The role of prophages in *Pseudomonas aeruginosa*

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by

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To Gran, the kindest and wisest woman I have ever known. You always encouraged me to go to university and I’m so glad that I did.

Abstract

Pseudomonas aeruginosa is a common opportunistic respiratory pathogen of individuals with cystic fibrosis (CF), capable of establishing chronic infections in which the bacterial population undergoes extensive phenotypic and genetic diversification. The Liverpool Epidemic Strain (LES) is a widespread hypervirulent and transmissible strain that is capable of superinfection and is linked to increased morbidity and mortality, relative to other *P. aeruginosa* strains. The LES has six prophages (LESφ1-6) within its genome, of which three are essential to the competitiveness of this strain. Temperate bacteriophages are incredibly common in bacterial pathogens and can contribute to bacterial fitness and virulence through the carriage of additional genes or modification of existing bacterial genes, lysis of competitors, or by conferring resistance to phage superinfection. Furthermore, the LES phages are detected at high levels in the CF lungs and have been implicated in controlling bacterial densities. The aims of this study were to (i) further characterise the LES phages and their induction, (ii) determine the extent to which the LES phages contribute to bacterial phenotypic and (iii) genetic diversification and (iv) determine how the LES phages affect host competitiveness, using a variety of *in vitro* and *in vivo* infection models.

LES phages are continuously produced by spontaneous lysis and this study found that environmental factors that are common to the CF lung, such as oxidative stress, pharmaceutical chelating agents and antibiotics, can alter phage production by clinical LES isolates. Characterisation of the phages highlighted differences between the phages with regards to their lytic cycles and ability to propagate in different environments.

P. aeruginosa undergoes extensive phenotypic diversification in an artificial sputum model (ASM) of infection, similar to that observed in chronic CF infections. Hypermutability, loss of motility and auxotrophy were phenotypes observed in bacteria evolved for approximately 240 bacterial generations in ASM in the presence and absence of the LES phages. However, the LES phages accelerated this process; loss of twitching motility occured earlier in populations evolved in the presence of phages. Sequencing of evolved populations revealed a high level of genetic diversification, with genes involved in motility, quorum sensing and genetic regulation experiencing loss of function mutations in parallel populations. In phage treated populations, LESφ4 had disruptively integrated into motility and quorum sensing genes, suggesting that temperate phages can provide an alternative (and quicker) route to adaptation.

LES prophage carriage is important for bacterial competitiveness; PAO1 LES Phage Lysogens (PLPLs) successfully invaded a phage-susceptible population *in vitro* from when initially rare. Strain invasiveness was dependent on the LES prophage; LESφ4 lysogens were more invasive than PLPLφ2 or PLPLφ3, whereas carriage of all three prophages accelerated bacterial invasion. PLPLφtriple could also invade a susceptible competitor population in a rat model of chronic lung infection, although not as successfully as *in vitro*. These data suggest that prophage carriage is important for LES competitiveness and that phage-mediated lysis of phage-susceptible competitors may explain why LES is adept at superinfection.

The study indicates that the LES phages are important drivers of bacterial diversification and evolution and confer a competitive advantage to their bacterial host. This may help explain why the LES is so successful, and the high prevalence of polylysogeny in bacterial pathogens.

Declaration

This thesis is the result of my own work. The material presented here has not been presented and is not being presented, either wholly or in part for any other degree or qualification. Some of the technical procedures were carried out in collaboration with other people and reference has been made to specific data from other colleagues where appropriate.

This project was co-supervised by Professors Steve Paterson, Craig Winstanley and Aras Kadioglu at the University of Liverpool, and Professor Michael Brockhurst at the University of York. All experimental work was carried out in the research laboratory of Professor Craig Winstanley at the Institute of Infection and Global Health, University of Liverpool.

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Abbreviations

AMOVA – analysis of molecular variance

ANOVA – analysis of variance

ASM – artificial sputum medium

CAZ – ceftazidime

CFTR – cystic fibrosis transmembrane conductance regulator

CFU – colony forming unit

CIP - ciprofloxacin

CF – cystic fibrosis

COL – colistin

CRISPR – clustered regularly interspaced short palindromic repeats

EDTA - ethylenediaminetetraacetic acid

FOL – frequency of lysogeny

Gm – gentamicin

ICTV – international committee on taxonomy of viruses

INDEL – insertion/ deletion polymorphism

(I)PCR – (inverse) polymerase chain reaction

LB – lysogeny broth

LES – Liverpool epidemic strain

MEM – meropenem

MGE – mobile genetic element

MIC – minimum inhibitory concentration

MOI – multiplicity of infection

Nflx – norfloxacin

ORF – open reading frame

PBR – phage to bacterium ratio

PBS – phosphate-buffered saline

PCA – principal component analysis

PFU – plaque forming unit

PLPL – PAO1 LES phage lysogen

QS – quorum sensing

r_{ij} – selection rate constant

SDW – sterile distilled water

Sm – streptomycin

SNV – single nucleotide variant

Tet - tetracycline

TOB – tobramycin

TSA – tryptone soy agar

TZP – tazobactam/ piperacillin

WT – wild-type

Chapter 1 General Introduction

1.1 Microbial infections in cystic fibrosis

1.1.1 Cystic Fibrosis (CF)

Cystic fibrosis (CF) is a genetic disorder caused by mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR), an ion channel protein that is required for chloride ion transport across epithelial cell membranes. To date, nearly 2000 mutations have been identified in the CFTR gene (Consortium, 2011), and the majority of these have been linked to CF, with disease severity dependent on the type of mutation (Riordan *et al.*, 1989). Defective or absent CFTR proteins cause an ionic imbalance, which results in the production of abnormally thick, sticky mucus. This has pathological effects on multiple organs throughout the body, including the pancreas and digestive system, endocrine and reproductive systems.

However, it is the respiratory system that is most affected; individuals are prone to bacterial infections, and whilst there is an immune response, it does not result in successful clearance, leading to chronic inflammation and lung damage. Inflammation is associated with reduced pulmonary function (Sagel *et al.*, 2002) and thought to be predictive of structural lung disease (Mott *et al.*, 2012).

It is still not completely understood why individuals with CF are initially so susceptible to bacterial infections, but contributory factors include decreased mucociliary clearance (Zahm *et al.*, 1997) and the abnormal composition of airway secretions. High liquid surface absorption of CF sputa causes depletion of the lubricating periciliary liquid layer (a component of airway surface liquid), and this prevents mucus transport and subsequent bacterial clearance (Matsui *et al.*, 1998). Furthermore, the viscosity of CF mucus impairs neutrophil chemotaxis and functionality (Matsui *et al.*, 2005), and the pH imbalance resulting from defective ion transport inhibits the natural bactericidal activity of airway surface liquid (Pezzulo *et al.*, 2012).

Airway inflammation is observed in very young children with CF, even in the absence of any detectable infection (Khan *et al.*, 2011). Inflammatory markers have even been detected in the lung of a foetus with CF (Verhaeghe *et al.*, 2007), which suggests that an inflammatory phenotype is a feature of CF, and not just a secondary consequence of microbial infections. CFTR is a negative regulator of pro-inflammatory cytokines (Vij *et al.*, 2009) and is required for the process of autophagy (Luciani *et al.*, 2010), hence defects trigger an innate immune response and chronic inflammation.

The clinical course of respiratory disease in CF is characterised by episodes of acute illness, termed pulmonary exacerbations. Exacerbations cause an increase in respiratory (and frequently systemic) symptoms, and require aggressive intravenous or inhalation antimicrobial therapy (Bhatt, 2013). An increased frequency of exacerbations is associated with a reduced 5-year survival rate (Liou *et al.*, 2001), and a poorer (and declining) baseline lung function (de Boer *et al.*, 2011). Numerous studies have linked changes in the microbial community to exacerbations, but demonstrating causality is difficult. The exact triggers remain unknown, but the treatment is always aggressive antimicrobial therapy.

1.1.1.1 Oxidative stress

Oxidative stress results as an imbalance between oxidants and antioxidants, and CF patients (particularly adults) have higher levels of oxidative stress markers than healthy control subjects, in part due to depleted antioxidant levels (Back *et al.*, 2004), but also as a consequence of inflammation. Activated immune cells (particularly neutrophils) produce reactive oxygen species; indeed, pulmonary inflammation has been associated with increased levels of oxidative stress in children with CF (Hull *et al.*, 1997), and oxidative stress increases after exacerbations (Reid *et al.*, 2007). High levels of oxidative stress are thought to cause injury to cells and contribute to the decline in lung function (Brown & Kelly, 1994), but one must also be aware that the toxicity of ROS is not limited to only human cells, and that high levels of oxidative stress are likely to impact on the microbial communities within the lung.

1.1.2 Common CF pathogens

The abnormal nature of the CF lung means that the CF lung is frequently colonised by multiple microbes, including opportunistic pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and the *Burkholderia cepacia* complex. Chronic *P. aeruginosa* and *S. aureus* are the most common airway infections in adults with CF, occurring in 51% and 21% of adults, respectively (Registry, 2014). For decades, culture-based methods have been used to determine the microbiology of CF airway infection, but more recently, culture-independent methods such as PCR and microbiome analyses have added to the array of microbes that are associated with the CF airways, although the role of many of these in pathogenesis remains unclear.

There is evidence that the microbiology of the CF lung has changed over the past few decades, presumably due to changes in infection control and treatment strategies. Whilst the incidence of *H. influenzae* and *S. aureus* has decreased, MRSA has increased, and there has been a rise in the incidence of rare or previously undetected species such as atypical mycobacteria, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* (Millar *et al.*, 2009).

The (relatively) recent advent of molecular techniques such as 16S rDNA gene sequencing has helped identify putative pathogens that were not previously associated with CF (Bittar *et al.*, 2008), including in children undergoing exacerbations who were culture negative (Harris *et al.*, 2007). A study characterising unusual bacterial airway isolates found that the majority belonged to species that were not known to colonise CF patients, or were in completely novel taxa (Coenye *et al.*, 2002). Chronic colonisation of individuals with *Pandoraea* species (Coenye *et al.*, 2000) and *Inquilinus limosus* (Wellinghausen *et al.*, 2005) has been described, but the clinical implications of this are not known. These studies highlight the complexity of the microbiology of the CF lung.

In addition to the presence of multiple bacterial species, fungi and viruses are often frequently detected. *Aspergillus fumigatus* is the most prevalent fungal species; over 1/5 of adults are chronically colonised, and incidence increases with age (Mortensen *et al.*, 2011). Use of a variety of fungal culture methods has shown that the fungal

community in CF airways is diverse yet stable, with a variety of yeast and filamentous fungal species (Masoud-Landgraf *et al.*, 2014). Like healthy children, children with CF suffer from acute respiratory infections. Interestingly, the frequency and duration of infections, and types of viruses detected, do not differ to healthy children, but the severity of lower respiratory tract disease is increased (van Ewijk *et al.*, 2008). It has been suggested that exacerbations are triggered by respiratory viral infections (Wark *et al.*, 2012), specifically rhinoviruses (Goffard *et al.*, 2014), and viruses have also been associated with an increase in *P. aeruginosa* density (Wark *et al.*, 2012).

Community profiling studies have been beneficial in furthering our understanding of the polymicrobial nature of the CF lung. Pyrosequencing of the whole bacterial community present in sputum samples has shown that the community is diverse and relatively stable over time, and antibiotic treatment has little effect, particularly on the dominant pathogens such as *Pseudomonas* and *Burkholderia* (Fodor *et al.*, 2012). A similar study that classified patients into clinically stable or declining found, similarly, that stable patients had a diverse bacterial community. However, antibiotic treatment drove loss of diversity in the declining patients, and *P. aeruginosa* became the dominant pathogen, to the exclusion of all others in some patients (Zhao *et al.*, 2012). Such studies can identify species as clinically important; for example, the *Streptococcus milleri* group (SMG) consists of several groups and was not previously recognised as a serious pathogen in CF. However, a longitudinal study that investigated bacterial airway communities and patient clinical status found that the community dynamics shifted prior to the onset of exacerbations, with the SMG increasing to become the dominant pathogen, evidence for a role as a serious CF pathogen (Sibley *et al.*, 2008).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram negative bacterium that is found in a variety of environments, both clinical and environmental (Wiehlmann *et al.*, 2007). It is an opportunistic pathogen, causing disease where normal host defences have been compromised, for example in immunocompromised individuals, individuals with severe burns, or contact-lens wearers (Lyczak *et al.*, 2000), and it is capable of causing both acute and chronic infections. This makes it a significant nosocomial

pathogen; it is the most common nosocomial causative agent of pneumonia, and the second most common in the case of urinary tract infections (Weinstein *et al.*, 2005).

Despite being an opportunistic pathogen, *P. aeruginosa* is capable of expressing a large collection of virulence factors, specific to the type of infection (Rumbaugh *et al.*, 1999). A recent study compared gene expression in acute and chronic murine wound mouse models, and identified fitness and virulence determinants using a library of transposon-insertion mutants. However, despite differential gene expression between the two types of infection, the genes required for fitness and virulence were similar between the two, with the exception of flagellar motility, which is only required in acute infections (Turner *et al.*, 2014).

1.2.1 Virulence in CF

As discussed in the previous section, *P. aeruginosa* virulence factor expression differs between acute and chronic infections. The general consensus is that *P. aeruginosa* requires expression of multiple virulence factors to colonise and establish an infection, but that these can then have a fitness cost due to their immunogenicity, resulting in a switch to a chronic infection. Cell surface factors such as flagella and pili are required for motility (Bradley, 1980; Köhler *et al.*, 2000) and adhesion to respiratory epithelial cells (Doig *et al.*, 1988; Lillehoj *et al.*, 2002). *P. aeruginosa* also produces numerous secreted factors with toxic effects, including phenazines (Cezairliyan *et al.*, 2013; Mahajan-Miklos *et al.*, 1999), proteases (Kida *et al.*, 2008; Woods *et al.*, 1982), phospholipase C (Ostroff *et al.*, 1989), CFTR inhibitory factor (MacEachran *et al.*, 2007), exotoxins (Woods *et al.*, 1982), hydrogen cyanide (Gallagher & Manoil, 2001) and rhamnolipids (Read *et al.*, 1992). Levels of secreted exoproteins in CF sputa are higher during exacerbations (Jaffar-Bandjee *et al.*, 1995) which may partly explain the morbidity associated with exacerbation. However, because virulence factors cause pulmonary inflammation (Wieland *et al.*, 2002), loss or downregulation of their expression represents an immune evasion strategy. For example, neutrophil elastase has been shown to trigger reduced flagellin synthesis (Palmer *et al.*, 2005; Sonawane *et al.*, 2006), and chronic CF isolates often display loss of cell surface factors (Mahenthiralingam *et al.*, 1994; Workentine *et al.*, 2013).

The exopolysaccharide alginate is a virulence factor that is associated specifically with later stages of chronic infection, as overproduction leads to the development of a “mucoidy” phenotype. The mucoid phenotype does not elicit an immune response and suppresses host apoptosis, which may help prevent clearance (Cobb *et al.*, 2004). Indeed, mucoid *P. aeruginosa* are more resistant to immune-mediated killing (Baltimore & Mitchell, 1980; Cabral *et al.*, 1987; Leid *et al.*, 2005) and form biofilms that are very resistant to antimicrobial treatment (Hengzhuang *et al.*, 2011). Biofilm growth, however, is not limited to mucoid strains, and biofilm formation can be viewed as a virulence factor in chronic infection, as biofilms are intrinsically refractory to antibiotic treatment (Bjarnsholt *et al.*, 2009; Singh *et al.*, 2000).

Chronic colonisation with *P. aeruginosa* has been associated with a gradual worsening of lung disease (Kosorok *et al.*, 2001) and is a major predictive factor for morbidity and mortality (Emerson *et al.*, 2002). Mucoid strains in particular are associated with a more rapid decline in lung function (Demko *et al.*, 1995).

1.2.2 Antimicrobial therapy of *P. aeruginosa* infection in CF

CF respiratory infections are polymicrobial, so patients are often receiving a cocktail of antibiotics. However, as *P. aeruginosa* is a common and serious pathogen, therapy in CF is often primarily targeted at preventing or treating *P. aeruginosa* infections. Whilst prophylaxis to prevent colonisation is not advised (Döring *et al.*, 2012), antibiotic eradication therapy is recommended for initial/ intermittent colonisation, and has been shown to be successful in clearing early-onset *P. aeruginosa* infections (Ratjen *et al.*, 2010; Treggiari *et al.*, 2011). A Cochrane review examining antibiotic eradication strategies included 7 trials over a period of 20 years, and concluded that nebulised or oral and nebulised antibiotics could successfully eradicate early *P. aeruginosa* infection. However, differences in the methodologies of the individual trials meant that they could not recommend an optimal treatment regimen, and the authors emphasised the need for trials comparing different eradication strategies (Langton Hewer & Smyth, 2014).

Once chronic colonisation has established (which it invariably does after a few years of intermittent colonisation), maintenance therapy is recommended to suppress infection and prevent exacerbations, with either daily inhaled colistin, or four week

on-off inhaled aminoglycoside therapy (Döring *et al.*, 2012). Exacerbations can be treated with intravenous antibiotics, either as monotherapy or in combination, with the exception of fluoroquinolones, which are administered orally (Smyth & Elborn, 2008).

1.2.3 Infection and transmission in CF

Until relatively recently, it was thought that individuals with CF were colonised with environmentally-acquired, patient-specific strains of *P. aeruginosa*. Common strains between patients were identified (Grothues *et al.*, 1988; Wolz *et al.*, 1989), but transmission was thought to be a rare event, occurring only after prolonged contact.

P. aeruginosa was thought to have a non-clonal (i.e. freely recombining) population (Curran *et al.*, 2004; Kiewitz & Tümmler, 2000). However, clonal strains have been identified, and a survey of the global population of *P. aeruginosa*, using a range of clinical and environmental isolates, identified multiple dominant clones (Wiehlmann *et al.*, 2007) in a variety of geographical and environmental origins. Clone C in particular is very prevalent in CF centres across the UK (Hall *et al.*, 2014; Martin *et al.*, 2013; Scott & Pitt, 2004) but there is little evidence that it is transmitted between patients, and its prevalence likely reflects its abundance in the environment.

Transmission between patients was first considered as a serious mode of strain acquisition after the observation of widespread ceftazidime resistance in *P. aeruginosa* isolates in a paediatric CF clinic in Liverpool. Genotyping revealed that a large number of children carried the same strain (Cheng *et al.*, 1996), which became known as the Liverpool Epidemic Strain (LES). Compared with other *P. aeruginosa* genotypes, the LES is associated with worsened health and quality of life (Ashish *et al.*, 2012a) and increased antibiotic resistance (Ashish *et al.*, 2012b). Whilst a strict patient segregation policy has successfully reduced the proportion of *P. aeruginosa* infections that are LES (Ashish *et al.*, 2013b), it remains the most common clone in those infected with *P. aeruginosa* (Martin *et al.*, 2013), and has recently been identified in North America (Aaron *et al.*, 2010). The characteristics of the LES are discussed further in section 3.1.1.

Since the LES, other transmissible strains have been identified, although they are not all associated with increased virulence. Several Australian epidemic strains have been identified, and a survey of *P. aeruginosa* isolates from multiple patients in Australian CF centres found high clonality, with more than 60% of patients infected with a shared genotype (Kidd *et al.*, 2013). The Australian epidemic strains AES-1 and AES-2 accounted for most of these and were thought to be acquired by patient to patient transmission. These two strains have been shown to produce more proteases than other, non-clonal isolates (Tingpej *et al.*, 2007), which may be a contributory factor in the increased morbidity associated with these strains (Tingpej *et al.*, 2010) and increased mortality associated with infection with AES-1 (Griffiths *et al.*, 2012).

There are numerous examples of other transmissible strains identified worldwide (Fothergill *et al.*, 2012), including two further epidemic strains in the UK, the Manchester (MES) and Midland (Mid 1) strains (Scott & Pitt, 2004). Unlike the LES, there is little evidence to suggest that infection with the MES or Mid 1 has clinical significance; Mid 1 is not linked with antibiotic resistance or worsened disease state (Chambers *et al.*, 2005) and MES does not elicit an increased inflammatory response, relative to other non-epidemic strains (Jones *et al.*, 2003), although a small study did find that infection with transmissible strains (of which MES was the most common) was associated with an increased frequency of antibiotic treatment and hospitalisation (Jones *et al.*, 2002).

1.2.4 The genetics of *P. aeruginosa*

P. aeruginosa has a large, circular chromosome (ranging from 5 – 7 million base pairs (Mbp)), with a relatively high GC content of 65-67%, and can carry additional extra-chromosomal plasmids. PAO1 was the first whole genome to be sequenced, and this revealed a 6.3 Mbp, high-complexity genome encoding 5,570 predicted open reading frames (ORFs) and a very high proportion (8.4%) of regulatory genes (Stover *et al.*, 2000). Since then, numerous other *P. aeruginosa* genomes have been published, allowing identification of the genes that are shared between strains, and those that are strain specific.

The core genome of *P. aeruginosa* is highly conserved (Mathee *et al.*, 2008; Wolfgang *et al.*, 2003), although the degree of conservation depends on which strains

are included; a recent study used 12 reference genomes, and found that when PA7 (an outlier strain) was included, the core genome size dramatically decreased (Ozer *et al.*, 2014). A comparison of a panel of strains from both clinical and environmental origins found that virulence genes are highly conserved between strains, suggesting that environmental strains have the capacity to cause infections (Wolfgang *et al.*, 2003).

The core genome is interspersed with blocks of DNA in a mosaic-like fashion which account for the majority of inter-strain diversity (Klockgether *et al.*, 2011), termed the accessory genome. The accessory genome represents between 7 and 18 % of the *P. aeruginosa* genome, has a much greater proportion of uncharacterised genes than the core genome and is enriched for horizontally acquired elements such as integrative and conjugative elements (ICEs) and phages (Ozer *et al.*, 2014).

Examination of the accessory genome of clinical isolates could be a useful strategy to determine genes required for virulence. The accessory genome of clinical isolates is quite strain-specific (Pohl *et al.*, 2014) and consists of many sequences not present in PAO1 (Shen *et al.*, 2006) or other *P. aeruginosa* strains. It includes sequences from other genera, evidence of interspecies HGT (Pohl *et al.*, 2014) and lots of genes that are associated with mobile genetic elements such as bacteriophages (Pohl *et al.*, 2014; Shen *et al.*, 2006). The accessory genome of CF epidemic strains is enriched for functions thought to aid survival in the CF lung, including antibiotic metabolism and redox genes (Dettman *et al.*, 2013).

When the genome sequence of LESB58 was published (the first historical isolate of LES), the most striking feature was the number of horizontally acquired elements, including five genomic islands and six prophages. Furthermore, a signature-tagged mutagenesis study found that genes in three of the prophages and one of the novel genomic islands were essential for LES competitiveness in a rat model of chronic lung infection (Winstanley *et al.*, 2009).

The genome sequence of LESB58 identified 350 genes with no homology to any other *P. aeruginosa* strain (at the time), and showed that LESB58 carries almost all known *P. aeruginosa* virulence genes (Winstanley *et al.*, 2009). The presence of

these known virulence genes, in addition to the horizontally acquired novel DNA may explain the evolutionary success of the LES. However, prediction of virulence from genome sequence is not straightforward; a comparison of strain virulence in a *Caenorhabditis elegans* model found little correlation between the presence of virulence genes and virulence, suggesting that virulence depends on the specific combination of virulence factors (Lee *et al.*, 2006). Furthermore, strains or isolates can exhibits difference in virulence between different animal models (Fothergill *et al.*, 2012).

The genome is constantly changing, and the *P. aeruginosa* genome undergoes extensive diversification through single nucleotide variations (SNVs), small insertions and deletions (INDELS) and horizontal acquisition of novel DNA, something that will be discussed in Chapter 5. These features of the *P. aeruginosa* genome contribute to its ability to rapidly adapt to new or changing environments.

1.2.4.1 DNA mismatch repair (MMR) system

DNA replication is not an error free process, and various systems exist to repair errors and DNA damage. The mismatch repair (MMR) system of *Escherichia coli* repairs post-replicative DNA errors and oxidative DNA damage (Wyrzykowski & Volkert, 2003), and consists of several proteins, including MutS, which recognises mismatched bases and small INDELS, and MutL, which forms a complex with MutS and mediates the activation of the endonuclease MutH. An *uvrD* (*mutU*)-encoded helicase unwinds the error-containing DNA strand (Li, 2008). Mutations in the genes encoding these proteins can lead to a heritable “hypermutator” phenotype, where a failure to repair mismatches leads to an elevated mutation rate.

P. aeruginosa encodes orthologues for *mutS*, *mutL* and *uvrD*, but no homologue has been detected for *mutH* (Oliver *et al.*, 2000; Stover *et al.*, 2000). Despite sequence similarity, the *P. aeruginosa* MMR genes do not complement MMR-defective *E. coli*, suggesting a degree of difference between the two systems (Oliver *et al.*, 2002). However, mutations in the MMR genes of *P. aeruginosa* result in a hypermutator phenotype, and each gene in this system has been shown to be capable of restoring a wild-type mutation rate in at least one clinical hypermutable isolate (Oliver *et al.*,

2002), so there is no doubt that defects in the *P. aeruginosa* MMR system have a similar effect on cell phenotype to that observed in *E. coli.*

Hypermutable *P. aeruginosa* is frequently isolated from the CF lung (Cramer *et al.*, 2011; Feliziani *et al.*, 2010), but the reasons underlying this high frequency are still unclear and will be discussed further in section 4.1.2.3.

1.2.5 Cell surface structures

P. aeruginosa, like many other bacteria, express proteinaceous appendages on their cell surface, such as pili and flagella. These interact with the environment surrounding the cell and have numerous roles, including motility, biofilm formation and adhesion to host cells, both of which may be important when colonising new hosts.

1.2.5.1 Pili

Type IV pili are long, thin filaments that are made up of pilin protein subunits which are manufactured with a leader peptide sequence that is cleaved before pilus assembly. There are two main types of type IV pili in bacteria, type IVa and type IVb, which differ in the length of both pilin and leader peptide sequence and have two distinct assembly systems. *P. aeruginosa* is unusual in that both types of pili have been described (Bernard *et al.*, 2009; Carter *et al.*, 2010b; de Bentzmann *et al.*, 2006; Giltner *et al.*, 2011), but type IVb is not associated with motility and is much less well characterised.

Numerous genes are involved in type IVa pilus biogenesis and retraction (Figure 1.1), and mutations in these genes can lead to different phenotypes, ranging from complete absence of pili, to hyperpiliation.

PilA is the major structural protein (termed the major pilin), and mutations in *pilA* can result in a total absence of pili. Within *P. aeruginosa* there are 5 subtypes of pili, based on the *pilA* allele, with PAO1 classed as group II, and another common reference strain, PA14 (originally an acute infection isolate) as group III (Kus *et al.*, 2004). Interestingly, group I is overrepresented in clinical CF isolates (Kus *et al.*, 2004), and LES is group I (Giltner *et al.*, 2011). Because of this, it has been

suggested that group I is a CF specific virulence factor. A recent study identified a potential mechanism for this; type I pilins are O-glycosylated, which confers resistance to pulmonary-surfactant protein A-mediated opsonisation and phagocytosis in a mouse model of acute lung infection (Tan *et al.*, 2015).

Each pilus type also has a specific set of minor pilin alleles, and it is thought that compatibility between the major and minor pilins is necessary for full functionality (Giltner *et al.*, 2011). The minor pilins form the pilus shaft, along with PilA (Figure 1.2) and function to prime pilus assembly (Nguyen *et al.*, 2015).

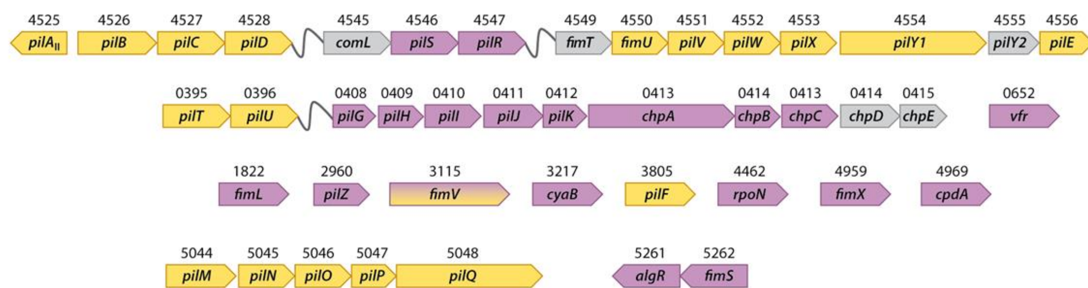


Figure 1.1 Genes involved in type IV pilus formation and twitching motility in *P. aeruginosa* strain PAO1. Each gene is represented by a coloured arrow: yellow represents structural, purple represents regulatory, and grey represents genes associated with type IV but lacking a pilus-related phenotype. The number above each gene is the PA accession number. Figure adapted from (Burrows, 2012).

Type IV pili are produced by an apparatus that consists of four subcomplexes (Figure 1.2). PilB, PilC, PilT and PilU putatively form the motor (Burrows, 2012); PilB is an ATPase that provided the required energy for pilus assembly, whereas PilT and PilU are retraction ATPases, required for pilus depolymerisation (Chiang *et al.*, 2005). The major and minor pilins subcomplex forms the pilus itself, which is extruded through the secretin PilQ, localised by the lipoprotein PilF (Koo *et al.*, 2008). The PilFQ outer membrane subcomplex is linked to the inner membrane subcomplex by the PilMNOP/FimV alignment subcomplex (Burrows, 2012). PilY1 is a non-pilin protein for which numerous roles have been identified, including twitching motility (Bohn *et al.*, 2009; Heiniger *et al.*, 2010), survival in a mouse model of infection (Bohn *et al.*, 2009), calcium dependent regulation of pilus retraction (Orans *et al.*, 2010) and adhesion to human epithelial cells (Heiniger *et al.*, 2010). A recent study found that the minor pilins are required to traffic PilY1 to the cell surface, and because of its role in adhesion and anti-retraction, the authors suggested that PilY1

may be localised at the tip of the pilus, but this remains to be determined (Nguyen *et al.*, 2015).

Type IV pili are required for several forms of motility, including twitching (1.2.6.3), swarming (1.2.6.1) and the more recently discovered walking (Gibiansky *et al.*, 2010) and slingshot motilities (Jin *et al.*, 2011). Type IV pili-mediated motility enables exploration of novel, solid-surface environments, but type IV pili also function synergistically with flagella to detach from surfaces and disperse (Conrad *et al.*, 2011). Type IV pili also have a major role in adhesion, and can bind to host epithelial cells (Bucior *et al.*, 2012), DNA (van Schaik *et al.*, 2005) and solid surfaces (Giltner *et al.*, 2006), and adhesion is a necessary step in surface-attached biofilm formation (1.2.7). Furthermore, type IV pili are the receptor for many bacteriophages (Ceyssens *et al.*, 2009b; James *et al.*, 2012). More recently, it was discovered that type IV also play a role in sensing the environment; pili bind to solid surface and are retracted, triggering signal transduction through the Chp chemosensory pathway and subsequent upregulation of many genes, including those involved in virulence and twitching motility (Persat *et al.*, 2015).

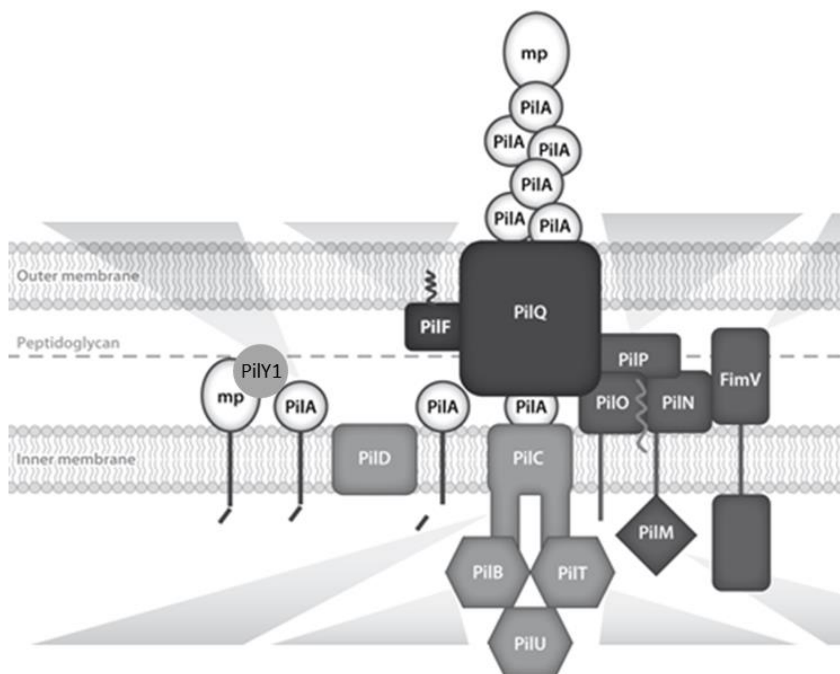


Figure 1.2 Diagrammatic representation of the type IV pilus complex of *P. aeruginosa* and its location within the cell. The pilus is formed from the major pilin protein PilA, and the minor pilins (mp) FimU, PilV, PilW, PilX, and PilE. Figure adapted from (Burrows, 2012).

1.2.5.2 Flagella

Flagella are whip-like appendages that are required for chemotaxis and motility in less solid environments, especially swimming and swarming motility. *P. aeruginosa* has a single polar flagellum, consisting of subunits of flagellin protein (encoded by the *fliC* gene) and powered by a motor. The motor is powered by a flow of protons across the cell membrane and consists of two subunits, the stator (a static structure) and a rotor, which fits within the stator and causes the flagellum to rotate (Blair, 2003). *P. aeruginosa* is unusual in that it encodes two different stators that have different roles with regards to motility; both are required for swarming motility, but either is sufficient for swimming (Toutain *et al.*, 2005).

There are two main types of flagella in *P. aeruginosa* (a and b) that differ in their FliC protein with regards to antigenicity, weight of flagellin subunits (Allison *et al.*, 1985) and amino acid sequence (Spangenberg *et al.*, 1996). A small-scale study found no difference in the distribution of flagella type in strains from different clinical infections, or in the immunogenicity of the flagellin protein (Shanks *et al.*, 2010), so there are no obvious clinical implication of flagella-type.

Flagella are thought to be important virulence factors as they are required for host colonisation (Montie *et al.*, 1982), due to their role in motility and adhesion. Flagella help *P. aeruginosa* adhere to hydrophobic surfaces (Bruzaud *et al.*, 2015) and the flagellin protein adheres to Muc1 mucin, a protein secreted by respiratory epithelial cells (Lillehoj *et al.*, 2002).

Furthermore, type IV pili and flagella are thought to act synergistically to adhere to host epithelial cells; type IV pili bind preferentially to N-glycans on the apical surface, and flagella to heparin sulphate on the basolateral surface of polarised epithelium, and it is this that mediates invasion of host cells (Bucior *et al.*, 2012).

Flagellin is detectable in the sputa of CF patients (Balloy *et al.*, 2014), which is concerning as flagellin is known to be highly immunogenic, activating toll-like receptor 5 which triggers an inflammatory innate immune response (Hayashi *et al.*, 2001a; Zhang *et al.*, 2005). Flagellin also causes hyper-secretion of mucins by human epithelial cells, which likely has clinical implications (Mohamed *et al.*, 2012).

There is also evidence that flagellin can modulate the host immune response, as it induces proliferation of myeloid-derived suppressor cells which dampen T cell immune responses (Rieber *et al.*, 2013). However, loss of flagella is also an effective strategy for avoiding the immune response (Patankar *et al.*, 2013) and has been widely documented in CF isolates (Mahenthiralingam *et al.*, 1994; Workentine *et al.*, 2013), including LES (Winstanley *et al.*, 2009).

1.2.6 Motility

1.2.6.1 Swarming

Swarming motility is a complex, flagella-driven multicellular form of motility. Bacteria "swarm" across the top of a surface, something that can easily be visualised in the laboratory using semi-solid media, although the conditions must be tightly controlled to avoid other forms of motility. For example, a high concentration of agar promotes twitching motility, and a low viscosity promotes swimming.

Swarming bacteria form tendrils, long branches that radiate out from the centre, with the area between the tendrils remaining uncolonised due to inhibitory signals produced by neighbouring tendrils (Caiazza *et al.*, 2005). The swarming pattern is strain specific (Tremblay & Déziel, 2008) and the differences between PAO1 and PA14 are illustrated in Figure 1.3.

Both flagella and type IV pili are required for swarming in *P. aeruginosa*, with flagella responsible for swarm expansion, and type IV pili playing a role in controlling the swarm, slowing expansion through pilus-pilus interactions between cells and directing movement away from harmful compounds such as antibiotics in the environment (Anyan *et al.*, 2014). Bacterial production of surfactants such as rhamnolipids is also necessary, as these function to reduce the surface tension (Köhler *et al.*, 2000) and modulate the swarming pattern (Caiazza *et al.*, 2005). There is evidence that rhamnolipid production is controlled by quorum sensing (QS) (1.2.8), so functional QS is required for swarming motility (Köhler *et al.*, 2000). However, another study found that the QS control of swarming motility was dependent of the carbon source and that rhamnolipid production was not essential (Shrout *et al.*, 2006)

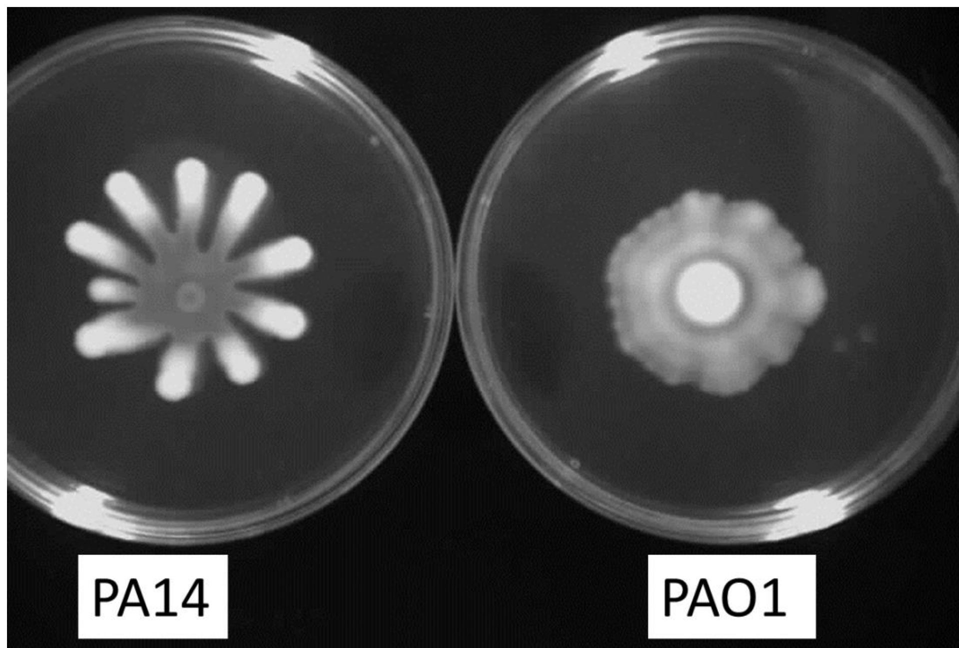


Figure 1.3 Swarming motility in the laboratory reference strains PA14 (left) and PAO1 (right). Swarming media was inoculated with a single colony and incubated for 16 hours at 37 °C.

The role of swarming in colonisation and virulence is unclear. A study comparing over 200 isolates from different infections (but not CF) identified a positive association between the ability to swarm and both protease production and the likelihood of being positive for the type III secretion system, both of which are well-known virulence factors, but this is only a correlational effect (Murray *et al.*, 2010). However, when actually swarming, bacteria upregulate certain virulence genes and are more resistant to antibiotics (Overhage *et al.*, 2008a), so swarming itself could be considered a virulence factor.

1.2.6.2 Swimming

Swimming is a form of motility that occurs in aqueous environments by rotation of the flagellum, which propels the bacterium forward. Flagella are essential for swimming motility, as evidenced by the complete lack of motility exhibited by *fliC* mutants, which do not produce flagella (Rashid & Kornberg, 2000).

There is evidence that it is swimming motility, rather than the flagellum itself, that is required for full virulence, as paralysis of the flagellum results in attenuation of virulence in a burned mouse model of infection (Arora *et al.*, 2005). Flagellin is immunogenic and the presence of flagella is required for nonopsonic phagocytosis

(Mahenthiralingam & Speert, 1995), so it was previously thought that loss of flagella was an immune evasion strategy, and that loss of swimming motility was a (perhaps unfortunate) consequence. However, another study with flagellated but paralysed cells demonstrated that it is loss of motility, not loss of the flagellum, that mediates resistance to phagocytosis (Amiel *et al.*, 2010).

1.2.6.3 Twitching

Twitching is a form of motility that occurs on solid surfaces by extension and retraction of the type IV pili. Bacterial movement appears jerky under the microscope, hence the name, "twitching" motility. It can be assayed by examining bacterial colony expansion at the plastic-agar interface of a petri dish; bacteria that twitch spread out from the initial inoculation point and can be visualised by staining (Figure 1.4).

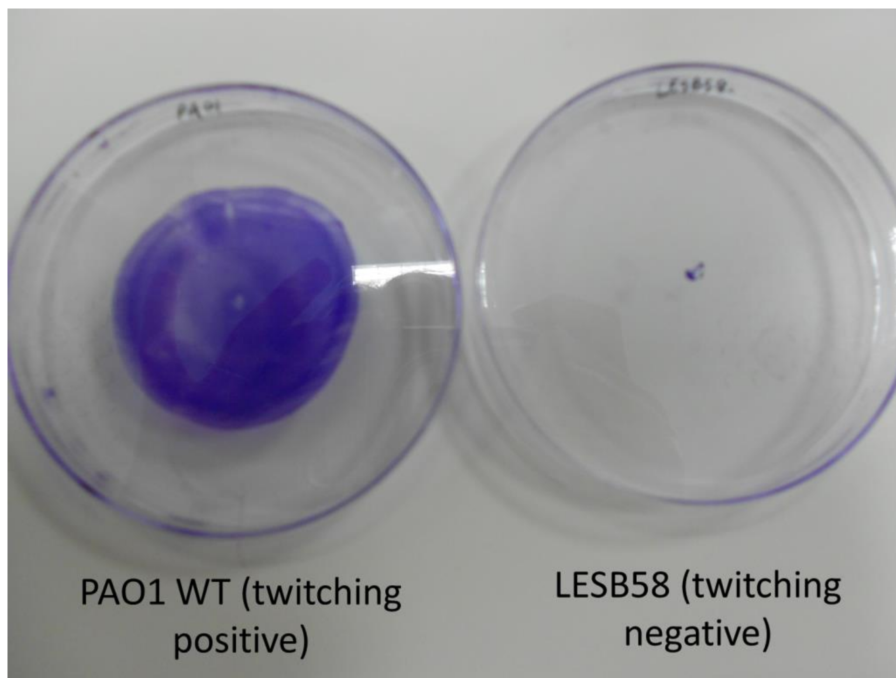


Figure 1.4 Twitching motility of *P. aeruginosa* strains PAO1 (left; twitching proficient) and LESB58 (right; twitching deficient). A single colony was stab-inoculated to the LB-agar-petri dish interface and incubated for 24 hours at 37 °C, prior to staining with crystal violet.

P. aeruginosa assembles and extends the type IV pilus, which attaches to a surface and is pulled taut. The pilus is retracted by rapid depolymerisation, and it is retraction that propels the cell forward (Skerker & Berg, 2001). It is thought that the

pilin subunits are recycled within the cell (Skerker & Berg, 2001), continuously being assembled and disassembled to enable twitching motility.

Because type IV pili have numerous roles, especially in adhesion and biofilm formation, it is difficult to elucidate the role of twitching motility itself in virulence. Studies with mutants that still produce pili but are unable to twitch can be useful in this respect. For example, *pilU* and *pilT* mutants, which are deficient in pilus retraction display reduced cytotoxicity in epithelial cells (Comolli *et al.*, 1999) and reduced colonisation and virulence in the murine cornea (Zolfaghar *et al.*, 2003), suggesting that twitching is necessary for full virulence, not just the presence of pili.

1.2.7 Biofilm formation

Biofilms consist of bacteria in a self-produced matrix of polysaccharides, proteins and DNA (Høiby *et al.*, 2010b), often attached to a surface. Biofilms can consist of a single bacterial species, but biofilms containing multiple bacterial species, fungi and archaea have all been described. The sessile nature of cells within the biofilm, and the physical protection afforded by the matrix means that biofilms are intrinsically resistant to attack from antibiotics, host immunity and phage predation. Analysis of quorum sensing signals in CF sputa indicates that *P. aeruginosa* likely grows as a biofilm in the CF lung (Singh *et al.*, 2000), and *P. aeruginosa* biofilm growth has been extensively studied in the laboratory, using the traditional surface-attached biofilm mode of growth (Hentzer *et al.*, 2001; Klausen *et al.*, 2003b; O'Toole & Kolter, 1998).

Extracellular DNA is an important component of *P. aeruginosa* biofilms (Barken *et al.*, 2008), as are polysaccharides that are produced by the bacteria. *P. aeruginosa* produces three extracellular polysaccharides: alginate, Psl and Pel (Franklin *et al.*, 2011). Surface attached PAO1 biofilms grow as mushroom-like structures, and alginate plays a key role in trapping cells in the cap of these structures, in addition to maintaining the viability of these cells (Ghafoor *et al.*, 2011), whereas Pel mediates interactions between cells within the structures. Psl is important for initial surface attachment of cells (Ghafoor *et al.*, 2011), and its production sets up a positive feedback loop, whereby Psl stimulates production of the secondary messenger molecule cyclic di-GMP, which is turn leads to increased Psl production and biofilm

formation (Irie *et al.*, 2012). Lack of production of these polysaccharides, alone or in combination, results in changes to the biofilm architecture (Ghafoor *et al.*, 2011).

Type IV pili are also though to play an important role in surface-attached biofilm formation. Their role in initial surface adhesion is not clear; some studies have shown that pilus deficient mutants can still adhere to surfaces and form biofilms (Klausen *et al.*, 2003b), but under certain conditions they cannot (Chiang & Burrows, 2003). Other cell appendages can play a role in initial adhesion, including novel adhesins encoded by the *cupA* genes (Vallet *et al.*, 2001). Mutations in the *cupA* genes of pilus-deficient PAO1 reduced surface adhesion and biofilm formation, and there may be numerous other undiscovered adhesins with a role in biofilm formation.

Type IV pilus-mediated twitching motility may be more important for biofilm formation than the adhesive properties of type IV pili, and is necessary for microcolony formation, an early step in biofilm formation where cells twitch across the surface and aggregate together (O'Toole & Kolter, 1998). Furthermore, experiments with mixture of fluorescently-tagged wild-type and non-piliated mutants found that whilst the twitching-deficient strain could form the stalks of the aforementioned mushroom-like structure, twitching motility was required to move up the stalk to form the cap (Klausen *et al.*, 2003a).

Strains that produce defective pili (hence are non-motile) are still capable of biofilm formation. Hyperpiliated mutants (defective for twitching, swimming and swarming) have been identified that form highly adherent biofilms (Déziel *et al.*, 2001), and hyperpiliated *pilT* mutants form very dense biofilms, suggesting that twitching motility is required for normal structure (Chiang & Burrows, 2003).

Mature biofilms undergo dispersal, where planktonic cells are released from the biofilm. It has been hypothesised that dispersal of *P. aeruginosa* biofilms in the CF lungs may cause exacerbations, but there is little evidence to support or refute this hypothesis. A filamentous bacteriophage has also been shown to be critical for biofilm dispersal in PAO1 (Rice *et al.*, 2008; Webb *et al.*, 2004; Webb *et al.*, 2003) and a panel of chronic CF isolates (Kirov *et al.*, 2007). The bacteriophage is known as Pf4 in PAO1 (a member of the *Inoviridae* (1.4.3.2)) and normally exists as a

prophage, but can develop into a superinfective form that is capable of causing cell lysis of cells within biofilms (Webb *et al.*, 2003) and drives the emergence of bacterial morphological variants (Webb *et al.*, 2004). When Pf4 is deleted, the microcolonies formed are much smaller, and do not undergo the same cell death and hollowing in the centre that is observed in WT PAO1 biofilms, but complementation with exogenously added superinfective Pf4 restores cell lysis. Moreover, Pf4 is required for full virulence in a mouse model of acute pneumonia, although the exact mechanism for this was not elucidated (Rice *et al.*, 2008). Both the presence of reactive oxygen and nitrogen species and a hypermutator phenotype result in increased/ earlier production of the superinfective form (Hui *et al.*, 2014), and a recent deep-sequencing study of *P. aeruginosa* biofilms found that the Pf4 phage undergoes rapid evolution over a short-timescale, accumulating multiple mutations in the *cI* repressor gene that normally functions to maintain the phage in prophage form (McElroy *et al.*, 2014).

Despite the fact that biofilms are traditionally thought to be surface attached, aggregates of *P. aeruginosa* have been detected in CF sputa and removed lungs, suggesting that *P. aeruginosa* may grow as unattached biofilms in the CF lungs (Bjarnsholt *et al.*, 2009). As most of the experimental work characterising biofilms has been done with surface attached biofilms, the role of certain factors, particularly those involved in surface attachment is thrown into question. Novel *in vitro* approaches are being used to model *P. aeruginosa* growth in the CF lung, most notably artificial sputum (1.3.1), in which *P. aeruginosa* forms “biofilm-like structures”. The structure of these biofilms is strain specific and dependent on the concentrations of free DNA and mucin (Haley *et al.*, 2012), both of which contribute to the viscosity of CF sputum.

Regardless of the types of biofilms formed in the CF lung, there are still clinical implications. When the reference strain PA14 is grown as microcolonies, over 40 genes are differentially expressed compared to when at stationary phase in LB media (Fung *et al.*, 2010). Gene expression is altered in biofilm bacteria (relative to planktonic), particularly in response to antibiotics (Whiteley *et al.*, 2001). Through altered gene expression and the physical protection afforded by the matrix, biofilms

are refractory to antibiotics (Mah & O'Toole, 2001) and the immune system (Kharazmi, 1991; Leid *et al.*, 2005), leading to persistence in the lungs.

1.2.8 Quorum sensing

Quorum sensing (QS) is a chemically-mediated cell to cell communication system, whereby bacteria produce and detect QS molecules, termed autoinducers. Autoinducers accumulate in the environment, until they reach a threshold density, upon which the gene expression of the cell changes in response to the high density of cells, towards cooperative behaviours. QS has been linked to biofilm formation (Davies *et al.*, 1998) and virulence factor production (Whiteley *et al.*, 1999). This is presumably because expression of virulence factors, for example, is wasted if the cell density is low as they cannot reach a high enough concentration to have an effect on the host.

Four different QS systems have been identified in *P. aeruginosa*, but they affect each other in a complex network of regulation. They are the Las, Rhl, PQS and recently discovered IQS systems (Lee *et al.*, 2013). Both the Las and Rhl systems consist of an autoinducer protein (encoded by *lasI* and *rhlI*, respectively) that produce N-acyl-homoserine lactone molecules that bind to the transcriptional regulators LasR and RhlR (encoded by *lasR* and *rhlR* genes) (Gray *et al.*, 1994; Pearson *et al.*, 1995). LasR and RhlR both regulate hundreds of genes (Schuster *et al.*, 2003) and LasR also positively regulates *lasI,* setting up a positive feedback loop, in addition to *rhlRI* (Latifi *et al.*, 1996), although this regulation of the Rhl system by the Las system is unidirectional.

The PQS system is a chemically distinct system, utilising the signalling molecule 2-heptyl-3-hydroxy-4-quinolone (also known as the Pseudomonas Quinolone Signal) (Pesci *et al.*, 1999). The PQS system is positively regulated by the Las system (McGrath *et al.*, 2004; Pesci *et al.*, 1999) and itself (McGrath *et al.*, 2004; Wade *et al.*, 2005) and negatively regulated by the Rhl system (McGrath *et al.*, 2004). PQS also has a dual function in iron acquisition, as it can chelate iron and positively regulates siderophore production (Diggle *et al.*, 2007b).

The IQS is the most recently discovered QS system, produced by the *ambABCDE* gene cluster, and is co-regulated by Las QS system and the phosphate limitation stress response. Furthermore, IQS is required for full virulence in an acute murine infection model (Lee *et al.*, 2013).

Mutants defective in QS are frequently isolated from chronic CF respiratory infections, particularly *lasR* mutants (Smith *et al.*, 2006a), a phenomenon that is discussed further in Chapter 5.

1.3 Models of CF

P. aeruginosa, like many bacteria, behaves differently when grown in different environments, and many different models, both *in vitro* and *in vivo*, have been developed in an attempt to model the growth and evolution of *P. aeruginosa* in the CF lungs.

1.3.1 Synthetic sputum models

CF sputum can be used as a growth medium by dissolving sterile, lysophilised sputum in buffer, creating a medium that supports high densities of *P. aeruginosa* (Palmer *et al.*, 2005) because of the high concentration of amino acids that bacteria use as a growth source (Barth & Pitt, 1996; Palmer *et al.*, 2005). However, CF sputum is in limited supply and the chemical composition is unlikely to be consistent between different batches. Because of this, several synthetic sputum models have been developed for the study of *P. aeruginosa* and other CF pathogens.

Artificial sputum media (ASM) was one of the first synthetic sputum models to be developed (Sriramulu *et al.*, 2005). ASM contains a high concentration of free DNA, mucin, amino acids, salts and lipids, resulting in a medium that has a similar viscosity and chemical composition to CF sputum (Sriramulu *et al.*, 2005). When grown in ASM, *P. aeruginosa* forms tight microcolonies that are not surface attached (Haley *et al.*, 2012; Kirchner *et al.*, 2012; Sriramulu *et al.*, 2005), thought to be how *P. aeruginosa* grows in the CF lung (1.2.7). Bacteria grown in ASM undergo phenotypic diversification (Sriramulu *et al.*, 2005; Wright *et al.*, 2013), exhibiting changes in colony morphology (Sriramulu *et al.*, 2005), pyocyanin production and antibiotic susceptibility (Wright *et al.*, 2013). Furthermore, gene expression is altered

in ASM, with an upregulation of genes involved in biofilm formation, QS and phenazine biosynthesis (Fothergill *et al.*, 2014).

ASM can be filtered to remove contaminating bacteria without damaging the heat-sensitive components, but a modified version (ASMDM) contains less DNA and more mucin, considered by the authors to better represent the CF lung (Fung *et al.*, 2010). The additional mucin makes filtration difficult, so antibiotics are added to ASMDM to inhibit growth of contaminants, but this is potentially concerning as subinhibitory antibiotic concentrations can have a wide range of effects on bacterial cells, including altered gene regulation (Davies *et al.*, 2006).

Other synthetic sputum models include synthetic CF sputum medium (SCFM), which contains amino acids but lacks DNA and mucin (Palmer *et al.*, 2007), and a more recent version, SCFM2, which is supplemented with DNA, mucin, N-acetyl glucosamine and lipids to better represent CF sputum (Turner *et al.*, 2015). The addition of these components probably makes SCFM2 more alike to ASM than its precursor.

Support for the use of synthetic sputum as a model for the CF lung comes from a recent transposon-sequencing study that determined the genes required for *P. aeruginosa* fitness in both CF and synthetic sputa (they used SCFM2). The essential genes were very similar for both (Turner *et al.*, 2015), suggesting that synthetic sputum is a good *in vitro* model to study *P. aeruginosa*.

1.3.2 *In vivo* models

As *P. aeruginosa* is an opportunistic pathogen, infection of healthy animals usually leads to rapid clearance (Southern Jr *et al.*, 1970), unless the dose is sufficiently high or the animal is immunocompromised, in which case an acute infection is established and the animal quickly dies (Morissette *et al.*, 1995). However, this is not representative of chronic *P. aeruginosa* infections in CF, which are characterised by a slow decline in health, often over many years (Kosorok *et al.*, 2001). Although natural animal models of CF do not exist, attempts have been made to engineer CFTR deficient animals in order to replicate the CF disease phenotype.

Numerous murine CF models have been used that differ in CFTR functionality and range from a defective to completely absent CFTR, but the pathophysiology of lung disease is not reflective of that in humans and they are not intrinsically susceptible to respiratory bacterial infections (Fisher *et al.*, 2011). The more recently developed ferret and pig models are more promising. CFTR defective pigs develop CF-like lung disease as adults (Ostedgaard *et al.*, 2011; Stoltz *et al.*, 2010), following colonisation with multiple bacterial species (Stoltz *et al.*, 2010). Furthermore, CF pigs were unable to successfully eradicate bacteria when artificially infected, mimicking the defective clearance observed in CF humans (Stoltz *et al.*, 2010). CFTR knock-out ferrets are susceptible to bacterial respiratory infections soon after birth and also demonstrate many other symptoms seen in CF humans (Sun *et al.*, 2010).

CFTR models are expensive and not readily available, so many animal models used in the study of *P. aeruginosa* infections in CF use phenotypically healthy animals, but the mode of infection is designed to make the resulting infection as close to chronic respiratory infection as possible. It was discovered that by embedding *P. aeruginosa* in agar beads and introducing the beads into the rat lung by intubation, a chronic lung infection could develop, resulting in a similar pathophysiology to chronic *P. aeruginosa* infections in individuals with CF (Cash *et al.*, 1979). The rat agar bead model is still in use and has been used to study the growth and fitness of several *P. aeruginosa* strains (Kukavica-Ibrulj *et al.*, 2008b).

A major issue with these models is the artificial nature of infection. A pulmonary mouse model was developed that used an alginate-overproducing (mucoid) CF isolate, and the self-produced alginate protected the bacteria from the immune system, negating the need for artificial embedding (Hoffmann *et al.*, 2005). However, such a model is obviously limited in the bacterial strains that can be used. Furthermore, implantation into the lungs by intubation bypasses the host's normal defences, and analysis of *P. aeruginosa* in the paranasal sinuses and lungs of CF children suggests that *P. aeruginosa* colonises and "pre-adapts" to the upper respiratory tract before seeding down into the lungs and establishing chronic infection (Hansen *et al.*, 2012). A recently developed murine model utilised a more natural infection route, as free bacteria were administered intranasally. Bacteria colonised the nasopharynx and although no bacteria were detected in the lungs 2

weeks post-infection, after 4 weeks bacteria were present in the lungs, suggesting the nasopharynx acts as an infection reservoir, leading to adaptation and re-seeding of the lungs (Fothergill *et al.*, 2014).

1.3.3 Other *P. aeruginosa* infection models

An *ex vivo* porcine lung infection model has recently been developed as an alternative model that bridges the gap between *in vivo* and *in vitro* models of chronic *P. aeruginosa* infection. It involves bacterial inoculation of sections of fresh pig lung tissue suspended in ASM, and the lung tissue provides the spatial structure that is lacking in ASM (Harrison *et al.*, 2014). The extent of tissue damage can then be evaluated to estimate the virulence of different strains. A major advantage of this model is that it is not subject to the same ethical constraints as *in vivo* models, as it uses pig carcasses from the food industry.

