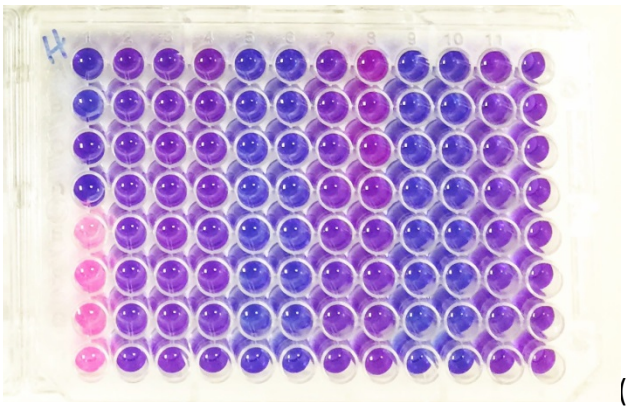
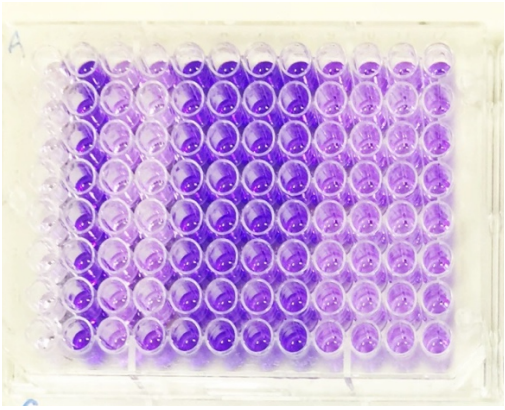
(a)  (b) 

Figure 2.7.1. Visual outcomes of both biofilm assays in 96 well plates.
On the left (a) the dark blue resazurin is metabolised into the pink resorufin by metabolically active cells at an excitation wavelength of 540nm and an emission wavelength of 590nm; and on the right (b) the stained biomass of the crystal violet assay solubilised in ethanol is analysed spectrophotometrically at 550nm.

These assays were intended to quantify different aspects of the biofilm: cell viability and biofilm biomass, respectively. The assays are compared in some more depth in Section 3.4.

2.8 DISC DIFFUSION ASSAY

Antibiotic sensitivity testing was performed by disc diffusion assay according to EUCAST protocols (252). Disc diffusion assay is one of the most widely used assays for performing antibiotic sensitivity testing in hospitals.

In summary, isolates were grown overnight on nutrient agar. An inoculum suspension was then prepared by taking a sweep of bacterial colonies with a sterile loop and suspending in a solution of 0.85% sodium chloride visually adjusted to 0.5 McFarland standard. McFarland standards are a visual scale of turbidity that corresponds to specific concentrations of colony forming units (CFU) per ml. This value has been standardized for gram-negative rods like *E. coli* or *P. aeruginosa* to roughly 1.5 x 10^8 CFU/ml (244, 253). A sterile cotton swab is then dipped into the inoculum and spread evenly across Mueller Hinton agar plates with an automatic plate rotator.

Antimicrobial discs (Oxoid) were applied firmly onto the surface of the agar within 15 minutes of inoculation of the plates when were then inverted and incubated at 37°C overnight for 16-20 hours. After overnight incubation, inhibition zones are read with a ruler at roughly 30cm from the naked eye. Zones of inhibition are judged to be where there is no growth of bacterial

colonies and these are compared to cut-offs established by EUCAST to determine resistance or sensitivity (252). *Pseudomonas aeruginosa* ATCC ® 27853 ™ is a laboratory strain where sensitivity cut-offs are published and was used as a quality control measure for each group of disc diffusion assay testing.

2.9 VARIABLE NUMBER TANDEM REPEAT TYPING OF *PSEUDOMONAS AERUGINOSA*

Variable number tandem repeat (VNTR) analysis was performed on isolates for selected experiments to allow for determination of Pa strain. This was initially described by Turton et al. (2010) using the numbers of tandem repeats at nine distinct loci to determine strain with a high level of accuracy (254). This methodology has since been adopted by a number of centres across the U.K., including the Royal Brompton Hospital (255), to track Pa infection in CF patients over time and to allow typing of strains to analyse cross-infection and outbreaks. Isolates used in this experiment were grown overnight on nutrient agar slopes and shipped to Public Health England, where the experiments were performed with the assistance of the team there.

Nine loci were used for strain typing according to the above method (ms61, ms172, ms207, ms209, ms211, ms214, ms217, ms222, and ms213). The Liverpool strain (NCTC 13415) was used as a control in each batch of experiments

For DNA extraction, a sterile plastic loop was used to suspend colonies from a nutrient agar slope in 100 µl PCR water in a 0.5ml Eppendorf tube which was vortexed for 10 seconds. Samples were them spun in a centrifuge at 15,500 g/rcf for 2 minutes. 2µl of supernatant was then pipetted into 9 wells of a plate for all loci to be amplified. Extracted DNA was stored in the refrigerator at 2-8°C for up to 2 days before PCR was performed.

For the PCR, master mixes had been prepared for amplification of each locus and stored in a clean freezer at -20°C. These reagents were thawed prior to use and made up in the PCR laboratory. The QIAGEN HotStarTaq® Plus Master Mix Kit was used with addition of primers and template. Reactions were set up at room temperature and PCR program was initiated with a 5 minute heat activation step at 95°C.

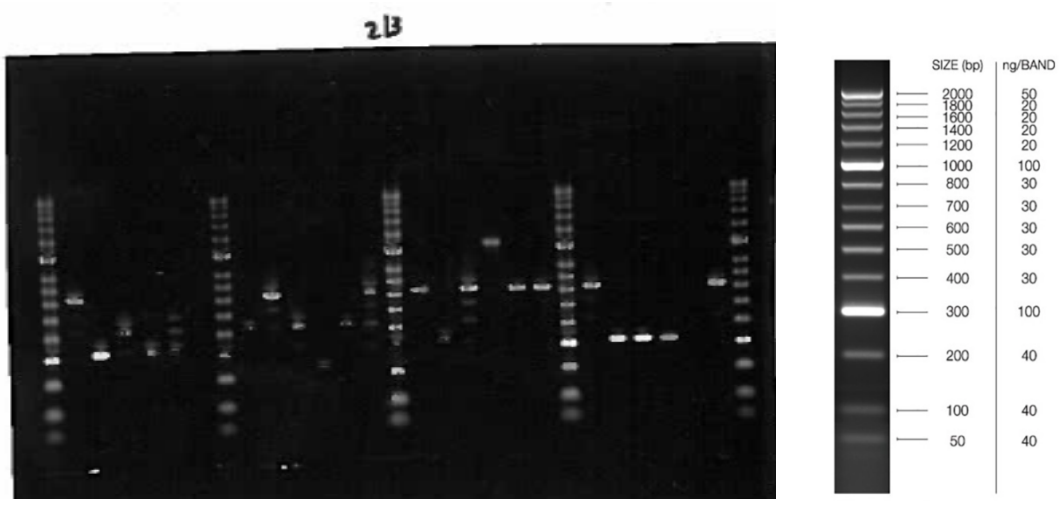
For sizing of amplicons at loci ms172, 211, 213, 214, 217 and 222, agarose gel electrophoresis was performed by running PCR products on a 1.5 % (w/v) agarose gel. 5 µl of each sample was loaded onto each agarose gel, as well as the same amount of the 50 bp HyperLadder (used every 6-8 wells to aid size readings). Electrophoresis was run at 2 hours at 150 V or until dye in loading buffer had migrated a sufficient distance. Gels were stained with GelRed (3 x 0.1 M NaCl) for 30 minutes and viewed under UV transillumination for measuring the number of repeats at each locus based on size tables.

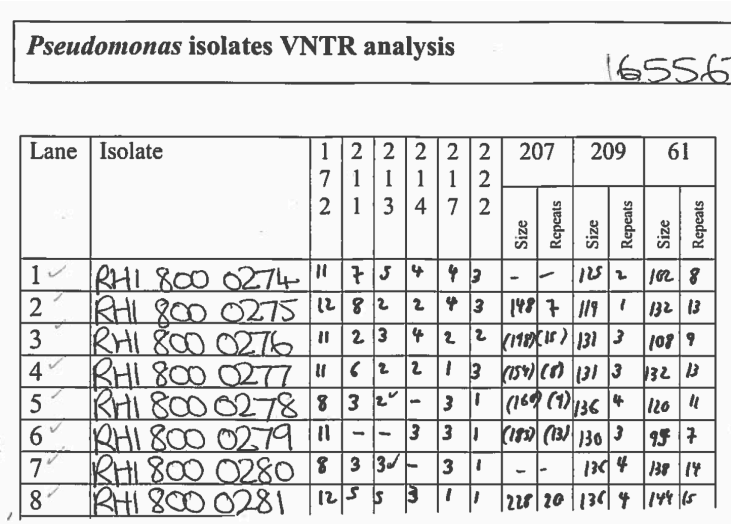
For loci ms207, 209 and 61, fluorescent primers were used and plates were submitted and analysed by the Genomic Services and Development Unit. Results were provided as FSA files and analysed using PeakScanner software to determine the size of each peak, allowing quantification of the number of repeats at the three remaining loci.

Zoë Payne from PHE helped with the VNTR typing as she performed the full analysis for the majority of the strains presented herein. The use of an external laboratory was required for the analysis of the three loci analysed by quantification of fluorescent primers (ms207, 209 and 61), as this required a different set of personnel and equipment within PHE.

An example of the agarose gel electrophoresis with a results table is shown in Figure 2.8.1 (a). Example summary data combining the gel electrophoresis and the fluorescent primers for loci 207, 269 and 61 are shown in Figure 2.8.1. (b).

(a)





***Pseudomonas* isolates VNTR analysis** 16556

Lane	Isolate	172	211	213	214	217	222	207		209		61	
								Size	Repeats	Size	Repeats	Size	Repeats
1	RHI 800 0274	11	7	5	4	4	3	-	-	125	2	162	8
2	RHI 800 0275	12	8	2	2	4	3	148	7	119	1	132	13
3	RHI 800 0276	11	2	3	4	2	2	(198)	(15)	131	3	108	9
4	RHI 800 0277	11	6	2	2	1	3	(154)	(8)	131	3	132	13
5	RHI 800 0278	8	3	2	-	3	1	(169)	(9)	136	4	120	11
6	RHI 800 0279	11	-	-	3	3	1	(182)	(13)	130	3	95	7
7	RHI 800 0280	8	3	3	-	3	1	-	-	134	4	138	14
8	RHI 800 0281	12	5	5	3	1	1	228	20	134	4	144	15

(b)

Figure 2.9.1. VNTR assay output and summary data.
(a) Example agarose gel electrophoresis with 4 strains after being stained with GelRed and viewed under UV transillumination. The amount of repeats at the given loci was determined by size on the 50bp HyperLadder, shown on the right (256). For each locus, a given weight using the 50bo HyperLadder corresponded to a different number of tandem repeats. Some of the data were difficult to interpret if there was no signal at a given locus (eg. 6th locus on the first strain on the left and 5th locus on the fourth strain on the right). (b) Summary data for VNTR reads of 8 isolates. Despite information at certain loci being indiscernible (Lane 6 shown by – at loci 211 and 213, among several other examples).

All of these results shown in the above figure were sufficient for clustering in the dendogram software, shown later in Chapter 6.

2.10 STATISTICAL ANALYSIS

All statistical analysis and graphs were performed using GraphPad Prism software (USA). Tests of normality were performed for all data using the Shapiro-Wilk test unless otherwise stated and data were said to be normally if an alpha of 0.05 was reached. For work in the microtiter plates, a method called 'robust regression followed by outlier elimination' (ROUT) was used to detect outliers if the data were normally distributed. With this method, an average false discovery rate for outliers is below 1% with data following a Gaussian distribution (257). All other statistical tests have been described in the relevant chapters.

3 CHAPTER 3: BACTERIOPHAGE USE ON *PSEUDOMONAS AERUGINOSA* BIOFILMS

3.1 INTRODUCTION

In CF, timely antibiotic administration (in combination with enhanced airway clearance and better nutrition), has contributed to increased longevity and improved quality of life in patients with this disease (258). In cases of chronic infection, especially with Pa, this success has required chronic daily suppressive therapy nebulised into the lungs. Antibiotic nebulisation suppresses the growth of a given pathogen with the objective of limiting inflammation and avoiding hospitalisation. Indeed, meta-analyses have demonstrated that nebulised antibiotics against Pa reduce bacterial load, thereby reducing inflammation and slowing the decline in lung function (259). Unfortunately, this daily nebulisation rarely clears well-established infection and the disease course of patients with CF and chronic infection is punctuated by periods of exacerbation that require courses of oral antibiotics and admissions to hospital for more aggressive therapy. For this reason, there has been a focus on both understanding the reasons that antibiotics fail, but also on potentiating their effects in the airways to allow for enhanced bacterial killing.

Though there have been many proposed shortcomings of conventional antibiotic therapy, here, I will focus on those that have been attributed to biofilms. Within the CF lung, Pa exists as aggregates that are suspended within the sputum matrix (100, 260). This biofilm lifestyle as aggregates contributes to survival of the bacteria by providing physical protection of the bacteria from immune cells and from antimicrobial therapies. It is known that the minimum inhibitory concentration (MIC) of planktonically grown bacterial can be many orders of magnitude higher for the same bacteria when grown in biofilms (261). In many instances, however, it is simply not possible to achieve these concentrations in the airway (161-163).

The reasons that bacterial biofilms are so tolerant to high levels of antimicrobials is multi-factorial and specific to both the bacterial species and antibiotics used to target them. Mah et al. (2003) demonstrated a distinct genetic mechanism employed by Pa, which allowed it to selectively sequester aminoglycosides at the surface of the biofilm by virtue of the production of periplasmic glucose polymers (262). This aminoglycoside sequestration was also nicely demonstrated in a study that used confocal microscopy to show that the antibiotic

tobramycin, by acting on actively dividing cells, can only target the outermost cells of a biofilm (155). In this same study, it was shown that the use of colistin, an antibiotic working by an entirely different mechanism - solubilising the outer membrane of bacterial cells (and thus with no requirement for metabolic activity to exert its effect) - is able to target cells deeper within the biofilm. By employing dual mechanistic action, the combination was seen to be effective in diffuse killing within the biofilm structure.

Strategies combining antibiotics with other agents to tackle biofilm infections are in active development and have been the subject of several comprehensive reviews, from the administration of antimicrobial peptides (263, 264), to liposomal preparations of antibiotics that have the ability for enhanced penetration into biofilms (158, 159). While the former strategy of using antimicrobial peptides is intended to potentiate the killing effects of antibiotics- by facilitating access through bacterial cell walls-, the latter seeks to allow greater penetrance of the antibiotics into deeper layers of the biofilm. Promisingly, a nebulised liposomal amikacin preparation that demonstrated increased biofilm penetrance in pre-clinical trials has made its way into clinical use and is efficacious in Pa infection, although it has not demonstrated superiority to nebulised tobramycin, which is often the treatment of choice in clinical practice (265).

There is a modest but expanding body of evidence demonstrating potential of bacteriophage to combat Pa infection in CF lungs. Previous studies have shown efficacy of anti-Pa phage in planktonic (241, 266), and chronic/biofilm models of murine lung infection (242). Although these results are promising, it is difficult to generalise results from studies with different bacteriophage and strains, even when clinical Pa strains from CF airways have been used. Pabary et al. (2016) have shown increased survival and lowered inflammatory response in a model of murine lung infection using several of the specific phages used in these experiments and the experiments described in this section are a natural extension of benchwork providing a stronger evidence base as this therapy makes the leap from laboratory to human therapy (241).

Some lytic phage specific to Pa employ the use of exopolysaccharide degrading enzymes that can disperse biofilms, which may prove beneficial in chronic, biofilm infections (267). Indeed, use of one such enzyme, alginate lyase - which degrades alginate (exopolysaccharide

produced by many types of bacteria, but particularly mucoid Pa), allows for greater bacterial killing when added to standard antibiotics (268). This specific enzyme has been seen to be produced by phage specific to *Azotobacter vinelandii* as early as 1977 (269) and has also been isolated from anti-Pa phage (267). It is believed that endogenous production of this enzyme by phages could permit penetration into deeper layers of the bacterial aggregates seen within the CF lung. The use of such phage encoded exopolysaccharide depolymerases has been put forth as promising antibiotic adjuvants in their own right (270).

Furthermore, in contrast to most conventional antibiotics which require actively dividing bacterial cells, bacteriophage have the ability to lyse metabolically dormant cells. A study investigating certain *S. aureus* phages demonstrated infection and lysis of metabolically dormant persister cells *in addition* to exopolysaccharide degradation of the biofilm matrix (271). As another proposed mechanism, a T7 bacteriophage engineered to produce AHLs involved in quorum-sensing for a wide range of bacterial species was seen to effectively inhibit biofilm formation in a mixed species biofilm of Pa and *E. coli* (272). Thus, from the ability to penetrate deep into the biofilm, to targeting cells that are not actively dividing, to the interruption of QS pathways necessary for biofilm formation and maintenance, phage therapy has the potential to enhance the amount of bacterial killing achieved by antibiotics alone.

Here, I wished to test the efficacy of bacteriophage in eradicating pre-formed biofilms in a wide range of different clinical strains, especially when added as an adjunct to standard antimicrobial treatment. Two commonly used antibiotics in Pa lung infection in CF patients are ceftazidime and tobramycin. Both antibiotics are bactericidal (as opposed to bacteriostatic) and are often used together in the treatment of lung infection in an inpatient setting and have distinct mechanisms of action. Tobramycin, which acts on the 30s and 50s ribosomal subunits of actively dividing cells, prevents the formation of a functional ribosome. Ceftazidime, a third-generation cephalosporin, breaks the peptidoglycan cross-linkages in the bacterial cell wall in order to kill bacteria. The action of phage is distinct, as described above, but without extensive research into the 4 specific phage that comprise the cocktail being used for our experiments, it is not known whether these phages will be able to penetrate biofilms or kill metabolically dormant cells, rendering them useful adjuncts to antibiotic therapy. It was a distinct possibility that, when used together, antibiotics and bacteriophage could

compete for the same cells on the exterior of the biofilm and that no added benefit would be observed.

3.2 HYPOTHESIS

The lytic bacteriophage cocktail used in these experiments, when used on Pa isolates that have been selected based on planktonic susceptibility to bacteriophage, will be effective against biofilm-grown bacteria and will enhance the efficacy of an antibiotic combination of two commonly used anti-pseudomonal antibiotics.

3.3 AIMS AND OBJECTIVES

Our main aim for this part of the research was to test efficacy of a 4-phage cocktail of antipseudomonal phage on a wide variety of CF isolates from airway cultures when grown in biofilm culture and to ascertain the additional effect of co-administering this treatment with antibiotics.

In order to quantity the efficacy, I had the following objectives:

- To ascertain the level of planktonic susceptibility to a 4-phage cocktail in Pa isolates from a range of different patients
- To grow these Pa isolates as biofilm and quantify reduction in biofilm using two assays: (1) a cell viability assay using resazurin salt; and (2) a crystal violet assay to quantify biomass
- To demonstrate reproducibility of the above data
- To assess correlation of the two biofilm killing assays used
- To investigate the relationship between planktonic and biofilm sensitivity to bacteriophage
- To determine whether there is enhanced biofilm eradication using the bacteriophage cocktail as an adjunct to two commonly used antibiotics: ceftazidime and tobramycin

3.4 MATERIALS AND METHODS

SELECTION OF PA STRAINS

The Microsoft Access database of Pa isolates from the Royal Brompton Hospital (RBH) was used to select isolates from 16 different CF patients. Isolates were selected on the basis of recorded sensitivity to both ceftazidime and tobramycin according to the microbiology laboratory of the hospital. These isolates were not rescreened for sensitivity prior to starting these experiments. All isolates were further examined by plaque assay and screened for their sensitivity to at least the neat bacteriophage cocktail ($1x10^9$ PFU/ml). On the basis of this, and experimental logistics, I selected 16 different Pa isolates for this chapter's experiments.

PLAQUE ASSAY

The standard plaque assay is the cornerstone of bacteriophage sensitivity testing and has been previously described (Section 2.5) (273). An overnight culture of the 16 selected Pa isolates was placed in nutrient broth by introduction of a sweep of bacterial colonies from nutrient agar (as described in section 2.1) and grown overnight. Each overnight bacterial culture was diluted in fresh nutrient broth to an optical density (OD) of 0.05. One hundred µL was added to 3mL of semi-solid nutrient agar (Oxoid), poured over sterile nutrient agar plates (Oxoid) and allowed to dry in the safety cabinet for 20-30 minutes. 5µL aliquots of each phage cocktail dilution (neat phage to 10^{-6} dilution, PBS was used as a negative control) were spotted onto the surface of the Pa inoculated plates in rows for three technical replicates per agar plate. Plates were then kept in the safety cabinet until the spots had dried before incubating the plates overnight (16-24 hours) at 37°C.

BIOFILM ASSAYS

For this experiment, I used standard 96-well plates for two different, high-throughput assays that were described previously (Section 2.6). In brief, 200 µL of overnight culture in Nutrient Broth (Oxoid) was diluted to an OD of 0.05 and then pipetted into each well of a 96 well plate. Plates were incubated at 37°C for 24 hours static to allow cells to adhere to the sides and bottom of the wells. Wells were then emptied by pipetting and washed with a phosphate buffered saline (PBS) solution before 180 L of Mueller Hinton broth (Sigma Aldrich) was added to all wells. Twenty (20) µl of each treatment was applied to the wells. There were three different treatment protocols:

i) An antibiotic mixture of ceftazidime and tobramycin, both at 2x the published breakpoints for sensitivity (final concentration in wells was 16 mg/ml ceftazidime + 8 mg/ml of tobramycin);

ii) The bacteriophage cocktail on its own (final concentration $1x10^8$ PFU/ml); and

iii) The antibiotic combination of ceftazidime and tobramycin as described above + the phage cocktail (final concentration $1x10^8$ PFU/ml of phage, 16 mg/ml ceftazidime and 8 mg/ml tobramycin).

For the no treatment wells, 20 µl PBS was added as this had been used to make antibiotic and phage dilutions. Plates were then incubated overnight (a further 16-20 hours) at 37° C before final biofilm measurements were obtained.

As described in Section 2.6, subsequent analysis involved two biofilm assays. For resazurin, all wells were emptied of free-floating cells by microchannel pipette and washed with PBS before introduction of 180 µL of MHB, then 20 µL of resazurin 0.02 % w/v working solution. Plates were covered with aluminium foil and incubated at 37°C for 3-4 hours before being transferred to the plate reader for analysis using emission and excitation wavelengths of 540 and 590 nm, respectively ($FI_{540/590}$). Plates were then emptied by tipping out contents and washed twice by submersion and inversion in water before the remaining liquid was removed by inversion. I added 180 µL of 0.1% crystal violet solution to each well for 15 minutes before 2 further inversion and washing steps in water. 200 µL of 95% ethanol was then added to each well to solubilise the biofilms and then left for 30 minutes before absorption readings with the spectrophotometer (OD_{550}).

I repeated these experiments on 5 strains that represented a spectrum of treatment effects at 2 different time points for statistical analysis to ensure reproducibility of data prior to expanding the experimental protocol to include all 16 strains.

STATISTICAL ANALYSIS

All statistical analysis and graphs were performed using GraphPad Prism software (USA). Tests of normality were performed for all data using the Shapiro-Wilk test unless otherwise stated and data were said to be normally if an alpha of 0.05 was reached. For all work in the microtiter plates, 'robust regression followed by outlier elimination' (ROUT) was employed to

detect outliers if the data were normally distributed. Previously published data shows a false discovery rate for outliers below 1% if the data falls under a Gaussian distribution with this method (257). Within an individual strain, 2-way ANOVAs were performed to compare outcomes between treatment groups, correcting for multiple comparisons. Although data for the replicates in each treatment group were parametrically distributed, the summary data for the 16 different clinical strains was non-parametric. The Wilkoxon signed-rank test was therefore used for comparison between treatment groups.

COMPARISON OF RESAZURIN AND CRYSTAL VIOLET BIOFILM ASSAYS

I had chosen two biofilm assays for work in this section for different quantification of biofilm properties. The crystal violet assay is widely used in biofilm work and gives a readout of biomass from stained cells and cellular debris attached to the side of the wells. However, it is easy to see how quantification of biomass as a stand-alone measure could miss amount of cells killed by antimicrobial treatment. Two ensure that these two reads were indeed measuring separate parameters, comparison is shown graphically in Figure 3.4.1. It is important to note that in this graph, outputs for both assays have been represented on the same graph to allow tracking sensitivity trends, although there is no relationship between the right and left sided Y axes.

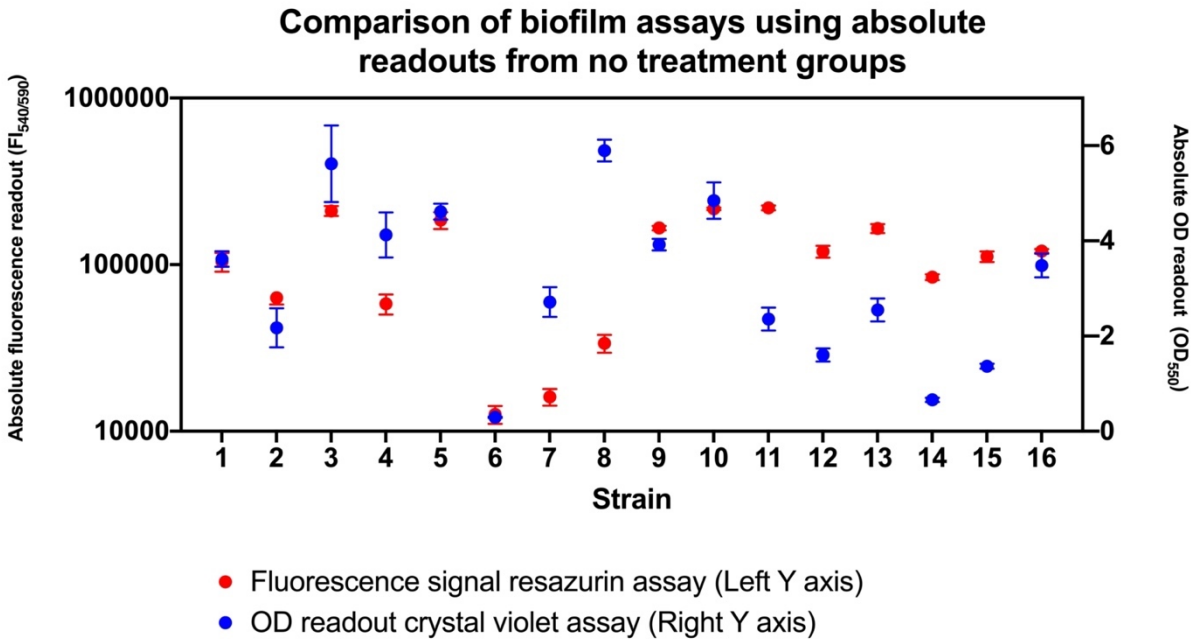


Figure 3.4.1. Comparison of the absolute reads from no treatment wells of both biofilm assays.

Results are shown for the 16 strains used in this section. The resazurin assay (red) is represented on the left hand Y axis, while the crystal violet assay (blue) is represented on the

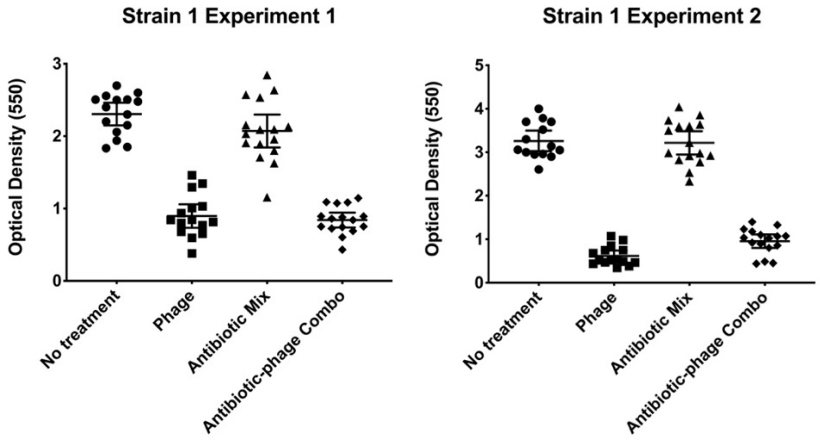
right hand Y axis. Outputs for both assays have been graphed together, although there is no correlation between the values of the left and right hand Y axis. Error bars represent mean ± SEM.

Fluorescent values for the resazurin assay did not correlate highly with the readout of the crystal violet assay for the no treatment groups. Some strains had biofilms with very high biomass with crystal violet, but a comparatively low read for cell viability (e.g. Strain 8, Figure 3.4.1), while others had a much higher read for cell viability but did not have a large biomass (e.g. Strain 14, Figure 3.4.1). The Pearson correlation coefficient was 0.35 with a p-value of 0.2, which did not meet significance.

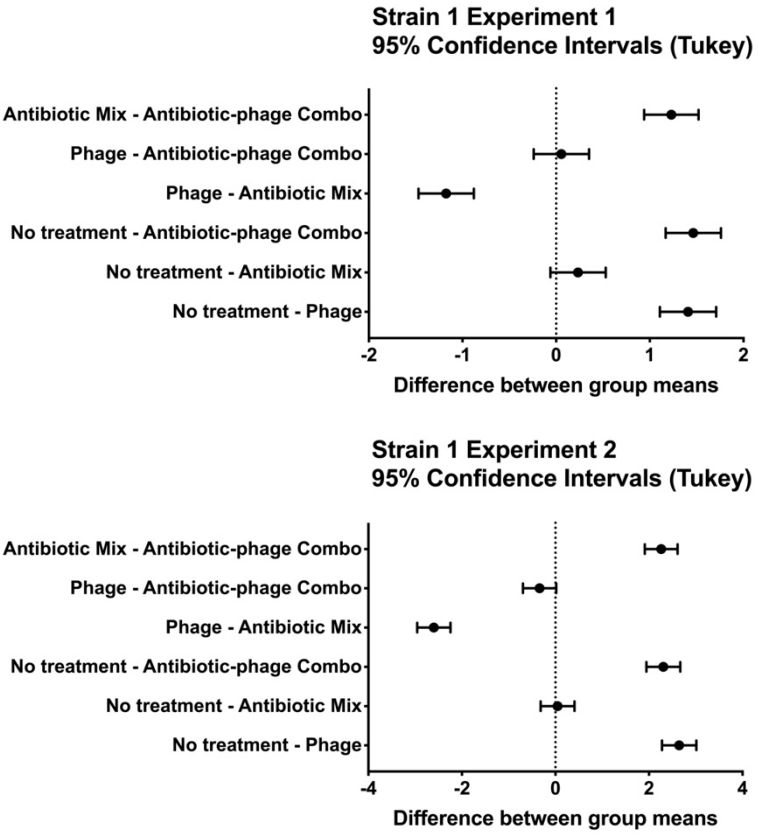
The challenge in working with both of these outcome measures were that the outputs were so different according to strain, which can be appreciated in Figure 3.4.1. This made comparisons between strains difficult. Due to the variability in amount of biofilm biomass and biofilm cell viability between strains, a decision was made to express these values as percent reduction from the negative control (i.e. no treatment wells) of each strain. In so doing, I standardised the treatment effects for each strain to itself, enabling us to compare treatment effects across the 16 strains.

ASSESSMENT OF REPRODUCIBILITY

Before embarking on the high throughput method including all 16 strains, I sampled 5 strains at 2 different time points to establish the reproducibility of the results. I selected 5 strains that showed a variety of different planktonic sensitivity levels to the phage, and also a diversity of treatment effects with both of the biofilm assays (response to antibiotics, response to phage, response to the antibiotic combination). The 5 strains selected for analysis and repetition of the assay were 1, 6, 8, 13 and 16. An example of the outcomes for the crystal violet assay in strain 1 (the strain with the most variability) is summarised in Figure 3.4.2.



(a)



(b)

Figure 3.4.2. Example reproducibility from strain 1 in two subsequent experiments using the same protocol.
(a) Raw OD values using the crystal violet assay are plotted here (n=16 technical replicates for each treatment group). Note the difference in the Y axis for the plotted OD values in the 2 replicates of the experiment. The second experiment showed greater staining with crystal violet and more consistent values. (b) Difference between group means showing 95% confidence intervals of the Tukey Test. Although the second experiment showed more staining with wider spread of data, it is the same treatment groups that reach significance.

The results shown in Figure 3.4.2 summarise one of the 5 strains analysed and although there is high variability of results in terms of absolute values (OD values for crystal violet assay presented here), they show the same trends, yielding the same statistical results between treatment groups on separate occasions. Comparisons of outcomes of the crystal violet assay for all 5 strains at 2 different time points can be seen in Figure 3.4.3.