Other models exist to assess *P. aeruginosa* virulence, including *Caenorhabditis elegans* (nematode) (Tan *et al.*, 1999), *Drosophila melanogaster* (fruit fly) (Apidianakis & Rahme, 2009), *Galleria mellonella* (wax moth) larvae (Miyata *et al.*, 2003), plants (Rahme *et al.*, 1997) and *Dictyostelium discoideum* (amoeba) (Cosson *et al.*, 2002), but these models result in acute infection, which is not representative of *P. aeruginosa* infections in CF and involves different fitness requirements (Turner *et al.*, 2014).

1.4 Bacteriophages

Bacteriophages, or phages as they are commonly known, are viruses that infect bacteria. The name bacteriophage is derived from the Greek word "phagein", which translates as "to eat" i.e. bacteria-eater. Like all viruses, phages encode the genetic material necessary for their replication, but require a host cell for the metabolic requirements of replication.

Phage genomes can be single or double stranded RNA or DNA, enclosed within a protein (or more rarely, lipid) capsid head, although filamentous phages of the genus *Inovirus* are an exception to this rule and are described in more detail in (1.4.3.2). The genetic material is protected within the capsid to enable survival in the environment, and phages may also have a tail, to facilitate bacterial infection.

1.4.1 Life cycles

Phages have two main life cycles, the lytic and lysogenic. Some phages only follow one lifecycle e.g. obligately lytic phages are only capable of a lytic lifecycle, but others can follow either, and are known as temperate phages.

1.4.1.1 Lytic

The lytic life cycle involves phage replication and the production of phage virions, which are then released from the bacterial host by cell lysis, causing the inevitable death of the host. An overview of the lytic and lysogenic cycles is described in Figure 1.5, using the model temperate phage λ, which infects *E. coli*, because it is well-characterised and is similar to the phages used in this study.

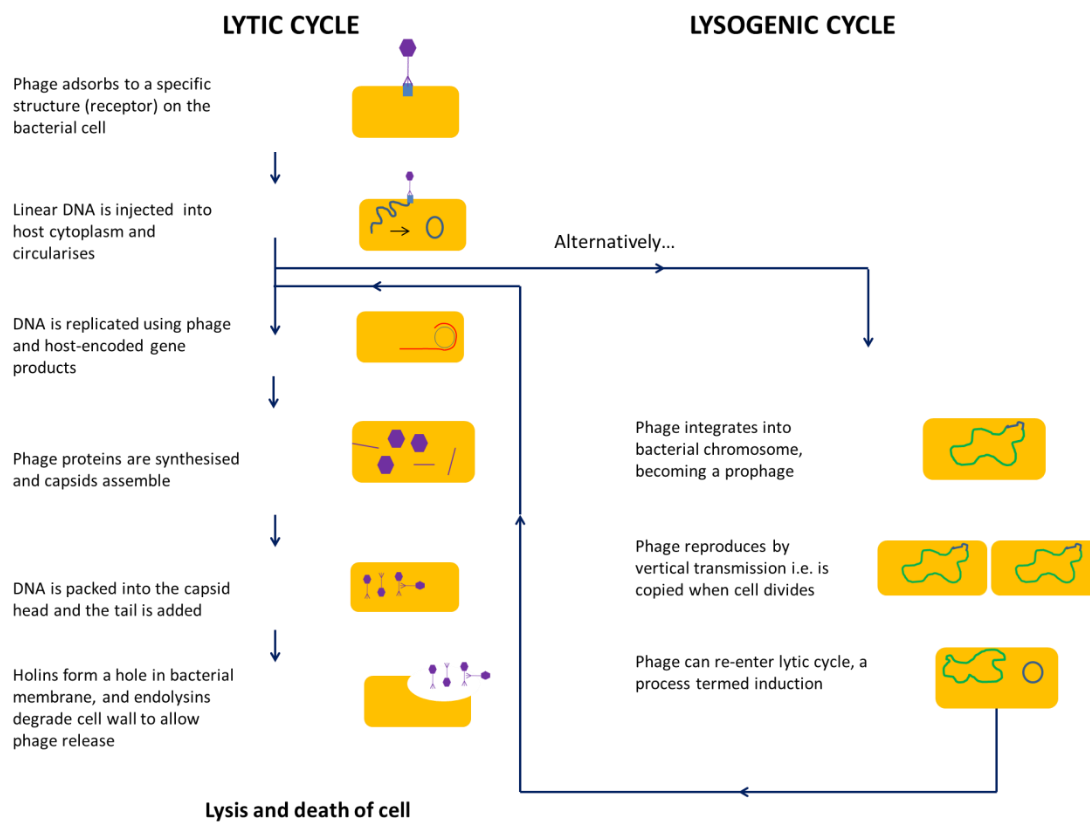


Figure 1.5 Diagrammatic representation of the lytic and lysogenic cycles of bacteriophages, based on the model *E. coli* phage λ.

Phage gene expression is regulated to ensure that the lytic life cycle proceeds correctly. For example, λ has immediate early, delayed early and late genes; the immediate early genes are required for the default lytic life cycle to proceed (and prevent the lysogenic life cycle), delayed early genes enable phage DNA replication

and late genes enable virion assembly (Oppenheim *et al.*, 2005). Transcription of the phage genome is controlled by phage-encoded regulatory and transcription antiterminator proteins. In λ, the regulatory protein Cro and antiterminator protein N are expressed early on. Cro is the essential lytic regulator, preventing synthesis of cI (a repressor of the lytic cycle that is expressed in lysogeny). The N protein allows expression of the early genes to proceed (Greenblatt *et al.*, 1998) by preventing pausing of the replication complex at the transcription terminators. This allows the late gene regulator Q to be produced, and when it reaches a threshold density (Kobiler *et al.*, 2005), it acts also as an antiterminator, allowing transcription of the late genes (Grayhack & Roberts, 1982). This mechanism of transcriptional control means that the genes required for DNA replication and phage particle formation are expressed at temporally discrete points in the phage life cycle, so the viral particles can be correctly assembled and are mostly infective upon lysis.

1.4.1.2 Lysogenic

Temperate phages are capable of following an alternative life cycle upon infection of a cell, known as the lysogenic cycle (Figure 1.5). The phage integrates into the bacterial cell chromosome, where it becomes known as a prophage, and the bacterium becomes known as a lysogen. The prophage can remain dormant in the cell for many generations, replicating with the host chromosome and being vertically transmitted to the daughter cell.

1.4.1.2.1 Lysis/ lysogeny decision

The decision of whether to enter the lytic or lysogenic pathway upon infection of the cell is largely dependent on the metabolic condition of the cell, and lysogeny is more likely in starving cells (Lieb, 1953). This is thought to be a survival tactic by the phage when resources are limited, allowing the phage to survive times of hardship; resource limitation can result in cells that are incapable of supporting lytic phage reproduction, and too low a density of neighbouring susceptible cells to support continued phage propagation (Stewart & Levin, 1984). Mathematical modelling of different phage strategies suggests that lysogeny will be favoured in fluctuating or isolated environments, and that the probability of a temperate phage establishing lysogeny is close to the probability of collapse of the local environment, thus

maximising the potential for growth but minimising the possibility of extinction, a form of bet-hedging (Maslov & Sneppen, 2015). The regulation of the lysis/ lysogeny decision and environmental factors that influence it will be discussed further in Chapter 3.

1.4.1.2.2 Phage integration

The establishment of lysogeny necessitates the phage to integrate itself into the bacterial chromosome, and there are two main strategies for this, integrase and transposase. Integrase is a phage-encoded, site-specific enzyme that mediates recombination between a specific sequence in the bacterial chromosome called the bacterial attachement site (*attB*) and a sequence in the phage genome known as the phage attachment site (*attP*). λ and two of the phages in this study (LESφ2 and LESφ3) use integrase, and so have a specific integration site in the bacterial genome.

Transposase is an enzyme that is utilised by transposable phages, including the well characterised *E. coli* phage Mu and one of the phages in this study (LESφ4). Mu-like prophages have been observed in a diverse range of bacteria (Morgan *et al.*, 2002) and are commonly identified infecting *P. aeruginosa* (1.4.3.1.1.1). Mu integrates into the host chromosome by a conservative transposition event (Harshey, 1984), facilitated by transposase. Because of the homology-independent nature of transposition (Shapiro, 1979), Mu can integrate randomly in the bacterial chromosome (Bukhari & Zipser, 1972), occasionally disrupting coding genes as a result, hence its name Mu, short for "mutator". The *P. aeruginosa* phage D3112 is also capable of insertional mutation (Rehmat & Shapiro, 1983).

1.4.1.2.3 Induction

Even if lysogeny is established, the lytic cycle can resume at any point, in a process known an induction. In the case of λ, the phage excises itself from the bacterial chromosome and re-enters the lytic cycle, producing virions and lysing the cell (Figure 1.5). Integrase is again required for excision, in combination with another phage-encoded enzyme, excisionase (Cho *et al.*, 2002).

Mu does not excise from the genome like λ, but instead remains in the same location within the genome and copies itself by replicative transposition (Shapiro, 1979). Mu

is not cleaved from the chromosome until late in the lytic cycle (Ljungquist & Bukhari, 1977), a characteristic also shared by the *P. aeruginosa* transposable phage D3112 (Rehmat & Shapiro, 1983).

Induction is known to be triggered by DNA damaging agents, including particular antibiotics, UV light and reactive oxygen species. It occurs as part of the bacterial SOS response, which is a global cellular response to non-lethal DNA damage that culminates in increased DNA repair, inhibition of cell division and prophage induction. The SOS response in *E. coli* involves activation of the RecA protease, which cleaves LexA, a repressor of genes involved in the SOS reponse (Little & Mount, 1982). The presence of activated RecA also results in autocleavage of the cI repressor, a phage-encoded protein that is essential for maintenance of lysogeny, triggering a cascade of genes involved in the λ lytic cycle (Ptashne, 2004). Prophage induction is thought to be a phage survival strategy, enabling a phage to escape a cell that is at risk of death (Refardt & Rainey, 2010).

Mitomycin C is a DNA cross-linking agent (Iyer & Szybalski, 1964) and a potent phage inducer, so is used commonly in prophage induction studies (Castellazzi *et al.*, 1972; Jiang & Paul, 1996; Ramirez *et al.*, 1999). Fluoroquinolone antibiotics such as norfloxacin and ciprofloxacin inhibit DNA synthesis (Hooper, 2001), and consequently induce the SOS response in *E. coli* (Phillips *et al.*, 1987). In *P. aeruginosa*, exposure to ciprofloxacin (which is used in the management of *P. aeruginosa* infections) has been linked with upregulation of phage-related genes (Cirz *et al.*, 2006) and increased production of phage virions (Fothergill *et al.*, 2011). *P. aeruginosa* phages are detected at high levels in CF sputa, most likely as a result of phage induction of lysogenic bacteria (James *et al.*, 2014); environmental factors affecting phage production are explored in Chapter 3.

Induction can also occur in the absence of any known environmental triggers, and is known as spontaneous induction. This often occurs at low but detectable levels in bacterial cultures, and varies between strains (Fothergill *et al.*, 2011; James *et al.*, 2012).

1.4.1.2.4 Prophage degradation

Some prophages accumulate mutations or deletions in genes required for lysis, and become known as cryptic, or defective prophages. However, some defective prophages are capable of forming infectious phage particles when in the presence of a helper phage. This has been described in the opportunistic pathogen *Enterococcus faecalis*; it has 7 prophages, one of which is dependent on another for encapsidation in order to produce infectious phage particles (Matos *et al.*, 2013).

Cryptic or defective prophages are common in bacterial genomes, although they can be difficult to detect, especially after extensive degradation and so their prevalence may actually be underestimated. A re-examination of a pathogenic *E. coli* strain questioned whether phages that are identified as defective from sequence data are defective at all. *E. coli* 0157 Sakai has 18 prophages, and genome sequence analysis found defects in all but one, for example in genes required for excision or morphogenesis. Despite this, half of the phages could excise and replicate, and many of those could form functional phage particles, even those that were defective in morphogenesis, strong evidence of phages complementing each other (Asadulghani *et al.*, 2009).

1.4.1.2.5 Benefits of prophage carriage

Prophages carry multiple genes, some of which can confer a fitness benefit to the bacterium (discussed in more detail in Chapter 3), and this may help explain the high numbers of prophages observed in many bacterial genomes (Hayashi *et al.*, 2001b; Matos *et al.*, 2013; Wang *et al.*, 2010b; Winstanley *et al.*, 2009). Pathogenic strains have a higher proportion of phage-related genes than non-pathogenic strains (Busby *et al.*, 2013) and many serious pathogens carry multiple prophages (Hayashi *et al.*, 2001b; Winstanley *et al.*, 2009), suggesting that prophages may make a significant contribution to bacterial pathogenicity.

1.4.2 Phage classification

There is no universal gene in bacteriophages that is akin to the 16S rRNA gene of bacteria that can be used to determine phylogenies, so classification is based primarily on morphology and genetic material (King *et al.*, 2011), but phages can

also be grouped according to characteristics such as host range, genome size, life cycle, sequence similarity and genome organisation (Ackermann, 2009). Following advances in technology, *de novo* peptide sequencing of major phage proteins has been used to determine relatedness between *P. aeruginosa* phages (Ceyssens *et al.*, 2009b), representing yet another potential mechanism for phage classification.

In the most recent report by the International Committee on Taxonomy of Viruses (ICTV), there were over six orders, 87 families, 19 subfamilies and 348 genera of viruses (including, but not limited to, bacteriophages). Phages are grouped firstly by their genetic material (i.e. ss/ds DNA/ RNA), and within that, grouped into orders based on morphology, which are further subdivided into families, sub-families genera and species (King *et al.*, 2011). Phage taxonomy is constantly evolving, and the ICTV recently published a report clarifying multiple changes to the system of virus classification and taxonomy that had been decided following the last official report in 2011 (Adams *et al.*, 2013).

1.4.2.1 Mosaicism

Temperate phage genomes are highly mosaic, consisting of modules that are interchangeable between phages by recombination (Hendrix *et al.*, 1999). Mosaicism obviously makes phage classification very difficult, as two phages could share 90% similarity for the tail genes for example, but very low sequence similarity across the rest of the genome. The dsDNA phages in particular are difficult to classify; multiple dsDNA phages from diverse bacterial genera have been shown to share sequence similarity, suggestive of a shared ancestry and extensive horizontal gene transfer (Hendrix *et al.*, 1999).

Many phages encode their own recombinases (Lopes *et al.*, 2010), enzymes that catalyse recombination between homologous stretches of DNA. The Rad52-like recombinase of λ allows more “relaxed” recombination than the bacterial recombinase RecA (i.e. recombination between more diverse sequences), suggesting a key role for phage recombinases in the generation of mosaicism. Bioinformatic analysis identified evidence of homologous recombination in numerous phage genomes, but interestingly not between lytic and temperate phages (De Paepe *et al.*, 2014). Within the LESB58 prophage pool, several of the prophages share regions

with high sequence similarity, but otherwise are not homologous across the rest of the genome, suggestive of recombination-driven mosaicism (Winstanley *et al.*, 2009).

1.4.3 *Pseudomonas aeruginosa* phages

There are 128 *Pseudomonas* phage genomes in the EMBL-EBI database (at the time of writing) and many more as prophages in bacterial genomes (McWilliam *et al.*, 2013). *P. aeruginosa* phages isolated from geographically diverse locations can be closely related and classified into a limited number of subgroups (Ceyssens *et al.*, 2009b), although a more recent study of phages isolated in Mexico suggested that the diversity of *P. aeruginosa* phages may be larger than previously thought, as 12 different "species" were identified, six of which were novel (Sepúlveda-Robles *et al.*, 2012).

1.4.3.1 Caudovirales

The majority of known phages infecting bacteria and archaea are members of the order *Caudovirales*, consisting of the three families of dsDNA tailed phages, *Siphoviridae*, *Myoviridae* and *Podoviridae* (King *et al.*, 2011). Classification of *Caudovirales* into families is based on tail morphology; *Siphoviridae* have long, non-contractile tails, *Myoviridae* have long, contractile tails, and *Podoviridae* have short tails. All the phages used in this study belong to the family *Siphoviridae*, which is discussed in more detail in the following section.

1.4.3.1.1 Siphoviridae

The majority of *Pseudomonas* phages are *Siphoviridae* (Ackermann, 2007; Knezevic *et al.*, 2009) with the long, non-contractile tails. The genome organisation of *Siphoviridae* tends to be quite conserved; for example, *E. coli* lambdoid phages have head and tail genes at the 5' end, genes involved in replication and recombination in the middle, and genes involved in host lysis at the 3' end (Casjens, 2005).

1.4.3.1.1.1 Transposable phages

Within the *Siphovirida*e family of phages that infect *Pseudomonas*, there exists a group of 100+ transposable phages, (Ceyssens & Lavigne, 2010), including the well-

characterised D3112 (Wang *et al.*, 2004). They use the type IV pilus as a receptor to infect the bacterium and encode transposases for integration and replicative transposition, like *E. coli phage* Mu. Like Mu, D3112 replicates across the genome, maintaining its original location until just before lysis, and can insert randomly into the genome (Rehmat & Shapiro, 1983).

However, their genome organisation is λ-like (Wang *et al.*, 2004; Winstanley *et al.*, 2009) and they are classed as *Siphoviridae* (like λ) based on virion morphology. The genome is organised 5' to 3' with the lysis and lysogeny genes, transposition and replication genes, and structural genes (Cazares *et al.*, 2014). A comparative genomics study was recently undertaken for 12 *P. aeruginosa* transposable phages, and revealed a core genome conserved between phages, but also highly variable regions (accessory genome) that represent 6-10% of the phage genomes, hypothesised to include genes important for enhancing bacterial host fitness (Cazares *et al.*, 2014).

1.4.3.2 Inoviridae

The *Inoviridae* are a family of ssDNA filamentous bacteriophages not assigned to the order *Caudovirales*. Pf1 was the first identified filamentous phage infecting *P. aeruginosa*; it infects the cell via the type IV pilus, and like other filamentous phages is unusual in that is does not cause cell lysis, but instead extrudes virions through the cell poles (Bradley, 1973; Hill *et al.*, 1991). Pf1-like phages have since been discovered to be widespread, detectable in over 50% of *P. aeruginosa* strains as prophages (Knezevic *et al.*, 2015). They are separated into 4 main clades: Pf4, which is the most abundant and found in PAO1, Pf5 (in PA14), Pf7 (in PA7) and Pf-LES (LES isolates, also referred to as LESφ6) (Knezevic *et al.*, 2015).

The *Inoviridae* are of interest because of their role in bacterial virulence and biofilm formation (1.2.7), but this is dependent on the prophage accumulating mutations and developing into a superinfective, replicative form that is capable of cell lysis (McElroy *et al.*, 2014; Rice *et al.*, 2008). Pf4 and Pf5 are intact prophages in the PAO1 and PA14 genomes, respectively (Knezevic *et al.*, 2015), and consequently can develop into the replicative forms (Mooij *et al.*, 2007; Webb *et al.*, 2004). However, other Pf1-like phages, including Pf-LES cannot be confirmed as intact by

sequence analysis (Knezevic *et al.*, 2015), and the replicative form has not been demonstrated, so their contribution (if any) to virulence is undetermined.

1.4.4 Mechanisms of phage resistance

The majority of phage infections result in cell lysis, so it is unsurprising that bacteria have numerous mechanisms to prevent or terminate phage infection. The first line of defence is to prevent phage adsorbtion; phages require a specific receptor, so rendering these absent or defective, through mutations, phase variation etc. prevents infection. For example, type IV pili are common phage receptors: in a panel of 15 diverse *P. aeruginosa* phages, 10 required type IV pili for infection (Ceyssens *et al.*, 2009a). Mutations that result in defective or absent pili, such as in the *pilA* gene, normally leads to loss of motility and phage resistance, but it is not always that simple. For example, mutations in *pilU* or *pilT* result in retraction deficient, hyperpiliated mutants that cannot twitch. However, a *pilU* mutation does not lead to phage resistance (Whitchurch & Mattick, 1994). Other common phage receptors include the flagellum and lipopolysaccharide (Garbe *et al.*, 2010; Hosseinidoust *et al.*, 2013b; Jarrell & Kropinski, 1976).

Once phages have absorbed to cells, superinfection exclusion (Sie) systems can prevent phage DNA injection. The Sie proteins are often encoded by resident prophages in the bacterial genome and protect from infection against related phages (Chapter 3). For DNA phages making it past this point, chromosomal or plasmid-borne restriction modification systems may cleave phage DNA or methylate it, making it resistant to restriction, thus inhibiting the phage lytic cycle (Gingeras & Brooks, 1983). Furthermore, the CRISPR-Cas system acts to recognise and destroy phage sequences to which it has had prior exposure.

The CRISPR-Cas system is a recently discovered form of acquired immunity to bacteriophages found in many bacterial and archaeal species (Barrangou *et al.*, 2007), including 36% of clinical *P. aeruginosa* strains (Cady *et al.*, 2011). In *P. aeruginosa*, two types of CRISPR-Cas have been discovered, the type I-E (similar to *E. coli*) and the more prevalent type I-F (*Yersinia*-like) (Cady *et al.*, 2011). Experiments with strain PA14, which has a functional type I-F system, and a panel of phages demonstrated that the bacteria acquired CRISPR-mediated resistance in

response to phage challenge (Cady *et al.*, 2012). The type I-E system is also active in *P. aeruginosa* (Pawluk *et al.*, 2014). The CRISPR-Cas system and phage counter-resistance mechanisms are described in Chapter 3.

The last resort is an abortive infection system (Abi), whereby programmed death of the bacterium occurs prior to cell lysis and phage release, thus protecting bacterial kin from phage infection (Chopin *et al.*, 2005; Fineran *et al.*, 2009). However, no specific Abi system has (to my knowledge) been described in *P. aeruginosa*.

1.5 Summary

P. aeruginosa is a significant respiratory pathogen in individuals with CF, and chronic infections are difficult to eradicate. *P. aeruginosa* adapts easily to its environment, in part due to the large accessory genome of this species which includes numerous phage-derived sequences. The Liverpool Epidemic Strain is a successful transmissible strain of *P. aeruginosa*, and genome sequencing of isolate LESB58 revealed six prophages contained within the genome, several of which were necessary for LES competitiveness *in vivo*. LES bacteriophages are present at high densities in CF sputa, but the role of these bacteriophages in driving *P. aeruginosa* diversification, and their effects on bacterial fitness have yet to be elucidated. Novel *in vitro* and *in vivo* infection models enable us to model chronic *P. aeruginosa* infection in the CF lung and test hypotheses.

1.6 Objectives of study

- Characterise the LES phages φ2-4 further by reannotating their genomes and determining environmental factors that influence their production.
- Determine the extent to which LES phages drive *P. aeruginosa* diversification using an experimental evolution approach, coevolving the well-characterised laboratory strain PAO1 with and without LES phages φ2-4 in an artificial sputum model of infection.
- Compare phage-driven diversification *in vitro* to that observed in a rat model of chronic lung infection.

- Consider how LES prophage carriage affects the ability of bacteria to invade a population of phage susceptible bacteria, both *in vitro* and in a rat model of chronic lung infection.

Chapter 2 Materials and methods

2.1 Bacterial strains and growth conditions

The strains of *Pseudomonas aeruginosa* used in this study and their origin are listed in Table 2.1. All strains were stored in Lysogeny Broth (LB) (Appendix A), with the addition of 25% (v/v) glycerol, and frozen at -80˚C. When required, a small aliquot of frozen cells was plated onto Columbia agar (Oxoid) and incubated overnight at 37˚C. When liquid cultures were required, a single colony was transferred into a glass universal containing a 5 ml aliquot of LB and incubated at 37˚C, with shaking at 180 r.p.m. until desired growth was achieved.

Table 2.1 Bacterial strains used in this study and sources

Name	***Pseudomonas aeruginosa* strain**	**LES prophages present in genome**	**Description**	**Origin**
PAO1 (aka PAO1$^{\varphi-}$)	PAO1	None	Wild-type PAO1	Winstanley strain collection: well studied laboratory reference strain (Stover *et al.*, 2000)
PAO1 GFP a.k.a. PAO1 Gm^{R}	PAO1	None	Mini-Tn*7* transposon marker inserted at a neutral and site-specific location on chromosome. Expresses green fluorescent protein (GFP) and gentamicin resistance.	Winstanley strain collection. Constructed by Dr. Chloe James (unpublished)
PAO1 DsRed-Express	PAO1	None	Mini-Tn*7* transposon inserted at a neutral and site-specific location on chromosome. Expresses DsRed-Express protein and gentamicin resistance.	Winstanley strain collection. Constructed by Dr. Chloe James (unpublished)
PAO1Δ*mutS*	PAO1	None	Displays hypermutable phenotype	Winstanley strain collection
PAO1*pilA*$^{-}$ ::Tet^{R}	PAO1	None	Mini-Tn5*lux* transposon mutant (tetracycline resistance). Insertion in *pilA* gene results in absence of typeIV pili.	(Taylor & Buckling, 2010)
PA14	PA14	None	Wild-type PA14	Winstanley strain collection: well-characterised laboratory reference strain (He *et al.*, 2004)
PLPLφ2	PAO1	LESφ2	LESφ2 lysogen	Winstanley strain collection. Constructed by Chloe James (James *et al.*,

				2012).
PLPLφ2 GFP	PAO1	LESφ2	Marker as for PAO1 GFP. LESφ2 lysogen	Winstanley strain collection. Constructed by Chloe James (unpublished)
PLPLφ2 DsRed-Express	PAO1	LESφ2	Marker as for PAO1 DsRed-Express. LES φ2 lysogen	
PLPLφ3	PAO1	LESφ3	LESφ3 lysogen	Winstanley strain collection. Constructed by Chloe James (James *et al.*, 2012).
PLPLφ3 GFP	PAO1	LESφ3	Marker as for PAO1 GFP. LESφ3 lysogen	Winstanley strain collection. Constructed by Chloe James (unpublished)
PLPLφ3 DsRed-Express	PAO1	LESφ3	Marker as for PAO1 DsRed-Express. LESφ3 lysogen	
PLPLφ4	PAO1	LESφ4	LESφ4 lysogen	Winstanley strain collection. Constructed by Chloe James (James *et al.*, 2012).
PLPLφ4 GFP	PAO1	LESφ4	Marker as for PAO1 GFP. LESφ4 lysogen	Winstanley strain collection. Constructed by Chloe James (unpublished)
PLPLφ4 DsRed-Express	PAO1	LESφ4	Marker as for PAO1 DsRed-Express. LESφ4 lysogen	
PLPLφtriple	PAO1	LESφ2, 3 and 4	Triple LES phage lysogen	Winstanley strain collection. Constructed by Chloe James (James *et al.*, 2012).
PLPLφtriple GFP	PAO1	LESφ2, 3 and 4	Marker as for PAO1 GFP. Triple LES phage lysogen	Winstanley strain collection. Constructed by Chloe James (unpublished)
PLPLφtriple DsRed-Express	PAO1	LESφ2, 3 and 4	Marker as for PAO1 DsRed-Express. Triple LES phage lysogen	
LESB58	LES	LESφ1:6 (full complement)	Clinical strain; CF isolate	Winstanley strain collection (Winstanley *et al.*, 2009)
LESB65	LES	LESφ1:4, 6	Clinical strain; CF isolate	Winstanley strain collection (Fothergill *et al.*, 2007; Jeukens *et al.*, 2014)
LES400	LES	LESφ1:6 (full complement)	Clinical strain; CF isolate	Winstanley strain collection (Jeukens *et al.*, 2014; Parsons *et al.*, 2002)
LES431	LES	LESφ1,3:6	Clinical strain; Isolate from non-CF parent of child with CF	Winstanley strain collection (Jeukens *et al.*, 2014; McCallum *et al.*, 2001)

2.1.1 Quantification of viable bacteria in a sample

Bacterial cultures were serially diluted 10-fold and 6 x 20 µl spots pipetted onto Columbia agar for each dilution and allowed to dry. Plates were incubated overnight, the resulting colonies counted, and the colony forming units (c.f.u.) ml^{-1} calculated. If bacterial colonies were required for further processing, then a spread-plate method was used, to ensure numerous, well-separated colonies. 75 µl of a dilution was spread evenly across the surface of the plate with a sterile L-shaped spreader and allowed to dry. All platings were conducted in triplicate.

2.1.2 Determination of the minimal inhibitory concentration (MIC) of antibiotics on bacterial strains

Antibiotic powders were purchased from Sigma-Aldrich (unless otherwise stated) and dissolved in 1 ml sterile distilled water (SDW), with the exceptions of norfloxacin, which was dissolved in 1% (v/v) glacial acetic acid and water, and nitrocefin and rifampicin, which were dissolved in 100 mg ml^{-1} dimethyl sulfoxide (DMSO). SDW was added and the antibiotic solution sterile filtered through a 0.2 µm syringe filter (Millipore). The concentrated stocks (100, 10 or 1 mg ml^{-1}) were frozen at -20˚C until required, or made fresh, depending on the manufacturer's recommendations. Stocks were diluted in Mueller Hinton broth (MHB (Oxoid)) to obtain required working concentrations.

2.1.2.1 Broth microdilution method

Two-fold serial dilutions of MHB/antibiotic were dispensed into rows of a sterile 96-well clear, flat-bottomed microtitre plate (50 µl per well). Overnight liquid bacterial cultures were diluted to the turbidity equivalent of a McFarland standard 0.5 (Beckton Dickinson), followed by a further 10^{-2} dilution, and 50 µl of this was added to each well and pipetted up and down to mix thoroughly. A growth control (MHB and bacteria only) and a sterility control (MHB only) were included on each plate. Plates were incubated for 18 hours at 37 ˚C and the MIC (i.e. lowest antibiotic concentration at which growth is inhibited) was determined by manual inspection of wells. Four wells per strain were tested on each plate, and each strain was tested on three individual plates.

2.1.2.2 Determination of the MIC for two antibiotics used in conjunction

The MICs of two antibiotics when used in combination may differ due to synergism or antagonism. To determine the MICs of two antibiotics used together, the checkerboard broth microdilution method was used. As in (2.1.2.1), all dilutions were performed in MHB. Two-fold serial dilutions of antibiotic 1 were dispensed into the rows of a 96 well plate, and two fold serial dilutions of antibiotic 2 were dispensed into the columns. Each well contained 25 µl of each antibiotic. Bacteria were added to the wells and controls included on each plate (2.1.2.1). The combination MIC was determined as the lowest concentration for each antibiotic at which no bacterial growth was observed. One strain was tested per plate, and nine replicate plates were tested for each strain, necessary because of the inherent unreliability of this test (White *et al.*, 1996).

2.1.2.3 Disk diffusion method

For high-throughput testing of multiple isolates, the standardised disc diffusion method was used, according to the British Society of Antimicrobial Chemotherapy (BSAC) guidelines (Andrews & Howe, 2011). Antibiotic susceptibility was determined by referring to the published breakpoints for *P. aeruginosa* (EUCAST, 2013). All isolates were tested in duplicate.

2.2 Use of artificial sputum medium (ASM) to culture bacteria

2.2.1 Preparation of ASM

ASM was prepared as described in (Kirchner *et al.*, 2012)(Appendix A) and stored at 4˚C for a maximum of eight weeks.

2.2.2 Bacterial culture in ASM

To set up initial cultures, 50 µl mid-exponential phase bacteria were inoculated into 5 ml ASM in a glass universal. Cultures were incubated at 37 ˚C with slow shaking (65 r.p.m.) for 96 hours.

2.2.3 Degradation of ASM biofilms

Biofilms were homogenised with Sputasol (Oxoid), a sputum liquefying agent. To prepare, 7.5 ml of Sputasol was diluted in 92.5 ml SDW. This was added in equal

volume to the bacterial culture, and the tube vortexed for 30 seconds, followed by 30 minutes incubation at 37 ˚C with rapid shaking (200 r.p.m.), to disrupt the biofilms. Unused Sputasol was stored at 4˚C for a maximum of 4 days.

2.3 General PCR methodology

2.3.1 Crude DNA preparation using boiled bacterial colonies

A small portion of a single colony was transferred into 50 µl SDW in a 0.2 ml PCR tube. The suspension was heated to 100˚C for 5 minutes and the resulting template used immediately in the standard PCR.

2.3.2 Standard polymerase chain reaction (PCR)

Standard PCR was carried out in 96 well PCR plates or 0.2 ml PCR tubes, depending on the number of samples. Each reaction contained: 1X Green GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 0.5 *U* GoTaq DNA polymerase (all Promega), 10 µM each dNTP (Bioline), 300 nM each primer and 5 µl crude DNA template. SDW was added to a final volume of 25 µl. Green GoTaq Flexi Buffer contains two dyes that migrate during gel electrophoresis, so addition of loading dye for gel electrophoresis was not required. All primer sequences and PCR cycling conditions are described in Table 2.2. PCR cycling was carried out in an Eppendorf MasterCycler Gradient.

Table 2.2. Oligonucleotide primers used in this study

Name	5'→3' sequence	Product size (bp)	Target gene/ region	Cycling conditions	Study	Reference
P2F	CTCCACTTCTCGGTTGCTTC	206	LES phage 2	95°C, 4 min then 30 cycles: 95°C, 30 s; 58°C, 30 s; 72°C, 30 s; final extension step, 72°C, 7 min	Any PCR assay involving detection of free phages or determining prophage complement of bacterial strain	This study
P2R	ACTAGCCCCGTATCCGAGTT					
P3F	TCAGGAAAACCTTGCCATTC	384	LES phage 3			
P3R	GTCTTCTGGTGGTCGGTGAT					
P4F	AGTTACGCCTGCTGGTGAGT	506	LES phage 4			
P4R	CCTCAGTCGTGCCTTCTTTC					
2totF	AGTAGCCGACCCAGACCTTT	141	LES phage 2	95°C, 10 min; then 40 cycles: 95°C, 10 s; 60°C, 15 s; 72°C 30s	qPCR to detect relative copies of free LES phages	(James *et al.*, 2012)
2totR	ATGGAAGCAACCGAGAAGTG					
3tot1F	CGCAGGTACCACCAGACTTT	122	LES phage 3			
3tot1R	CATGTCCAGCAGGTTCAAAA					
4tot1F	GCTCATGAGTGGCTGACAAC	105	LES phage 4			
4tot1R	TCTTGGGCAGAGAACCATTC					
PS1	ATGAACAACGTTCTGAAATTCTCTGCT	249	*P. aeruginosa oprI* lipoprotein gene	94°C, 5 min then 30 cycles: 94°C, 1 min; 60°C, 1 min; 72°C, 2 min; final extension step, 72˚C, 5 min.	qPCR of free phages; confirmation of degradation of *Pseudomonas* DNA	(De Vos *et al.*, 1993)
PS2	CTTGCGGCTGGCTTTTTCCAG					
lasRF	GTGCCGAATCCATATTTG	854	*lasR* gene	95°C, 4 min then 30 cycles: 95°C, 30 s; 48°C, 30 s; 72°C, 30 s; final extension step, 72°C, 7 min.	Amplification and Sanger sequencing of *lasR* gene	(Fothergill *et al.*, 2007)
lasRR	CCTTCCCTATATATCTGC					
7leftF	TTCGAGTTGGATCCGGCC	247	*fimU* LJ (left phage-host junction)	95°C, 4 min then 30 cycles: 95°C, 30 s; 55°C, 30 s; 72°C, 30 s; final extension step, 72°C, 7 min.	Confirmation of LESφ4 integration sites in PAO1	This study
7leftR	GTCGTGCTGTGCTGATCTTT					
7fimUrF	CGCTCATTCCGTGCCAATTA	358	*fimU* RJ (right phage-host junction)			
7fimUrR	TCAATGCGATGCTGCAGTAC					
7leftF		328	*fimU* intact insertion site			
7fimUrR						

11leftF	CTTCTTCAAGGCCAAGGGGT	458	*pilV* LJ			
11leftR	CGATGGCGATACGGTGATGA					
11rightF	GACGAGGTGCTTAGACGGAG	610	*pilV* RJ			
11rightR	ATCATGGACAGGCCCGATTG					
11leftF		349	*pilV* intact integration site			
11rightR						
Nar1F	GTATCCGATTCCATCCTGGGGAA	Variable	5' end of LES phage 4	94°C, 3 min then 30 cycles: 94°C, 1 min; 60°C, 2 min; 72°C, 4 min; final extension step, 72°C, 7 min.	Inverse PCR to determine LESφ4 integration sites	This study
NarIR	GGATGACCACTTGTACGCCAAGC					

2.3.3 Visualisation of PCR products

PCR products were visualised by agarose gel electrophoresis. Molecular grade agarose (Bioline) was dissolved in 0.5X TBE buffer (Appendix B). A 2% (w/v) agarose gel was used for PCR products below 650 bp, and a 1% (w/v) gel used for larger DNA fragments, or visualisation of intact genomic DNA. The agarose was melted by heating and cooled (to avoid vapour formation) prior to the addition of ethidium bromide to a final concentration of 0.1 μg ml^{-1}. 4 μl of PCR product was added to each well, and a 1 KB^{+} marker (Invitrogen) was run alongside for size determination of products. For PCR reactions using the colourless GoTaq Flexi Buffer, 5 parts DNA were mixed with 1 part loading dye (Invitrogen), prior to loading. Electrophoresis was performed for 45-90 minutes at 100V and DNA was visualised under an UV transilluminator (Syngene InGenius).

2.3.4 DNA extraction and purification from gels

After agarose gel electrophoresis, required bands were cut from the gel using a disposable scalpel. DNA was extracted from the gel slice using the Qiaquick gel extraction kit (Qiagen). The gel slice was weighed and dissolved in buffer QG (3 volumes buffer to 1 volume gel) by heating to 50˚C for 10 minutes. Isopropanol was added (1 volume isopropanol to 1 volume gel) and the mixture added to a spin column to bind the DNA. The column was centrifuged at 13 000 r.p.m. for 2 minutes and 500 μl buffer QG added to remove traces of agarose. The column was centrifuged again and 750 μl buffer PE (contains ethanol) was added to wash the DNA. The column was allowed to stand for 2 minutes before centrifugation at 13,000 r.p.m. for 1 minute. The column was centrifuged for a further minute to remove traces of buffer PE and DNA was eluted by pipetting 30 μl buffer EB directly onto the centre of the membrane and allowing to stand for 2 minutes, followed by centrifugation at 13 000 r.p.m. for 1 minute.

2.3.5 Determination of DNA concentration and quality

DNA quality was assessed by spectrophotometric analysis, using a Nanodrop ND-1000 spectrophotometer. The A_{230}, A_{260} and A_{280} of 2 μl of DNA were measured, and the $A_{260/280}$ and $A_{260/230}$ ratios calculated. DNA was deemed acceptable quality if the $A_{260/280}$ was 1.8 (±0.1) and the $A_{260/230}$ was above 1.6. DNA quantity in the sample

was estimated by fluorimetric analysis, as this measures only DNA, and is not influenced by other nucleic acids that may be present in the sample. The double-stranded DNA broad range assay (Invitrogen) was used to estimate DNA concentration. 10 µl DNA was added to 190 µl Qubit buffer (prepared according to the manufacturer's instructions), which contains a dye that binds to DNA and emits a fluorescent signal. Samples were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes. The fluorimetric signal was measured using the Qubit fluorometer and DNA concentration was estimated by comparison to known standards.

2.4 Bacteriophages used in this study and general protocols

All bacteriophages used in this study were initially isolated from *P. aeruginosa* strain LESB58 and purified. LESφ2, LESφ3 and LESφ4 were the only free LES phages used in this study as they were the only LES phages to be successfully isolated on strain PAO1 (James *et al.*, 2012). Where phage production by bacterial strains is investigated, the bacterial strains used are detailed in Table 2.1.

2.4.1 Separation of free infective bacteriophages from bacterial host

Bacterial cultures were filtered through a 0.2 µm filter (Millipore) to remove cells and debris, unless otherwise stated. The phage-containing filtrate was stored at 4˚C.

2.4.2 Preparation and storage of high titre phage stocks

High titre phage stocks were prepared previously (James *et al.*, 2012) and were stored at 4 ˚C. The titre was checked regularly. To achieve very high titre phage stocks, 500 µl sterile 4X PEG/NaCl solution (Appendix B) was added to 1500 µl high titre phage stock in a sterile 2 ml microfuge tube and inverted slowly to mix. Tubes were incubated overnight at 4˚C before centrifugation at 13,000 *g* for 15 minutes at 4 ˚C, and the supernatant discarded. The pellet was resuspended in 120 µl PBS with overnight incubation at 4˚C.

2.4.3 Determination of bacteriophage titre

2.4.3.1 Standard plaque assay

100 µl mid-exponential phase (OD_{600} 0.5) *P. aeruginosa* PAO1 was added to 100 µl of diluted bacteriophage suspension and vortexed. 5 ml of molten top agar (LB + 0.4% agar (w/v)) was added, and the tube inverted three times, slowly so as to minimise bubble formation. The mixture was overlaid onto a standard LB agar (1% w/v) plate and allowed to set, before incubation overnight at 37˚C. Plaques were counted and plaque forming units (p.f.u.) ml^{-1} calculated as an estimate of the number of infective phages present. Three technical replicates were performed per bacteriophage dilution.

2.4.3.2 Spot assay

Where a more high-throughput method was required, a modification of the traditional plaque assay was used. PAO1 was mixed with top agar and overlaid onto LB agar. 20 µl spots of phage dilutions were pipetted onto the surface and allowed to dry, before overnight incubation.

2.4.3.3 Quantifying levels of each phage separately

The methods described in section 2.4.3.1 allow an accurate estimate of the total number of infective virions present in a sample, but cannot distinguish between bacteriophages. To obtain counts and differentiate between the different phages, two approaches were taken, quantitative real-time PCR (qPCR), and direct PCR of plaques. qPCR gives relative, rather than absolute numbers of phages present and does not indicate phage viability. Direct PCR of plaques was used when phage copy number was too low to be accurately estimated by qPCR. This method allows direct quantification of the number of infective phages of each type present in a sample but is time consuming and labour intensive.

2.4.3.3.1 Quantitative real-time PCR (qPCR) method

2.4.3.3.1.1 DNase I treatment of phage supernatants

Phage were separated from bacteria and DNase treated to remove contaminating bacterial/ prophage DNA. 17 µl phage supernatant was mixed with 2 µl incubation

buffer and 1 µl DNAse I recombinant (Roche) and incubated at 37 °C for 10 minutes. The reaction was stopped by the addition of EDTA to a final concentration of 8 mM, and heating to 75 °C for 10 minutes. A PCR assay was carried out using primers targeted to the bacterial outer membrane lipoprotein gene (*oprI)*; a negative result confirmed total degradation of bacterial DNA. The phage supernatant was then heated to 100˚C for 5 minutes in order to burst the phage capsids and release the phage DNA.

2.4.3.3.1.2 qPCR assays

Copy numbers of each phage were estimated by comparison to a standard curve. Standards were produced by PCR amplification of bacteriophage supernatants (2.3.2). The amplified DNA was separated by gel electrophoresis and the correct sized DNA fragment extracted from the gel and purified (2.3.4). DNA concentration was estimated (2.3.5) and copy number calculated using the following formula:

$$number\ of\ copies = \frac{(amount\ DNA\ (ng) * 6.022 * 10^{23})}{(amplicon\ length\ (bp) * 6.6 * 10^{11})}$$

A 10-fold dilution series of each standard was prepared for each primer pair, ranging from a concentration of 10^2 – 10^7 copies μl^{-1}. The set-up of the qPCR reaction was automated with the Qiagility liquid handling robot (Qiagen). Each reaction contained: 1X SYBR green mastermix (Qiagen), 1 µM each primer, 1 µl DNase-treated phage supernant (or standard) and DEPC-treated water, to a final volume of 25 µl. Reactions took place in 0.1 ml strip-tubes (Qiagen), which were placed in a Rotor-Gene 6000 (Corbett). Cycling conditions are detailed in Table 2.2. Data were analysed with the accompanying software. Negative controls and standards were included in each run, with 2 replicates per concentration. Three technical replicates per sample were performed each run and averaged, and each sample was tested on 3 separate runs.

2.4.3.3.2 PCR amplification from plaques

A plaque assay was performed to obtain numerous, well separated plaques. To identify the phage responsible for each plaque, crude DNA was prepared from the plaque using a modification of the bacterial colony boil preparation; a pipette tip was

placed directly into the centre of the plaque and the material transferred into SDW, prior to boiling. The resulting template was used in a standard PCR reaction with primers targeted to the phage genomes (Table 2.2). The p.f.u. ml^{-1} per phage was calculated as the total p.f.u.ml^{-1}, multiplied by the proportion of plaques caused by the phage. 76 plaques were tested for each biological replicate, with a total of three biological replicates.

2.4.4 Factors affecting phage production in original bacterial host strain

To test the effect of substances on phage production by bacterial strains, mid-exponential phase cultures were exposed to a sub-inhibitory concentration of the substance for 1 hour (2.4.4.1, 2.4.4.2, 2.4.4.3). Cultures were then diluted 1:10 into fresh media and incubated for a further two hours, to allow bacterial recovery and phage lysis. Phages were separated from bacteria and quantified. Norfloxacin (known to induce the lytic cycle of the phages used in this study) was used as a positive control and added at the MIC for the bacterial strain (2.1.2.1). Four biological replicates were carried out for each strain/ substance combination.

2.4.4.1 Antibiotics

Antibiotics were added singly, or in combination, at the MIC for the bacterial strain.

2.4.4.2 Ethylenediaminetetraacetic acid (EDTA)

EDTA was dissolved in water and autoclaved, and added to a final concentration of 5 mM, both alone, and in combination with norfloxacin.

2.4.4.3 Hydrogen peroxide

Hydrogen peroxide solution (Sigma-Aldrich) was added to a final concentration of 0, 2.5, 5 or 50 mM. To verify that hydrogen peroxide did not affect bacterial viability at the concentrations used, bacterial viable cell counts were calculated after 1 hour incubation with hydrogen peroxide and compared to the control.

2.4.5 Phage one-step growth curves

1 ml mid exponential phase PAO1 was mixed with a 1 ml aliquot of purified phage preparation, at a concentration of 2×10^5 p.f.u. ml^{-1} (an approximate MOI of 2 x 10^-

^{3}), inverted slowly to mix, and incubated at 37 ˚C for 5 minutes to allow phage adsorption. The mixture was centrifuged at 6,000 *g* for 2 minutes and the pellet resuspended in 20 ml LB, pre-warmed to 37 ˚C. The bacterial suspension was incubated at 37 ˚C, with shaking at 180 r.p.m., and every 10 minutes, 200 µl was removed, centrifuged at 6,000 *g* for 2 minutes to pellet the bacteria, and the phage in the supernatant quantified. The unadsorbed phage in the discarded supernant from the first step were quantified, and the frequency of infected bacteria calculated as the MOI multiplied by the proportion of phage adsorbed. The number of phage per infected cell was calculated for every time point by dividing the total number of phages by the number of infected cells. The growth curve for each phage was repeated 4 times, on separate days.

2.4.6 Measurement of the frequency of lysogeny of bacteriophages upon bacterial infection

A single colony of PAO1 was inoculated into 5 ml ASM and incubated for 24 hours and the culture was homogenised. The OD_{600} was adjusted to 1.0 (5 x 10^{8} c.f.u.ml^{-1}) and bacteriophage added to a multiplicity of infection (MOI) of 10. The mixture was incubated on ice for 30 minutes, with regular inversions, to allow phage adsorption. It was then transferred to 37˚C for 20 minutes, with shaking at 65 r.p.m. to allow the cells to make the lysis/ lysogeny decision. The mixture was then transferred to ice for 5 minutes, followed by centrifugation at 4˚C at 12,000 *g* for 2 minutes. The supernatant was discarded and the pellet washed twice with ice cold 10 mM $MgSO_4$. The pellet was resuspended in 1 ml ice-cold LB and the cells serially diluted down to 10^{-5}, on ice. Dilutions were plated onto a *P. aeruginosa* PAO1 soft-agar overlay (2.4.3.2) for infective centres (ICs). An IC is observed as a plaque, and is defined as any cell that has been infected by a phage, regardless of the decision made by the cell. Both lytic and lysogenic infections result in plaques; when a cell becomes a lysogen, the high rate of spontaneous entry into the lytic cycle results in lysis of surrounding cells, hence a plaque is formed. In addition, dilutions were plated onto Columbia agar for colony forming units. Only uninfected cells and lysogens formed colonies; cells that entered the lytic cycle lysed. The colonies observed were then screened for the presence of prophage to determine the number of lysogens; colonies were patched onto a PAO1 soft agar lawn and incubated overnight. Spontaneous

lysis of the surrounding area was indicative of a lysogen (Figure 2.1). The frequency of lysogeny was calculated with the following equation:

$$\textit{Frequency of lysogeny} = (\textit{Number of lysogens/infective centres}) * 100$$

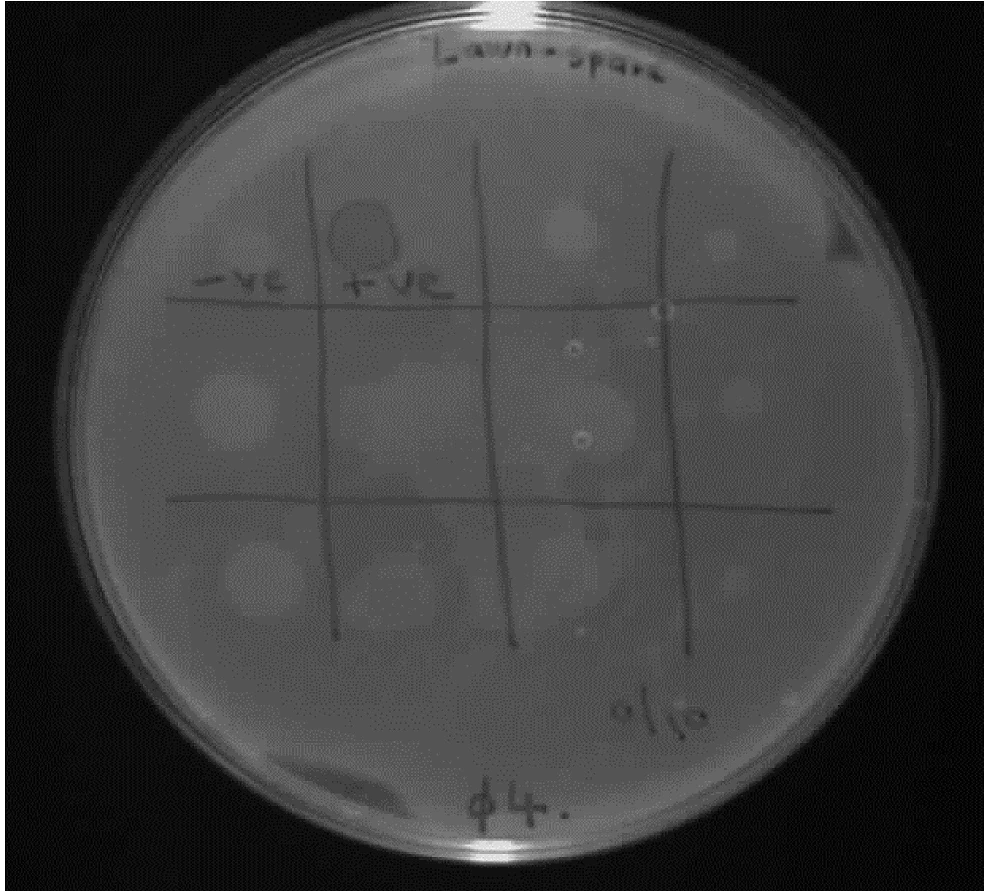


Figure 2.1 Screening of colonies for the presence of prophage. Single colonies were patched onto the PAO1 soft agar lawn (1 per grid-square) and incubated overnight. PAO1 was used as a negative control, and the corresponding PLPL as a positive control. The zone of lysis surrounding the positive control can be easily distinguished.

2.4.7 Phage amplification efficiency in different bacterial growth media

25 ml glass universal tubes containing 5 ml LB or ASM were each inoculated with strain PAO1 and incubated for 24 hours at 37°C with shaking at 180 r.p.m. (LB) or 65 r.p.m. (ASM). Phage were added to the stationary phase cultures to a final titre of 1×10^5 p.f.u. ml^{-1} (M.O.I. = 5×10^{-5}) and cultures incubated for a further 24 hours. Cultures were homogenised and the bacteriophages separated and enumerated. The

fold change in titre (day^{-1}) was calculated for each phage under each condition. Six replicates were performed for each phage in each bacterial growth medium.

2.5 Experimental coevolution of *P. aeruginosa* and bacteriophages in ASM

2.5.1 Experimental design

Replicate populations were set up, with each population defined as a culture of *P. aeruginosa* PAO1 in ASM (2.2.2). Six populations were assigned to each of two main treatments; a control treatment (PAO1 alone) and a LES phage treatment (PAO1, plus the three LES phages (LES φ2, LES φ3 and LES φ4)). Three additional control treatments (each with addition of a single phage) were included in the experimental design, but were not subjected to the same follow-up experiments as the two main treatments (sections 2.5.3 to 2.5.7.5). Bacteria were incubated for 24 hours prior to addition of the phages. Phage were added to a MOI of 0.1 (with equal numbers of each phage), and were added only once, at the beginning of the experiment. The cultures were incubated for a further 3 days and the biofilms homogenised. 50 µl of the homogenate was transferred into 5 ml ASM and the process was repeated, for a total of 30 transfers. Every transfer, 1 ml of homogenate from each population was stored at -80 ˚C in 25 % (v/v) glycerol-LB. The experimental design is summarised in Figure 2.2.

2.5.2 Quantification of bacterial and phage numbers throughout the experiment

At every other transfer, the numbers of viable bacterial cells and total free phages in the biofilm homogenate were quantified. In addition, at every 5th transfer, bacteria were screened for the presence of prophage, using multiplex PCR with three sets of primers (Table 2.2), targeted to each phage. Free phage levels were measured separately for each phage in end-point populations using qPCR (2.4.3.3.1).

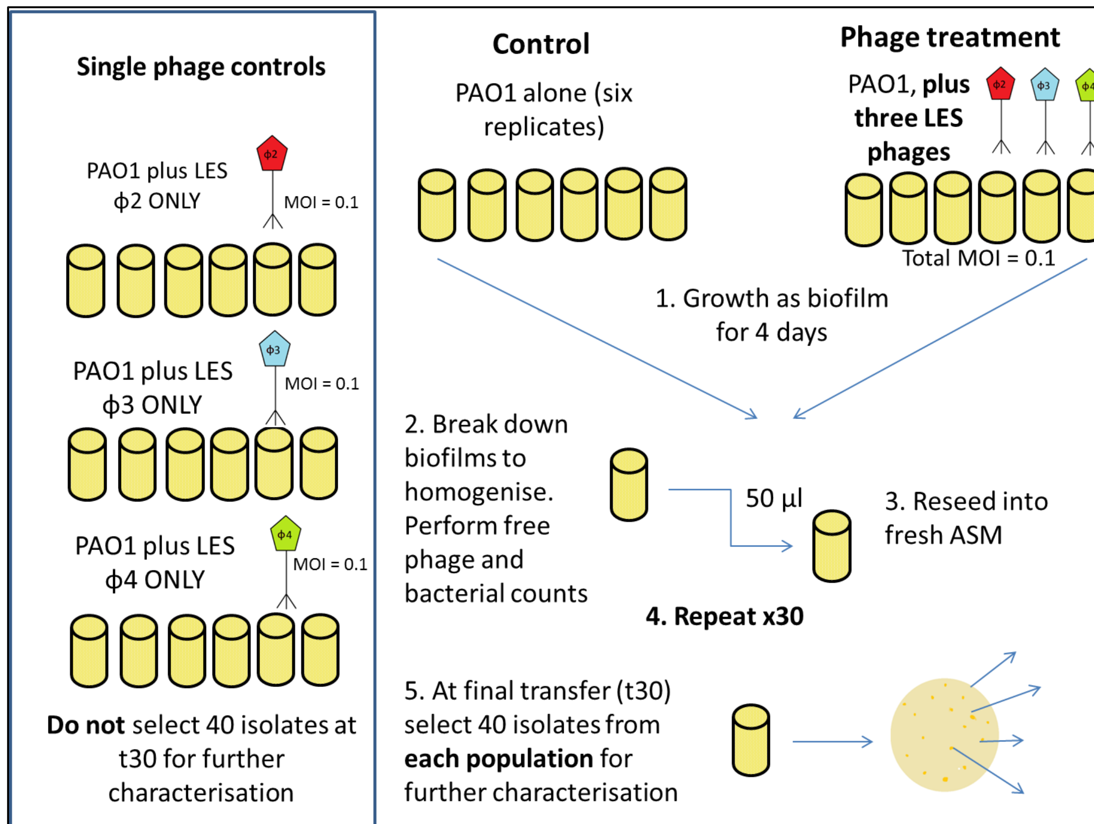


Figure 2.2 Bacteria-phage coevolution in ASM: experimental design

2.5.3 Endpoint assessment of colony morphology diversity

After 30 transfers, cultures were spread-plated to obtain >300 well-separated colonies. Colony morphology was independently classified by two researchers into one of five morphologies: Smooth and straw-coloured, small (<0.5mm) and straw-coloured, raised centre, fuzzy spreader or wrinkly spreader. Simpson's dominance index, *D* (the complement of Simpson's diversity index) was calculated for each population as a measure of the morphological diversity within a population (Hill *et al.*, 2003) using the following formula:

$$D = \frac{1 - (\sum n(n-1))}{N(N-1)}$$

where n = number of colonies displaying a particular morphology, and N = the total number of colonies (all morphologies).

2.5.4 Endpoint isolation of individual colonies

Forty colonies were selected randomly from each population, by dividing plates into portions with a grid, and picking all colonies within each square, moving in a predetermined direction across the plate, until the quota was reached. Isolates were streaked onto *Pseudomonas* isolation agar (Appendix A) to confirm that they were *Pseudomonas* spp. and stored in sterile 96-well plates, in 25% (v\v) glycerol-LB. Each isolate was characterised with multiple phenotypic tests (2.5.5).

2.5.5 Phenotypic characterisation of endpoint isolates

The ancestral PAO1 strain was used as a negative control for all phenotypic tests. All phenotypic tests were performed in duplicate.

2.5.5.1 Auxotrophy

A single colony was patched onto M9 minimal media (Appendix A) and incubated at 37 °C. Isolates displaying no growth after 18 hours were classed as auxotrophic.