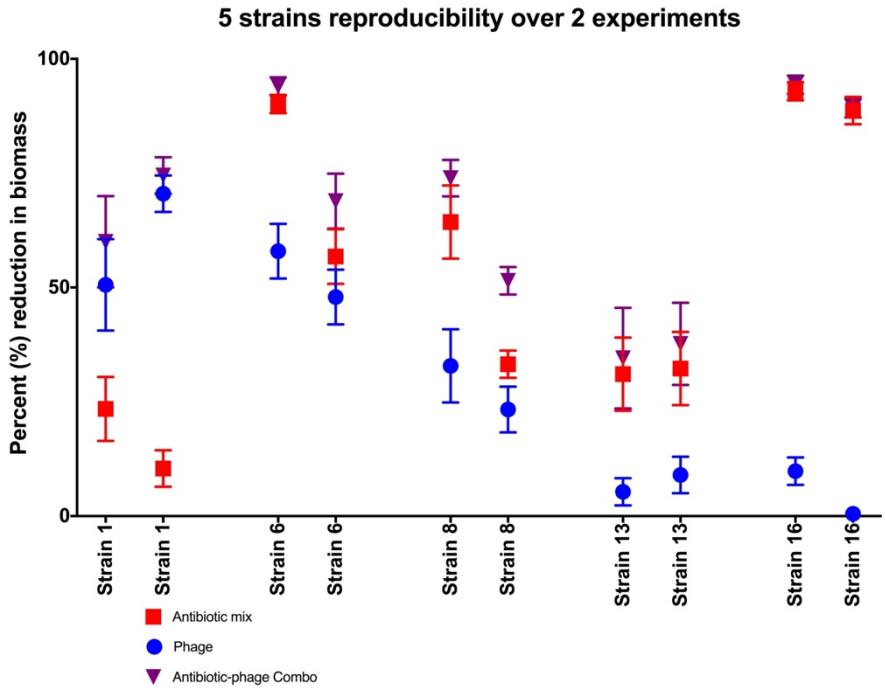


Figure 3.4.3. Reproducibility in 5 Pa strains at different time points (biological replicates). The percent reduction in biomass compared to no treatment wells when using the crystal violet assay is compared at two different time points for 3 different treatment groups (phage alone, antibiotic mix alone, and antibiotic mix + phage). N=16 technical replicates, means SEM shown Though the absolute values in terms of percentage did change, the trends remained the same on the 2 occasions.

There was good reproducibility of data on 2 separate occasions with the same experimental protocol. These results were discussed with Dr. Susan Lehman and Dr. Sandra Morales of Ampliphi Biosciences Corporation, and after consideration of results for all 5 strains, our team decided that there was sufficient reproducibility of the data to proceed to the next phase of analysis involving all 16 strains.

3.5 RESULTS

Although examining the use of bacteriophage as a stand-alone treatment was not itself an aim of this chapter, the plaque assay underpins much of the work involving phage and is itself

likely to form the basis for the selection of patients for future clinical trials. It was useful for us to be able to see the level of variation in this assay at different timepoints and also to see how it related to results obtained in the biofilm assays. The work of this chapter also forms the basis for the work described in subsequent chapters. Thus, results for the plaque assays and correlation of plaque assay sensitivity with the amount of biofilm eradication is presented first, with the adjunctive use of bacteriophage with antibiotics discussed later.

The Plaque Assay

The plaque assay for each of the strains was performed in biological triplicate. In most instances, the results that were obtained were similar; however, in some instances, results of the plaque assay differed by many orders of magnitude. This distribution of results can be seen in Figure 3.5.1.

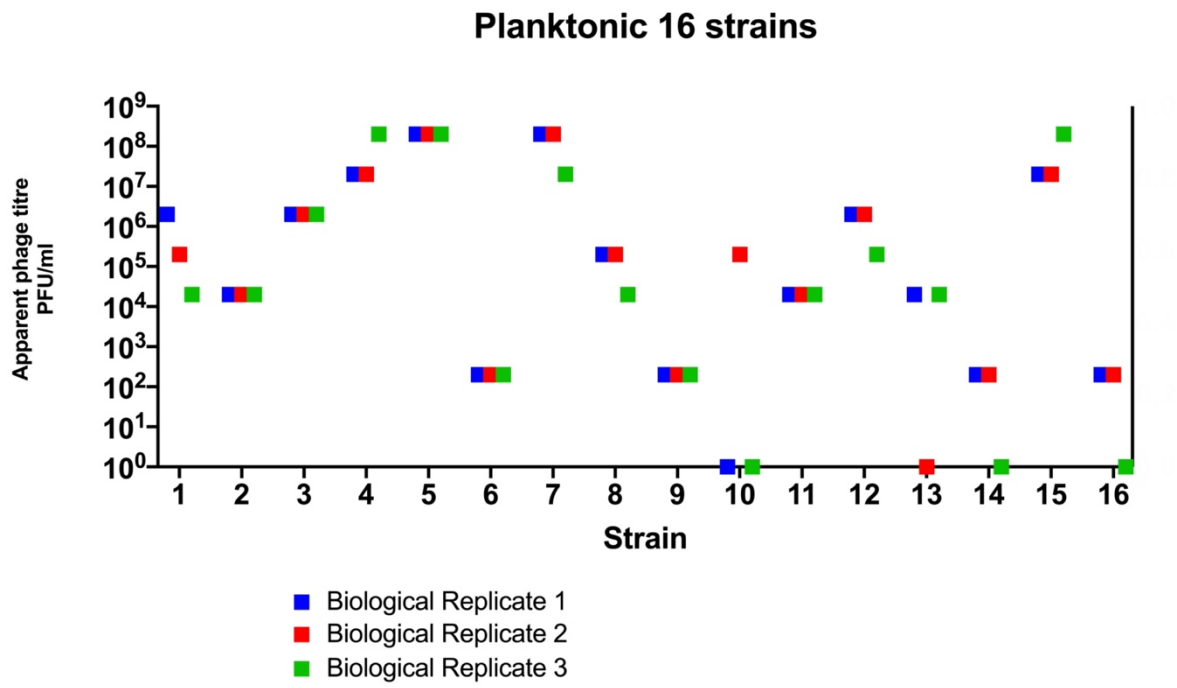


Figure 3.5.1. Distribution of the three biological replicates of the plaque assay experiments. Results show the three replicates for each strain to determine sensitivity to the bacteriophage cocktail. Increasing bacteriophage sensitivity is shown on the Y axis in ascending PFU/ml.

The apparent phage titre is highly variable in different biological replicates from certain strains (notably 10, 13 which differ by >2 orders of magnitude). Additionally, although inclusion for these experiments required sensitivity to at least the neat phage cocktail, strain

10, and strains 13, 14 and 16 showed complete resistance to the phage cocktail at 2 and 1 time point, respectively.

COMPARING PLANKTONIC AND BIOFILM SUSCEPTIBILITY TO PHAGE

In general, the results of the plaque assay were not predictive of biofilm reduction. The results depended on clinical strain and also on the type of assay being used, with the resazurin outcome measure (immunofluorescence expressed as a percent of the no treatment wells) approximating cell viability and the crystal violet outcome measure (OD expressed as a percent of the no treatment wells) approximating biomass reduction. These results are summarised in Figure 3.5.2.

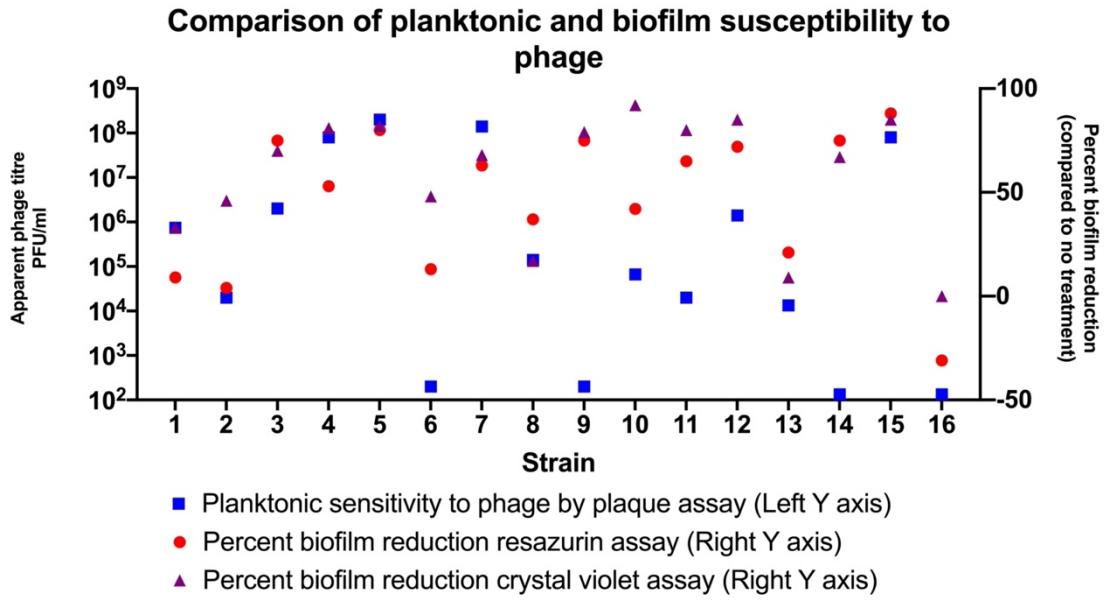


Figure 3.5.2. Comparison of planktonic and biofilm susceptibility to phage.
Planktonic sensitivity to phage as assessed by plaque assay (blue square) is shown on the logarithmic scale on the left sided Y axis. Percent reduction in biofilm cell viability (red circle - assessed by IF resazurin assay) and biofilm biomass (purple triangle - assessed by crystal violet OD readings) are plotted on the right sided Y axis. Results of the cell viability assay for strain 16 actually increased with addition of phage and this was statistically significant using a paired t-test (p= 0.0021, 95% CI 6.7-17.9% increase in cell viability).

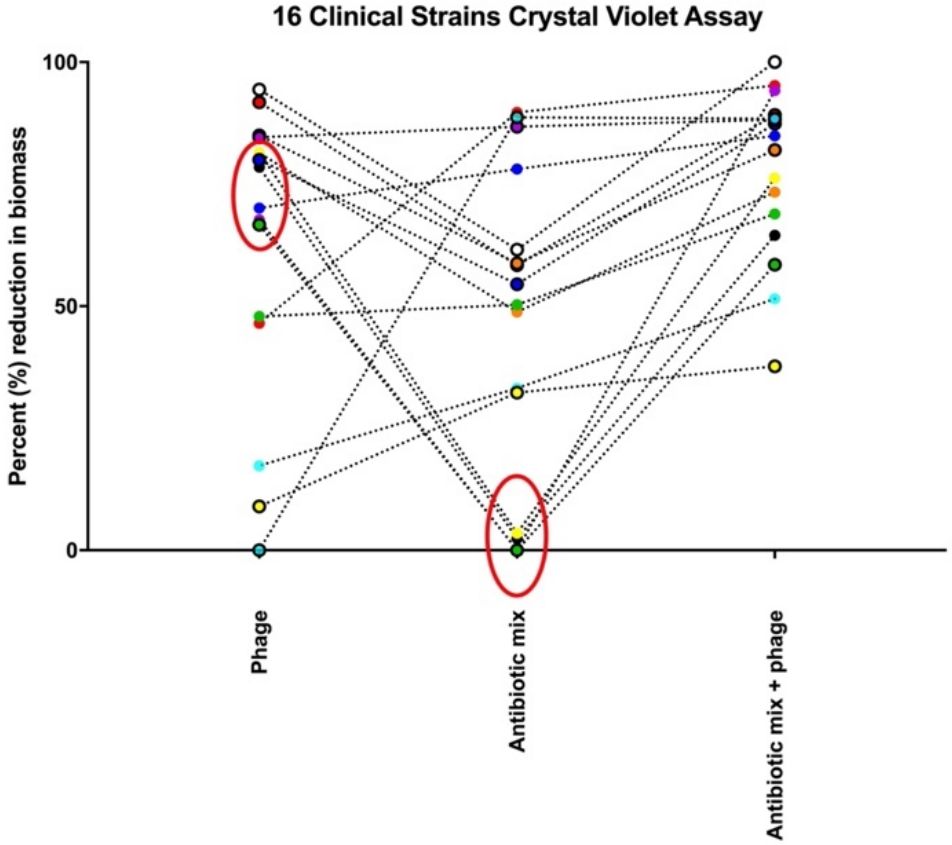
Across the group of 16 strains, bacteriophage cocktail monotherapy resulted in a decrease in cell viability (resazurin) of 46% (95% CI 28.0-64.6%) and in biomass of 59% (95% CI 43.0-74.7%) and these means were not significantly different from one another. Though in general, results of the resazurin assay and of the crystal violet assay correlated well with each other (Pearson coefficient 0.80, p=0.0002), there were more instances of greater biofilm biomass reduction

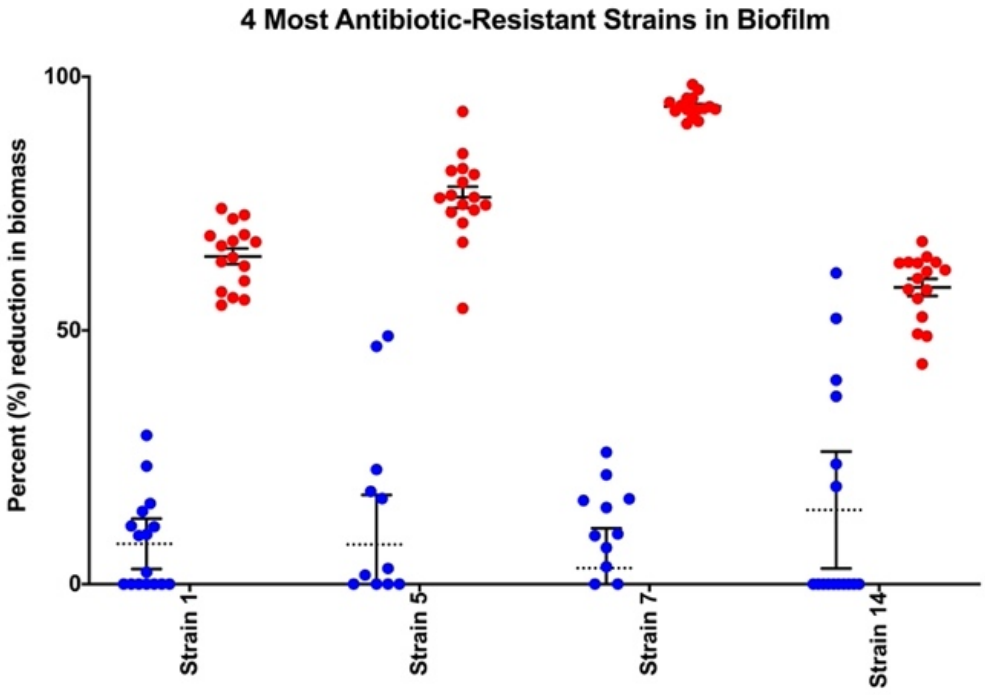
than cell viability (strains 1, 2, 6, 10). The level of planktonic sensitivity to bacteriophage did not correlate with outcomes of either biofilm assay (Pearson's coefficient of 0.36 and 0.41 for the resazurin and crystal violet assays, respectively). Though for many cases, planktonic sensitivity did translate to biofilm reduction (strains 3, 4, 5, 12, 15), there were instances of planktonic sensitivity that did not equate to much reduction of biofilm (strain 1) and certain instances where the phage was not particularly efficacious planktonically, but demonstrated enhanced efficacy in biofilm reduction (strains 9 and 14).

BACTERIOPHAGE AND ANTIBIOTICS IN BIOFILM

Comparing the results of the biofilm assay between the antibiotic combination group and the bacteriophage group yielded a spread of different responses, which are summarised in Figure 3.5.3.

(a)





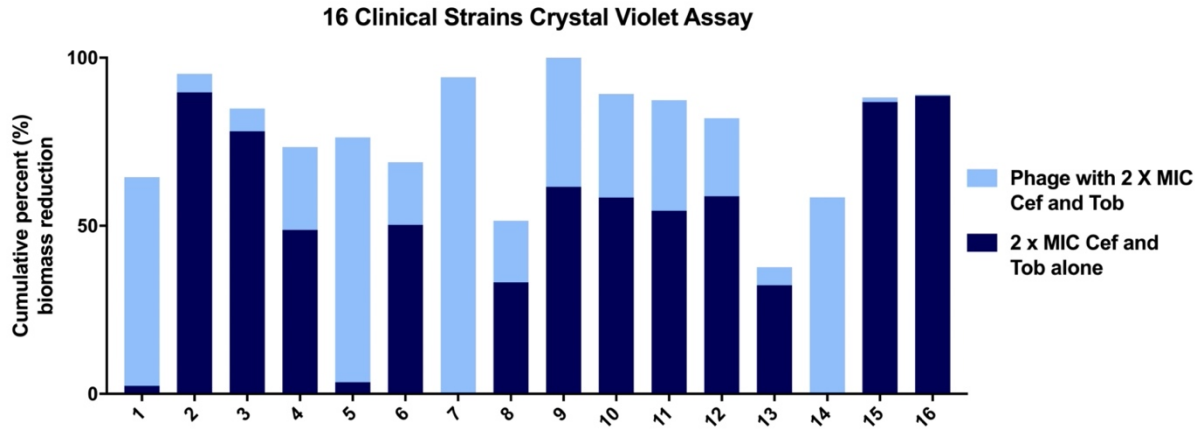
(b)

Figure 3.5.3. Biofilm readout for the 16 strains with focus on the 4 most antibiotic resistant strains.
(a) Percent reduction in biofilm biomass as assessed by the crystal violet assay for 16 clinical Pa strains. Each strain is represented by a different coloured circle and is joined by dotted black lines across treatments. Phage, antibiotic mix as well as combination treatment groups are shown here graphically for comparison. Coloured circles represent means of 16 technical replicates. The 4 most antibiotic resistant strains are circled in red and show high susceptibility to phage as a single therapeutic agent). (b) Comparison between the 4 most antibiotic resistant strains seen in (a) when looking at reduction in biomass compared to antibiotic-phage combination. Error bars are means ± SEM, N=16 .

I note that there is a large spread of responses that vary according to strain. In terms of biofilm reduction as assessed by the crystal violet assay, some strains are more sensitive to the antibiotic combination, some to the bacteriophage, others to both and some to neither. But for the 4 most antibiotic resistant strains, these same 4 strains show high levels of biofilm reduction when exposed to the bacteriophage cocktail.

The next portion of analysis, and the main objective of this chapter, looks at the use of bacteriophage as an adjunct to standard antibiotic therapy in biofilm eradication across all 16 strains. Summary results for the 16 clinical strains are shown in Figure 3.5.4.

(a)



(b)

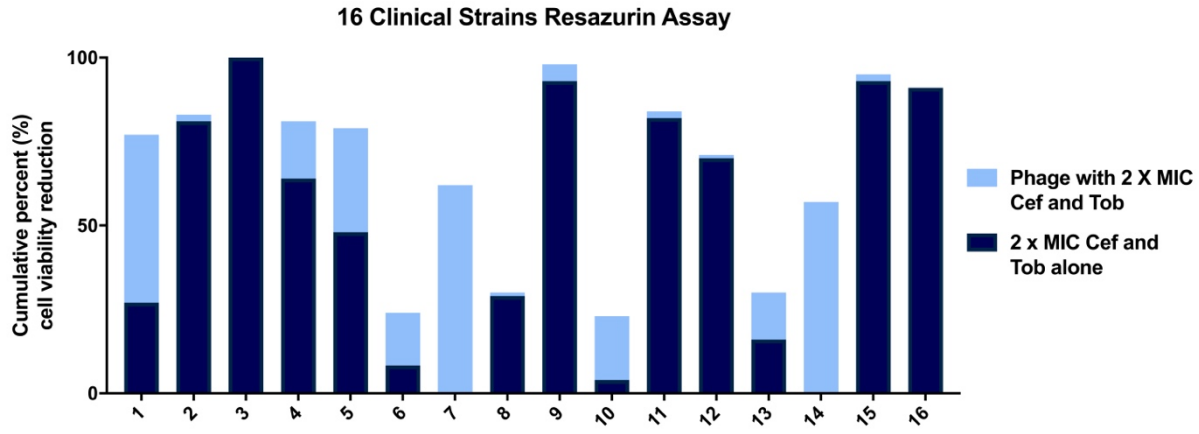


Figure 3.5.4. Percent reduction in biofilm biomass and cell viability with antibiotic combination and phage.
Dark blue shows the amount of biofilm reduction with the antibiotic combination alone, with the added light blue showing the additional reduction in killing when the bacteriophage was added. (a) Percent reduction of biofilm biomass when the phage cocktail was added to 2xMIC of both antibiotics. The addition of the phage cocktail resulted in a further 1.7-fold reduction in cell viability across the 16 strains ($p < 0.001$ using the Wilcoxon signed rank test) compared with the dual antibiotics alone. (b) Percent reduction in metabolic activity of biofilm cells when the phage cocktail was added to 2xMIC of both antibiotics. The addition of the phage cocktail resulted in a further 1.3-fold reduction in cell viability across the 16 strains ($p < 0.001$ using the Wilcoxon signed rank test) compared with the dual antibiotics alone.

The 4 strains that demonstrated the greatest level of biofilm reduction with the addition of the phage cocktail (strains 1, 5, 7 and 14) were the same 4 that were shown in Figure 3.5.3 as being the most antibiotic resistant but also the most sensitive to the bacteriophage independently.

3.6 DISCUSSION

In this section, I looked at phage as an adjunctive therapy to standard antibiotics. Due to the fact that antibiotics are the mainstay to target lung infection in CF and are associated with improved patient outcomes, phages are unlikely to be given as a stand-alone treatment. In exploring bacteriophage alongside antibiotic therapy, I demonstrated efficacy of a bacteriophage cocktail when used as an adjunct to a standard antibiotic combination against pre-established biofilms across a spectrum of different clinical Pa strains isolated from CF patient airway cultures. Though there are many reviews summarising the scientific literature looking at the efficacy of bacteriophage in both the prevention of biofilm establishment (274) and in biofilm eradication (275-277) of a wide range of clinically relevant organisms, this research is important in that it shows efficacy in an array of clinical strains found in a specific disease process alongside clinically relevant antibiotics and concentrations.

The fact that the 4 strains demonstrating the highest level of resistance to both ceftazidime and tobramycin demonstrated high levels of sensitivity to the bacteriophage cocktail does indicate that these agents are potentially targeting different cell populations. This is encouraging for an adjunctive therapy as it indicates that the agents would not necessarily be competing for access and killing of the same cell populations, but might be, in fact, broadening the spectrum of populations eradicated. The hope is that by using agents that work in different ways, the success of antimicrobial therapy would be increased and that this may translate to improved patient outcomes in CF.

In an historic sense, the precedent of using bacteriophage clinically as an adjunct to antibiotic treatment was employed as early as 1946, when used with penicillin to fight Staphylococcal infection, although at this time there was not a good understanding of bacteriophage structure or biology (231). I used both ceftazidime and tobramycin, two standard antibiotics that are often used together in Pa lung infection in CF, at concentrations that could be achieved with either nebulisation or intravenous administration. In the absence of clinically relevant bacteriophage concentrations, I selected one concentration of bacteriophage for use in these experiments and applied it broadly to the 16 clinical strains. In some cases, however, either the antibiotic combination on its own or the bacteriophage on its own was too efficacious at biofilm eradication to be able to contribute a meaningful signal. In some strains, I saw that the biofilms were tolerant to both phage and antibiotics. It was interesting that

there was a disconnect between planktonic sensitivity to the phage combination and the efficacy in biofilm reduction. Because all isolates had been selected on the basis of sensitivity to both ceftazidime and tobramycin in our hospital microbiology laboratory, this disconnect is also observed with antibiotics and is the subject of further discussion in Chapter 6 of this thesis.

The biofilm assays themselves should be the subject of some scrutiny as they both are crude measures of one aspect of biofilm. It was our hope that by using two assays to measure different biofilm components (in this case, biofilm cell viability and biomass), that the result would be a more robust representation of the biofilm structure. The resazurin assay, for instance, is intended and used as a cell viability assay; however, a more accurate description would be one of detecting cell metabolic activity. And whether metabolic activity equates to cell viability in a biofilm is a question that deserves attention. Addition of sub-inhibitory concentrations of aminoglycosides has, at least in some *in vitro* work, been shown to result in thicker, more robust biofilms (278), a fact that led researchers in this study to conclude that biofilm formation was a specific and direct response to antimicrobial treatment. Without either visualising these cells with microscopy using a live/dead stain, or solubilising the biofilm and performing colony counts, I was not able to see whether the increase in metabolic activity was from an increased number of cells, or (as I expect) a stable number of existing cells increasing their metabolic activity in direct response to a threat in the environment in order to enhance bacterial survival.

The time course of the experiments is another variable that deserves discussion. I selected 24 hours for the initial growth of our biofilms to allow cells to adhere to the 96-well plates, but there are experiments using much longer timepoints, which could be useful in analysing a treatment that would likely be given over some weeks and not at a single time point. For instance, some of the early work looking at bacteriophage T4, which infects and lyses *E. coli* cells, showed both bacterial and phage evolution that resulted in population stability over periods as long as 52 weeks, that were worrying with regards to the development of lytic phage therapy (279). Further studies with *E. coli* showed that resistance could *rapidly* evolve to T4 phage by changes in lipopolysaccharide on the bacterial outer membrane (280). It was then shown that mutations conferring resistance of T4 phage in *E. coli* can occur in several different loci. Despite these numerous and varied mechanisms of resistance, researchers also

saw that evolution to evade the predatory effects of phage came at a cost, as these changes rendered the bacteria less competitive (281). Nonetheless, these studies illustrate the fact that (i) the relationship between phage and bacteria is complex and (ii) I do not know whether the repeated and sustained use of bacteriophage will continue to show efficacy of this therapy or whether, as in some of the above studies, a population equilibrium would be reached.

Despite these valid concerns surrounding our models and methodology and ability to extrapolate results and apply them to a clinical context, our results are promising. The 4-phage cocktail used in these experiments showed efficacy across a variety of clinically relevant Pa strains both planktonically and within biofilm models. Notably, it was shown to augment the benefit seen in the eradication of pre-formed biofilms with a clinically relevant antibiotic combination, indicating that this is a therapy with great potential and deserving of future studies.

4 CHAPTER 4: EXPLORING ANTIBIOTIC AND BACTERIOPHAGE INTERACTIONS AT SUB-INHIBITORY CONCENTRATIONS IN PLANKTONIC AND BIOFILM CULTURE

4.1 INTRODUCTION

In the previous section, I used high concentrations of antibiotics (double the established breakpoints published in EUCAST guidelines (252) for ceftazidime and tobramycin) in combination with a high phage titre ($1x10^8$ PFU/ml – one 10-fold serial dilution from the neat phage) in an attempt to achieve greater levels of biofilm eradication in 16 different Pa strains. The combination of ceftazidime and tobramycin, two anti-Pseudomonal antibiotics commonly used together in clinical practice, was more effective when combined with a bacteriophage cocktail, in eradicating pre-formed biofilms when used at these high concentrations. In general, however, the results showed that it was either the bacteriophage cocktail or the antibiotic combination that led to the majority of the biofilm eradication. It was difficult to draw any conclusions about what effect combination therapy would have on Pa at different concentrations or even in different conditions.

Determining the effects of combined bacteriophage-antibiotics is potentially complex and there is a body of preclinical experimentation that alludes to this. Part of the reason for this is that many classes of antibiotics interfere with bacterial cell physiology in ways that are crucial for phage infection, and hence there is a distinct possibility that combining phage with ceftazidime or tobramycin will reveal non-additive or even inhibitory/antagonistic results. Tobramycin, for instance, is an aminoglycoside antibiotic that targets the bacterial ribosome 30S subunit, and ribosomal function is essential for virion replication and the cell lysis of bacteriophage (282). Ceftazidime, a ß-lactam, works at the cell wall, which is where the bacteriophage adsorb to surface proteins, necessary for injecting genetic material into the cell. Exposing Pa to an antibiotic to which it is susceptible can, in theory, block subsequent phage infection and phage-mediated killing (282). One study illustrating this point looked at 4 phages infecting *Ralstonia solanacearum*, and demonstrated that phage viron production was completely blocked with bacterial exposure 20 minutes prior to the addition of phage with twice the observed MIC of rifampicin (283). Of note, there were two other phages this group tested, for which there was simply a reduction of phage titre at 4 x MIC values. This

observation led the authors to conclude that there are phages that carry their own RNA polymerases, a finding which could have relevance to future phage selection. There are also numerous Pa specific examples of phage-antibiotic antagonism that highlight this as a risk. One early study revealed that burst sizes (the amount of phage virion progeny released at cell lysis) of anti-Pseudomonal phage PM2 was reduced to less than 1% of that seen in control conditions with simultaneous addition of rifampicin at a concentration of 0.3 μg/ml (284). Subsequent work decades later, also with Pa, demonstrated that 100 μg/ml of rifampicin added 10 minutes prior to lytic phages PAK_P3 and PAK_P4 completely inhibited phage replication (285).

But as much as there is *in vitro* work contributing to the concept of phage-antibiotic antagonism, there is a similar body of evidence supporting additivity or synergy. Gu Liu et al. (2020) recently looked at many different antibiotic classes combined with phage against a highly drug resistant *E. coli* in checkerboard assay and found that phage could render this MDR bacteria susceptible to antibiotics at concentrations to which it had previously demonstrated resistance (286). Interestingly, these experiments demonstrated synergism with phage and ceftazidime and additivity of effects with kanamycin, an aminoglycoside antibiotic in the same class as tobramycin. However, it should be noted that other experiments exploring the use of anti-*Staphylococcus aureus* phage and kanamycin demonstrated antagonism (287). For Pa, a study quantifying the number of phage present after exposure of 48 hour biofilms to phage in conjunction with 5 different classes of antibiotics for 48 hours concluded that the antibiotics tobramycin and ciprofloxacin blocked virion production when concentrations approached 8 x MIC, while for ceftazidime and colistin, phage production was still seen at these concentrations (288).

Furthermore, there is no reason that the interactions between antibiotics, phage and bacteria should be consistent across planktonic and biofilm modes of growth. One study, for instance, demonstrated that for 17 clinical Pa strains with resistance to a bacteriophage in planktonic assay, this same phage was able to replicate within the biofilms of 8 of the 17 strains (289). In the previous section, I discussed biofilms in some more depth, including the fact that some phage has been seen to produce enzymes that degrade the biofilm matrix (290, 291), allowing access to different populations of bacteria from deeper within the structure. I do not know whether the 4 phage used in our cocktail produce such enzymes, but if they do, it is possible

that (i) they might targeting cells embedded deeper into the biofilm and (ii) that they might liberate cells from the biofilm and allow antibiotics to exert their bactericidal effects. Theoretically, phage could reduce biomass by freeing up biofilm bacteria, in the absence of virion production/ cell lysis. If the above is true, this might have been the reason that our phage cocktail broadened the coverage achieved with conventional antibiotics in the previous section, and could also mean that additivity of effect will more frequently be demonstrated in biofilm as opposed to planktonic culture.

On the other hand, many cells in biofilm culture have access to fewer nutrients than their planktonic counterparts, and there is laboratory evidence showing that many phages are less efficacious and produce fewer viral progeny in conditions of reduced bacterial metabolic activity (292). The process of lytic phage infection failing to lead to cell lysis (usually in conditions of bacterial starvation) is termed pseudo-lysogeny and this is a potential reason for reduced efficacy in biofilms. Importantly, however, when nutritional conditions improve and metabolic activity of the bacteria resumes, lysis proceeds as intended (293).

My concern in drawing conclusions from the previous section, beyond the limitations of *in vitro* testing, were that in the lung environment, gradients of antimicrobial concentrations invariably exist, from supra-inhibitory to sub-inhibitory, not only accounting for the geography and heterogeneity of the lung environment, but also for time due to the pharmacokinetic properties of antibiotic clearance. For this section, I therefore wished to evaluate the effects of varying sub-inhibitory levels of antibiotics individually when combined with varying sub-inhibitory levels of bacteriophage in planktonic and biofilm culture. Without a sufficient body of results for clinical trials involving phage in the context of most human infections, including CF lung infection, such knowledge could not only help identify patients who are more likely to benefit from phage therapy (for example, in the case of synergy with antibiotic compounds used as chronic suppressive therapy), but may also help in post-hoc analysis for clinical trials that are currently underway (for example, performing sub-analyses on patients in whom certain classes of antibiotics have been used during the trial period).

4.2 HYPOTHESIS

After strain-specific selection of sub-minimum inhibitory concentrations for ceftazidime, tobramycin and bacteriophage in both planktonic and biofilm cultures, I will discover individual interactions for phage resulting in varying efficacy rates.

4.3 AIMS AND OBJECTIVES

- To identify what effect sub-inhibitory concentrations of ceftazidime and tobramycin have in reducing growth in planktonic culture and biofilm biomass, when combined with sub-inhibitory concentrations of bacteriophage for 10 clinical and 1 laboratory Pa strain
- To determine whether there are instances of synergy or inhibition and whether there are trends as to type of culture or type of antibiotic when such conditions arise
- To establish whether antibiotic and phage combination therapy is more efficacious in biofilm than in planktonic culture or vice versa
- To establish whether antibiotic-phage combination therapy is more efficacious with ceftazidime or tobramycin, commonly used antibiotics with differing mechanisms of action

4.4 MATERIALS AND METHODS

SELECTION OF PA STRAINS

The Microsoft Access database of Pa isolates from the Royal Brompton Hospital (RBH) was used in the previous section to select isolates from 16 different CF patients. All strains had been selected based on sensitivity to both ceftazidime and tobramycin as assessed by the microbiology department at RBH, as well as to the neat bacteriophage cocktail, as assessed by the plaque assay described in the previous section. I randomly selected 10 of the previously examined 16 strains to perform more focused analysis in this section. The strains selected were laboratory strain PA01, as well as clinical Strains 1, 3, 4, 5, 7, 9, 10, 11, 14 and 15.