2.5.5.2 Biofilm formation

Overnight cultures were diluted 1:100, and 150 µl added to each well (*n*=8 per isolate) of a clear-bottomed 96 well plate. Plates were incubated statically for 24 hours at 37 ˚C. Liquid was removed and the wells washed gently with PBS (3 times) to remove all planktonic bacteria. Plates were dried at 60 ˚C for 90 minutes to fix the biofilms, and biofilm mass stained by addition of 200 µl of 0.25 % crystal violet (w/v) for 10 minutes at room temperature. Crystal violet was removed by gentle washing with tap water, and the crystal violet stain resolubilised in 125 µl 95% ethanol. The absorbance of each well was measured at a wavelength of 590 nm. The mean A_{590} was calculated for each isolate on the plate, and normalised to the ancestor by dividing by the A_{590} value for PAO1 on that plate. Two independent biological replicates were performed for each isolate. Only the first 10 isolates were tested per population, due to time constraints.

2.5.5.3 Hypermutability

10-fold serially diluted overnight cultures grown in LB were spotted onto LB agar plates containing 300 µg ml^{-1} rifampicin. Plates were incubated overnight at 37 °C.

Isolates were classed as hypermutable if growth on rifampicin was comparable to PAOΔ*mutS*, a known hypermutator.

2.5.5.4 Motility

2.5.5.4.1 Swarming

A small portion of a colony was lightly touched onto the surface of a swarming plate (Appendix A). Plates were incubated for 16 hours at 37 ˚C and the diameter of the swarm measured at the widest point. An isolate with a diameter <6 mm was considered swarming deficient.

2.5.5.4.2 Swimming

A small portion of a colony was lightly touched to the surface of a swimming plate (Appendix A). Plates were wrapped loosely in clingfilm to prevent dehydration, and incubated without inversion for 14 hours. The visible diameter of bacterial growth was measured at the widest point. An isolate with a diameter <20 mm was considered swimming deficient.

2.5.5.4.3 Twitching

A colony was stabbed through to the bottom of a LB agar plate and incubated for 24 hours. The agar was removed with forceps and 10 ml 0.25 % (w/v) crystal violet added to the plate for 30 minutes, to stain the area of bacterial growth. The crystal violet was removed and plates rinsed with water. The diameter of bacterial growth was measured at the widest point. An isolate with a diameter <10 mm was considered twitching motility impaired.

2.5.5.5 Phage resistance

To test bacterial resistance to the phages used in this study, a modified version of the phage spot assay was used. 20 µl of 10-fold diluted overnight culture was mixed with 200 µl agar overlay and poured into a well containing 1 ml bottom agar, in a 25 well square petri dish (Fisher Scientific). High titre ($>1x10^{10}$ pf.u. ml^{-1}) preparations of each phage were spotted on top. Isolates were classed as resistant to a phage if there was no evidence of bacterial lysis after overnight incubation at 37 ˚C.

2.5.5.6 Prophage complement

Isolates were tested for the presence of the three prophages with a multiplex PCR assay (2.3.2, primers described in Table 2.2), using crude DNA templates (2.3.1).

2.5.5.7 Growth in LB

Overnight cultures were diluted 1:100 and 200 µl added to wells (n=4 per isolate) of a clear-bottomed 96 well plate. The plate was held at 37 ˚C, with shaking at 100 r.p.m. in an Omega fluostar plate reader. The OD_{600} was measured every 5 minutes and the doubling time in the exponential phase of growth calculated for each well. Two biological replicates were performed per isolate. Only the first 10 isolates were tested per population, due to time constraints.

2.5.5.8 Antibiotic susceptibility

Antibiotic susceptibility was determined using the disc diffusion assay (2.1.2.3) to six anti-pseudomonals, chosen because they are core components of the CF treatment regime. They were: piperacillin/ tazobactam, ceftazidime, meropenem, ciprofloxacin, tobramycin and colistin.

2.5.6 Detection of clinically antibiotic resistant isolates at a low abundance in whole populations

To investigate the possibility of the existence of clinically antibiotic resistant isolates at a low abundance in a population, a modified version of the agar dilution method for testing MICs was developed. Clinical antibiotic resistance is defined as the ability to grow in the presence of antibiotics that are in clinical use, at concentrations higher than the breakpoint levels of clinical resistance, which are updated regularly (EUCAST, 2013). Whole population cultures were adjusted to the turbidity equivalence of a McFarland standard 0.5 (Beckton Dickinson), and plated onto MHA to obtain c.f.u.ml^{-1}. 100 µl of undiluted culture was plated directly onto MHA plates containing antibiotics at the breakpoint levels for clinical resistance (EUCAST, 2013). After overnight growth at 37 °C, colonies growing on antibiotic-containing media were calculated as a proportion of total population counts. PAO1 grown in ASM was included as an additional negative control, to control for any effects caused by growth in a biofilm.

2.5.6.1 Beta-lactamase assay of beta-lactam resistant isolates

Isolates were passaged twice through antibiotic free media to ensure basal (as opposed to induced) beta-lactamase levels were being studied. Cultures were grown to an OD_{600} 0.3, and nitrocefin (Merck-Millipore) was added to a final concentration of 51.6 µg ml^{-1}. As the λ_{max} of nitrocefin changes from 390 nm (in the absence of beta-lactamase) to 486 nm upon hydrolysis, the absorbance at both wavelengths was measured every 5 minutes, for 35 minutes. The $A_{486/390}$ ratio was calculated for every time point as an indicator of beta-lactamase production. Five biological replicates were performed for each isolate.

2.5.7 Genotypic characterisation of endpoint populations

2.5.7.1 DNA extraction and pooling

DNA was extracted from all 40 isolates within each population, normalised and pooled, and the pooled DNA from each population was sequenced, allowing an estimation of the frequency of variants in the population. DNA was extracted from overnight cultures using the Wizard genomic DNA extraction kit (Promega) and eluted in Qiagen elution buffer. DNA for each isolate was quantified in triplicate (2.3.5) and normalised to the lowest concentration in the pool, resulting in equal amounts of DNA for isolates across the population.

2.5.7.2 DNA sequencing

Library preparation (500 bp paired-end) was done by the Liverpool Centre for Genomic Research (CGR). Samples were sequenced on a HiSeq 2000 (Illumina), with multiplexing of 3 samples per flow cell lane.

2.5.7.3 Bioinformatics analysis

Bioinformatics analysis was performed by Dr. Sam Haldenby, CGR. Briefly, single-nucleotide variants (SNVs) were called by mapping to the reference strain PAO1-UW. Pre-existing variants in our PAO1 strain were filtered out after resequencing of this strain, as PAO1 is known to demonstrate substantial diversity between laboratories (Klockgether *et al.*, 2010). Prophage positions in the PAO1 genome were determined by examination of contig ends.

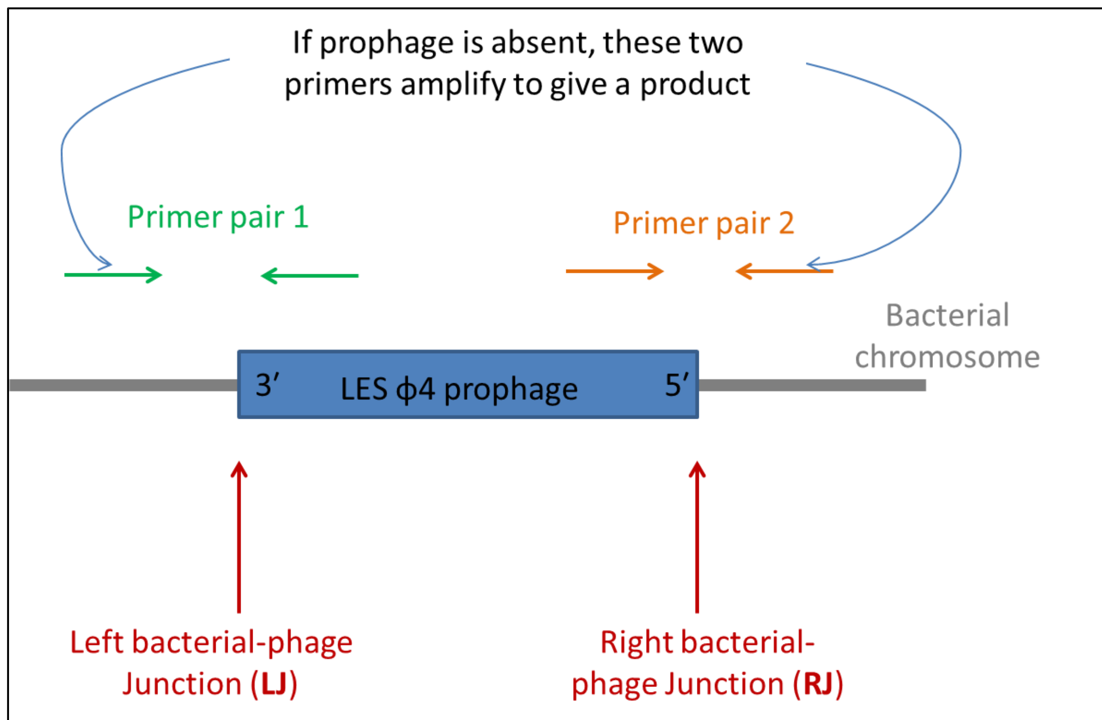
2.5.7.4 PCR confirmation of single nucleotide polymorphisms (SNPs) in the *lasR* gene

An 854 bp region, encompassing the entire *lasR* gene (719 bp) and a small region of the adjacent *rsaL* gene was amplified using standard PCR, using the LasRF and LasRR primer pair (Table 2.2). Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and Sanger sequenced (Source Bioscience) using the reverse primer. Sequence quality was assessed by inspection of the sequence chromatogram using Finch TV (Geospiza). Good quality sequence was similarity searched using the nucleotide basic local alignment search tool (BLASTn), against *P. aeruginosa* PAO1 sequences in the non-redundant nucleotide database. All other parameters were set to the default. Alignments were inspected to identify variants.

2.5.7.5 PCR confirmation of phage integration sites

PCR amplification was conducted to confirm phage integration sites, and quantify the exact frequency at which a specific integration site occurred in a population. Dual PCR amplification was used to amplify both the left and right phage-bacterial chromosomal junctions, and the principle is summarised in Figure 2.3. Primer pair 1 targeted the left bacterial-phage junction, with the forward primer designed to bind to the bacterial gene and the reverse primer to the 3′ end of the phage. Primer pair 2 was designed to target the right junction, with the forward primer designed to bind to the 5′ end of the phage and the reverse primer to the bacterial gene. Positive amplification yielding two products confirmed occupation of the locus by LESφ4. Positive amplification between the forward and reverse primer of primer pair 1 and 2, respectively, yielding a single product, indicated an intact integration site. If an isolate was PCR positive for prophage, but had an intact integration site, the phage was assumed to have integrated elsewhere in the PAO1 genome. Primers were designed to span the *fimU* integration locus in population 7, and the *pilV* integration locus in population 11. All 40 endpoint isolates were PCR screened to obtain each integration frequencies, in addition to 20 isolates per time point, per population, at transfers 1, 5 and 15.

Figure 2.3 Diagram to illustrate the concept of PCR confirmation of LES φ4 integration sites in the PAO1 genome.



2.5.7.6 Inverse PCR to determine LES φ4 phage integration site

Inverse PCR (IPCR) was used to determine LESφ4 integration sites in the single-phage control treatment (as sequence data was not available), allowing a comparison of phage integration sites in the presence and absence of other LES phages. The principle of IPCR is summarised in Figure 2.4

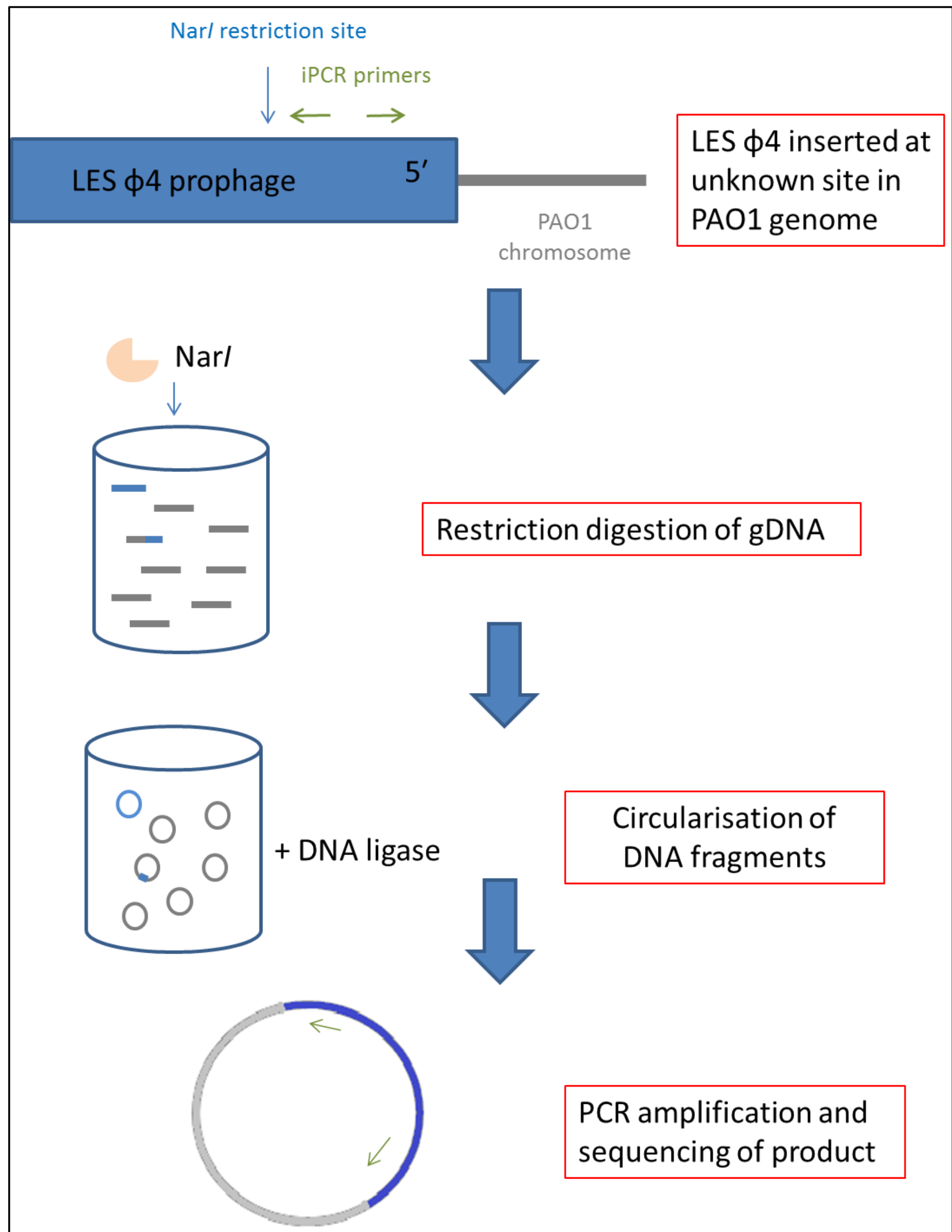


Figure 2.4 Diagram to illustrate the concept of inverse PCR to determine LESφ4 integration sites in the PAO1 genome.

2.5.7.6.1 Restriction endonuclease digestion

Genomic DNA was extracted (2.5.7.1) and digested with *Nar*I restriction enzyme (New England Biolabs), selected as it was known to cut 775 bp into the 5' end of LESφ4, and also at numerous points throughout the PAO1 genome. Digestion was performed according to manufacturer's recommendations. The enzyme was inactivated by heating to 65 ˚C for 20 minutes, and the digested DNA purified using the QIAquick PCR purification kit (Qiagen), and quantified.

2.5.7.6.2 Recircularisation of DNA fragments

Ligation is favoured at low DNA concentrations and large reaction volumes. To this end, DNA was diluted to <1 ng μl^{-1} and 45 µl diluted DNA mixed with 5 µl 10X ligase buffer and 3 µl T4 DNA ligase (Stratagene). The mix was incubated at 18 °C overnight, then heated to 100 °C for 3 minutes, to introduce nicks in the circularised DNA. The DNA was purified again, as before, and eluted in a small (30 µl) volume to ensure the DNA was as concentrated as possible.

2.5.7.6.3 PCR amplification of DNA

Primers were designed using the free software FastPCR, which has a tool to allow design of primers for IPCR. Primers were designed to face outwards (as opposed to conventional primers which face inwards) at the 5' end of the phage, in the region between the end of the phage and the first restriction enzyme cut site. Each PCR reaction contained: 500 nM each primer, 10 µM each dNTP (Bioline), 10 µl Q5 reaction buffer, 10 µl high GC enhancer, 1 *U* Q5 high-fidelity DNA polymerase (all New England Biolabs), 5 µl ligated DNA and DEPC treated water, to a final volume of 50 µl. Q5 DNA polymerase was selected due to its low error rate and its improved ability to amplify long and difficult sequences, relative to other polymerases. Primers and cycling conditions are described in (Table 2.2).

2.5.7.6.4 Sanger sequencing of DNA amplicons to determine phage integration site

The entire PCR reaction was subjected to agarose gel electrophoresis. The most visible band was selected and the DNA extracted (2.3.4). The amplicon was Sanger sequenced (Source Bioscience) using the reverse primer. BLASTn analysis revealed a small portion of sequence with high similarity to the end of the phage (*c* repressor gene), and the remainder of the sequence matched to the phage integration locus in the PAO1 genome.

2.6 Direct competition of evolved populations with ancestor in ASM

Endpoint evolved populations were competed against antibiotic labelled PAO1 strains, so that the competitors could be easily separated. Whole-populations were competed, rather than individual isolates, as it is known that in genetically diverse

populations, the fitness of individual isolates are not representative of population level-fitness (Wong *et al.*, 2012). Populations were competed against their "ancestor", PAO1::Gm^R, and to control for the competitive effect of phages, populations were also competed against a strain that does not produce the receptor required for phage infection, PAO1 *pilA*$^-$::Tet^R. To confirm the absence of any fitness costs of the antibiotic gene cassette, wild-type PAO1 was competed against both antibiotic marked strains in LB.

Prior to initiation of the competition, both competitors were acclimatised to the competition medium (ASM). Biofilm homogenate from endpoint populations, and stocks of the ancestral competitor strains, stored at -80 ˚C, were defrosted. Approximately 5 x 10^5 bacterial cells were inoculated into 1 ml of ASM in a sterile 24 well tissue culture plate and incubated for 24 hours. Cultures were homogenised with Sputasol and diluted 5-fold. The OD_{600} for each competitor was measured and adjusted to 0.4. Competitions were initiated with a 50:50 starting ratio of each competitor; 50 µl of each competitor was inoculated into 5 ml ASM and incubated under conditions identical to 1 cycle of the coevolution experiment (2.5.1). The exact input cell density of each competitor was quantified by plating onto Columbia agar. After 4 days, biofilms were homogenized and diluted cultures plated onto both non-selective media (LB agar) and the appropriate antibiotic media (LB agar containing 10 µg ml^{-1} gentamicin or 50 µg ml^{-1} tetracycline), in order to quantify the density of each competitor. The density of the evolved PAO1 population was calculated by subtracting the density on antibiotic media from the density on non-selective media. Five independent biological replicates were performed for each competition.

2.6.1 Estimation of bacterial fitness and calculation of the selection rate constant

The Malthusian parameter was calculated for each competitor, and describes the average rate of increase per day. The following equation was used:

$$m_i = (\ln(\text{final cell density}/\text{initial cell density}))/\ \text{number of days}$$

and has units of day^{-1}. The selection coefficient (S_{ij}) is routinely used to compare the relative fitness of one strain to another, when in competition, by comparing the ratio

of the competitors' Malthusian parameters. However, the selection coefficient relies on an increase in competitor density over the course of the competition. Due to the natural bacterial growth curve in ASM, cell densities were lower at the end of the competition than at the beginning, hence the selection coefficient could not be calculated. An alternative method is to calculate the selection rate constant (r_{ij}), which measures the subtractive difference of the Malthusian parameters of the two competitors:

$$r_{ij} = m_i - m_j$$

where *i* is the evolved competitor population and *j* is the ancestral PAO1 competitor, with units of day^{-1}.

2.7 Experimental coevolution of *P. aeruginosa* and LES bacteriophages in a rat model of chronic lung infection

The rat model of chronic lung infection is a well established model for *P. aeruginosa* infection (Kukavica-Ibrulj *et al.*, 2008b; Van Heeckeren & Schluchter, 2002), and involves infection (by intubation) with agar beads that contain the strain(s) of interest. This experiment was done in collaboration with Irena Kukavica-Ibrulj, in the lab of Professor Roger Levesque, at Laval University, Québec. The experimental design consisted of two groups of rats: a control group, which were infected with PAO1 only, and a phage-treated group, which were infected with PAO1, plus LES phages φ2, φ3 and φ4.

2.7.1 Agar bead preparation

PAO1::Sm^R was grown to an OD_{600} of 1.0 in tryptone-soy broth (TSB, Difco). 2 x 10^{10} cells were centrifuged at 7,200 r.p.m. for 3 minutes, and washed 3 times in sterile PBS. Cells were resuspended in 1 ml PBS (controls), or in 1ml PBS containing LES φ2, φ3 and φ4, each at a concentration of 6.7 x 10^8 (phage treatment). The phage suspension in PBS was prepared according to (2.4.2), and gave an approximate total MOI of 0.1. The bead-making protocol results in some loss of both bacterial and phage titre (approximately 3 logs), hence it was necessary to determine the actual MOI once the beads had been made. However, we

determined that the loss of bacteria and phage throughout the process was proportional.

The bacterial suspension was added to 10 ml molten 2% (w/v) agar-PBS, preheated to 48 ˚C, and vortexed vigorously. The mixture was poured slowly into the centre of an Erlenmeyer flask containing 200 ml sterile mineral oil (Sigma) held at 48 ˚C, with constant magnetic stirring. Ice was added to the sides of the flask to cool, and the stirring continued for 5 minutes. The stirring was ceased and the flask left at room temperature for 10 minutes to allow the beads to settle at the bottom of the flask.

The mineral oil was siphoned off using a vacuum pump, and the beads washed with an equal volume of PBS, followed by centrifugation at 10,000 r.p.m at 4 ˚C for 20 minutes. Residual mineral oil was removed, and this wash-step repeated a further two times to remove all the mineral oil. All but 50 ml of the PBS was removed, and the remaining PBS-bead suspension transferred into a sterile falcon tube and allowed to settle for 10 minutes. The majority of the PBS was removed, to leave approximately 7.5 ml of beads, suspended in an equal volume of PBS. The beads were stored at 4˚C prior to infection.

2.7.1.1 Quantification of bacteria in the beads

1 ml of the bead suspension was diluted into 9 ml PBS and homogenised (on ice) for 30 seconds, using a PTA 20S Polytron homogeniser (Kinematica). Bacterial counts in the bead suspension were determined by plating on TSA containing 300 μg ml^{-1} streptomycin.

2.7.1.2 Quantification of bacteriophages in the beads

Bacteriophages were quantified without centrifugation or filtration of the sample, to ensure that all bacteriophages were detected, even those that may have adsorbed to bacterial cells/ agar. The actual MOI was calculated by calculating the ratio of phage to bacterial counts in the beads.

2.7.2 Rat infection

Ethical approval for animal experiments was obtained from the Animal Care Committee, Laval University. Male Sprague-Dawley rats, weighing between 300-

350 g, were housed in individual cages, with food and water *ad libitum*. Rats were weighed, and anaesthetised with 2% (v/v) isofluorane-oxygen, followed by an intra-peritoneal ketamine injection to maintain anaesthesia. Rats were intubated using an 18-gauge venous catheter, and a 1 ml tuberculin syringe used to deliver between 2 x 10^7 and 4 x 10^7c.f.u.s into the lungs (in a volume of between 100 and 120 μl). Rats were sacrificed after 10 days, or earlier if showing signs of morbidity, with 0.2 ml Euthanyl (Bimeda-MTC), administered by intra-peritoneal injection. Lungs were removed and suspended in 10 ml sterile PBS, on ice. Lungs were homogenised as described for the beads (2.7.1.1), and the c.f.u.s in the lungs determined by plating onto TSA containing 300 μg ml^{-1} streptomycin. Total free infective phages in the lung homogenate were quantified using the methods described in 2.4.1 and 2.4.3.2. The phage to bacterium ratio was calculated as the total number of p.f.u.s in the lungs, divided by the total number of c.f.u.s.

2.7.3 Characterisation of multiple endpoint isolates

40 bacterial isolates were selected from each rat (as for the coevolution experiment in ASM (2.5.4)) and characterised for the presence of prophage (2.5.2) and twitching motility phenotype (2.5.5.4.3).

2.8 *In vitro* bacterial invasion assays

2.8.1 Experimental design

PAO1 LES Phage Lysogens (PLPLs) are isogenic to PAO1 but contain bacteriophage(s) integrated into their genome i.e. they are lysogens (Table 2.1). The ability of bacteria harbouring prophages to both invade, and resist invasion to a population of phage susceptible bacteria was tested using fluorescently labelled PLPLs and non-lysogenic PAO1 (termed PAO1$^{φ-}$). Each strain was labelled separately with both a green fluorescent protein (GFP) (Chalfie, 1995) and red fluorescent protein (DsRed-Express) marker (Lambertsen *et al.*, 2004), to allow the effect of marker to be controlled. We tested three single prophage PLPLs (LESφ2, LESφ3 and LESφ4), and the PLPL harbouring all three prophages (PLPLφtriple), against PAO1$^{φ-}$. Bacteria were competed from initial starting ratios of 1:9 (PLPL to PAO1$^{φ-}$, and vice versa), and bacterial densities of each competitor were calculated

every 8 hours, over a period of 24 hours. At the end of the experiment, bacteria were plated and colonies were PCR screened for the presence of prophage.

2.8.2 Growth in fluorometer and estimation of bacterial densities

Both competitors were grown to mid-exponential phase (OD_{600} 0.5) in LB. Cultures were centrifuged at 12,000 *g* for 2 minutes, the supernatant discarded and the pellet resuspended in 10 times the initial volume of prewarmed M9-CaGlu (Appendix A), selected for its lack of autofluorescence, relative to other media. Competitions were carried out in black polystyrene, clear-bottomed, 96-well microtitre plate (Corning), to minimise cross-talk between wells and background fluorescence. $PAO1^{\varphi-}$-GFP and PLPL-DsRed-Express were added to a well in the correct ratio, to a final volume of 200 µl, and the mixture pipetted up and down to mix well. This repeated in another well for the reverse markers, with 6 technical replicates for each mixture. The plate was incubated at 37˚C with shaking at 200 r.p.m. in a Fluostar Omega plate reader (BMG Labtech). Fluorescence intensity (FI) of both GFP and DsRed-Express in each well was measured every 8 hours using 485 nm and 544 nm excitation filters and 520 nm and 590 nm emission filters, for GFP and DsRed-Express, respectively. Readings were normalised to an unlabelled PAO1 negative control to control for bacterial autofluorescence. To allow non-destructive sampling of bacterial densities over the 24 hour period, densities were estimated by comparing FI of each well to a standard curve of FI against bacterial density. To produce the standard curve, PAO1 GFP and PAO1 RFP were mixed together, with different ratios in each well (i.e. 10 µl GFP/ 190 µl DsRed-Express, 20 µl GFP/ 180 µl DsRedExpress etc.). Standards were included on every plate. At each time point, the FI for each set of filters was plotted against the bacterial cell densities (predetermined for each time point by plating for c.f.u.s), and the line of best fit was calculated using Sigma plot.

Three independent biological replicates were performed for each competition. The Malthusian parameters of each competitor and the selection rate constant (2.6.1) were calculated separately for each marker in each competition, at t_{24} only.

2.8.3 Estimation of prophage fitness (i.e. frequency) in bacterial population

At the end of the competition (t_{24}), mixture were plated onto Columbia agar and incubated overnight at 37 ˚C. Single colonies were patched onto fresh Columbia agar

and PCR tested for prophage complement following overnight incubation. 23 colonies were tested per competition.

2.8.4 *In vivo* bacterial invasion assays

Bacterial invasion assays were conducted *in vivo* using the rat model of chronic infection, as described previously (2.7). Using a similar experimental design to the *in vitro* invasion assays, PLPLφtriple was competed with PAO1$^{φ-}$, but the competitors were labelled with antibiotic resistance instead of fluorescence markers. Two groups of rats were used, with a competitive ratio of 1:9 (lysogen to non-lysogen) in one group, and a ratio of 1:1 in the second. The protocol followed was as described in (2.5), except for the differences outlined below:

2.8.5 Agar bead preparation

PAO1$^{φ-}$Sm^R and PAO1φtripleGm^R were each grown to an OD_{600} of 1.0. For the first group (1:9 competitive ratio), 2 x 10^9 cells PAO1φtriple Gm^R and 1.8 x 10^{10} cells PAO1$^{φ-}$ Sm^R were centrifuged and washed separately, and the cell suspensions pooled together and mixed thoroughly, just prior to the addition to the agar-PBS. This was repeated for the second group (1:1 competitive ratio), but using 1 x 10^{10} cells of each competitor.

2.8.6 Determination of bacterial cell counts

To determine the counts of each competitor, in both the beads and in the lungs, dilutions were plated on TSA containing 300 µg ml^{-1} streptomycin or 10 µg ml^{-1} gentamicin, in addition to TSA containing no antibiotic. Selection rate constants were calculated as described in section 2.6.1, using cell densities in the bead inoculum and lungs as start and end densities, respectively.

2.9 Statistical analysis

2.9.1 Basic statistical tests

All statistical analyses were performed in R (R, 2013), unless otherwise stated. Parametric tests were used where possible; data were checked for homogeneity of variance by examination of a box-plot, and for normality by examination of a quantile-quantile (QQ) plot. Non-normal data were transformed (using a $\log_{10}$ or

arcsine transformation) and re-checked for normality. Data that still violated the assumptions of the parametric test were analysed using the non-parametric equivalent.

2.9.2 Principal component analysis (PCA)

Phenotypic data from the host-phage coevolution experiment resulted in large, multivariate datasets. To explore the underlying structure of the data and elucidate the factors that are responsible for much of the variance, PCA was used as an exploratory data analysis tool, using the ade4 package (Dray, 2007) in R. Complete datasets (i.e. data for all 40 isolates within each population) were available for all but two of the variables (biofilm formation and doubling time in LB). For the incomplete variables, only the first 10 isolates of every population were tested, due to the large amount of time and work required to test these specific phenotypes. PCA in R cannot proceed with missing values, but numerous methods exist to allow missing values to be ignored or replaced. Multiple imputation was chosen as a method to replace the missing data, using the mi package (Su *et al.*, 2011) in R, as it enables existing data to be retained. Data were plotted with dudi PCA scatter plots, and the independent variables (phage-treatment and population) overlaid, using the `s.class` function.

2.9.3 Analysis of molecular variance

Analysis of molecular variance (AMOVA) involves partitioning the diversity between various genetic haplotypes (where a haplotype is defined as a collection of specific alleles) into within and between population components. The diversity is defined as the average distance between two randomly chosen haplotypes. Whilst such an approach has traditionally been applied to genetic data, it is possible to apply this statistical method to phenotypic data (Mowat *et al.*, 2011), by defining haplotypes as a collection of specific phenotypic traits. This was applied to the dataset of phenotypic traits produced from the host-phage coevolution experiment. Isolates were classified as different haplotypes based on the combination of phenotypic traits displayed. For each trait, isolates were scored a 0 if they displayed the same phenotype as the ancestor, or a 1 if they displayed a mutant phenotype. A haplotype in this instance was defined as a unique combination of phenotypic traits, and the frequency of each haplotype in each population was determined. An

Euclidean distance matrix was created to determine the evolutionary distance between the various haplotypes. AMOVA was performed using the ade4 package in R (Dray, 2007). The distance matrix was partitioned into sub-matrices for the various subdivisions of the data, including treatment (control or phage-treated), population and individual isolates. The sums of squares were computed and analysed in a nested ANOVA framework, termed AMOVA. This yielded φ statistics (analogous to the F statistic of ANOVA). Significance was tested by Monte-Carlo permutation tests (49 permutations).

2.10 Reannotation of LES phage genomes

2.10.1 Assigning protein functions using PSI-BLAST

Predicted protein sequences within the LESφ2,φ3 and φ4 prophage regions of LESB58 were subjected to protein similarity searches using the Position-Specific-Iterated-BLAST (PSI-BLAST) algorithm, with the aim of improving the functional annotation of the prophage genomes. Predicted open-reading frames (ORFs) were obtained from the supplementary data of the LESB58 published genome paper (Winstanley *et al.*, 2009). Protein sequences were subjected to BLASTP and PSI-BLAST searches against the non-redundant protein sequences database, using the default parameters. Only hits to proteins with an annotated function were considered. If the initial BLASTP search revealed only hits to hypothetical proteins, an iterative search was carried out using the position-specific scoring matrix (PSSM) derived from the initial BLASTP search and subsequent multiple sequence alignment of significant protein hits. Iterations were continued until either detection of multiple sequences with a similar function, or convergence. All BLASTP hits were critically analysed by manual inspection of alignments. As PSI-BLASTING can give false positives, a reverse PSI-BLAST strategy was employed to indicate whether relationships were likely to be meaningful; the hit sequence was subjected to a PSI-BLAST search, and if the phage protein sequence was not returned as a hit, then it was assumed to be a false positive. For hits to more divergent proteins, multiple related sequences in a protein family plus the query protein were aligned with COBALT and Clustalx and visualised in Jalview, to search for conserved domains and residues. The likelihood of homology was assessed by considering numerous factors, including the quality of alignment, PSI-BLAST scores, presence of

conserved domains, and the context of the gene function with regards to its location on the phage genome. Phage genes were grouped into gene modules according to function, and the phage genome visualised using DNA plotter (Carver *et al.*, 2009).

2.10.2 Identification of phage-encoded anti-CRISPR genes

Protein sequences with known anti-CRISPR (clustered regularly interspaced short palindromic repeats) activity were obtained from (Bondy-Denomy *et al.*, 2013; Pawluk *et al.*, 2014). A tblastn search was conducted for each anti-CRISPR protein sequence against the translated (in all 6 reading frames) genome sequence of LESB58. In addition, the nucleotide sequences of LES prophage 2, 3 and 4 were blastn searched against *P. aeruginosa* PA14 genome, to identify putative CRISPR spacers. Exact matches were searched against the CRISPRdb database (Grissa *et al.*, 2007) to determine whether PA14 harbours spacers from previous phage infection that would potentially confer immunity to the LES phages.

Chapter 3 Characterisation of the LES phages

3.1 Introduction

3.1.1 The Liverpool epidemic strain

The Liverpool epidemic strain (LES) was the first transmissible strain of *Pseudomonas aeruginosa* identified, and is associated with a worsened morbidity and mortality, relative to infection with other *P. aeruginosa* strains (Al-Aloul *et al.*, 2004). The LES was discovered after multiple patients in a CF children's centre were found to be infected with a common beta-lactam- resistant strain (Cheng *et al.*, 1996), and challenged the previous notion that all *P. aeruginosa* strains were acquired from the environment and unique to each CF individual. The LES has an unusual phenotype, with premature expression of quorum sensing (QS) -related *rhl* and *las* regulatory genes, and subsequent overproduction of secreted virulence factors related to QS such as pyocyanin and elastase (Fothergill *et al.*, 2007; Salunkhe *et al.*, 2005). LES is a biofilm hyperproducer (Kukavica-Ibrulj *et al.*, 2008b), displays enhanced survival on dry surfaces (Panagea *et al.*, 2005), is capable of superinfection (McCallum *et al.*, 2001), and displays enhanced antimicrobial resistance (Ashish *et al.*, 2012b; Fothergill *et al.*, 2007; Salunkhe *et al.*, 2005). These factors may have contributed to its successful spread; in 2004, LES was identified as the most common clone in CF centres in England and Wales in 2004 (Scott & Pitt, 2004), and a recent UK-wide study of *P. aeruginosa* isolates using variable number tandem repeat (VNTR) analysis found that it is still the most common clone in CF patients (Martin *et al.*, 2013). The LES has even spread as far as North America (Aaron *et al.*, 2010).

3.1.2 LES bacteriophages

Genome-sequencing of LESB58, the first isolate of LES identified, revealed some clues as to how the LES has established itself as such a successful pathogen. The LES genome contains six prophages (named LESφ1-6), of which four are not found in strain PAO1. Table 3.1 describes the main characteristics of the six LES phages. A signature-tagged mutagenesis approach demonstrated that insertions in three of the prophages (LESφ2, LESφ3 and LESφ5) greatly reduced LES competitiveness in a rat model of chronic lung infection (Winstanley *et al.*, 2009). These were in genes

encoding the DNA replication protein DnaC of LESφ2, a hypothetical protein of LESφ3, and a putative lytic enzyme of LESφ5, suggesting that perhaps active phage replication is necessary for bacterial competitiveness.

LES phagesφ2-5 are novel, but display a degree of mosaicism, with regions of similarity to other known phages, and to each other (Figure 3.1). LESφ2 has a large region that is homologous to part of bacteriophage F10, of which a small part is also homologous to a small region of LESφ3. LESφ3 also displays similarity to LESφ5, sharing a 7.5 kb region with 99.8% identity. LESφ4 and LESφ5 are similar to the sequenced phages D3112 (Wang *et al.*, 2004) and D3 (Kropinski, 2000), respectively. All of the active LES phages encode integrases, and therefore integrate by site-specific replication, with the exception of LESφ4, which encodes a transposase, rendering it capable of replication by transposition, and therefore able to integrate at multiple locations throughout a bacterial genome. Although LESφ4 is transposable, like the well characterised *Escherichia coli* phage Mu (Morgan *et al.*, 2002), its genome organisation is more reminiscent of *E. coli* phage λ. LESφ2 and LESφ3 also appear similar to lambdoid phages.

Examination of the phage genomes does not reveal any obvious genes that may be involved in bacterial virulence or competitiveness, but the phage genomes are poorly annotated and the annotations have not been updated since the LES genome was published, despite the fact that a lot of phage functional proteomics data has since become available.

Table 3.1 Prophages identified in *Pseudomonas aeruginosa* strain LESB58. Data from Winstanley *et al*. (2009).

LES prophage	Characteristics	Number of genes	Related phages in reference strain PAO1	Known related phages	Phage morphology
φ1	Defective prophage, predicted to encode pyocin R2	19	Defective prophage gene cluster encoding pyocin R2	Pyocin gene clusters predicted to have evolved from phage tail genes	
φ2	Active prophage, encodes integrase	44	None	F10	*Siphoviridae*
φ3	Active prophage, encodes integrase	53	None	F10 (also homology to LES φ2)	*Siphoviridae*

φ4	Active prophage, encodes transposase	48	None	D3112	*Siphoviridae*
φ5	Active prophage, encodes integrase	65	None	D3	
φ6	Active prophage, encodes integrase	12	Pf4 (filamentous phage implicated in biofilm dispersal)	Filamentous phage Pf1 (family: *Inoviridae*)	

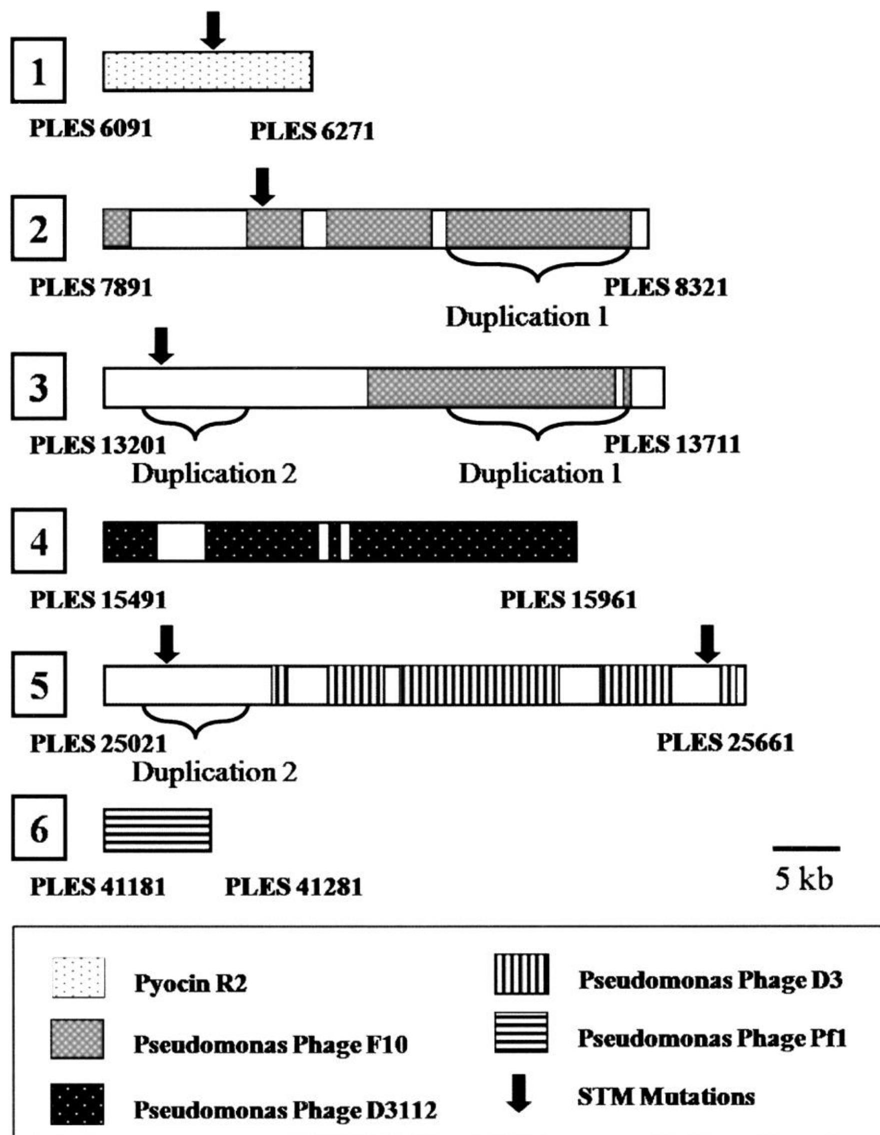


Figure 3.1 LESB58 prophages and regions of genome similarity to other known phages. Duplicated regions between the phages are labelled. Black arrows denote mutations that were found to significantly reduce competitiveness of LESB58 in a model of chronic infection. Figure adapted from (Winstanley *et al.*, 2009).

A recent study characterised three of the LES phages (LESφ2, 3 and 4), and found that they can all successfully infect the laboratory reference strain PAO1 (James *et al.*, 2012). Moreover, the LES phages could integrate into the PAO1 genome and

form stable lysogens, although the level of spontaneous phage lysis was higher than in the native bacterial host LESB58. It was observed that the phages each produce characteristic plaques on PAO1. LESφ2 produces very small, clear plaques, LESφ3 produces large plaques with a turbid centre, and LESφ4 produces small, clear plaques with a turbid halo. All three phages were found to require the type IV pili of the bacterial cell for infection, which is required for twitching motility and surface-attached biofilm formation. Examination of the host range of the phages observed that 6/32 clinical isolates of *P. aeruginosa* were susceptible to LES phage infection. Interestingly, several clinical isolates, as well as the laboratory reference strain PA14, were resistant to LESφ2 and LESφ3, but susceptible to LESφ4, yet the mechanism underlying this difference was not explored.

3.1.3 LES isolates have different prophage complements

Since the LES was discovered, numerous isolates have been identified, all exhibiting differences in phenotype, virulence, and prophage complement. The best characterised are the recently sequenced LES400, LESB65 and LES431 (Jeukens *et al.*, 2014), alongside LESB58, which is the oldest LES isolate, and the first to be genome sequenced. LES400 has retained all six prophages, but is a *lasR* mutant (Parsons *et al.*, 2002), resulting in low virulence in mouse and *Caenorhabditis elegans* models of infection (Carter *et al.*, 2010a). LES431 is missing LESφ2 and demonstrates high virulence in both infection models. Interestingly, LES431 was first isolated from the lungs of a parent of a child with CF, an unusual occurrence (McCallum *et al.*, 2002). LESB65 is missing LESφ5 (Fothergill *et al.*, 2007), and displays moderate virulence in the mouse model, capable of actually establishing stable colonisation in the lungs and nasopharynx (Fothergill *et al.*, 2014). However, the presence (or absence) of the prophages is not necessarily responsible for the phenotypes and virulence levels observed.

Studies that involve collection of large numbers of LES isolates in multiple patients over time, have observed that loss (Mowat *et al.*, 2011) or degradation (Williams *et al.*, unpublished) of one or more of the prophages are common phenomena. A screening of 26 LES isolates found that LESφ5 was missing in 69% of isolates, and that LESφ2 was missing in 3 isolates (Winstanley *et al.*, 2009). A similar pattern was

observed for sequenced LES and LES-like isolates, with loss LESφ5 and LESφ2 in multiple isolates, loss of LESφ5 being the most common (Jeukens *et al.*, 2014).

3.1.4 Phage contribution to bacterial fitness

3.1.4.1 Phage-encoded fitness factors

Prophages often encode genes that are not directly involved in viral replication and that can confer a benefit to their bacterial host. Such genes are often found in regions of DNA termed morons, defined as independent transcriptional units of DNA that are not part of the essential phage genome, and are expressed whilst the phage is in the prophage state (Juhala *et al.*, 2000). One example is genes that encode superinfection exclusion proteins, which block incoming phage DNA, hence enabling avoidance of lytic phage infection and consequent cell death. Such as system has been observed in multiple phage-host systems, including *Salmonella* phage P22 (Hofer *et al.*, 1995), *E. coli* T-even phages (Lu & Henning, 1994), lactococci (Akçelik, 1998) and streptococci (Sun *et al.*, 2006). LES phagesφ2, φ3 and φ4 are known to display a hierarchy of superinfection exclusion, with LESφ2 lysogens resistant to infection by LESφ3 and LESφ4, but not vice versa (James *et al.*, 2012), suggesting that LESφ2 encodes at least one protective superinfection exclusion gene.

Morons can contain genes that enhance the virulence of their bacterial host, either directly e.g. phage-encoded toxins, or indirectly, by enhancing the fitness of bacteria, which may result in increased virulence (Hacker & Carniel, 2001). For example, the *bor* gene of *E. coli* phage λ plays an important role in bacterial immune evasion; it encodes an outer membrane protein that aids cell survival in animal serum (Barondess & Beckwith, 1990; Barondess & Beckwith, 1995). Another example is the *gipA* gene found in the late operon of the Gifsy-1 bacteriophage of *Salmonella enterica* serovar Typhimurium. *gipA* was observed to be massively upregulated in the small intestine in a mouse model of infection, and was identified as important for survival and/or growth in the Peyer's patches (Stanley *et al.*, 2000). Both of these phage-encoded fitness factors were identified as probable morons based on their expression whilst in the prophage state, and the presence of terminator stem loops (Hendrix *et al.*, 2000).

Prophages frequently encode multiple morons; phage 933W, isolated from the pathogenic *E. coli* strain O157:H7, harbours the aforementioned *bor* gene, as well as the *stk* gene, which encodes a serine/ threonine kinase that is predicted to affect the signal transduction pathways of eukaryotic cells (Plunkett *et al.*, 1999). It also posseses a *lom* homologue, a gene that encodes an outer membrane protein necessary for adhesion to human epithelial cells (Vaca Pacheco *et al.*, 1997).

3.1.4.2 Phage virulence factors expressed upon prophage induction

Some prophage-encoded virulence factors are only expressed/released when the prophage enters the lytic cycle, whereas others are increased, through an increase in phage replication and/ or increased release in virulence proteins through cell lysis. Shiga-toxin production in *E. coli* Stx phage lysogens is massively upregulated upon phage induction (Wagner *et al.*, 2001b), and substances that cause prophage induction, such as quinolone antibiotics, are associated with increased toxin production and disease in the mammalian host (Zhang *et al.*, 2000). A recent experiment with an *E. coli* Stx phage mutant incapable of induction and cell lysis observed that toxin production whilst in the prophage state was very low; phage induction and lysis was necessary for virulence in a mouse model of infection (Tyler *et al.*, 2013).

The prophage-encoded PblA and PblB platelet binding proteins of *Streptococcus mitis* strain SF100 play an important role in the pathogenesis of this strain, which causes endocarditis (Bensing *et al.*, 2001). A study found that deletion of prophage genes necessary for cell lysis (holin and lysin genes) resulted in reduced platelet binding. It was observed that prophage-mediated cell lysis was necessary for release of the proteins from the cell cytoplasm, allowing them to bind to the outer membrane of other bacteria (Mitchell *et al.*, 2007). Furthermore, phage lysin interacts with fibrinogen, further enhancing platelet binding (Seo *et al.*, 2010).

3.1.5 Phage classification and genomics

In the absence of a universal gene in bacteriophages, and the pervasive genetic mosaicism that is observed between phages, bacteriophage classification is difficult (Lawrence *et al.*, 2002), and is currently based on nucleic acid type, and morphology (Di Serio *et al.*, 2014). LES phages φ2, 3 and 4 are all of the order *Caudovirales*, and

have been classified by their morphology into the *Siphoviridae* family, due to the presence of long, non-contractile tails.

3.1.5.1 Phage genome organisation

The *Siphoviridae* are notorious for their genetic mosaicism, and demonstrate high levels of horizontal transfer (Brüssow & Desiere, 2001). This can be observed by comparison of bacteriophage genome sequences, where blocks of homologous sequences are often interspersed with regions of low sequence similarity (Casjens, 2005). LES phages φ2, φ3 and φ4 are all λ-like, based on their modular genome organisation, and the similarity of these modules to λ. Like many bacteriophage genomes (Hatfull, 2008), there are a large number of genes that are of unknown function.

3.1.5.2 Using protein similarity searches to reannotate phage genomes

When the LES genome was published in 2009, details of phage genomes were included in the supplementary data. The coding sequences (CDS) were predicted using Glimmer3 and genes that were not present in PAO1 or PA14, which would include the phage genes, were manually annotated using BLASTP. Since the LES genome was published, the amount of information in the databases has increased substantially, including characterisation of several transposable *P. aeruginosa* bacteriophages, a category to which LESφ4 belongs. Genomes require regular reviewing and re-annotation every 3-5 years, in line with knowledge and database expansion. Such an approach has been taken for several bacterial genomes (Camus *et al.*, 2002; Dandekar *et al.*, 2000) and has identified novel virulence factors in an uropathogenic strain of *E. coli* (Luo *et al.*, 2009). A similar approach applied the LES phage genomes would be useful to further our understanding of the genome architecture of the phages, and to potentially identify genes that may contribute to bacterial fitness.

3.1.6 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system

The CRISPR-Cas system of bacteria is a recently discovered form of adaptive immunity to bacteriophages and other mobile genetic elements (MGEs), such as

plasmids (Marraffini & Sontheimer, 2008). The CRISPR genes consist of repetitive sequences, interspersed with short regions of DNA that are homologous to MGEs (Bolotin *et al.*, 2005; Mojica *et al.*, 2005), termed spacers. These spacers were found to confer resistance to bacteriophages harbouring a similar sequence (Barrangou *et al.*, 2007). Spacers are acquired after infection of the cell with foreign DNA; the DNA is incorporated into the CRISPR region, and the bacteria are thereafter able to mount a defence to incoming DNA with the appropriate protospacers (i.e. regions of foreign DNA that match the spacer sequences in the bacterial chromosome). Spacers are acquired preferentially from MGEs (as opposed to host chromosome) via multiple mechanisms, including a preference for replicating DNA and stalled replication forks, with bacterial confinement of stalled replication forks by Chi sites to avoid self-acquisition (Levy *et al.*, 2015).

The CRISPR genes are usually found in close proximity to the *cas* (CRISPR associated) genes. The spacers are transcribed and cleaved by the Cas protein(s) to form small CRISPR RNAs (Brouns *et al.*, 2008; Carte *et al.*, 2008), and the CRISPR-Cas complex targets and cleaves the foreign DNA (Garneau *et al.*, 2010). Such as system is analogous to the RNA interference system of viral defence in eukaryotic cells (Makarova *et al.*, 2006).

3.1.6.1 Anti-CRISPR phage proteins

Given the widespread nature of CRISPR-Cas systems, it seemed highly likely that bacteriophages would have a counter defence strategy. Indeed, metagenomic analysis of the phage population obtained after experimental coevolution between *Streptococcus thermophilus* and phage 2972 revealed SNPs in or near the proto-spacer adjacent motif region in the phage genome, rendering the phage immune to one of the bacterial CRISPR-Cas systems (Sun *et al.*, 2013). However, this is likely a poor strategy, as it is known that upon recognition of imperfectly matching DNA (i.e. mutated phage proto-spacers), the CRISPR-Cas acquisition system is “primed”, and moves along the phage DNA, selecting multiple spacers, hence counteracting phage resistance (Datsenko *et al.*, 2012) .

Recently, a more sophisticated phage resistance mechanism has been observed in numerous related transposable *Pseudomonas* phages. These phages produce anti-

CRISPR proteins that allow them to successfully infect *P. aeruginosa* strain PA14, which has an active type I-F CRISPR system. When these genes were cloned into a high copy plasmid and overexpressed in the bacterial strain, CRISPR-sensitive phages were then able to infect. The mechanism by which the anti-CRISPR proteins act was not elucidated, although the effect was thought to occur after the formation of the CRISPR-Cas complex (Bondy-Denomy *et al.*, 2013). A more recent study identified more anti-CRISPR proteins, this time active against the type I-E CRISPR system, that inhibit the CRISPR-Cas complex (Pawluk *et al.*, 2014). These are recent studies, and it is thought that there are many more phage anti-CRISPR genes that have not yet been discovered.

3.1.7 Phage growth characteristics

Bacteriophages have innately different growth characteristics, and upon lytic infection of a bacterial host will display a characteristic growth curve. The growth characteristics of a bacteriophage impact on its fitness and evolutionary success, and are likely to also affect host ecology.

The growth characteristics of bacteriophages can be assessed using one-step growth curves, whereby the phage lytic cycle is observed from the beginning (initial adsorption to bacteria) to the end (release of phage virions by bacterial cell lysis) by measurement of free phage over time. Two important characteristics can be determined using such can approach; the latent period, and the burst size (Ellis & Delbrück, 1939). The latent period refers to the time spent in the bacterial host, between adsorption to the bacterium, and lysis of the cell. The burst size is the number of virions released per infected cell. The longer the phage latent period, the more time available to accumulate virions within the cell, hence the larger the burst size, but with this comes the cost of less time available to find and infect new hosts. When attempting to maximise fitness, there exists a clear trade-off between latent period and burst size. Theoretical models predict an optimal latent period exists, one that is intermediate, resulting in an intermediate burst size (Wang *et al.*, 1996). Experiments with isogenic *E. coli* λ lysogens, that differ only in their lysis time (a proxy for latent period), support the theoretical work. There was a clear, positive relationship between lysis time and burst size, and experimental support for the

trade-off; an intermediate lysis time was associated with higher phage fitness (Wang, 2006).

Trade-offs exist for other aspects of the phage life-cycle. For example, there is a trade-off between phage adsorption rate and latent period, although, interestingly, competition experiments between phages revealed that adsorption rate has a greater impact on phage fitness than the latent period (Shao & Wang, 2008). There is also a trade-off between the multiplication rate in the bacterial host and survival outside the host (De Paepe & Taddei, 2006), most likely because building virions capable of surviving for lengthy periods in the environment is more metabolically costly.

Phage growth characteristics and the optimal strategy are hugely influenced by the bacterial host(s) and growth environment. Host availability, and the quality of those hosts (i.e. the metabolic status of the cell) both affect the optimal lysis time (Abedon *et al.*, 2001; Abedon *et al.*, 2003). Host physiology is also known to affect phage burst size. Bolger and colleagues measured the burst size of phages upon infection of cultures that had been in stationary phase for different lengths of time. Burst size varied with time in stationary phase. Unexpectedly, the burst size was larger in five day versus three day old cultures, a fact that was attributed to the cells transitioning to a biofilm mode of growth (Bolger-Munro *et al.*, 2013). It is important to note that what appears optimal in a laboratory environment may not be optimal in the natural environment. For example, a high adsorption rate can be detrimental in a biofilm mode of growth (Gallet *et al.*, 2009), presumably due to adsorption to cellular debris etc.

Most studies looking at phage life-history traits have been performed with obligately lytic phages. Temperate phages have an added dimension of complexity in that they have the option of integrating into the host chromosome and delaying the lytic cycle until a later date. This is the equivalent of having a longer latent period, and also of increasing the burst size, as phage progeny are produced by vertical transmission (i.e. binary fission of bacterial cell, resulting in prophage replication), as well as when the cell enters prophage-mediated lysis (Abedon, 2009).

3.1.8 Lysis / Lysogeny decision and phage fitness

Upon infecting a host cell, temperate phages usually take one of two routes, and enter the lytic cycle, or, alternatively, integrate into the host chromosome as a prophage. Ultimately, the decision should be based upon what is likely to maximise phage fitness. Extensive research has been conducted with *E. coli* λ into what factors affect the lysis/lysogeny decision, as the regulation of it is well understood. Briefly, lysogeny results when the CII lysogenic regulator accumulates within the cell to above a certain threshold, switching off the default lytic cycle and stimulating the synthesis of Int, which is required for phage integration (Oppenheim *et al.*, 2005). The regulation is more complex than this, but that is sufficient information for the purposes of this chapter. Conditions that prevent CII degradation favour lysogeny. For example, nutrient starvation increases the frequency of lysogeny (FOL), as starving cells lack the enzymes that degrade CII (Lieb, 1953). $MgSO_4$ starvation has a similar effect, although the molecular mechanism for this was not determined (Kourilsky 1973). A high multiplicity of infection also increases the FOL (Lieb, 1953); Kourilsky concluded that this was due to an unknown phage-encoded pro-lysogeny factor, and the more phage that infect the cell, the more likely it is that the critical threshold concentration will be reached (Kourilsky, 1973). The lysis/ lysogeny decision appeared to be quite stochastic in λ, but a recent study suggested that it may simply be the result of pre-existing variation in bacterial cells; smaller cells are more likely to become lysogens, as the necessary regulatory molecules, such as CII, reach the threshold concentration faster when in a smaller volume (St-Pierre & Endy, 2008).

3.1.9 Factors affecting phage production by lysogens

3.1.9.1 Induction

It is known that DNA-damaging agents that induce the SOS response in the bacterial cell, such as UV light and mitomycin C can cause prophage induction. The focus of research has now shifted to other, more biologically relevant inducers, as well as prophage induction via SOS independent pathways.

Los *et al.* studied phage production in different *E. coli* Stx lysogens under a range of conditions. The DNA-damaging antibiotic norfloxacin and mitomycin C both caused

prophage induction, but sodium chloride did not. Temperature affected the efficiency of induction (Łoś *et al.*, 2009). Hydrogen peroxide (H_2O_2), a DNA damaging agent that is present in the CF lung (Jobsis *et al.*, 2000), resulted in a rapid, but more limited induction, occurring in only a fraction of the population (Łoś *et al.*, 2009). Stx lysogens produce increased levels of toxin when in the presence of H_2O_2, and also when co-cultured with human neutrophils, which produce H_2O_2 (Wagner *et al.*, 2001a). A transcriptomics study of *Burkholderia cenocepacia* (another CF pathogen) growing as a biofilm found that exposure to high doses of H_2O_2 led to upregulation of multiple phage-related genes (Peeters *et al.*, 2010). A similar finding was observed for *P. aeruginosa* strain PAO1 (Chang *et al.*, 2005).