PLANKTONIC AND BIOFILM ASSAYS

Selected strains stored in glycerol solution on glass beads were removed from the -80°C freezer and grown overnight in an incubator at 37°C on Nutrient agar (Oxoid). A sweep of colonies removed by plastic loop was then grown in Nutrient broth (Oxoid), to reach

logarithmic growth phase. I used standard 96-well plates for both planktonic and biofilm experiments (ThermoFisher Scientific).

For planktonic culture, 180 µL of overnight culture was diluted in Mueller Hinton broth (Sigma Aldrich) to an OD of 0.1 and then pipetted into each well of a 96 well plate. Twenty (20) µL of solution for treatment groups was administered so that final target concentrations were reached and final volume in the wells = 200 µL. For negative control wells, PBS was used as this was used for phage and antibiotic dilutions.

Results were evaluated spectrophotometrically at 18 hours after treatment overnight in the incubator at 37°C shaking at 200 rpm. Treatment groups (n= 6 technical replicates each) were compared to each other and standardized across strains to account for differences in OD measurements according to the following equation:

Percent reduction (%) in OD = $((OD_{control/no\ treatment} - OD_{treatment})/OD_{control/no\ treatment}) \times 100$

For biofilm assays, 200 µL of overnight culture was diluted to an OD of 0.1 in Nutrient broth and then pipetted into each well of a 96 well plate. Plates were incubated at 37°C static for 24 hours to allow cells to adhere to the sides and bottom of the wells. Wells were then emptied and washed with a phosphate buffered saline (PBS) solution before 180µL of Mueller Hinton broth (Sigma Aldrich) was added to all wells along with different treatments so that final volume in the wells = 200 µL. For negative control wells, PBS was used as this had been used for phage and antibiotic dilutions. Plates were then incubated at 37°C overnight (a further 16-20 hours) shaking at 200 rpm before final biofilm measurements were obtained using the crystal violet assay, as described in Methods section 2.6.

Treatment groups consisting of 6 technical replicates each were compared to each other and standardized across strains to account for differences in biomass measurements according to the following equation:

Reduction (%) of biomass with CV assay =

$((OD_{control/no\ treatment} - OD_{treatment})/OD_{control/no\ treatment}) \times 100$

ANTIMICROBIAL CHECKERBOARD ASSAY

I used a checkerboard assay, which has been previously described in detail, whereby serial dilutions of two different antimicrobial compounds are administered to bacterial culture in a 96 well plate (294, 295). This is shown, modified for the purposes of my experiments, in Figure 4.4.1.

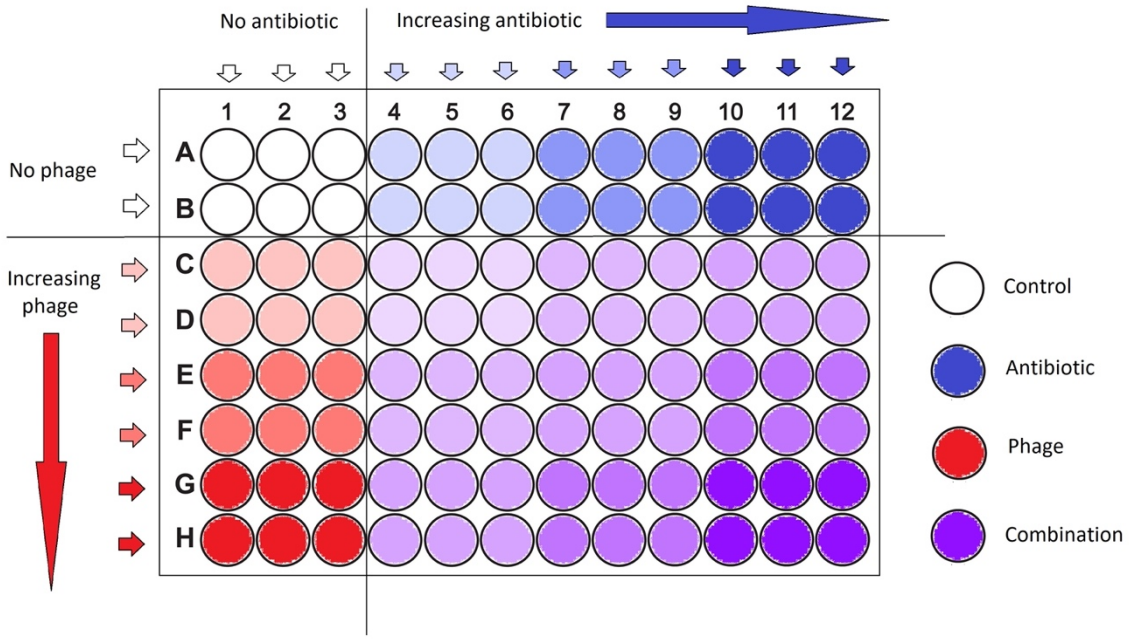


Figure 4.4.1. Example setup of a synergy checkerboard assay.
This depiction of the checkerboard assay shows testing of two antimicrobial compounds (in this case, an antibiotic compound and a bacteriophage preparation) in increasing concentrations moving across the 96-well plate from left to right and down the plate from top to bottom. Modified from (296).

Due to the amount of variability seen in treatment groups in the experiments performed in the previous chapter, I opted for 6 technical replicates in each condition and 3 biological replicates of the experiments performed using separate cultures from sweeps of colonies on different days.

COEFFICIENT OF DRUG INTERACTION (CDI)

A coefficient of drug interaction (CDI) was developed for the assessment of interaction between two different antimicrobial compounds and was used in accordance with previous published data (297). It is calculated based on the following formula:

$$CDI = AB/(A \times B)$$

According to the OD readings for each treatment group, AB is the ratio of the antibiotic-phage combination groups to the negative control group; A or B is the ratio of the single agent (antibiotic or phage) group to the negative control group. A CDI value of <1 indicates that the combination of A+B is synergistic, which values <0.7 are said to be strongly synergistic.

STATISTICAL ANALYSIS

All statistical analysis and graphs were performed using GraphPad Prism software (USA). Tests of normality were performed for all data using the Shapiro-Wilk test unless otherwise stated and data were said to be normally distributed if an alpha of 0.05 was reached. For all work in the microtiter plates, 'robust regression followed by outlier elimination' (ROUT) was employed to detect outliers if the data were normally distributed. Previously published data shows a false discovery rate for outliers below 1% if the data falls under a Gaussian distribution with this method (257).

Within an individual strain, one-way ANOVAs were performed to compare outcomes between treatment groups, correcting for multiple comparisons. If the mean effects of combination treatment groups for the 3 biological replicates were statistically different using one-way ANOVAs, CDIs were calculated to determine whether the combination should be classified as synergistic (S) or was simply additive (A). Experimental conditions in which the effect of combination therapy was significantly worse than either single phage *or* antibiotic therapy were classified as inhibitory (I). Where results of group means were not statistically different, the combination treatment was said to have no effect (N).

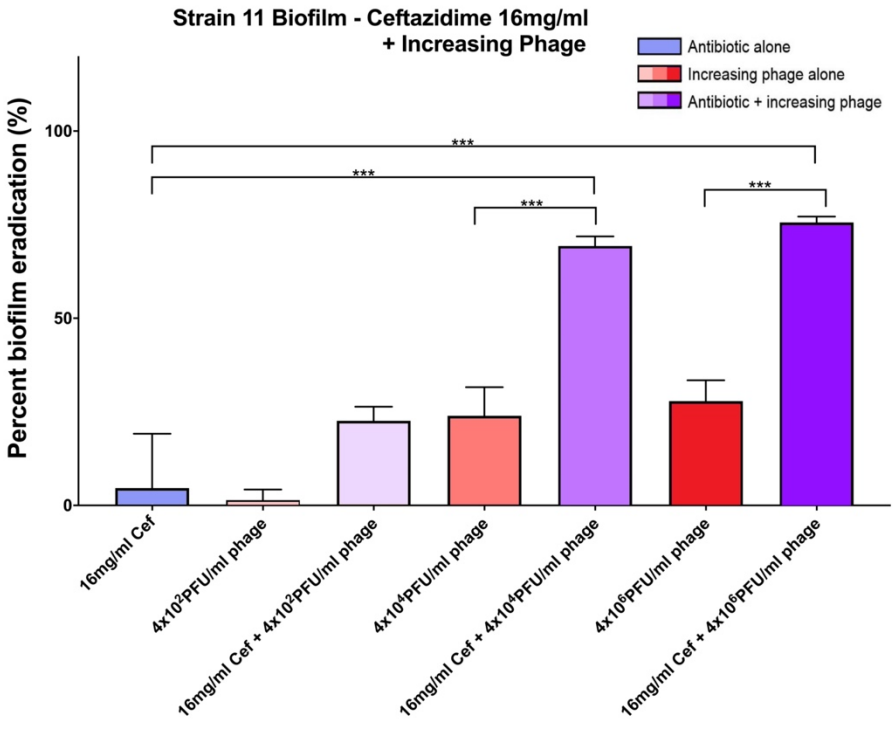
For comparing the relative success of combination therapy based on antibiotic choice and type of culture, combination therapy was classified as either beneficial (additive or synergistic) or not of benefit (no effect or antagonistic). Following conversion to a binomial outcome (success or failure), the modified Wald method was used to calculate confidence intervals of the proportion of successful outcomes for ceftazidime and tobramycin, and for planktonic and biofilm culture (298).

ASSAY OPTIMISATION AND CHOICE OF ANTIMICROBIAL CONCENTRATIONS

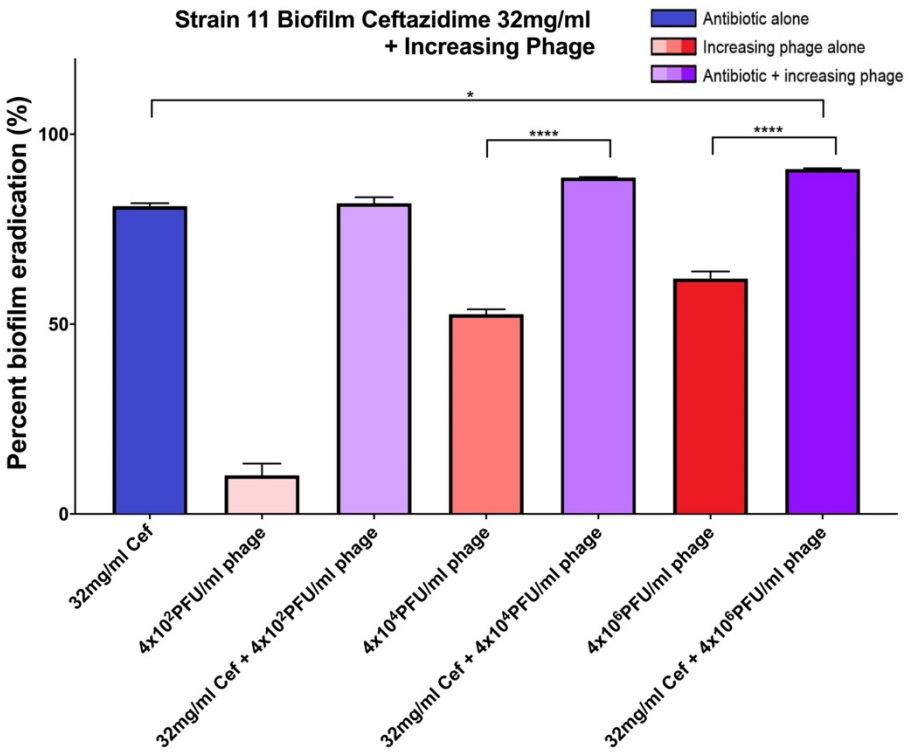
I spent some weeks finding optimal antibiotic concentrations to test as each strain demonstrated different breakpoints for both ceftazidime and tobramycin. For some strains, for instance, not much antibiotic killing was observed at 1/16 the published MIC for

tobramycin (0.25 mg/ml), but 1/8 the published MIC (0.5 mg/ml) achieved near total eradication of the Pa in the broth of planktonic culture. Other strains, especially in biofilm culture, demonstrated hugely varying rates of sensitivity to the antimicrobials used in this section, from sub-MIC values to many times the published MIC. I capped our biofilm treatments at 8x the published MIC for Pa – 64 mg/ml ceftazidime and 32 mg/ml tobramycin, respectively. For phage, given that the neat cocktail provided to us was at a concentration of $1x10^9$ PFU/ml, I opted for three concentrations: $1x10^6$ PFU/ml, $1x10^4$ PFU/ml and $1x10^2$ PFU/ml, representing 3, 5, and 7 log_{10} serial dilutions, respectively.

Initial results demonstrated that there were too many parameters to be able to successfully categorise the effects of combination therapy in a meaningful way. Excessive antibiotic or excessive phage prevented any additivity or synergy being demonstrated, as seen in Figure 4.4.2 (b). Results for one clinical strain demonstrating synergy in biofilm culture are shown in Figure 4.4.2.



(a)



(b)

Figure 4.4.2. Checkerboard output for Strain 11 in biofilm culture with ceftazidime and phage. (a) Results of adding three different concentrations of bacteriophage to 16 mg/ml ceftazidime showed that there was a synergistic effect at $1x10^4$ PFU/ml and $1x10^6$ PFU/ml. The added biofilm eradication at $1x10^2$ PFU/ml did not reach significance. (b) Results for adding the three concentrations of bacteriophage to 32 mg/ml ceftazidime showed that there was an over 80% reduction in biofilm biomass using the ceftazidime alone, which made any additional benefits to the addition of phage more difficult to appreciate. Results shown here comprise 6 technical replicates. One-way ANOVAs correcting for multiple comparisons; * <0.05, ** <0.01, ***<0.001, ****<0.0001.

The above figure demonstrates the importance of choosing antibiotic and phage concentrations, and although the additional effects in terms of biofilm biomass reduction were seen at both concentrations of ceftazidime, the efficacy of the ceftazidime on its own at a concentration of 32 mg/ml meant that statistical significance was lost when combining the means of all 3 biological replicates. I therefore opted to choose one concentration of both antibiotic and phage on which I performed statistical analysis and discerned the effects of combination therapy. For antibiotics in planktonic and biofilm culture, I opted for the first two-fold dilution below the observed MIC (either eradication of planktonic growth or eradication of biofilm biomass), as long as this value achieved <75% reduction in OD from positive controls to be able to optimally appreciate the effects of combination therapy. For

bacteriophage, I used the same principles, but as I worked with concentrations of 10-fold serial dilutions, I used the first dilution below the breakpoint (either eradication of planktonic growth or eradication of biofilm biomass), as long as this value achieved <75% reduction in OD from positive controls to be able to optimally appreciate the effects of combination therapy. For biofilm culture, there were instances where highest antibiotic and phage concentrations did not achieve >75% biofilm eradication. In these instances, I assessed the highest concentration tested. Applying these criteria to Strain 11, as seen in Figure 4.4.2, resulted in a selection of ceftazidime at 16 mg/ml and bacteriophage at $1x10^6$ PFU/ml (for this strain, I did not identify a phage concentration that was seen to eradicate >75% of the biofilm on its own, so the highest concentration tested was used).

4.5 RESULTS

The checkerboard assay was organised such that rapid visual assessment of additivity or inhibition, both in planktonic and biofilm culture, was sometimes possible. Results for the crystal violet assay in a strain in which there is a loss of phage efficacy when combined with tobramycin as compared to phage alone is shown as an example in Figure 4.5.1.

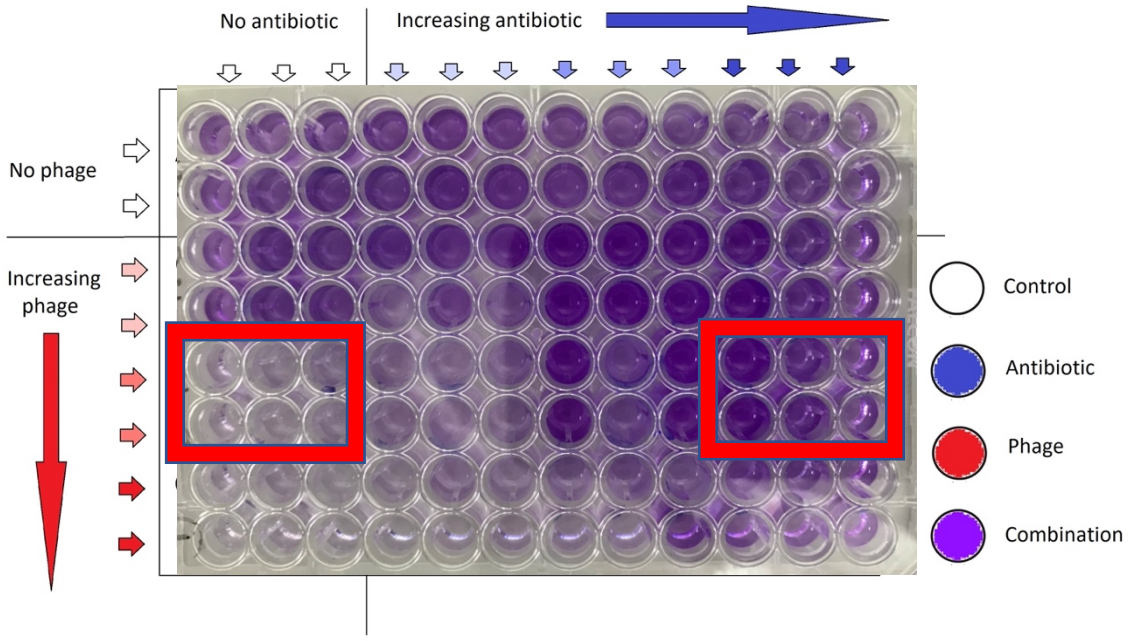


Figure 4.5.1. Photo of the crystal violet assay from one biological replicate for Strain 15. This demonstrates the inhibitory effects of tobramycin on phage. Moving across the plate left to right is increasing tobramycin. Moving down the plate is increasing phage. Each treatment group has 6 technical replicates and the two treatment groups that best demonstrate this phenomenon are boxed in red (left box = phage alone; right box = phage +

tobramycin). In this photo, it is easily seen that bacteriophage is efficacious at eradicating biofilm, as shown by less purple crystal violet staining. With the addition of tobramycin, however, the bacteriophage is no longer able to exert the same effect, as shown by the darker purple crystal violet staining denoting increased biofilm biomass.

Broadly speaking, there were three outcomes of the experiments in this section which are depicted below: additive (Figure 4.5.2), inhibitory (Figure 4.5.3) and no effect (Figure 4.5.4).

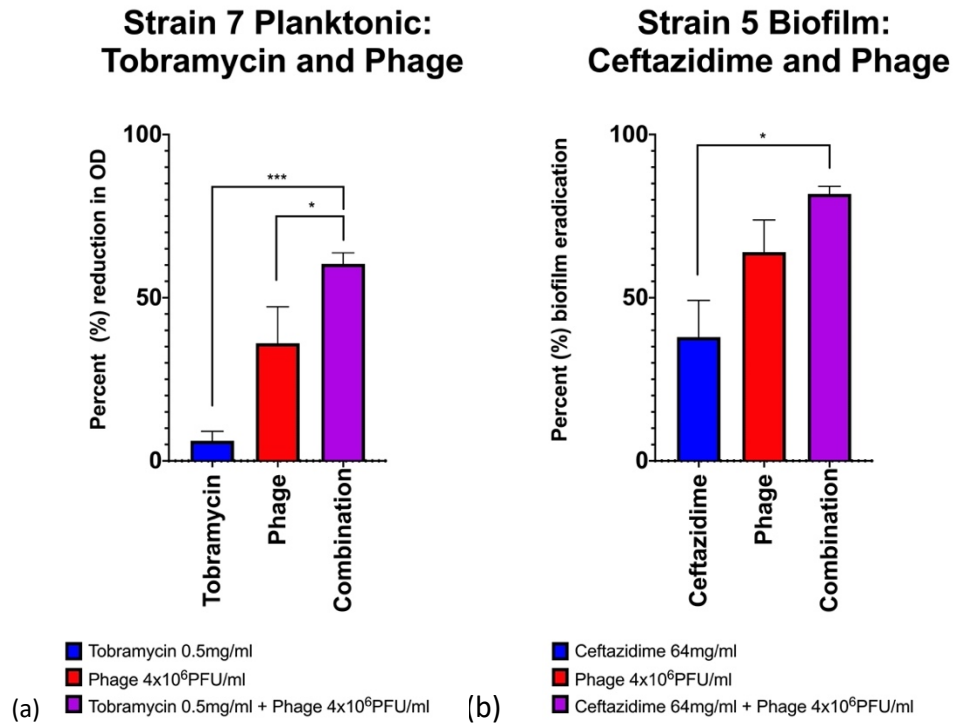


Figure 4.5.2. Two different clinical strains demonstrating the additive effects of combining individual antibiotics and phage.
Results depicted are for 3 biological replicates combining 6 technical replicates in each group. One-way ANOVAs correcting for multiple comparisons; * <0.05, ** <0.01, ***<0.001. (a) Strain 7 demonstrates synergy of effect in the addition of phage at $1x10^6$PFU/ml to tobramycin at 0.5mg/ml in planktonic culture. (b) Strain 5 demonstrated the additive effects in the addition of phage at $1x10^6$PFU/ml with ceftazidime 64mg/ml in biofilm culture.

In the case of tobramycin-phage in planktonic culture for Strain 7 (Figure 4.5.2 a.), effects were seen to be strongly synergistic, CDI = 0.43±0.07. In the case of ceftazidime-phage in biofilm culture for Strain 5 (Figure 4.5.2 b.), group means were statistically different, but results were simply additive. When group means showed a significant reduction in the efficacy with combination therapy compared to single therapy (antibiotic or phage alone), results were said to be inhibitory/antagonistic and examples are shown in Figure 4.5.3.

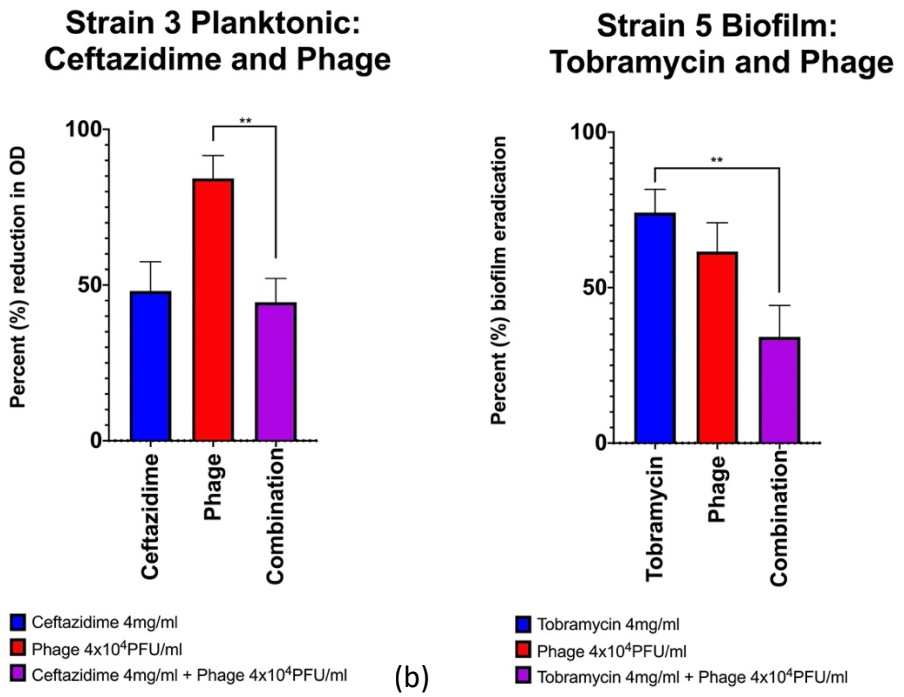


Figure 4.5.3. Two strains demonstrating different types of inhibition when combining individual antibiotics and phage.
(a) Strain 3 in planktonic culture demonstrated antagonism of the action of phage when ceftazidime at 4mg/ml was added. (b) Strain 5 in biofilm culture demonstrated antagonism of the action of tobramycin when phage at $1x10^4$PFU/ml was added. Results depicted are for 3 biological replicates combining 6 technical replicates in each group. One-way ANOVAs correcting for multiple comparisons; * <0.05, ** <0.01, ***<0.001.

It is important to note that if the effects were inhibitory, this could have meant either that (i) the antibiotic reduced the efficacy of phage or (ii) the phage reduced the efficacy of the antibiotic.

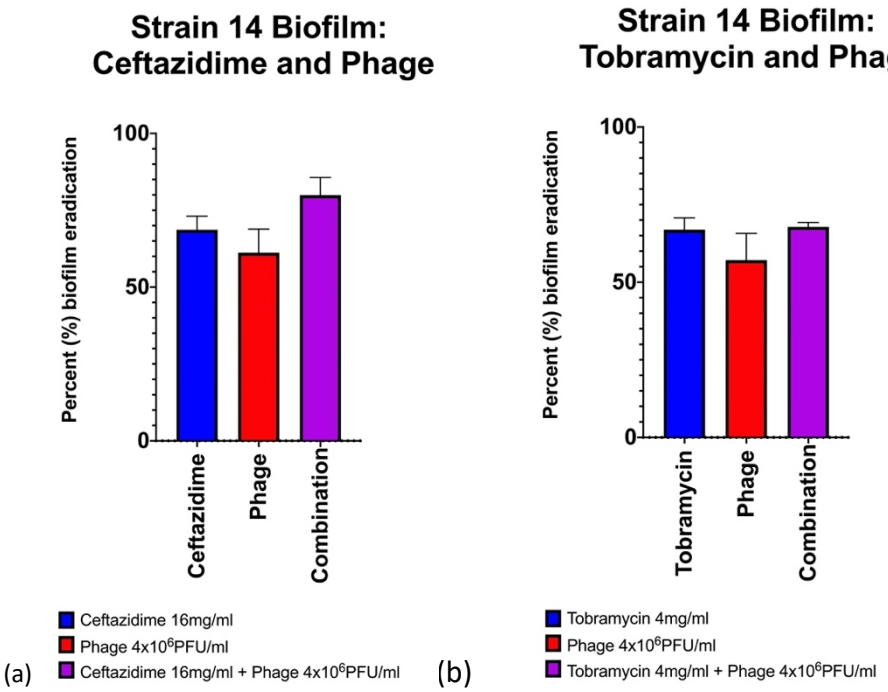


Figure 4.5.4. Example of biofilm results for Strain 14 combining phage with ceftazidime or tobramycin.
(a) There was no significant reduction in biomass when phage at $1x10^6$PFU/ml was added to ceftazidime at 16mg/ml. Although the addition of phage appears to favour more biofilm eradication than with the ceftazidime alone, results did not meet significance. (b) Results for Strain 14 showing that there is no additive effect of the biofilm eradicating effects of bacteriophage $1x10^6$PFU/ml when added with tobramycin 4mg/ml. Results depicted are for 3 biological replicates combining 6 technical replicates in each group. One-way ANOVAs correcting for multiple comparisons; * <0.05, ** <0.01, ***<0.001.

Many individual strain results lost significance when means of the 3 biological replicates were combined. This is the case for ceftazidime-phage in biofilm assay for Strain 14 (Figure 4.5.3 a.). A summary of all outcomes for individual strains for combination antibiotic therapy in planktonic and biofilm culture are shown in Table 4.5.1.

Table 4.5.1. Summary data for checkerboard experiments across 10 clinical strains + PA01.

Antibiotic	**Ceftazidime**		**Tobramycin**	
Culture type	**Planktonic**	**Biofilm**	**Planktonic**	**Biofilm**
PA01	A	S	A	N
Strain 1	N	N	N	N
Strain 3	I*	N	N	N
Strain 4	A	N	N	N
Strain 5	A	A	A	I#
Strain 7	A	A	S	N
Strain 9	N	N	N	N
Strain 10	N	A	N	N
Strain 11	A	S	A	N
Strain 14	A	N	A	N
Strain 15	A	A	N	I*

The above summarises the results for both planktonic and biofilm assays, for which there were 3 biological replicates comprising 6 technical replicates in each treatment group. A = additional benefit for combination of antibiotic + phage; N = No added benefit from combining antibiotic + phage (N denotes that group means were not statistically different); S = synergistic benefit to adding antibiotic + phage; I = inhibitory effect of combination of antibiotic + phage. Colour coding to enable simpler visualisation; dark green = synergistic; light green = additive; yellow = no significant benefit; red = antagonistic. * indicates antibiotic was antagonistic to efficacy of phage; # indicates that phage was antagonistic to the effects of the antibiotic.

With planktonic cultures, combination antibiotic-phage therapy resulted in a higher rate of bacterial eradication 54.5% of the time (95% CI; 34.7-73.1%), whereas in biofilm culture this was only seen 27.3% of the time (95% CI; 13.2-48.2%). For individual antibiotics, combination ceftazidime-phage therapy resulted in a statistically higher rate of bacterial eradication 59.1% of the time (95% CI; 38.7-76.7%), whereas for tobramycin this was 22.7% (95% CI; 10.1-43.4%). Notably, in biofilm, there were no strains that demonstrated a significant additivity of effect with phage-tobramycin therapy, whereas 6/11 strains tested (54.5%) demonstrated additivity or synergy with ceftazidime-phage combination therapy. In the cases where synergy was demonstrated, CDIs were as follows: Strain PA01 with ceftazidime-phage in biofilm culture, CDI = 0.44±0.10; Strain 7 with tobramycin-phage in planktonic culture, CDI = 0.43±0.07; and Strain 11 with ceftazidime-phage in biofilm culture, CDI = 0.58±0.12. Two of the 3 instances in which phage-antibiotic antagonism was seen were due to reduced efficacy of phage when combined with the antibiotic. There was one instance where combination

treatment led to reduced antibiotic efficacy (Strain 5, tobramycin-phage in biofilm depicted in Figure 4.5.2 b.).

4.6 DISCUSSION

I demonstrate in this section varying interactions between phage and ceftazidime and tobramycin individually in planktonic and biofilm culture. On the more extreme ends of the spectrum, there were instances of both strong synergy (CDI <0.7) and antagonism for both ceftazidime and tobramycin in both planktonic and biofilm culture. Promising results for combination therapy with a given antibiotic in planktonic culture did not necessarily translate to significant reductions in biofilm biomass. It is worth noting that there were no instances of additivity seen in biofilms with tobramycin for our 10 clinical and 1 laboratory strain. In using a variety of clinical Pa strains from CF airway cultures, working out strain-specific MICs during the experimental set up, using 2 different antibiotics employing different modes of action, and utilizing both planktonic and biofilm culture methods, I had intended to see if a pattern would emerge. Ultimately, however, the 11 strains described in these experiments were very diverse and the final message is one of individual strains interacting differently with antibiotics and phage in different growth conditions.

There has been a large focus in the literature on conditions that inhibit the action of phage, and such studies have largely looked at the application of antibiotic prior (i.e. as pre-treatment) to the administration of phage (283, 285). There is indeed a growing body of research suggesting that the order of application can drastically alter the outcome of antibiotic-phage experiments. One such study investigated the ability of phage treatment to enhance the activity of antibiotics against biofilm-forming *Staphylococcus aureus*, and found that when phage treatment was administered prior to antibiotics, results were additive or synergistic, as opposed to when phage was administered after antibiotics, when results were seen to be antagonistic (299). When designing my experimental protocol, timing of phage and antibiotic administration had been intended to be simultaneous; however, in practice, I had been administering the antibiotic compounds prior to the addition of phage on my 96-well plates and time lags between antibiotic and phage administration could have been as high as 15 minutes for individual plates. In this way, I may have been unintentionally reducing the efficacy of the bacteriophage in both planktonic and biofilm culture, giving the antibiotics time to exert effects leading to antagonism of phage (which was seen in 2 experimental

conditions: once in planktonic culture with ceftazidime and once in biofilm culture with tobramycin; Table 4.5.1). Notably, in only 1 out of the 3 instances of antagonism seen did the combined administration of phage worsen the efficacy of antibiotics alone (this was seen in biofilm culture with tobramycin in Strain 5, Figure 4.5.2 b.).

One obvious criticism of these experiments is the failure of much *in vitro* work to adequately simulate the complexity of bacterial infection in a living organism. The fact that bacteria exist in polymicrobial communities within the lung environment is one such factor (300). This is thought to be one of the reasons for the disconnect between laboratory-based methods of antibiotic sensitivity testing (both in planktonic and in biofilm cultures) compared to clinical outcomes (301). In one experiment using *E. coli* and Pa, the appropriate phage could eradicate each monoculture biofilm, whereas the two bacteriophages applied together were not effective in a mixed biofilm (302). Yet another factor is the environment in which these organisms are grown in the laboratory. In my experiments, organisms were grown in Mueller Hinton broth, which is rich in nutrients essential for the replication and survival of Pa. This provides conditions that are vastly different to the human body environments in which Pa can live – from the lung (where the 10 clinical isolates in this section came from) in the case of lung infection or pneumonia, to urine in the case of a urinary tract infection, to blood serum in the case of bacteraemia. Interestingly, Gu Liu *et al*.'s experiment (2020) involving MDR *E. coli* also tested the combined effects of antibiotic and phage in urine and serum to simulate some of the complexity of human infection, observing a reduction in the efficacy of both antibiotics and phage, which they attributed to lower bacterial densities in urine and serum when compared with broth culture. In some cases, however, they described an attenuation of the synergistic effects that had been seen with certain antibiotics when combined with phage in broth culture (286).

On the basis of the findings in this section, it is difficult to draw conclusions that might affect the design and interpretation of clinical trials exploring phage therapy; however, based on the finding that the aminoglycoside tobramycin was less likely to result in a favourable outcome when combined with phage, it is important that further in vitro work be carried out to investigate this, similar to work that has been carried out exploring antibiotic-phage combinations in other organisms (286, 287). Furthermore, it would be reasonable to perform sub-group analyses for patients involved in upcoming trials with phage to determine the

clinical effect in those patients on chronic suppressive therapy with aminoglycosides and whether this differs to patients on different suppressive therapy (for instance, with inhaled levofloxacin or colistin).

5 CHAPTER 5: ASSESSING IMPACTS OF PHAGE EXPOSURE ON BACTERIAL BEHAVIOURS: A PILOT STUDY

5.1 INTRODUCTION

Pa's large genome (5.5-7 Mb) encodes for an array of proteins including efflux pumps, cell surface proteins, cell membrane transporters and virulence factors amongst others (303). Differential up- and down-regulation of such genes translates to a phenotypic versatility and resilience when faced with environmental changes and challenges, such as those posed by the administration of antimicrobials. This adaptability in the heterogeneity of the lung environment results in niches of phenotypically distinct bacterial communities, which collectively enable Pa to persist despite attempts at eradication. In previous chapters, I looked at this population heterogeneity through the biofilm aggregate structure (with fluctuating levels of nutrients, oxygen, and antimicrobials as well as waste products). The resultant phenotypic heterogeneity was discussed earlier in reference to the reservoirs of bacterial populations within the biofilm that have the ability to withstand high levels of antibiotics. But biofilms are one of many adaptations that Pa can undergo.

One common phenotypic change is proliferation of a mucoid phenotype – through copious production of the exopolysaccharide alginate, which provides a physical barrier to antimicrobials and host defences (110). Early Pa acquisition is typically associated with wild-type strains from environmental sources that are non-mucoid and more easily eradicated with aggressive antibiotic therapy (72). Phenotypic change such as conversion to mucoidy is one example of a survival mechanism that can be driven by factors inherent in the cystic fibrosis lung (110, 111), but also by external factors, such as antibiotic administration, aimed at its eradication (112).

One factor that is believed to underpin this Pa adaptability described above is the spontaneous mutation rate of the bacterial genome due to alterations in the DNA mismatch repair (MMR) system. Strains with a high rate of spontaneous mutation (or hypermutator strains), where mutation rates are up to 1,000-fold above levels that would otherwise be expected, have been discovered in CF lungs (304). One seminal study revealed that 37% of patients with chronic Pa infection were infected with a hypermutator strain (305). The MMR system is an inhibitor of recombination of sufficiently divergent DNA sequences, some of

which can be found in the genomes of related bacteria (306). When this system is suppressed or down-regulated, there is also an increased rate of horizontal gene transfer from other populations of bacteria (307). By way of summary, it seems that these strains are better able to produce a phenotypically diverse set of progeny as a result of (i) an elevated mutation rate and (ii) acquisition of genes from external sources. High spontaneous mutation rates have also been seen in other CF lung pathogens, including *Staphylococcus aureus* and *Haemophilus influenzae*, and this is therefore believed to be a predominant mechanism of adaptability in pathogens that form the basis of human disease (308). Correspondingly, antibiotic sensitivity testing has also demonstrated higher rates of antimicrobial resistance (AMR) in strains with a high endogenous spontaneous mutation rate (304). It is, therefore, important to acknowledge the role that the aggressive therapies prescribed in a clinical setting may play in selecting for isolates with a genetic predisposition for mutation and adaptability. Aggressive antimicrobial therapy administered to combat lung infection and decline poses a strong selection pressure for driving bacterial phenotypic change.

Transcriptomics is the field that deals with the entire set of RNA transcripts for a particular cell or organism at a given time point (309). The use of such high-throughput methods has the potential to analyse the expression of all transcripts at a given time-point and compare them between different physiological conditions or treatments. The use of transcriptomic analysis on Pa has yielded some very interesting – and clinically insightful - findings with regards to standard antibiotic treatments. One study using transcriptomic technology demonstrated that subinhibitory concentrations of imipenem (a carbapenem, one of the ß-lactam antibiotics) in Pa culture resulted in upregulation of *ampC*, which encodes beta-lactamase production (310). Furthermore, researchers in this study demonstrated an elevated expression of genes involved in the expression of alginate, one of the biofilm exopolysaccharides involved in the phenotype of mucoidy. Transcriptomic analysis of Pa has also been carried out on cells exposed to ceftazidime, another ß-lactam (one treatment in the experiments carried out in Chapters 3 and 4), and found increased expression of genes relating to mutagenesis (311) and a decrease in the production of a range of QS-regulated virulence factors involved in biofilm formation and maintenance (312). For the aminoglycosides (of which tobramycin, one of the antibiotics used in Chapters 3 and 4, is a

member), the MexXY efflux system has been shown in several independent transcriptomic analyses to be induced by sub-inhibitory concentrations in wildtype Pa (313, 314).

Pa, therefore, can modulate the expression of genes in a response that is tailored to the antimicrobial being administered. In these experiments, I wished to explore the impact of bacteriophage on phenotypic expression and see how this translated to gene transcription in both biofilm and planktonic culture. To select an appropriate strain for this experiment, I first performed phenotypic analysis using a Congo Red agar method to identify strains that changed phenotypic expression with topical application of phage. For the second part of this section, I was given a small budget for a transcriptomic pilot involving one Pa strain in 4 different experimental conditions. For the transcriptomic analysis, I wished to explore how bacteriophage might be able to influence bacterial phenotype through the expression of different genes, quantified by RNA transcript readouts for one clinical strain.

5.2 HYPOTHESIS

i) Topical application of phage may drive phenotypic change in mucoid status in some Pa strains visible with congo red staining;

ii) Differences will be detectable at the transcriptomic level for genes involved in virulence/phenotypic change in both planktonic and biofilm cultures for one Pa strain which has been exposed to sublethal concentrations of phage, after careful strain selection based on a) susceptibility to the phage cocktail in both planktonic and biofilm culture and; b) phenotypic change observed after topical application of phage on Congo Red agar

5.3 AIMS AND OBJECTIVES

- Observe presence of phenotypic differences with the topical application of bacteriophage and antibiotic combination across 16 clinical strains using the Congo Red agar assay
- For any strains that demonstrate this phenotypic change, carry out further work in planktonic and biofilm culture for the selection of one strain treated with sub-inhibitory concentrations of phage for transcriptomic analysis
- Perform RNA extraction for the chosen strain in planktonic and biofilm culture with and without the addition of phage for analysis by the Imperial BRC Genomics Facility

- Involve an external statistician to generate a list of genes with significantly increased and decreased expression within each experimental protocol
- Investigate whether the list of genes identified by the statistician fit into known biological pathways when mapped onto the genome of PA01 with online software

5.4 METHODS

CONGO RED AGAR

Using Congo Red dye in agar was first described by Freeman *et al*. in 1979 to visualise the production of exopolysaccharides in coagulase negative staphylococci (315). Pa is known to produce exopolysaccharide *pel*, which is a Congo-red-binding component of the biofilm matrix (316). Coomassie Brilliant Blue is also a dye that has been used to indicate increased biofilm formation; however, in this case the dye binds to proteins and DNA-rich components of the biofilm matrix (317).

An overnight culture of all 16 clinical strains (OD600 = ~1.0) was grown in a tryptone broth medium (10 g/L tryptone; Oxoid) and then spotted in 15 µL drops onto Congo Red plates (10 g/L tryptone, 40 µg/mL Congo Red, 20 µg/mL Coomassie Brilliant Blue, and 1.5% agar) and left in the safety cabinet for 15 minutes to dry (Congo Red and Coomassie Brilliant Blue purchased from ThermoFisher Scientific and Tryptone and agar purchased from Oxoid). Ten (10) µL of either PBS for control, bacteriophage (neat cocktail = $1x10^9$ PFU/ml) in descending log_{10} serial dilutions, or an antibiotic solution (ceftazidime 16 mg/ml + tobramycin 8 mg/ml) in 2-fold serial dilutions were spotted onto the dried overnight bacterial spot and dried for a further 15 minutes in the safety cabinet before inversion and incubation in the dark overnight at 37° C.

These experiments were repeated 3 times to ensure predictable phenotypic expression with phage.

SELECTION OF STRAIN

Three strains (1, 10 and 13) demonstrating phenotypic change when grown on Congo Red agar in the presence of phage were selected for further analysis of overnight growth curves. All wells of a 96 well plate were inoculated with 180 µL of the respective strains, along with 20 µL of PBS for control wells, and bacteriophage from the neat cocktail ($1x10^9$ PFU/ml) to the 10^{-6} serial dilution ($1x10^3$ PFU/ml).

Strain 10 was selected on the basis of overnight growth curves in planktonic culture on the plate reader (OD_{600} readings every 15 minutes, set at 37° C and shaking at 200 rpm for 18 hours).

SELECTION OF PHAGE CONCENTRATIONS AND TIME FOR HARVEST

I selected a phage concentration for Strain 10 when grown in both types of culture that did not eradicate growth, but led to a discernibly lower OD (for planktonic culture) and lower staining with crystal violet (for biofilm culture). This concentration was $1x10^6$ PFU/ml and an 8 hour time-point was chosen for harvest in both culture models.

RNA EXTRACTION AND GENERATION OF DATA

Overnight culture of Strain 10 was grown in Nutrient broth (Oxoid) overnight at 37°C shaking at 200 rpm. For the planktonic assay, 180 µL of culture diluted to an OD of 0.05 was added to 6 wells of a 96 well plate. In 3 wells, 20 µL PBS was added as a negative control and in the 3 remaining wells, 20 µL of bacteriophage ($1x10^6$ PFU/ml) was added before harvest at 8 hours. For biofilms, 6 wells were filled with 200 µL of overnight culture diluted to an OD of 0.05 and grown static for 24 hours to allow cells to adhere to the sides of the wells. At 24 hours, wells were emptied by pipette, cleaned twice with PBS, and then 180µL of Nutrient broth was added to all 6 wells. In 3 wells, 20 µL PBS was added as a negative control and in the 3 remaining wells, 20 µL of bacteriophage ($1x10^6$ PFU/ml) was added for a further 8 hours before harvest. For biofilm harvest, planktonic growth was removed by pipette and remaining cells were scraped from the side of the wells with a cell scraper.

I performed RNA extraction using the Qiagen RNEasy Mini Kit according to the manufacturer's instructions (Qiagen, Toronto, Canada). In brief, biological samples (12 samples, 3 in each experimental group; planktonic control and planktonic + phage, biofilm control and biofilm + phage) were taken from a 96-well plate and lysed and homogenized in the presence of a guanidine-thiocyanate–containing buffer, which denatured and inactivated any RNAses to ensure purification of intact RNA. Ethanol was added to optimise binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA bound to a silica-based membrane and contaminants were washed away. RNA was then eluted in 30–100 µl water. The concentration of RNA was determined by measuring the absorbance at 260

nm (A260) in a spectrophotometer and all 12 samples had values of >0.15, indicating purity (318).

Samples were then stored on dry ice and transported to the Imperial BRC Genomics Facility (IGF) for sequencing. The following is a description provided by the IGF on the analysis they performed on the 12 RNA samples:

Each RNA sample was quantified using Qubit and run on a Tapestation to assess sample integrity. For each pool of libraries, Tapestation was used to assess fragment distribution and presence of artefacts. Each submitted pool was also quantified by Qubit and/or qPCR prior to sequencing. Sequencing was performed using a HiSeq4000. A total of 1 lane(s) was run as 75 base-Paired End. Sequencing data were quality checked using FastQC, and alignment performed with TopHat2. The final analysis generated a gene count table comprising differentially expressed genes and PCA plots files using HTSeq and DESeq2 respectively. The

FastQC files were used in subsequent statistical analysis.

STATISTICAL ANALYSIS

The following is an account of the methodology used by Dr. Sara Fontanella (Department of Medicine, National Heart and Lung Institute, Imperial College London). She has written the following paragraph for inclusion in my work:

FASTQ files were aligned using the R package *Rsubread* (319) and, using the function *featureCounts*, we quantified mapped reads to genes. Statistical analysis was, then, performed using the edgeR (320) and limma (321) R/Bioconductor packages. To adjust for differences in library-size, counts-per-million (CPM) were computed for each gene. Moreover, to filter out noise, genes with an expression level below 0.5 CPM in less than 7 samples were removed from the analysis and CPM data were normalized per the trimmed mean of M-values (TMM) normalization method (322). Similarities between samples were, then, assessed using multidimensional scaling (MDS) on the log2 (CPM). After pre-processing, limma-voom (323) was used for differential expression analysis of the gene-level RNA-seq data. The linear models (324) were fitted to the log2(CPM) and the Benjamini–Hochberg (FDR) (325) multiple-testing correction at 5% was used for all DE genes. The end result of this statistical analysis is

the generation a list of genes that have significantly increased or decreased expression for a given experimental condition.

BIOLOGICAL PATHWAY ANALYSIS

With the results generated by Dr. Sarah Fontanella in the previous section, upregulated and downregulated genes were fed into DAVID, an online software that is free to use and developed and maintained by the NIH in the USA (326). I analysed only transcripts with a minimum 2-fold change expression (q value <0.05). Without the complete genome of the clinical Strain 10 known, we used PA01 as the reference genome in this analysis.

5.5 RESULTS

CONGO RED AGAR

There were 3 strains that demonstrated significant phenotypic change with topical application of sub-inhibitory concentrations of bacteriophage on Congo Red agar, and these are shown in Figure 5.5.1.

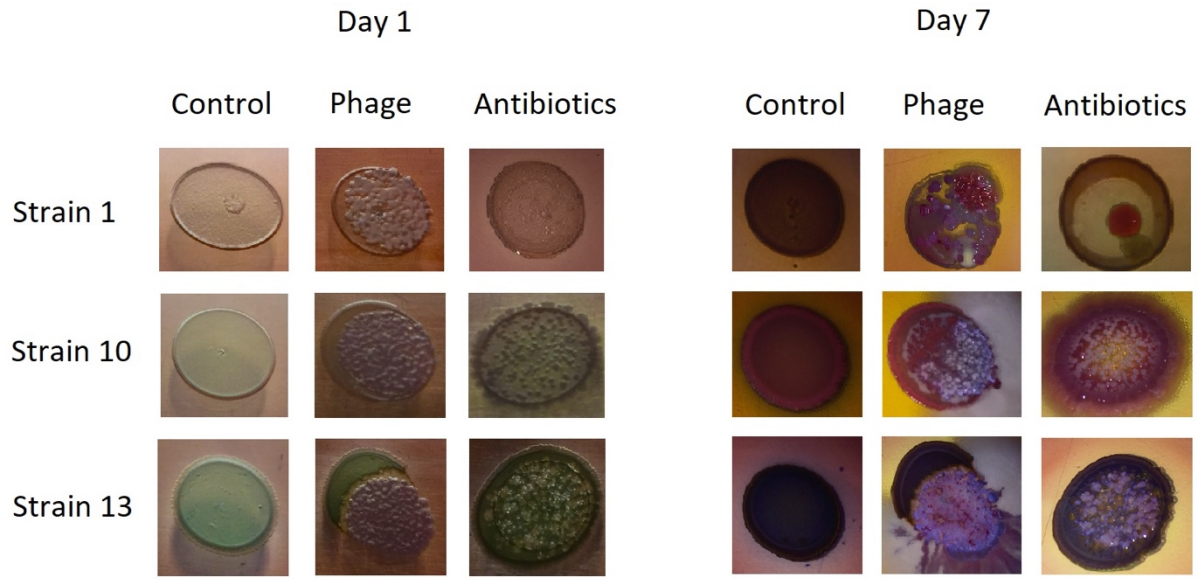


Figure 5.5.1. Congo Red agar on Day 1 and 7 for 3 strains that demonstrated phenotypic change with phage.
(n=3 experiments). Results are shown in 3 columns – Control, with only topical application of PBS solution used to dilute phage and antibiotic mixture; phage, in which the first sub-inhibitory dilution of the neat phage solution is shown (original solution $1x10^9$ PFU/ml); and Antibiotics, in which the first sub-inhibitory dilution of the combination of ceftazidime and tobramycin (original mixture ceftazidime 16 mg/ml + tobramycin 8 mg/ml). Increased red staining with phage and antibiotics shows increased production of exopolysaccharides, and

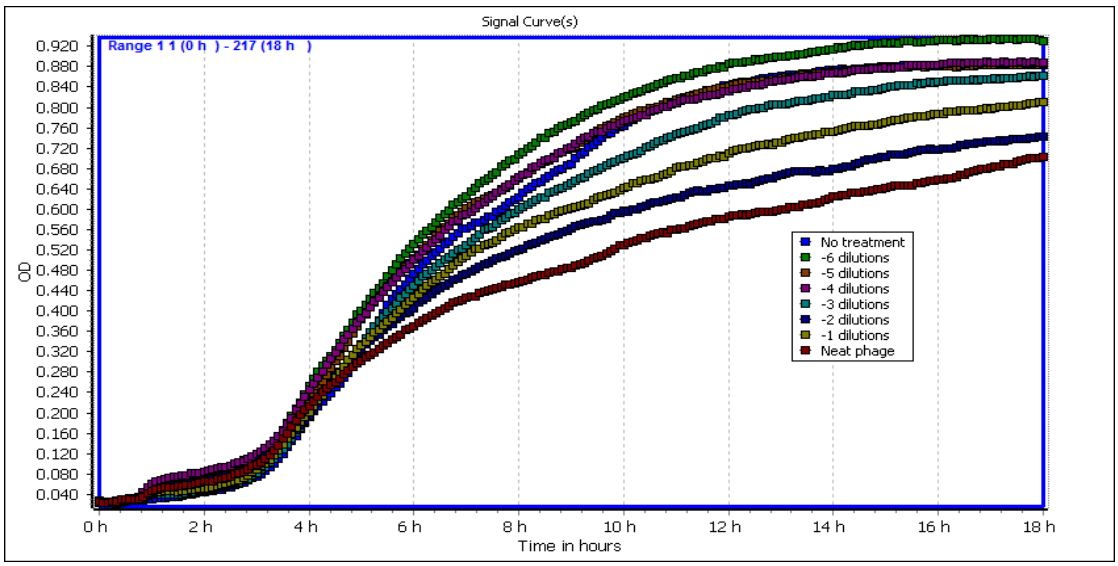
increased alginate production is suspected to be the white substance on day 7 for the phage panel in all 3 strains.

The 3 strains that demonstrated consistent phenotypic change on Congo Red agar across 3 experiments were strains 1, 10 and 13. These strains demonstrated increased productions of exopolysaccharides involved in biofilm formation as well as a white substance suspected to be alginate as it had a mucoid sheen when examined under light. These 3 strains were subjected to further analysis with bacteriophage at different dilutions for growth curve analysis with the plate reader.

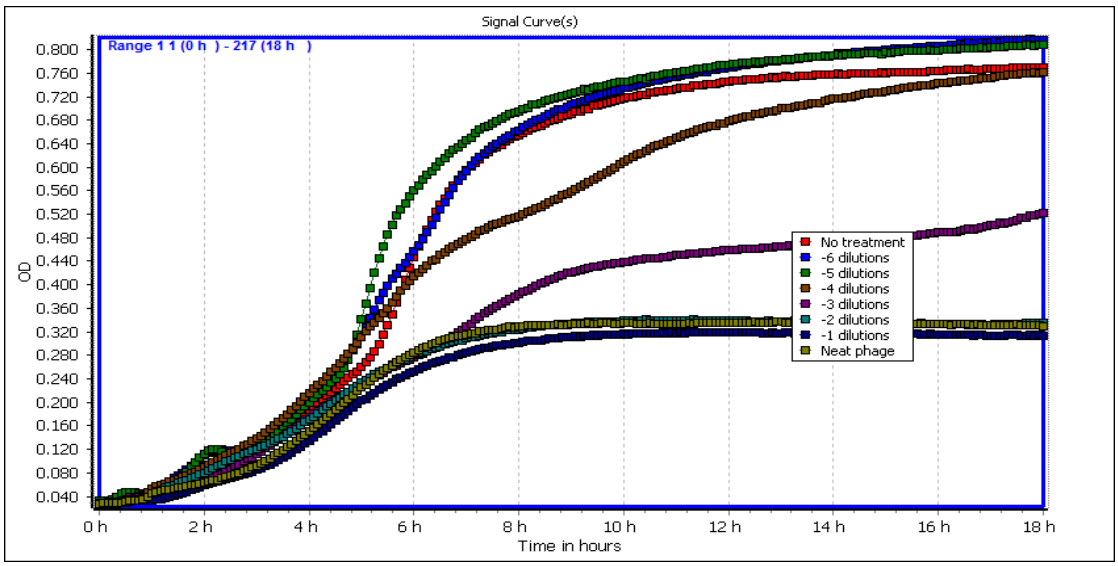
SELECTION OF STRAIN

Growth curves for Strains 1, 10 and 13 are shown in Figure 5.5.2 a., b., and c., respectively.

(a)



(b)



(c)

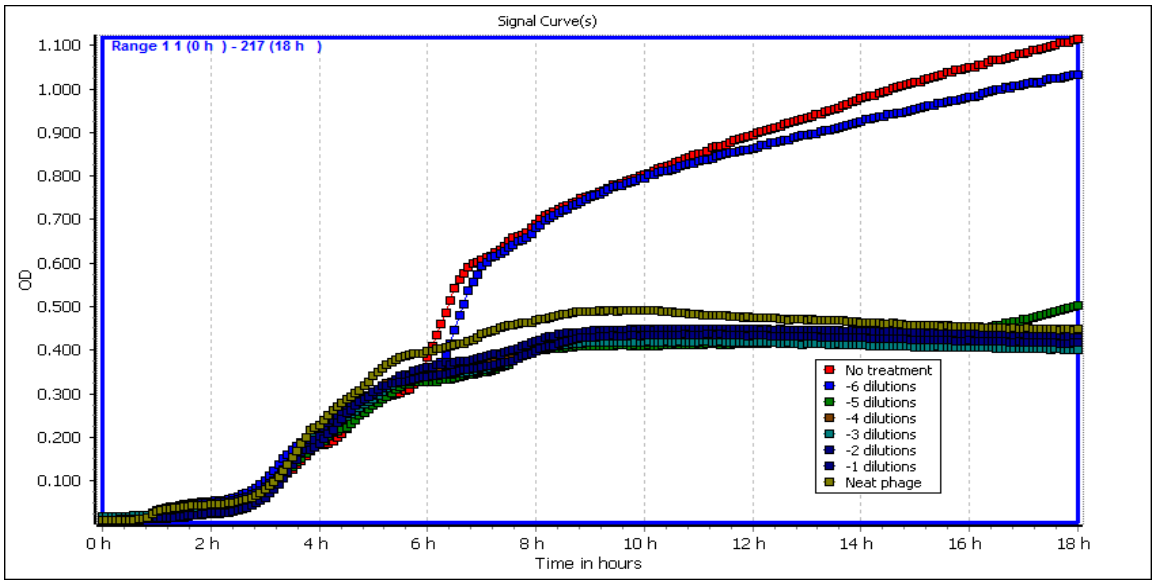


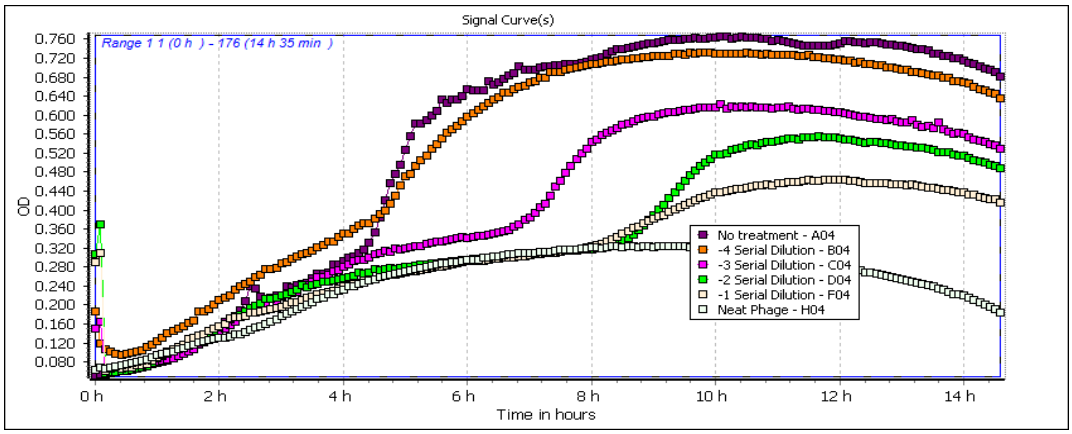
Figure 5.5.2. Overnight growth curves for Strains 1 (a), 10 (b) and 13 (c).
Coloured dots are OD_{600} readings taken every 15 minutes and are averages of 8 treatment wells. (a) The treatment effects of the different dilutions of the phage cocktail were difficult to discern in this overnight growth curve for Strain 1. (b) Strain 10 demonstrated a significant treatment effect at 10^{-3} dilutions ($1x10^{6}$ PFU/ml); however, there was still Pa growth extending to the end of the 18 hour period. (c) Strain 13 demonstrated sensitivity to the phage cocktail at all but the 10^{-6} dilutions.

On the basis of these overnight growth curves, I selected Strain 10 for further analysis in the transcriptomic pilot project. Of the three strains demonstrating phenotypic change with subinhibitory topical application of bacteriophage, it was the only strain for which there was a clearly distinct OD readout for different dilutions of phage, where it was clear that the bacteria were still actively dividing.

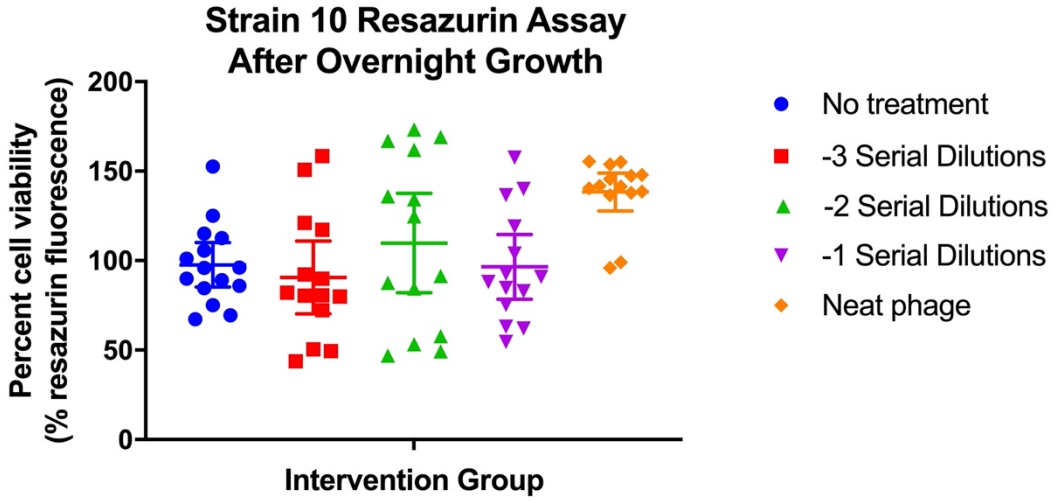
SELECTION OF PHAGE CONCENTRATIONS AND TIME POINT FOR HARVEST

Further work for selection of concentrations and time points for harvest are shown in Figure 5.5.3.

(a)



(b)



(c)

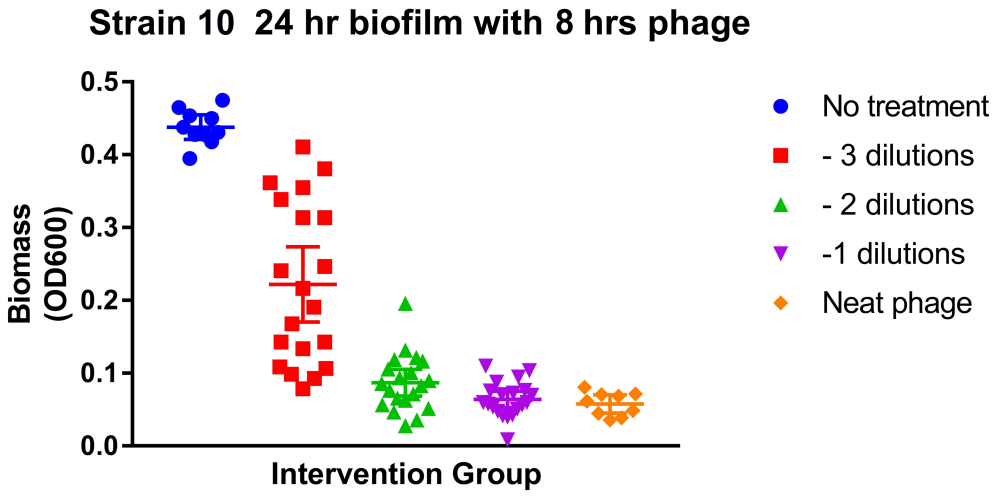


Figure 5.5.3. Analysis of Strain 10 with growth curve and resazurin assay, as well as crystal violet biofilm assay.
Results show the neat phage cocktail ($1x10^9$ PFU/ml) as well as 3 ten-fold serial dilutions in different culture assays. (a) Growth curve for Strain 10 is shown, once again demonstrating a treatment effect at 10 dilutions ($1x10^6$ PFU/ml). Coloured dots are averages of 16 replicates on the 96-well plate. (b) A resazurin cell viability assay at the end of 18 hours overnight incubation demonstrating the disconnect between OD readouts and amount of fluorescent

signal. N=16, error bars are means ± SD (c) Twenty-four hour biofilms subjected to lytic phage for 8 hours showing biomass quantification with crystal violet assay. N=16, error bars are means ± SD.

Based on the above information, I selected an 8 hour time point for harvest in both planktonic and biofilm assays at a concentration of $1x10^6$PFU/ml. Notably, the increased cell viability immunofluorescent signal compared to negative (no treatment) control wells for Strain 10, which had been mentioned in Chapter 3, is illustrated nicely after overnight planktonic growth in Figure 5.5.3 b.

RNA EXTRACTION

The figure in this section were provided by the Imperial BRC Genomics Centre on the 12 purified RNA samples that I had provided to them. The quality readout is summarised in Figure 5.5.4.

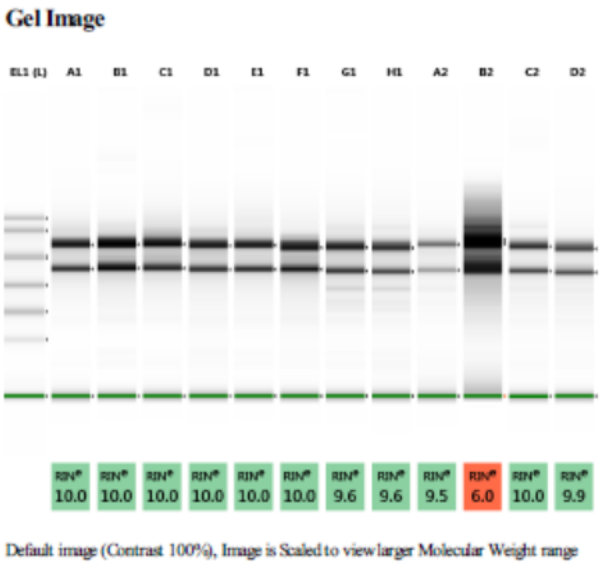


Figure 5.5.4. RNA integrity numbers (RINs) for different treatment groups.
The above figure shows the RNA integrity numbers (RINs) for the different treatments (N = 12 columns, 3 columns per treatment with 4 treatment groups: A1-C1 planktonic no phage, D1-F1 planktonic with phage, G1-A2 biofilm no phage, B2-D2 biofilm with phage). Apart from one sample (column B2 on gel) with RIN of 6, which is deemed acceptable, all other RINs were good quality (between 7-10).

The output file received from the Imperial BRC Genomics Centre was in the format of a FastQc file, which was shared with Dr. Sara Fontanella for work in the following section.

WORK WITH STATISTICIAN

The work in this section was completed by Dr. Sara Fontanella in the statistics department. Her work identified the genes with significant (2-fold or more change in expression, q value <0.05). Differential expression in all 4 experimental conditions can be seen in the heatmap with dendogram in Figure 5.5.5.