One study proposed a role for the quorum-sensing molecules, acyl homoserine lactones (AHLs) in prophage induction (Ghosh *et al.*, 2009). They observed that when added to soil samples, AHLs caused an increase in the total viral abundance of the sample. They then tested this on the *E. coli* - λ model system, and found that AHLs cause an increase in the numbers of free λ, although with no significant decrease in bacterial cell counts. This was true for a *recA* mutant, suggesting that this is SOS independent. However, a recent study challenged the conclusion that the increase in phage-production was due to induction; in fact, AHLs cause a downregulation of bacterial phage receptors, leading to reduced superinfection and a subsequent build-up of free phages (Høyland-Kroghsbo *et al.*, 2013).

EDTA and citrate are found in many pharmaceuticals due to their role as chelating agents, and increase phage and toxin production in *E. coli* lysogens. This is not SOS-dependent, but occurs due to disruption of the cell membrane through magnesium ion chelation. This effect is synergistic with the known inducer norfloxacin, resulting in much higher levels of phage production than with norfloxacin alone (Imamovic & Muniesa, 2012). Interestingly, the effect of EDTA on phage production was greater in wild-type STEC strains than with mitomycin C.

3.1.9.2 Repression

Sub-inhibitory concentrations of certain antibiotics have been found to reduce spontaneous phage production in certain lysogens, including the LES (Fothergill *et al.*, 2011). Inhibitors of protein translation, including azithromycin, and the cell wall

inhibitor fosfomycin, reduce Shiga toxin production in *E. coli* (McGannon *et al.*, 2010). Similarly, azithromycin (among other antibiotics) decreases both phage and toxin production in strain O104:H4 (Bielaszewska *et al.*, 2012). A study involving a panel of 4 LES isolates and six commonly used anti-pseudomonals found that azithromycin suppresses phage production in two of the isolates, as does tobramycin, an aminoglycoside that inhibits protein synthesis (Fothergill *et al.*, 2011).

3.1.10 Factors affecting phage amplification

When estimating phage fitness, the traditional approach is to measure phage amplification (growth) in a naïve bacterial host, under laboratory conditions. However, this is never representative of the actual environment of the phage and host, and there are numerous factors that affect phage fitness in the natural environment. The CF lung environment in which the LES phages are found is nutritionally rich, viscous (Denton, 1960) and contains high concentrations of free DNA (Chernick & Barbero, 1959) and mucin glycoproteins (Boat *et al.*, 1976). *P. aeruginosa* grows as a biofilm and this may affect susceptibility to phage infection, through the simple physical protection afforded by the biofilm substratum. In addition, as *P. aeruginosa* becomes adapted to the CF airways in chronic infection, common phage receptors are lost (Smith *et al.*, 2006b), although chronic isolates are not always more resistant to phage (Friman *et al.*, 2013).

3.2 Objectives:

1. To use protein sequence similarity searches to update the annotation of the LES phage genomes.
2. To measure phage production by the original bacterial host strain (LES) in the presence of environmental factors common to the CF lung.
3. To assess the lytic growth of the LES phages in a novel host background, and factors that affect this, including bacterial growth environment.
4. To assess the frequency of entry into the lytic or lysogenic cycle for the LES phages, in conditions that are relevant to the CF lung.

3.3 Results

3.3.1 Reannotation of LES phage genomes

3.3.1.1 LESφ2

Using BLASTP, it was possible to assign a putative function to a further 5 ORFs (Appendix C). A large number of ORFs return only hits to hypothetical proteins, a common issue when annotating phage genomes. PALES_08001, previously annotated only as a phage-related protein, returned significant hits to several DNA binding proteins. It possessed a conserved domain at its C terminus that is characteristic of the phage pRha superfamily of DNA binding proteins. ORF PALES_08061 shared 100% identity to an antitermination protein Q in *P. aeruginosa*, and returned a hit to a superfamily of such proteins (PF06530), as well as significant hits to multiple bacteriophage proteins in the same superfamily. PALES_08161 returned a hit to a bacterial recombinase in the initial BLASTP search, and subsequent PSI-BLAST iterations returned hits to multiple recombinases across different bacterial species, some phage encoded.

Two ORFs in the late gene region returned BLASTP hits to structural phage proteins, and an iterative search using PSI-BLAST indicated that a further three ORFs in the late gene region are homologous to phage structural and tail proteins, including those from phages of *P. aeruginosa*, *Burkholderia* spp. and *Raoultella planticola*. Based on their position in the phage genome, it is likely that this is their function.

3.3.1.2 LESφ3

PSI-BLAST analysis, as well as identification of some conserved protein domains of the LES φ3 genomes, enabled us to assign a putative function to a further 11 ORFs. PALES_13341 had a specific CDD hit to ParBc (Pfam02195), a ParB-like nuclease domain. The ParB protein domain is known to cleave single-stranded DNA, but can also nick super-coiled plasmid DNA and act as an exonuclease. PALES_13381 had high scoring hits to *P. aeruginosa* proteins labelled as transcriptional regulators, due to the presence of a multi-domain transcriptional regulator COG2932. A specific CDD hit was also obtained for the peptidase S24 LexA-like protein, responsible for repair of single-stranded DNA in bacterial cells upon activation of the SOS response,

suggesting that this is a LexA-like transcriptional regulator. The top hit of the ORF next to this, PALES_13391, was a DNA-binding protein, yet further analysis using the CDD revealed specific hits to two conserved domains; at the N terminus of the protein to the phage regulatory protein Rha (pfam09669), and at the C terminus to the phage anti-repressor (KilAC domain) protein (pfam03374).

3.3.1.3 LESφ4

Using BLASTP and PSI-BLAST, it was possible to assign a putative function to a further 13 ORFs, in addition to a confident prediction of function for a further 3 as anti-CRISPR genes (3.3.2.2), based on the recently published protein sequences of known anti-CRISPR genes (Bondy-Denomy *et al.*, 2013; Pawluk *et al.*, 2014). The majority of newly-annotated ORFs were structural or phage-tail proteins. Several proteins were annotated based on their high levels of similarity (≥90%) to the recently characterised *Pseudomonas* phage LPB1 that was originally isolated from hospital sewage (Ceyssens *et al.*, 2009a). Using the new functional annotations, genes were classed into modules. Two clear functional regions of early and late genes can be observed (Figure 3.2).

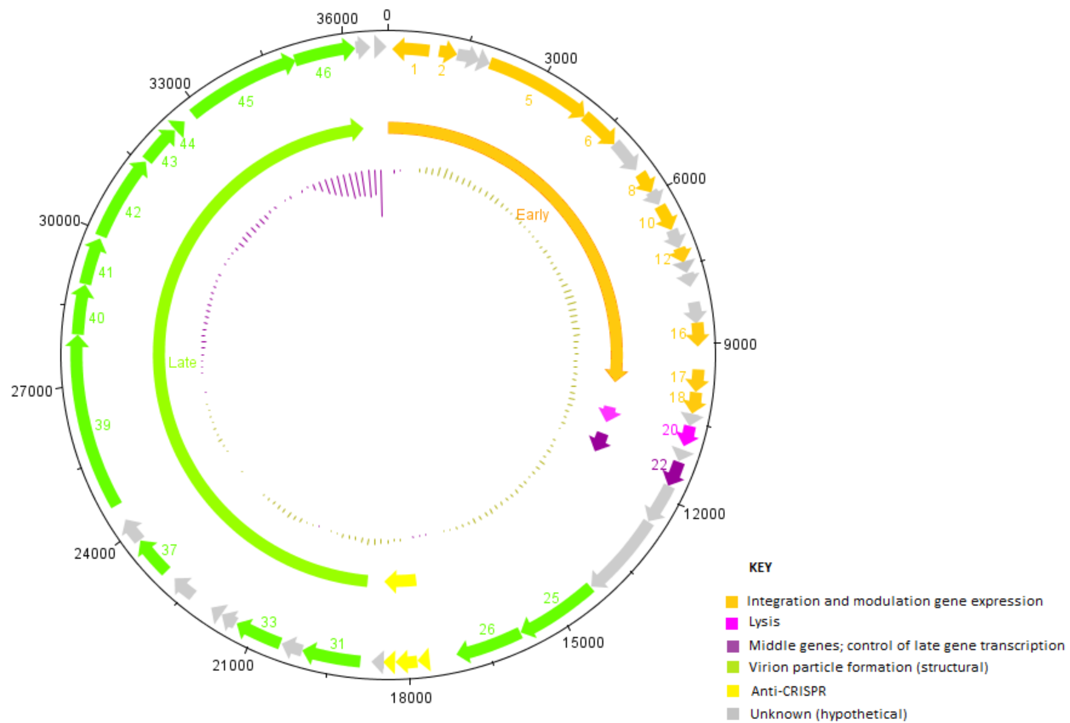


Figure 3.2 Genome organisation of LES phage 4. Circles represent (from inside out): GC content, classification according to timing of gene expression and classification according to predicted function.

3.3.2 Identification of anti-CRISPR phage-encoded proteins

3.3.2.1 Putative spacers targeted to LESφ4 observed in PA14 genome

As LESφ4 is known to be capable of infection of PA14, a strain which possesses two CRISPR systems, we searched for evidence that (a.) PA14 has been exposed to LES φ4 or a similar sequence in the past, and had spacers present in its genomes capable of recognising the phage and (b.) that LESφ4 encodes proteins capable of protecting itself from the PA14 CRISPR response. The LESφ4 genome was BLASTN searched against the PA14 genome to identify potential spacers present in the PA14 CRISPR region. The result was positive; a short sequence matching exactly (E = 4e-11) to one of the 2 known CRISPR regions of PA14 (between 2936004 and 2936036 bp) was observed, suggesting that PA14 has had prior exposure to a LESφ4-type phage.

Despite their inability to infect PA14, no putative spacers were identified in the PA14 genome matching to LESφ2 or 3, suggesting that an alternative bacterial resistance mechanism is preventing phage infection.

3.3.2.2 LESφ4-encoded anti-CRISPR proteins

Two recent publications identified numerous anti-CRISPR proteins in *Pseudomonas* transposable phages that show activity against either the type I-E or type I-F CRISPR system of *P. aeruginosa*, including phage D3112, which is known to share similarity with LESφ4. BLASTP searches of these protein sequences against the LESφ4 genome revealed that four of the anti-CRISPR proteins are highly similar to proteins predicted to be encoded by the LES φ4 genome. These include protein ACR3112-3, active against the type I-E CRISPR system (94% identity, E = 9e-25), as well as three that are active against the type I-F CRISPR system, gp33 (88% identity, E = 8e-84), gp35 (96% identity, E = 8e-96) and gp36 (92% identity, E = 2e-20). It is worth noting that gp33 and gp35 both match to either end of PALES_15761 (known as orf29b).

3.3.3 Phage production by original host LES in the presence of various environmental factors relevant to the CF lung environment

The effect of various environmental conditions on LES phage production was investigated. Such conditions were chosen because they are known to affect phage production in other bacterial species, and are conditions that bacteria are likely to experience in the CF lung environment. Phage production after treatment was compared to a control, as there is a low level of spontaneous phage production.

3.3.3.1 H_2O_2 increases LES phage production

The presence of H_2O_2 significantly increases phage production in isolate LESB58, (One-way ANOVA; $F_{3,12} = 45.43$, $p < 0.001$). The mean PFU ml^{-1} values of H_2O_2 treated cultures are significantly higher than the control at all concentrations used (Dunnett's post-hoc test: 2.5 mM, $p < 0.05$, 5 mM and 50 mM, $p < 0.001$). The effect is dose-dependent; as the concentration of H_2O_2 increases, so do the numbers of phage produced (Figure 3.3). Bacterial viability was not affected at the doses used (data not shown).

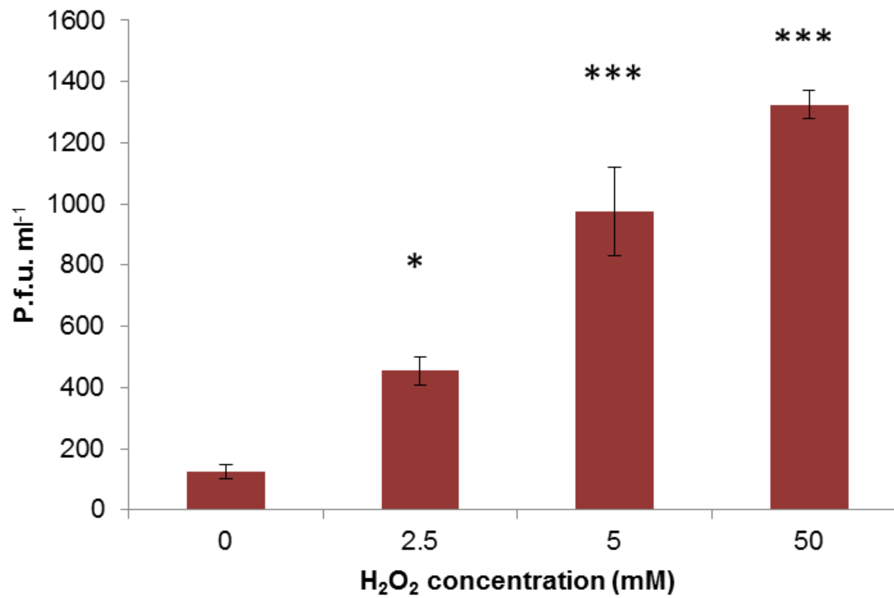


Figure 3.3 Effect of increasing H_2O_2 concentration on production of free plaque-forming phages by isolate LESB58. Error bars ±1 SEM. Asterisks denote statistical significance.

3.3.3.2 EDTA does not affect phage production and does not act synergistically with norfloxacin

EDTA has no effect on phage production (Figure 3.4); no difference was observed between the control and EDTA treated cultures (Mann-Whitney: $W = 13$, $P > 0.05$). Norfloxacin, a known inducer, increased phage production. However, no synergy was detected between norfloxacin and EDTA, as evidenced by no significant difference in phage production between norfloxacin alone, and norfloxacin in combination with EDTA (Mann-Whitney: $W = 15$, $P > 0.05$).

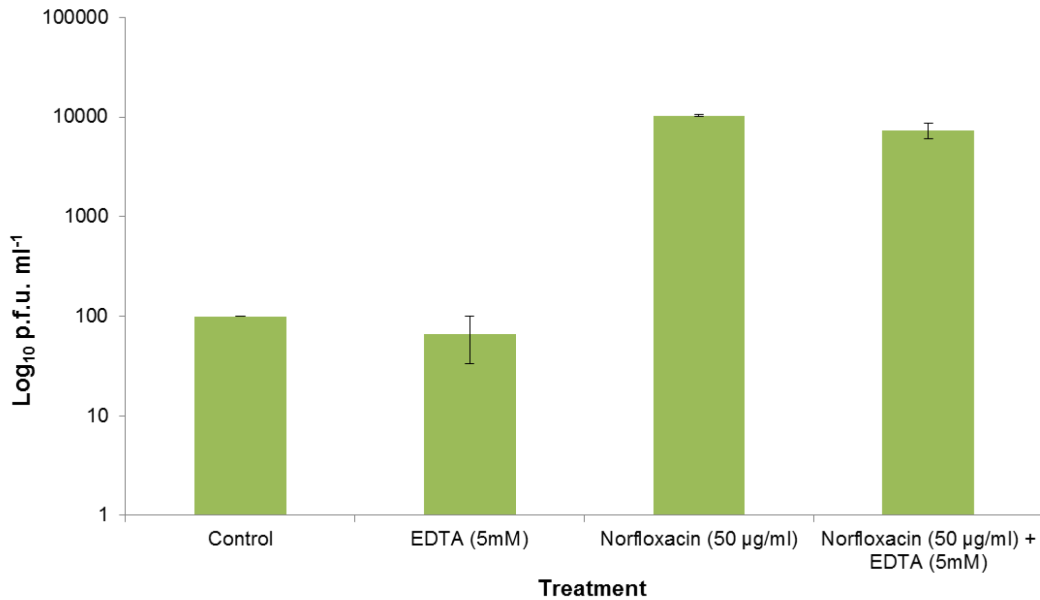


Figure 3.4 Effect of EDTA, both alone, and in combination with the known inducer norfloxacin, on production of free, plaque-forming phages by isolate LESB58. Error bars ± 1 S.E.M.

3.3.3.3 Antibiotics

3.3.3.3.1 Commonly used antibiotics suppress LES phage production

Certain antibiotics used in the management of CF are known to increase or decrease phage production in 4 LES isolates (Fothergill *et al.*, 2011). However, this previous dataset did not include the antibiotics fosfomycin and ceftazidime, or any combination of antibiotics. The effect of these antibiotics on phage production was tested in 4 LES isolates, using the same methodology as described previously (Fothergill *et al.*, 2011), so that the results may be directly comparable. In addition, the effect of the antibiotics ceftazidime and colistin in combination was determined; colistin is known to greatly suppress phage production (Fothergill *et al.*, 2011), but whether this effect is additive when in combination with another antibiotic was unknown. The MICs were determined for isolate LESB58, and were found to be: 100 µg ml^{-1} for fosfomycin, 64 µg ml^{-1} for ceftazidime, and 192 and 3.4 µg ml^{-1} for ceftazidime and colistin, respectively, when used in combination (determined using the checkerboard method). Data were analysed separately for each isolate with a Kruskal-Wallis test, and post-hoc pair-wise comparisons performed using the Nemenyi test with Chi-squared approximations, with the PMCMR package (Pohlert, 2014) in R.

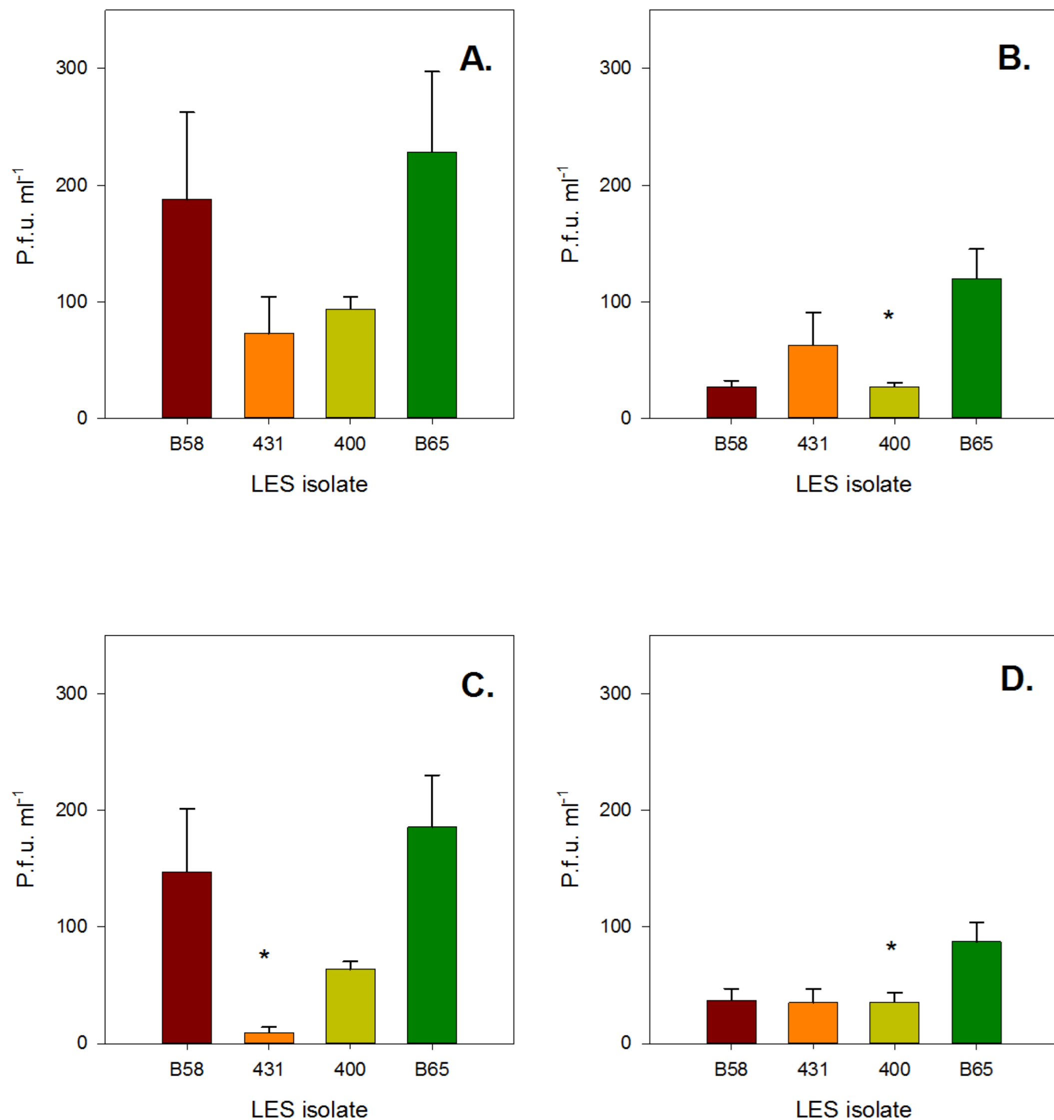


Figure 3.5 Effect of antibiotic treatment on phage production by a panel of clinical LES isolates. (A.) Control treatment (spontaneous phage induction), (B.) Ceftazidime monotherapy, (C.) Fosfomycin monotherapy and (D.) Ceftazidime and colistin combination therapy. Antibiotics were at added at the MIC to exponentially growing cultures (n = 7) and incubated for 1 hour, followed by 1 hour recovery and phage enumeration. Error bars + 1 S.E.M. Asterisks denote a statistically significant difference compared to the control value for that isolate.

The effect of the different antibiotics varied between the different LES isolates (Figure 3.5); all three treatments resulted in significantly less phage production ($p < 0.05$) in just one of the four isolates; fosfomycin suppressed phage production in isolate LES431, and ceftazidime supressed phage production in isolate LES400, both when used alone, or in combination with colistin. There were no obvious additive effects on phage production when two antibiotics were used in combination.

3.3.3.3.2 Individual LES phage suppression with colistin

Colistin is known to suppress total LES phage production (i.e. numbers of virions capable of causing plaques on the indicator host PAO1). However, it is not known whether the effect varies between phages. To determine this, we PCR-tested plaques and calculated the relative proportion of plaques caused by each phage, both after colistin treatment, and in untreated controls. The proportion of plaques caused by each phage varied between the phages, even in the absence of antibiotics (Figure 3.6). Approximately equal proportions of plaques were caused by LESφ2 or LESφ4, with relatively few caused by LESφ3. Colistin greatly reduced the numbers of free phages produced (data not shown), in line with previous findings (Fothergill *et al.*, 2011). However, the relative frequencies of plaques caused by each phage was not significantly different in the colistin treatment compared to the control ($\chi 2$=0.06, d.f. =2, $P > 0.05$), suggesting that the LES phages are affected equally by colistin treatment.

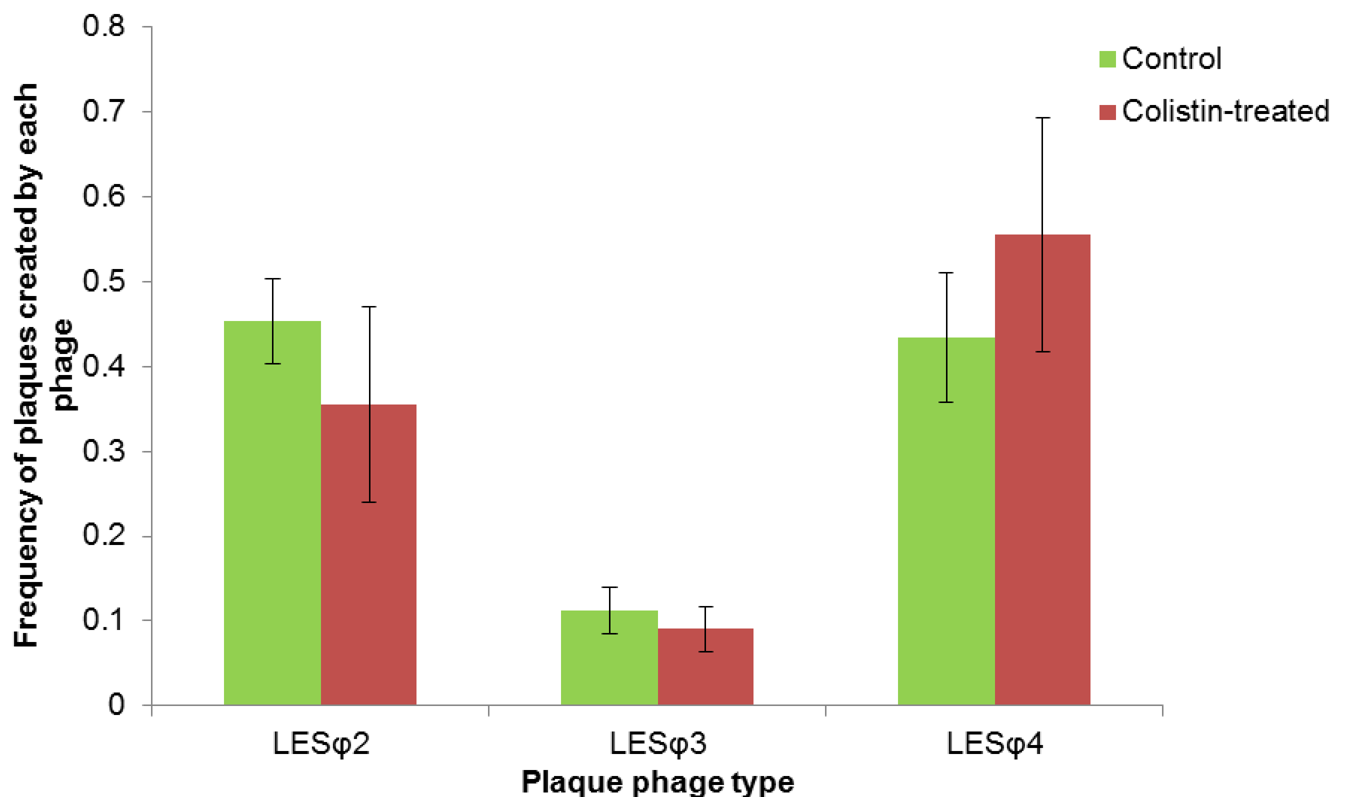


Figure 3.6 The proportion of plaque-forming virions produced by LESB58 (in the presence of absence of colistin) that can be attributed to each LES phage. Plaques were PCR tested using a multiplex PCR assay. Total phage production was lower in the colistin treatment (data not shown). Error bars ±1 S.E.M.

3.3.4 LES prophages in a novel host background

3.3.4.1 One-step growth curves of LES phages in PAO1

The lytic growth of LESφ2-4 on the naïve bacterial host PAO1 was measured under laboratory conditions, using a one-step growth curve. This allowed determination of two major growth parameters, latent period and burst size, and for comparisons to be made between the phages.

LESφ2 has a mean (±1 S.E.M) latent period of 88(±6) minutes and a burst size of 27(±4) (Figure 3.7).

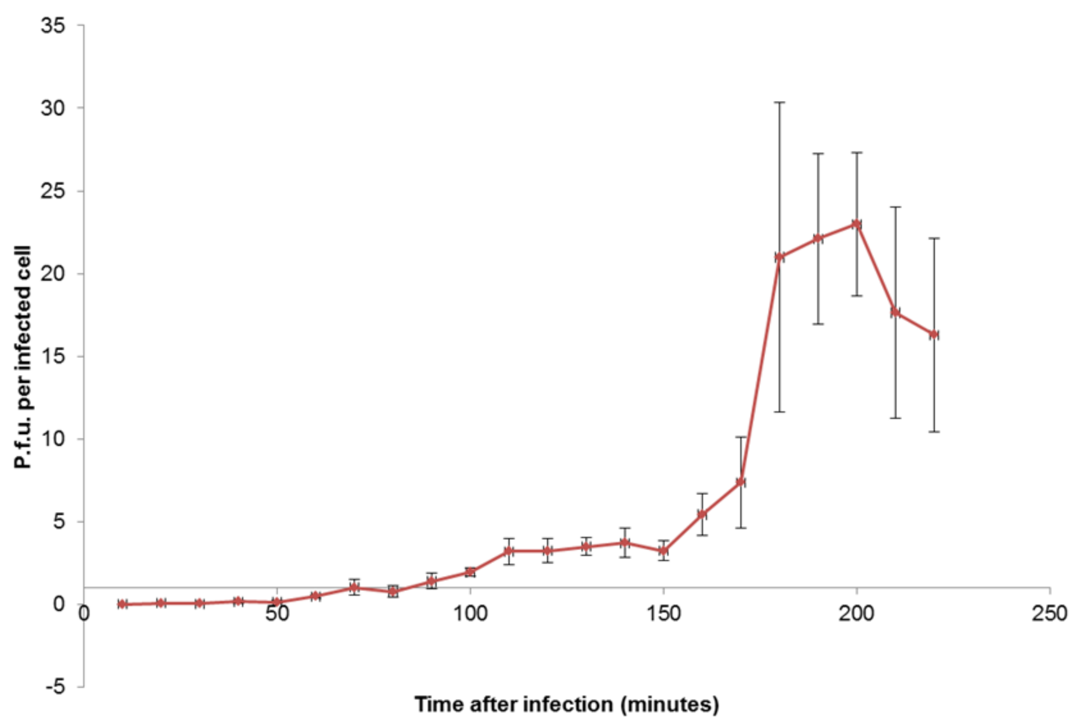


Figure 3.7 One step growth curve of LESφ2 on exponentially growing *P. aeruginosa* strain PAO1. Each data point represents the mean of 4 separate experiments. Error bars denote ±1 S.E.M.

LESφ3 displays both the shortest latent period, and the largest burst size of the three LES phages. It has a latent period of 47(±3) minutes and a burst size of 77(±14)

(Figure 3.8).

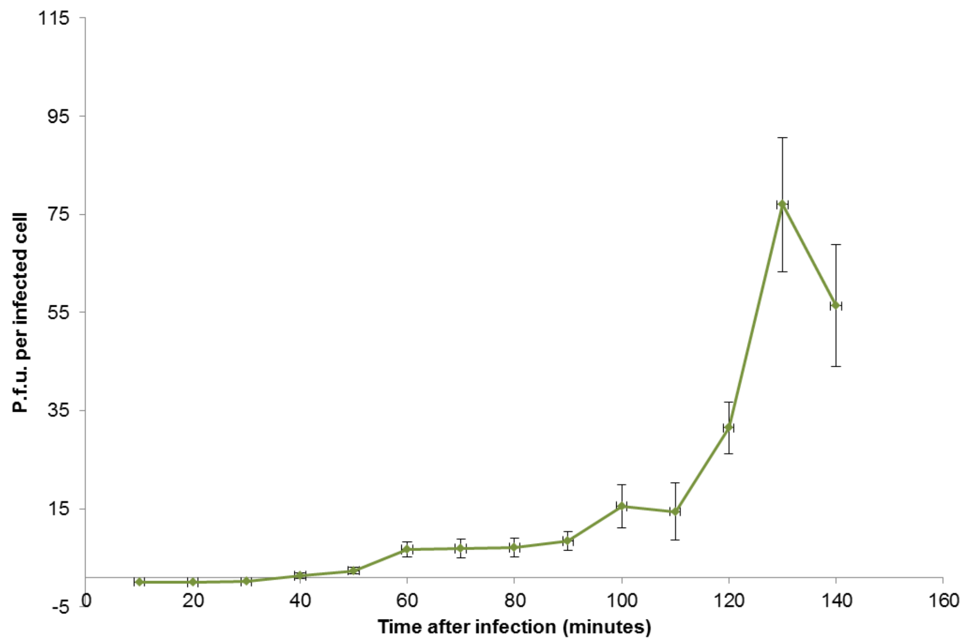


Figure 3.8 One step growth curve of LESφ3 on exponentially growing *P. aeruginosa* strain PAO1. Each data point represents the mean of 4 separate experiments. Error bars denote ±1 S.E.M.

LESφ4 displays a long latent period of 93(±12) minutes and a relatively small burst size of 13(±2) (Figure 3.9).

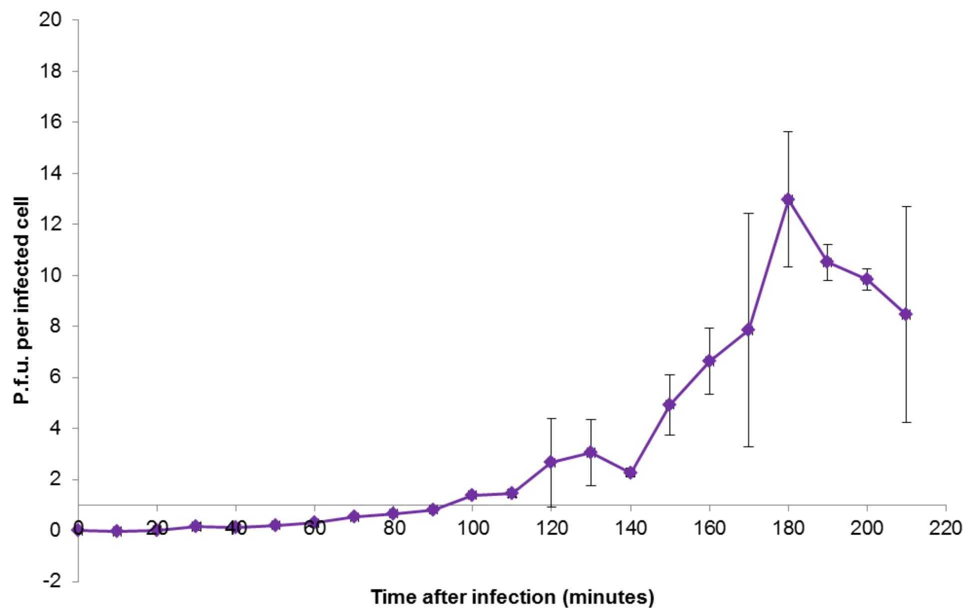


Figure 3.9 One step growth curve of LESφ4 on exponentially growing *P. aeruginosa* strain PAO1. Each data point represents the mean of 3 separate experiments. Error bars denote ±1 S.E.M.

3.3.4.2 LES phage amplification in different growth media

The majority of phage growth experiments are performed with exponentially growing cells in rich, liquid media. However, it is known that growth rate and the potential for phage amplification is dependent on many factors, including the physiological state of the cell, and the bacterial growth environment. A simple assay was performed to test the ability of the LES phages to replicate in two different growth media: LB, the simple laboratory broth, and ASM, a medium designed to mimic cystic fibrosis sputum in order to simulate the lung environment as much as possible. From a small starting MOI of 5×10^{-5}, free phage were enumerated after 24 hours of co-incubation with PAO1, and the amplification (fold change in titre day^{-1}) of each phage was calculated. There were large differences, both between the different LES phages, and between the different media (Figure 3.10). A $\log_{10}$ transformation was applied to the data, and after the interaction term was found to be non-significant ($P = 0.69$), a simple, additive model was fitted to the data, with phage type (3 levels) and media (2 levels) as the 2 main factors. A significant effect was observed for both phage type ($F_{2,32} = 44.2$, $MSE = 43.7$, $P < 0.001$) and media ($F_{1,32} = 30.7$, $MSE = 30.4$, $P < 0.001$). Post-hoc comparisons with TukeyHSD tests revealed that LESφ2 had a lower fold change in titre than LESφ3 ($P < 0.001$) and LESφ4 ($P < 0.001$), and LESφ3 amplified less than LESφ4 ($P < 0.05$). LESφ2 was unable to amplify over the 24 hour period; a loss of virions was actually observed in LB, possibly through non-specific binding to bacterial debris. LESφ3 amplified quite poorly in LB, yet interestingly showed a huge increase in titre in ASM, making it comparable to LESφ4. Of the three phages, LESφ4 was the most successful, amplifying well in both media.

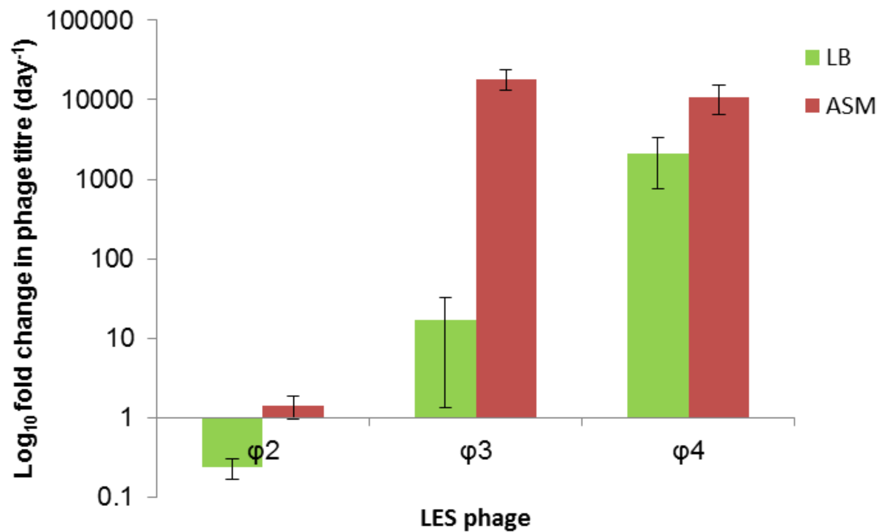


Figure 3.10 Fold change in free phage titre (p.f.u. ml^{-1}) after 24 hours co-incubation with strain PAO1, in different media. LES phages were added to bacteria that had already reached stationary phase. Error bars ±1 S.E.M.

3.3.4.3 Frequency of lysogeny upon infecting PAO1 is high in ASM

Upon infecting a bacterial cell, temperate phages tend to follow one of two routes: lysis, or lysogeny. In order to quantify how frequently this decision is made either way upon infection of PAO1 with the LES phages, we used a modified assay to determine the frequency of lysogeny (FOL). The FOL is defined as the frequency of infections of PAO1 that result in phage integration into the bacterial genome, as opposed to entry into a lytic infection. The limitations of the assay mean that a FOL of below 10% cannot be detected. The FOL of the three LES phages was determined for single-phage infection of exponentially growing PAO1, at an MOI of 0.1, and was below the limit of detection (i.e. <10%) for all three phages. The FOL was subsequently determined in conditions designed to mimic the CF lung environment i.e. bacteria in the stationary phase of the growth cycle, and a high MOI. Such conditions are known to increase the FOL for *E. coli* λ (Kobiler *et al.*, 2002; Kourilsky, 1973; Lieb, 1953), and it was hypothesised that this would also be the case for the LES phages. Indeed, this is what was observed. The FOL was high for all three LES phages (Table 3.2), but the highest was for LESφ3, with on average over half of cell infections resulting in lysogeny. Because of the substantial variation between replicates, it is difficult to determine whether the FOL is significantly different between the phages, but the fact that the FOL is for all three is high in a simulated CF lung environment is an important finding.

Table 3.2 The frequency of lysogeny of LES phages 2, 3 and 4, upon infection of stationary phase *P. aeruginosa,* with a MOI of 10.

	Phage		
	LES φ2	**LESφ3**	**LESφ4**
Frequency of lysogeny (mean)	33	56	34
Range	11-47	36-81	10-47
Standard deviation	17	17	14

3.4 Discussion

In this study, the genomes of the LES phages φ2-4, originally published in 2009, were revisited, and reannotated using protein sequence similarity searches. This method was reliant on new data being made available in the time elapsed since the LES genome was published, and was very successful for LESφ4, due to functional proteomics studies with similar phages (Ceyssens *et al.*, 2008; Ceyssens *et al.*, 2009a). In all three phages, several structural proteins were identified; these tend to be well conserved at the amino acid level in many phages (Proux *et al.*, 2002), even those infecting different host species (Brüssow & Desiere, 2001).

The majority of the ORFs with newly-assigned functions in the LESφ2 and LESφ3 genomes were involved in lysis/ lysogeny and control of replication. An antitermination protein Q was identified in both: this was initially identified in λ (Yang & Roberts, 1989), and prevents termination of host RNA polymerase at the terminator, therefore enabling expression of the next set of genes. This protein regulates both early and late phage gene operons. LESφ2 also encodes a putative recombinase protein. Phage recombinases are required for genome replication (Mosig *et al.*, 2001) and DNA repair (Stahl *et al.*, 1997) and are quite common: a 2010 study used Hidden Markov Models of known phage recombinases to screen 465 phage genomes for the presence of homologues and found them in 133 genomes (Lopes *et al.*, 2010). Phage recombinases play a key role in horizontal gene transfer, allowing shuffling of genes, even between functional temperate and defective phages (De Paepe *et al.*, 2014), and so may play an important role in driving phage (and consequently bacterial) evolution. Given the high degree of mosaicism between the LES phages, it is possible such phage-mediated recombination between the LES phages occurred in the past.

A cluster of ORFs in LESφ3 (PALES_13431 – PALES_13461) were newly annotated and appear to be part of the lysis/ lysogeny module; they include the antiterminator protein Q, a transcriptional regulator and a holin. Interestingly, PALES_1345, in the middle of this block of genes, shares 100% identity with an mRNA interferase of present in multiple *Pseudomonas* spp., and 73% amino acid identity with HicA, an mRNA interferase first identified in *P. fluorescens* (Silby *et al.*, 2009), and the bacteriostatic toxin component of a toxin-antitoxin system that is found in many prokaryotes (Jørgensen *et al.*, 2009). Many hypotheses for their function have been put forward to explain their prevalence, including a stress survival response for bacteria (Christensen *et al.*, 2003; Wang *et al.*, 2011) and stabilisation of genetic elements (Wozniak & Waldor, 2009), among others.

A further two ORFs in the LESφ3 genome that were of previously unknown function, PALES_13381 and 13391, were annotated as a LexA-like transcriptional regulator, and phage regulatory/ anti-repressor protein, respectively. Work with *Salmonella* Gifsy phages observed that the phages are induced by binding of phage anti-repressor protein, rather than cleavage of the repressor molecule, as in λ, and that this anti-repressor protein is under control of LexA. Even more interestingly, the anti-repressors could bind to the repressors in other phages; the authors speculated that this could lead to co-ordination of phage induction in polylysogenic strains (Lemire *et al.*, 2011). Clearly, the mechanism by which the LES phages are induced and the interactions between the phages are of future interest.

Reannotation of LESφ4 identified mainly structural genes, but also one ORF involved in host lysis, encoding the Rz protein. LESφ2 also carries a putative gene for an Rz protein, located in the same relative position as in the genome of λ, next to the holin genes. The Rz protein forms a protein complex that completes host cell lysis by fusing the inner and outer bacterial membrane (Berry *et al.*, 2008). However, arguably the most significant finding was the three ORFs that encode putative anti-CRISPR proteins; this may explain the ability of LESφ4 to infect PA14, as well as several clinical strains of *P. aeruginosa* (James *et al.*, 2012), where LESφ2 and LESφ3 could not, despite using the same receptor to infect cells. Anti-CRISPR proteins were found in a large group of transposable *Pseudomonas* phages (Bondy-Denomy *et al.*, 2013; Pawluk *et al.*, 2014), and the high sequence similarity and

conservation of their location in the genome between phages is suggestive of a fitness benefit to the phages. This finding also raises important questions regarding polylysogeny; anti-CRISPR proteins are expressed whilst the phage is in the prophage state, thereby silencing the host CRISPR response; would this then allow infection by other phages that would be typically CRISPR sensitive? This is a new and fascinating field of research and warrants further study.

A recently published study by our group used qPCR to measure the levels of free LES phages in the sputa of LES-infected patients, as well as bacterial density. The levels of free phage were found to be very high, outnumbering bacteria 10-100 fold, and the free phage to bacterium ratio negatively correlated to bacterial density, suggesting a role for the LES phages in the control of *P. aeruginosa* densities in the CF lung (James *et al.*, 2014). Given that the *P. aeruginosa* populations are dominated by, if not exclusively, LES in these patients, this suggests that the phages are produced by prophage induction, rather than lytic infection of susceptible strains. We investigated the influence of various aspects of the CF lung environment on phage production, in an attempt to elucidate what might be causing such high levels of phage production in the lung. H_2O_2, a known inducer of *E. coli* phages (Łoś *et al.*, 2010) increased phage production in LES φ2-4 in a dose-dependent manner. However, selecting the correct dosage of H_2O_2 to use for this study (i.e. a biologically realistic concentration) was difficult, because there are (to my knowledge) no studies accurately quantifying reactive oxygen species (ROS) concentrations in CF sputum. Some studies even question the common belief that H_2O_2 (and other reactive oxygen species) are elevated in the CF lung, finding no difference in H_2O_2 concentration in the breath condensate of CF and non-CF individuals (Ho *et al.*, 1999; Worlitzsch *et al.*, 1998). Another study found that during exacerbations, children with CF have abnormally high levels of H_2O_2 that subsequently decrease after antibiotic treatment (Jobsis *et al.*, 2000), yet there was no comparison with healthy control subjects. All these studies rely on H_2O_2 in breath condensate as a reliable proxy for levels in the lower lung, yet obstructive mucus plugs may trap air in the lower lungs and prevent in being exhaled. However, if H_2O_2 (and probably other ROS also) cause dose-dependent phage induction, elevated H_2O_2 levels (for example, during exacerbations) could have an effect on the host-phage dynamics in the CF lung. Despite this, a recent study by our group found no increase

in LES phage densities during patient exacerbations {James, 2014 #475}. There is also evidence that H_2O_2-mediated prophage induction is a form of interspecific competition; *Streptococcus pneumoniae* is able to displace *Staphylococcus aureus* by producing H_2O_2, causing prophage induction and cell lysis (Selva *et al.*, 2009).

Previous work studying the effect of anti-pseudomonals on phage production in LES isolates observed that whilst quinolone antibiotics, such as ciprofloxacin, increase the levels of free phage, many others, including azithromycin and colistin actually suppress phage production. The range of anti-pseudomonals used was extended in this study, and it was found that both fosfomycin and ceftazidime suppress phage production, each in only one of the four LES isolates tested. Fosfomycin blocks a critical enzymatic step in cell wall biosynthesis (Kahan *et al.*, 1974) and ceftazidime inhibits penicillin binding proteins, causing cell filamentation and subsequent lysis (Hayes & Orr, 1983). Whilst these mechanisms of action would have no obvious inhibitory effects on phage production, antibiotics have global effects; sub- inhibitory concentrations of antibiotics with different modes of action are capable of modulating transcription of multiple genes in several bacterial strains (Goh *et al.*, 2002).

The CF lung is a complex environment, and there are likely to be many factors, other than antibiotics and H_2O_2, that affect phage production. One candidate is cigarette smoke, as it contains many known mutagens. A study showed that benzo(a) pyrene diol epoxide, a chemical detected in the vaginal secretions of female smokers, causes prophage induction in lactobacilli, although other chemicals in the panel, including nicotine and tar, did not (Pavlova & Tao, 2000). The effect cigarette smoke may have on phage production on bacteria in the CF lung has not been investigated, and is potentially important, given that the lungs will have the highest exposure to cigarette chemicals (relative to other organs) in smokers, or individuals exposed to passive smoking.

Given that mutations in prophage genes encoding proteins involved in cell lysis affect the competitiveness of LES, it is likely that environmental factors that affect phage production will also affect bacterial dynamics, potentially influencing disease progression. In Shiga-toxigenic *E. coli*, the relationship between toxin production

and disease is clear (Boerlin *et al.*, 1999; Zhang *et al.*, 2000). As antibiotics affect toxin production, the choice of antibiotic has an impact on disease progression; the phage suppressing antibiotic azithromycin had a protective effect, whilst the phage inducing-agent ciprofloxacin worsens disease in gnotobiotic piglets infected with O157:H7, compared to untreated controls (Zhang *et al.*, 2009). Similarly, clinical *S. aureus* isolates from CF patients undergo prophage induction upon exposure to ciprofloxacin, leading to the upregulation of phage-related virulence factors (Goerke *et al.*, 2006). Regardless of the effect on bacterial virulence/ competitiveness, antibiotics or environmental conditions that cause phage induction should still be viewed with caution, given their potential for mediating horizontal gene transfer.

The fitness of LESφ2-4 in both laboratory and CF-like (ASM) environments has been considered. The three phages display quite different lytic growth curves when infecting PAO1 in the exponential growth phase. One-step growth curves are a method used frequently to estimate phage growth, and LESφ4 showed the lowest growth rate, having both the longest latent period and smallest burst size. However, this was not predictive of its fitness and ability to amplify in stationary phase cultures over an extended period of time; LESφ4 amplified more than LESφ2 and LESφ3 in LB, and more than LESφ2 in ASM. LESφ2 did poorly in both media, but actually decreased in titre in LB. There are likely many explanations for the differences observed; ability to infect and replicate in stationary phase cultures, ability of phages to penetrate the biofilm (in ASM), and the phage adsorption coefficient. Whilst phage must be able to adsorb to bacteria to initiate an infection, a high adsorption coefficient can be detrimental, especially in a biofilm environment (Gallet *et al.*, 2009). A study of lytic phages infecting *Pseudomonas* biofilms found that one of the phages adsorbed to both the biofilm substratum and culture plate walls, reducing its initial infection efficiency (Pires *et al.*, 2011). Conversely, adhering reversibly to surfaces can be advantageous; lytic bacteriophages are known to be able to adhere to mucus, and by doing so may form part of the mammalian immune system; by adhering to mucosal surfaces, phage have greater access to incoming bacteria (Barr *et al.*, 2013). This is a relatively new idea, and has not been empirically tested in temperate phages, or lytic phages in the lung. However, a recent metagenomic analysis of the temperate phage populations of *P. aeruginosa* from CF and non-CF bronchiectasis found that phage-encoded Ig-like domains were very prevalent. Phage

binding to human glycoproteins was proposed as a mechanism to enable phage infection and propagation within the lung (Tariq *et al.*, 2015), a hypothesis that was supported by a previous finding that Ig-like domains were widespread in both lytic and temperate phages, across all three families of *Caudovirales* (Fraser *et al.*, 2006). Attempts to assay mucin and DNA binding for the LES phages, using a modified *in vitro* assay of the one described by Barr *et al.* were unsuccessful, due to large inter-assay variation.

The fitness of the LES phages has only been considered in isolation, on a short-term basis. Given the poly-lysogenic nature of the LES, there are likely to be phage-phage interactions. Co-infecting bacteriophages face competition for resources; phage fitness is seriously impaired when co-infecting for *E. coli* lambdoid phages (Refardt, 2011). A study of the levels of free LES phages in the sputa of LES-infected patients found that although LESφ2 and LESφ4 usually dominate and are found at high numbers, when LESφ2 was not present in one patient, high levels of free LESφ3 were found (James *et al.*, 2014). In addition, there is a superinfection hierarchy between the LES phages (James *et al.*, 2012), which could affect the fitness of phages when infecting naïve bacterial strains.

Many theories as to the role of bacteriophages focus on their lytic activity. For example, bacteriophages can control bacterial densities (Shapiro *et al.*, 2010) and act as allelopathic agents, lysing phage-susceptible competitors (Brown *et al.*, 2006; Gama *et al.*, 2013; Joo *et al.*, 2006). However, in a model CF-lung environment, the frequency of LES phage infections resulting in lysogeny is very high, suggesting that this is perhaps the norm in the CF lung. This finding is similar to that observed in λ. There is evidence that environmental conditions can select for a more or less responsive lytic switch in λ; Refardt & Rainey coevolved *E. coli* and λ under two different regimes, designed to favour either lysis or lysogeny. λ evolved under the latter had a significantly higher probability of lysogeny than the ancestor, or phage in the former regime (Refardt & Rainey, 2010). Furthermore, the issue of polylysogeny remains to be explored. It is known that for λ, a high number of infecting phages per cell increases the likelihood of lysogeny, due to phage-encoded pro-lysogeny factors (Kourilsky, 1973; Lieb, 1953) However, studies with the *E. coli* – λ model system only ever consider a single phage, but there are multiple LES phages. It remains to

be determined what effect would multiple phage genotypes have on the probability of lysogeny, if any.

3.5 Summary

- Reannotation of the published genomes of LESφ2, LESφ3 and LESφ4 enabled the functionality of more ORFs in each of the LES phage genomes to be predicted, in particular for LESφ4.
- LESφ4 encodes putative anti-CRISPR proteins, which in related phages are active against the type I-E and I-F CRISPR immunity systems of *P. aeruginosa.*
- H_2O_2, a chemical present in the CF lung causes an increase in LES phage production, but EDTA, a chemical found in many pharmaceuticals, does not.
- Two commonly used anti-*Pseudomonas* drugs, fosfomycin and ceftazidime, suppress LES phage production, but only in one isolate from four tested. Antimicrobial combination therapy with two phage-suppressing drugs does not have an additive effect. Colistin, a strong phage suppressor, has an equally negative effect on each of the LES phages, φ2, φ3 and φ4.
- LES phages φ2, φ3 and φ4 each have a characteristic growth curve upon lytic infection of naïve strain PAO1, with LESφ4 displaying the lowest fitness. However, over an extended period of time, LESφ4 demonstrates the highest amplification in both LB and ASM.
- The frequency of phage infections resulting in lysogeny is low in exponentially growing cultures, but high in a model CF lung environment.

Chapter 4 The role of the LES phages in driving bacterial phenotypic diversification in ASM and *in vivo*

4.1 Introduction

4.1.1 *P. aeruginosa* growth in the CF lung

4.1.1.1 Biofilm formation

Microscopy of CF sputa, as well as analysis of quorum sensing (QS) signals support the widely accepted hypothesis that *P. aeruginosa* grows as a biofilm in the CF lung (Singh *et al.*, 2000). Schwab *et al.* observed biofilm-like masses of *P. aeruginosa* cells in CF lung tissue (Schwab *et al.*, 2014), but Bjarnsholt *et al.* performed a more detailed analysis of the spatial distribution; by examining different regions of lung tissue from explanted lungs, as well as lungs from deceased individuals, they found that *P. aeruginosa* aggregates were common in the conductive zone of explanted lungs, and had spread to the respiratory zone in those individuals who died as a result of the infection (Bjarnsholt *et al.*, 2009).

P. aeruginosa biofilm growth *in vivo* is clearly very difficult to study, but many studies have used artificial sputum to model the CF lung environment. Interestingly, *P. aeruginosa* forms biofilm-like structures in this media that are not surface attached (Haley *et al.*, 2012; Sriramulu *et al.*, 2005), and it is thought that this is how it may exist in the CF lungs. When grown in a biofilm for a short period, *P. aeruginosa* undergoes rapid diversification, giving rise to subpopulations that have a specific role in the biofilm structure (Boles *et al.*, 2004).

4.1.1.2 Chronic infection

P. aeruginosa initial airway colonisation follows a distinct pattern: transient/ intermittent detection, followed by continuous culture positive detection of non-mucoid, antibiotic-susceptible strains, followed by progression to chronic infection (Burns *et al.*, 2001) and the appearance of mucoid colonies (Li *et al.*, 2005), at which stage the infection is almost impossible to eradicate. Chronic infection is usually diagnosed by continuous culture-positive sputum samples and the presence of antipseudomonal antibodies in patient sera (Brett *et al.*, 1992), although this is not

definitive. Chronic infection is associated with a worsened clinical status (Lee *et al.*, 2003) and the emergence of certain phenotypes that are common to chronic CF infections but not environmental or acute infection isolates of *P. aeruginosa*, presumably representing the adaptations to a uniquely challenging environment.

4.1.2 Phenotypes of *P. aeruginosa* isolated from chronic CF infections

Isolates obtained from chronic *P. aeruginosa* infections often have characteristic phenotypes, including auxotrophy, hypermutability, resistance to anti-pseudomonals, loss of motility, quorum–sensing deficiency and mucoidy (Hogardt & Heesemann, 2010), all representing a divergence from the so called "wild-type" which causes acute infections. Indeed, such chronic infection isolates actually have a reduced ability to cause acute infections (Lorè *et al.*, 2012). Some of the most commonly affected phenotypes are discussed in the following subsections:

4.1.2.1 Auxotrophy

An auxotroph is a mutant microorganism that has nutritional requirements that differ from the wild-type from which it is derived. The most common example of auxotrophy is the inability of cells to grow in the absence of certain amino acids. CF sputum is especially rich in amino acids (Barth & Pitt, 1996), so amino acid synthesis may become unnecessary or even undesirable, as it is metabolically expensive. The presence of auxotrophic mutants has been correlated with the severity of disease (Taylor *et al.*, 1993; Thomas *et al.*, 2000), although this may occur indirectly through an increase in the sputum amino acid content in severe disease. However, a correlation between amino acid concentration and the numbers of auxotrophic mutants was not observed (Thomas *et al.*, 2000). A recent study investigated the metabolic adaptation of *P. aeruginosa* isolates in more detail, and found that whilst isolates developed more efficient use of the majority of compounds, it was largely dependent on the cost of synthesis, with a correlation between the cost of a compound and the efficiency with which the bacteria utilised it (Behrends *et al.*, 2013).

4.1.2.2 Biofilm formation

Biofilm production is often altered in chronic CF isolates; some display enhanced biofilm formation (Head & Yu, 2004), whereas other non-mucoid isolates display reduced surface biofilm formation in comparison to PAO1, in addition to an altered biofilm architecture (Lee *et al.*, 2005a). However, mucoid isolates, which over-produce the polysaccharide alginate and are considered a hallmark of chronic infection, are associated with enhanced biofilm formation; upon examination of CF sputum, the mucoid cells in a mixed population of *P. aeruginosa* can be observed in aggregates, surrounded by an extracellular matrix (Yang *et al.*, 2008). Production of another biofilm-associated exopolysaccharide, Psl, is increased early on in chronic infection (via multiple pathways), resulting in enhanced biofilm formation (Huse *et al.*, 2013). Biofilm formation is a clinical concern, not least because isolates can be more resistant to antimicrobials when growing as a biofilm (Aaron *et al.*, 2002; Hill *et al.*, 2005; Kirchner *et al.*, 2012). Such tolerance is likely due to a combination of factors, including anaerobic conditions that make many antipseudomonals ineffective (Borriello *et al.*, 2006), and upregulation of the *blrR* gene, a regulatory gene that mediates antimicrobial resistance in biofilms (Liao & Sauer, 2012).