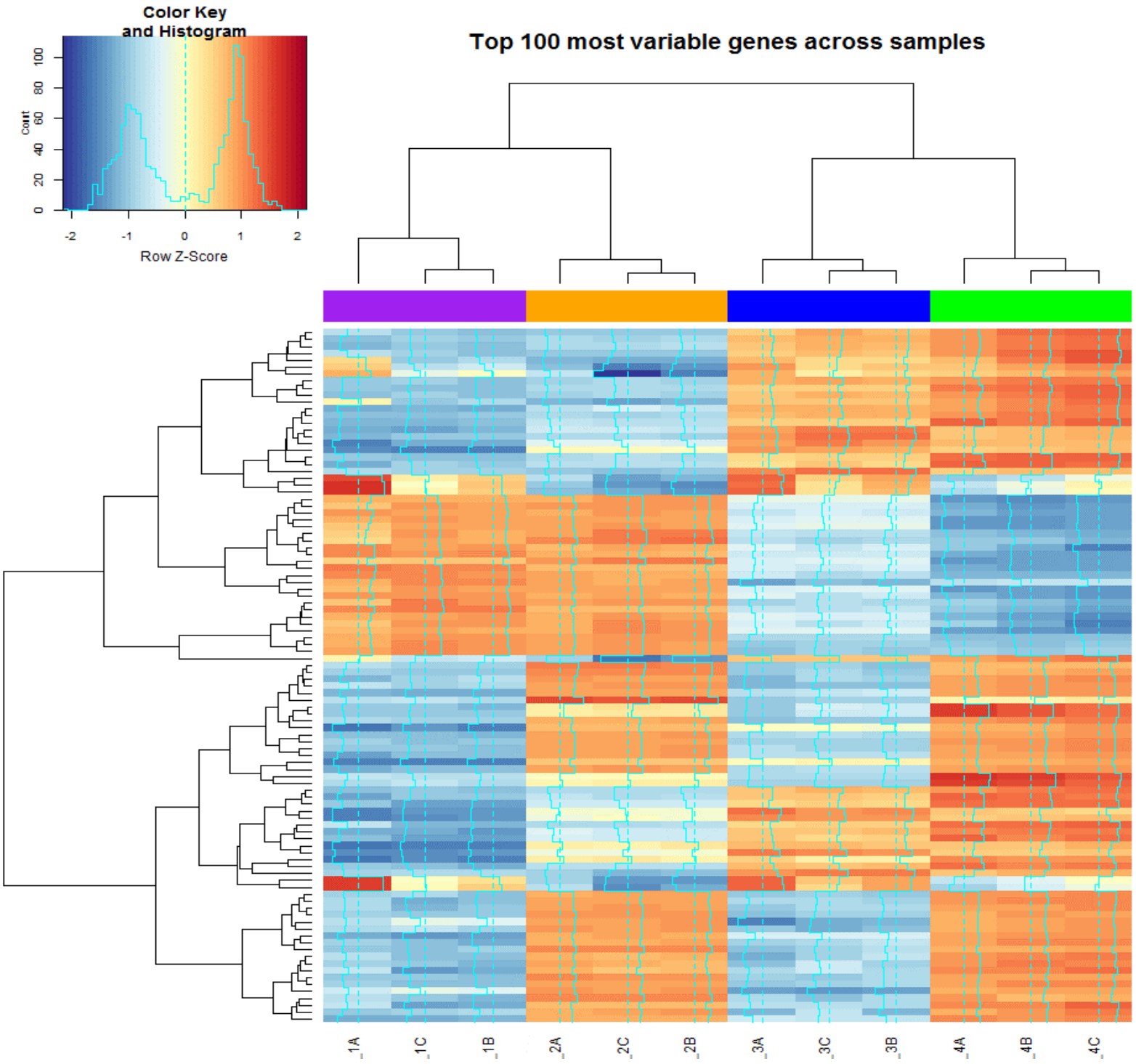


Figure 5.5.5. Heatmap with cluster dendrogram demonstrating top 100 differentially expressed genes for different treatment groups.

Groups are planktonic control (purple, 1A-C), phage-exposed planktonic Pa (orange, Phage 2A-C), biofilm control (blue, 3A-C) and phage-exposed biofilm Pa (green, Phage 4A-C). Red-orange colour indicates increased expression and blue indicates decreased expression. Individual genes are represented by horizontal lines. Pa gene expression was similar between the 3 replicates in all groups.

Following phage exposure, a minority of Pa genes (total number of genes = 5570) showed increased expression in planktonic (n=95), biofilm (n=107) and both (n=59) conditions subjected to phage when compared to controls. Decreased expression was seen in 93, 38 and 14 genes respectively.

BIOLOGICAL PATHWAY ANALYSIS

Using these readouts with DAVID online software significant changes in multiple pathways in phage treated samples. In both bacteriophage treatment groups, genes involved in alginate biosynthesis (*algX*, *algJ*, *algF*, *algE*, *algB*; p=0.0003), those involved in a two-component regulatory system involved in reacting to environmental changes (*algB, pfeR, phoB, phoR, pfeS*; p=0.03), genes involving antibiotic resistance (*arnD, arnA, pvdT*; p=0.03) and genes involved in the transport of molecules across the cell membrane (*aqpZ, pfeA, fepD, fliI, hitB, norB, potC, potA, pvdT, xqhA, phoB, phoR*; p=0.04) were all upregulated. Downregulated were genes associated with the cell inner membrane and the transport of molecules across the cell membrane (*nuoA, nuoH, nuoD, cyoB, lepA, fliP, mexA, ponA, kdpA, rne, mucA, sdhD, tolQ, cmaX*; p=0.003), and genes involved in the cell outer membrane (*omlA, oprL, oprF, cupC3*; p=0.03).

5.6 DISCUSSION

In these experiments, I saw that phenotypic expression changed for 3 of the 16 (18.8%) clinical strains with topical application of subinhibitory bacteriophage on Congo Red agar. Phenotypic change was also seen with topical antibiotic administration; however, I did not observe conversion to a more mucoid phenotype with topical antibiotics. I was given a sum of money sufficient to carry out a small pilot study to generate a transcriptomic data set in 4 different conditions for one Pa clinical strain. In these experiments, I demonstrate significant changes in the phenotypic and transcriptomic profile of one clinical Pa strain in response to sub-inhibitory concentrations of bacteriophage in biofilm and planktonic culture.

The transcriptomic results showing upregulation of genes involved in alginate biosynthesis do support the phenotype of mucoidy seen on Congo Red agar. Additionally, downregulation of outer membrane proteins could represent a survival response to phage in these experiments. Although a small pilot study on one strain under very specific conditions, the results of these experiments highlight the potential for bacteriophage therapy to drive phenotypic diversity and evolution through in exerting selective pressures on bacteria.

Notably, I was restricted by costs in the number of treatment groups I could include in this analysis and it is therefore difficult to know whether the findings are specific to the phage and Pa strain used in these experiments or are able to be generalised across other clinical strains for subinhibitory concentrations of phage. It is possible that the observed transcriptomic responses of Pa to phage seen in these experiments are general responses seen in Pa when subjected to any environmental pressure. It is therefore important to carry out further transcriptomic analysis on a range of Pa strains in response to an array of lytic phage to see if there are commonalities that can be seen. In the setup of these experiments, it would be essential to include controls that have been subjected to other substances that reduce bacterial growth to a similar extent to phage to tease out what are phage specific adaptive responses and what are general survival mechanisms employed by Pa in response to any antimicrobial agents. These results, however nonspecific due to the experimental limitations, are in line with studies showing upregulation of genes involved in alginate production with agents that threaten bacterial survival, studied most frequently with antibiotics (310, 327).

Selection pressure towards mucoidy is not a new finding with regards to phage influencing bacterial phenotype. A study involving *Pseudomonas fluorescens* found that in co-evolution with the lytic phage phi2, there was a selection of a mucoid phenotype as an adaptive response that conferred the bacteria partial resistance to the phage (328). There are indeed other theoretical ramifications in the bacterial evolution of phage resistance having a potential for negative impact in clinical practice. Porin proteins OmpF and OmpC in enteric bacteria like *Klebsiella pneumonia*, for example, play a role in both the permeation and action of ß-lactam antibiotics (329) and also the adsorption of phage (330), a finding that has led some to hypothesise about the potential negative action that the evolution of phage resistance could have on the action of this antibiotic class (331).

Although it is reasonable to see examples of such phenotypic change as a cautionary finding, there is also a growing body of research demonstrating that many of the changes bacteria undergo to evade viral infection come at a cost to overall fitness, including reduced virulence and resensitisation to antibiotics (331). Seed et al. (2014) found that *Vibrio cholerae* isolates from patients in Haiti and Bangladesh demonstrated significantly reduced virulence potential when predated upon by lytic phage (332). In *E. coli* infected with bacteriophage T2, studies have similarly shown that evolution of phage resistance results in fitness trade-offs (333-335).

While it is important to recognise the contribution from the body of *in vitro* work described above, we must also recognise the disconnect between laboratory test and clinical outcome. Nowhere is this more apparent than in the examination of the mucoid phenotype as, counterintuitively, it has also been shown that mucoid isolates are more antibiotic sensitive than their non-mucoid counterparts in laboratory culture, though they clearly are selected for due to survival advantage in the lung (117). A study observing mucoid conversion of *E. coli* in the presence of phage concluded that transient phenotypic changes like mucoidy are less costly to the bacteria than are “fixed” mutations, like cell wall changes, as bacteria reverted back to non-mucoid phenotypes once the concentration of phage declined (336). Bacterial changes like mucoidy, therefore, seem to be transient and reversible. Interestingly, simulations investigating predator-prey interactions between bacteria and phage have shown that the selection pressure exerted by phage supports both the evolution of phage-resistance with simultaneous coexistence of phage sensitive bacteria (337). In a biofilm, for example, one might expect different populations of bacteria with differing profiles of gene expression. The further level of complexity within human disease states (biofilms, polymicrobial communities, exposure to immune cells) is not generally taken into account with *in vitro* work and so clinical trials examining phage efficacy will be the arbiter of success or failure of this therapy.

6 CHAPTER 6: TRACKING VARIABILITY IN SENSITIVITY TO PHAGE AND ANTIBIOTICS OVER TIME

6.1 INTRODUCTION

In previous chapters, I have looked at the adaptability of Pa and have seen how biofilms are a significant barrier to effective treatment of CF lung infection. I have explored how bacteriophage, by employing a distinct mechanism of action, targets populations not effectively killed by physiologically achievable concentrations of two commonly used anti-Pseudomonal antibiotics. In the last chapter, I looked at phenotypic change within Pa isolates that translated to a meaningful transcriptomic response and underlined, once again, the survival strategies of this organism.

In clinical practice, these mechanisms of adaptability result in populations of cells that have a survival advantage and can withstand repeated exposure to inhaled medications (antimicrobials and mucolytics), as well as cells and molecules involved in host defence. However, antibiotic therapy is a cornerstone of care in CF that is widely attributed to improvements in quality of life as well as longevity (144). As patients age and the antimicrobial burden increases, the rates of AMR in CF lung infection are high (145), which limits the agents at our disposal. This is worrying because AMR has been associated with worse patient outcomes in Pa infection (146) and has been seen to increase over time using registry data from a number of different countries (147).

What this level of phenotypic diversity and adaptability translates to in a laboratory setting is poor reproducibility of assay results for Pa clinical strains. High levels of phenotypic diversity are seen to exist within Pa isolates from a single patient. One study looked at 169 Pa clonal isolates collected from one patient during periods of stability and during pulmonary exacerbations and found extremely high levels of phenotypic variability (even within isolates of the same colony morphotype) for parameters such as colony morphology, motility, quorum sensing, antibiotic resistance, and growth profiles (338). Foweraker et al. (2005) demonstrated extremely high levels of phenotypic variability in Pa isolates from CF patients experiencing a pulmonary exacerbation, which translated to vastly different antibiograms, even from the same sputum sample (339). This within-patient diversity, wherein multiple

distinct but related bacterial populations derived from an ancestral clone evolve in parallel over time, is thought to be one of the great barriers in the development of a laboratory test that more accurately predicts antibiotic response in the clinical setting (340). The above results in a poor correlation of laboratory antibiotic sensitivity testing results and clinical outcomes in CF (98, 341).

We have previously reported in a murine model that antipseudomonal bacteriophages resulted in a reduction in both bacterial load and inflammatory response (241). These are promising findings that indicate the great potential of these viruses as therapy in human disease. Although support for phage treatment in CF lung infection is predominantly anecdotal (206, 342), there is a clinical trial underway in the U.S. using an inhaled anti-Pa phage preparation in CF patients (343). Further rigorously conducted clinical trials are sure to follow suit, regardless of the outcome. Nonetheless, several questions remain around study design and patient selection. For example, although it is intuitive that clinical efficacy will require patients with phage-sensitive organisms, heterogeneity in the sensitivity signal would present a barrier to patient selection and could dilute a signal of efficacy. In addition, any clinical safety signal related to bacterial lysis would be missed in those harbouring phage-resistant strains. To aid future patient selection, I have determined whether bacteriophage sensitivity patterns are stable or variable over time.

I looked at the variability in sensitivity to commonly prescribed antibiotics for a given patients at different time points within a year and compared this with the sensitivity to the phage cocktail. In this way, I hoped to be able to identify in what way patients for upcoming clinical trials involving phage would qualify for enrolment and whether multiple isolates from different time points would be advised. I also examined the rates of bacteriophage susceptibility in isolates demonstrating an MDR pattern. For these experiments, I adopted a definition for MDR that is widely used in the literature (resistance to $\geq$1 antibiotic in $\geq$3 antibiotic classes (344), though I are aware that other definitions exist (1).

I hypothesised that longitudinal, intra-subject variability in bacteriophage susceptibility patterns would occur, as has been described for conventional antibiotics. The confirmation

of this would help with the development of selection criteria for the recruitment of patients to future studies involving phage.

6.2 HYPOTHESIS

Pa will demonstrate variability in response to our bacteriophage cocktail and to antibiotics over time.

6.3 AIMS AND OBJECTIVES

- To identify a group of CF patients who are chronically colonized with Pa.
- To test their Pa isolates at different time points (more than one month apart) for sensitivity to the phage cocktail as well as to 8 standard anti-pseudomonal antibiotics.
- If variability was observed, to determine whether this is due to the presence of >1 Pa strain or whether variability occurs independently of co-infection with different strains.
- To establish the level of phage sensitivity in the isolates that demonstrate an AMR pattern on their antibiogram.
- To help decide what inclusion criteria should exist for future clinical trials involving phage with regards to sensitivity testing of Pa.

6.4 MATERIALS AND METHODS

DATABASE AND STRAIN REPOSITORY

Clinical Pa strains in these experiments were provided by the RBH microbiology laboratory and were stored in our repository as described in Section 2.2. In brief, samples from CF patients were taken directly from sweeps of bacterial colonies grown on agar with a plastic inoculation loop. Strains were stored on glass Microbank™ beads in a -80° C freezer. All isolates had been identified by MALDI-TOF analysis (345) in the microbiology department at the hospital as being Pa. This database includes details (including antibiotic sensitivities, VNTR analysis and details of co-colonising organisms, where available) of all CF patients receiving care at RBH.

CHOICE OF PATIENTS AND ISOLATES

I accessed the Microsoft Access database and repository of organisms from CF airway cultures at RBH and selected 24 patients with 4 or more *P. aeruginosa* isolates throughout 2016. I selected patients who had isolates at least one month apart to capture the widest spread of data throughout the period tested.

Once isolates had been selected from the database, the corresponding vial was briefly removed from the freezer and placed on dry ice. A bead was selected with a sterile 22 G needle and colonies were seeded on nutrient agar for overnight growth in the incubator at 37° C.

ANTIBIOTIC SENSITIVITY TESTING BY DISC DIFFUSION

Antibiotic sensitivity testing was performed by disc diffusion assay according to EUCAST protocols (252). This is described in more detail in Chapter 2.

In summary, isolates were grown overnight on nutrient agar (Oxoid). An inoculum suspension was then prepared by taking a sweep of bacterial colonies with a sterile loop and suspending in a solution of 0.85% sodium chloride visually adjusted to 0.5 McFarland standard. A sterile cotton swab is then dipped into the inoculum and spread evenly across Mueller Hinton agar plates with an automatic plate rotator.

Antimicrobial discs are applied firmly onto the surface of the agar within 15 minutes of inoculation of the plates and inverted and incubated at 37°C overnight for 16-20 hours. After overnight incubation, inhibition zones are read with callipers and a ruler at roughly 30 cm from the naked eye. Zones of inhibition are judged to be where there is no growth of bacterial colonies and these are compared to cut-offs established by EUCAST to determine resistance or sensitivity (252). *Pseudomonas aeruginosa* ATCC ® 27853 ™ is a laboratory strain where sensitivity cut-offs are published and was used as a quality control measure for each group of disc diffusion assay testing.

PLAQUE ASSAY

Please refer to Chapter 2 for details of the plaque assay. In brief, selected isolates were grown as a lawn on semi-solid agar with serial 10-fold dilutions of a topical bacteriophage preparation topically applied to the lawn. Sensitivity was demonstrated at a given

concentration if plaques (lysis) was visible on the bacterial lawn after overnight incubation at 37C.

VNTR TYPING

Variable number tandem repeat (VNTR) analysis was performed on isolates for selected experiments to allow for determination of Pa strain. This was initially described by Turton et al. (2010) using the numbers of tandem repeats at nine distinct loci to determine strain with a high level of accuracy (254). Isolates used in this experiment were grown overnight on nutrient agar slopes and shipped to Public Health England, where the experiments were performed by myself with the assistance Zoe Payne. This process is summarised in more detail in Chapter 2.6

Nine loci were used for strain typing according to the above method (ms61, ms172, ms207, ms209, ms211, ms214, ms217, ms222, and ms213). The Liverpool strain (NCTC 13415) was used as a control in each batch of experiments

For sizing of amplicons at loci ms172, 211, 213, 214, 217 and 222, agarose gel electrophoresis was performed by running PCR products on a 1.5 % (w/v) agarose gel. 5 µl of each sample was loaded onto each agarose gel, as well as the same amount of the 50 bp HyperLadder (used every 6-8 wells to aid size readings). Electrophoresis was run at 2 hours at 150 V or until dye in loading buffer had migrated a sufficient distance. Gels were stained with GelRed (3 x 0.1 M NaCl) for 30 minutes and viewed under UV transillumination for measuring the number of repeats at each locus based on size tables.

For loci ms207, 209 and 61, fluorescent primers were used and plates were submitted and analysed by the Genomic Services and Development Unit. Results were provided as FSA files and analysed using PeakScanner software to determine the size of each peak, allowing quantification of the number of repeats at the three remaining loci.

VARIABLE NUMBER TANDEM REPEAT DENDOGRAM

Data were analysed using BioNumerics software (Applied Maths, Belgium) using Euclidian distance over the first eight loci. The last (ninth) locus may vary in repeat number between

colonies from the same CF sputum sample and was not included in the analysis. Dendogram images were provided from Public Health England.

STATISTICAL ANALYSIS

Statistical analysis was performed

All statistical analysis and graphs were generated using Prism 8.0 (GraphPad, San Diego, CA, USA). For parametric data, standard t-tests were used to compare means. A Mann–Whitney U test was used to compare median proportions of isolates with variable responses to antimicrobials for those with more than one Pa strain to those with one strain. Patient level data were also analysed using a Fisher's exact test with sensitivity data converted to categorical outcomes (sensitive vs. resistant).

LUNG FUNCTION TESTS

For the 23 adults in this dataset, European Coal and Steele reference equations were used to determine FEV1 (346). The one paediatric patient whose isolates were used in this dataset used the Rosenthal equation as reference for determination of FEV1 % predicted (347).

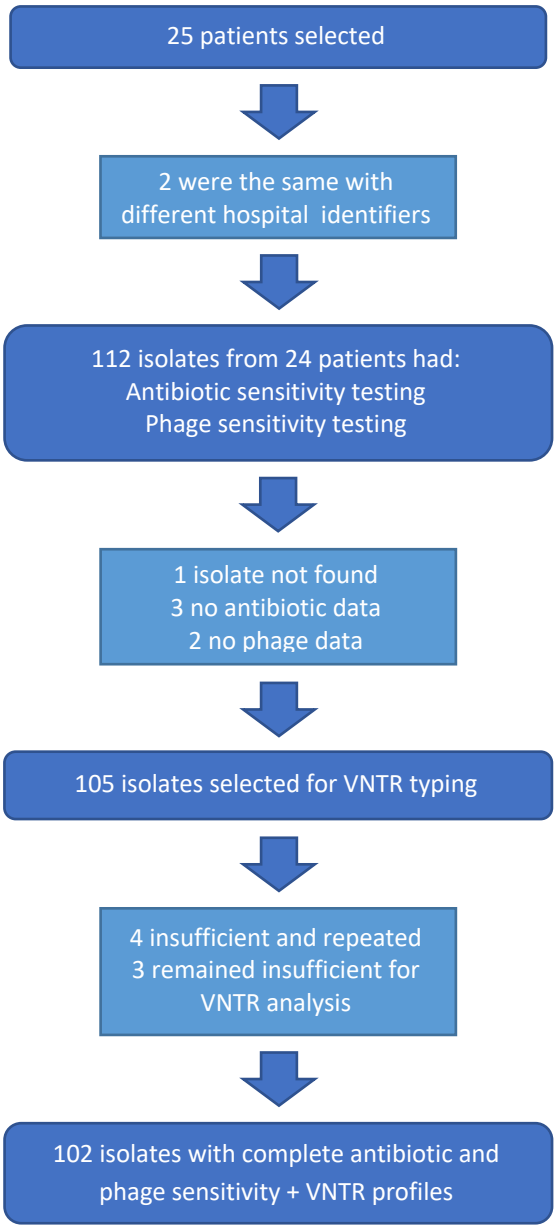
6.5 RESULTS

Isolates from 25 CF patients with chronic Pa were chosen based on availability of ≥3 isolates ≥1 month apart over ≥8 months in 2016. However, upon collection of isolates, I discovered that 2 were the same patient with different hospital identifiers; the 2nd of the two sample sets were excluded from further analysis. From the remaining 24 patients, a total of 112 isolates were selected for testing, of which 102 had matched antibiotic and bacteriophage sensitivity profiles. The remainder either did not grow in one of the experiments (5 isolates), grew another organism (identified by MALDI-TOF analysis on 3 isolates that did not yield expected amplicons on VNTR analysis) and in one case, the isolate could no longer be located in our repository when I went to perform VNTR analysis. Isolates from individuals were coded and anonymised so that there could be no selection of similar morphotypes to bias results.

As the typing of the different Pa isolates represented a large foundation of this study, these results are presented in their own section, with data comparing variability in antibiograms with phage sensitivity data at different time points presented later. The data from the VNTR Pa strain typing were used to inform some of the analysis in the later sections.

Variable Number Tandem Repeat Analysis

There were 105 isolates of 112 that underwent VNTR analysis. There were 5 cases in which there was missing antibiotic or phage sensitivity data or in which the isolate could no longer be found in our repository. In 4 of 105 instances (3.8%), experiments were repeated if data were insufficient to accurately type a given isolate and in 3 of these instances, data were insufficient to VNTR type the isolate. These data are represented in the CONSORT diagram, below:



Prior to obtaining the completed results from the VNTR analysis, there were sometimes clear phenotypic differences that were appreciated once the antibiograms had been read and the isolates had been de-anonymised. I have shown as an example a photograph of two isolates from the same patient on the antibiotic sensitivity disc diffusion assay in Figure 6.5.1.

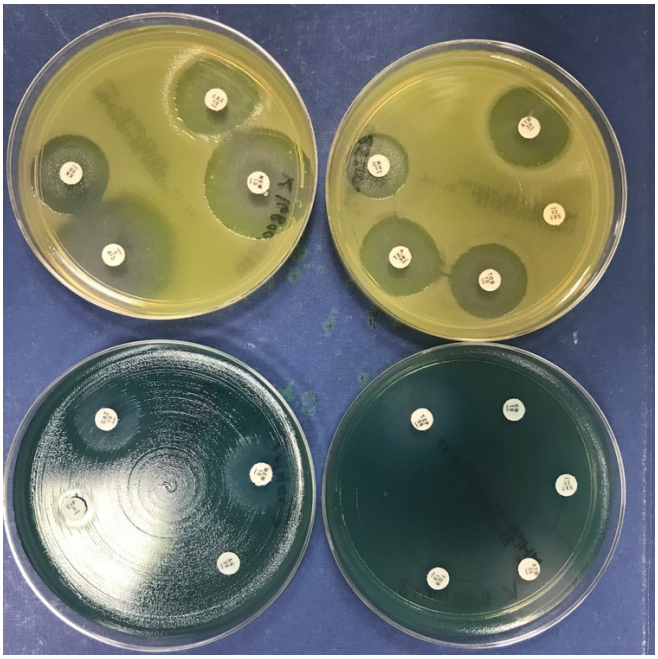
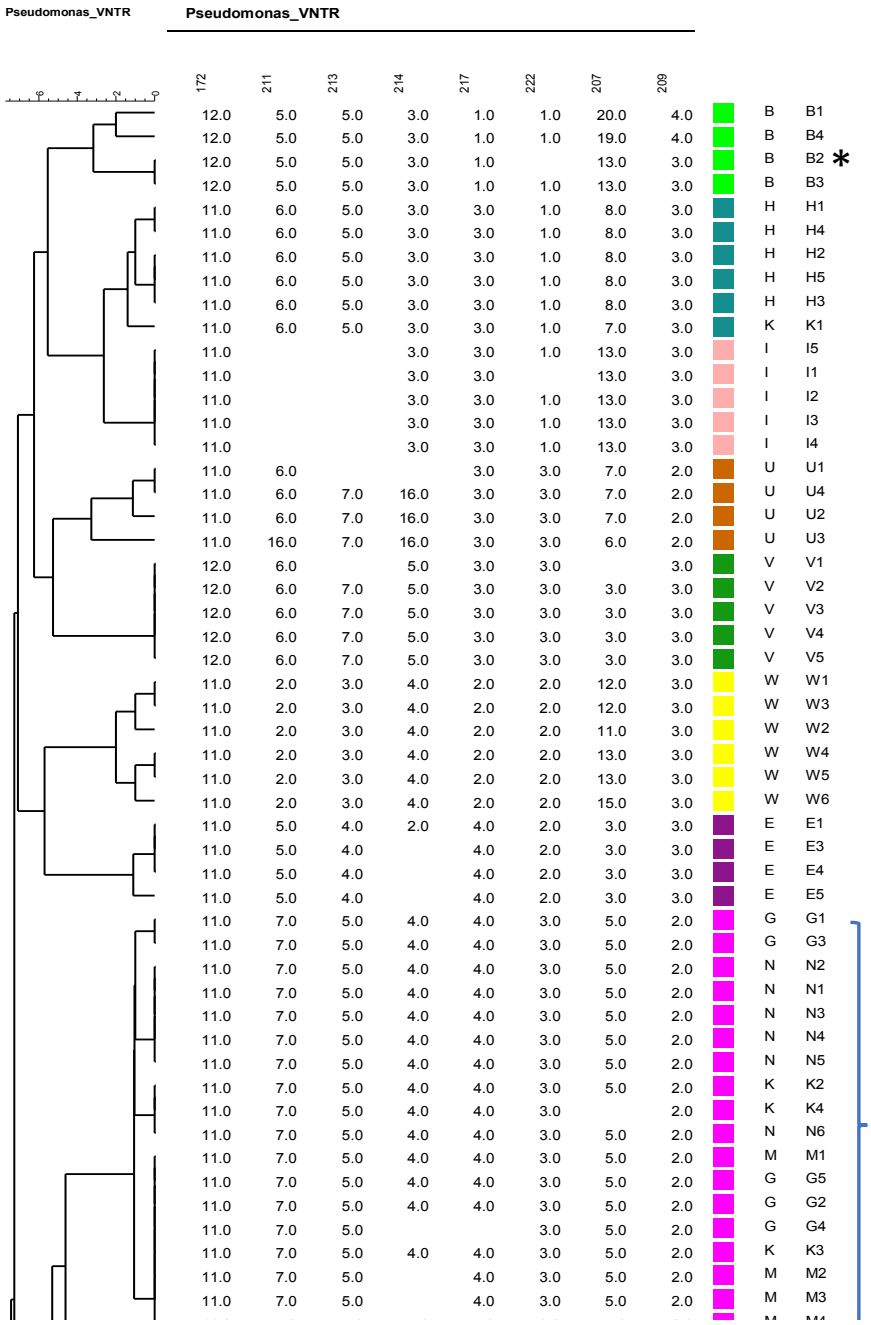


Figure 6.5.1. Differential phenotypic expression of Pa isolates at different time points.
The above Figure shows 2 isolates from patient K with differential production of pyocyanin (lower two agar plates showing high levels of pyocyanin). VNTR analysis later demonstrated this as two distinct Pa strains, which can be seen in the dendogram in Figure 6.5.2.

This experiment was not designed to observe differences in phenotypic expression beyond resistance profiles to antimicrobials and it is therefore possible that other differences in pyocyanin expression, as illustrated in Figure 6.5.2, were observable within the same genetic strains. This was, however, a stark visual difference that was observed for patient K and was documented. Phenotypic change is discussed in more lengths in Chapter 5.

Clustering of the VNTR profiles by all but 3 patients can be seen in the dendogram in Figure 6.5.2 demonstrating colonization with a single genetic strain of Pa. The most commonly shared strain was the LES, which was seen in 4 patients.



Liverpool strain (LES)

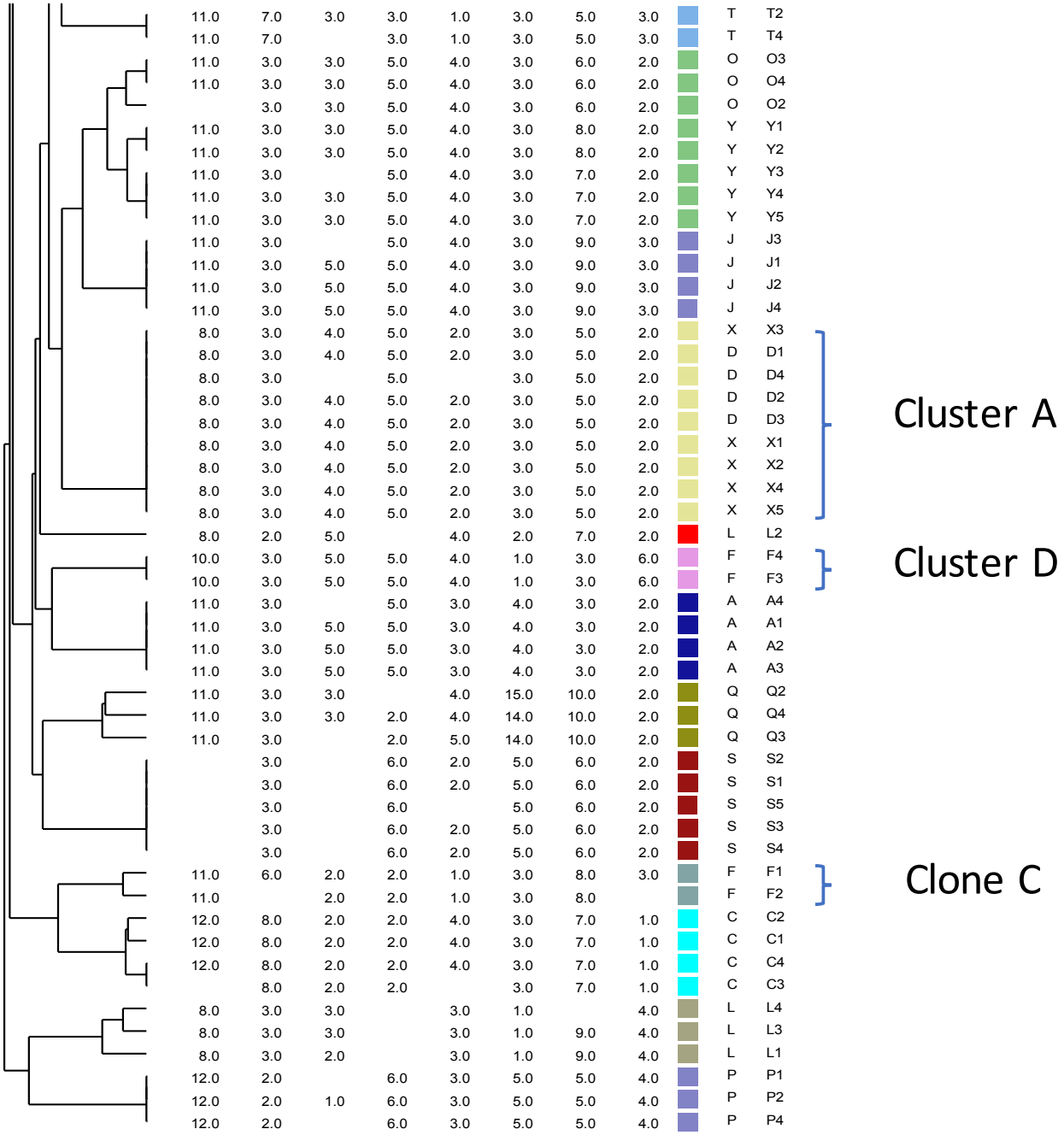


Figure 6.5.2. Dendrogram showing VNTR (Variable Number Tandem Repeat) profiles for 102 isolates from 24 patients analysed during this study.
Isolates underwent VNTR analysis using the method described by (254). Isolates in the same family (using Euclidian distance over the first eight loci) are denoted by different colours. Patients are represented by letters.
* Isolates from patient B exhibited considerable variation in the repeat number at the seventh locus, perhaps due a small DNA insertion gain/loss between isolates, reflecting the existence of strain variants.

On the basis of VNTR, 21 of 24 (87.5%) patients isolated only a single genetic Pa strain over the period tested. In 3 (12.5%) patients (patients F, L and K) had isolates with 2 distinct VNTR profiles in their serial samples. There were no patients that isolated more than one Pa strain

at a given time point. Furthermore, there were no patients who isolated two strains and then converted back to the original strain over the study period. Patients G, K, M and N all were identified as being infected with LES, and patients D and X identified Cluster A.

PATIENT AND ISOLATE BREAKDOWN

Baseline patient characteristics, including age, sex and FEV1, as well as VNTR, combined with isolate characteristics, are summarized in Table 6.5.1. From the 24 patients, a total of 112 isolates were selected for testing (median and range of 4 (3-6) per subject), of which 102 (91%) had complete antibiotic/bacteriophage sensitivity profiles and VNTR data. Isolates without either antibiotic/phage sensitivity data or missing VNTR data (10) were excluded from the final analysis.

Table 6.5.1. Properties of selected patients and their Pa isolates.