4.1.2.3 Hypermutability

Hypermutable strains have an elevated mutation rate, due to a defect in DNA repair or error avoidance, most commonly the methyl-directed mismatch repair system (Oliver *et al.*, 2000). Mutations in the *mutS, mutL, mutH* or *uvrD* genes can all cause this phenotype (Oliver *et al.*, 2002). Hypermutators are rare in acute *P. aeruginosa* infections (Oliver *et al.*, 2000), but common in chronic infections such as CF, with frequencies as high as 42% of isolates (Feliziani *et al.*, 2010). However, there is certainly variation between patients; one study found 38% of isolates to be hypermutable in one individual, whereas in another individual, only one hypermutator was isolated at the beginning of the study. Isolates obtained from this individual at later timepoints were all non-mutators (Cramer *et al.*, 2011). Similar variation is observed in LES-infected patients; whilst the majority of LES isolates are non-mutable, in some individuals, hypermutable LES isolates are found at high frequency (Mowat *et al.*, 2011).

A high mutation rate is often considered detrimental (Giraud et al., 2001b), due to the risk of accumulating deleterious mutations; indeed, when mutator and non-mutator strains are competed *in vitro* and *in vivo*, in a mouse model of chronic lung infection, the mutator strains display lower fitness (Montanari *et al.*, 2007). However, when additional selective pressures are present, a hypermutable phenotype can be advantageous, enabling quicker adaptation to antibiotic pressure (Alcalá-Franco *et al.*, 2012) or a change in nutrient source (Le Bars *et al.*, 2014). The CF lung is a heterogenous environment, and a high mutation rate can confer increased fitness in novel or diverse environments (Weigand & Sundin, 2012). Similarly, experimental evolution with *E. coli* mutator strains revealed the mutator phenotype to be important for colonising and adapting to a new environment (Giraud et al., 2001a), and they have a selective advantage in an *in vivo* infection (Labat *et al.*, 2005).

The prevalence of hypermutators has clinical significance; they are strongly correlated with increased antibiotic resistance (Maciá *et al.*, 2005; Plasencia *et al.*, 2007) and increased morbidity (Ferroni *et al.*, 2009). There is some evidence that hypermutators become more common as chronic infection progresses; they are more common in lengthy infections/ older patients (Ciofu *et al.*, 2005) and are at a higher frequency in end-stage chronic infections (Ciofu *et al.*, 2010).

Phage pressure is a possible reason for the high frequency of hypermutators in chronic *P. aeruginosa* infections; bacteria evolving in the presence of lytic phage are more likely to be hypermutable, and, consequently, more likely to develop resistance to phage (Pal *et al.*, 2007).

4.1.2.4 Motility

Environmental isolates of *P. aeruginosa* are usually motile, yet chronic CF isolates have frequently lost motility (Lee *et al.*, 2005a; Mahenthiralingam *et al.*, 1994; Smith *et al.*, 2006b); a recent study found approximately half of CF isolates to be motility mutants (Workentine *et al.*, 2013). Similarly, a study of end-stage mutator strains found that approximately 1/3 of chemotaxis genes were downregulated, suggesting reduced motility (Hoboth *et al.*, 2009).

Piliation and flagellation are proposed to be important for colonisation: type IV pili are necessary for twitching motility, biofilm development (Klausen *et al.*, 2003b) and phage transduction (Craig *et al.*, 2004); flagella are necessary for swimming and the multi-cellular swarming motility (Verstraeten *et al.*, 2008), enabling rapid host colonisation. However, once infection is established, such appendages become disadvantageous, as they require energetic investment and are targets of bacteriophages (Ceyssens *et al.*, 2009b; Chibeu *et al.*, 2009) and the immune system (Patankar *et al.*, 2013; Patel *et al.*, 1991). The LES phages are known to require the type IV pili for infection (James *et al.*, 2012), and the lytic pilus-specific phage PP7 is known to drive loss of twitching (Hosseinidoust *et al.*, 2013a) and swimming motility in *P. aeruginosa* (Brockhurst *et al.*, 2005; Hosseinidoust *et al.*, 2013a). Bacteriophages can also drive loss of motility in alternative ways; temperate *P. aeruginosa* bacteriophages are known to inhibit motility through CRISPR-Cas mediated interactions with the host cell (Zegans *et al.*, 2009) and through the expression of phage proteins that prevent pilus biogenesis (Chung *et al.*, 2014).

4.1.2.5 Antibiotic resistance

Antibiotic resistance is a particularly problematic phenotype that is associated with chronic infection. Patients are constantly exposed to high levels of antimicrobials, but without clearance of the infection. This presumably exerts strong selective pressures on the *P. aeruginosa* populations residing in the lung, leading to the development of antimicrobial resistance.

P. aeruginosa naturally has a high level of intrinsic antibiotic resistance and is resistant to many beta-lactams, fluoroquinolones and aminoglycosides. However, there are several antipseudomonals that the "wild-type" is susceptible to, but to which resistance will often develop in chronic CF infection. Resistance occurs by two mechanisms: adaptive and acquired.

Adaptive resistance involves changes in patterns of gene expression and can be induced by the environmental conditions, such as presence of extracellular DNA (Mulcahy *et al.*, 2008) and antibiotics (Daikos *et al.*, 1990; Fernández *et al.*, 2010), or behaviour e.g. biofilm mode of growth (Høiby *et al.*, 2010a) or swarming motility

(Overhage *et al.*, 2008b). However, adaptive resistance is not heritable and will not persist long-term after removal of the stimulus.

Acquired resistance, in contrast, is heritable, and can be mutational or transferred horizontally on genetic elements, such as plasmids and phages. There is a strong link between hypermutability and mutational resistance (Plasencia *et al.*, 2007)**,** and it may help explain why CF isolates demonstrate such high levels of antibiotic resistance. Mutations in genes associated with efflux pumps or target enzymes are common, as are mutations leading to over production of beta-lactamases; all lead to clinically meaningful resistance.

4.1.2.6 Loss of social behaviour

Many cooperative behaviours in bacteria are controlled by quorum sensing (QS), but "cheats", that either do not produce, or respond to the signalling molecules can arise. They benefit from "public goods" produced by non-cheats, and by not producing these costly molecules themselves, may gain a fitness advantage and invade the population (Diggle *et al.*, 2007a). Given that QS regulates the expression of multiple virulence factors, loss of QS is associated with attenuated virulence (Pearson *et al.*, 2000). Loss of social behaviour is common in chronic CF isolates; they are often QS mutants (Marvig *et al.*, 2014; Mowat *et al.*, 2011; Smith *et al.*, 2006b), and there is evidence that levels of QS molecules, and the associated reduced secretion of exoproducts, are reduced in accordance with the length of infection (Jiricny *et al.*, 2014; Lee *et al.*, 2005a).

4.1.3 Phenotypic diversity of chronic isolates

There have been numerous studies characterising multiple isolates from sequential sputum samples, sometimes in multiple individuals, and it has become apparent that whilst there is a trend towards the phenotypes mentioned in the previous section, chronic *P. aeruginosa* populations display incredible phenotypic and genetic diversity, even within a host. Wilder *et al.* studied a panel of isolates from 8 individuals, and found that whilst 7/8 had a large proportion of QS deficient isolates, they were also incredibly diverse, suggesting diverse selective pressures (Wilder *et al.*, 2009). Indeed, a study of multiple LES isolates, in multiple samples, from multiple individuals, found huge phenotypic diversity, with greater diversity within

individuals than between them (Mowat *et al.*, 2011), a finding that also holds true for non-LES isolates (Ashish *et al.*, 2013a). Workentine *et al.* considered a larger range of phenotypes in multiple samples from just one individual, and observed huge phenotypic diversity within samples (Workentine *et al.*, 2013). However, the route of evolution towards these "chronic" phenotypes is not well understood.

One proposed explanation for *P. aeruginosa* diversity in the CF lung is the spatial heterogeneity of the environment. Sequencing of multiple isolates of the DK1 lineage (commonly isolated in CF clinics in Denmark) obtained from a single patient over time suggested the presence of three sublineages, each with different mutation rates and phenotypic characteristics, and limited parallel evolution between them. The authors speculated that this was due to spatial separation of the lineages, a conclusion that was supported to some extent by analysis of fluids and tissues from different sites within the respiratory tract (Markussen *et al.*, 2014).

The phenotypic heterogeneity of the population in the lungs that occurs through adaptive radiation in chronic infections has important implications for the treatment of *P. aeruginosa*. For example, traditional antibiotic susceptibility testing based on one isolate will give little clue to the rest of the population and result in potentially ineffective antibiotic treatment being administered. There is a need to understand which factors drive this diversification and enable the long-term persistence of *P. aeruginosa* in the lungs.

4.1.4 *P. aeruginosa* diversification in Artificial Sputum Media (ASM)

ASM has been used previously to study bacterial diversification, but only in short-term experiments. *P. aeruginosa* strain LESB58 undergoes extensive diversification when cultured through one cycle of growth (7 days) in ASM in the presence of antibiotics, including altered antibiotic sensitivity, colony morphology and pyocyanin production (Wright *et al.*, 2013). PA14 has been studied in synthetic CF sputum (an alternative to ASM) in the presence of mucin (to change the viscosity) or ciprofloxacin. Evolved isolates, and whole-populations were each competed against the ancestor, but individual genotype fitness was not predictive of population fitness. This indicates diversity within the population, and suggests that that fitness of individual isolates would be a poor measure of assessing the fitness of a population

(Wong *et al.*, 2012). Similarly, when grown as a biofilm for an extended period of time (although not in ASM), PAO1 undergoes genetic diversification, particularly in genes expected to confer a phenotypic change, including motility and alginate synthesis genes (McElroy *et al.*, 2014).

4.1.5 Bacteriophages as drivers of phenotypic diversity

Bacteriophages are known to drive bacterial diversification through numerous mechanisms. Lytic bacteriophages obligately kill their hosts and so place a strong antagonistic selective pressure on bacteria to avoid infection. The "kill the winner" hypothesis posits that the competition specialists in a bacterial population (often, but not necessarily the most abundant) become targets of bacteriophages. The subsequent reduction in the "winners" selects for diversity in the population (Winter *et al.*, 2010). Experiments with the *P. fluorescens*/bacteriophage ϕ2 host – parasite model system (Brockhurst *et al.*, 2007) have provided evidence of how bacteriophages can drive bacterial diversification through antagonistic coevolution, and how this can be influenced by the environment. The diversity between replicate populations is high when bacteria coevolve with phage, but the total diversity (including within population diversity) is not, a fact that is largely driven by the emergence of different phage-resistant variants in different populations (Buckling & Rainey, 2002b). However, phage can drive reduced diversity in spatially heterogenous (but not homogenous) environments, largely through reductions in bacterial densities, which results in less competition for resources and hence less diversification (Brockhurst *et al.*, 2004). The effects of extreme environments and bacterial dispersal have also been investigated using this system; phage increase the diversity in stressful environments (Benmayor *et al.*, 2008), but strongly reduce diversity when in combination with dispersal (compared to the effects of dispersal alone) (Vogwill *et al.*, 2011). In addition, phage can drive the emergence of hypermutable variants (Pal *et al.*, 2007); such a high mutation rate may further accelerate the process of diversification.

Lytic phage can drive diversification through selection for resistant phenotypes. For example, phage-resistant mutants of *P. syringae* have defective type IV pili (Lythgoe & Chao, 2003), a common target of bacteriophages. When *Escherichia coli* and phage PP01 underwent coevolution, phage-resistant variants with altered cell

membranes, as well as some mucoid variants, were observed (Mizoguchi *et al.*, 2003). The colony morphology diversity of *P. aeruginosa* is increased when coevolved with the lytic pilus-binding phage PP7 because of the emergence of resistant variants. Phage-treated populations are also more diverse with regards to motility, phage resistance and preferred environmental niche (Brockhurst *et al.*, 2005).

Most experiments focussing on phage-driven diversification have neglected the effect of temperate phages. Temperate phage can increase the diversity of a bacterial population through the carriage of additional genes during lysogeny, and may lessen the antagonistic selection pressure (relative to lytic phages) through the option of lysogeny. These factors will likely influence phage-driven host diversification.

4.2 Objectives

1. Establish an experimental evolution set-up, with the well-characterised laboratory reference strain *P. aeruginosa* PAO1, in the presence or absence of LES bacteriophages φ2, φ3 and φ4 in ASM.
2. Evaluate the usefulness of ASM as a model of chronic CF infection by comparing the phenotypic trajectory of *P. aeruginosa* in ASM with that in CF lung infections. To achieve this, the phenotypes observed in evolved isolates from the endpoint of this experiment will be compared with those specific to chronic CF isolates, as described in the literature.
3. Quantify and compare the phenotypic diversification of PAO1 in the presence and absence of the LES phages.
4. Address whether end-point bacterial populations are better adapted than the ancestor to the ASM environment with *in vitro* competition experiments.
5. Contrast the phenotypic evolution observed in ASM with an *in vivo* model of chronic *P. aeruginosa* infection.

4.3 Results

Six replicate microscosms (hereafter referred to as populations) were included per treatment. Replicate populations 1-6 were assigned to the control treatment (PAO1 alone in ASM), and 7-12 to the phage treatment (PAO1 + LES φ2, φ3 and φ4).

4.3.1 Bacterial / phage dynamics

4.3.1.1 Bacterial and free phage counts are high and stable through time

Colony forming and plaque- forming units (ml^{-1}) in the biofilm homogenate were quantified at every other transfer. Bacterial counts were high (~10^8 c.f.u. ml^{-1}) and very similar between treatments (Figure 4.1). Free, infective phages were detected at high levels in all populations, with a median phage to bacterium ratio of 1.3 (interquartile range: 0.65 to 2.3).

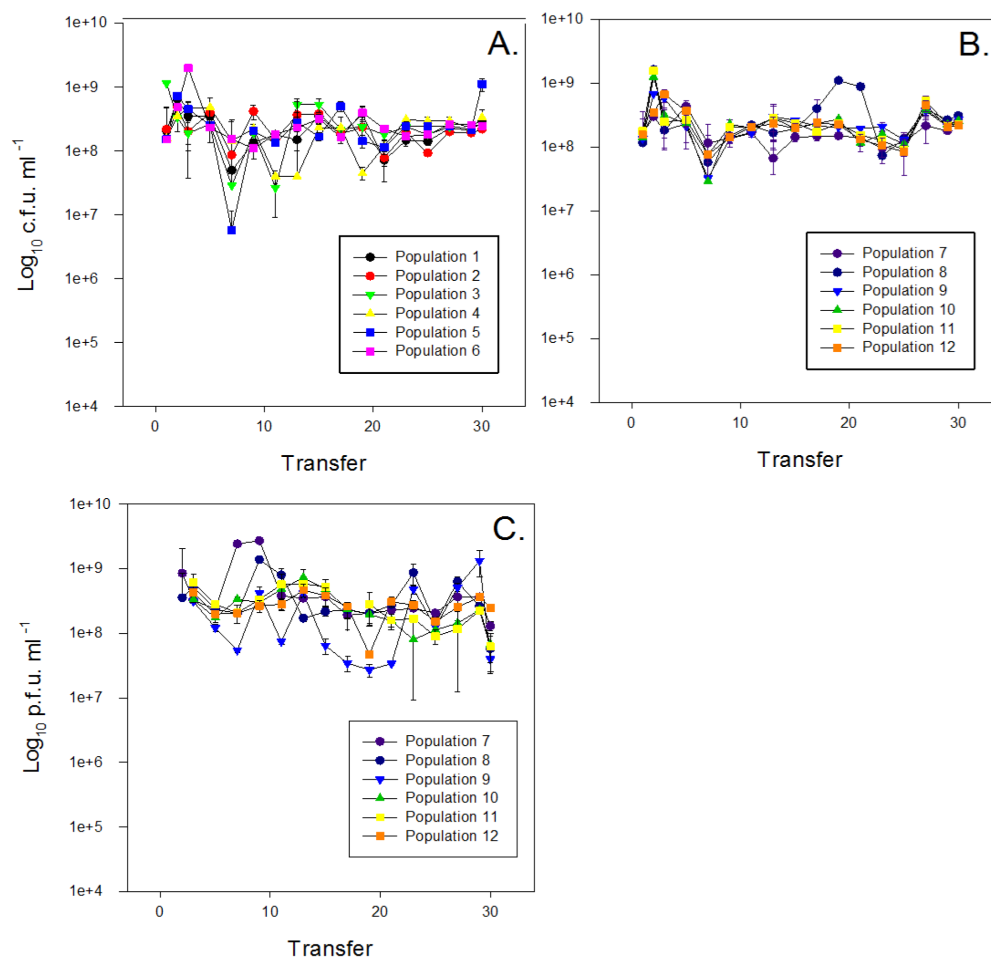


Figure 4.1 Bacterial densities and abundance of free phages over the course of the challenge experiment.(A.) Bacterial densities of the control (phage-free) populations, (B.) Bacterial densities of the phage-treated populations and (C.) Densities of free LES phages. Phage were enumerated by plaque assay, using ancestral PAO1 as the indicator host. Datapoints represent the nean of two technical replicates. Error bars ±1 S.D.

4.3.1.2 Prophage dynamics

Every 5 transfers, 15 colonies per population (phage-treatment only) were screened for prophage complement (40 colonies at the final transfer), using a multiplex PCR assay targeted to each of the LES phages (φ2-4) (Figure 4.2). LESφ4 dominated; three of the populations were almost exclusively single LESφ4 lysogens, and a further two were mostly double lysogens, with LESφ4 in combination with LESφ2 or LESφ3. Population #9 showed a different trend over time, with a large number of non-lysogens, in addition to a small number of LESφ3 single lysogens, and some triple lysogens. The frequencies of the different lysogens remain relatively stable over time. PCR sampling of each control population was negative for LESφ2-4 at every time point.

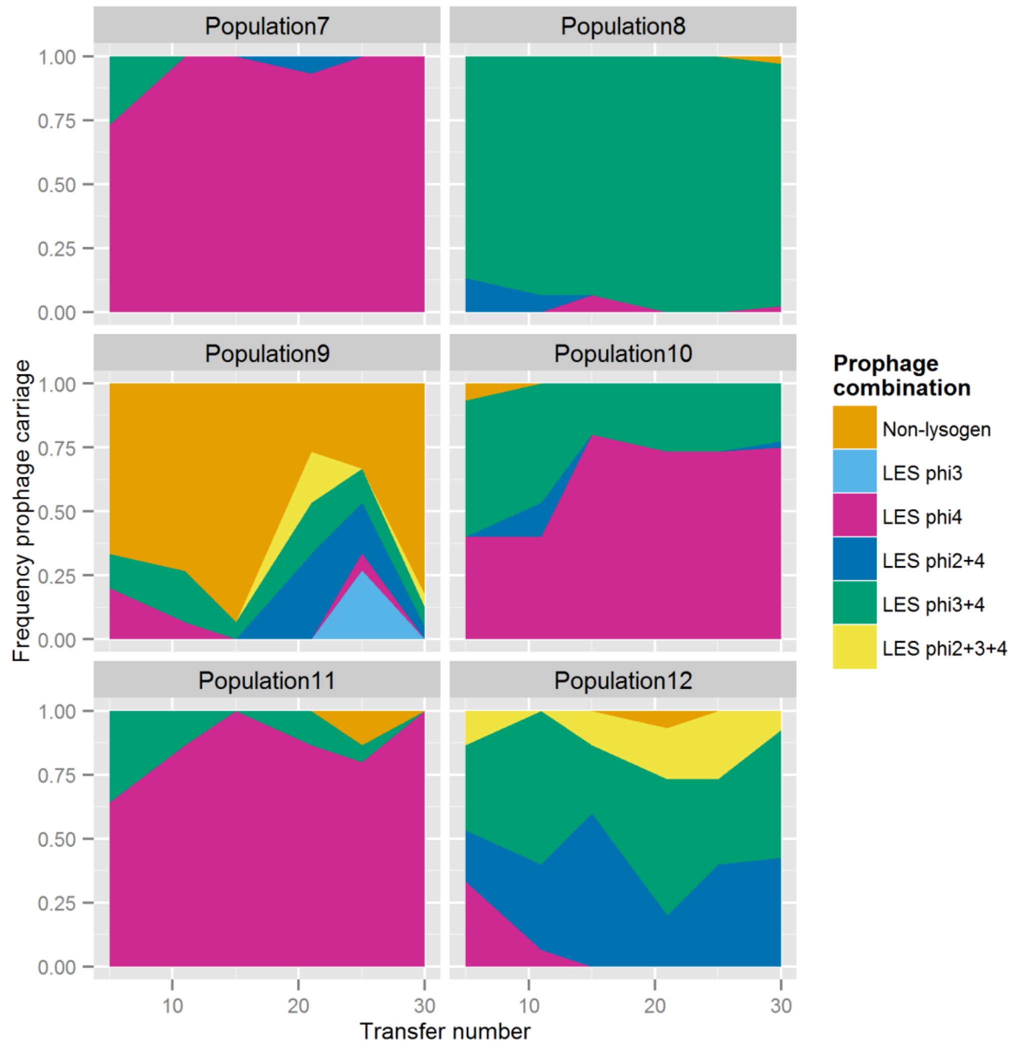


Figure 4.2 Distribution of lysogens in phage-treated populations. A minimum of per 15 isolates per timepoint (every 5 transfers) were PCR screened for the presence of LES φ2, 3 and 4. Area plots (1 per population) show the percentage of isolates testing positive for LES phages, either singularly or in combination.

4.3.1.3 Quantification of individual free phages in endpoint populations

Free phage were quantified at every other transfer by plaque assay, yet this does not differentiate between the three phages. To address this, a qPCR approach was taken to calculate the relative numbers of each LES phage in the DNAse-treated supernatant of end-point populations. LESφ2 was not detected in two of the populations (#7 and #11) but found at high levels in the remaining four populations. (Figure 4.3). LESφ4 and LESφ3 were detected in all populations and the density of LESφ4 was higher than the other phages in all populations. The level of LESφ3 was quite variable between populations, and did not correlate well with the prophage

complement data (i.e. a low frequency of LESφ3 in prophage form does not necessarily lead to a low level of LESφ3 in free form).

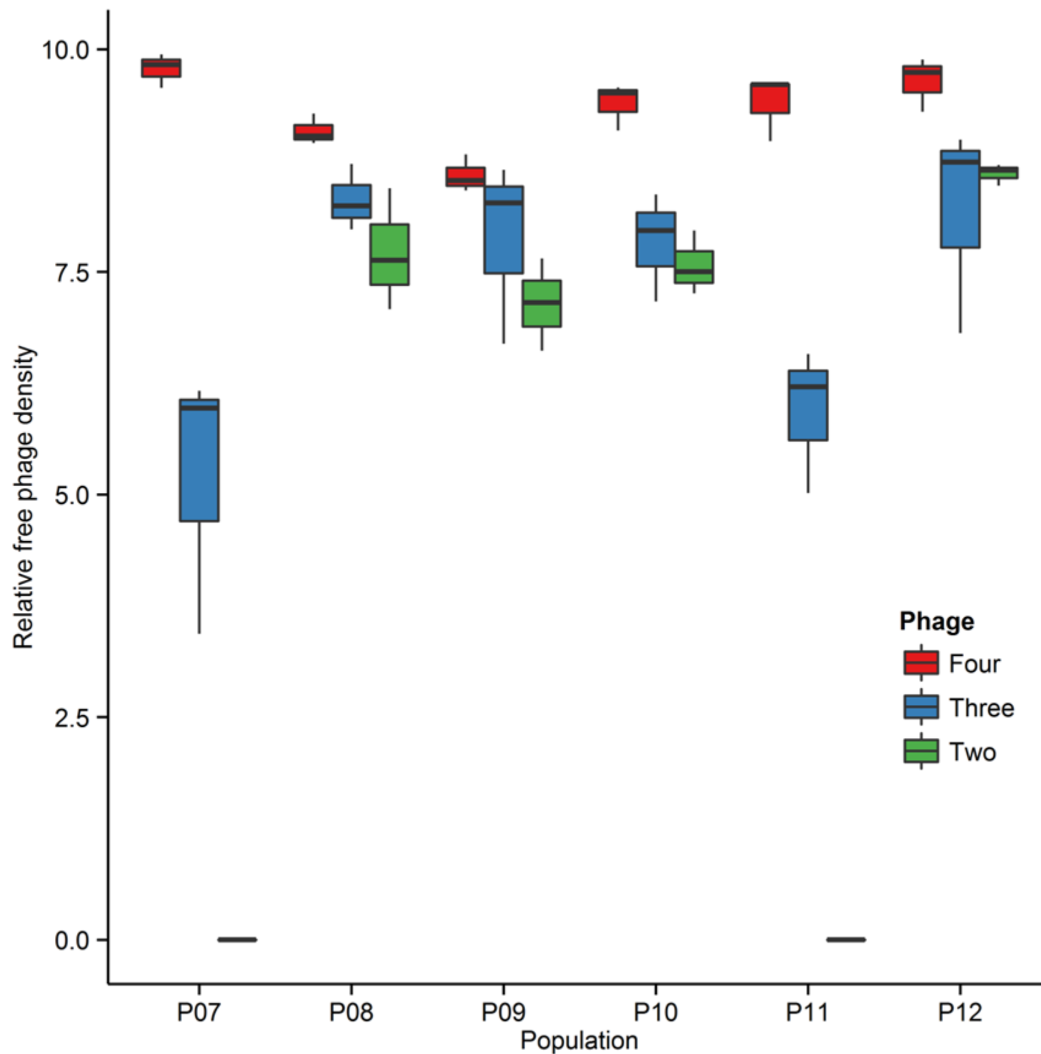


Figure 4.3 Relative densities of each free LES phage in endpoint, phage-treated populations.Relative density is calculated as the $\log_{10}$(copies μl^{-1}+1), as determined by qPCR. Copy numbers per μl were calculated by comparison to a standard curve of known concentrations. The thick horizontal line denotes the median for each population.

4.3.2 Phenotypic diversity of endpoint isolates

4.3.2.1 Exploratory principal component analysis (PCA) of multivariate phenotype data

At the end of the challenge experiment, the 40 isolates selected from each population were characterised for multiple phenotypes. Given the multivariate nature of this dataset, a PCA was performed in order to visualise the data and identify any

relationships between variables, or any obvious differences between the two treatments (Figure 4.4). Data for seven phenotypes were included: swimming, swarming and twitching motility, hypermutability, growth rate, biofilm formation and auxotrophy. The first principal component (which represents the maximum variation in the data) is strongly associated with twitching motility, suggesting that this phenotype explains a large amount of variation in the data.

Two phenotypes, swimming motility and growth rate in LB, are strongly diametrically opposed, suggesting that a slow growth rate is associated with less motility. When the independent variable (treatment) is overlaid on the PCA plot (middle panel), it is apparent that there is a large degree of overlap between isolates from both treatments. There is a degree of clustering for some populations (bottom panel, points coloured according to population), but for others, the isolates are spread across, suggesting a large amount of diversity within the population.

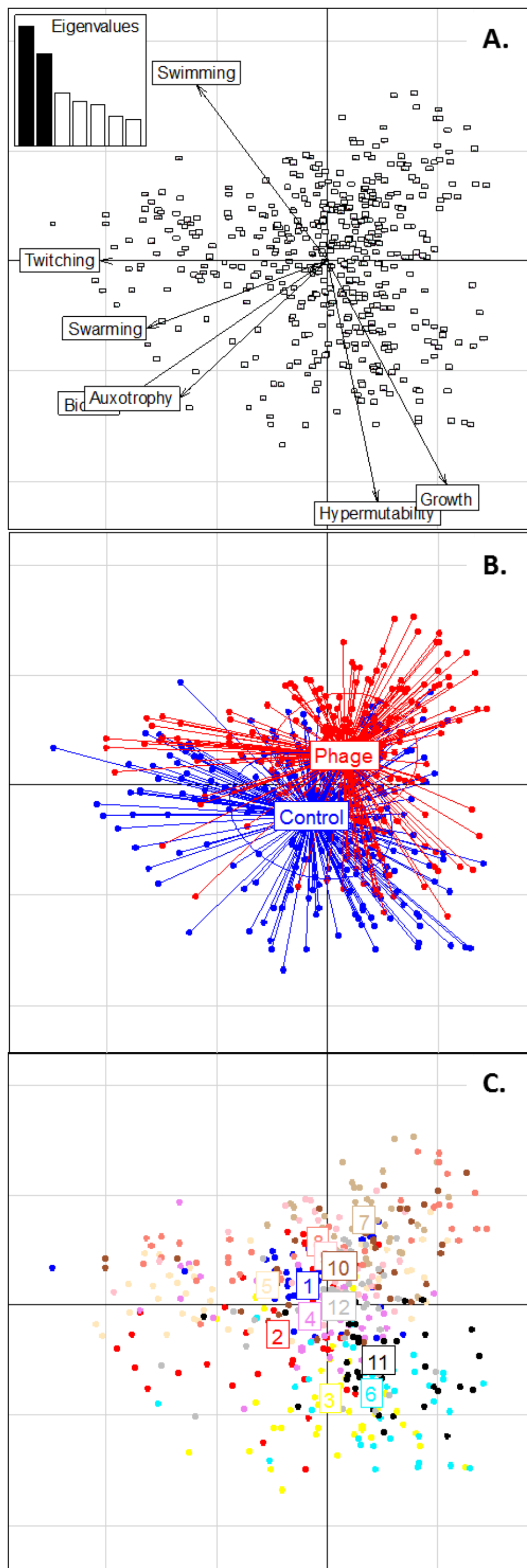


Figure 4.4 Exploratory principal component analysis of multivariate phenotype data.(A.) variable factor map, (B.) individual factor map, labelled with experimental treatment and (C.) individual factor map, labelled with population number. Data for the biofilm and growth variables were incomplete and were predicted with a multiple imputation approach. The Eigenvalues for the first and second components were 2.07 and 1.59, respectively. The “Biofilm” label is partially obscured by the “Auxotrophy” label on the variable factor map.

4.3.2.2 Loss of wild-type phenotype

The PCA highlights the diversity that is seen between isolates in terms of different phenotypes. Scaled continuous measurement data were required for the PCA, but for certain phenotypes it is possible to easily categorise isolates in a dichotomous fashion i.e. wild-type (similar to ancestral PAO1) or mutant (loss/alteration of phenotype, similar to characterised mutant). To this end, we scored isolates as wild-type or mutant (using defined cut-off measurements) for the following five phenotypes: twitching, swimming and swarming motility, ability to synthesise amino acids, and spontaneous mutation rate. Many isolates were impaired for multiple phenotypes, and there was considerable variation in the proportion of mutants in each population (Figure 4.5). There was no difference between the control and phage treatments for any of the phenotypes (Wilcoxon rank-sum test, $P > 0.05$).

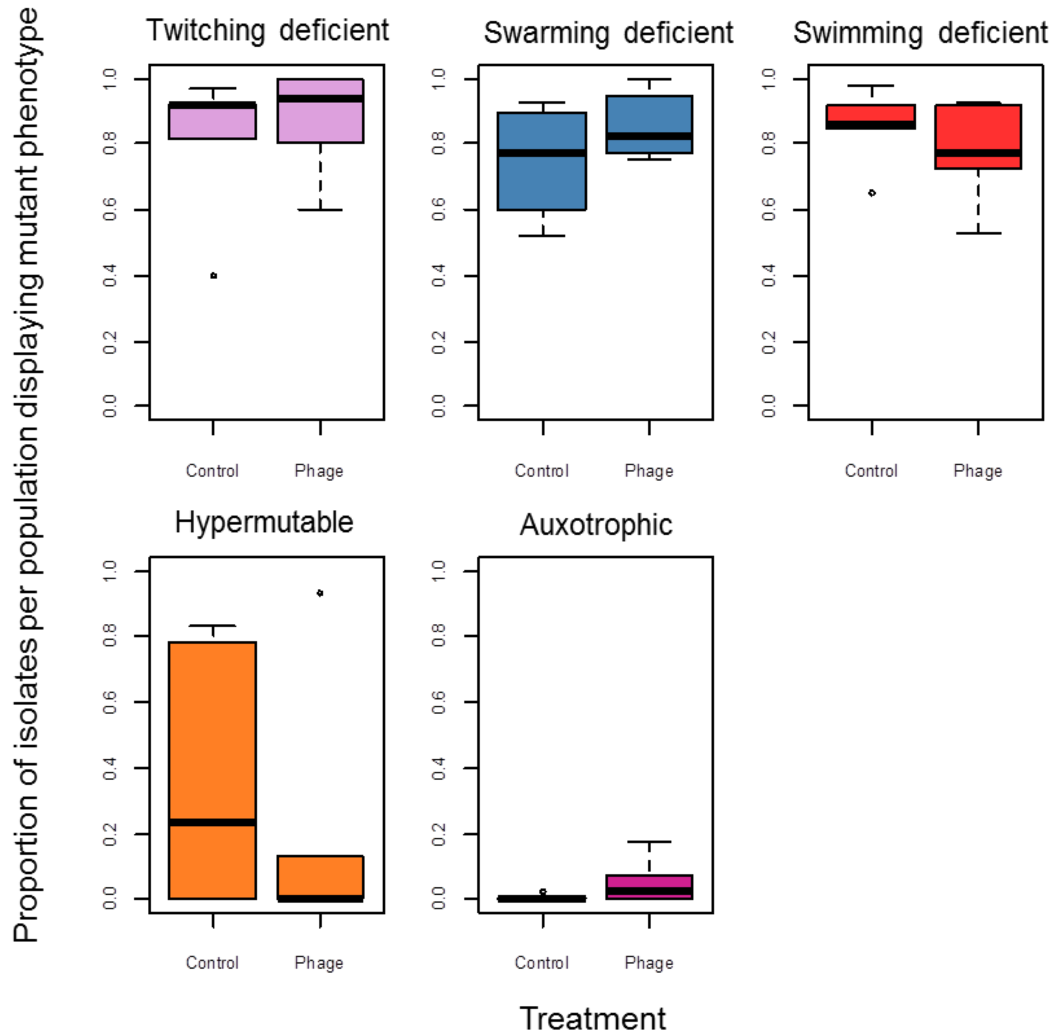


Figure 4.5 Boxplots of the proportion of isolates per population displaying the mutant phenotype. Thick black horizontal lines represent the median, the box represents the upper and lower quantiles, and the circles represent outliers. 40 isolates were tested per populations (6 populations per treatment).

4.3.2.3 Colony morphology diversity

300 isolates from each population were classified as one of 5 morphological sub-types: smooth and straw coloured, small (<0.5mm) and smooth, raised centre, fuzzy spreader or wrinkly spreader. All populations showed evidence of morphological diversification, but whilst there were large variations in inter-population morphological diversity (Figure 4.6), there was no difference in overall diversity between control and phage-treated populations (Wilcoxon rank-sum test: $W = 19.5$, $P = 0.87$).

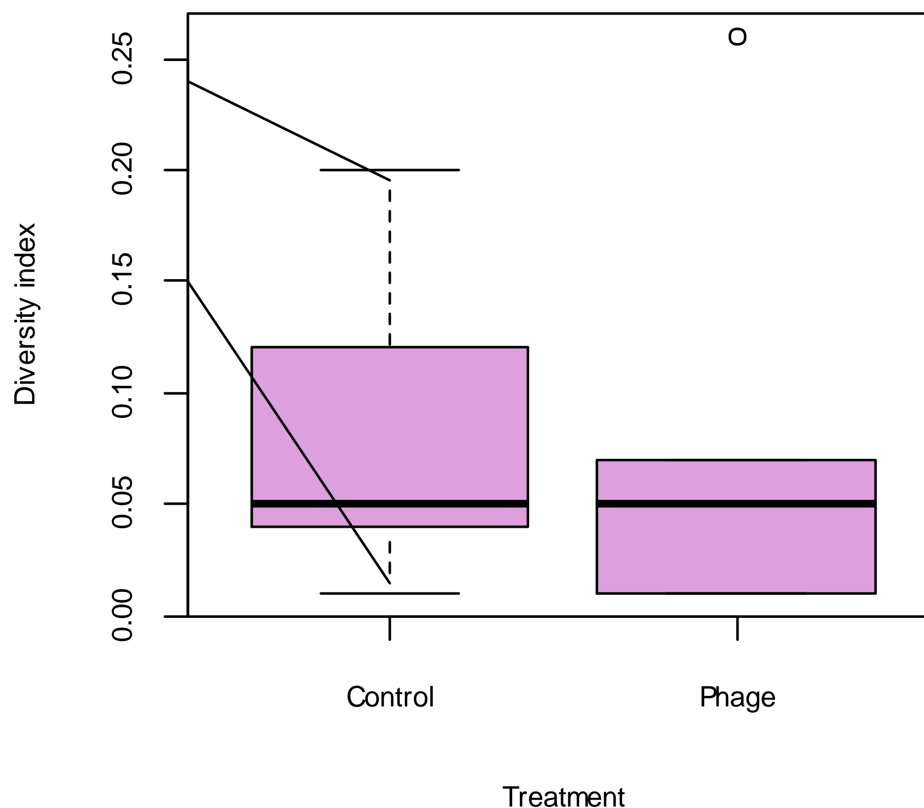


Figure 4.6 Boxplot of the morphological diversity of bacterial populations (n=6). Thick black horizontal lines represent the median, the box represent s the upper and lower quantiles, and the circles represent outliers. Colonies were assigned to 1 of 5 morphologies and Simpson's dominance index (1-(Simpson's index)) calculated as an estimate of population diversity, where 0=no diversity and 1=high diversity. 40 isolates were assessed per population.

4.3.2.4 Phage resistance

Endpoint isolates were tested for resistance to each of the LES phages. Phage resistance was widespread in both treatments (Figure 4.7). The majority of isolates were resistant to all three phages in all but one population (#5), in which 93% of isolates remained susceptible to all three phages.

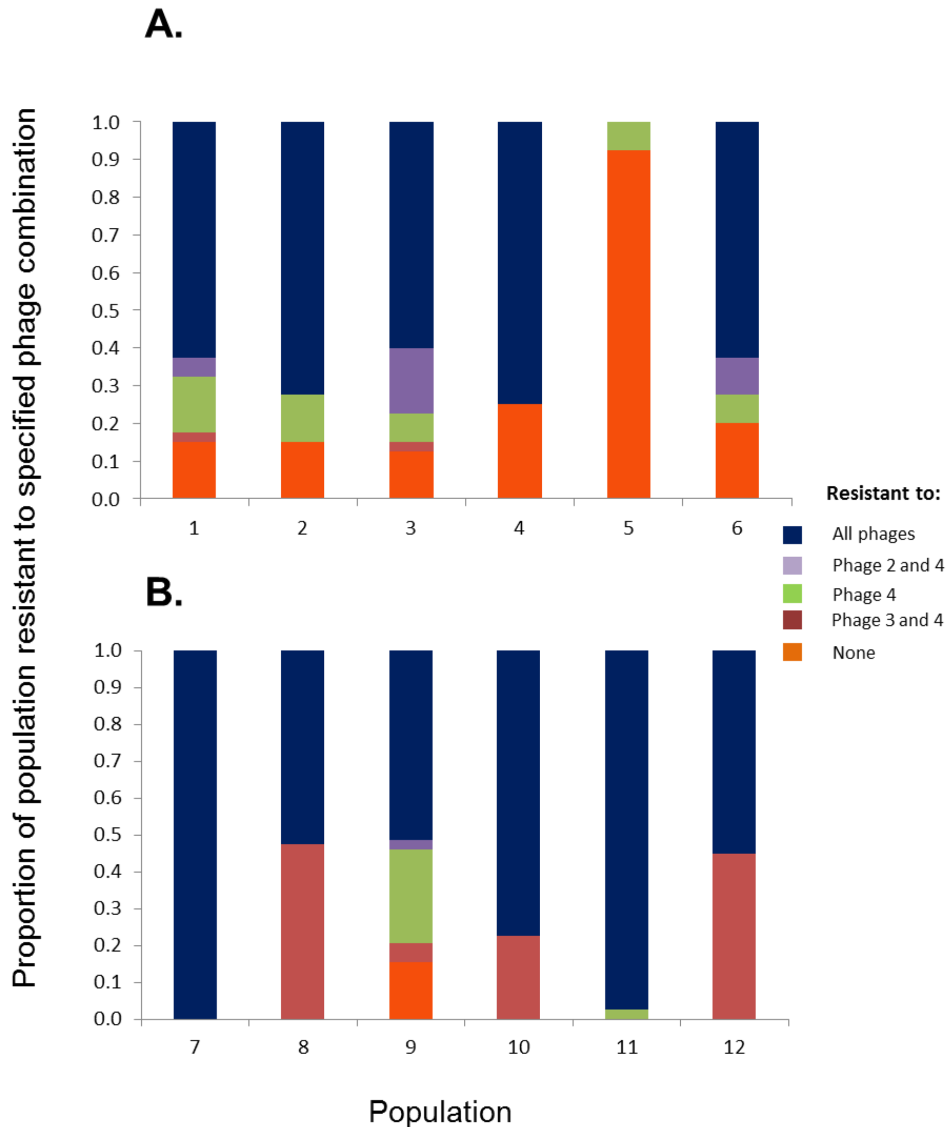


Figure 4.7 Phage resistance of endpoint isolates to LESφ2-4. (A.) Control and (B.) phage-treated populations. Bars are coloured according to the particular phage combination to which resistance was observed. High titre pure phage stocks were spotted onto a soft-agar lawn of each isolate and scored as resistant if no lysis was observed after overnight incubation at 37˚C.

4.3.2.5 Biofilm formation

In order to assess whether isolates evolved in ASM had altered in their capacity to form biofilms, a simple *in vitro* assay was used to measure surface-attached biofilm formation. Due to the inherent variability of this assay, a large number of replicates are required, so it was not feasible to test all the isolates; instead, the first 10 from each population were tested. After staining of the biofilm with crystal violet and solubilisation, the A_{590} was measured as a proxy of biofilm formation, and normalised to the ancestor. Overall, there was no trend towards increased or decreased biofilm formation (Figure 4.8). There was large variability within many

populations, most notably population #3, which has a median biofilm formation 1.5 times that of the control, but included poor and hyperbiofilm producers in the population, resulting in a large range.

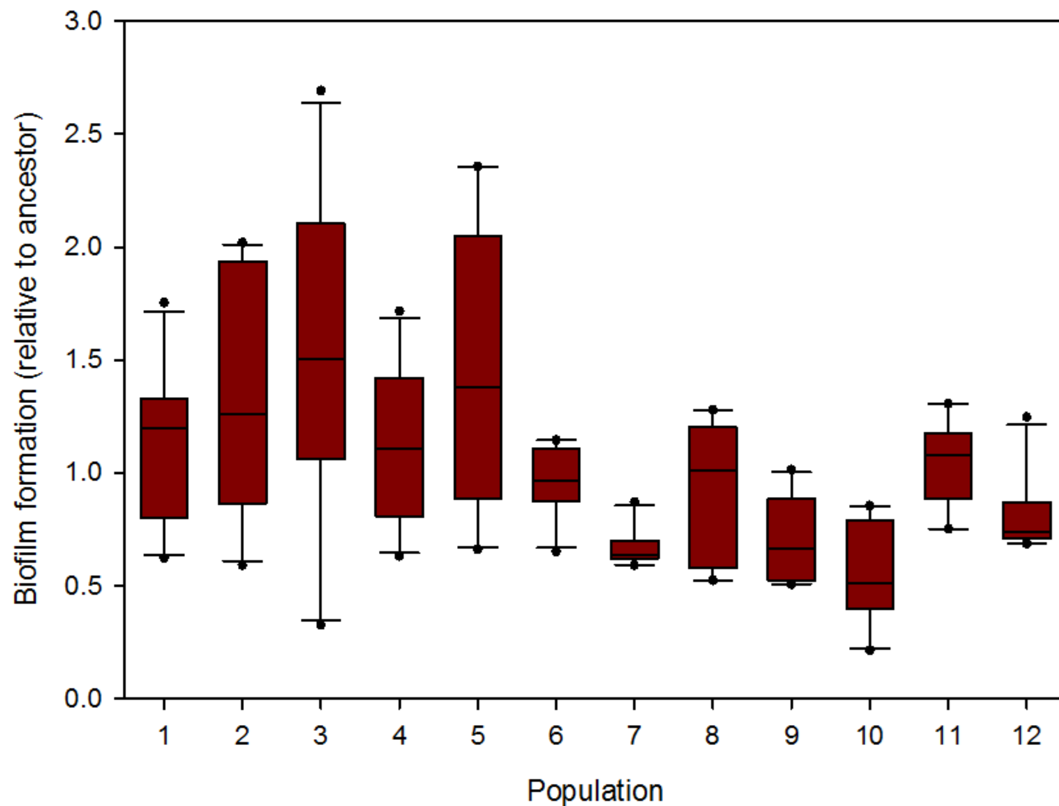


Figure 4.8 Biofilm formation of isolates obtained from endpoint populations. Biofilm formation was assessed by crystal violet staining of a surface-attached biofilm, followed by solubilisation and measurement at A_{590}. Technical replicates were normalised the PAO1 control on the same plate. Two biological replicates were performed for each isolates, and 10 isolates were assayed per population. Boxes represent the interquartile range of the population, the middle black line the median, the whiskers the 5th and 95th percentiles, and the black circles outliers.

4.3.2.6 Antibiotic resistance

Disc diffusion assays revealed all 40 isolates from every population to be as susceptible to the selected panel of antipseudomonals as the ancestor. The method was then adapted to look for clinically resistant isolates that may be present at a low frequency in the entire population, by plating entire populations on agar containing antibiotics at clinical resistance breakpoint levels.

The frequency of antibiotic resistant isolates varied according to population and to antibiotic, with certain populations containing a relatively high frequency of isolates displaying clinical resistance (Figure 4.9); tazobactam-piperacillin resistance was displayed in 0.08% of the population in population 11. Resistance occurred in a higher frequency of cells in this population than in the mutator strain, PAO1Δ*mutS*. Resistance to ceftazidime and tazobactam-piperacillin arose more frequently than resistance to other antibiotics. No meropenem resistant isolates were identified.

In order to assess the extent to which the mutator phenotype of the isolates is responsible for the high frequencies of antibiotic resistant cells, the proportion of hypermutators in a population was plotted against the (arcsine transformed) frequency of resistant isolates (Figure 4.10). This was done for ceftazidime and tazobactam-piperacillin, as these were the antibiotics to which the highest level of resistance was detected. A correlation was detected between the frequency of hypermutators and tazobactam-piperacillin resistance (Spearman's rank correlation coefficient: $\rho = 0.69$, $p < 0.05$), and between the frequency of hypermutators and ceftazidime resistance (Spearman's rank correlation coefficient: $\rho = 0.72$, $p < 0.01$).

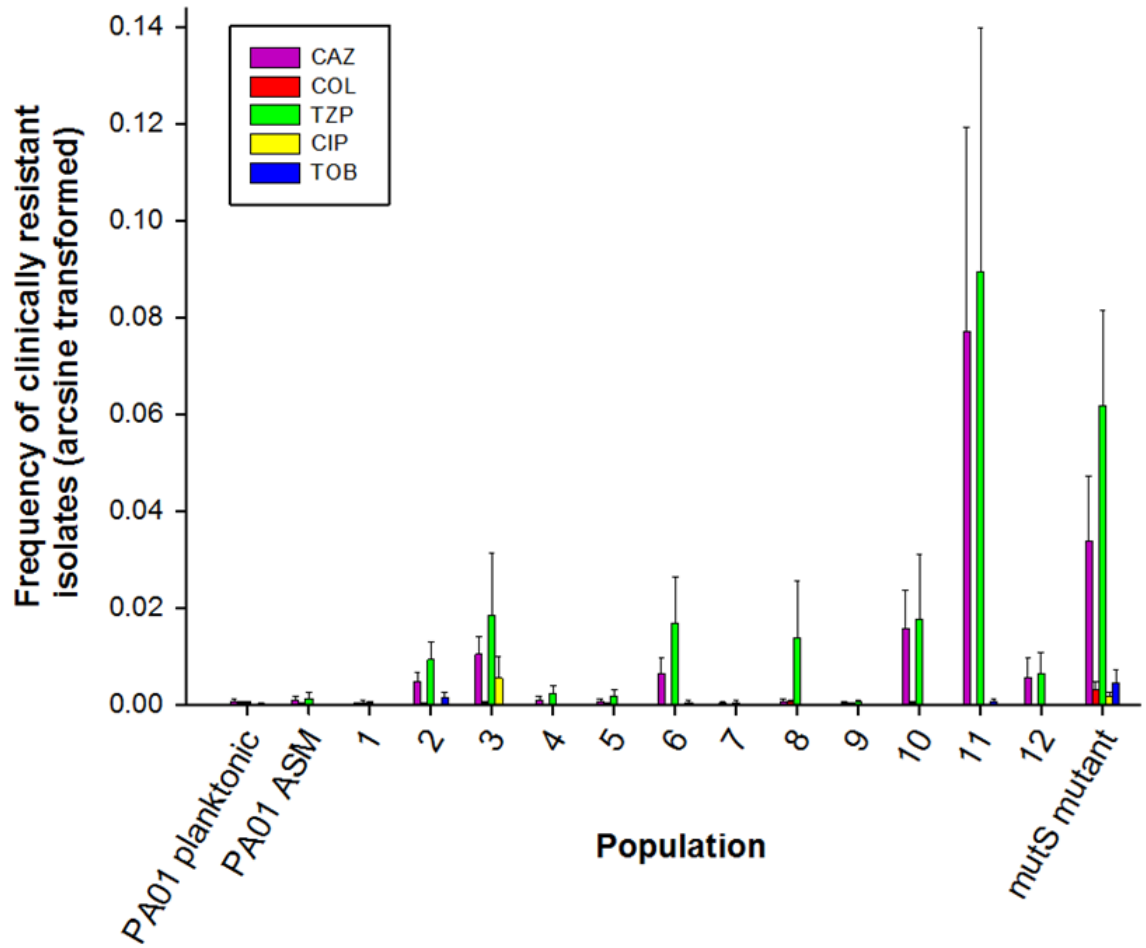


Figure 4.9 Frequency of bacterial cells in whole populations displaying clinical resistance to a panel of anti-pseudomonals (arcsine transformed). Frequencies were calculated after plating endpoint populations onto antibiotic free media, and media containing antibiotics at the clinical resistance breakpoint level. Planktonic PAO1 and PAO1 grown in ASM were included as negative controls. PAO1 Δ*mutS* was included as a reference. Error bars ±1 S.E.M.

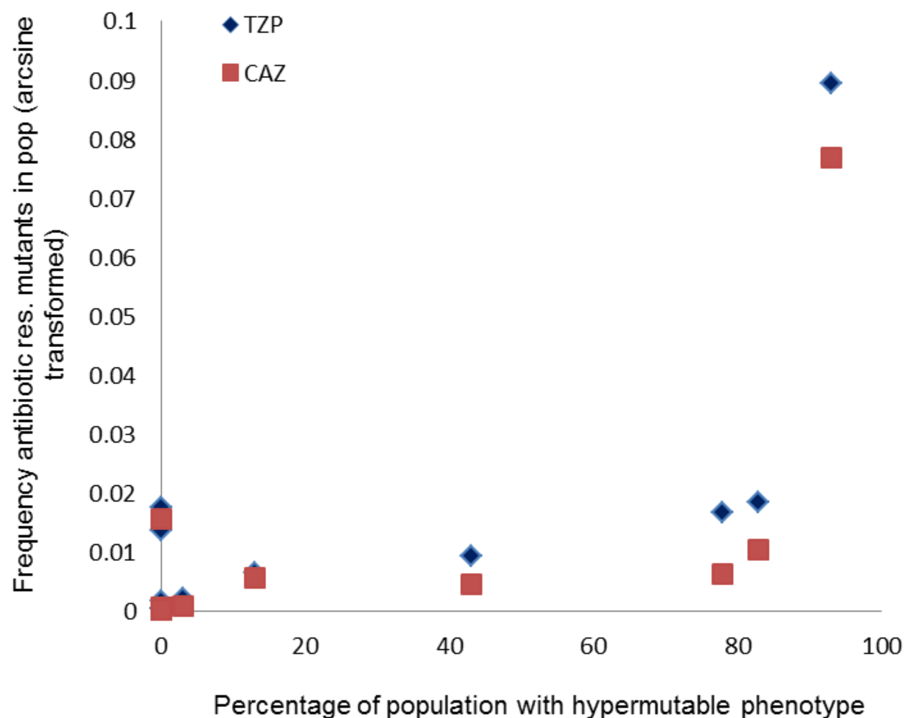


Figure 4.10 The correlation between the frequency of hypermutators in a population and the frequency of isolates (arcsine transformed) demonstrating clinical resistance to the antibiotics ceftazidime and piperacillin/tazobactam (*n*=12).

In order to further characterise the nature of the spontaneously occurring antibiotic resistance, resistant colonies growing on ceftazidime or tazobactam-piperacillin from population #11 were isolated and MICs to both antibiotics determined (n = 20). The MICs were at least two-fold higher than the clinical resistance breakpoints, and there was a high degree of cross-resistance; all isolates found to be resistant to one antibiotic were also resistant to the other (Table 4.1). This suggests a resistance mechanism common to both antibiotics. Ceftazidime and piperacillin are both beta-lactam antibiotics, and certain beta-lactamase enzymes are capable of degrading both, even in the presence of tazobactam (an inhibitor). In order to clarify if beta-lactmases were being produced by these isolates, a beta-lactamase assay was conducted on five randomly selected isolates from each antibiotic medium, using nitrocefin, a chromogenic substrate for beta-lactamases.

Beta-lactamase production was observed in all the isolates tested, to varying degrees, with no clear difference between isolates originally isolated from media containing ceftazidime or tazobatam-piperacillin (Figure 4.11).

Table 4.1 Minimum inhibitory concentrations (as determined by broth microdilution) of PAO1 isolates displaying resistance to ceftazidime or tazobactam-piperacillin, to each antibiotic.

Isolated from	MICs CAZ (mg L^{-1})	MICs TZP (mg L^{-1})
CAZ	32-128	128-256
TZP	32-64	128

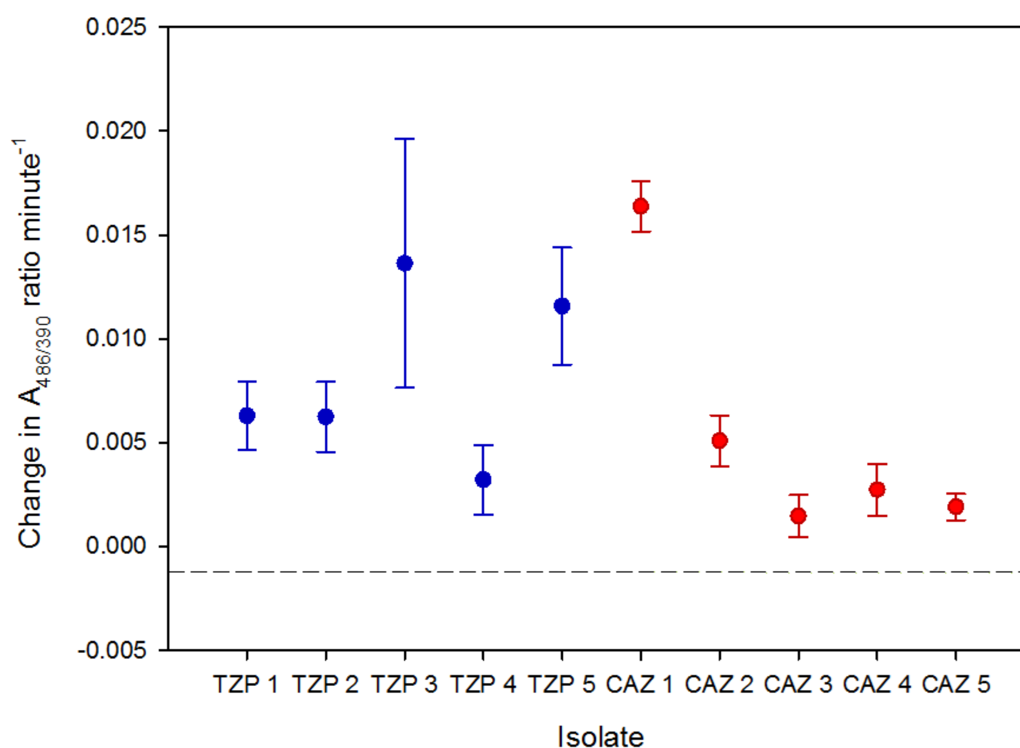


Figure 4.11 Beta-lactamase production in ceftazidime and tazobactam-piperacillin resistant isolates. Activity was measured using nitrocefin, in a chromogenic assay by calculating the change in $A_{486/390}$ per minute. The dashed line indicates the value of the negative control (PAO1). The MIC of CAZ required for all isolates tested that were originally isolated from CAZ was 64 µg ml^{-1}.

4.3.2.7 Analyis of Molecular Variance (AMOVA)

4.3.2.7.1 Haplotype diversity

Isolates were classified as different haplotypes based on the combination of phenotypic traits displayed. For each trait, isolates were scored a 0 if they displayed the same phenotype as the ancestor, or a 1 if they displayed a mutant phenotype. A haplotype in this instance was defined as a unique combination of phenotypic traits, and the frequency of each haplotype in each population was determined.

57 different haplotypes were identified in this study, based on 8 phenotypic traits (Figure 4.12). The haplotype of the ancestor (haplotype 5) was very rare in evolved populations; only 8/480 isolates shared the ancestral haplotype, all of which were in the control populations. The most common haplotype (haplotype 4) was found in almost a quarter (111) of isolates across all but one population, and was characterised by loss of all three types of motility and resistance to all three LES phages, but displaying WT phenotypes of normal mutation rate and ability to synthesise amino acids.

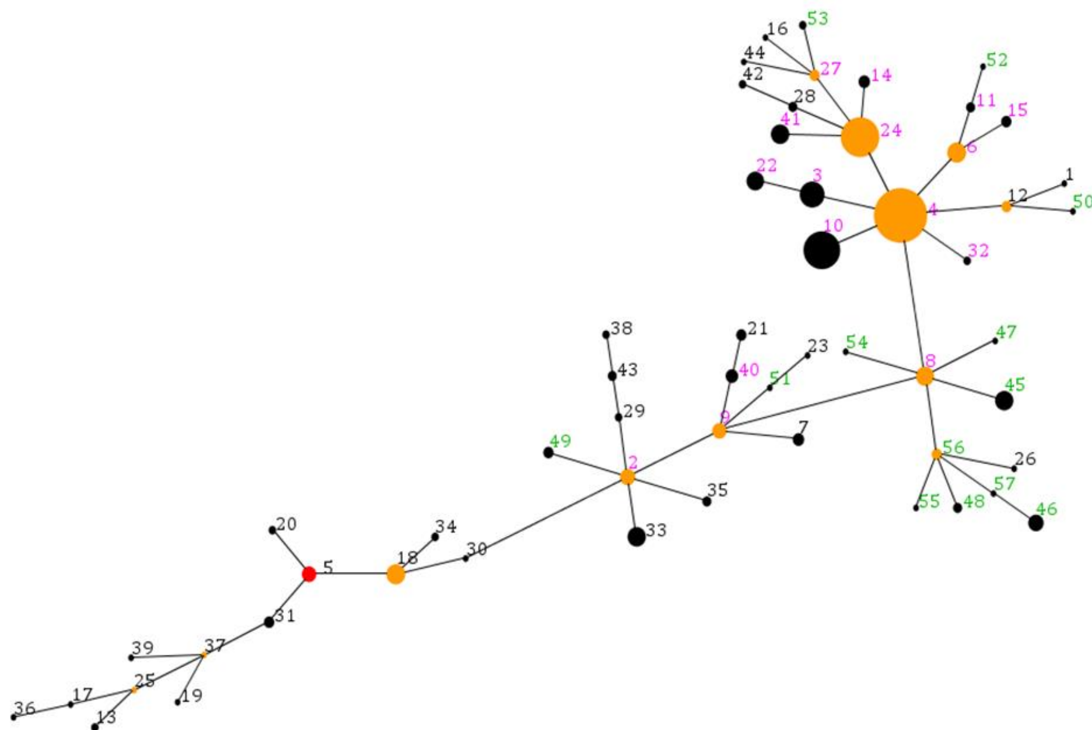


Figure 4.12 eBURST diagram reflecting diversity of phenotypic isolates obtained from end-point populations. A haplotype was defined as a unique combination of 8 phenotypic traits (auxotrophy, resistance to each LES phage, hypermutability and swimming, swarming and twitching motility). Each

dot represents a single haplotype and the size of the dot reflects abundance. The ancestral haplotype is labelled in red. Sub group founders are labelled in yellow. Haplotypes found only in the control treatment are labelled in black, haplotypes found only in the phage treatment are labelled in green, and haplotypes observed across both treatments are labelled in pink. Haplotype numbers are arbitrary.

The ancestral haplotype (haplotype 5) is phenotypically identical to PAO1. It is relatively low in abundance and is found only in the control treatment. Haplotype 41 is the most divergent to the ancestor in that it differs in every phenotype.

4.3.2.7.2 AMOVA

The phenotypic distance between haplotypes was calculated, and the distance matrix was partitioned into sub-matrices for the various subdivisions of the data, including treatment (control or phage-treated), population and individual isolates. The proportion of variance attributable to each sub-division was estimated. Treatment group explained only 2% of the total variance; the majority of the variance (74%) was within populations (Table 4.2).

Table 4.2 Analysis of Molecular Variance of the phenotypic diversity of haplotypes identified in endpoint populations. Variance was partitioned into within and between populations, and between treatments. Significance was tested by Monte-Carlo permutation tests (49 permutations).

Variance component	σ^2 Variance	% of total variance (100)	*P*	Alternative hypothesis
Within populations	0.773	74	<0.02	Less than
Between populations (within treatment)	0.183	24	<0.02	Greater than
Between treatments	0.015	2	0.26	Greater than

4.3.3 Competitive assays of evolved populations

To deduce whether evolved (endpoint) populations had adapted to the ASM environment, whole populations were competed against the ancestral PAO1 isolates, in conditions identical to one transfer of the challenge experiment, and the selection rate constant (r_{ij}) was calculated for each competition. The r_{ij} was >0 for all competitions which indicates that the evolved populations are more competitive in ASM than the ancestor.

The mean r_{ij} of the phage-treated populations was higher than the controls (1.58 versus 1.10 day^{-1}). To remove the competitive effect conferred by the LES phages (through prophage-mediated lysis), populations were also competed against PAO1 *pilA*$^-$, which is resistant to the LES phages. (Figure 4.13).

All evolved populations from both treatments were fitter relative both to PAO1 and *pilA*$^-$ (Figure 4.13; one-sample t-test of each treatment-competitor combination against an expected value of 0 (no selection); all significant at an alpha-level of 0.0125). However, when PAO1 *pilA*$^-$ was the competitor, there was a reduction in the mean selection rate constant for both treatments. No difference was observed between PAO1 *pilA*$^-$ and ancestral PAOl (mean $r_{ij} \pm 1$ SEM = 0.26 ± 0.31), suggesting that the observed difference is not due to an inherent adaptive benefit of the *pilA* mutation in ASM. A two way ANOVA was conducted to compare the effect of treatment (phage or no phage) and competitor (PAO1 or *pilA*$^-$) on selection rate constant. There was no effect of treatment ($F_{1,20} = 2.64$, MSE = 0.18, $p = 0.12$) but the main effect of competitor was significant ($F_{1,20} = 35.8$, MSE = 2.46, $p < 0.001$). The interaction of the 2 factors was significant ($F_{1,20} = 8.54$, MSE = 0.59, $p < 0.01$), so one way ANOVAs were conducted to assess the effect of treatment in the presence of each competitor separately. At an α level of 0.025, the effect of treatment was statistically significant when the competitor was PAO1, ($F_{1,10} = 7.12$, MSE = 0.71, $p < 0.025$), but not when the competitor was *pilA*$^-$ ($F_{1,10} = 1.53$, MSE = 0.06, $p = 0.24$).

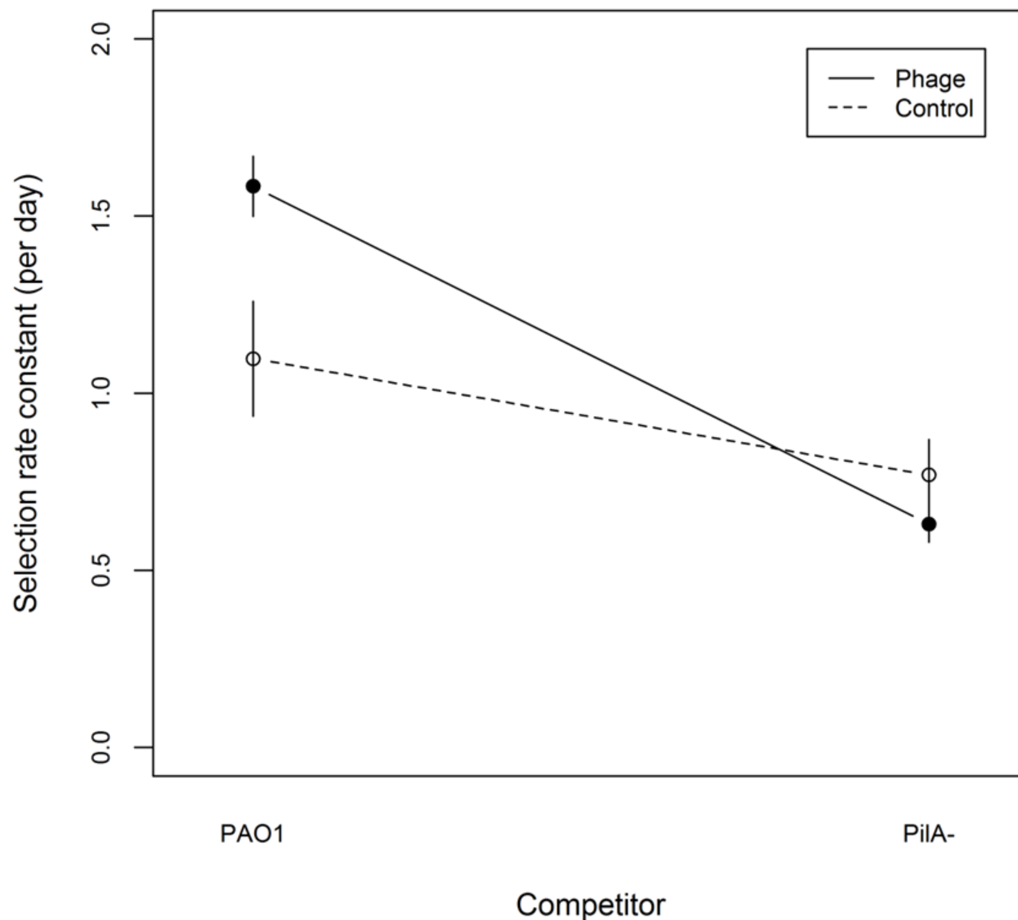


Figure 4.13 Interaction plot displaying treatment and competitor effects on population fitness. The selection rate constant was determined as a proxy for population fitness for each population when in competition with either ancestral PAO1, or an isogenic phage-resistant competitor, PAO1*pilA*$^{-}$. Data points represent the mean ± 1 S.E.

4.3.4 *In vivo* coevolution of PAO1 and LESφ2-4

In order to compare the trajectory of *P. aeruginosa* population evolution and role of temperate phages in an ASM biofilm model with behaviour *in vivo*, a well-established rat chronic lung infection model was used. The experimental set-up was replicated in two groups of 6 rats, which were infected by intubation with agar beads containing PAO1 and free LES phages φ2-4. The experiment ran for 10 days before the rats were sacrificed. Levels of bacteria and free phages in the lungs were quantified. There was a large inter-rat variation in bacterial numbers in the lungs, but the mean bacterial cell counts between the two treatments were quite similar

(geometric mean = 3.8 x 10^6 (control) and 5.4 x 10^6 (phage treatment)). Free phage were detected in the lung homogenate of all phage-treated rats, with the phage to bacterium ratio ranging from 0.004 to 0.4. There was no clear relationship between bacterial densities and the ratio of free phage to bacteria (Figure 4.14). No free phage were detected in any of the control rats.

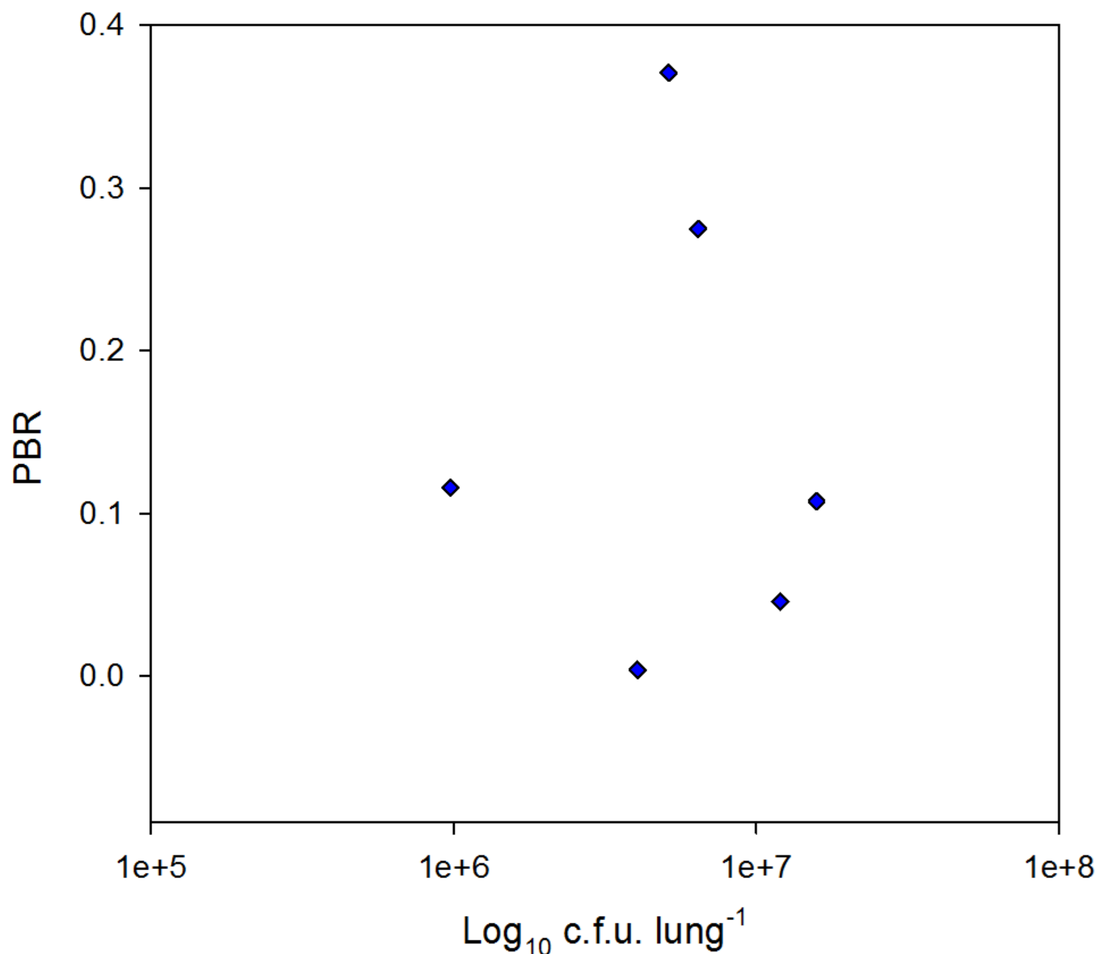


Figure 4.14 The relationship between $\log_{10}$ bacterial densities and the ratio of p.f.u.s to c.f.u.s (PBR) after 10 days in the rat lung. Each individual datapoint represents one replicate (rat) in the phage-treatment only.

Bacterial colonies were screened for their morphology, but no diversity was observed, with all colonies retaining the "smooth" ancestral morphotype. Forty bacterial isolates were selected randomly from each rat and subjected to PCR analysis for prophage complement, and the twitching motility phenotype. LESφ2 and LESφ3 single lysogens were common, yet single LESφ4 lysogens were rare (Figure 4.15). LESφ4 occurred more frequently as double lysogens, in combination with

LESφ2 or LESφ3. Indeed, polylysogeny was a common phenomenon, with double lysogens of all phage combinations, as well as triple lysogens, being detected. All isolates (from both treatments) retained full twitching motility, comparable to the ancestor.

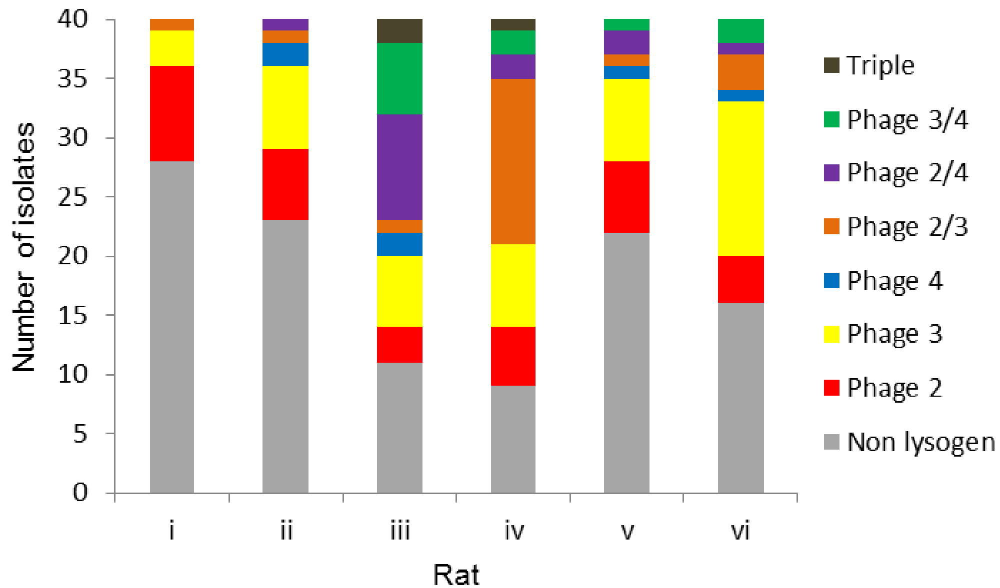


Figure 4.15 Prophage complement of PAO1 isolates obtained after 10 days *in vivo* growth, in the presence of free LES φ2-4 bacteriophages. Bars are coloured according to the number of isolates carrying each specific prophage combination.

4.4 Discussion

In this study, the well-characterised reference strain PAO1 was evolved in two different models of the CF lung, ASM, and the rat model of chronic infection, both in the presence and absence of LES phages φ2, φ3 and φ4. Extensive phenotypic diversification was detected in endpoint populations evolved in ASM, with the emergence of many phenotypes that are common to chronic CF isolates, yet there was no difference between phage-treated and control populations, suggesting that this diversification can occur in the absence of bacteriophages. The majority of the diversity was actually within populations, something that has been observed in many studies of CF isolates (Ashish *et al.*, 2013a; Mowat *et al.*, 2011; Wilder *et al.*, 2009; Workentine *et al.*, 2013).

The parallel phenotypic trajectories that are evident between *P. aeruginosa* evolving in the CF lung and in ASM suggest that ASM could have useful applications as a

model for bacterial behaviour in the CF lung. Despite the lack of antibiotic or immune system pressures in this model, similar phenotypes are detected, suggesting that a large majority of bacterial adaptation in the CF lung may simply be driven by growth in a biofilm. In contrast, no phenotypic diversification was observed in the rat model of chronic lung infection. This may be partly because only two phenotypes were tested (due to time constraints), reducing the likelihood that diversity would be detected. Twitching motility was chosen, as this had a strong correlation with the 1st principal component, in addition to colony morphology diversity, as this is used frequently to assess general diversity in a population (Brockhurst *et al.*, 2004; Buckling & Rainey, 2002b; Korona *et al.*, 1994; Rainey & Travisano, 1998). The lack of diversification is more likely due to the short timescale of the experiment (10 days, in contrast to 120 days in ASM), but the high costs and ethical implications of animal models limit the length of evolution experiments. Despite being proposed as a model of chronic infection (Cash *et al.*, 1979; Kukavica-Ibrulj *et al.*, 2008a), there is no evidence that *P. aeruginosa* grows as a biofilm in the rat agar bead model, and the method of bypassing the host defences by implantation of the beads is unrealistic. In contrast, a novel mouse model of infection that involves a more natural intranasal administration of *P. aeruginosa* has demonstrated that LES isolate B65 can adapt to, and colonise, the nasopharynx, with subsequent "seeding down" to the lungs (Fothergill *et al.*, 2014). It is though that the upper respiratory tract acts as a reservoir of *P. aeruginosa* in CF, allowing bacteria to "pre-adapt" to the lungs (Hansen *et al.*, 2012), which could be an important step in the establishment of chronic infection. The nasopharyngeal mouse model seems to be a more realistic process of infection than the rat model, and can be maintained for a longer period of time (28 days). However, our attempts at establishing colonisation with this model using strain PAO1 were unsuccessful, as mice either cleared the infection completely, or developed an acute infection. At present, ASM offers the best option for conducting long-term evolution experiments.

Bacterial and phage numbers were monitored over time during coevolution in ASM. Bacterial densities were relatively stable, and similar between all populations. Total free phage densities also did not fluctuate markedly over time. The continued existence of phage suggested maintenance of susceptible bacteria (Schrag & Mittler, 1996), allowing continued phage production through lytic infection, and/or phage

production through prophage-mediated lysis. PCR amplification of colonies revealed that LESφ4 was the most dominant prophage, and LESφ2 and LESφ3 lysogens were rare. Likewise, qPCR revealed that LESφ4 was the most abundant free phage in endpoint populations. Interestingly, LESφ2 densities remained relatively high in the populations in which it had not been driven extinct, suggesting continued lytic cycling of LESφ2, or the existence of a small sub-population of LESφ2 lysogens undergoing high rates of spontaneous lysis. Indeed, previous research has shown that PAO1 LESφ2 lysogens have higher rates of spontaneous phage production than the other LES phages (James *et al.*, 2012) and that LESφ2 is frequently the most abundant free phage in the sputa of LES infected individuals (James *et al.*, 2014).

Despite the relatively short timescale of the *in vivo* infection, the LES phages were able to establish lysogeny at quite high frequencies. However, the comparative evolutionary successes of the LES phages in the rat model of chronic infection were in complete contrast to in ASM, with LESφ2 and LESφ3 lysogens common, yet LESφ4 lysogens rare. That the pattern of dominance is so different to ASM suggests that the environment plays an important role in the order that the LES phages can establish lysogeny. LESφ4 in particular does badly *in vivo* when compared to ASM, but there are a host of possible explanations for this. LESφ4 may confer an advantage to bacteria in the ASM environment, and not *in vivo*, or there may be an inhibitor of successful infection *in vivo* that is specific to LESφ4. It has previously been observed that insertions in the LESφ2 and LESφ3 prophages in isolate B58 reduce bacterial competitiveness in this model, suggesting that they are beneficial *in vivo* (Winstanley et al., 2009), which could explain their prevalence in this experiment. How insertions in the prophages affect bacterial competitiveness, and whether these findings would be applicable to LESB58 infections in CF, remain unknown.

Large numbers of ASM-evolved endpoint isolates were phenotypically similar to chronic CF isolates, demonstrating elevated mutation rate and loss of motility. Previous studies of CF isolates have found loss of swimming, swarming and twitching motility (Workentine *et al.*, 2013), and it has been proposed that this is an immune avoidance strategy (Mahenthiralingam *et al.*, 1994). However, the lack of any immune system in our model suggests that loss of motility may evolve, at least

in part, because of other selective pressures: maybe they are simply a waste of energy in a sessile environment? The CF lung chemical environment may also influence motility; when *P. aeruginosa* is grown in processed CF sputum, the large majority of cells are non-motile. The same effect in not seen when the sputum is heat-treated, suggesting that there is some heat-labile, inhibitory component of sputum (Palmer *et al.*, 2005). However, the researchers did not identify it, and it is unknown if ASM has the same effect.

Perhaps surprisingly, loss of motility in endpoint populations was equally frequent in the phage treatment as in the controls. The majority of isolates had greatly reduced swarming, a complex type of motility regulated by multiple genes. Swarming cells upregulate production of virulence factors such as proteases and the type III secretion system (including related effector proteins), and demonstrate resistance to several antipseudomonals (Overhage *et al.*, 2008b). The logical conclusion is that loss of swarming motility will result in lowered virulence. Functional type IV pili and flagella are required for swarming motility (Köhler *et al.*, 2000), and as most isolates were twitching and swimming motility deficient (suggestive of defective type IV pili and flagella, respectively), it is unsurprising that such high levels of swarming deficient mutants were observed.

Given that the type IV pili act as a receptor for the LES phages, the antagonistic selection pressure (i.e. the threat of lysis) may drive loss of this receptor, as described for lytic phages (Hosseinidoust *et al.*, 2013a). However, temperate phages do not obligately kill, and lysogeny offers an alternative phage resistance mechanism, albeit only to similar phages. Lysogeny can have the added benefit of enhancing the fitness of bacteria through prophage-encoded genes (Barondess & Beckwith, 1990; Barondess & Beckwith, 1995; Stanley *et al.*, 2000; Vaca Pacheco *et al.*, 1997; Wang *et al.*, 2010b). The fact that pili are lost in the control populations suggests that this is not merely phage driven, although by studying populations through time, we could assess if the rate at which twitching motility is lost differs in the two populations.

The loss of twitching motility explains the high levels of phage resistance detected across both treatments. Population 5 was the only one to retain a high frequency of motile isolates, and also had a high frequency of isolates fully susceptible to all three

phages. It is also interesting to note the 15% of isolates in population 9 that remained fully susceptible to all three phages; it is possible that the spatial structure of the ASM environment/ biofilm allows escape from phage predation (Schrag & Mittler, 1996). Such spatial heterogeneity is also a feature of the CF lung.

Previous studies have suggested a role for type IV pili in biofilm formation (Klausen *et al.*, 2003b; O'Toole & Kolter, 1998), yet they are defective or not produced in this model, in which PAO1 forms biofilms. However, despite the reported link between type IV motility and biofilm formation, a study examining clinical isolates observed no correlation between biofilm production and motility (Head & Yu, 2004), although the authors did not control for growth rate. Another comparison of chronic infection isolates observed reduced biofilm formation in microtitre plate assays, as well as differences in biofilm architecture to that of PAO1, and the more late-stage the infection, the more pronounced the differences. PAO1 formed a smooth biofilm, covering a surface, yet many isolates formed patchy aggregates that did not cover a surface. The heterogeneities were largely due to differences in motility, with motile isolates forming biofilms more akin in structure to PAO1 (Lee *et al.*, 2005a).

Despite high frequencies of non-motile isolates in this study, biofilm formation was not strongly negatively affected. There were huge heterogeneities between isolates in biofilm formation, but no overall trend towards increased or decreased biofilm formation. The natural assumption is that growth in a biofilm will result in adaptation towards increased biofilm formation, but this is not necessarily the case. *In vivo,* biofilm formation may protect cells from aggressor such as antibiotics, other bacteria and the immune system, but these are lacking in this *in vitro* model. Being a better biofilm producer in this model is not necessarily advantageous; in order for individuals to pass on their genes, they must survive the 4 days until the transfer into fresh media, and if biofilm formation is not necessary for this, it will not necessarily be selected for.

A high frequency of hypermutators were observed in some evolved populations, but again, there were large differences between populations, ranging from 0 to over 90% hypermutators. A high mutation rate is thought to be detrimental due to the risk of deleterious mutations, but can sweep a population by hitch-hiking with beneficial

mutations (Chao & Cox, 1983), and can be advantageous in a novel environmental, accelerating adaptation. The long-term *E. coli* selection experiment in the laboratory of Richard Lenski observed that hypermutators arose in several populations and swept to a high frequency. This was attributed to hitch-hiking, but, surprisingly was not linked to increased fitness (Sniegowski *et al.*, 1997). The mutator phenotype may confer intrinsic fitness benefits; PAO1 *mutS* and *mutL*-deficient mutator isolates form biofilms with significantly enhanced microcolony growth (Conibear *et al.*, 2009), and *mutS* mutants overproduce the H_2O_2-degrading enzyme catalase, increasing their resistance to oxidative stress (Torres-Barceló *et al.*, 2013). The hypermutable phentotype is linked with antibiotic resistance (Plasencia *et al.*, 2007; Waine *et al.*, 2008), and a clear correlation was observed between the frequency of hypermutators in a population and the frequency of clinically resistant isolates. Characterisation of these isolates identified increased basal β-lactamase levels, a resistance mechanism that has been identified previously in mutator strains (Mandsberg *et al.*, 2009). The finding that clinically resistant isolates are present at low frequency in hypermutable populations, even in the absence of antibiotic treatment, may be of concern in chronic CF infections.

Hypermutators aren't necessarily maintained in the long-term; once a population has become adapted to its environment, the focus is then on reducing the genetic load, with a lowering of the mutation rate (Wielgoss *et al.*, 2013). Hypermutability through time was not recorded in this study, so it is unknown when the mutator phenotype first arose in each population, or whether mutator lineages were previously present in the non-mutator populations, although sequence data may go some way to answering those questions.

Despite the emergence of many phenotypes associated with chronic infection isolates, no mucoid isolates, which are often noted as a hallmark of chronic infection, were identified. This may be due to the short timescale of this study and lack of other selective pressures, such as the host immune system, other microbes or antibiotics. The mucoid phenotype is associated with resistance to opsonisation (Baltimore & Mitchell, 1980) and increased antimicrobial resistance (Govan & Fyfe, 1978), but is rarely identified in non-CF *P. aeruginosa* (Govan & Deretic, 1996). Growth in a biofilm compounds this problem, as mucoid biofilms are much more difficult to

eradicate (in comparison to non-mucoid biofilms) (Hengzhuang *et al.*, 2011; Hentzer *et al.*, 2001). There is also evidence that alginate, the polysaccharide that is overproduced in mucoid strains, protects the biofilms from the immune system (Leid *et al.*, 2005).

The diversification observed in ASM over what is a relatively short timescale could be driven by growth in a biofilm. *P. aeruginosa* diversifies when grown as a biofilm, and this self-generated diversity helps protect against oxidative stress (Boles *et al.*, 2004). The authors suggested that this was due to the "insurance effect", which posits that biodiversity has a buffering effect over time on productivity, as well as raising the overall mean productivity of an ecosystem (Yachi & Loreau, 1999). However, the extent to which the data supported the insurance hypothesis was questioned by Cooper and colleagues (Cooper *et al.*, 2005), and the insurance hypothesis with regards to bacterial biofilms has still not been fully tested.

Aside from diversity, the heterogeneity observed between replicate populations is also striking, suggesting that they are following divergent evolutionary trajectories. This is often observed in experimental evolution (Buckling & Rainey, 2002a; Lythgoe & Chao, 2003), but also between patients (Ashish *et al.*, 2013a; Mowat *et al.*, 2011). This suggests that not all the diversity observed *P. aeruginosa* populations in different patients can be explained by the infecting genotype or innate host differences, and highlights our lack of understanding of the processes driving this diversification.

It is interesting that no differences in diversity were detected between the two treatments. Phages have previously been shown to drive the development of phenotypes associated with chronic infection in *P. aeruginosa*. For example, when coevolved with the pilus-specific phage PP7, PAO1 displayed impaired twitching motility and reduced biofilm formation, although sequencing of an impaired isolate did not reveal SNPs in any genes known to be involved in twitching motility. Phage-resistant isolates evolved with an additional, LPS-specific phage also had lowered motility and a reduced growth rate, but a higher production of virulence factors such as pyoverdine and pyocyanin, when compared to single-phage or phage free controls (Hosseinidoust *et al.*, 2013a). Further analysis of these phage resistant isolates

revealed that they were more resistant to nonopsonic phagocytosis, presumably due to loss of required receptors, and had increased resistance to oxidative stress (Hosseinidoust *et al.*, 2013b). Clearly, selection for phage-resistance can have far-reaching consequences for bacterial phenotypes, even affecting virulence; phage resistance in the fish pathogen *Flavobacterium columnare*, is associated with reduced virulence *in vivo* (Laanto *et al.*, 2012).

As the LES phages are temperate, they are unlikely to impose such antagonistic selection pressures on the bacteria, as are seen with obligately lytic phages. Sequence data may give us more information about the coevolutionary interactions between PAO1 and the LES phages, and this is something that will be addressed in the following chapter.

Such phenotypic diversification is not necessarily a consequence of adaptation to the ASM environment, but when competed against the ancestral PAO1, all the evolved populations had equal or increased fitness, relative to the ancestor. Interestingly, phage-treated populations were more competitive than the controls, but only when competed against the phage-susceptible competitor. This suggests that the LES phages may confer a competitive advantage to their hosts that is dependent on prophage-mediated lysis of the competitor, again something that will be investigated further in Chapter 6.

4.5 Summary

- PAO1 and LES bacteriophages φ2, φ3 and φ4 can stably coexist in an ASM model of the CF lung, but there are differences in the evolutionary success of the phages, with LESφ4 largely dominating in prophage form.
- PAO1 undergoes extensive diversification in ASM, with many isolates developing phenotypes commonly observed in chronic CF isolates. Crucially, this diversification is not LES phage-driven, but occurs in the presence or absence of phages.
- Low frequencies of clinically antipseudomonal-resistant isolates were detected in hypermutable populations. Ceftazidime and piperacillin-tazobactam resistant isolates produced high levels of β-lactamases.

- All evolved populations had equal or increased fitness relative to the ancestor. Phage treated populations were more competitive than controls, but only when the competitor was phage-susceptible.
- The evolutionary success of the LES phages is very different in a rat model of chronic infection, with LESφ2 and LESφ3 prophages dominating. However, no bacterial phenotypic diversity was detected after 10 days.

4.6 Declaration

The coevolution experiment was initially designed by Dr. Chloe E. James (in conjunction with Prof. M. A. Brockhurst and Prof. C. Winstanley), and was carried out by myself and Dr. James. The majority of the phenotypic characterisation of endpoint populations and all data analyses were performed by myself.

The *in vivo* work was done in collaboration with Dr. Irena Kukavica-Ibrulj at the lab of Prof. Roger C. Levesque, Laval University.

Chapter 5 The role of the LES phages in driving bacterial genetic diversification in ASM

5.1 Introduction

5.1.1 Evolution of *P. aeruginosa* in the CF lung

Isolates of *P. aeruginosa* from the lung of chronically infected CF patients tend to display characteristic phenotypes, as discussed in the previous chapter, which are often underpinned by mutations in several key genes. In the era of low-cost, high throughput genome sequencing, numerous studies have attempted to characterise the evolutionary trajectory of *P. aeruginosa* over the course of infection, and identify the genetic changes that enable adaptation to the CF environment.

Sequencing of the same lineage over the course of an infection can identify genes important for adaptation, as can examination of multiple lineages for parallel evolution. Taking a longitudinal approach, Smith *et al*. sequenced two isolates from one patient that were 7.5 years apart, in addition to PCR testing of intermediate isolates. Interestingly, the majority of mutations that occurred over time were loss of function (LOF) mutations, with virulence factors in particular a target; genes involved in the type III secretion system, twitching motility, biofilm formation and production of proteases (all known virulence factors) were altered (Smith *et al.*, 2006b). It is thought that these virulence factors are required for establishing infection, as a transposon mutagenesis study of PA14 found that insertions in virulence genes resulted in reduced fitness in a mouse colonisation model, and reduced systemic dissemination in neutropenic mice (Skurnik *et al.*, 2013). However, the selective pressures of a chronic infection may be different; bacteria are under constant attack from the immune system and antibiotics, and subjected to oxidative stress and competition from co-infecting microorganisms. It is hypothesised that loss of virulence factors may be an immune evasion strategy (Smith *et al.*, 2006b), but it is difficult to pinpoint the exact factors that are driving within-host evolution.

Some researchers have attempted to shortlist genes involved in adaptation to the CF lung. Marvig *et al*. compared multiple isolates of the DK2 lineage of *P. aeruginosa*, a common transmissible clone that is widespread through CF centres in Denmark. By

identifying genes that underwent parallel evolution, they were able to identify 65 "pathoadaptive" genes, which are defined as genes that maximise pathogen fitness. Broadly, these were genes involved in cell envelope formation (linked with immune evasion), antibiotic resistance, or regulation of other genes. Interestingly, when the researchers looked at lineages that has displaced others, they were found to have more pathoadaptive mutations (Marvig *et al.*, 2013), suggesting that such mutations may be important to the evolutionary success of a lineage. In a follow up study, a similar approach was taken, but studying isolates from children (i.e. recent infections) to identify genes that are involved in early adaptation to the CF lung. This strategy identified 52 pathoadaptive genes, including genes involved in antibiotic resistance, motility, secreted products and transcriptional regulation. Again, most were predicted LOF mutations (Marvig *et al.*, 2014).

Mutations in regulatory genes are a common theme (Dettman *et al.*, 2013; Jeukens *et al.*, 2014; Marvig *et al.*, 2014), and may be an obvious target because of their pleiotropic effects. The anti-sigma factor MucA regulates alginate production, and mutations in *mucA* can cause overproduction of alginate (Pulcrano *et al.*, 2012), resulting in the mucoidy phenotype that is associated with chronic infection. However, MucA has pleiotropic effects, regulating motility, rhamnolipid production, QS signal production and production of virulence factors (Rau *et al.*, 2010; Wu *et al.*, 2004), suggesting it may facilitate early adaptation to the CF lung environment. Similarly, the 2-componenet regulator RetS has been implicated in the switch from acute to chronic infection. Inactivation of the *retS* gene results in reduced expression of genes involved in initial colonisation and virulence, such as motility and type II and III secretion system genes, and upregulation of genes involved in biofilm formation, resulting in lowered virulence in an acute model of infection (Goodman *et al.*, 2004). The QS regulatory gene *lasR* is another regulatory gene that is commonly mutated in chronic isolates (Ciofu *et al.*, 2010; Smith *et al.*, 2006b; Yang *et al.*, 2011), although it has been suggested that this doesn't happen until the late stages of chronic infection (Bjarnsholt *et al.*, 2010). The QS systems control expression of multiple virulence factors (Latifi *et al.*, 1995; Passador *et al.*, 1993), and mutants cause less severe lung pathology in a rat model of chronic infection (Wu *et al.*, 2001) and reduced virulence in acute murine models of infection (Pearson *et al.*, 2000;

Rumbaugh *et al.*, 1999). *lasR* mutants rise in prevalence as an infection progresses, and are associated with increased decline in lung function (Hoffman *et al.*, 2009).

A recent study attempted to determine the genetic basis for the evolutionary success of the LES, using 32 North American *P. aeruginosa* isolates that had been typed as LES. By estimating the strength of selection acting upon individual genes, those that were involved in adaptation to the CF lung could be predicted, and they included regulatory, secretion and transport genes. The researchers then considered genes that contribute most to the divergence of LES from other non-LES strains, and that may explain its evolutionary success. Genes involved in biofilm formation, arginine metabolism, protein secretion and cation transport were highlighted as important, as they were overrepresented in the accessory genome of the LES (Dettman *et al.*, 2013). A comparison of various LES and LES-like isolates found that SNPs were overrepresented in regulatory and virulence genes, suggesting strong selection (Jeukens *et al.*, 2014).

Transcriptomics data can provide additional information on genes that might be important for CF lung adaptation. Huse *et al.* selected clonal isolates over the course of an infection in three individual patients, and measured changes in gene expression against length of infection, and also against other clones. Multiple genes were expressed differently in the different clones, including genes that are commonly mutated, such as those involved in motility and virulence. When tracking gene expression in a single clone over time, 24 genes were found to be up or down-regulated in the same way in the different clones, and these similar patterns are indicative of parallel evolution. Five of the consistently down-regulated genes were involved in type-IV pilus formation, whereas genes involved in biofilm formation were among those consistently upregulated (Huse *et al.*, 2010). This upregulation was linked to a phenotypic change in a follow-up study; strains demonstrated increased Psl production, and, consequently, increased biofilm production (Huse *et al.*, 2013).

Despite the trend towards evolution in particular genes, as with phenotypes, there exists extensive diversity within a *P. aeruginosa* population, and between patients. In a similar study to Smith *et al.*, isolates from two patients that each harboured

different clones of *P. aeruginosa* were sequenced. The genes that were mutated in the Smith *et al.* study were not found to be under selection, and there were substantial differences between the two clones, with regards to phenotypic and genetic diversification (Cramer *et al.*, 2011) . A recent study with hypermutators identified parallel evolution in multiple sub-lineages in antibiotic resistance and catabolic genes, but also huge genetic diversity (Feliziani *et al.*, 2014). It has been argued that this genetic diversification occurs during initial adaptation to the lung but quickly plateaus; in 12 DK2 isolates, taken from 6 patients over 3 decades there were several mutations in key regulatory genes early on in infection, with limited change afterwards, suggesting that the bacteria had reached an adaptive peak in the fitness landscape (Yang *et al.*, 2011). However, this observation may be a peculiarity of the DK2 lineage, as chronic LES infections display huge phenotypic and genetic diversity, with little stability over time (Mowat *et al.*, 2011), something that is also observed in chronic non-LES infections (Ashish *et al.*, 2013a; Workentine *et al.*, 2013).

The spatial structure of the lung can influence the evolution of *P. aeruginosa* in the lung, and it is plausible that bacteria occupy different niches in the lung, potentially evolving independently. Multiple lineages do occur: a recent study of DK1 (another transmissible that infects multiple patients in Danish CF centres), found that despite showing little overall genetic change (a handful of SNPs over a 32 year infection), the DK1 population could still be classified into three sub-lineages, each differing in their evolutionary rates, prevalence of pathoadaptive mutations, and phenotypes. Limited parallel evolution was detected between the three sublineages, suggesting that perhaps they occupy different niches. Indeed, there was some evidence that the sublineages were spatially separated in the CF lung (Markussen *et al.*, 2014). Likewise, a recent paper highlighted the existence of multiple sub-lineages in several LES-infected individuals, with greater genetic diversity within patients than between (Williams *et al.*, 2015).

5.1.2 Role of hypermutators in genome evolution and adaptation

As discussed in the previous chapter, several of the replicate PAO1 populations evolved in ASM (across both treatments) had a high frequency of isolates displaying the hypermutator phenotype. Given the prevalence of hypermutators in chronic

infections (Oliver *et al.*, 2000), and the fact that a high mutation rate is likely to have an effect on genome evolution and adaptation, several studies have attempted to address the effect of mutation rate on adaptation to the CF lung. A study using the same isolates from Smith *et al*. (2006) compared hypermutators and non mutators. Unsurprisingly, hypermutators had more mutations, but mutations were similarly distributed in adaptive genes between both hypermutators and non mutators, suggesting a generalised effect of adaptation (Mena *et al.*, 2008). Most fixed SNPs in hypermutator lineages are neutral (Feliziani *et al.*, 2014). Development of a hypermutator phenotype is often preceded by mutations in *mucA* and *lasR* (Ciofu *et al.*, 2010), and hypermutators dominate towards the end stage of chronic infection (Hogardt *et al.*, 2007), suggesting that a high mutation rate is more adaptive in the later stages of chronic infection, although they have been observed as early as 2 years after colonisation (Cramer *et al.*, 2011). Hypermutators are associated with development of antibiotic resistance (Ferroni *et al.*, 2009) and downregulation of several virulence-associated traits (Hogardt *et al.*, 2007).

5.1.3 Bacteriophages as drivers of genome evolution

Bacteriophages drive the phenotypic evolution of their hosts, but the underlying genomic change can occur by multiple mechanisms. Bacteriophages can have a direct effect on the genome of their host, for example through carriage of extra genes (in the case of prophages), or indirectly, as seen in the emergence of phage resistance mutations that occur during antagonistic coevolution with lytic phage.

Temperate bacteriophages (like the LES phages in this study) can drive host genome evolution through gene disruption, transduction or by acting as anchor points for major chromosomal rearrangements, in addition to the carriage of extra genes, as mentioned above.

5.1.3.1 Phage-mediated gene disruption

Gene disruption frequently occurs through insertional inactivation; Staphylococcal phage L54a inserts into the lipase- encoding *geh* gene, resulting in a loss of phenotype, termed negative lysogenic conversion (Lee & Iandolo, 1986). Another *Staphylococcus aureus* phage, φ13, integrates into the 5' end of the *hlb* gene by site and orientation-specific integration, causing a loss of *hlb*-encoded beta-toxin

expression (Coleman *et al.*, 1991). *Escherichia coli* phage Mu (short for mutator) was the first identified example of a bacteriophage causing mutations in the host chromosome, after lysogens were observed to display differences in their nutritional requirements, through phage-mediated disruption in gene function (Taylor, 1963). As a transposable phage, Mu can integrate randomly (Bukhari & Zipser, 1972), unlike many other phages, including λ, which utilise a site-specific integration system. Transposable phages infecting *Pseudomonas* are commonplace, and include one of the subjects of this study, LESφ4, the closely related D3112 (Wang *et al.*, 2004), and related phage B3 (Braid *et al.*, 2004). A comparison of two recently sequenced phages to other *P. aeruginosa* transposable phages highlights a common genome architecture, similar to that of phage Mu, despite sharing a low sequence similarity (Cazares *et al.*, 2014). D3112 has been shown to cause mutations in PAO1 through insertional inactivation (Rehmat & Shapiro, 1983), yet the potential impact of phage-mediated gene disruption on bacterial evolution has been widely neglected.

5.1.3.2 Phage transduction

Transduction is the transfer of genetic material between bacterial genomes by a bacteriophage, of which there are two types, specialised and generalised. Specialised transduction is mediated by temperate phages, and occurs when the phage excises imprecisely from the bacterial genome, taking with it adjacent bacterial gene(s), which are transferred to another bacterial host upon phage infection and lysogenisation. Specialised transducing *E. coli* λ phages have been shown to transduce the gene for the β-subunit of RNA polymerase (Kirschbaum & Konrad, 1973), genes involved in DNA replication (Hansen & von Meyenburg, 1979), genes encoding ribosomal proteins (Jaskunas *et al.*, 1975) and *recA* (McEntee & Epstein, 1977). Specialised transducing phages are not confined to *E. coli*, and have been identified in *Salmonella enterica* serovar Typhimurium (Chan *et al.*, 1972), *Bacillus subtilis* (Zahler *et al.*, 1977) and *P. aeruginosa* (Cavenagh & Miller, 1986).

Generalised transduction occurs during the lytic cycle of phages, so is not confined to only temperate phages. Prior to cell lysis, phage capsules are packaged with phage DNA, but bacterial DNA can be mistakenly incorporated. The fact that any part of the bacterial chromosome can be packaged gives rise to the name “generalised transduction”. Unlike in specialised transduction, the virions contain little or no

phage DNA. Upon infection of another cell, the DNA is released into the cell cytoplasm and can potentially recombine with the host chromosome. Temperate phage P22 in *S.* Typhimurium is capable of generalised transduction (Ebel-Tsipis *et al.*, 1972), like over 90% of temperate phages of the *S.* Typhimurium complex (Schicklmaier & Schmieger, 1995). Generalised transduction can affect bacterial evolution; the veterinary antibiotic carbadox-mediated phage induction of a multi-drug resistant strain of *S.* Typhimurium causes generalised transduction of antibiotic resistance genes (Bearson *et al.*, 2014). Generalised transducing phages of *P. aeruginosa* have been identified, including the recently characterised φPA3, originally isolated from sewage. It is capable of infection of clinical CF isolates, and can transduce mutations in *las* and *rhl* between PAO1 isolates (Monson *et al.*, 2011). There is evidence that generalised transduction may play a role in the evolution of bacteria in natural *P. aeruginosa* populations; the environmental phage UTI can transduce DNA between environmental *P. aeruginosa* strains (Ripp *et al.*, 1994). Furthermore, transduction can occur between lysogens in environmental freshwater test chambers, which are similar to the natural environment (Saye *et al.*, 1990). The same study compared transduction frequencies between lysogenic and non-lysogenic *P. aeruginosa*, and found the transduction was highest between lysogen and non-lysogen, as opposed to between two lysogens.

5.1.3.3 Phage-mediated chromosomal inversions

Prophages can act as anchor points for chromosomal inversions and other major genomic rearrangements. Sequencing of a pathogenic *Streptococcus pyogenes* isolate identified two major chromosomal inversions, one of which was caused by homologous recombination between two related prophages, and the other which the authors hypothesised occurred after a phage integration event which caused an “unbalancing” of the genome (Nakagawa *et al.*, 2003). There is evidence of a prophage-mediated chromosomal inversion in *Enterococcus faecium*, but despite the notion that major chromosomal rearrangements would have a negative impact on fitness, no such effect was detected (Lam *et al.*, 2012).

Most studies look at isolates taken from one or more patients, at one or more timepoints, but it is impossible to determine the drivers of evolution. An experimental evolution approach can simplify things by removing factors (such as

immune system, antibiotic pressure etc.), and reintroducing in a controlled manner, comparing evolution in the presence or absence of such factors.

Phages are found at high levels in the CF lung (James *et al.*, 2014), yet their contribution to *P. aeruginosa* evolution is largely ignored. The populations generated in the previous study (Chapter 4) provide the ideal opportunity to study genetic diversification in an artificial sputum environment, in the presence or absence of temperate bacteriophages.

5.2 Objectives

1. Identify genetic variants occurring in endpoint ASM populations by sequencing pooled DNA of the 40 phenotypically characterised isolates for each population.
2. Identify genes that are important for adaptation to the ASM environment by looking for those most commonly mutated, and those predicted to have an effect on amino acid sequence, in addition to those that are mutated between parallel populations. Contrast genes identified as adaptive in the presence and absence of LES phages.
3. Identify integration sites of the LES phages in the evolved PAO1 isolates.

5.3 Results

In the previous chapter, PAO1 was cultured long-term in ASM, in the presence or absence of LESφ2-4, and the phenotypic diversity within and between populations was assessed by phenotypic characterisation of 40 isolates from each population. DNA was extracted for the same 40 isolates per population, pooled and sequenced. Using this pooling strategy, it is possible accurately determine the frequency of different variants within each population, which can then be compared with the phenotype data. Bioinformatics analysis was performed by Dr. Sam Haldenby, CGR.

5.3.1 Single nucleotide variants (SNVs) across both treatments

Deep sequencing of populations confirmed genetic diversification in both the phage-treated and control populations, but there were no clear differences between the two groups. Between 21-185 single nucleotide variants (SNVs) or small insertion/ deletion polymorphisms (INDELS) were identified in each population. Most variants

were detected at a low frequency in a limited number of populations, but a scoring system of variants enabled identification of SNVs that are more likely to be biologically meaningful. Using this method (developed by Sam Haldenby), scores were weighted by predicted severity of effect on protein, the number of variants identified per gene and frequency of all variants across all populations. The highest scoring mutated genes are summarised in Table 5.1. There is a strong correlation between the frequency of isolates with the hypermutator phenotype at t_{30}, and the number of SNVs detected in the population (Figure 5.1: Pearsons product-moment correlation coefficient; $r = 0.94$, $P < 0.001$).

Table 5.1 Detection of variants in pooled populations. The number of variants across all populations is reported, both as a total (all populations) and split according to treatment (control or phage). The frequency of each variant was estimated for each population (pool of 40). The most prevalent variant was defined as the variant observed in the highest number of isolates over all populations. Mutated genes were scored by frequency of variants detected, prevalence of each variant and severity of predicted effect on the resulting protein (scoring system developed by Sam Haldenby). Highest scoring mutated genes are located at the top of the table. Only SNVs scoring >3.5 are reported. "DEL" = deletion, "*dN*" = non-synonymous mutation.

				Control treatment		Phage challenged					
Gene	**Protein function**	**Total number of variants detected**	**Score (max 120)**	**Number of variants**	**Populations in which variants observed**	**Number of variants**	**Populations in which variants observed**	**Type of mutation (most prevalent variant only)**	**Total populations in which observed**	**Mean frequency of variant in populations where observed**	**Predicted effect**
hutU	Urocanate hydratase (role in histidine metabolism)	1	70.0	1	4/6	1	3/6	DEL	7/12	1	Single nucleotide deletion and frameshift (severe)
lasR	Transcriptional regulator (role in regulation of quorum sensing)	4	38.6	1	1/6	4	4/6	d_N	3/12	0.91	Glutamic acid replaced with STOP codon – protein truncation (severe)
glpR	Glycerol-3-phosphate regulon repressor	4	36.5	3	6/6	3	5/6	DEL	9/12	0.37	Deletion and frameshift (severe)
PA4394	Conserved hypothetical protein	1	30.0	1	2/6	1	1/6	DEL	3/12	1	Single nucleotide deletion and frameshift (severe)
cysB	Transcriptional regulator	18	26.7	11	6/6	8	6/6	d_N	1/12	0.55	Glutamic acid → valine (moderate)
PA3271	Probable sensory transduction histidine kinase	12	24.4	6	6/6	6	6/6	d_N	1/12	0.65	Phenylalanine →leucine (moderate)
braB	Branched chain amino acid transporter	12	24.2	6	5/6	8	6/6	d_N	6/12	0.40	Threonine → alanine (moderate)
amrZ	Alginate and motility regulator Z	9	20.9	6	5/6	3	3/6	DEL	1/12	0.60	Single nucleotide deletion and frameshift (severe)
fha1	Predicted component of typeVI secretion system	1	19.3	1	1/6	1	3/6	DEL	4/12	0.96	10 bp deletion and frameshift (moderate)
PA4954 (*motA*)	Flagellar motor component	7	19.3	4	4/6	4	4/6	d_N	2/12	0.38	Leucine → phenylalanine (moderate)
mutS	DNA mismatch repair protein	5	16.0	3	2/6	2	2/6	d_N	1/12	0.93	Cysteine → arginine (moderate) C295R(37)
pilY1	Type IV fimbrial biogenesis protein	4	13.7	3	2/6	1	1/6	DEL	1/12	0.88	(severe) -992(35)
mvfR	Transcriptional regulator	6	12.2	1	1/6	5	4/6	d_N	1/12	0.93	Alanine → threonine (moderate) A25T(37)
aer	Aerotaxis receptor	7	9.9	3	3/6	4	4/6	d_N	1/12	0.58	G380S(23)
opdE	Membrane protein	1	9.3	0	0/6	1	1/6	d_N	1/12	0.93	Tryptophan replaced with STOP codon (severe) W190*(37)
PA5233	Predicted flagellar basal body-associated protein	4	8.3	3	3/6	1	1/6	d_N	1/12	0.58	Glutamic acid replaced with STOP codon (severe) E109*(23)
acsA	Acetyl-coenzyme synthetase	4	7.8	0	0/6	4	4/6	d_N	1/12	0.53	Asparagine → threonine (moderate) NS566T(21)
PA2230	Conserved hypothetical	2	6.3	1	1/6	1	1/6	d_N	1/12	1	Threonine → isoleucine

	protein										(moderate) T43I(40)
rpoS	Sigma factor	3	6.3	2	2/6	1	1/6	d_N	1/12	0.75	Asparagine → serine (moderate) N129S(30)
PA2229	Conserved hypothetical protein	3	6.0	2	1/6	1	1/6	d_N	1/12	0.95	Leucine → methionine (moderate) L167M(38)
PA0671	Hypothetical protein	1	5.8	1	1/6	0	0/6	DEL	1/12	0.58	-160?(23)
PA3521	Probable outer membrane protein precursor	1	5.8	1	1/6	0	0/6	DEL	1/12	0.58	-469?(23)
bifA	Predicted signal transduction protein	4	5.5	2	2/6	2	2/6	d_N	1/12	0.48	Threonine → proline (moderate) T377P(19)
motB	Chemotaxis protein motB	4	5.1	2	3/6	3	3/6	d_N	3/12	0.13	Deletion (moderate) VKR12-(15)
PA0061	Hypothetical protein	1	5.0	1	1/6	0	0/6	DEL	1/12	0.50	FS? (severe) -84?(20)
braC	Branched-chain amino acid transport protein	7	4.9	3	3/6	4	3/6	d_N	1/12	0.30	Glutamic acid → lysine (moderate) E259K(12)
PA1442	Conserved hypothetical protein	2	4.8	2	2/6	0	0/6	DEL	1/12	0.40	-11?(16)
PA5017	Conserved hypothetical protein	3	4.8	1	1/6	2	2/6	d_N	1/12	0.28	Serine → proline (moderate) S438P(11)
PA2827	Conserved hypothetical protein	1	4.6	0	0/6	1	1/6	d_N	1/12	0.93	Proline → serine (moderate) P111S(37)
PA1047	Probable esterase	1	4.5	1	1/6	0	0/6	DEL	1/12	0.45	(severe)-278(18)
PA0690	Hypothetical protein	2	4.4	1	1/6	2	1/6	d_N	1/12	0.35	Threonine → isoleucine (moderate) T597I(14)
hutC	Histidine utilization repressor	5	4.1	3	3/6	2	2/6	DEL	1/12	0.20	(severe) -81(8)
vqsM	AraC-type transcriptional regulator	1	4.1	1	1/6	0	0/6	d_N	1/12	0.83	Isoleucine → leucine (moderate) I62L(33)
algL	Poly(beta-d-mannuronate) lyase precursor	1	4.0	1	1/6	0	0/6	d_N	1/12	0.80	Alanine → threonine (moderate) A341T(32)
PA3157	Probable acetyltransferase	1	4.0	1	1/6	1	1/6	DEL	2/12	0.20	(severe) -372?(16)
PA2462	Hypothetical protein	4	3.9	0	0/6	1	1/6	DEL	1/12	0.28	(severe) -5315(11)
antB	Anthranilate dioxygenase small subunit (anthranilate metabolism)	1	3.9	1	3/6	1	2/6	d_N	5/12	0.16	Threonine → methionine (moderate) T57M(31)
pilT	Twitching motility protein	3	3.9	3	4/6	0	0/6	d_N	1/12	0.60	Leucine → proline (moderate) L193P(24)
fleQ	Transcriptional regulator	2	3.6	2	2/6	0	0/6	d_N	1/12	0.60	Glycine → serine (moderate) G454S(24)

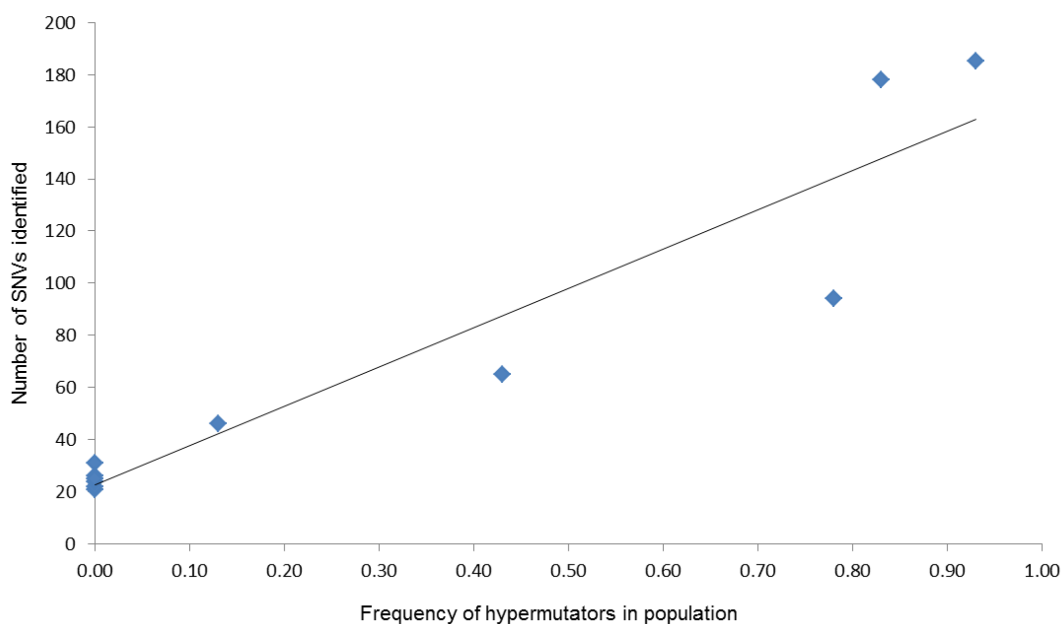


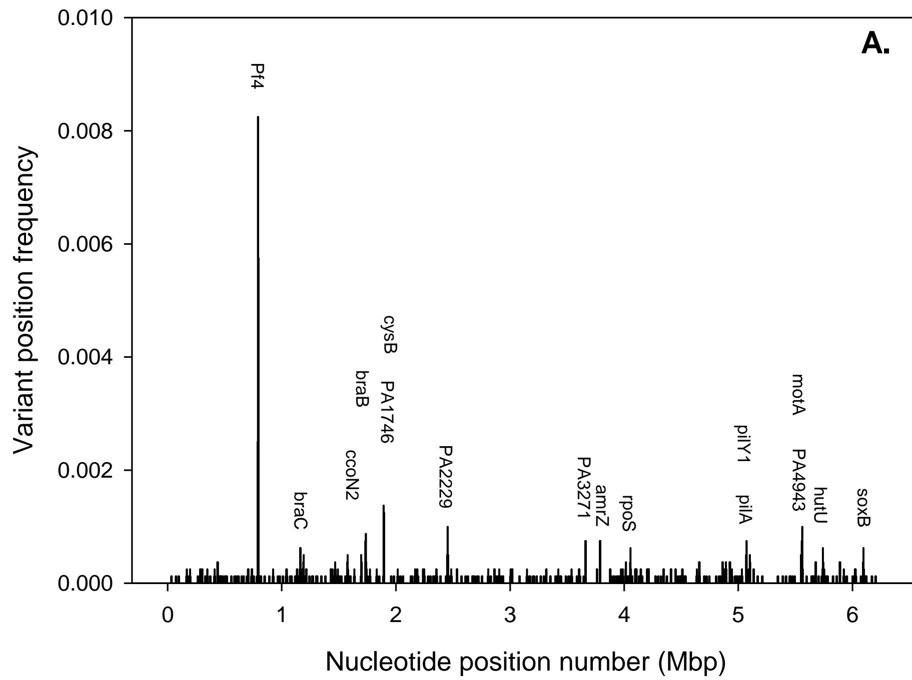
Figure 5.1 Correlation between the frequency of isolates displaying a hypermutator phenotype, and the number of SNVs in endpoint populations.