Patients:	**Number**	**24**
	Age (median (range)) in years	31 (14-57)
	Male: Female ratio	12:12
	Best FEV_1 (% predicted median and range) for 2016	62 (23-120)
	Number of isolates per patient (median (range))	4 (3-6)
	Number of subjects with exclusively non-mucoid strains	3/24 (12.5%)
	Number of subjects with exclusively mucoid strains	6/24 (25%)
	Number with >1 VNTR type over time period examined	3
	Shared strains: Liverpool Epidemic Strain*	4
	Cluster A*	2
Isolates:	**Number of isolates (total)**	112
	Number with antibiotic and phage sensitivity + VNTR	102
	Number (%) of non-mucoid/mucoid isolates	44 (43%): 58 (57%)
	Number sensitive to neat phage cocktail	76/102 (75%)
	Number sensitive to dilute phage cocktail (10^{-3})	51/102 (50%)
	Number with resistance to ≥1 antibiotic	88/102 (86%)
	Number qualifying as MDR**	51/102 (50%)
	Number of MDR isolates sensitive to neat phage	40/51 (78%)
	Number of MDR isolates sensitive to dilute phage (10^3 dilution)	26/51 (51%)

*including the 3 patients who isolated more than one strain by VNTR analysis. Isolates were said to be MDR if an isolate demonstrated resistance to at least one antibiotic in ≥3 antibiotic classes (ß-lactam, aminoglycoside, fluoroquinolone)

There was a large amount of variation in all patient parameters, from age to best FEV1 during the period of the study. On consideration of the individual isolates, there was a roughly equal

distribution of mucoid and non-mucoid strains. Notably, exactly half of the isolates demonstrated an MDR profile on their antibiogram and 78% of these isolates demonstrated lysis with the neat phage cocktail, while 51% demonstrated sensitivity to the phage at a 10^{-3} dilution. I took the best FEV1 value throughout the course of 2016 for each patient, but our dataset was too small to detect any statistically significant difference between FEV1% predicted in patients in whom I had isolated MDR strains (13 of 24). Additionally, there was no statistically significant difference in sensitivity to the bacteriophage in mucoid and non-mucoid strains.

ANTIBIOGRAM PROFILES AND PHAGE SENSITIVITY

Each of the chosen isolates underwent disc diffusion assays for 8 common anti-pseudomonal antibiotics (amikacin, aztreonam, ceftazidime, ciprofloxacin, gentamicin, meropenem, piperacillin-tazobactam, and tobramycin) as well as having sensitivity to the bacteriophage cocktail assessed by plaque assay. I considered the group with only a single VNTR strain and that with 2 strains both separately and combined (Table 6.5.2).

Table 6.5.2. Inter-patient antibiogram and plaque assay variability.

	N patients			
Antimicrobial	**Stably Sensitive**	**Variable**	**Stably Resistant**	**Stable/ variable (%)**
Bacteriophage (neat cocktail)	10 (*1)	10 (*2)	1	52 /48 (*33/67)
Bacteriophage (10^{-3} dilution)	1	17 (*3)	3	17 /83 (*0/100)
Amikacin	6	7 (*2)	8 (*1)	67 /33 (*33/67)
Aztreonam	8 (*1)	6 (*2)	7	71 /29 (*67/33)
Ceftazidime	9	6 (*3)	6	71 /29 (*0/100)
Ciprofloxacin	1	9 (*3)	11	57 /43 (*0/100)
Gentamicin	4	9 (*2)	8 (*1)	57 /43 (*33/67)
Meropenem	8	6 (*2)	7 (*1)	71 /29 (*33/67)
Piperacillin Tazobactam	10	6 (*2)	5 (*1)	71 /29 (*33/67)
Tobramycin	14 (*1)	3 (*2)	4	86 /14% (*33/67)

Numbers are presented for patients with a single VNTR strain and separately in parentheses with an asterisk, *, to demonstrate the spread of data for those with >1 VNTR strain.

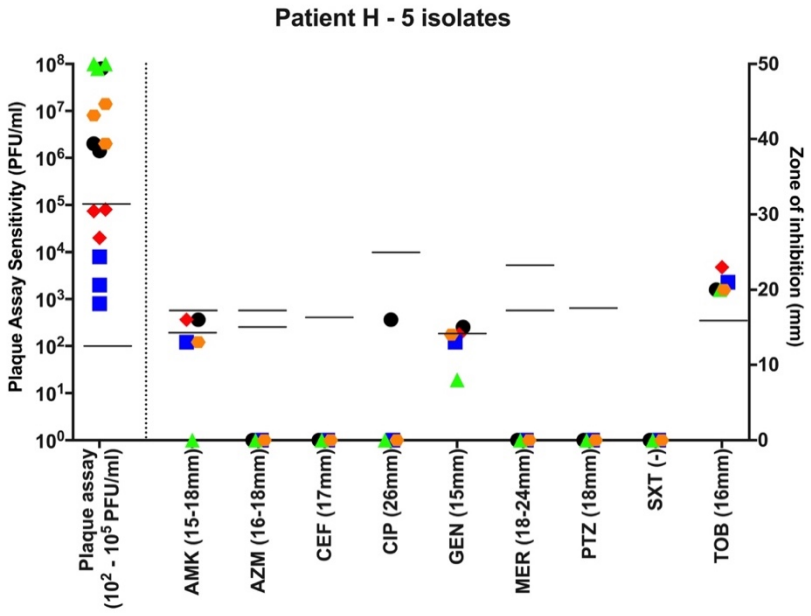
There was a large degree of variability in sensitivity profiles to the bacteriophage cocktail at both concentrations. Amongst the 21 patients who had a single Pa strain, 10 (48%) demonstrated stable sensitivity to the neat phage (apparent phage titre >$2x10^2$ PFU/ml) and only 1 patient (5%) had stably resistant isolates at this concentration. At the lower phage concentration of 10^{-3} serial dilutions (or phage titre of > $2x10^5$ PFU/ml), greater variability was seen with only 1 patient (5%) showing stable sensitivity, the majority (n=17; 81%) demonstrating variable sensitivity and 3 (14%) being stably resistant. As expected, patients were much more likely to be sensitive to the neat phage than to the dilution (p=0.004 using Fisher's exact test).

There was also a high degree of variability in antibiogram results from disc diffusion assay for isolates in this experiment, both when looking at individuals with a single VNTR strain, and with those with >1 strain. The variability was seen for all tested antibiotics. Tobramycin was the antibiotic with the most consistent antibiogram, whereas ciprofloxacin was the antibiotic that demonstrated the greatest degree of variability. It was not possible to examine individual isolates with regard to the timing of antibiotic administration as in this group, I sometimes only had dates for intravenous antibiotics administered in hospital, whereas patients often reported having taken a course of oral antibiotics at ill-defined points prior to being seen in clinic. However, 18/24 (75%) were documented to have received ciprofloxacin, while 22/24 (92%) were documented to have received inhaled or intravenous tobramycin over the time period of this study suggesting that the variability observed may not have been related to exposure.

I did not consider it appropriate to directly compare the variability of isolates to bacteriophage and antibiotics based on differences in the testing assays. However, having data from multiple antibiotics allowed us to compare a median proportion of isolates demonstrating variable responses to drugs between subjects with one as compared to two VNTR; as expected, variability was higher in the latter (67%) but was substantial even in the group with a single strain (29%)(p<0.001 Mann-Whitney-U)

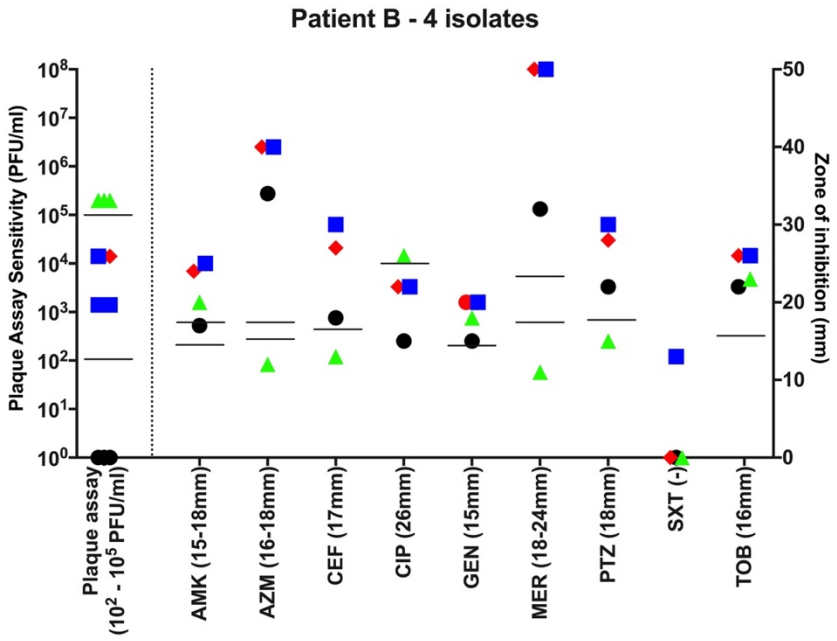
Example plaque assays and antibiogram results chosen to demonstrate different sensitivity patterns are shown in Figure 6.5.4. The first three of these (Figure 6.5.4 a, b and c) have a single VNTR type: patient H shows stability in responses to antibiotics and neat phage; patient

B shows substantial variability in sensitivity to both phage and antibiotics; and patient D demonstrates a more subtle variability in sensitivity to both antimicrobial choices Fig. 6.5.4 c. shows an example profile of patient H, colonized with 1 strain showing a consistent antibiogram, but vastly different response to the phage cocktail at different timepoints (resistant at the first two time points, but sensitive at subsequent time points). The fourth subject (patient F, Figure 6.5.4 d) harboured two VNTR types, demonstrating variability to both phage and antibiotics. Much of patient F's variability was seen to relate to these strain differences. The two other patients (K and L) in whom more than one VNTR strain was identified had variability that mapped less clearly to the different strains (patient K is presented in Figure 6.5.5).

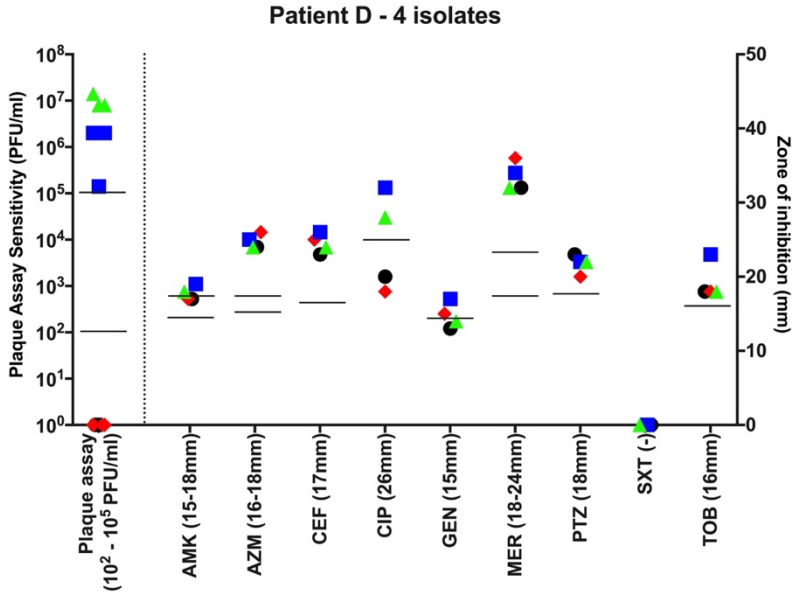


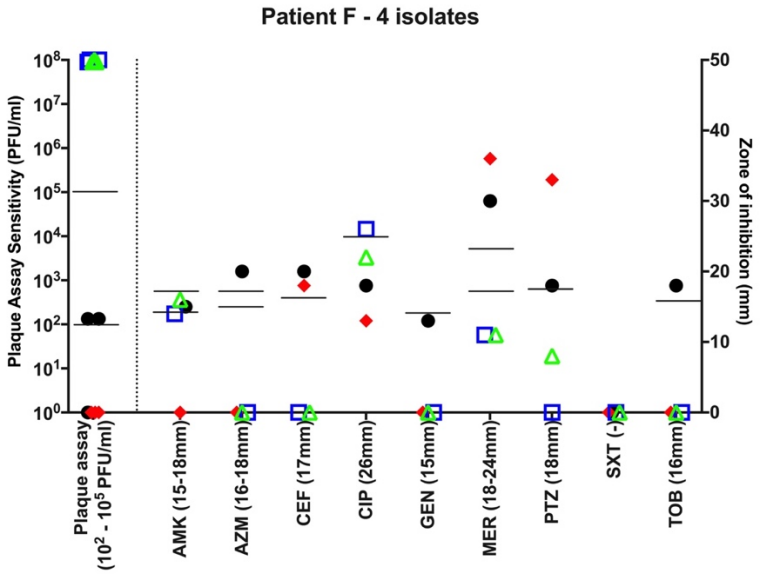
(a)

(b)


Patient B - 4 isolates
Plaque Assay Sensitivity (PFU/ml)
Zone of inhibition (mm)
Plaque assay (10² - 10⁵ PFU/ml)
AMK (15-18mm)
AZM (16-18mm)
CEF (17mm)
CIP (26mm)
GEN (15mm)
MER (18-24mm)
PTZ (18mm)
SXT (-)
TOB (16mm)

(c)


Patient D - 4 isolates
Plaque Assay Sensitivity (PFU/ml)
Zone of inhibition (mm)
Plaque assay (10² - 10⁵ PFU/ml)
AMK (15-18mm)
AZM (16-18mm)
CEF (17mm)
CIP (26mm)
GEN (15mm)
MER (18-24mm)
PTZ (18mm)
SXT (-)
TOB (16mm)

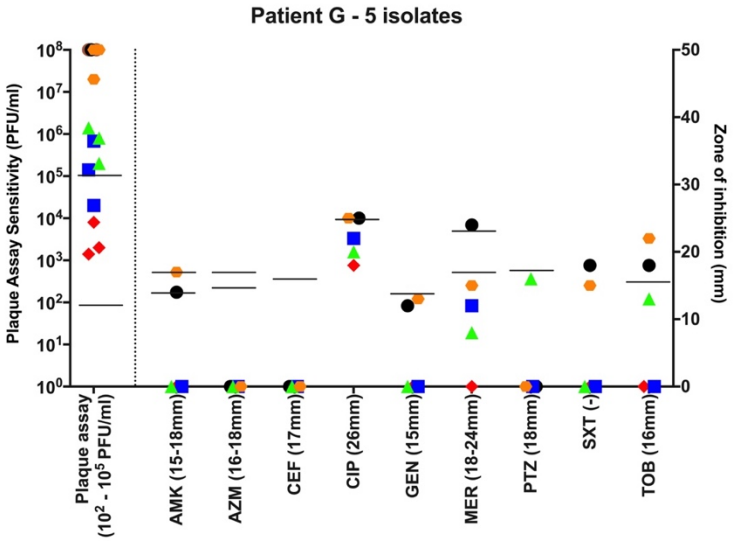


(d)

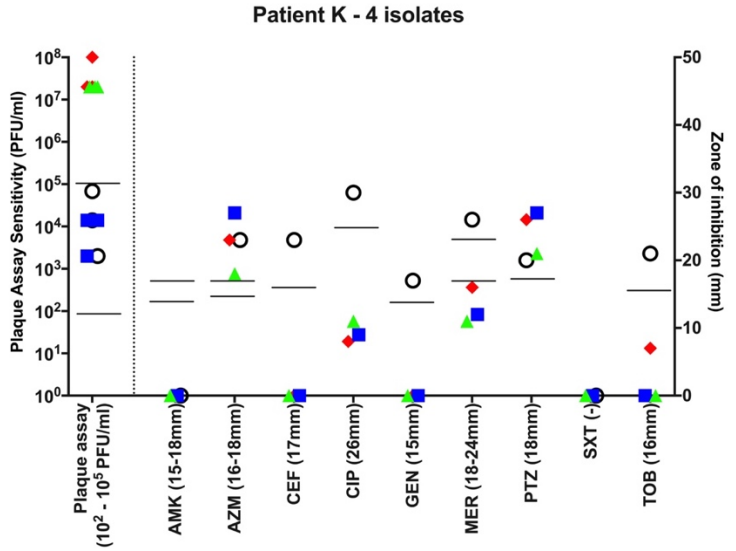
Figure 6.5.1. Example antibiograms and bacteriophage sensitivity profiles for 4 different patients chosen to illustrate variability in results.
Isolates are graphically represented by different symbols chronologically throughout the year. The first isolate is represented by ●, with subsequent isolates ◆, ■, ▲, ⬢. Open symbols represent different strains, identified by VNTR analysis. Abbreviations: AMK: amikacin; AZM: aztreonam; CEF: ceftazidime; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; PTZ: piperacillin-tazobactam; SXT: co-trimoxazole (no EUCAST breakpoint); TOB: tobramycin. EUCAST sensitivity cutoffs by disc diffusion assay are indicated by the bars. Biological triplicates of phage assay are represented individually and as bars at both neat phage (>2x10^2 PFU/ml) and dilute phage (>2x10^5 PFU/ml).
a) Patient H is an example of a patient colonized with MDR Pa, sensitive only to tobramycin and phage at all 5 time points throughout 2016. (b) Patient B represents a patient harboring a Pa strain showing high variability to all antibiotics as well as the phage cocktail. Of note, this patient's first isolate was resistant to the phage cocktail, while subsequent isolates throughout the year showed susceptibility. (c) Patient D harboured a Pa strain resistant to the phage cocktail at the first two timepoints in the year, but sensitive throughout the rest of 2016. (d) Patient F was one of three from whom two distinct Pa strains were identified. The first two isolates in the year are one strain, while the subsequent two are another. There are distinct antibiograms for the two strains, as well as a distinct pattern of susceptibility/resistance to the phage cocktail.

Results from patients with the Liverpool Epidemic Strain

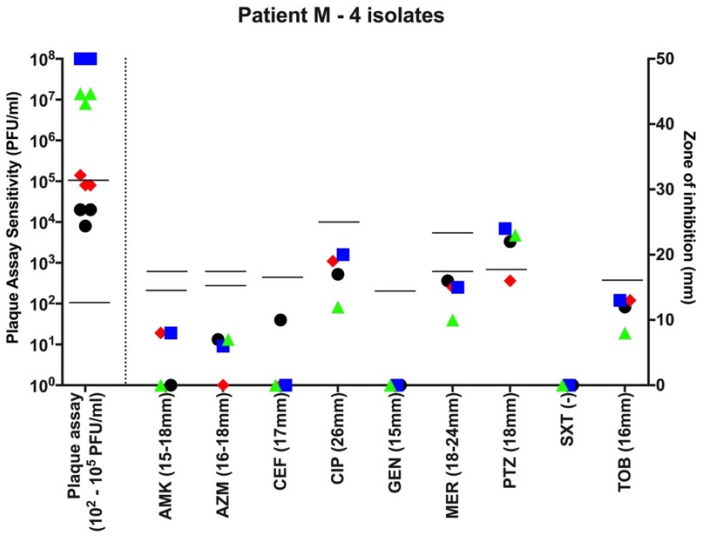
In our dataset, there were 4 patients in whom I identified the LES, making it the most commonly shared strain. Summary data from these 4 patients is shown in Figure 6.5.5. Two other patients were infected with Cluster A, a strain which often shows no epidemiological link, but has been isolated from hospital and environmental sources across the UK (255).


Patient G - 5 isolates
Plaque Assay Sensitivity (PFU/ml)
Zone of inhibition (mm)
Plaque assay (10² - 10⁵ PFU/ml)
AMK (15-18mm)
AZM (16-18mm)
CEF (17mm)
CIP (26mm)
GEN (15mm)
MER (18-24mm)
PTZ (18mm)
SXT (-)
TOB (16mm)

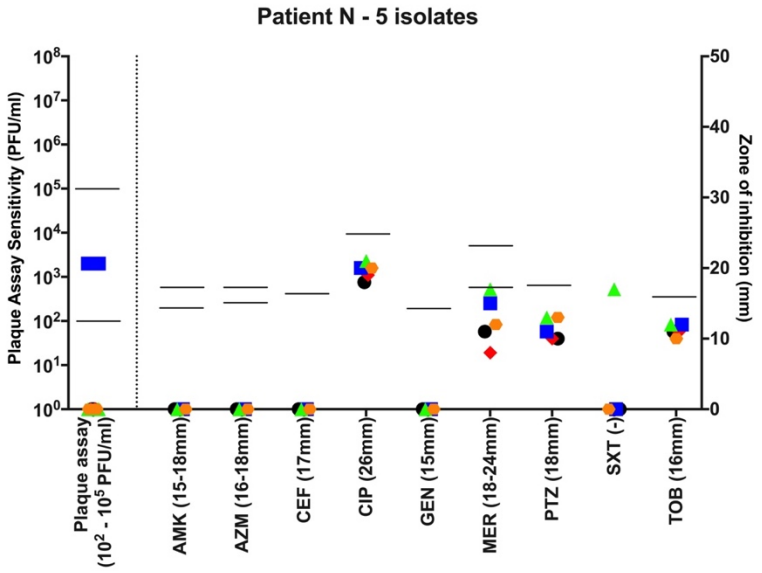
(a)


Patient K - 4 isolates
Plaque Assay Sensitivity (PFU/ml)
Zone of inhibition (mm)
Plaque assay (10² - 10⁵ PFU/ml)
AMK (15-18mm)
AZM (16-18mm)
CEF (17mm)
CIP (26mm)
GEN (15mm)
MER (18-24mm)
PTZ (18mm)
SXT (-)
TOB (16mm)

(b)


Patient M - 4 isolates
Plaque Assay Sensitivity (PFU/ml)
Zone of inhibition (mm)
Plaque assay (10² - 10⁵ PFU/ml)
AMK (15-18mm)
AZM (16-18mm)
CEF (17mm)
CIP (26mm)
GEN (15mm)
MER (18-24mm)
PTZ (18mm)
SXT (-)
TOB (16mm)

(c)



(d)

Figure 6.5.2. Antibiograms and bacteriophage sensitivity profiles for 4 patients with the Liverpool Epidemic Strain.
Isolates are graphically represented by different symbols chronologically throughout the year. The first isolate is represented by ●, with subsequent isolates ◆, ■, ▲, ⬢, if there were 5 isolates through the course of 2016. Open symbols represent different strains, identified by VNTR analysis. Abbreviations – AMK: amikacin; AZM: aztreonam; CEF: ceftazidime; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; PTZ: piperacillin-tazobactam; SXT: co-trimoxazole (no EUCAST breakpoint); TOB: tobramycin. EUCAST sensitivity cutoffs by disc diffusion assay are indicated by the bars. Biological triplicates of phage assay represented individually and bars at both neat phage (>2×10^2 PFU/ml) and dilute phage (>2×10^5 PFU/ml).
(a, b, c, d) Patients G, K, M and N were colonized with the LES. All strains qualified as MDR based on antibiograms and 14/18 (78%) LES isolates showed some lytic activity to phage using the lower sensitivity cutoff. Using the higher sensitivity cutoff of 10^3 serial dilutions, 9/18 (50%) showed sensitivity to phage. (a) Patient G had isolates that are broadly resistant to antibiotics - sensitive to tobramycin at 2 and to meropenem at 1 time points, respectively, but broadly sensitive to phage at all time points; (b) Patient K was colonized by a different strain at the first time point, but all subsequent isolates were LES with lytic activity to phage; (c) Patient M's LES strain was sensitive only to piperacillin-tazobactam but sensitive to phage at all time points; and (d) Patient N was colonized with LES resistant to all antibiotics tested. Only 1 of this patient's 5 isolates showed lytic activity to the neat phage cocktail and no isolates were deemed sensitive using the higher cutoff.

In line with published literature, all LES isolates qualified as MDR based on antibiogram (348). There were, however, isolates demonstrating sensitivity to several individual antibiotics (eg. in patient G, one isolate was sensitive to ciprofloxacin, tobramycin and meropenem with one other sensitive to ciprofloxacin, tobramycin and amikacin; in patient K, three LES isolates were sensitive to both aztreonam and piperacillin-tazobactam; and in patient M, three isolates

were sensitive to piperacillin-tazobactam). Importantly, all but one isolate showed sensitivity to the undiluted bacteriophage cocktail, while lytic activity was retained at a 10^{-3} dilution in 53% (9 of 17) of the LES isolates. There was not a shared pattern in the 2 patients with Cluster A.

6.6 DISCUSSION

This study looked at antibiogram and bacteriophage plaque assay data for Pa isolates from CF patients over a period of time and, as such, contributes a unique perspective to what is known about variability in antimicrobial sensitivity data in chronic infection within this population. High rates of variability in antibiotic susceptibility testing have been seen in other studies (339, 349), although to our knowledge, this study is unique in employing a longitudinal approach. Notably, Foweraker et al. (35), found that even Pa colonies with shared morphotypes from a single time point had vastly different antibiograms. I are also not aware of any studies taking this longitudinal approach and applying it to bacteriophage sensitivity profiles, which therefore provides a novel perspective on this emerging therapy. As I expected for this group of CF patients with chronic Pa infection, I have demonstrated significant variability in both antibiotic and bacteriophage susceptibility over 8-12 months. The main aim of this study was to assess a suitable time point for recruiting into a clinical trial and our results suggest that this should be based on contemporaneous isolates rather than evidence of historic sensitivity to phage.

In the majority of cases, the variability in sensitivity was not the result of different strains at time points tested; most patients had only a single VNTR type at all time points. Three patients did isolate 2 distinct strains, an observation which is well-recognised in CF. Although reported rates of infection by more than one strain vary, one Danish study using a different methodology looked at Pa infection in CF patients over a period of 30 years and identified 454 distinct strains from 195 patients at 51 different centres (350). For comparison, Public Health England has typed Pa isolates from CF patients at RBH annually since 2010 and has found that 192 of 1068 patients (18%) harboured more than one strain type over that period (personal communication, PHE). Ours is a small group of patients, making statistical comparisons difficult, but there was a high degree of variability as shown in Table 6.5.3. I cannot say for certain whether these patients were infected by both strains simultaneously or whether they acquired a new Pa strain which became dominant during the period of the

study. However, no patient cultured more than one strain at a given time point and there was no change from one strain to the other and back again, suggesting that the latter may be more likely.

The cohort with the same VNTR type identified in each of their samples provides the cleanest group to assess variability to both antimicrobial drugs and phages. As there are no established breakpoints for bacteriophage sensitivity, I have not compared rates of variability with the two therapies. I used the bacteriophage solution provided to us at its highest concentration (4×10^8 PFU/mL) and also assessed lysis to more dilute ($\log_{10}$) concentrations. For ease, I have here compared strains based on lysis to both the neat cocktail and $>10^{-3}$ dilutions, although both are arbitrarily chosen values with, as yet, no clinical relevance. As would be expected, the lower concentration cut-off led to more patients falling into the variable and resistant groups than did the higher concentration; when looking at the more concentrated phage, roughly half demonstrated stable sensitivity.

In the absence of clinical trials, it is not possible for us to infer a minimum effective dose (MED) for bacteriophage. Randomized, placebo-controlled studies are required to determine the breakpoint values for phage susceptibility testing and whether a MED can be established. There are also relevant questions about whether co-administration of antibiotics and bacteriophage would result in antagonism or synergy, with several comprehensive reviews on this topic that summarise the experimental data (282, 351) and is itself the subject of the experiments in Chapter 4.

Although sensitivity data varied for all of the antibiotics tested, it was particularly variable for the fluroroquinolone ciprofloxacin and most stable for the aminoglycoside tobramycin. Both these antibiotics had been administered to our cohort during the study, although design limitations meant that the treatment episodes in individuals could not be aligned with sampling times. Longitudinal variability in sensitivity could be the result of antibiotic use exerting a selective pressure on these organisms. High levels of antibiotic resistance for ciprofloxacin have been previously reported from registry data in different countries (147), and this antibiotic is often used in short (2-3 week) courses. It was reassuring to us that tobramycin, a mainstay of intravenous and nebulized anti-pseudomonal therapy in CF, was the antibiotic in our panel that yielded the most stable and promising sensitivity results, being

administered as a chronic, inhaled therapy by 92% of this cohort. These usage patterns, distinct in their own way, may explain some of this variability.

The reason for the variability in bacteriophage sensitivity in these chronic Pa isolates is intuitively much less straightforward since phage had not been administered as therapy during the study period. I have not undertaken analysis of phage susceptibility from multiple colonies from a single sputum sample as was performed by Foweraker *et al*. for antibiotics, nor are I aware of other research looking into this. But lysogenic phages infecting Pa and incorporating into the host genome as prophages is something that is well documented in many clinical strains, highlighting the fact that there is an ongoing and constant interaction between viruses and bacteria (199-201). Indeed, lysogenic phages are known to provide reservoirs of genes and have been demonstrated to be a driving force behind horizontal gene transfer and the acquisition of genes encoding antimicrobial resistance in the CF lung (352). So although the patients in this experiment had not been exposed to phage therapy during the period tested, the Pa populations in the lung may have been exposed to resident phage, necessitating up and down-regulation of genes involved in antiviral defence. Full genomic sequencing of the isolates used in this experiment could quantify the level of prophage DNA in the bacterial genome to help answer this question.

Finally, it has been hypothesized that the disconnect between laboratory sensitivity testing and clinical outcome may be the result of sampling populations of bacteria in different geographic locations within the heterogenous CF lung environment. It is well recognized that a sputum sample is generated from only a small portion of the airway tree and substantial geographical heterogeneity in infection and inflammation has previously been documented (353). One study looked at explanted lungs from CF patients and showed that Pa isolates from different lobes of the lung differed phenotypically and phylogenetically, demonstrating that bacterial evolution happens both in tandem and in geographic isolation (354). This may, at least in part, explain the variability in response seen for both phage and antibiotics.

The second contribution of this study (though not the primary outcome) is to the growing body of evidence that supports the development of new antimicrobial agents such as bacteriophage in the battle against Pa. The rates of sensitivity in MDR strains was promising enough to merit giving this field some space for focus and the development of well-designed

clinical trials. Although rates of variability were high – often many orders of magnitude difference was observed in PFU/ml using the plaque assay as a sensitivity assay for a single isolate – the overall message was one of broad levels of lytic activity across multiple isolates from the same patient over time. One unintended finding was that all patients with LES, notoriously antibiotic resistant, demonstrated sensitivity to the phage. Whether or not this will translate into clinical efficacy within the context of CF is something that remains to be seen, but anecdotal results for other pathogens are starting to show promise (206) and with the pressure of increasing MDR, this will likely expand into trials using phage.

I demonstrated high levels of MDR Pa in a group of 24 CF patients with chronic infection at a busy London hospital. In our dataset, roughly half of all isolates qualified as MDR, which is much higher than rates that have been reported elsewhere. The Cystic Fibrosis Foundation in the US has found that the level of MDR Pa in the adult population is 16.9% (1), but their definition of MDR is different in that it specifies resistance to *all* antibiotics in at least 2 antibiotic classes. Due to the fact that I have only tested 8 common anti-pseudomonal agents, I opted for a practical and widely accepted definition, which I believed would indicate more sensitively when choice of antimicrobials is being limited by resistance profiles (resistance to at least one antibiotic in at least 3 different antibiotic classes, which in this study were fluoroquinolones, ß-lactams and aminoglycosides) (344). Patients in this study were selected based on not only chronic infection, but a large number of isolates spread throughout 2016 stored in our repository. This selection pressure resulted in a group with a high burden of antibiotic administration, which likely resulted in the high rates of AMR seen in this study**.** However, variability data for phage were similar for both the MDR and non-MDR patient groups, which suggests that these individuals may be representative of CF populations broadly speaking. I were encouraged by the rates of phage susceptibility in this difficult to treat subgroup.

7 CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

7.1 PHAGE IN BIOFILMS

Efficacy of a 4-phage cocktail in reducing biofilm biomass and biofilm cell viability was demonstrated when combined with a standard anti-Pseudomonal antibiotic combination (ceftazidime and tobramycin) in 16 different clinical strains. The strengths in the experimental design included clinically relevant antibiotics in concentrations that are achievable in the airway and the inclusion of 16 different Pa strains from Pa airway cultures.

There are, however, obvious limitations to the use of the biofilm models used in these experiments. Cells adhering to the sides of a plastic 96-well plate in broth culture are likely not representative of the aggregates seen suspended within mucus in the lung; however, this is a drawback of most *in vitro* testing. This disconnect between what goes on in the laboratory and in human disease is certainly apparent in the field of microbiology, where conventional antibiotic sensitivity testing fails to predict patient response in CF (98, 355).

Great emphasis has been shifted to finding a laboratory test more reflective of the lifestyle of different pathogenic bacteria, although the use of conventional biofilm models has also not yielded improved clinical predictive values (356, 357). There are *in vitro* strategies to synthesize the conditions of the lung more accurately in an attempt to develop a microbiology assay that will be more reflective of clinical practice, from flow cells that create biofilms in dynamic conditions with a consistent provision of nutrients over time (358), to the creation of an artificial sputum medium in which biofilm aggregates grow suspended within mucin (359, 360).

7.2 PHAGE AND ANTIBIOTICS AT SUB-INHIBITORY CONCENTRATIONS

In order to better qualify the effects of phage and antibiotics at different sub-inhibitory concentrations, checkerboard experiments were designed to test the combination of phage-ceftazidime and phage-tobramycin in planktonic and biofilm culture. Results varied according to both antibiotic and type of culture. There were both instances of strong synergy (CDI <0.7) and antagonism for both ceftazidime and tobramycin in both planktonic and biofilm culture. Results for combination therapy in planktonic culture did not necessarily translate to

significant reductions in biofilm biomass. Notably, there were no instances of additivity seen in biofilms with tobramycin for all strains tested.