There were several variants that occurred in more than one population. One example is a single variant in the *hutU* gene, coding for urocanate hydratase. The same frameshift mutation had reached fixation in 7/12 populations. A similar occurrence was observed for the QS regulatory *lasR* gene; four variants were detected in *lasR*, of which two had a predicted "severe" effect and were found at a very high frequency in the population. One of the variants was present in 3 populations (one in the control and two in the phage-treatment). To confirm that this was not an artifact of the sequencing, the *lasR* gene was PCR amplified in each of these populations (using pooled population DNA), and Sanger sequenced. This confirmed the Illumina data: a C to A transversion corresponded with a predicted change in the amino acid sequence, from a glutamic acid to a STOP codon. The *lasR* gene of population 11, in which the other high scoring variant was found, was also Sanger sequenced, and a 4 base pair insertion was identified, resulting in a predicted frameshift.

Many of the top scoring mutated genes were regulatory genes, including the aforementioned *lasR*, a transcriptional activator and key component of one of the major quorum sensing (QS) systems of *P. aeruginosa*. Other high scoring mutated regulatory genes included *glpR, cysB, amrZ, vqsM* and *fleQ*. Mutations in genes with

key roles in bacterial motility, including *motA, motB, pilT* and *pilY1* were also common.

As an additional approach to identify areas of the bacterial genome that may be under selection, the frequency of variants across all populations observed at each position in the PAO1 genome was plotted separately for each treatment (Figure 5.2). There is a clear peak in the frequency of mutations in the region of the endogenous filamentous prophage of PAO1 (Pf4), in both treatments. Other locations were identified as hot spots for mutations in both treatments, but to a lesser degree, including the regulatory genes *rpoS* and *cysB,* and the *braB* and *braC* genes, that are involved in the Na^+ dependent and Na^+ independent branched amino acid transport systems, respectively. There are some mutational hotspots present (above the threshold) in one treatment but not the other, most notably in the pilus-related genes *pilY1* and *pilA*, only present in the control treatment. High frequencies of twitching impaired mutants were detected across populations in both treatments (Chapter 4), yet there are very few variants with mutations in known pilus-related genes in the phage treatment.



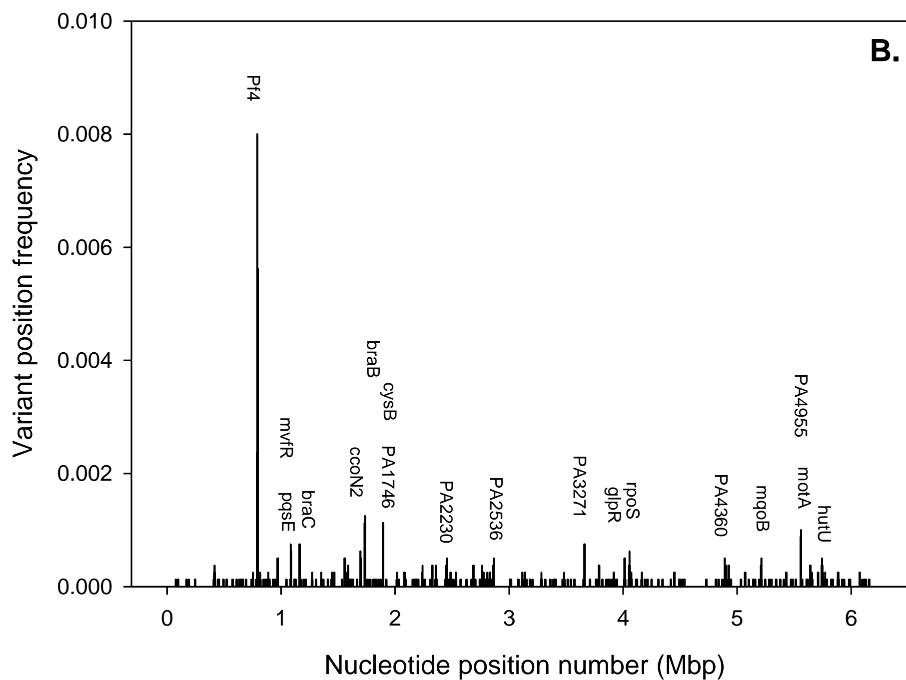


Figure 5.2 Variant position frequency across the PAO1 genome for (A.) control populations combined and (B.) phage-treated populations combined. Variant position frequency is the frequency of variants observed in a sliding window of 8 kb, but gives no information as to the number of times each variant is observed. Genes/ regions in the PAO1 chromosome with a variant position frequency >0.0005 are labelled. Data were produced by Dr. Sam Haldenby.

5.3.2 LES phage integration sites in the PAO1 genome

The LES phages are always found at the same positions in different LES isolates, with the exception of LESφ4, which is very rarely identified in an alternative additional genomic location (Haldenby *et al*, unpublished). Whilst LESφ2 and LESφ3 have been previously shown to share the same integration site in PAO1 and LESB58, LESφ4 is known to be capable of inserting into several different locations in PAO1(James *et al.*, 2012). The integration sites of the LES phages, and the relative frequency of those in each population, were determined by analysis of the sequence data by Sam Haldenby. LESφ2 and LESφ3 were always in the same position (relative to their integration site in LESB58), but LESφ4 integrated at numerous locations across the PAO1 genome (Appendix D). The integration sites identified were classified according to gene function (Figure 5.4), and the majority are genes involved in QS or motility.

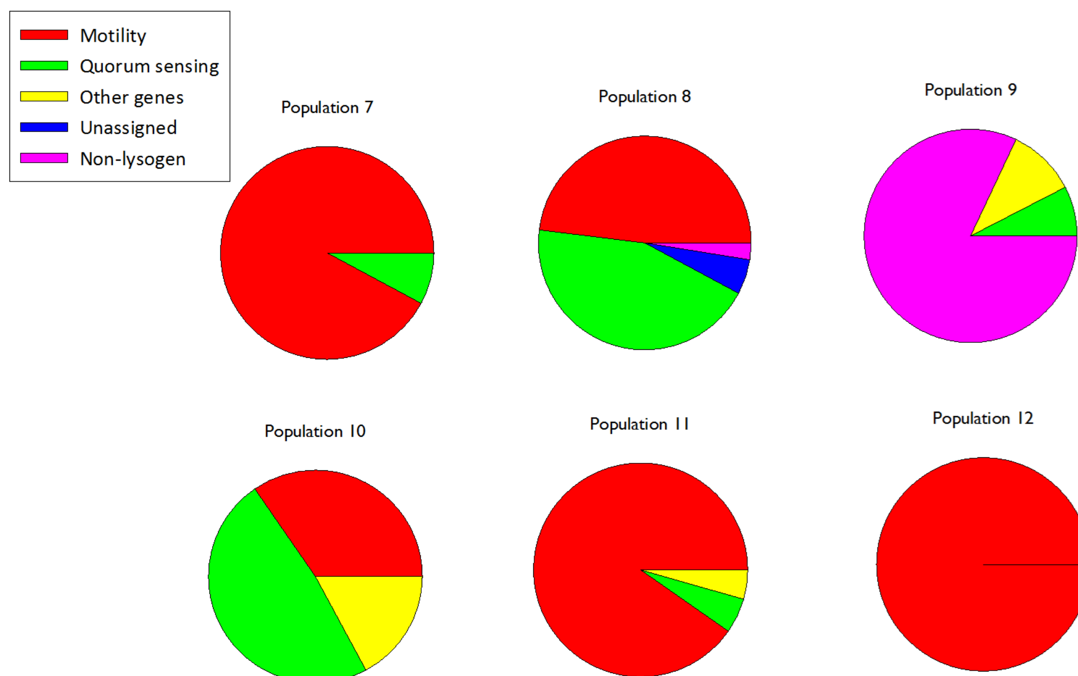


Figure 5.3 Frequency of bacteria in each phage-treated population with LESφ4 integrated into specific loci. Integration loci were grouped according to general function. The relative abundances of different integration sites in each populations were estimated by Sam Haldenby, and the frequency in the whole population determined as a proportion of the frequency of LES φ4 lysogens.

In two of the populations, populations 7 and 11, LESφ4 was found to have integrated into the pilus-related *fimU* and *pilV* genes, respectively, in a high frequency of the

population. As designed, the sequence data in this study only provide an estimate of the frequency of bacteria harbouring LESφ4, and give but a snapshot in evolutionary time. PCR primers were designed to flank these integration sites, enabling determination of the exact frequency of each integration site in its respective population, in addition to the frequency of this integration site over time.

PCR assays of the 40 endpoint isolates from populations 7 and 11 (using primers specific to each integration site) confirmed the presence of LESφ4 integrated at each site in the PAO1 genome at high frequency. The temporal data shows a similar trend in each population, with an increase in frequency over time to near fixation (Figure 5.4).

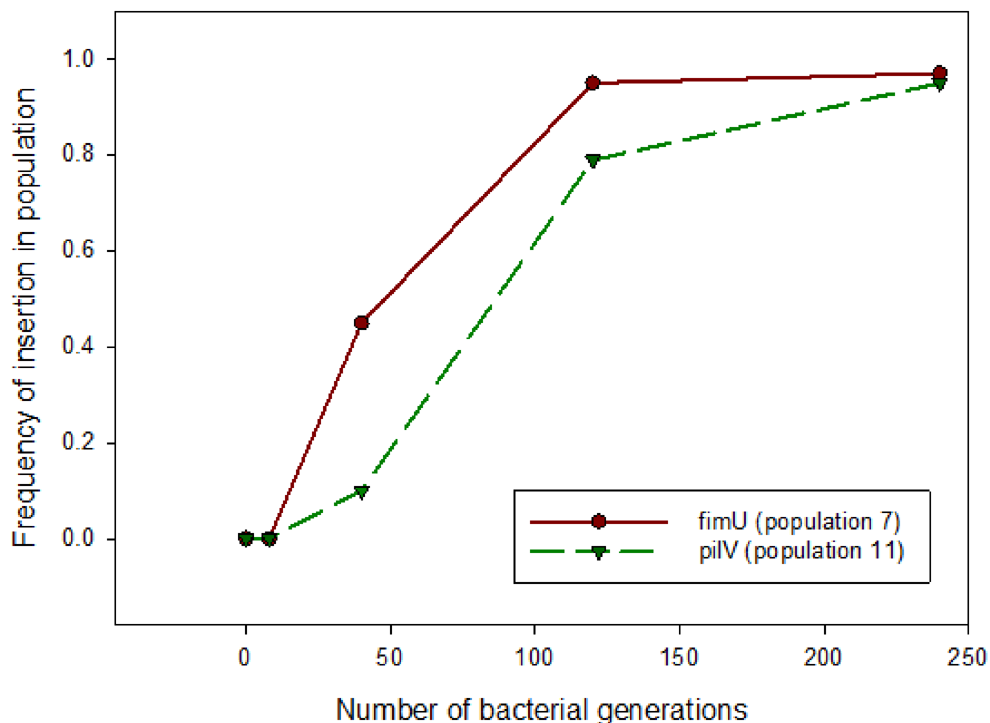


Figure 5.4 Frequency of bacterial isolates with LESϕ4 integration at specific loci. 40 isolates at multiple time-points throughout the ASM experiment were PCR tested in two phage-treated replicate populations (7 and 11) for the presence of LESϕ4 prophage at a specific site in the *fimU* and *pilV* genes, respectively.

5.3.3 Temporal loss of twitching motility

Twitching motility mutants are found at a high frequency in endpoint populations from both treatments. Loss of the twitching motility phenotype has previously been

associated with the presence of bacteriophages (Hosseinidoust *et al.*, 2013a), so it was questioned whether there was any difference in the rate at which this occurred. Isolates obtained from each population at earlier time points were tested for the twitching motility phenotype. Loss of twitching motility is accelerated in phage-treated populations (Figure 5.5); at transfer 5 (approximately 40 bacterial generations), there was a significantly higher frequency of twitching impaired isolates in the phage-treated populations than the controls (Wilcoxon-Mann-Whitney; $W = 21.0$, $P < 0.01$).

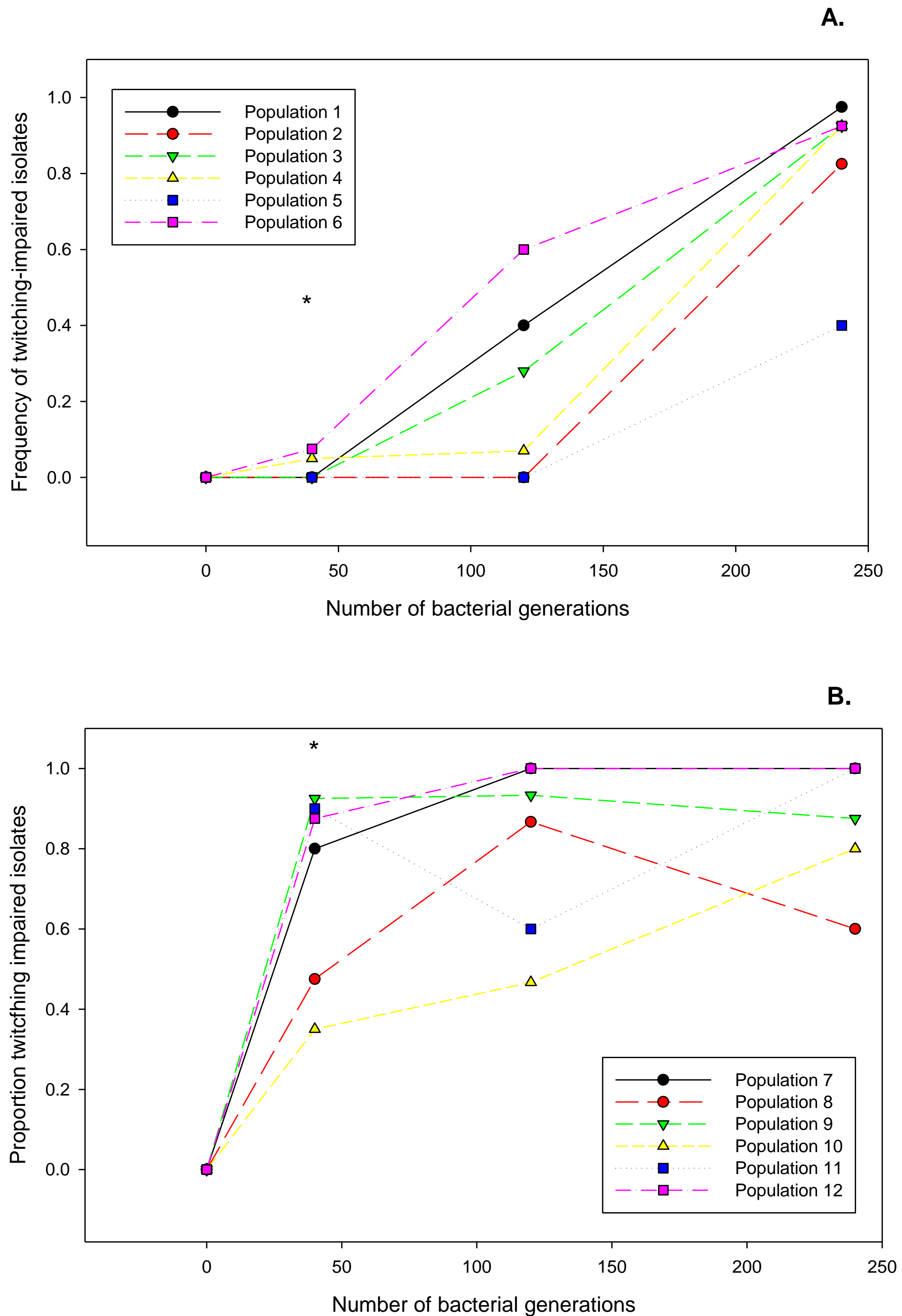


Figure 5.5 Temporal variation in the frequencies of twitching impaired mutants in ASM populations. (A.) control and (B.) phage treated populations (LESφ2-4). 40 isolates were assayed per population. Asterisks denote a statistically significant difference between treatments at a particular time point (*P* < 0.05).

5.3.4 Distribution of SNVs in pilus-related genes between treatments

Twitching motility is impaired in a high frequency of isolates in both control and phage-treated populations, but loss of motility is accelerated in phage-treated populations, potentially through LESφ4 disruptive integration into pilus-related genes. It became apparent that mutational hotspots in pilus-related genes, including *pilY1* and *pilA*, were present only in the controls (Figure 5.2), leading to the hypothesis that LESφ4 integration can act as an alternative (and potentially quicker) route along the evolutionary trajectory, towards the impaired twitching motility phenotype at which both treatments converge.

To test this hypothesis, the number of different SNVs per population in pilus-related genes were counted. Pilus-related genes are defined as those that encode structural or regulatory pilus components, and are known to affect twitching motility phenotype, as defined in (Burrows, 2012). The distribution of variants in pilus-related genes across the two treatments was compared.

More SNVs in pilus-related genes were detected in control populations; the median number of SNVs in each population was 3.5 and 0.5 for control and phage treatments, respectively (Table 5.2). The distribution of the two treatments was significantly different (Mann-Whitney; $W = 52.5$, $P < 0.05$). However, when phage integration sites are included as a variant (i.e. in addition to SNVs), the result became non-significant ($P > 0.05$).

Many of the pilus-related gene variants in the controls were in genes that were the phage integration loci in phage-treated populations. Interestingly, population 9 had low carriage of LESφ4, and of the 18% of the population that were LESφ4 lysogens, none had integrations in pilus-related genes. However, this population still had a high frequency of twitching impaired mutants (88%), which corresponded with 88% of isolates carrying a mutation in *pilY1* gene. Other phage-treated populations (of which three had no mutations in pilus-related genes) generally had high levels of twitching impairment, which suggests that the LESφ4 integration into pilus-related genes causes loss of phenotype. Populations 8 and 10 had the lowest frequency of LESφ4

integrations into pilus-related genes (Figure 5.4), and also the lowest frequencies of twitching impaired mutants (Figure 5.5).

Although the data are only correlational, there is reasonable evidence that loss of motility in populations is a result of the phage integrations or mutations in pilus-related genes that were detected. However, there are some instances where the sequence and phenotype data do not correlate well. For example, no SNVs in pilus-related genes were detected in the control population 5, yet 40% of the population were impaired in twitching motility.

Table 5.2 Comparison of the numbers of different variants in pilus-related genes between populations. Two types of variants were identified; those caused by single-nucleotide polymorphisms, and those caused by LES φ4 integrations. The number of variants does not give any information about the frequency of those variants in the population. Phenotype data (i.e. the frequency of twitching impaired mutants in the population) is included for reference.

Treatment	Population number	Number of SNVs in pilus-related genes	Number of different LESφ4 integration sites in pilus-related genes	Total number of variants	Frequency of twitching impaired mutants
Control	1	2	0	2	0.98
	2	6	0	6	0.83
	3	3	0	3	0.93
	4	4	0	4	0.93
	5	0	0	0	0.40
	6	5	0	5	0.93
Phage-treated	7	0	1	1	1.0
	8	0	1	1	0.60
	9	1	0	1	0.88
	10	0	1	1	0.80
	11	1	1	2	1.0
	12	1	2	3	1.0

5.3.5 Temporal loss of twitching motility and phage integration sites in single phage controls

In addition to the two treatments (phage treated and phage-free control) that have been widely discussed in the present and previous chapter, there were additional single-phage control treatments that ran alongside the ASM evolution experiment, but the endpoint populations were not characterised in detail. Each single-phage control treatment consisted of 6 replicates, with the addition of either LESφ2, LESφ3 or LESφ4 at the beginning. The single-phage controls allow a comparison of bacterial and phage evolution in the presence or absence of the other LES phages.

The LES phages each require type IV pili for infection (James *et al.*, 2012), hence LESφ4 disruptive integration into pilus-related genes may act as a protective superinfection exclusion mechanism against LESφ2 and LESφ3, which may explain the high frequency of pilus-related LESφ4 integration sites and rapid loss of motility. To explore this possibility further, LESφ4 single-phage control populations (P4 i-vi) were characterised for the twitching motility phenotype, and the frequency of lysogens through time. Although single-phage control populations were not sequenced, IPCR was used to attempt to determine the integration sites of LESφ4 in 12 isolates (2 per population, randomly selected).

All isolates tested in populations P4 i-vi were LESφ4 lysogens, at every timepoint (data not shown). The frequency of twitching impaired isolates was high, reaching 100% of the population in five of the six replicates at t30. In the remaining population (P4i) however, the frequency of twitching impaired isolates increased initially, but then decreased over time (Figure 5.6).

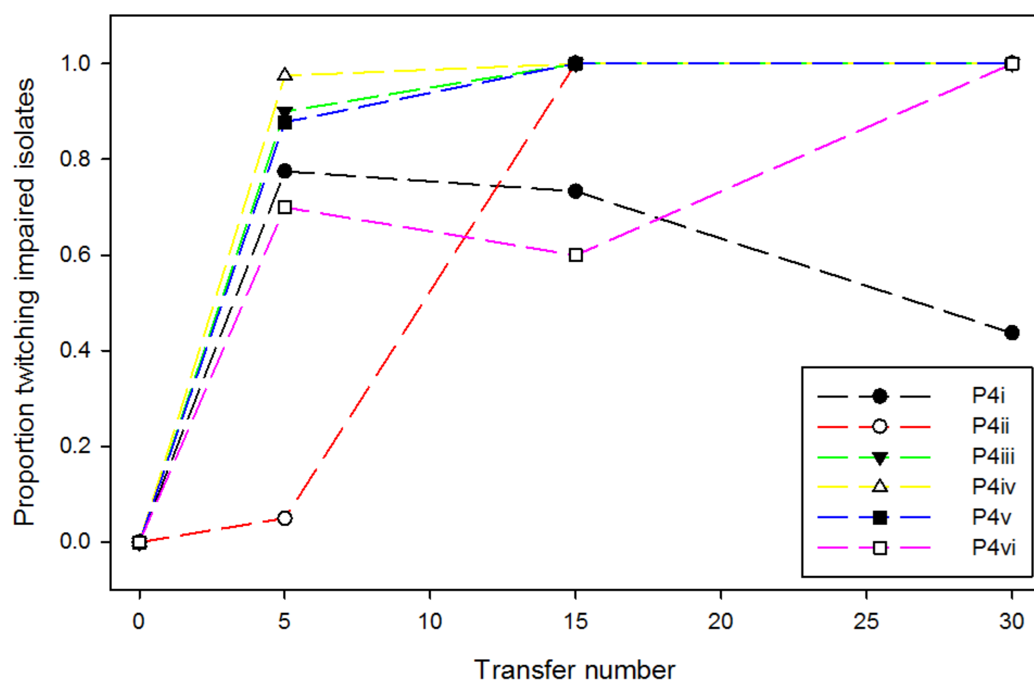


Figure 5.6 Temporal variation in the frequencies of twitching impaired mutants in single-phage control (LESφ4 only) populations.

IPCR was performed on two randomly selected isolates from each population P4 i-vi. The method was largely unsuccessful, and the integration sites of many of the isolates could not be determined, as either no PCR product was obtained in the final step, or it was at too low a concentration for sequencing. The results obtained are described in (Table 5.3).

Table 5.3 Inverse PCR to determine integration sites of LES φ4 in the PAO1 genome. Two isolates were tested in each of the six replicate LESφ4 only treatment populations.

Population	Isolate number	Integration site (gene)	Gene function(s)
P4i	1	*pqsB*	Part of the *pqsABCDE* locus (role in QS)
P4i	2	Undetermined	N/A
P4ii	1	Undetermined	N/A
P4ii	2	*phzG1 / phzS* intergenic region	Intergenic region, unknown function
P4iii	1	*pilW*	Type IV fimbrial biogenesis protein
P4iii	2	*pilW*	Type IV fimbrial biogenesis protein
P4iv	1	Undetermined	N/A
P4iv	2	Undetermined	N/A
P4v	1	Undetermined	N/A
P4v	2	Undetermined	N/A
P4vi	1	Undetermined	N/A
P4vi	2	Undetermined	N/A

5.4 Discussion

In this study, the genomes of the 40 phenotypically characterised isolates per population were sequenced using a pooling strategy. Such a strategy has a major advantage over sequencing a single isolate per population, as it enables one to detect genetic diversity in a population. However, it is a challenge to identify mutations that are adaptive, as opposed to those that are neutral, and may simply become fixed through genetic drift. Using the scoring system developed by Dr. Sam Haldenby, it was possible to identify mutations that are likely to be adaptive, based on their prevalence throughout all populations and their predicted severity. Genes that are at a high frequency in multiple populations (an example of parallel evolution) and genes with more severe mutations (e.g. frameshift and non-synonomous mutations) will have a high score, and these are genes that are likely to be under selection. This scoring method was chosen over the commonly used dN/dS ratio, where genes that are under selection are expected to have a higher proportion of non-synonomous mutations, relative to the number of synonomous mutations. This is because the dN/dS ratio is suitable only for distantly diverged sequences, and the assumptions are violated when applied to a single population (Kryazhimskiy & Plotkin, 2008).

Some of the highest scoring mutated genes were those involved in QS, notably *lasR*, which as a regulatory gene will likely have pleiotropic effects on other genes. Mutations in *lasR* are observed frequently in chronic CF isolates (Bjarnsholt *et al.*, 2010; Smith *et al.*, 2006b; Yang *et al.*, 2011), resulting in down-regulation of many virulence genes. *In vitro*, LOF mutations in *lasR* confer a growth advantage with amino acids (D'Argenio *et al.*, 2007), and reduced cell lysis during the death phase of growth in culture (Heurlier *et al.*, 2005). PAO1 QS mutants also demonstrate altered biofilm formation in ASM (Haley *et al.*, 2012; Sriramulu *et al.*, 2005). The fact that multiple non-synonymous mutations in *lasR*, capable of reaching fixation in four populations were observed suggests that loss of LasR function is advantageous in this model. However, the same SNV reached fixation in three of the populations, and true parallel evolution of this degree seems highly unlikely. However, it is possible: parallel evolution to the level of individual nucleotides has been observed in short-term experimental evolution of *P. aeruginosa* in a biofilm (McElroy *et al.*, 2014). A

possible explanation in this study is that this particular variant was present at a low frequency in the input PAO1 culture. A single colony was grown in liquid culture and used to seed all the populations, so it is feasible that a spontaneous mutation occurred during growth, resulting in inoculation of ASM populations with a low frequency of mutants. However, even if this is the case, the fact that the SNV reached fixation in these population suggests some kind of adaptive benefit. An alternative explanation is cross-contamination between populations, but this seems highly unlikely upon examination of other SNVs in those populations.

Mutations in regulatory genes were a common theme; in addition to *lasR,* mutations were identified in *glpR*, *cysB* and *amrZ*. Early adaptation to an environment through mutations in regulatory genes has been observed in experimental evolution experiments with *E. coli* (Wang *et al.*, 2010a; Zambrano *et al.*, 1993) and in CF *P. aeruginosa* isolates (Yang *et al.*, 2011). In *E. coli* and *Citrobacter freundii* strains isolated from the gut and experimentally evolved in rich media, the two most common mutations were in the global regulators *rpoS* and *arcA*, with mutations in *rpoS* mostly LOF. These mutations were associated with improved amino acid utilisation, suggesting that changes in global regulators can be short-cuts to rapid adaptation (Saxer *et al.*, 2014).

Genes involved in metabolism also scored highly, with a frameshift mutation in *hutU* that reached fixation in 7/12 populations, a strong indicator for positive selection. The product of *hutU* is an enzyme required in an early step in both the four and five-step histidine catabolism pathways, both of which are active in PAO1 (Gerth *et al.*, 2012). Transposon mutagenesis of PA14 found that *hutU* mutants show reduced biofilm formation (Müsken *et al.*, 2010), and HutU expression is altered during growth in biofilm (Fung *et al.*, 2010; Patell *et al.*, 2010), and in response to phage infection (Lavigne *et al.*, 2013) and low oxygen concentrations (Alvarez-Ortega & Harwood, 2007). It is possible that HutU may have other functions other than histidine catabolism.

Analysis of the frequency of variants across the genome identified hotspots of mutations in the *braB* and *braC* genes, involved in the low affinity and high affinity branched chain amino acid transport systems, respectively. *braC* is part of a 5 gene

operon necessary for the Na+ independent high-affinity LIV-I transport system (Hoshino & Kose, 1990) specific for alanine and threonine in addition to branched chain amino acids. Mutants in *braC* have reduced utilisation of several amino acids (Johnson *et al.*, 2008), but what effect the mutations observed in this study will have on the cell phenotype is unknown.

The highest frequency of variants was observed in the Pf4 region of the PAO1 genome. Pf4 is a Pf1-like filamentous prophage , implicated in host virulence (Rice *et al.*, 2008) and the formation of small colony variants (Webb *et al.*, 2004). During growth in a biofilm, a superinfective, lytic form of Pf4 arises that causes the cell death necessary for biofilm differentiation and dispersal (Rice *et al.*, 2008; Webb *et al.*, 2003). A recent deep-sequencing study of *P. aeruginosa* after short-term growth as a biofilm revealed a high rate of evolution in the Pf4 genome (McElroy *et al.*, 2014). Similar to this study, they also observed mutations in type IV pili genes, and speculated that this may be a resistance mechanism to avoid superinfection by lytic Pf4.

Impaired twitching motility is frequently observed in chronic CF isolates (Lee *et al.*, 2005b; Mahenthiralingam *et al.*, 1994; Smith *et al.*, 2006b) and was observed at high frequencies in most of the endpoint populations in this study (Chapter 4). Interestingly, the genetic mechanisms underlying loss of twitching motility appear to be different between the two treatments. Control populations had significantly more SNVs in pilus-related genes than phage-treated populations, and the SNVs observed in the phage-treated populations could not explain the high levels of twitching motility (with the exception of population 9, in which a SNV in *pilY1* was detected at high frequency). However, LESφ4 was integrated into pilus-related genes at high frequency in several populations. PCR analysis confirmed the two most common LESφ4 integration loci, and temporal analysis revealed that both integration loci had risen in frequency in the population over the course of the experiment, reminiscent of a selective sweep. It is known that the majority of bacteria in both populations were LESφ4 lysogens from early on in the experiment, so the increase in lysogens with LESφ4 integrated at these loci suggests either a strong selective advantage for the bacteria, genetic drift or hitchhiking with other beneficial alleles. The fact that loss of twitching motility evolves repeatedly indicates the former.

Type IV pili are the target of many bacteriophages (Ceyssens *et al.*, 2009a; Chibeu *et al.*, 2009), including LESφ2, φ3 & φ4 (James *et al.*, 2012), so loss may be driven by antagonistic pressure imposed by phages, or may be an adaptation to the ASM environment. Type IV pili have previously been identified as necessary for surface-attached biofilm formation (O'Toole & Kolter, 1998; Yoon *et al.*, 2002), although this can be dependent on the nutrients available (Klausen *et al.*, 2003c). However, in ASM, *P. aeruginosa* form biofilm-like aggregates (Haley *et al.*, 2012; Sriramulu *et al.*, 2005) similar to those observed in the CF lung (Hassett *et al.*, 2002), and the necessity for type IV pili in the formation of such structures is unclear. There is no apparent fitness cost to lack of type IV pili in this model; when ancestral PAO1 is competed against the phage-resistant PAO1 *pilA*$^{-}$ (does not produce type IV pili), no difference is observed in the Malthusian parameters of the two strains (Chapter 4).

Loss of twitching motility is accelerated in the LES phage-treated populations, apparently through LES φ4 integration into pilus-related genes. As LESφ2 and LESφ3 also require type IV pili for infection, this has the added effect of rendering cells resistant to infection from phage infection. Considering the majority of temperate phage infections result in lytic infection, this could be advantageous to the bacteria (and also LESφ4), functioning as a superinfection exclusion mechanism. However, total loss of twitching motility in 5/6 LESφ4-only control populations (P4i-vi) suggests that this is not driven solely by antagonistic selective pressures imposed by LESφ2 and LESφ3. However, the presence of Pf4, the endogeneous filamentous phage (which enters through the type IV pili and develops into a superinfective lytic form) means that phage-driven selection is still a plausible explanation.

A recent study investigating superinfection exclusion by a related transposable phage, D3112, found that the phage encoded a small protein that inhibits the polar localisation of the ATPase PilB, preventing the normal polymerisation of the pilin subunits and subsequent pilin production. The authors also observed that this conferred resistance to lysogens from other pilus-specific phages (Chung *et al.*, 2014). A BLASTP search of the LESφ4 predicted protein sequences revealed that LESφ4 does not encode this protein. Another study with the related phage DMS3 observed lack of swarming motility and reduced biofilm formation in PA14

lysogens, but only in bacteria with a functional CRISPR-Cas system. An interaction between the CRISPR-Cas system and the phage inhibited motility, which the authors proposed may be a strategy by the bacteria to limit dissemination of phage throughout the bacterial population (Zegans *et al.*, 2009). Together with previous data, this study raises the possibility that motility inhibition is widespread throughout *Pseudomonas* transposable phages, but acting via different mechanisms. Bacterial motility is an important virulence factor, and the potential of phage infection as a means of inhibiting motility and reducing virulence has been explored as a therapeutic option. This has been successful to a degree; D3112 infection and twitching motility inhibition of the virulent *P. aeruginosa* strain PA14 leads to reduced bacterial numbers and virulence in mouse and fly models of acute infection (Chung *et al.*, 2012).

Negative lysogenic conversion by bacteriophages has been identified before, but whether or not it confers a fitness benefit (or deficit) to host bacteria has not really been considered. Bacteriophages can insert into intergenic regions, tRNA or protein coding genes, but integration into the latter two does not normally cause LOF, as certain bacteriophages (those that insert by site site-specific recombination, such as λ) duplicate the sequence from the 3' end of the inserted gene, so a functional gene product is still transcribed (Campbell, 1992). However, gene disruption can still occur if this process does not happen, or if it is faulty; an analysis of 13 *Lactobacillus johnsonii* prophages found that two integrated into tRNA genes, and one caused gene disruption, as there was no reconstitution of the gene product (Ventura *et al.*, 2003). Because D3112 (the close relative of LESφ4) replicates by transposition, the hypothesis was that it caused mutations through integration into coding regions. To test this, PAO1 D3112 lysogens were plated onto pyruvate-fluoroacetamide agar; only amidase mutants are able to grow, as amidase-mediated hydrolysis of fluoroacetamide produces fluoroacetate, which is toxic to cells when growing on pyruvate as a carbon source. Phage infection and integration increased the frequency of occurrence of amidase mutants 40 to 65-fold, and analysis of the lysogens found that D3112 had integrated into different locations in the *ami* regions, suggesting that it is not a hotspot and that D3112 inserts randomly (Rehmat & Shapiro, 1983). Even though LESφ4 is observed integrated frequently into motility genes, it is uncertain whether LESφ4 can insert anywhere, but confers an adaptive benefit to lysogens with

loss of function in the genes, or whether LESφ4 preferentially inserts at certain loci. Like D3112, LESφ4 was not observed at the same site between populations, suggesting that integration site is at least partly random, and lysogens with LESφ4 integrated into type IV pilus genes have a selective advantage.

P. aeruginosa evolution in the CF lung is somewhat unusual in that it can be dominated by LOF mutations (Hogardt & Heesemann, 2010). The majority of mutations in the study of isolates from chronic infections by Smith *et al*. were LOF, and when the mechanistic basis for this was investigated, it was found to be largely through transposon insertions, gene deletions and STOP codon or frameshift mutations, all of which would result in predicted loss of protein function (Smith *et al.*, 2006b). Similarly, in this study, LOF mutations dominated, especially in QS and motility genes. Large chromosomal inversions (LCIs) can also cause LOF, and genome analysis of the highly prevalent *P. aeruginosa* clone C, which is the most common non-epidemic clone identified in CF centres (Hall *et al.*, 2014; Scott & Pitt, 2004), found that LCIs had disrupted the LPS biosynthesis gene *wbpM*, the type IV pilus-related *pilB* and the DNA repair gene *mutS*, resulting in impaired biosynthesis of the O-antigen, loss of twitching motility and hypermutability, respectively. It was suggested that inactivation of these genes by LCIs was advantageous was selected for over the course of evolution (Kresse *et al.*, 2003). Transposable phages such as LESφ4 offer an alternative mechanism for achieving LOF mutations and the associated change in phenotype.

A large number of SNVs were detected across all endpoint populations, yet, as with the phenotypic data, there were no obvious differences between the two treatments (with the exception of the mechanisms underlying changes in type IV pilus-related genes). This was associated with no difference in fitness between phage-treated and control populations when competed against a phage-resistant competitor, which removes any advantage conferred by prophage-mediated lysis of competitors (Chapter 4). This is perhaps surprising, given that temperate bacteriophages can carry genes that increase host fitness; indeed, LESφ2 and LESφ3 are necessary for the competitiveness of their host, LESB58 (Winstanley *et al.*, 2009). Such benefits are either dependent on lysis of the competitor, host-specific or not applicable in this simplified *in vitro* system.

Experiments with lytic phages have shown, conversely, that coevolution can have a negative effect on bacterial fitness, through resistance mutations that have an associated cost (Brockhurst *et al.*, 2005; Buckling *et al.*, 2006; Koskella *et al.*, 2012). A recent study found that bacteria that are coevolved with a lytic bacteriophage have lower fitness in the growth medium than bacteria evolved in the absence of phage. Bacteria evolved in the absence of phage demonstrated greater genetic divergence and adaptation to the media, showing increased fitness relative to the ancestor in pairwise competitions, and parallel evolution in a set of genes posited to be important for adaptation to the abiotic environment. However, when these populations were then coevolved with phage, the fitness benefit of these mutations was lost. In the reverse, coevolved populations were then evolved in the absence of phage, but they did not show an increase in fitness. Together, these results suggest some type of epistatic interaction between growth promoting and phage resistance mutations (Scanlan *et al.*, 2015). The lack of difference between controls and phage-treated populations in this study, both in terms of fitness and sequence data, suggests a lack of antagonistic pleiotropy, or epistatic interactions. Such a finding is unsurprising, given that the LES phages are temperate, hence the same strong antagonistic selective pressures are unlikely to apply.

5.5 Summary

- SNVs were detected in all populations, across both treatments, and the highest scoring genes (those most likely to be under positive selection) were involved in motility, QS or genetic regulation.
- A high frequency of variants was observed in the Pf4 region of the PAO1 chromosome.
- LESφ4 integrated at different sites in the PAO1 chromosome, but in the majority of lysogens had integrated into protein-coding genes involved in motility or QS. The two integration sites that were most common in endpoint isolates (both motility genes) rose in prevalence over the course of the experiment.
- Twitching motility mutants are at a high frequency in endpoint populations in both treatments, but loss of twitching motility occurs faster in the phage-treatment.

- More SNVs were detected in twitching motility-related genes in the control populations, suggesting that LESφ4 can act as an alternative (and more rapid) mechanism to achieving LOF in motility genes.
- When PAO1 was coevolved with LESφ4 as the only phage, twitching motility was still lost rapidly. In the few isolates tested, LESφ4 had integrated into protein-coding genes involved in motility and QS, and intergenic regions. This suggests that LESφ4-mediated loss of motility is not driven by the presence of LESφ2 and LESφ3 in the triple phage treatment.

5.6 Declaration

All sequence data were subjected to quality control and analysed by Dr. Sam Haldenby, Centre for Genomic Research, University of Liverpool. SNVs were called and the variant position frequency determined, and phage integration sites were estimated by examination of contig ends.

Chapter 6 Allelopathy: temperate phages as a strategy for bacterial invasion

6.1 Introduction

6.1.1 Bacterial competition

Bacteria face constant competition for resources, and inter-microbial competition is thought to play a large role in driving the evolution of bacterial populations. Essential resources are limiting factors to bacterial growth (Monon, 2012), hence competition for them is fierce. There are two main types of competition: scramble and contest. Scramble, or exploitative competition, is competition to utilise a finite resource that is available to all competitors. It is passive in that it does not have direct, antagonistic effects on other competitors, but rather deprives them of the resource. A proposed analogy is young boys scrambling for pennies dropped on the floor, with the winners those that get there first (Hölldobler & Wilson, 1990). Contest, or interference competition, is direct, antagonistic interaction between competitors, for example, the production of toxins that harm competitors. Returning to the analogy, contest competition can be likened to the boys having a fight, with the winner taking the pennies (Hölldobler & Wilson, 1990). It is likely that bacteria utilise both types of competition to proliferate in the CF lung, but there is little research addressing this specifically.

The CF lung is a polymicrobial environment, and there is likely to be competition between, as well as within, species. There is evidence for interspecies competition between *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Colonisation by *S. aureus* is negatively associated with *P. aeruginosa* colonisation, and in culture, *S. aureus* reaches lower densities when in co-culture with *P. aeruginosa* than in monoculture. This also extends to a rat dialysis membrane peritoneal model, and it was observed that *P. aeruginosa* was not only capable of lysing *S. aureus*, but also downregulates expression of iron-acquisition genes. This suggests that *P. aeruginosa* uses *S. aureus* as an iron source (Mashburn *et al.*, 2005), and as iron is a limiting resource that is sequestered by the host, this is strong evidence for interspecific interference competition.

P. aeruginosa populations in chronic CF infections are highly diverse, with several strains or lineages often coexisting (Williams *et al.*, 2015), and it is likely that there will be competition between lineages. The LES is very prevalent in the UK and North America (Aaron *et al.*, 2010) and is capable of superinfection i.e. it can replace resident *P. aeruginosa* strains in the lung (McCallum *et al.*, 2001). In a rat model of chronic lung infection, LES isolate B58 is more competitive than strain PAO1 (Kukavica-Ibrulj *et al.*, 2008b), yet the mechanisms underlying this competitiveness have not been fully elucidated. A signature-tagged mutagenesis study of LESB58 highlighted some of the genes and regions that were necessary for LESB58 competitiveness, including several prophages (Winstanley *et al.*, 2009). One hypothesis is that LES uses its prophages as an allelopathic agent, lysing resident *P. aeruginosa* in the lung, enabling LES to invade and dominate the *P. aeruginosa* population in the CF lung.

6.1.1.1 Allelopathy

Allelopathy is a form of interference competition, and can be defined as the production of agents (e.g. chemicals) that kill or inhibit the growth or reproduction of competitors. Allelopathy has been widely studied in plants, and it is argued that it plays a large role in the invasion of certain plant species (Fitter, 2003). Allelopathy has also been identified in bacteria, most notably in the production of bacteriocins. Bacteriocins are antimicrobial, proteinaceous compounds, produced by bacteria and capable of killing closely related species. Among others, they have been identified in *P. aeruginosa* and *E. coli*, where they are known as pyocins and colicins, respectively. Bacteriocin production is an example of a spiteful interaction (Gardner *et al.*, 2004): harm occurs to the producer (cell lysis is often required to release the toxin) and to the recipient (probable death). Bacteria that carry the immunity gene are protected from the harmful effects of the bacteriocin, and close relatives of the producer are more likely to carry the immunity gene. Despite the high cost to the producing cell, bacteriocin production is maintained in the population because of kin selection (Hamilton, 1964); the bacteriocin targets non-relatives, conferring a fitness benefit to close relatives of the producer (which carry the immunity gene). The producer receives an indirect fitness benefit through increased fitness of its relatives, as the relatives can pass on its genes. Under this social theory, bacteriocin production

is expected to be favoured under intermediate levels of kinship, where kinship is defined as the relative frequencies of the "producer" and "recipient" genotypes. At low levels of kinship, the density of producers, and consequently levels of bacteriocins are low, and the bacteriocin is unable to reach a critical concentration to affect competitors. When kinship is high i.e. high density of producers, the density of susceptible competitors is low, hence there is not enough fitness benefit to justify the inherent cost (Gardner *et al.*, 2004). There is experimental evidence for this in *P. aeruginosa*, as when pyocin producing PAO1 and a sensitive competitor were competed *in vitro* (with different initial starting ratios), fitness was highest when densities of each competitor were similar (Inglis *et al.*, 2009).

It has been suggested that temperate bacteriophages can also act as allelopathic agents (Stewart & Levin, 1984). Like bacteriocins, phages can kill susceptible recipient cells, are costly to produce (cell lysis of the producer is required), and closely related bacterial cells are more likely to carry the phage and associated superinfection exclusion genes, hence relatives are protected from lytic phage infection. Brown and colleagues compared bacteriocins and phages as allelopathic agents, using mathematical modelling to predict outcomes and experimental data to validate. Bacteriocins were superior as a strategy to resist invasion i.e. when the density of producing cells was high, but phage were superior as an invasion strategy, largely because phages are capable of amplification: upon lytic infection of a susceptible cell, more phages are produced. Amplification is key to the success of this strategy; when the experiment was repeated with a phage-susceptible competitor that was still killed upon infection, but was not capable of releasing infective phages, the producing strain was unable to invade. Prophage carriage was inferior as a strategy to resist invasion because the density of susceptible cells was too low for the phage to successfully multiply (Brown *et al.*, 2006).

Numerous other examples have been described in the literature of phages acting as allelopathic agents. Bossi *et al*. co-cultured Gifsy lysogens and non-lysogens of *Salmonella enterica* serovar Typhimurium, and found that lysogens dominated at the end of the experiment. The same did not happen when the experiment was repeated with a lysogen in which the phage was unable to excise, suggesting that the success of the lysogen was through prophage-mediated lysis of the competitor, as opposed to

an inherent fitness benefit conferred by the phage (Bossi *et al.*, 2003). The authors also observed that the majority of non-lysogens became lysogens, something that was also observed in a more recent study with *E. coli* phage λ, suggesting that whilst phage can act as allelopathic agents, lysogenic conversion means that any advantage is transient (Gama *et al.*, 2013).

Another example of phages acting as allelopathic agents has been described in *Bordetella bronchiseptica*. The starting densities of isogenic lysogens and non lysogens were manipulated, and lysogens were able to invade from when initially rare, with a reversal in the 1:10 initial starting ratio of lysogen to non-lysogen after 24 hours. Lysogens were also able to resist invasion and still increase in frequency when initially dominant, increasing from a ratio of 10:1 to 1000:1 over a 24 hour period (Joo *et al.*, 2006). Lysogen invasion of phage-susceptible populations has recently been demonstrated *in vivo*; *Enterococcus faecalis* lysogens that produce composite phages outcompete non-lysogens in a mouse intestinal colonisation model (Duerkop *et al.*, 2012).

6.1.1.1.1 Factors influencing the efficacy of allelopathy

The spatial structure of the environment is known to have an effect on the fitness benefits to be gained by using phage as an allelopathic agent. In competitions of *E. coli* λ lysogens and non-lysogens, with different starting densities of each competitor, differences were observed between competitions that took place in structured (soft agar) or unstructured habitats (broth). The lysogen to non-lysogen ratio increased in structured habitats at all initial starting density ratios, but the opposite was observed in an unstructured habitat. In an unstructured habitat, the lysogen to non-lysogen ratio *decreased* over the course of the experiment at all starting densities, a finding that contradicts other similar experiments (Brown *et al.*, 2006; Joo *et al.*, 2006). When the experiment was performed with reverted antibiotic resistance markers, lysogens were no longer at a disadvantage, which the authors suggested was due to the effect of the antibiotic resistance mutation on phage development e.g. amplification efficiency or probability of lysogeny (Gama *et al.*, 2013). In addition, the λ phage is known to propagate poorly in unstructured environments, because it has very small side tail fibres. Together, these factors mean that the initial finding that lysogens do poorly in unstructured habitats is not

necessarily the case. The better performance of lysogen in structured habitats can be explained by an increased concentration of phages in the surrounding area, whereas in unstructured habitats, phages diffuse away. A high probability of lysogeny was also observed, for the same reason, as a high concentration of phages in one area (high MOI) favours lysogeny (Lieb, 1953).

The pathogenic properties of the phage itself, or the presence of multiple phages, can also affect bacterial success in competitions. Lysogen invasion of *S*. Typhimurium susceptible populations is more marked in double lysogens than single lysogens. In addition, invasion occurs faster when the prophage carried is P22, instead of Gifsy, which may be attributable to the larger burst size of P22 (Bossi *et al.*, 2003). Similarly, Joo *et al*. found that the phage pathology can affect the invasive properties of lysogens (Joo *et al.*, 2006). A recent study compared the fitness of PAO1 strains containing two of the LES phages, LESφ2 and LESφ4, either alone or in combination in competition with an isogenic non-lysogen. The experiments were conducted *in vivo*, using the popular *Galleria mellonella* (wax moth) larvae model. As well as differences in the competitive abilities of lysogens harbouring each of the phages (LESφ4 lysogens were more competitive than LESφ2 lysogens), the double lysogen was significantly more competitive than either of the two single lysogens (Burns *et al.*, 2014), suggesting that polylysogeny may be a useful strategy with regards to interference competition, possibly by delaying the emergence of resistance to phage lysis in susceptibles, as after lysogenic conversion by one phage, bacteria would remain susceptible to other phage(s).

6.1.1.2 Apparent competition

The use of temperate phages by bacteria to outcompete phage-susceptible competitors is also a form of apparent competition. Apparent competition is a form of parasite –mediated competition between two hosts that are both capable of infection by the same parasite. It was first described between two species of the flour beetle, *Tribolium castaneum* and *T. confusum*. Despite being competitively inferior, *T. confusum* is capable of outcompeting *T. castaneum* through the carriage of the sporozoan parasite, *Adelina tribolii*, which has negative effects on the latter, yet no such effects on the former (Park, 1948). There are relatively few empirical example of apparent competition, but examples in plants (Recart *et al.*, 2013) and animals

(Tompkins *et al.*, 2000) have been described in the literature. The importance of apparent competition in microbial interactions is largely unknown.

6.2 Objectives

1. Determine the effect that LES prophage carriage has on bacterial invasiveness *in vitro*, where invasiveness is defined as the ability of a bacterial strain to increase in proportion when in competition with another strain when initially rare.
2. Consider the ability of bacterial LES phage lysogens to resist invasion by a phage-free strain.
3. Contrast the effects of single or mutiple prophage carriage on bacterial invasiveness and resistance to invasion, and compare the dynamics of invasion between carriage of different prophages.
4. Repeat bacterial competitions *in vivo*, to determine whether any effects of prophage carriage on bacterial fitness persist in a more natural, spatially structured environment.

6.3 Results

6.3.1 *In vitro* invasion assays

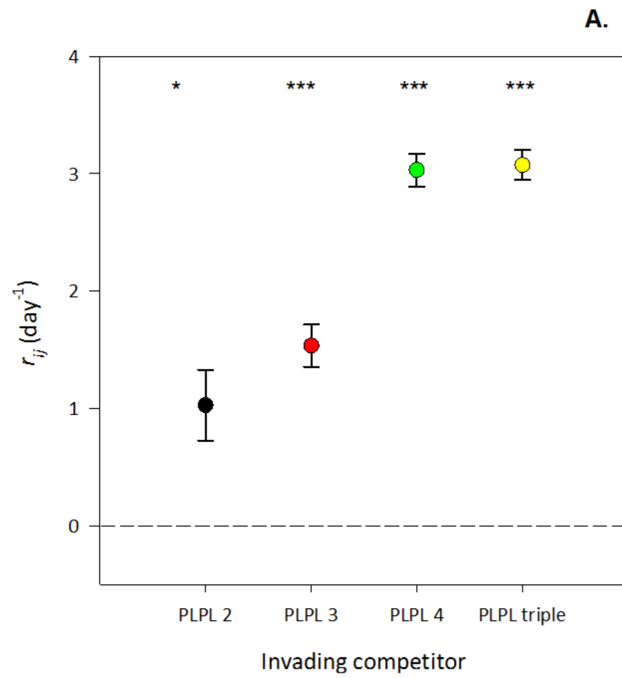
The aim of this study was to observe whether carriage of LESφ2, LESφ3, or LESφ4 leads to enhanced bacterial fitness when in competition with a phage susceptible strain. To achieve this, PAO1 LES Phage Lysogens (PLPLs) were competed in liquid media for 24 hours with an isogenic phage susceptible competitor, which does not carry any of the LES prophages, and is hereafter referred to as PAO1$^{\varphi-}$. To differentiate between competitors, strains were labelled with both GFP and DsRed-Express, separately (James *et al.,* unpublished). Reverse markers were used to control for any effect of marker, with the exception of PLPLφ2, for which only the GFP-labelled version was used, due to an issue of the DsRed-Express marker in the PLPLφ2 host background.

Two different starting ratios of the two competitors were implemented: 1:9 and 9:1 (PLPL: PAO1$^{\varphi-}$), in order to test the ability of PLPLs to invade a population of phage-susceptible bacteria, or defend against invasion by said population,

respectively. The density of each competitor was measured at 8-hourly intervals. At the end of each competition, the difference in the Malthusian parameters of the two competitors was calculated, returning a value that is known as the selection rate constant (r_{ij}). A r_{ij} value of 0 indicates no selection, but a r_{ij} value above 0 indicates positive selection for the PLPL.

6.3.1.1 PLPL invasion of a phage susceptible population

Over 24 hours, all PLPLs were able to successfully invade the phage susceptible population of PAO1$^{\varphi-}$ (Figure 6.1), as indicated by positive r_{ij} values. Selection rate constants were compared with a two-way ANOVA, fitting invading strain (5 levels, including a control with a r_{ij} of 0, expected if there was no selection) and marker (2 levels) as the two factors. The interaction term was not significant ($P = 0.17$) so a simple additive model was fitted to the data. A significant effect was observed for PLPL ($F_{3,16} = 14.8$, MSE = 4.42, $P < 0.001$) but not marker ($F_{1,16} = 1.24$, MSE = 0.37, $P = 0.28$). Tukey HSD tests were utilised to compare the means of the different PLPLs. PLPLφtriple was significantly more invasive than PLPLφ2 ($P < 0.01$) or PLPLφ3 ($P < 0.001$), but not PLPLφ4 ($P = 0.87$). PLPLφ4 was also significantly more invasive than PLPLφ2 ($P < 0.01$) or PLPLφ3 ($P < 0.001$). There was no difference between PLPLφ2 and PLPLφ3 ($P = 0.99$).



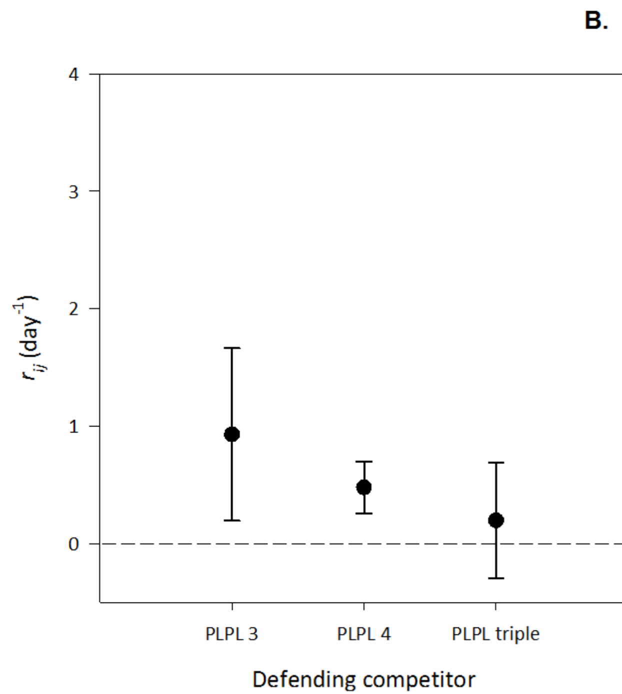


Figure 6.1 Selection rate constant values for competition between PLPL and PAO1$^{\varphi-}$ where the starting ratio of PLPL to PAO1$^{\varphi-}$ is (A.) 1:9 and (B.) 9:1. Error bars ± 1 S.E.M. Asterisks denote statistical significance (* *P*<0.05; ** *P*<0.01; * *P*<0.001), when compared to a hypothesised value of 0 (no selection).**

6.3.1.2 PLPL resistance to invasion by PAO1$^{\varphi-}$

The ability of PLPLs to resist invasion was also investigated. However, there was a clear effect of marker; the mean r_{ij} for PLPL gfp/ PAO1 DsRed-Express

competitions was much higher than the reverse (1.8 vs. 0.53 day^{-1}). This is because the threshold bacterial density for detection of the fluorescent protein was slightly higher for DsRed-Express than GFP, possibly because the available filters on the fluorometer were not optimal. This meant that at low densities, $PAO1^{\varphi-}$ was not detected, resulting in an artificially high selection rate constant. Because of this issue, data from competitions where $PAO1^{\varphi-}$ was labelled with DsRed-Express were excluded, which means that PLPLφ2 must necessarily be excluded from the analysis (as an issue with the DsRed-Express marker in the PLPLφ2 background meant that this strain was not used in competitions).

PLPLφ3, PLPLφ4 and PLPLφtriple can all resist invasion to $PAO1^{\varphi-}$ (Figure 6.1), with no difference between the PLPLs (one-way ANOVA; $F_{3,8} = 0.78$, $P = 0.54$). Post-hoc comparison to a control with a value of 0, expected if there was no selection, was non-significant ($P > 0.05$), suggesting a lack of selection in either direction for PLPLs when initially dominant.

6.3.1.3 Dynamics of PLPL invasion over time

Selection rate constants are a measure of the difference between the Malthusian parameters of two strains over a 24 hour period, using bacterial densities at the end and beginning of competition. However, this tells us only the outcome of the competition, but nothing of the dynamics of the interaction between competitors. By measuring the ratio of two competitors at 8-hourly intervals in PLPL invasion assays, a more detailed picture of the dynamics of this invasion, including differences between the PLPLs, can be constructed.

For each competition, the fluorescence intensity (FI) was measured at 4 time points, and used to estimate the ratio of the two competitors. The ratio of PLPL to $PAO1^{\varphi-}$ continues to rise for all PLPLs until t_{16}, after which the ratio stabilises in PLPLφ2 and PLPLφ3 (Figure 6.2). However, the ratio of PLPL to $PAO1^{\varphi-}$ continues to rise

for PLPLφ4 and PLPLφtriple over the course of the competition.