Strengths of the experiments in this section included the use of a variety of clinical Pa strains from CF airway cultures, and in the body of work that established strain-specific MICs during the experimental set up, enabling the checkerboard assays to yield significant results. Evaluating each antibiotic individually (from 2 different antibiotic classes and using 2 distinct modes of action), and utilizing both planktonic and biofilm culture methods, I had intended to see if a pattern would emerge; however, for the 11 strains described in these experiments, the final message is one of individual strains interacting differently with antibiotics and phage in different growth conditions. These experiments contribute to the body of work evaluating lytic phage therapy in combination with different classes of antibiotics.

Together, results of such experiments could inform the analysis of clinical trials with phage, examining the effects in subgroups of patients in whom different classes of antibiotics have been used. Ultimately, such analysis could reveal whether specific phage-antibiotic combinations are more or less efficacious when used together.

7.3 Phage Affecting Phenotype

Phenotypic change in 3 of the 16 different clinical strains was seen on Congo Red agar. In addition to increased *pel* binding, an exopolysaccharide involved in Pa biofilms, there was also a trend towards increased mucoidy. I was given funds for a pilot study involving one strain in 4 different experimental conditions for transcriptomic analysis and showed increased transcription of genes involved in alginate production in both biofilm and planktonic Pa when exposed to phage.

While not possible to draw conclusions beyond the one clinical strain studied in the transcriptomic analysis, this exploratory dataset serves as a platform for further evaluation of the changes in gene expression undertaken by Pa in response to lytic phage infection. This may serve to inform the design of future studies investigating the transcriptomic effect of sub-inhibitory concentrations of other antimicrobial agents, as well guide in establishing therapeutic strategies incorporating lytic phages.

7.4 Phage and Antibiotic Sensitivity Over Time

Variability in antibiograms and sensitivity to phage was seen at different timepoints throughout a year and this was not due to differences in Pa strains. The rates of bacteriophage-induced bacterial lysis in Pa strains, including those with MDR, provide further support for the application of this therapy in CF lung infection. Specifically, these susceptibility profiles demonstrate the peril of choosing antimicrobial therapy at one timepoint as there is a large degree of change (sensitive/resistant) to both individual antibiotics as well as phage. Clinical trials exploring phage therapy should either include susceptibility data from contemporaneous airway cultures *or* should include testing of more than one historic isolate in order to recruit patients likely to benefit maximally from this therapy.

These experiments expand our understanding of the longitudinal phenotypic diversity of Pa populations within individual patients and how this translates to varying rates of resistance to antimicrobials in general. In future clinical trials, efficacy read-outs seem intuitively most likely in subjects with susceptible strains, although the lessons of the in vitro–clinical disconnect for antibiotics should not be overlooked. A direct relationship between laboratory susceptibility testing and clinical response may not be assumed and an assessment of this should underpin future trial design.

On the basis of our findings, I suggest (a) isolates tested during screening for eligibility should be from current rather than historical samples, (b) several colonies from an airway sample should be tested and (c) susceptibility should be assessed at more than a single time point. It would perhaps be most informative from both efficacy and safety perspectives to design a trial with enough participants to include patients in whom in vitro testing indicates phage resistance as a control group, albeit phage adaptation in vivo may still be possible.

7.5 Final Thoughts

The development of AMR and our inability to effectively eradicate many lung pathogens within CF has led to the development of alternative antimicrobial therapies such as bacteriophage. These viruses have adapted over millennia to effectively infect and lyse a broad range of bacterial pathogens and the efficacy of a cocktail of 4 naturally occurring anti-Pa phages has been demonstrated for a range of phenotypically distinct clinical strains within

the experiments described in this thesis. Beyond the *in vitro* efficacy of this treatment in MDR isolates and with clinically relevant antibiotics, there were instances of antagonism and of phenotypic change, which are reminders that the scientific community must approach this therapy with a strong evidence base and a degree of caution. Ultimately, the diversity of both bacteriophages and individual Pa strains means that it is very difficult to draw firm conclusions without clinical trial data.

But even prior to proceeding to clinical trial, where basic considerations like efficacy and side effects will be considered, there remain many unanswered questions that are outside the scope of this thesis. The optimal or therapeutic dosing of phage is a topic deserving attention, as is the duration and frequency of therapy and optimal route of administration. The latter is especially pertinent to cystic fibrosis lung infection, where it is uncertain whether oral or intravenous phage could be as efficacious as a nebulised route for maximising Pa killing. Studies have laid the groundwork for the use of nebulisers as a delivery system (361). There are different approaches using both nebuliser solutions and dry powder formulations of anti-Pa phage in development that have looked at both structural integrity of phage (via EM) when subjected to a nebuliser system (362) and in a dry powder formulation that saw only a 1.3 log reduction in phage titre after 12 months of storage (228). The time is ripe for such questions to be addressed, as there are currently clinical trials for Pa infection in CF lung infection underway and more are sure to follow. Results from a phase 1a/2b trial in the U.S. looking at both safety and efficacy of a nebulized preparation of anti-Pa bacteriophages in patients with chronic infection are eagerly awaited (NCT04596319) (343).

REFERENCES

1. Cystic Fibrosis Foundation. 2018 Patient Registry Annual Data Report. 2018.
2. UK Cystic Fibrosis Trust. 2017 Registry Annual Data Report. 2018.
3. Bombieri C, Seia M, Castellani C. Genotypes and phenotypes in cystic fibrosis and cystic fibrosis transmembrane regulator-related disorders. Semin Respir Crit Care Med. 2015;36(2):180-93.
4. Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RCea. Demonstration That Cftr Is a Chloride Channel by Alteration of Its Anion Selectivity. Science. 1991;253(5016):202-5.
5. Anderson DH. Cystic fibrosis of the pancreas and its relation to celiac disease: A clinical and pathologic study. Am J Dis Child. 1938;56(2):344-99.
6. O'Sullivan BP, Freedman SD. Cystic fibrosis. The Lancet. 2009;373(9678):1891-904.
7. Cohen TS, Prince A. Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med. 2012;18(4):509-19.
8. Gibson LE, Cooke RE. A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis. Pediatrics. 1959;23(3):545-9.
9. Quinton PM. Physiological basis of cystic fibrosis: a historical perspective. Physiol Rev. 1999;79(1 Suppl):S3-S22.
10. Dobra R, Edmondson C, Hughes D, Martin I, Davies JC. Potentiators and Correctors in Paediatric Cystic Fibrosis Patients: A Narrative Review. Paediatr Drugs. 2018;20(6):555-66.
11. US CF Foundation, Johns Hopkins University, The Hospital for Sick Children. The Clinical and Functional TRanslation of CFTR (CFTR2) [Available from: https://cftr2.org.
12. Boyle MP, De Boeck K. A new era in the treatment of cystic fibrosis: correction of the underlying CFTR defect. Lancet Respir Med. 2013;1(2):158-63.
13. Flume PA, Liou TG, Borowitz DS, Li H, Yen K, Ordonez CL, et al. Ivacaftor in subjects with cystic fibrosis who are homozygous for the F508del-CFTR mutation. Chest. 2012;142(3):718-24.
14. Hamosh A, Corey M. Correlation between Genotype and Phenotype in Patients with Cystic Fibrosis. New England Journal of Medicine. 1993;329(18):1308-13.
15. Castellani C, Cuppens H, Macek M, Cassiman JJ, Kerem E, Durie P, et al. Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. Journal of Cystic Fibrosis. 2008;7(3):179-96.
16. Bronsveld I, Mekus F, Bijman J, Ballmann M, de Jonge HR, Laabs U, et al. Chloride conductance and genetic background modulate the cystic fibrosis phenotype of Delta F508 homozygous twins and siblings. J Clin Invest. 2001;108(11):1705-15.
17. Davies JC. Modifier genes in cystic fibrosis. Pediatr Pulmonol. 2005;39:383-91.
18. Wanner A, Salathe M, O'Riordan TG. Mucociliary clearance in the airways. Am J Respir Crit Care Med. 1996;154(6 Pt 1):1868-902.
19. Fokkens WJ, Scheeren RA. Upper airway defence mechanisms. Paediatr Respir Rev. 2000;1(4):336-41.
20. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. J Clin Invest. 2002;109(5):571-7.
21. Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, William Davis C, et al. Evidence for Periciliary Liquid Layer Depletion, Not Abnormal Ion Composition, in the Pathogenesis of Cystic Fibrosis Airway Disease. Cell. 1998;95:1005-15.
22. Button B, Cai LH, Ehre C, Kesimer M, Hill DB, Sheehan JK, et al. A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. Science. 2012;337(6097):937-41.
23. Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur Respir J. 2004;23(1):146-58.
24. Boucher RC. Evidence for airway surface dehydration as the initiating event in CF airway disease. Journal of Internal Medicine. 2007;261(1):5-16.
25. Zabner J, Smith JJ, Karp PH, Widdicombe JH, Welsh MJ. Loss of CFTR Chloride Channels Alters Salt Absorptionby Cystic Fibrosis Airway Epithelia In Vitro. Molecular Cell. 1998;2:397-403.

26. Guggino WB. Cystic fibrosis and the salt controversy. Cell. 1999;96(5):607-10.
27. Hull J, Skinner W, Robertson C, Phelan P. Elemental content of airway surface liquid from infants with cystic fibrosis. Am J Respir Crit Care Med. 1998;157(1):10-4.
28. Reddy MM, Light MJ, Quinton PM. Activation of the epithelial Na+ channel (ENaC) requires CFTR Cl- channel function. Nature. 1999;402(6759):301-4.
29. Robinson M, Bye PT. Mucociliary clearance in cystic fibrosis. Pediatr Pulmonol. 2002;33(4):293-306.
30. Regnis JA, Robinson M, Bailey DL, Cook P, Hooper P, Chan HK, et al. Mucociliary clearance in patients with cystic fibrosis and in normal subjects. Am J Respir Crit Care Med. 1994;150(1):66-71.
31. Matsui H, Randell SH, Peretti SW, Davis CW, Boucher RC. Coordinated clearance of periciliary liquid and mucus from airway surfaces. J Clin Invest. 1998;102(6):1125-31.
32. Poulsen JH, Fischer H, Illek B, Machen TE. Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. Proc Natl Acad Sci U S A. 1994;91(12):5340-4.
33. Gustafsson JK, Ermund A, Ambort D, Johansson ME, Nilsson HE, Thorell K, et al. Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. J Exp Med. 2012;209(7):1263-72.
34. De Lisle RC. Pass the bicarb: the importance of HCO3- for mucin release. J Clin Invest. 2009;119(9):2535-7.
35. Boucher RC. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. Annu Rev Med. 2007;58:157-70.
36. Zahm JM, Pierrot D, Vaquez-Girod S, Duvivier C, King M, Puchelle E. The role of mucus sol phase in clearance by simulated cough. Biorheology. 1989;26(4):747-52.
37. Tarran R, Button B, Picher M, Paradiso AM, Ribeiro CM, Lazarowski ER, et al. Normal and cystic fibrosis airway surface liquid homeostasis. The effects of phasic shear stress and viral infections. J Biol Chem. 2005;280(42):35751-9.
38. Mets OM, Roothaan SM, Bronsveld I, Luijk B, van de Graaf EA, Vink A, et al. Emphysema Is Common in Lungs of Cystic Fibrosis Lung Transplantation Patients: A Histopathological and Computed Tomography Study. PLoS One. 2015;10(6):e0128062.
39. Lopez-Vidriero MT. Mucus as a natural barrier. Respiration. 1989;55 Suppl 1:28-32.
40. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. Respirology. 2003;8(4):432-46.
41. B. K, Bals R, Roos D, Korting HC, Griese M, Hartl D. Innate immune receptors on neutrophils and their role in chronic lung disease. Eur J Clin Invest. 2009;39(7):535-47.
42. Cantin AM, Hartl D, Konstan MW, Chmiel JF. Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. J Cyst Fibros. 2015;14(4):419-30.
43. Chow CW, Landau LI, Taussig LM. Bronchial mucous glands in the newborn with cystic fibrosis. Eur J Pediatr. 1982;139(4):240-3.
44. Ornoy A, Arnon J, Katznelson D, Granat M, Caspi B, Chemke J. Pathological confirmation of cystic fibrosis in the fetus following prenatal diagnosis. Am J Med Genet. 1987;28(4):935-47.
45. Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW. Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. J Infect Dis. 1997;175(3):638-47.
46. Verhaeghe C, Delbecque K, de Leval L, Oury C, Bours V. Early inflammation in the airways of a cystic fibrosis foetus. J Cyst Fibros. 2007;6(4):304-8.
47. Chmiel JF, Berger M, Konstan MW. The role of inflammation in the pathophysiology of CF lung disease. Clin Rev Allergy Immunol. 2002;23(1):5-27.
48. Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. Am J Respir Crit Care Med. 1999;160(1):186-91.
49. Tirouvanziam R, Gernez Y, Conrad CK, Moss RB, Schrijver I, Dunn CE, et al. Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. Proc Natl Acad Sci U S A. 2008;105(11):4335-9.

50. Hisert KB, Heltshe SL, Pope C, Jorth P, Wu X, Edwards RM, et al. Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. Am J Respir Crit Care Med. 2017;195(12):1617-28.
51. Hoffman L, Pope C, Jorth P, Hisert KB, Welsh M, Singh PK, et al. Short-Term And Long-Term Effects Of Ivacaftor Treatment On Sputum Microbiota In Cf Patients With The G551d Cftr Mutation. American Journal of Respiratory and Critical Care Medicine. 2016;193.
52. Auerbach H, Kirkpatrick J, Williams M, Colten H. Alternate-Day Prednisone Reduces Morbidity and Improves Pulmonary Function in Cystic Fibrosis. The Lancet. 1985;326(8457):686-8.
53. Eigen H, Rosenstein BJ, FitzSimmons S, Schidlow DV. A multicenter study of alternate-day prednisone therapy in patients with cystic fibrosis. Cystic Fibrosis Foundation Prednisone Trial Group. J Pediatr. 1995;126(4):515-23.
54. Lai HC, FitzSimmons SC, Allen DB, Kosorok MR, Rosenstein BJ, Campbell PW, et al. Risk of persistent growth impairment after alternate-day prednisone treatment in children with cystic fibrosis. N Engl J Med. 2000;342(12):851-9.
55. Ross KR, Chmiel JF, Konstan MW. The role of inhaled corticosteroids in the management of cystic fibrosis. Paediatr Drugs. 2009;11(2):101-13.
56. Balfour-Lynn IM, Welch K, Smith S. Inhaled corticosteroids for cystic fibrosis. Cochrane Database Syst Rev. 2019;7:CD001915.
57. Konstan MW, Byard PJ, Hoppel CL, Davis PB. Effect of high-dose ibuprofen in patients with cystic fibrosis. N Engl J Med. 1995;332(13):848-54.
58. Lands LC, Milner R, Cantin AM, Manson D, Corey M. High-dose ibuprofen in cystic fibrosis: Canadian safety and effectiveness trial. J Pediatr. 2007;151(3):249-54.
59. Konstan MW, Schluchter MD, Xue W, Davis PB. Clinical Use of Ibuprofen Is Associated with Slower FEV1 Decline in Children with Cystic Fibrosis. Am J Respir Crit Care Med. 2007;106:1084-9.
60. Lands LC, Stanojevic S. Oral non-steroidal anti-inflammatory drug therapy for lung disease in cystic fibrosis. Cochrane Database Syst Rev. 2019;9(9):CD001505.
61. Konstan MW. Ibuprofen therapy for cystic fibrosis lung disease: revisited. Curr Opin Pulm Med. 2008;14(6):567-73.
62. Chmiel JF, Konstan MW, Knesebeck JE, Hilliard JB, Bonfield TL, Dawson DV, et al. IL-10 attenuates excessive inflammation in chronic Pseudomonas infection in mice. Am J Respir Crit Care Med. 1999;160(6):2040-7.
63. Moss RB, Mistry SJ, Konstan MW, Pilewski JM, Kerem E, Tal-Singer R, et al. Safety and early treatment effects of the CXCR2 antagonist SB-656933 in patients with cystic fibrosis. J Cyst Fibros. 2013;12(3):241-8.
64. Bergmann U, Scheffer J, Koller M, Schonfeld W, Erbs G, Muller FE, et al. Induction of inflammatory mediators (histamine and leukotrienes) from rat peritoneal mast cells and human granulocytes by *Pseudomonas aeruginosa* strains from burn patients. Infect Immun. 1989;57(7):2187-95.
65. Konstan MW, Doring G, Heltshe SL, Lands LC, Hilliard KA, Koker P, et al. A randomized double blind, placebo controlled phase 2 trial of BIIL 284 BS (an LTB4 receptor antagonist) for the treatment of lung disease in children and adults with cystic fibrosis. J Cyst Fibros. 2014;13(2):148-55.
66. Mall MA. Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. J Aerosol Med Pulm Drug Deliv. 2008;21(1):13-24.
67. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Nature. 2012;487(7405):109-13.
68. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest. 2002;109(3):317-25.

69. Bonfield TL, Hodges CA, Cotton CU, Drumm ML. Absence of the cystic fibrosis transmembrane regulator (Cftr) from myeloid-derived cells slows resolution of inflammation and infection. J Leukoc Biol. 2012;92(5):1111-22.
70. UK Cystic Fibrosis Trust. 2020 Annual Data Report. 2021 December 2021.
71. Hoiby N, Frederiksen B, Pressler T. Eradication of early *Pseudomonas aeruginosa* infection. J Cyst Fibros. 2005;4 Suppl 2:49-54.
72. Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, et al. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J Infect Dis. 2001;183(3):444-52.
73. Tümmler B, Koopmann U, Grothues D, Weissbrodt H, Steinkamp G, von der Hardt H. Nosocomial Aqcuisition of *Pseudomonas aeruginosa* by Cystic Fibrosis Patients. Journal of Medical Microbiology. 1991;29:1265-7.
74. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. Antimicrob Agents Chemother. 2006;50(1):43-8.
75. Cheng K, Smyth RL, Govan JR, Doherty C, Winstanley C, Denning N, et al. Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. Lancet. 1996;348(9028):639-42.
76. Langton Hewer SC, Smyth AR. Antibiotic strategies for eradicating *Pseudomonas aeruginosa* in people with cystic fibrosis. Cochrane Database Syst Rev. 2014(11):CD004197.
77. Hansen CR, Pressler T, Hoiby N. Early aggressive eradication therapy for intermittent *Pseudomonas aeruginosa* airway colonization in cystic fibrosis patients: 15 years experience. J Cyst Fibros. 2008;7(6):523-30.
78. Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. Biotechnol Adv. 2019;37(1):177-92.
79. Breidenstein EB, de la Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol. 2011;19(8):419-26.
80. Hancock RE, Brinkman FS. Function of pseudomonas porins in uptake and efflux. Annu Rev Microbiol. 2002;56:17-38.
81. Sugawara E, Nestorovich EM, Bezrukov SM, Nikaido H. *Pseudomonas aeruginosa* porin OprF exists in two different conformations. J Biol Chem. 2006;281(24):16220-9.
82. Hancock RE. The bacterial outer membrane as a drug barrier. Trends Microbiol. 1997;5:37-42.
83. Daury L, Orange F, Taveau JC, Verchere A, Monlezun L, Gounou C, et al. Tripartite assembly of RND multidrug efflux pumps. Nat Commun. 2016;7:10731.
84. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs. 2009;69(12):1555-623.
85. Dupont P, Hocquet D, Jeannot K, Chavanet P, Plesiat P. Bacteriostatic and bactericidal activities of eight fluoroquinolones against MexAB-OprM-overproducing clinical strains of *Pseudomonas aeruginosa*. J Antimicrob Chemother. 2005;55(4):518-22.
86. Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plesiat P. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob Agents Chemother. 2004;48(5):1797-802.
87. El-Shaer S, Shaaban M, Barwa R, Hassan R. Control of quorum sensing and virulence factors of *Pseudomonas aeruginosa* using phenylalanine arginyl beta-naphthylamide. J Med Microbiol. 2016;65:1194-204.
88. Wright GD. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv Drug Deliv Rev. 2005;57(10):1451-70.
89. Wolter DJ, Lister PD. Mechanisms of beta-lactam resistance among *Pseudomonas aeruginosa.* Curr Pharm Des. 2013;19:209–22.
90. Rawat D, Nair D. Extended-spectrum beta-lactamases in Gram Negative Bacteria. J Glob Infect Dis. 2010;2(3):263-74.

91. Poole K. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2005;49:479-87.
92. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. 2000;406(6799):959-64.
93. Girgis HS, Hottes AK, Tavazoie S. Genetic architecture of intrinsic antibiotic susceptibility. PLoS One. 2009;4(5):e5629.
94. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martinez JL. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. Antimicrob Agents Chemother. 2010;54(10):4159-67.
95. Organization WH. Global Action Plan on Antimicrobial Resistance. 2015. Report No.: 978 92 4 150976 3.
96. Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol. 2005;13(1):20-6.
97. Wimpenny J, Manz W, Szewzyk U. Heterogeneity in biofilms. FEMS Microbiol Rev. 2000;24(5):661-71.
98. Smith AL, Fiel SB, Mayer-Hamblett N, Ramsey B, Burns JL. Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. Chest. 2003;123(5):1495-502.
99. Boudarel H, Mathias JD, Blaysat B, Grediac M. Towards standardized mechanical characterization of microbial biofilms: analysis and critical review. NPJ Biofilms Microbiomes. 2018;4:17.
100. Alhede M, Kragh KN, Qvortrup K, Allesen-Holm M, van Gennip M, Christensen LD, et al. Phenotypes of non-attached *Pseudomonas aeruginosa* aggregates resemble surface attached biofilm. PLoS One. 2011;6(11):e27943.
101. Hoiby N, Ciofu O, Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. Future Microbiol. 2010;5(11):1663-74.
102. Siryaporn A, Kuchma SL, O'Toole GA, Gitai Z. Surface attachment induces *Pseudomonas aeruginosa* virulence. Proc Natl Acad Sci U S A. 2014;111(47):16860-5.
103. Martin I, Waters V, Grasemann H. Approaches to Targeting Bacterial Biofilms in Cystic Fibrosis Airways. Int J Mol Sci. 2021;22(4).
104. Marvin Whiteley MB, M. G.; Bumharner, R. E.; Parsek, M. R.; Teitzel, G. M.; Lory, S.; and Greenberg, E. P. . Gene expression in *Pseudomonas aeruginosa* biofilms. Nature. 2001;413:860-4.
105. Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol. 2004;186(21):7312-26.
106. Banin E, Brady KM, Greenberg EP. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl Environ Microbiol. 2006;72(3):2064-9.
107. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunol Med Microbiol. 1999;26(3-4):189-95.
108. Mulcahy LR, Burns JL, Lory S, Lewis K. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol. 2010;192(23):6191-9.
109. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett. 2004;230(1):13-8.
110. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, et al. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol. 2012;10(12):841-51.
111. Mowat E, Paterson S, Fothergill JL, Wright EA, Ledson MJ, Walshaw MJ, et al. *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. Am J Respir Crit Care Med. 2011;183(12):1674-9.

112. Fernandez-Barat L, Ciofu O, Kragh KN, Pressler T, Johansen U, Motos A, et al. Phenotypic shift in *Pseudomonas aeruginosa* populations from cystic fibrosis lungs after 2-week antipseudomonal treatment. J Cyst Fibros. 2017;16(2):222-9.
113. Jorth P, Spero MA, Livingston J, Newman DK. Quantitative Visualization of Gene Expression in Mucoid and Nonmucoid *Pseudomonas aeruginosa* Aggregates Reveals Localized Peak Expression of Alginate in the Hypoxic Zone. mBio. 2019;10(6).
114. Lee B, Schjerling CK, Kirkby N, Hoffmann N, Borup R, Molin S, et al. Mucoid *Pseudomonas aeruginosa* isolates maintain the biofilm formation capacity and the gene expression profiles during the chronic lung infection of CF patients. APMIS. 2011;119(4-5):263-74.
115. Thomassen MJ, Demko CA, Boxerbaum B, Stern RC, Kuchenbrod PJ. Multiple of isolates of *Pseudomonas aeruginosa* with differing antimicrobial susceptibility patterns from patients with cystic fibrosis. J Infect Dis. 1979;140(6):873-80.
116. Shawar RM, MacLeod DL, Garber RL, Burns JL, Stapp JR, Clausen CR, et al. Activities of tobramycin and six other antibiotics against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob Agents Chemother. 1999;43(12):2877-80.
117. Srifuengfung S, Tiensasitorn C, Yungyuen T, Dhiraputra C. Prevalence and antimicrobial susceptibility of *Pseudomonas aeruginosa* mucoid and non-mucoid type. Southeast Asian J Trop Med Public Health. 2004;35(4):893-6.
118. Ciofu O, Fussing V, Bagge N, Koch C, Hoiby N. Characterization of paired mucoid/nonmucoid *Pseudomonas aeruginosa* isolates from Danish cystic fibrosis patients: antibiotic resistance, beta-lactamase activity and Ribo-Printing. J Antimicrob Chemother. 2001;48:391-6.
119. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, et al. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A. 2006;103(22):8487-92.
120. Jelsbak L, Johansen HK, Frost AL, Thogersen R, Thomsen LE, Ciofu O, et al. Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. Infect Immun. 2007;75(5):2214-24.
121. Bragonzi A, Paroni M, Nonis A, Cramer N, Montanari S, Rejman J, et al. *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am J Respir Crit Care Med. 2009;180(2):138-45.
122. Sutherland I. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology (Reading). 2001;147(Pt 1):3-9.
123. Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. J Immunol. 2005;175(11):7512-8.
124. Camilli A, Bassler BL. Bacterial small-molecule signaling pathways. Science. 2006;311(5764):1113-6.
125. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 2005;21:319-46.
126. Singh P, Schaefer A, Parsek M, Moninger TO, Welsh MJ, Greenberg EP. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature. 2000;407:762–4.
127. Schuster M, Greenberg EP. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int J Med Microbiol. 2006;296(2-3):73-81.
128. Venturi V. Regulation of quorum sensing in Pseudomonas. FEMS Microbiol Rev. 2006;30(2):274-91.
129. De Kievit TR, Gillis R, Marx S, Brown C, Iglewski BH. Quorum-Sensing Genes in *Pseudomonas aeruginosa* Biofilms: Their Role and Expression Patterns. Appl Environ Microbiol. 2001;67.
130. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. J Bacteriol. 2002;184:1140–54.

131. Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol. 1996;21(6):1137-46.
132. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol. 2006;59(4):1114-28.
133. Diggle SP, Cornelis P, Williams P, Camara M. 4-quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. Int J Med Microbiol. 2006;296(2-3):83-91.
134. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog. 2008;4(11):e1000213.
135. Finkel SE, Kolter R. DNA as a Nutrient: Novel Role for Bacterial Competence Gene Homologs. J Bacteriol. 2001;183.
136. Ueda A, Wood TK. Connecting Quorum Sensing, c-di-GMP, Pel Polysaccharide, and Biofilm Formation in *Pseudomonas aeruginosa* through Tyrosine Phosphatase TpbA (PA3885). PLoS Pathog. 2009;5(6):e1000483.
137. Derbyshire ER, Marletta MA. Structure and regulation of soluble guanylate cyclase. Annu Rev Biochem. 2012;81:533-59.
138. Arora DP, Hossain S, Xu Y, Boon EM. Nitric Oxide Regulation of Bacterial Biofilms. Biochemistry. 2015;54(24):3717-28.
139. Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J Bacteriol. 2006;188(21):7344-53.
140. Barraud N, Kelso MJ, Rice SA, Kjelleberg S. Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. Curr Pharm Des. 2015;21(1):31-42.
141. Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, et al. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. J Bacteriol. 2009;191(23):7333-42.
142. Chua SL, Hultqvist LD, Yuan M, Rybtke M, Nielsen TE, Givskov M, et al. In vitro and in vivo generation and characterization of *Pseudomonas aeruginosa* biofilm-dispersed cells via c-di-GMP manipulation. Nat Protoc. 2015;10(8):1165-80.
143. Chua SL, Liu Y, Yam JK, Chen Y, Vejborg RM, Tan BG, et al. Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. Nat Commun. 2014;5:4462.
144. Doring G, Flume P, Heijerman H, Elborn JS, Consensus Study G. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. J Cyst Fibros. 2012;11(6):461-79.
145. Pitt TL, Sparrow M, Warner M, Stefanidou M. Survey of resistance of *Pseudomonas aeruginosa* from UK patients with cystic fibrosis to six commonly prescribed antimicrobial agents. Thorax. 2003;58(9):794-6.
146. Hirsch EB, Tam VH. Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. Expert Rev Pharmacoecon Outcomes Res. 2010;10(4):441-51.
147. Lucca F, Guarnieri M, Ros M, Muffato G, Rigoli R, Da Dalt L. Antibiotic resistance evolution of *Pseudomonas aeruginosa* in cystic fibrosis patients (2010-2013). Clin Respir J. 2018;12(7):2189-96.
148. Sanders DB, Bittner RC, Rosenfeld M, Hoffman LR, Redding GJ, Goss CH. Failure to recover to baseline pulmonary function after cystic fibrosis pulmonary exacerbation. Am J Respir Crit Care Med. 2010;182(5):627-32.
149. Collinson J, Nicholson KG, Cancio E, Ashman J, Ireland DC, Hammersley V, et al. Effects of upper respiratory tract infections in patients with cystic fibrosis. Thorax. 1996;51(11):1115-22.
150. Johansen HK, Hoiby N. Seasonal onset of initial colonisation and chronic infection with *Pseudomonas aeruginosa* in patients with cystic fibrosis in Denmark. Thorax. 1992;47(2):109-11.
151. Van Ewijk BE, Wolfs TF, Aerts PC, Van Kessel KP, Fleer A, Kimpen JL, et al. RSV mediates *Pseudomonas aeruginosa* binding to cystic fibrosis and normal epithelial cells. Pediatr Res. 2007;61(4):398-403.

152. de Vrankrijker AM, Wolfs TF, Ciofu O, Hoiby N, van der Ent CK, Poulsen SS, et al. Respiratory syncytial virus infection facilitates acute colonization of *Pseudomonas aeruginosa* in mice. J Med Virol. 2009;81(12):2096-103.
153. Chattoraj SS, Ganesan S, Jones AM, Helm JM, Comstock AT, Bright-Thomas R, et al. Rhinovirus infection liberates planktonic bacteria from biofilm and increases chemokine responses in cystic fibrosis airway epithelial cells. Thorax. 2011;66(4):333-9.
154. Kieninger E, Vareille M, Kopf BS, Blank F, Alves MP, Gisler FM, et al. Lack of an exaggerated inflammatory response on virus infection in cystic fibrosis. Eur Respir J. 2012;39(2):297-304.
155. Herrmann G, Yang L, Wu H, Song Z, Wang H, Hoiby N, et al. Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*. J Infect Dis. 2010;202(10):1585-92.
156. Gomez-Junyent J, Benavent E, Sierra Y, El Haj C, Soldevila L, Torrejon B, et al. Efficacy of ceftolozane/tazobactam, alone and in combination with colistin, against multidrug-resistant *Pseudomonas aeruginosa* in an in vitro biofilm pharmacodynamic model. Int J Antimicrob Agents. 2019;53(5):612-9.
157. Baek MS, Chung ES, Jung DS, Ko KS. Effect of colistin-based antibiotic combinations on the eradication of persister cells in *Pseudomonas aeruginosa*. J Antimicrob Chemother. 2020;75(4):917-24.
158. Bassetti M, Vena A, Russo A, Peghin M. Inhaled Liposomal Antimicrobial Delivery in Lung Infections. Drugs. 2020;80(13):1309-18.
159. Rukavina Z, Vanic Z. Current Trends in Development of Liposomes for Targeting Bacterial Biofilms. Pharmaceutics. 2016;8(2).
160. Moriarty TEM, J. C.; and Elborn, J. S. Intrapulmonary concentrations of tobramycin and ceftazidime achieved after intravenous administration for the treatment of Cystic Fibrosis lung infections. Journal of Cystic Fibrosis. 2006;5(Supplement 1):S20.
161. Moriarty TF, McElnay JC, Elborn JS, Tunney MM. Sputum antibiotic concentrations: implications for treatment of cystic fibrosis lung infection. Pediatr Pulmonol. 2007;42(11):1008-17.
162. Conway SP. Nebulized antibiotic therapy: the evidence. Chron Respir Dis. 2005;2(1):35-41.
163. Chmiel JF, Aksamit TR, Chotirmall SH, Dasenbrook EC, Elborn JS, LiPuma JJ, et al. Antibiotic management of lung infections in cystic fibrosis. I. The microbiome, methicillin-resistant Staphylococcus aureus, gram-negative bacteria, and multiple infections. Ann Am Thorac Soc. 2014;11(7):1120-9.
164. Yang C, Chilvers M, Montgomery M, Nolan SJ. Dornase alfa for cystic fibrosis. Cochrane Database Syst Rev. 2016;4:CD001127.
165. Kaplan JB, LoVetri K, Cardona ST, Madhyastha S, Sadovskaya I, Jabbouri S, et al. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. J Antibiot (Tokyo). 2012;65(2):73-7.
166. Sharma K, Pagedar Singh A. Antibiofilm Effect of DNase against Single and Mixed Species Biofilm. Foods. 2018;7(3).
167. Pritchard MF, Powell LC, Menzies GE, Lewis PD, Hawkins K, Wright C, et al. A New Class of Safe Oligosaccharide Polymer Therapy To Modify the Mucus Barrier of Chronic Respiratory Disease. Mol Pharm. 2016;13(3):863-72.
168. Powell LC, Pritchard MF, Ferguson EL, Powell KA, Patel SU, Rye PD, et al. Targeted disruption of the extracellular polymeric network of *Pseudomonas aeruginosa* biofilms by alginate oligosaccharides. NPJ Biofilms Microbiomes. 2018;4:13.
169. Khan S, Tondervik A, Sletta H, Klinkenberg G, Emanuel C, Onsoyen E, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics. Antimicrob Agents Chemother. 2012;56(10):5134-41.
170. Tondervik A, Sletta H, Klinkenberg G, Emanuel C, Powell LC, Pritchard MF, et al. Alginate oligosaccharides inhibit fungal cell growth and potentiate the activity of antifungals against Candida and Aspergillus spp. PLoS One. 2014;9(11):e112518.

171. van Koningsbruggen-Rietschel S, Davies JC, Pressler T, Fischer R, MacGregor G, Donaldson SH, et al. Inhaled dry powder alginate oligosaccharide in cystic fibrosis: a randomised, double-blind, placebo-controlled, crossover phase 2b study. ERJ Open Res. 2020;6(4).
172. A Dose-finding Study of Inhaled OligoG vs Placebo in Patients With Cystic Fibrosis (SMR3372): US National Libarary of Medicine; 2020 [Available from: https://www.clinicaltrials.gov/ct2/show/NCT03698448?term=oligog&cond=Cystic+Fibrosis&draw=2&rank=4.
173. Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song Z, Johansen HK, et al. Azithromycin Blocks Quorum Sensing and Alginate Polymer Formation and Increases the Sensitivity to Serum and Stationary-Growth-Phase Killing of *Pseudomonas aeruginosa* and Attenuates Chronic P. aeruginosa Lung Infection in Cftr−/− Mice. Antimicrob Agents Chemother. 2007;51:3677–87.
174. Florescu DF, Murphy PJ, Kalil AC. Effects of prolonged use of azithromycin in patientswith cystic fibrosis: A meta-analysis. Pulmon Pharmaco Ther. 2009;22.
175. Abedon ST, Thomas-Abedon C, Thomas A, Mazure H. Bacteriophage prehistory: Is or is not Hankin, 1896, a phage reference? Bacteriophage. 2011;1(3):174-8.
176. Hankin EH. L'action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera. Ann Inst Pasteur. 1896;10:511.
177. d'Hérelle F. The Bacteriophage and its Behavior. J Immunol. 1926.
178. Breitbart M, Rohwer F. Here a virus, there a virus, everywhere the same virus? Trends Microbiol. 2005;13(6):278-84.
179. Koskella B. Bacteria-phage interactions across time and space: merging local adaptation and time-shift experiments to understand phage evolution. Am Nat. 2014;184 Suppl 1:S9-21.
180. Abrescia NG, Bamford DH, Grimes JM, Stuart DI. Structure unifies the viral universe. Annu Rev Biochem. 2012;81:795-822.
181. Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, et al. Virus Taxonomy: Springer Science & Business Media; 1995.
182. Bradley DE. Ultrastructure of bacteriophage and bacteriocins. Bacteriol Rev. 1967;31(4):230-314.
183. Ackermann HW. Bacteriophage electron microscopy. Adv Virus Res. 2012;82:1-32.
184. Ackermann HW. Under the Microscope: Bateriophage Taxonomy. Microbiology Australia. 2011:90-4.
185. Sayers EW, Cavanaugh M, Clark K, Pruitt KD, Schoch CL, Sherry ST, et al. GenBank. Nucleic Acids Res. 2021;49(D1):D92-D6.
186. Casasnovas JM. Virus-receptor interactions and receptor-mediated virus entry into host cells. Subcell Biochem. 2013;68:441-66.
187. Marsh M, Helenius A. Virus entry: open sesame. Cell. 2006;124(4):729-40.
188. Baranowski E, Ruiz-Jarabo CM, Domingo E. Evolution of cell recognition by viruses. Science. 2001;292(5519):1102-5.
189. Stehle T, Casasnovas JM. Specificity switching in virus-receptor complexes. Curr Opin Struct Biol. 2009;19(2):181-8.
190. Young R. Phage lysis: do we have the hole story yet? Curr Opin Microbiol. 2013;16(6):790-7.
191. Goldberg E, Grinius L, Letellier L. Recognition, attachment and injection. Mathews CKea, editor: American Society for Microbiology. Wahsington, DC, USA.; 1994.
192. Young R. Phage lysis: three steps, three choices, one outcome. J Microbiol. 2014;52(3):243-58.
193. Hadas H, Einav M, Fishov I, Zaritsky A. Bacteriophage T4 development depends on the physiology of its host Escherichia coli. Microbiology (Reading). 1997;143 (Pt 1):179-85.
194. Abedon ST, LeJeune JT. Why Bacteriophage Encode Exotoxins and other Virulence Factors. Evolutionary Bioinformatics. 2017;1.
195. Wagner PL, Waldor MK. Bacteriophage control of bacterial virulence. Infect Immun. 2002;70(8):3985-93.

196. Frobisher MJ, Brown JH. Transmissable Toxogenicity of Streptococci. Bulletin of the Johns Hopkins Hospital. 1927;41:167-73.
197. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996;272(5270):1910-4.
198. Holguin AV, Rangel G, Clavijo V, Prada C, Mantilla M, Gomez MC, et al. Phage PhiPan70, a Putative Temperate Phage, Controls *Pseudomonas aeruginosa* in Planktonic, Biofilm and Burn Mouse Model Assays. Viruses. 2015;7(8):4602-23.
199. Davies EV, James CE, Kukavica-Ibrulj I, Levesque RC, Brockhurst MA, Winstanley C. Temperate phages enhance pathogen fitness in chronic lung infection. ISME J. 2016;10(10):2553-5.
200. Rice SA, Tan CH, Mikkelsen PJ, Kung V, Woo J, Tay M, et al. The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. ISME J. 2009;3(3):271-82.
201. Ambroa A, Blasco L, Lopez-Causape C, Trastoy R, Fernandez-Garcia L, Bleriot I, et al. Temperate Bacteriophages (Prophages) in *Pseudomonas aeruginosa* Isolates Belonging to the International Cystic Fibrosis Clone (CC274). Front Microbiol. 2020;11:556706.
202. Marvin DA, Symmons MF, Straus SK. Structure and assembly of filamentous bacteriophages. Prog Biophys Mol Biol. 2014;114(2):80-122.
203. Martinez E, Campos-Gomez J. Pf Filamentous Phage Requires UvrD for Replication in *Pseudomonas aeruginosa*. mSphere. 2016;1(1).
204. Burgener EB, Sweere JM, Bach MS, Secor PR, Haddock N, Jennings LK, et al. Filamentous bacteriophages are associated with chronic Pseudomonas lung infections and antibiotic resistance in cystic fibrosis. Sci Transl Med. 2019;11(488).
205. Fong SA, Drilling A, Morales S, Cornet ME, Woodworth BA, Fokkens WJ, et al. Activity of Bacteriophages in Removing Biofilms of *Pseudomonas aeruginosa* Isolates from Chronic Rhinosinusitis Patients. Front Cell Infect Microbiol. 2017;7:418.
206. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. Nat Med. 2019;25(5):730-3.
207. Kutateladze M, Adamia R. Phage therapy experience at the Eliava Institute. Med Mal Infect. 2008;38(8):426-30.
208. Fruciano DE, Bourne S. Phage as an antimicrobial agent: d'Herelle's heretical theories and their role in the decline of phage prophylaxis in the West. Can J Infect Dis Med Microbiol. 2007;18(1):19-26.
209. da Costa Cruz J. Le traitement des dysenteries bacillaires par le bacteriophage. Compt rend Soc Biol. 1924;91:845.
210. d'Hérelle F. Le bactériophage et ses application Thérapeutique: Paris Doin; 1933. 30 p.
211. Compton A. Bacteriophage Treatment of Bacillary Dysentery. Br Med J. 1942;1(4249):719-20.
212. Kutter E. Bacteriophages: Biology and Applications. 1st Edition ed: CRC Press; 2004.
213. Eaton MD, Bayne-Jones S. Bacteriophage therapy. JAMA. 1934;103:1769-76.
214. Krueger AP, Scribner EJ. The Bacteriophage. Journal of the American Medical Association. 1941;116(20).
215. Morton HE, Engley Jr FB. Dysentery Baeteriophage. Review of the Literature on its Prophylactic and Therapeutic Uses in Man and in Experimental Infections in Animals. JAMA. 1945;127(10):584-91.
216. Blainey PC, Milla CE, Cornfield DN, Quake SR. Quantitative analysis of the human airway microbial ecology reveals a pervasive signature for cystic fibrosis. Sci Transl Med. 2012;4(153):153ra30.
217. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proc Natl Acad Sci U S A. 2012;109(34):13769-74.

218. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A. 2012;109(15):5809-14.
219. Dabrowska K. Phage therapy: What factors shape phage pharmacokinetics and bioavailability? Systematic and critical review. Med Res Rev. 2019;39(5):2000-25.
220. Kakasis A, Panitsa G. Bacteriophage therapy as an alternative treatment for human infections. A comprehensive review. Int J Antimicrob Agents. 2019;53(1):16-21.
221. Chanishvili N. Bacteriophages as Therapeutic and Prophylactic Means: Summary of the Soviet and Post Soviet Experiences. Curr Drug Deliv. 2016;13(3):309-23.
222. Hyman P, Abedon ST. Chapter 7 - Bacteriophage Host Range and Bacterial Resistance. Advances in Applied Microbiology. 2010;70:217-48.
223. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol. 2010;8(5):317-27.
224. Lusiak-Szelachowska M, Zaczek M, Weber-Dabrowska B, Miedzybrodzki R, Klak M, Fortuna W, et al. Phage neutralization by sera of patients receiving phage therapy. Viral Immunol. 2014;27(6):295-304.
225. Zaczek M, Lusiak-Szelachowska M, Jonczyk-Matysiak E, Weber-Dabrowska B, Miedzybrodzki R, Owczarek B, et al. Antibody Production in Response to Staphylococcal MS-1 Phage Cocktail in Patients Undergoing Phage Therapy. Front Microbiol. 2016;7:1681.
226. Semler DD, Goudie AD, Finlay WH, Dennis JJ. Aerosol phage therapy efficacy in Burkholderia cepacia complex respiratory infections. Antimicrob Agents Chemother. 2014;58(7):4005-13.
227. Davison PF. The Effect of Hydrodynamic Shear on the Deoxyribonucleic Acid from T(2) and T(4) Bacteriophages. Proc Natl Acad Sci U S A. 1959;45(11):1560-8.
228. Leung SSY, Parumasivam T, Gao FG, Carter EA, Carrigy NB, Vehring R, et al. Effects of storage conditions on the stability of spray dried, inhalable bacteriophage powders. Int J Pharm. 2017;521(1-2):141-9.
229. Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Tediashvili M, Lashkhi N, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One. 2009;4(3):e4944.
230. Pirnay JP, Merabishvili M, Van Raemdonck H, De Vos D, Verbeken G. Bacteriophage Production in Compliance with Regulatory Requirements. Methods Mol Biol. 2018;1693:233-52.
231. Mac NW, Filak L, Blevins A. Conjoined action of penicillin and bacteriophages. J Lab Clin Med. 1946;31(9):974-81.
232. Pires DP, Cleto S, Sillankorva S, Azeredo J, Lu TK. Genetically Engineered Phages: a Review of Advances over the Last Decade. Microbiol Mol Biol Rev. 2016;80(3):523-43.
233. Chen Y, Batra H, Dong J, Chen C, Rao VB, Tao P. Genetic Engineering of Bacteriophages Against Infectious Diseases. Front Microbiol. 2019;10:954.
234. Edgar R, Friedman N, Molshanski-Mor S, Qimron U. Reversing bacterial resistance to antibiotics by phage-mediated delivery of dominant sensitive genes. Appl Environ Microbiol. 2012;78(3):744-51.
235. Monteiro R, Pires DP, Costa AR, Azeredo J. Phage Therapy: Going Temperate? Trends Microbiol. 2019;27(4):368-78.
236. Kwiatek M, Parasion S, Rutyna P, Mizak L, Gryko R, Niemcewicz M, et al. Isolation of bacteriophages and their application to control *Pseudomonas aeruginosa* in planktonic and biofilm models. Res Microbiol. 2017;168(3):194-207.
237. Rohde C, Resch G, Pirnay JP, Blasdel BG, Debarbieux L, Gelman D, et al. Expert Opinion on Three Phage Therapy Related Topics: Bacterial Phage Resistance, Phage Training and Prophages in Bacterial Production Strains. Viruses. 2018;10(4).
238. Nikolich MP, Filippov AA. Bacteriophage Therapy: Developments and Directions. Antibiotics (Basel). 2020;9(3).

239. Wright A, Hawkins, C. H., Anggard, E. E., and Harper, D. R. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiot ic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. Clinical Otolaryngology. 2009;34:349-57.
240. Saussereau E, Vachier I, Chiron R, Godbert B, Sermet I, Dufour N, et al. Effectiveness of bacteriophages in the sputum of cystic fibrosis patients. Clin Microbiol Infect. 2014;20(12):O983-90.
241. Pabary R, Singh C, Morales S, Bush A, Alshafi K, Bilton D, et al. Antipseudomonal Bacteriophage Reduces Infective Burden and Inflammatory Response in Murine Lung. Antimicrob Agents Chemother. 2016;60(2):744-51.
242. Waters EM, Neill DR, Kaman B, Sahota JS, Clokie MRJ, Winstanley C, et al. Phage therapy is highly effective against chronic lung infections with *Pseudomonas aeruginosa*. Thorax. 2017;72(7):666-7.
243. Aslam S, Courtwright AM, Koval C, Lehman SM, Morales S, Furr CL, et al. Early clinical experience of bacteriophage therapy in 3 lung transplant recipients. Am J Transplant. 2019;19(9):2631-9.
244. Sutton S. Measurement of Microbial Cells by Optical Density. Journal of Validation Technology. 2011(Winter 2011):46-9.
245. McBirney SE, Trinh K, Wong-Beringer A, Armani AM. Wavelength-normalized spectroscopic analysis of Staphylococcus aureus and *Pseudomonas aeruginosa* growth rates. Biomed Opt Express. 2016;7(10):4034-42.
246. Shim H. [Available from: https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Kinetics/02%3A_Reaction_Rates/2.01%3A_Experimental_Determination_of_Kinetics/2.1.05%3A_Spectrophotometry.
247. Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. Curr Protoc Microbiol. 2005;Chapter 1:Unit 1B
248. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp. 2011(47).
249. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study of biofilms. Methods Enzymol. 1999;310:91-109.
250. Rampersad SN. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors (Basel). 2012;12(9):12347-60.
251. Labtech B. Gain Optimization for Microplate Readers. YouTube2018.
252. Matuschek E, Brown DF, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect. 2014;20(4):O255-66.
253. Harrison JJ, Stremick CA, Turner RJ, Allan ND, Olson ME, Ceri H. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. Nat Protoc. 2010;5(7):1236-54.
254. Turton JF, Turton SE, Yearwood L, Yarde S, Kaufmann ME, Pitt TL. Evaluation of a nine-locus variable-number tandem-repeat scheme for typing of *Pseudomonas aeruginosa*. Clin Microbiol Infect. 2010;16(8):1111-6.
255. Martin K, Baddal B, Mustafa N, Perry C, Underwood A, Constantidou C, et al. Clusters of genetically similar isolates of *Pseudomonas aeruginosa* from multiple hospitals in the UK. J Med Microbiol. 2013;62(Pt 7):988-1000.
256. Biosciences M. HyperLadder 50bp https://www.bioline.com/hyperladder-50bp.html2020 [Available from: https://www.bioline.com/hyperladder-50bp.html.
257. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics. 2006;7:123.
258. Turcios NL. Cystic Fibrosis Lung Disease: An Overview. Respir Care. 2020;65(2):233-51.
259. Smith S, Rowbotham NJ, Regan KH. Inhaled anti-pseudomonal antibiotics for long-term therapy in cystic fibrosis. Cochrane Database Syst Rev. 2018;3:CD001021.

260. Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. 2009;44(6):547-58.
261. Olson ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res. 2002;66(2):86-92.
262. Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature. 2003;426(6964):306-10.
263. Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. J Appl Microbiol. 2008;104(1):1-13.
264. Batoni G, Maisetta G, Esin S. Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. Biochim Biophys Acta. 2016;1858(5):1044-60.
265. Ehsan Z, Wetzel JD, Clancy JP. Nebulized liposomal amikacin for the treatment of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. Expert Opin Investig Drugs. 2014;23(5):743-9.
266. Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophages phiMR299-2 and phiNH-4 can eliminate *Pseudomonas aeruginosa* in the murine lung and on cystic fibrosis lung airway cells. mBio. 2012;3(2):e00029-12.
267. Glonti T, Chanishvili N, Taylor PW. Bacteriophage-derived enzyme that depolymerizes the alginic acid capsule associated with cystic fibrosis isolates of *Pseudomonas aeruginosa*. J Appl Microbiol. 2010;108(2):695-702.
268. Alkawash MA, Soothill JS, Schiller NL. Alginate lyase enhances antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. APMIS. 2006;114(2):131-8.
269. Davidson IW, Lawson CJ, Sutherland IW. An alginate lysate from Azotobacter vinelandii phage. J Gen Microbiol. 1977;98(1):223-9.
270. Yan J, Mao J, Xie J. Bacteriophage polysaccharide depolymerases and biomedical applications. BioDrugs. 2014;28(3):265-74.
271. Tkhilaishvili T, Lombardi L, Klatt AB, Trampuz A, Di Luca M. Bacteriophage Sb-1 enhances antibiotic activity against biofilm, degrades exopolysaccharide matrix and targets persisters of Staphylococcus aureus. Int J Antimicrob Agents. 2018;52(6):842-53.
272. Pei R, Lamas-Samanamud GR. Inhibition of biofilm formation by T7 bacteriophages producing quorum-quenching enzymes. Appl Environ Microbiol. 2014;80(17):5340-8.
273. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol. 2009;501:69-76.
274. Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol. 2009;17(2):66-72.
275. Tait K, Skillman LC, Sutherland IW. The efficacy of bacteriophage as a method of biofilm eradication. Biofouling. 2002;18(4):305-11.
276. Carson L, Gorman SP, Gilmore BF. The use of lytic bacteriophages in the prevention and eradication of biofilms of Proteus mirabilis and Escherichia coli. FEMS Immunol Med Microbiol. 2010;59(3):447-55.
277. Chegini Z, Khoshbayan A, Taati Moghadam M, Farahani I, Jazireian P, Shariati A. Bacteriophage therapy against *Pseudomonas aeruginosa* biofilms: a review. Ann Clin Microbiol Antimicrob. 2020;19(1):45.
278. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. 2005;436(7054):1171-5.
279. Horne MT. Coevolution of Escherichia coli and bacteriophages in chemostat culture. Science. 1970;168(3934):992-3.
280. Wright A, McConnel lM, Kanegasaki S. Lipopolysaccharide as a Bacteriophage Receptor. Randall L.L. PL, editor: Springer, Dordrecht; 1980.

281. Lenski RE, Levin BR. Constraints on the Coevolution of Bacteria and Virulent Phage: A Model, Some Experiments, and Predictions for Natural Communities. The American Naturalist. 1985;125(4):585-602.
282. Abedon ST. Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy? Antibiotics (Basel). 2019;8(4).
283. Matsui T, Yoshikawa G, Mihara T, Chatchawankanphanich O, Kawasaki T, Nakano M, et al. Replications of Two Closely Related Groups of Jumbo Phages Show Different Level of Dependence on Host-encoded RNA Polymerase. Front Microbiol. 2017;8:1010.
284. Riveros-Moreno V. Effect of rifampicin on bacteriophage PM2 biogenesis. FEBS letters. 1975;51(1-2):249-52.
285. Blasdel BG, Chevallereau A, Monot M, Lavigne R, Debarbieux L. Comparative transcriptomics analyses reveal the conservation of an ancestral infectious strategy in two bacteriophage genera. ISME J. 2017;11(9):1988-96.
286. Gu Liu C, Green SI, Min L, Clark JR, Salazar KC, Terwilliger AL, et al. Phage-Antibiotic Synergy Is Driven by a Unique Combination of Antibacterial Mechanism of Action and Stoichiometry. mBio. 2020;11(4).
287. Berryhill BA, Huseby DL, McCall IC, Hughes D, Levin BR. Joint antibiotic and phage therapy: addressing the limitations of a seemingly ideal phage for treating Staphylococcus aureus infections. BioRxiv. 2020.
288. Chaudhry WN, Concepcion-Acevedo J, Park T, Andleeb S, Bull JJ, Levin BR. Synergy and Order Effects of Antibiotics and Phages in Killing *Pseudomonas aeruginosa* Biofilms. PLoS One. 2017;12(1):e0168615.
289. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 1999;37(6):1771-6.
290. Latka A, Maciejewska B, Majkowska-Skrobek G, Briers Y, Drulis-Kawa Z. Bacteriophage-encoded virion-associated enzymes to overcome the carbohydrate barriers during the infection process. Appl Microbiol Biotechnol. 2017;101(8):3103-19.
291. Pires DP, Oliveira H, Melo LD, Sillankorva S, Azeredo J. Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. Appl Microbiol Biotechnol. 2016;100(5):2141-51.
292. Miller RV, Day M. Contribution of lysogeny, pseudolysogeny, and starvation to phage ecology. Abedon ST, editor. Cambridge, UK: Cambridge University Press; 2008.
293. Los M, Wegrzyn G. Pseudolysogeny. Adv Virus Res. 2012;82:339-49.
294. Orhan G, Bayram A, Zer Y, Balci I. Synergy tests by E test and checkerboard methods of antimicrobial combinations against Brucella melitensis. J Clin Microbiol. 2005;43(1):140-3.
295. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother. 1996;40(8):1914-8.
296. Pharma E. Antimicrobial Synergy Study – Checkerboard Testing 2021 [cited 2021 June 12, 2021]. Available from: https://emerypharma.com/biology/antimicrobial-synergy-study-checkerboard-assay/.
297. Zhou X, Zhang Y, Li Y, Hao X, Liu X, Wang Y. Azithromycin synergistically enhances anti-proliferative activity of vincristine in cervical and gastric cancer cells. Cancers (Basel). 2012;4(4):1318-32.
298. Agresti A, Coull BA. Approximate is Better than "Exact" for Interval Estimation of Binomial Proportions. The American Statistician. 1998;52(2):119-26.
299. Kumaran D, Taha M, Yi Q, Ramirez-Arcos S, Diallo JS, Carli A, et al. Does Treatment Order Matter? Investigating the Ability of Bacteriophage to Augment Antibiotic Activity against Staphylococcus aureus Biofilms. Front Microbiol. 2018;9:127.
300. Surette MG. The cystic fibrosis lung microbiome. Ann Am Thorac Soc. 2014;11 Suppl 1:S61-5.

301. Waters VJ, Kidd TJ, Canton R, Ekkelenkamp MB, Johansen HK, LiPuma JJ, et al. Reconciling Antimicrobial Susceptibility Testing and Clinical Response in Antimicrobial Treatment of Chronic Cystic Fibrosis Lung Infections. Clin Infect Dis. 2019;69(10):1812-6.
302. Kay MK, Erwin TC, McLean RJ, Aron GM. Bacteriophage ecology in Escherichia coli and *Pseudomonas aeruginosa* mixed-biofilm communities. Appl Environ Microbiol. 2011;77(3):821-9.
303. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. Pseudomonas genomes: diverse and adaptable. FEMS Microbiol Rev. 2011;35(4):652-80.
304. Macia MD, Blanquer D, Togores B, Sauleda J, Perez JL, Oliver A. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob Agents Chemother. 2005;49(8):3382-6.
305. Oliver A, Canton R, Campo P, Baquero F, Blazquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science. 2000;288(5469):1251-4.
306. Matic I, Rayssiguier C, Radman M. Interspecies gene exchange in bacteria: The role of SOS and mismatch repair systems in evolution of species. Cell. 1995;80(3):507-15.
307. Funchain P, Yeung A, Stewart J, Clendenin WM, Miller JH. Amplification of mutator cells in a population as a result of horizontal transfer. J Bacteriol. 2001;183(12):3737-41.
308. Oliver A, Mena A. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. Clin Microbiol Infect. 2010;16(7):798-808.
309. Yadav D, Tanveer A, Malviya N, Yadav S. Chapter 1 - Overview and Principles of Bioengineering: The Drivers of Omics Technologies. Barh D, Azevedo V, editors: Academic Press; 2018.
310. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, et al. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. Antimicrob Agents Chemother. 2004;48(4):1175-87.
311. Blazquez J, Gomez-Gomez JM, Oliver A, Juan C, Kapur V, Martin S. PBP3 inhibition elicits adaptive responses in *Pseudomonas aeruginosa*. Mol Microbiol. 2006;62(1):84-99.
312. Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, et al. Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2008;52(10):3648-63.
313. Hay T, Fraud S, Lau CH, Gilmour C, Poole K. Antibiotic inducibility of the mexXY multidrug efflux operon of *Pseudomonas aeruginosa*: involvement of the MexZ anti-repressor ArmZ. PLoS One. 2013;8(2):e56858.
314. Kindrachuk KN, Fernandez L, Bains M, Hancock RE. Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2011;55(5):1874-82.
315. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989;42(8):872-4.
316. Friedman L, Kolter R. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. J Bacteriol. 2004;186(14):4457-65.
317. Larimer C, Winder E, Jeters R, Prowant M, Nettleship I, Addleman RS, et al. A method for rapid quantitative assessment of biofilms with biomolecular staining and image analysis. Anal Bioanal Chem. 2016;408(3):999-1008.
318. QIAGEN. RNeasy Mini Handbook. t www.qiagen.com2019. p. t www.qiagen.com.
319. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Res. 2013;41(10):e108.
320. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40.
321. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.
322. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 2010;11(3):R25.

323. Law CW, Chen Y, Shi W, Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15(2):R29.
324. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.
325. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society: Series B (Methodological). 1995;57(1):289-300.
326. NIAID N. Functional Annotation Tool: DAVID Bioinformatics Resources 6.8 2018 [Available from: https://david.ncifcrf.gov/summary.jsp.
327. Khaledi A, Schniederjans M, Pohl S, Rainer R, Bodenhofer U, Xia B, et al. Transcriptome Profiling of Antimicrobial Resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2016;60(8):4722-33.
328. Scanlan PD, Buckling A. Co-evolution with lytic phage selects for the mucoid phenotype of Pseudomonas fluorescens SBW25. ISME J. 2012;6(6):1148-58.
329. Jaffe A, Chabbert YA, Semonin O. Role of porin proteins OmpF and OmpC in the permeation of beta-lactams. Antimicrob Agents Chemother. 1982;22(6):942-8.
330. Moreno F, Wandersman C. OmpC and LamB proteins can serve as substitute receptors for host range mutants of coliphage TuIa. J Bacteriol. 1980;144(3):1182-5.
331. Mangalea MR, Duerkop BA. Fitness Trade-Offs Resulting from Bacteriophage Resistance Potentiate Synergistic Antibacterial Strategies. Infect Immun. 2020;88(7).
332. Seed KD, Yen M, Shapiro BJ, Hilaire IJ, Charles RC, Teng JE, et al. Evolutionary consequences of intra-patient phage predation on microbial populations. Elife. 2014;3:e03497.
333. Bohannan BJ, Kerr B, Jessup CM, Hughes JB, Sandvik G. Trade-offs and coexistence in microbial microcosms. Antonie Van Leeuwenhoek. 2002;81(1-4):107-15.
334. Bohannan BJM, Lenski RE. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. Ecology Letters. 2000;3(4):362-77.
335. Jessup CM, Bohannan BJ. The shape of an ecological trade-off varies with environment. Ecol Lett. 2008;11(9):947-59.
336. Chaudhry W, Lee E, Worthy A, Weiss Z, Grabowicz M, Vega N, et al. Mucoidy, a general mechanism for maintaining lytic phage in populations of bacteria. bioRxiv. 2020.
337. Simmons M, Bond MC, Drescher K, Bucci V, Nadell CD. Biofilm structure promotes coexistence of phage-resistant and phage susceptible bacteria. BioRxiv. 2019.
338. Workentine ML, Sibley CD, Glezerson B, Purighalla S, Norgaard-Gron JC, Parkins MD, et al. Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. PLoS One. 2013;8(4):e60225.
339. Foweraker JE, Laughton CR, Brown DF, Bilton D. Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. J Antimicrob Chemother. 2005;55(6):921-7.
340. Clark ST, Guttman DS, Hwang DM. Diversification of *Pseudomonas aeruginosa* within the cystic fibrosis lung and its effects on antibiotic resistance. FEMS Microbiol Lett. 2018;365(6).
341. Hurley MN, Ariff AH, Bertenshaw C, Bhatt J, Smyth AR. Results of antibiotic susceptibility testing do not influence clinical outcome in children with cystic fibrosis. J Cyst Fibros. 2012;11(4):288-92.
342. Law N, Logan C, Yung G, Furr CL, Lehman SM, Morales S, et al. Successful adjunctive use of bacteriophage therapy for treatment of multidrug-resistant *Pseudomonas aeruginosa* infection in a cystic fibrosis patient. Infection. 2019;47(4):665-8.
343. A Phase 1b/2a, Multi-Center, Double-Blind, Randomized, Placebo-Controlled, Single and Multiple Ascending Dose Study to Evaluate the Safety and Tolerability of AP-PA02 Multi-Phage Therapeutic Candidate for Inhalation in Subjects With Cystic Fibrosis and Chronic Pulmonary Pseudomonas Aeruginosa (Pa) Infection: US National Library of Medicine; 2020 [Available from:

https://www.clinicaltrials.gov/ct2/show/NCT04596319?term=bacteriophage&cond=Cystic+Fibrosis&draw=2&rank=2.
344. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268-81.
345. Pusch W, Kostrzewa M. Application of MALDI-TOF mass spectrometry in screening and diagnostic research. Curr Pharm Des. 2005;11(20):2577-91.
346. Quanjer P. Standardized lung function testing. ReportWorking Party Standardization of Lung Function Tests. EuropeanCommunity for Coal and Steel. Bull Eur Physiopathol Resp. 1983.
347. Rosenthal M, Bain SH, Cramer D, Helms P, Denison D, Bush A, et al. Lung function in white children aged 4 to 19 years: I--Spirometry. Thorax. 1993;48(8):794-802.
348. Jeukens J, Boyle B, Kukavica-Ibrulj I, Ouellet MM, Aaron SD, Charette SJ, et al. Comparative genomics of isolates of a *Pseudomonas aeruginosa* epidemic strain associated with chronic lung infections of cystic fibrosis patients. PLoS One. 2014;9(2):e87611.
349. Fyfe JA, Govan JR. Chromosomal loci associated with antibiotic hypersensitivity in pulmonary isolates of *Pseudomonas aeruginosa*. J Gen Microbiol. 1984;130(4):825-34.
350. Cramer N, Wiehlmann L, Ciofu O, Tamm S, Hoiby N, Tummler B. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. PLoS One. 2012;7(11):e50731.
351. Tagliaferri TL, Jansen M, Horz HP. Fighting Pathogenic Bacteria on Two Fronts: Phages and Antibiotics as Combined Strategy. Front Cell Infect Microbiol. 2019;9:22.
352. Fancello L, Desnues C, Raoult D, Rolain JM. Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fibrosis sputum microbiota. J Antimicrob Chemother. 2011;66(11):2448-54.
353. Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, et al. Spatial distribution of microbial communities in the cystic fibrosis lung. ISME J. 2012;6(2):471-4.
354. Jorth P, Staudinger BJ, Wu X, Hisert KB, Hayden H, Garudathri J, et al. Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs. Cell Host Microbe. 2015;18(3):307-19.
355. Hurley MN, Amin AH, Bertenshaw AC, Bhatt J, Smyth AR. Results of antibiotic susceptibility testing do not influence clinical outcome in children with cystic fibrosis. Journal of Cystic Fibrosis. 2012;11:288-92.
356. Yau YC, Ratjen F, Tullis E, Wilcox P, Freitag A, Chilvers M, et al. Randomized controlled trial of biofilm antimicrobial susceptibility testing in cystic fibrosis patients. J Cyst Fibros. 2015;14(2):262-6.
357. Waters V, Ratjen F. Standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis. Cochrane Database Syst Rev. 2017;10:CD009528.
358. Crusz SA, Popat R, Rybtke MT, Camara M, Givskov M, Tolker-Nielsen T, et al. Bursting the bubble on bacterial biofilms: a flow cell methodology. Biofouling. 2012;28(8):835-42.
359. Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, Winstanley C. Use of artificial sputum medium to test antibiotic efficacy against *Pseudomonas aeruginosa* in conditions more relevant to the cystic fibrosis lung. J Vis Exp. 2012(64):e3857.
360. Sriramulu DD, Lunsdorf H, Lam JS, Romling U. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. J Med Microbiol. 2005;54(Pt 7):667-76.
361. Cooper CJ, Denyer SP, Maillard JY. Stability and purity of a bacteriophage cocktail preparation for nebulizer delivery. Lett Appl Microbiol. 2014;58(2):118-22.
362. Astudillo A, Leung SSY, Kutter E, Morales S, Chan HK. Nebulization effects on structural stability of bacteriophage PEV 44. Eur J Pharm Biopharm. 2018;125:124-30.