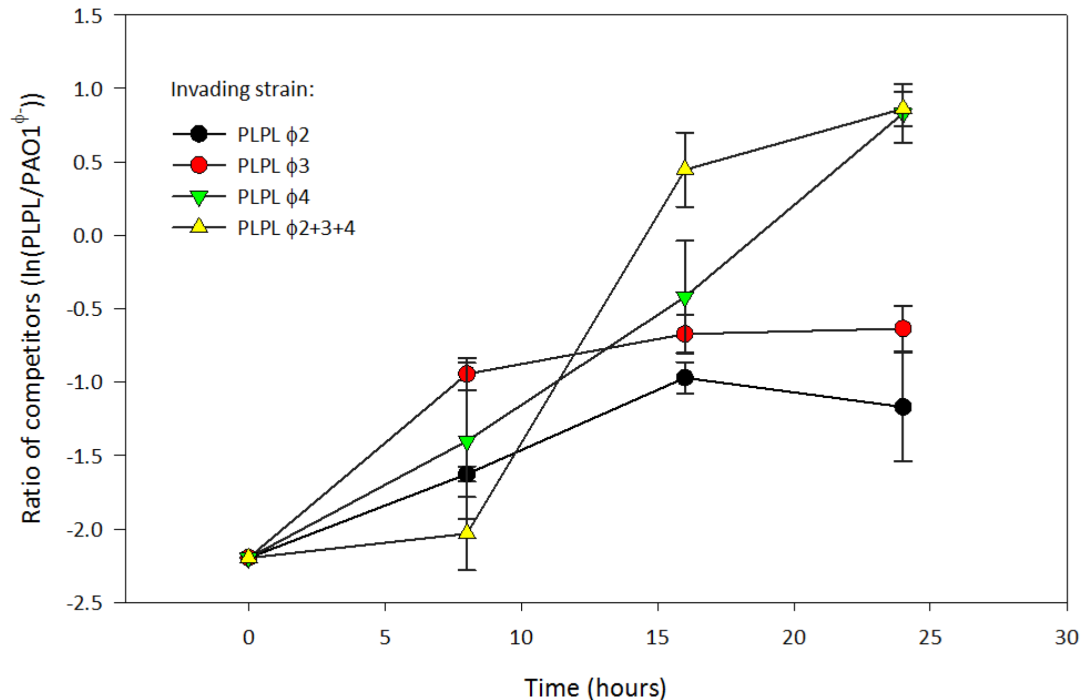


Figure 6.2 Ratio of PLPL to PAO1$^{φ-}$ over 24 hour coculture, with a starting ratio of 1:9 PLPL to PAO1$^{φ-}$. Error bars ±1 S.E.M. Three biological replicates were performed for each PLPL.

6.3.1.3.1 Statistical analysis

Because of this repeated measures design, the assumption of independence between data points is violated, hence the analysis must account for this to avoid temporal pseudoreplication. To this end, natural log-transformed data were analysed with a repeated measures ANOVA design by fitting a general linear model (GLM). Time and PLPL were fitted as fixed factors, fluorescent marker as a random effect, and competition ID as a random effect, nested within PLPL to account for the repeated measures. The effect of PLPL and time was significant, as was the interaction, but the random effect of marker was non-significant ($F_{1,71} = 0.73$, $P = 0.40$), so it was removed from the model in order to simplify. There was no increase in deviance, so the new model was preferred, under the principle of parsimony. Under the simplified model, the main effects of PLPL ($F_{3,72} = 4.40$, $P < 0.05$) and time ($F_{3,72} = 198.5$, $P <$ 0.001) were significant, as was the PLPL x time interaction ($F_{9,72} = 20.1$, $P < 0.001$). Post-hoc Tukey HSD tests were performed for the interaction effect, comparing all PLPLs to each other at each time point using the hierarchical model. PLPLφtriple

was significantly more invasive than all PLPLs at t_{16} (Table 6.1), whilst there was no difference between the three single phage PLPLs until t_{24}, when PLPLφ4 was equally invasive as PLPLφtriple, and more invasive than PLPLφ2 and PLPLφ3. Interestingly, carriage of three prophages appears to be associated with a minor fitness cost at an early stage of the competition, as the mean ratio of the two competitors was lowest for PLPLφtriple at t_8, and significantly lower than PLPLφ3 at the same time point.

Table 6.1 Tukey HSD post-hoc comparisons of Phage x Time interaction. *T* value and adjusted *p* values are displayed for each pairwise comparison.

PLPL invading		*Φ3*				*Φ4*				*Φtriple*			
Φ2	**Time**	**0**	**8**	**16**	**24**	**0**	**8**	**16**	**24**	**0**	**8**	**16**	**24**
	0	$t=0.0$, $p=1.0$				$t=0.0$, $p=1.0$				$t=0.0$, $p=1.0$			
	8		$t=-3.4$, $p=0.09$				$t=-0.99$, $p=1.0$				$t=2.4$, $p=0.59$		
	16			$t=1.5$, $p=0.98$				$t=-2.8$, $p=0.32$				$t=-7.1$, $p<0.001$	
	24				$t=-2.5$, $p=0.46$				$t=-9.9$, $p<0.001$				$t=-10.2$, $p<0.001$
Φ3	**0**					$t=0.0$, $p=1.0$				$t=0.0$, $p=1.0$			
	8						$t=2.4$, $p=0.59$				$t=-5.7$, $p<0.001$		
	16							$t=-1.3$, $p=1.0$				$t=5.6$, $p<0.001$	
	24								$t=-7.5$, $p<0.001$				$t=7.7$, $p<0.001$
Φ4	**0**									$t=0.0$, $p=1.0$			
	8										$t=-3.3$, $p=0.09$		
	16											$t=4.3$, $p<0.01$	
	24												$t=0.21$, $p=1.0$

6.3.1.4 Phage invasion

The previous experiments have been concerned only with the effect that a LES prophage has on the competitiveness of the bacterial strain. However, unlike other examples of allelopathy, such as bacteriocins, bacteriophages are ultimately selfish genetic elements, concerned only with their own replication. In this experiment, the competitor is fully susceptible to the LES phages and capable of becoming a lysogen, so it is interesting to consider the invasiveness of the phages themselves, and whether invasiveness of a bacterial strain has any effect on the evolutionary success of the phage itself. To assess this, bacterial colonies were isolated at the end of each competition and PCR-tested for prophage complement. The frequency of lysogens in the endpoint population was determined, although this does not differentiate between competitors. The frequency of lysogens was very high for all PLPL competitions under both starting ratios (Figure 6.3). For the PLPLφtriple competition, a breakdown is given of the different combinations of prophages detected (Table 6.2). The majority are triple lysogens, presumably the PLPL host genotype. Those remaining are not dominated by any particular phage; lysogens of all three LES phages are observed.

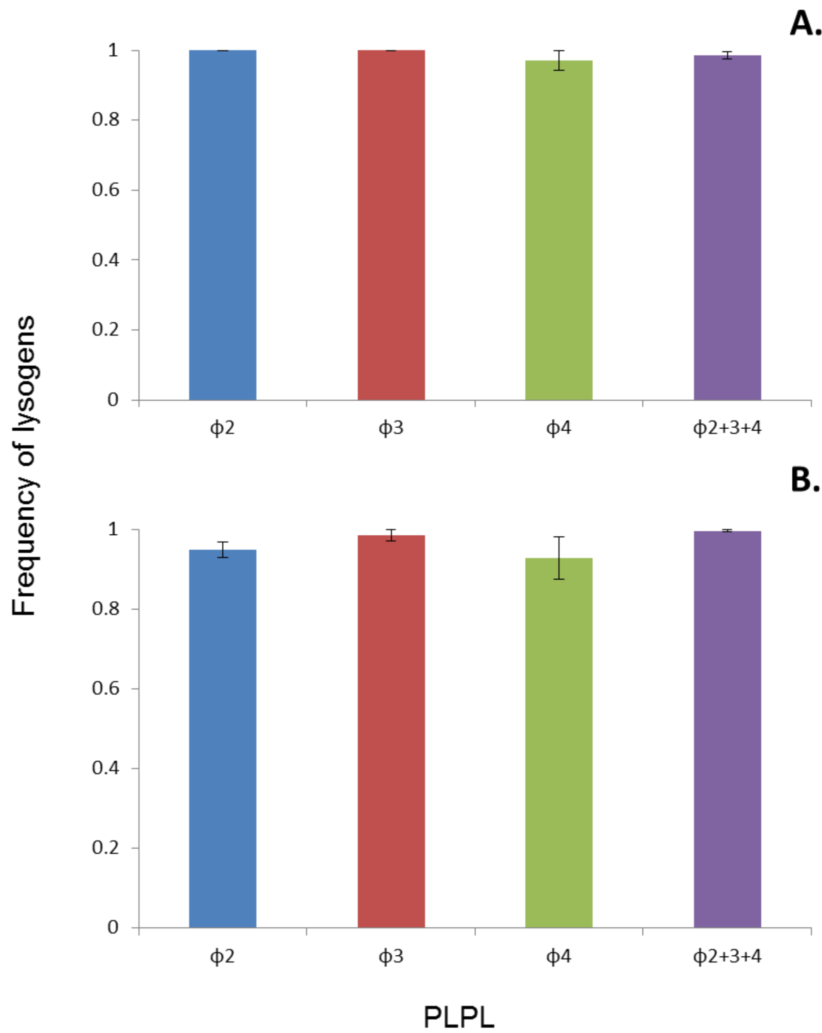


Figure 6.3 Frequency of bacterial lysogens after 24 hours of co-culture of various PLPL with PAO1$^{φ-}$ with an initial starting ratio of (A.) 9:1 and (B.) 1:9. Differentiation is not made between the two bacterial genotypes. Error bars ±1 S.E.M. Three biological replicates were performed for each strain, and a minimum of 46 separate colonies were tested per replicate.

In order to estimate the fitness of the phages themselves, selection rate constants were calculated for the phage. The Malthusian parameters were calculated for competitors carrying the phage (i.e. all bacteria that were PLPLs at the beginning, in addition to all those that were PAO1$^{φ-}$ at the beginning, but have subsequently become lysogens) and for competitors not carrying the phage (i.e. PAO1$^{φ-}$ that has maintained its phage-free status). The r_{ij} was calculated as follows:

$$r_{ij} = m_i - m_j$$

where *i* is the competitor carrying the phage, and *j* is the phage-free competitor. For simplicity, this was performed for PLPL single phage competitions only. The selection rate constant was high for all three phages, and there were no significant inter-phage differences (one-way ANOVA; $F_{2,6} = 1.2$, $P = 0.37$). The phage selection rate constant values are illustrated in (Figure 6.4), alongside the selection rate constants for the PLPL, which highlights the difference. The invasive ability of PLPLs differs depending on the prophage carried, but in terms of phage fitness, all the LES phages do equally well.

Table 6.2 Prophage complement of bacterial colonies obtained after 24 hours co-culture of PLPLφtriple and PAO1$^{\varphi-}$, at two different initial starting ratios of the two competitors. A minimum of 46 colonies were tested per marker, per replicate, and the results were pooled for both markers.

Frequency of bacteria harbouring specific prophage combination in total endpoint population						
	1:9 initial starting ratio (lysogen invading)			*9:1 initial starting ratio (lysogen defending)*		
	R1 (averaged for markers)	**R2**	**R3**	**R1**	**R2**	**R3**
Non lysogen	0.01	0.00	0.00	0.01	0.00	0.03
LESϕ2+3+4 (triple)	0.84	0.99	0.98	0.93	0.99	0.97
LESϕ2	0.09	0.00	0.01	0.00	0.00	0.00
LESϕ3	0.00	0.01	0.01	0.00	0.01	0.00
LESϕ4	0.00	0.00	0.00	0.01	0.00	0.00
LESϕ2+3	0.01	0.00	0.00	0.00	0.00	0.00
LESϕ2+4	0.01	0.00	0.00	0.16	0.00	0.00
LESϕ3+4	0.03	0.00	0.00	0.03	0.00	0.00

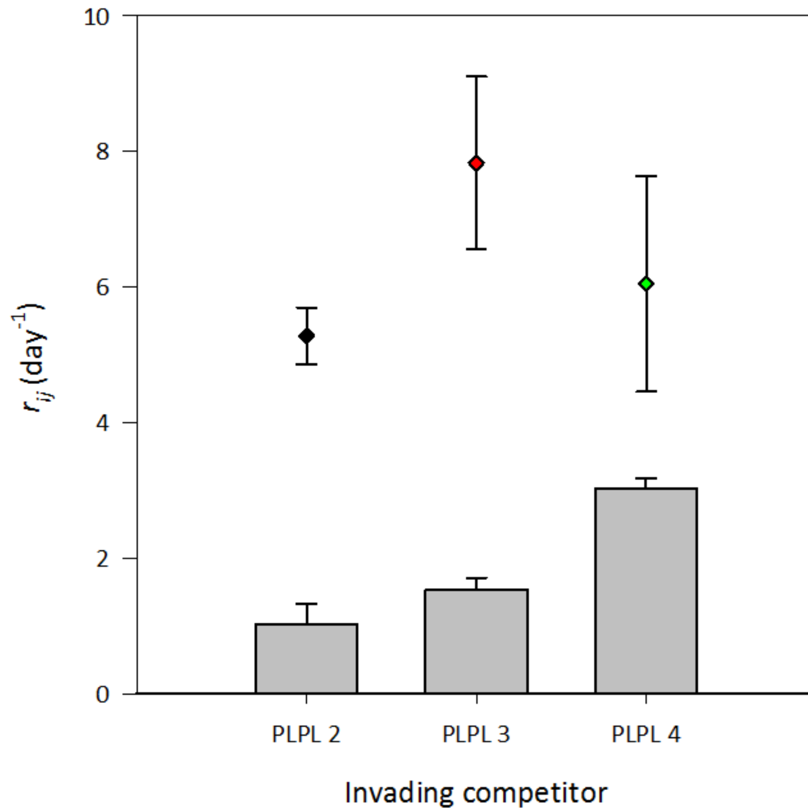


Figure 6.4 Selection rate constants of the LES phages (coloured diamonds), compared with the selection rate constants of bacterial strains that harbour the LES phages when competing against phage susceptible PAO1$^{\varphi-}$ (grey bars). Error bars ±1 S.E.M.

6.3.2 *In vivo* invasion assays

6.3.2.1 PLPLφtriple in competition with PAO1$^{\varphi-}$ at different starting densities

In order to test whether PLPLs were still capable of invading PAO1$^{\varphi-}$ *in vivo*, a similar experiment was conducted in a rat model of chronic lung infection, using a planned ratio of 1:9 PLPL to PAO1$^{\varphi-}$. PLPLφtriple was selected for this experiment, as it was the most successful, and is the most similar to the LES parent strain, with regards to prophage carriage. An additional experiment was set up with a planned initial starting ratio of 1:1, as an "insurance experiment", as bacterial competition experiments *in vivo* can be unsuccessful if the density of one competitor is very low. Instead of fluorescent markers, antibiotic markers were used, for easy separation of strains, both from each other, and from the rat microflora. PAO1$^{\varphi-}$ was labelled with a streptomycin resistance marker, and PLPLφtriple with a gentamicin resistance

marker. PAO1$^{\varphi-}$Sm^R grew equally well on LA with and without 300 µg ml^{-1} streptomycin (2 sample t-test; t = -0.24, P = 0.83) and did not grow on LA plates containing 10 µg ml^{-1}, and vice versa for PLPLφtripleGm^R (2 sample t-test; t = 1.13, P = 0.38). This suggests that there is no major fitness cost incurred by either marker.

PLPLφtriple was capable of invading a PAO1$^{\varphi-}$ *in vivo*, with positive r_{ij} values for all replicates, across both initial starting ratios (Figure 6.5). In the 1:9 initial starting ratio, two clear outliers, with very high r_{ij} values, can be observed (Figure 6.5). These values were obtained from two rats that had to be sacrificed after 2 days, as they were showing symptoms of acute infection (Table 6.3), including weight loss, chromodacryorrhea, piloerection, hunched posture and lethargy. Their lungs were harvested, the density of each competitor recorded, and the r_{ij} calculated. The very high r_{ij} values can be explained partly by the very high ratio of lysogens to non-lysogens at the end of the experiment, and also because of the timescale, as r_{ij} has units of per day. The bacterial load in the lungs was also very high (~100-fold higher than other rat lungs after 7 days). Due to acute nature of these infections, these rats were excluded from later analyses. Other rats were excluded from later analyses because they died after infection (Table 6.3). After exclusion of these outliers, the mean r_{ij}±1 S.E.M. was very similar between the 1:1 (0.24±0.01, n = 7) and 1:9 (0.26±0.02, n = 4) groups.

The lysogen was significantly fitter than the non-lysogen when at an initial starting ratio of 1:1 (1 sample t-test; t_6 = 20.3, P < 0.001) and when at an initial staring ratio of 1:9 (1 sample t-test; t_3 = 15.8, P< 0.01), as evidenced by a mean r_{ij} that was significantly higher than 0, which is the expected value if there was no difference in the fitness of the two competitors. There was no difference in the mean r_{ij} between the two groups (2 sample t test; t_6 = 1.08, P = 0.32).

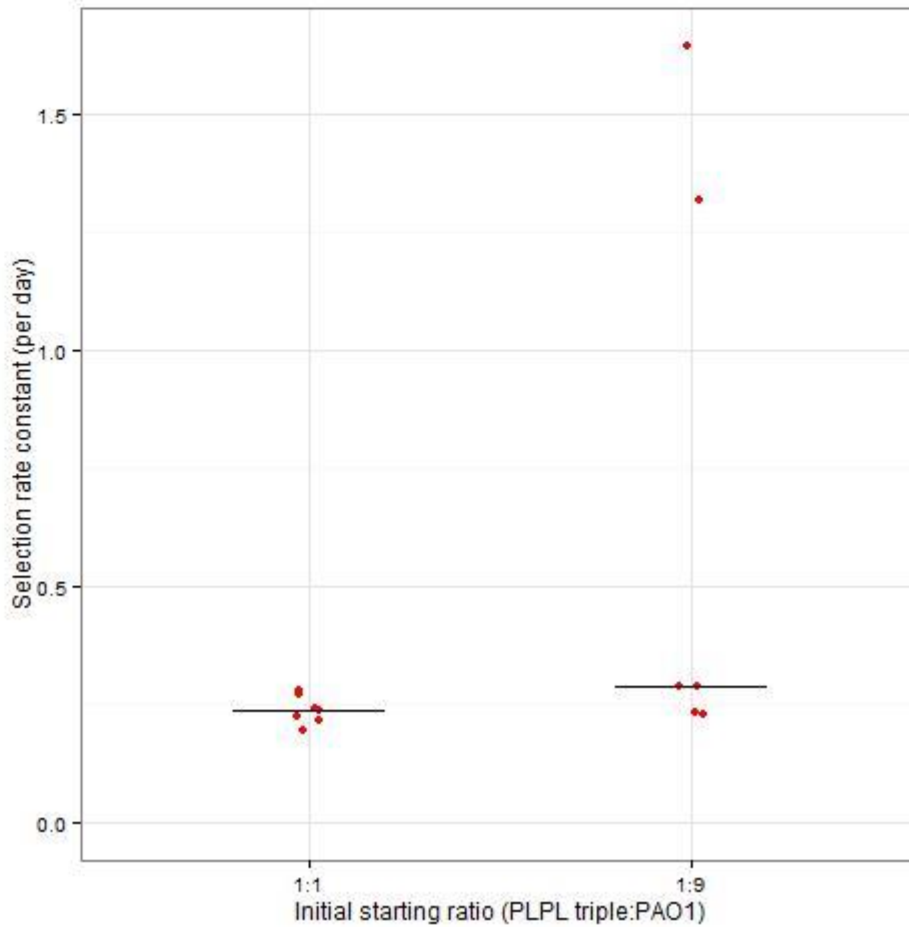


Figure 6.5 Selection rate constant value calculated for the outcome of competition *in vivo* between PAO1φ- and PLPLφtriple, when at two different starting ratios of the two competitors. Each data point represents the outcome of competition in an individual animal's lungs after 7 days, or less if the animal had to sacrificied due to ill health. Horizontal black lines denote the median.

Table 6.3 Summary of *in vivo* PLPL invasion experiment, summarised for each individual animal.

Planned initial starting ratio	Rat ID	End density $PAO1^{\varphi-}Sm^R$ in lungs	End density LESφtriple Gm^R in lungs	End ratio (Gm^R: Sm^R)	Start ratio (Gm^R: Sm^R) in beads	Change in rat body weight (%)	Length of competition (days)	Excluded from final statistical analyses?	Phage to bacterium ratio (PBR)
1:1	E1	1.5E+05	8.1E+05	5.4	1.0	-1	7		0.8
1:1	E2	2.8E+05	2.0E+06	7.1	1.0	+1	7		0.09
1:1	E3	1.4E+06	6.7E+06	4.8	1.0	-7	7		0.1
1:1	E4	3.8E+05	2.5E+06	6.7	1.0	-1	7		0.07
1:1	E5	1.0E+05	4.0E+05	3.9	1.0	-3	7		0.1
1:1	E6	N/A	N/A	N/A	1.0	N/A	2 (died)	Yes	N/A
1:1	E7	5.1E+04	2.6E+05	5.2	1.0	+11	7		0.3
1:1	E8	4.0E+04	1.8E+05	4.5	1.0	-2	7		2
1:9	I1	2.6E+08	4.1E+08	1.6	0.16	-14	2 (euthanised early after signs of acute infection)	Yes	1
1:9	I2	4.9E+06	4.1E+06	0.8	0.16	0	7		0.1
1:9	I3	2.1E+08	6.2E+08	3.0	0.16	-12	2; euthanised early after signs of acute infection	Yes	0.8
1:9	I4	N/A	N/A	N/A	0.16	N/A	Died after infection procedure	Yes	N/A
1:9	I5	N/A	N/A	N/A	0.16	N/A	1 (died)	Yes	N/A
1:9	I6	6.9E+05	5.7E+05	0.8	0.16	-2	7		0.8
1:9	I7	2.5E+07	1.4E+07	0.6	0.16	-10	7		0.9
1:9	I8	5.4E+06	3.0E+06	0.6	0.16	+1	7		0.1

6.3.2.2 Lysogenic conversion of PAO1$^{\varphi-}$ in vivo

In both competitions, total lysogens dominated at the end of the experiment (Figure 6.6), but were at a higher frequency in the 1:1 initial starting ratio group, likely due to the higher proportion of the PLPLφtripleGm^R at the end (Table 6.3). A breakdown of the prophage complements of the PAO1$^{\varphi-}$ Sm^R competitor is given in (Figure 6.7), and the majority maintain their prophage-free status over the 7 day competition. In those that became lysogens, LESφ2 and LESφ3 dominated, whereas LESφ4 lysogens were rare.

Levels of free phage detected in the lungs were relatively low, with a mean ±1 S.E.M PBR of 0.55±0.32 for the 1:1 and 0.47±0.22 1:9 initial starting ratio groups. There was no difference between the 2 groups ($\log_{10}$ +1 transformation; 2-sample t-test; t_6 = -0.11, p = 0.91).

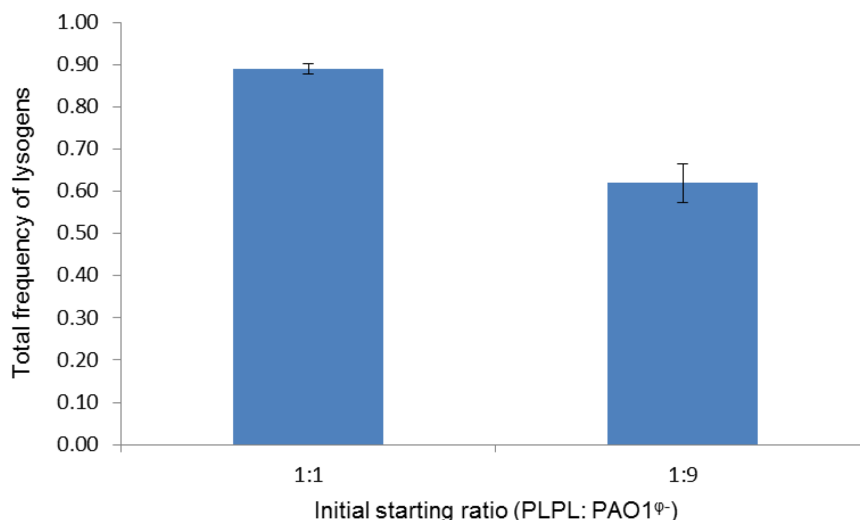


Figure 6.6 Frequency of bacterial lysogens in rat lungs after 7 days competition between PLPLφtriple and PAO1$^{\varphi-}$ at two starting ratios of competitors. Lysogens are defined as bacterial isolates carrying one of more LES phages. Error bars ±1 S.E.M.

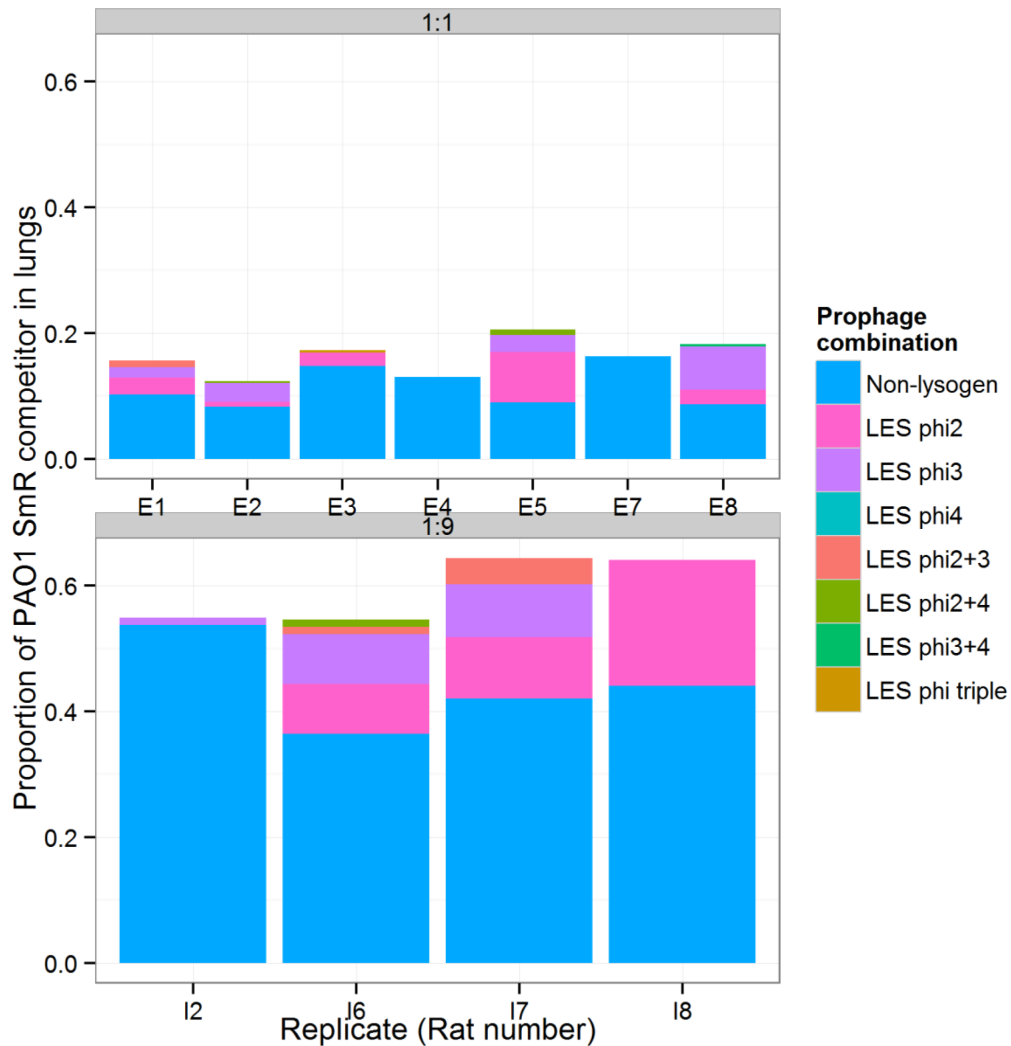


Figure 6.7 PAO1$^{φ-}$ as a proportion of total bacteria (both competitors) in rat lungs 7 days post-infection. Height of bars denote frequency of PAO1$^{φ-}$ and colours denote prophage complement of isolates. Data are reported separately for each rat and separated based on initial starting density of PLPLφtriple to PAO1$^{φ-}$ (top-panel, 1:1 starting ratio; bottom panel, 1:9 starting ratio).

6.4 Discussion

6.4.1 Inter-phage differences in PLPL invasiveness

The *P. aeruginosa* LES is widespread in individuals with CF (Aaron *et al.*, 2002), more competitive than strain PAO1 in an animal model of chronic lung infection (Kukavica-Ibrulj *et al.*, 2008b) and capable of superinfection (McCallum *et al.*, 2001). However, the mechanisms underlying its competitiveness have yet to be explored. The LES prophages are known to be critical to the competitiveness of this strain (Winstanley *et al.*, 2009), and the purpose of this work was to examine the

effect that prophage carriage has on bacterial competitiveness when in competition with an isogenic, phage-free (and phage-susceptible) competitor. PLPLs can invade PAO1$^{\varphi-}$ over a 24 hour period *in vitro* from being initially rare, which gives support to the hypothesis that prophages could be used as a bacterial superinfection strategy. There were differences depending on the prophage carried, which is interesting, as LES isolates are known to differ in their prophage complement (Fothergill *et al.*, 2012). Carriage of LESφ4 was associated with greater bacterial invasiveness than carriage of LESφ2 or LESφ3, but analysis of endpoint mixed populations found that for all competitions, the majority of bacteria were lysogens. This indicates that rapid lysogenic conversion of PAO1$^{\varphi-}$ may limit the efficacy of phage as strategy for invasion, something that has been discussed previously in the literature (Burns *et al.*, 2014; Gama *et al.*, 2013). The increased invasiveness of PLPLφ4 could be due to a variety of possible reasons, including the likelihood of establishing lysogeny (low likelihood of lysogeny would presumably delay the lysogenic conversion of PAO1$^{\varphi-}$, increasing potential for invasion) or differences in phage pathology; more pathogenic bacteriophage are associated with a better competitive outcome (Joo *et al.*, 2006). Competition experiments between *S.* Typhimurium P22 or Gifsy lysogens and non-lysogens have shown that whilst a similar proportion of lysogens are detected at the end for both lysogens, this occurs earlier for P22 lysogens, possibly because of the larger burst size of this phage (Bossi *et al.*, 2003). This is not the case for LESφ4; it has a smaller burst size and longer latent period than the other two phages (Chapter 3). The increased competitiveness of PLPLφ4 is somewhat surprising, especially given that the STM study of LES B58 found that mutations in LESφ2 and LESφ3, but not LESφ4, are associated with reduced competitiveness (Winstanley *et al.*, 2009). However, the results do agree with a recent study that found that LESφ4 carriage was associated with increased competitiveness in a *G. mellonella* model, compared to LESφ2 (Burns *et al.*, 2014).

One of the major limitations of this study is that a distinction was not made between increased competitiveness due to prophage-mediated lysis of competitors, or an intrinsic fitness benefit conferred by the phage. There are no differences in the growth rates of the PLPLs and PAO1 when cultured alone, which suggests that the prophages do not confer an intrinsic fitness benefit (*in vitro* at least). Despite this, it would be beneficial to repeat the experiments with a phage resistant competitor; if

the invasive ability is because of prophage-mediated lysis, PLPLs should not have a fitness benefit when competed against a phage resistant isogen of $PAO1^{\varphi-}$.

6.4.2 Carriage of multiple prophages accelerates PLPL invasion

In this study, carriage of multiple prophages was associated with faster invasion, as the ratio of PLPL to $PAO1^{\varphi-}$ was significantly higher for PLPLφtriple than any of the single phage PLPLs. It is possibile that carriage of multiple phages extends the usefulness of this strategy, as establishment of lysogeny results in resistance to phage lysis, and resistance to multiple phages should take longer to acquire. The presence of multiple phages could also increase the MOI and the likelihood of phage coinfection, which could affect how quickly cell lysis occurs. Coinfection of *E. coli* with multiple lytic phages results in a quicker time to lysis than infection by a single phage (Brewster *et al.*, 2012). On the other hand, there is evidence that a high MOI could actually be maladaptive for bacteria when competing *in vivo*. Experiments with *S*. Typhimurium P22 lysogens have shown that whilst lysogens are more invasive than non-lysogens in chicken macrophage cells, addition of exogenous P22 reduces invasion and cell survival, potentially through increased cell lysis and macrophage activation (Ahn & Biswas, 2014). Any results obtained *in vitro* must be interpreted cautiously, as there are many differences to the CF lung environment, including the immune system.

Despite the faster invasion time of PLPLφtriple, it was associated with an apparent cost early on, with the lowest mean ratio of all the PLPLs at t_8. Increased cell lysis and possibly slower growth due to the metabolic burden of extra DNA could pose a fitness cost, although no difference in the growth rates of the PLPLs alone was observed (James *et al.*, personal communication). A temperate bacteriophage has been associated with a fitness cost to its bacterial host under specific environmental conditions (DeBardeleben *et al.*, 2014), but there is little evidence to suggest that the carriage or extra DNA and risk of lysis represents a fitness cost, despite the widely held belief.

6.4.3 Phage invasion

As well as considering the fitness benefit conferred to bacteria by prophage carriage, the evolutionary success of the phage itself was considered. Bacteriophages are

ultimately selfish genetic elements, and whilst phage can increase their fitness by increasing the fitness of their host, spreading by vertical transmission, they can also spread by horizontal transmission. LESφ4 carriage was more beneficial to bacteria than LESφ2 or LESφ3, but the phages all did equally well, and the majority of bacteria were lysogens after 24 hours. This simply reconfirms that it is a short-lived strategy for the bacteria, as phage can quickly lysogenise the competing strain, removing any competitive advantage held by the initial lysogen. However, it is worth noting that strains used in this study, and indeed other similar studies are isogenic, and differ only in the presence of phage, and once phage has transferred to the initially phage free competitor, the competitors are equal. However, competing bacterial strains in the lungs are likely to differ in other ways, and a transient advantage afforded by prophage-mediated lysis could be enough for a strain to rise to dominance, using other competitive advantages.

6.4.4 Factors affecting efficacy of phage-mediated lysis as an invasion strategy

In addition to the problem posed by rapid lysogenic conversion, there are other factors that will reduce the efficacy of phage-mediated allelopathy further, including environmental influences that favour lysogeny rather than lysis (Gama *et al.*, 2013). The LES phages have a high frequency of lysogeny in an environment that simulates the CF lung (Chapter 3), hence phage-mediated allelopathy could be useless in the CF lung environment. In addition, chronic CF isolates are known to frequently display loss of common phage receptors such as type IV pili (Lee *et al.*, 2005a; Mahenthiralingam *et al.*, 1994; Smith *et al.*, 2006b; Workentine *et al.*, 2013), flagella (Lee *et al.*, 2005a; Mahenthiralingam *et al.*, 1994; Workentine *et al.*, 2013) and lipopolysaccharides (Hancock *et al.*, 1983), rendering bacteria phage resistant and this strategy obsolete. It is possible that phage-mediated allelopathy is a strategy associated with the early stages of chronic infection. Indeed, once chronic infection has established, that particular strain is rarely displaced (Cramer *et al.*, 2012), although LES is the exception, in that it is known to displace other *P. aeruginosa* strains, even after years of colonisation (McCallum *et al.*, 2001).

6.4.5 PLPL invasion *in vivo*

In order to determine whether the benefits of allelopathy extend to *in vivo*, the PLPL invasion experiment was repeated in a rat agar bead model of chronic infection. Over a seven day period, PLPLφtriple could successfully invade a population of PAO1$^{\varphi-}$, even from when initially rare. However, the extent of the invasion was more limited than *in vitro*; PLPLφtriple increased in frequency relative to PAO1$^{\varphi-}$ in both groups, but in the 1:9 initial starting ratio group didn't increase sufficiently to become the dominant strain. However, the two rats (I1 and I3, Table 6.3) which developed an acute infection and were necessarily sacrificed provide an interesting avenue for further study. After 2 days, PLPLφtriple was the dominating strain, but without doing further experiments, it is impossible to tell whether this was due to chance or if there is an alternative explanation. As this was not a time course experiment, nothing is known of the dynamics of the invasion, and it is possible that PLPLφtriple dominates early on and then declines, and was identified in these rats because they were sacrificed at an early time point. An alternative explanation is that PLPLφtriple was more invasive in these rats and that the competitive interactions between the two strains (and resulting bacterial lysis) was actually responsible for the acute nature of the infection. Obviously, two rats do not provide enough statistical power to draw any conclusions, and further experiments would be necessary, sacrificing rats at an earlier time point to determine whether PLPLφtriple dominates early on.

There are issues with the rat model of infection, and it is a concern that some of the rats developed acute infection, or died soon after the infection procedure. The infection is not supposed to be acute, but PAO1 is very virulent compared to other *P. aeruginosa* strains in a murine model of acute respiratory infection (Carter *et al.*, 2010a). Attempts to develop a mouse nasopahryngael carriage model were unsuccessful because of this, as mice either developed an acute infection and died, or failed to become colonised, despite this model having been used successfully previously for a different strain of *P. aeruginosa* (Fothergill *et al.*, 2014). In addition, the rats used are outbred, hence there will be more heterogeneity between them than there would be for inbred animals. There were also substantial differences in the age and size of the rats, which can not only affect infection outcome, but also the success of the infection procedure.

After 7 days, PAO1$^{\varphi-}$Sm^{R} isolates were screened for the presence of prophage. The majority had remained non-lysogens, although the mechanism for this was not investigated; it could be due to phage-resistance, or it simply lack of contact with free phages. Of those that became lysogens, the majority were LESφ2 or LESφ3 lysogens, similar to the results of the *in vivo* study in Chapter 4.

6.4.6 Implications for bacterial virulence and human health

There is considerable interest in factors that affect the virulence of *P. aeruginosa* when in the CF lung, in the hope that therapies could be developed that target bacterial virulence, rather than attempting to control bacterial numbers. Within-host competition for resources is predicted to increase virulence, as bacteria try to maximise resources. However, spiteful interactions that result in killing of one or more of the competitors has the opposite effect, reducing overall bacterial density and therefore virulence (Massey & Buckling, 2004). This is also dependent on levels of kinship. As described in 6.1.1.1, intermediate levels of kinship result in the highest fitness benefit to producing strains (Inglis *et al.*, 2009). In this paper, the authors also examined the effect of this on bacterial virulence in an insect model of infection. Total bacterial density and virulence was lowest at intermediate levels of kinship, presumably because of high cell death of the susceptible strain.

Spiteful interactions are not always associated with altered virulence. PAO1 lysogens are more competitive than non-lysogens in *G. mellonella*, presumably through prophage-mediated lysis, but this has no effect on virulence; time to death of larvae was the same for single (one competitor only) as for mixed infections (Burns *et al.*, 2014), although it is possible that time to death is not a sensitive enough measure to detect subtle differences in virulence. Models of infection must be treated with caution, as virulence defined and assayed in such models is not necessarily the same as virulence in CF, and there may be a completely different set of virulence factors involved in being a successful coloniser of individuals with CF.

The ability of prophages to act as allelopathic weapons raises interesting possibilities with regards to phage therapy. A previous study considered the intermicrobial interactions between the competitively superior *Streptococcus pneumoniae* and *Staphylococcus aureus*. *S. pneumoniae* produces H_2O_2 at concentrations that are

lethal to *S. aureus*, and the mechanism was found to be via prophage induction and cell lysis (Selva *et al.*, 2009). This raises the interesting possibility of killing bacteria by prophage induction, but this is really an imperfect strategy, potentially increasing horizontal gene transfer of virulence or antibiotic resistance genes. An alternative strategy would be to utilise apparent competition when targeting difficult-to-reach bacteria with lytic phages. Such a strategy has been used successfully to target the virulent *Mycobacterium avium*, which resides in macrophages. Treatment with lytic phage alone has no effect, as the phage is unable to enter the macrophages. To overcome this, non-pathogenic *M. smegmatis* was infected with the lytic phage and allowed to infect the macrophages, enabling the phage to access and kill *M. avium*, and also the pathogenic *M. tuberculosis* (Broxmeyer *et al.*, 2002).

6.5 Summary

- Bacteria carrying LES prophages are able to invade a phage-susceptible population of PAO1, even when initially rare.
- LES φ4 carriage is associated with greater bacterial invasiveness than LESφ2 or LESφ3, but carriage of all three is associated with faster invasion.
- Under all competitions *in vitro*, the rate of lysogenic conversion was high, and may limit the efficacy of temperate phage release as an allelopathic strategy.
- PLPLφtriple can invade PAO1$^{\varphi-}$ *in vivo*, but the competitive outcome depends on initial starting ratio of competitors.

6.6 Declaration

The *in vivo* work was done in collaboration with Dr. Irena Kukavica-Ibrulj at the lab of Prof. Roger C. Levesque, Laval University.

Chapter 7 General discussion

7.1 Lysogeny of bacterial pathogens

Prophages are frequently found in bacterial genomes, and are known to carry genes that alter the fitness or virulence of their host (Barondess & Beckwith, 1995; Stanley *et al.*, 2000) or protect host from lytic phage infection (Heo *et al.*, 2007; McGrath *et al.*, 2002). For example, *Escherichia coli* strain K12 harbours nine cryptic prophages, which have been shown to confer various benefits to their host, including enhanced biofilm and increased resistance to environmental stressors such as sub-lethal concentrations of antibiotics, acid and oxidative stress (Wang *et al.*, 2010b). Certain bacteriophages have been identified that alter the antigenicity of their host by modifying the O-antigen of LPS (Davies *et al.*, 2013; Verma *et al.*, 1991), which could prevent recognition by the host immune system. Prophage-derived elements are overrepresented in pathogen genomes (Busby *et al.*, 2013), and there is evidence that temperate phages are important in clinical infections, yet they still remain relatively understudied.

The LES is a successful transmissible strain that carries six prophages, some of which are necessary for bacterial competitiveness (Winstanley *et al.*, 2009). A recent study tracked bacterial and temperate phage dynamics in the lungs of LES-infected CF patients and identified high levels of lytic phage activity (James *et al.*, 2014), suggesting a role for temperate phages in controlling bacterial densties. This was the first study to examine bacteria-phage dynamics in the CF lung, and underlies their importance. A study comparing lung viral metagenomes found that the CF virome was very similar between individuals. The functional genes carried by the phage community differed between CF and non-CF individuals, presumably reflective of the differing environments, and CF phages encoded more genes involved in anaerobic aromatic catabolism genes (Willner *et al.*, 2009). A similar study found that antimicrobial resistance genes were overrepresented in the CF virome (Fancello *et al.*, 2011). These results indicate that bacteriophages could also have an important role in HGT between bacteria in the CF lung.

The aim of this investigation was to elucidate how the LES phages contribute to the success of the LES, by studying the genetics and ecology of these phages, in addition

to investigating their impact on the ecology and evolution of *P. aeruginosa*. Both *in vitro* and *in vivo* models were used, in order to replicate the conditions in the CF lung as far as possible.

7.2 Temperate phage genomics

Prophages can carry genes that increase the fitness of the bacterial strain; hence studying phage genomics can be a useful strategy to better understand their contribution to bacterial virulence and competitiveness. To this end, the LES phage genomes were re-annotated in this study, in order to identify any potential phage-encoded genes that may increase the fitness of LES, relative to other *P. aeruginosa* strains. Whilst this strategy helped to further understanding of the phage genomes themselves, it did not identify genes that may confer a benefit to the bacterial host. Phage genomes are often poorly annotated, due to a lack of functional data and poor sequence similarity between phages. Bacteriophages have a higher proportion of ORFan genes than bacteria (Edwards & Rohwer, 2005; Lima-Mendez *et al.*, 2007), a number that is still rising (Yin & Fischer, 2008). Despite assigning a putative function to several LES phage predicted proteins, based on sequence similarity, a large proportion remain unknown, as do their effects (if any) on the bacterial host. A proteomics approach, combining mass spectrophotometry with affinity purification was used in a recent study to elucidate protein-protein interaction (PPI) networks in *P. aeruginosa* phages for the first time, enabling the identification of multiple polypeptides that are expressed early in the lytic cycle (Van den Bossche *et al.*, 2014). Such an approach could be used to identify gene products encoded by the LES phages and identify interactions with the host cell and may deepen our understanding of the effect that the LES phages have on their host.

7.3 Phage-mediated allelopathy and implications for virulence

The fact that the LES prophage genes necessary for competitiveness are putatively involved in phage lysis (Winstanley *et al.*, 2009) suggests that active phage replication in required for full bacterial competitiveness. Phage-encoded bacterial fitness factors frequently require the lytic cycle to be expressed or released (Mitchell *et al.*, 2007; Tyler *et al.*, 2013), but analysis of the LES phage genomes did not identify any obvious virulence factors, and phage-mediated lysis can have other

benefits for the bacterial host. Temperate bacteriophages can act as allelopathic agents, facilitating invasion of a competitor population by production of infective phages that kill phage-susceptible bacteria (Brown *et al.*, 2006; Burns *et al.*, 2014; Gama *et al.*, 2013; Joo *et al.*, 2006). In this study, carriage of LES phages φ2-4 enabled PAO1 to invade an isogenic, phage-free competitor from when initially rare, *in vivo*. This supports the hypothesis that LES may use its bacteriophages as a strategy for superinfection, lysing and replacing resident *P. aeruginosa* strains.

The extent to which phage-mediated allelopathy occurs in the CF lung, and its implications for pathogenicity, remains undetermined. Although this study provides evidence that such a strategy can be successful *in vivo*, there is currently no empirical evidence that this occurs in the CF lung. There is indirect evidence that bacteriocin-mediated allelopathy occurs in the CF lung, as the majority clinical strains of *P. aeruginosa* and the *Burkholderia cepacia* complex produce bacteriocins that are inhibitory to multiple strains of both species (Bakkal *et al.*, 2010). It is known that several strains of *P. aeruginosa*, including LES (McCallum *et al.*, 2001) and the prairie epidemic strain (Parkins *et al.*, 2014) can replace other *P. aeruginosa* strains from the lung, so intraspecific competition seems likely.

7.4 Variable phage densities and effects on bacterial populations

Given that the LES phages can be used as a mechanism for intraspecific competition, it is significant that factors that are common to the CF environment, including H_2O_2 and antibiotics, alter LES phage production. In addition, there is evidence that the LES phages play a role in controlling bacterial densities in the CF lungs (James *et al.*, 2014), so altered levels of prophage-induced lysis could have potential implications for the clinical outcome of patients. Similar roles for phages regulating bacterial densities have been described in the swine faecal microbiome (Allen *et al.*, 2011) and environmental microbial communities (Shapiro *et al.*, 2010).

Changes in the level of phage production by bacterial lysogens may have other effects on bacterial populations. Temperate phages are frequently capable of transduction, and it is logical that increased phage production would lead to increased rates of transduction. This is a concern that has been identified in other bacterial genera; temperate phages of *Lactobacillus gasseri* are capable of

transduction and produced from their host strain at high levels by spontaneous induction (Baugher *et al.*, 2014). There is evidence that transducing phages can actually transduce genes likely to be beneficial to their bacterial hosts; in a large-scale study of 243 phages isolated from chicken meat, a quarter were capable of transducing antimicrobial resistance genes into *E. coli* (Shousha *et al.*, 2015). Furthermore, rates of transduction can be altered with antibiotic treatment; treatment of mice with antibiotics causes an enrichment of phage-encoded antibiotic resistance genes in the gut microbiome, which can be transferred to a naïve microbiome and cause an increase in the density of antibiotic resistant bacteria (Modi *et al.*, 2013). In summary, choice of antibiotics in CF therapy could have far-reaching implications, potentially affecting bacterial densities, altering the outcome of phage-mediated bacterial competitive interactions and changing the rate of HGT, all through altered phage production. The study by James *et al.* attempted to investigate the effect that antibiotic choice had on phage production, bacterial densities and patient clinical status, but unfortunately, the antibiotic treatment history of the study subjects was incomplete and so could not be used in the analysis (James *et al.*, 2014). A longitudinal study designed to address this specific issue would be needed to determine the importance of choice of antimicrobial therapy in relation to the role of temperate phages.

What effect is altered phage production likely to have on clinical outcome of an infection? Some studies have suggested that bacteriophages, or their components, can be immunogenic. Lytic *E. coli* phage T4 head proteins are immunogenic, and antibodies to it can be detected in the sera of 80% of individuals (Dąbrowska *et al.*, 2014). There are no (to my knowledge) studies investigating the effect of "naturally occurring" (as opposed to exogenously applied) phages on the normal (or CF) human immune response, and this represents an interesting avenue for future research. It is not simply phages that may elicit an immune response; mass phage-induced lysis can also be immunogenic, as the large quantities of endotoxin released from gram negative bacteria after phage treatment can elicit a severe (potentially fatal) immune response (Merril *et al.*, 1996).

Lessons can be learnt from temperate phages that can potentially be applied to further our understanding of lytic phages, which are increasingly being explored as

an alternative treatment to antibiotics. Certain antibiotics are likely to have similar effects on both temperate and exclusively lytic phages. For example, the antibiotic azithromycin reduces phage production in the LES (Fothergill *et al.*, 2011) and Stx toxin production in *E. coli* (McGannon *et al.*, 2010). This is presumably due to its mechanism of action (inhibiting protein synthesis), and it is likely to have the same effect on the lytic cycle of exclusively lytic phages.

7.5 Phages and bacterial adaptation

One of the interesting findings from this study is that LESφ4 can alter the trajectory of evolution by disruptive integration into protein coding genes. Loss of twitching motility occurred in both control and phage-treated populations, but it occurred faster in the phage treatment and by an alternative mechanism. Whilst phage-mediated gene disruption has been described before (Lee & Iandolo, 1986; Rehmat & Shapiro, 1983), it has not really been considered as a mechanism for bacterial adaptation, and the parallel evolution of loss of twitching motility does suggest it is adaptive. Theoretically, if the selective pressures were different, the genomic location of LESφ4 would be different. This finding raises interesting questions about LESφ4 in its native host background, LESB58. In the sequenced LESB58 isolate, LESφ4 is located between two genes encoding hypothetical proteins (Winstanley *et al.*, 2009) and is very rarely found in other locations (Haldenby *et al.,* unpublished), which suggests that LESφ4 does not play the same role in the evolution and adaptation of this strain.

In this study, there were discrepancies in the fitness of the LES phages in different environments. LESφ4 has higher fitness in ASM, and this could be for a variety of reasons, for example, increased infection efficiency of bacteria growing as a biofilm. Previous comparisons of lytic phage growth in ASM and liquid broth have highlighted differences in phage ability to infect biofilms (Garbe *et al.*, 2010) and shown that alginate limits the efficiency of phage infection.

The differences observed between the two models raise doubts about the conclusion of the LES genome paper that LESφ2 and LESφ3 are essential for bacterial competitiveness, and LESφ4 is not (Winstanley *et al.*, 2009), as such a conclusion is limited only to this infection model. Examining the prophage complement of LES CF

isolates can give more information, as prophages that increase host fitness are theoretically more likely to be retained. LESφ4 is always retained, but LESφ2 is frequently missing (Jeukens *et al.*, 2014), or degraded (Williams *et al.*, unpublished), as is LESφ3. This is supportive of the notion that LESφ4 confers a greater fitness benefit to its host. *P. aeruginosa* transposable phages are incredibly common, isolated as virions or detected as prophages in bacterial genomes (Cazares *et al.*, 2014). They have a large accessory genome, encoding genes that are putatively important for host fitness, and so their potential for affecting bacterial evolution is great.

7.6 Limitations of study

The majority of the experiments in this study were performed using LES phages in a PAO1 host background, rather than in the native host, LESB58. Such a strategy was adopted for multiple reasons. Firstly, LESB58 is difficult to manipulate genetically, and previous attempts to delete the prophage regions had failed (Fothergill *et al.*, personal communication). Secondly, PAO1 is a well characterised reference strain that was susceptible to three of the LES phages. In addition, PAO1 does not have any other endogenous prophages, other than a defective pyocin-encoding phage and Pf1-like filamentous phage (Stover *et al.*, 2000), similar to LESφ1 and LESφ6, respectively. However, there are obvious disadvantages to using PAO1 as a model strain, primarily that in a different host background, the LES phages may exert different effects. Also, PAO1 was not susceptible to LESφ5, so this was not considered in any experiments. This is not a huge issue though, as LES isolates have frequently lost LESφ5 (Jeukens *et al.*, 2014). Using PAO1 allowed construction of isogenic lysogenic and non-lysogenic strains, and means that the effect of phages in a naïve bacterial host can be considered, which is especially important for the invasion assays, for example, in which the assumption was that the competitor would be naïve to these phages.

7.7 Experimental evolution as a tool to understand drivers of evolution

In order to understand the evolution of *P. aeruginosa* in the CF lung, many researchers study sequential isolates from individuals that are chronically infected with *P. aeruginosa*, looking for correlations and patterns in the data in order to

identify factors that might influence the evolutionary outcome. However, to elucidate the drivers of *P. aeruginosa* diversification, an experimental approach is superior, as it enables the study of evolution in the presence or absence of certain factors, under controlled conditions. Experimental evolution was used in this study to determine the effect that LES phage have on *P. aeruginosa* diversification, as lytic phages are known to drive bacterial phenotypic diversity, largely through selection for resistant variants (Brockhurst *et al.*, 2005; Buckling & Rainey, 2002b; Mizoguchi *et al.*, 2003). Whole populations can be stored periodically through time and serve as a frozen "fossil record", which can be resurrected to test hypotheses. For example, temporal changes in the prevalence of the twitching motility phenotype, and the rise in frequency of LESφ4 integrated at two specific loci were both determined retrospectively, based on analysis of endpoint populations. Crucially, the ancestor of these populations is known (unlike *in vivo* studies), so any phenotypic or genetic changes are known to be a divergence from the ancestor, and competition experiments can be performed between evolved populations and the ancestor to determine if such changes are actually adaptive.

Whilst one of the major advantages of experimental evolution is that evolution can be studied in a reductive manner, this is also one of its major criticisms. In the absence of the myriad of factors that influence evolution (a potential network of interactions), then the validity of any conclusions drawn is questionable. The *P. fluorescens*- φ2 model system has been widely used to study evolutionary processes in chemostats containing laboratory media. A recent study compared host-phage coevolutionary dynamics in a more natural environment (soil), and found that fluctuating selection dynamics was the norm, compared with arms race dynamics *in vitro* (Gómez & Buckling, 2011). Another issue with using the closed microcosms in this study is that populations are completely isolated from each other. In reality, processes such as dispersal, causing gene flow between populations, are important evolutionary drivers in microbial populations (Kerr *et al.*, 2002; Venail *et al.*, 2008), although we do not know whether they are in *P. aeruginosa* populations in CF lungs. The spatial structure of the CF lung may limit gene flow and migration more than in other environments, but there is little empirical data on the spatial location of *P. aeruginosa* populations in the CF lung and the gene flow between them, although

recent evidence suggests the separate lineages can coexist for long periods (Williams *et al.*, 2015).

7.8 Polymicrobial interactions and polylysogeny

One of the key features of CF lung infections is that they are polymicrobial (Zhao *et al.*, 2012). In this study, all experimental approaches only considered *P. aeruginosa* in isolation. Even in a rat model of chronic lung infection, the lungs are essentially sterile prior to infection. Although *P. aeruginosa* is frequently the dominant species in the CF lung, it will be subject to numerous polymicrobial interactions, all of which may affect *P. aeruginosa* survival and evolution. Species interactions can affect social behaviours, altering levels of cooperation (Mitri *et al.*, 2011). For example, when *P. aeruginosa* is grown in the presence of another CF pathogen, *Staphylococcus aureus*, in an iron-limited environment, siderophore production is upregulated, and with that is an increased abundance of social cheats (Harrison *et al.*, 2008). If other species are capable of altering the outcome of intraspecific competition, then it is likely that they may also have an effect on the trajectory of evolution and the outcome of competitive interactions such as phage-mediated allelopathy. Moreover, the close relatedness of many bacterial species means that the potential for apparent competition is high. Indeed, cross infectivity between phages has been observed, and several temperate phages isolated from the *Burkholderia cepacia* complex are also able to infect *P. aeruginosa* (Langley *et al.*, 2003).

In addition to interspecific competition between bacteria in the CF lung, there will be a degree of within-host competition between bacteriophages. The *P. aeruginosa* evolution experiment in ASM in the presence or absence of phages was unique not only in that it utilised temperate phages (as most host-parasite coevolution experiments have been performed with lytic phages), but also in that multiple phages were used, both alone and in combination. In this study, the presence of additional phages altered the outcome of several experiments, and the impact of multiple prophages is something that should be considered more widely, particularly given that polylysogeny is frequently observed in bacterial pathogens (Figueroa-Bossi *et al.*, 2001; Matos *et al.*, 2013; Winstanley *et al.*, 2009).

The presence of an additional prophage reduces the baseline productivity of prophage, where the baseline productivity is the number of phages produced upon induction when the only prophage in a cell (Refardt, 2011). A similar phenonenom is observed in PLPLs, as phage productivity of PLPLφtriple is very similar to single phage PLPLs (James *et al.*, 2012), meaning that the production of each individual phage is clearly reduced. Moreover, the added productivity of two phages in a double lysogen does not equal one, suggesting some form of interference competition between them (Refardt, 2011). A study with double *E. coli* lysogens, carrying two Stx2 phages found that Shiga toxin production was actually reduced in the double lysogen compared to the single lysogen, as was phage production (Serra-Moreno *et al.*, 2008), suggesting that polylysogeny can have a stabilising effect, reducing the rate of spontaneous lysis. Interestingly, LES has a much reduced rate of phage production compared to PLPLφtriple (James *et al.*, 2012). It is a possible that the presence of additional prophages in the LES genome has a stabilising effect, or alternatively, that a more stable relationship has evolved over time in the LES host background. Clearly, a stabilisation effect could then have implications for the efficacy of phage-mediated allelopathy.

Polylysogeny could potentially affect the efficacy of phage therapy; the presence of prophages could inhibit lytic phage infection (through superinfection exclusion, phage-driven loss of receptors or within-host phage-phage interactions) and may reduce the therapeutic potential of phage therapy, something which is often proposed as an alternative to antibiotic treatment for *P. aeruginosa* lung infections.

Polylysogeny is a neglected phenomenom, but as more and more bacterial genomes are sequenced, and better software to detect phages is produced, we are realising that polylysogeny is incredibly common. In this study, and in a previous study (Burns *et al.*, 2014), polylysogeny has been shown to increase bacterial competitiveness. Recent evidence suggests that prophages undergo rapid degradation in the bacterial genome to become lysis-defective, but then are maintained by purifying selection, which is strongly suggestive that prophage genes contribute to bacteria fitness (Bobay *et al.*, 2014), and could explain why polylysogeny is so widespread.

7.9 Summary

Temperate phages of an epidemic strain of *P. aeruginosa* increase host fitness by acting as allelopathic agents, and can alter the trajectory of evolution by disruptive integration into protein coding genes. The LES phages are detected at high densities in CF sputum and they have been implicated in controlling bacterial densities. Many aspects of the CF lung environment alter levels of phage production, but it is still unclear what the clinical implications of this are. With the renewed interest in phage therapy to control bacterial infections, a clear understanding of prophages and how they interact with the host and each other is necessary to predict the success of such a treatment.

Appendix

A. Bacterial growth media preparation

All growth media were prepared with double-distilled water and sterilised by autoclaving at 121˚C for 15 minutes.

Lysogeny broth (LB) contains (litre^{-1}):

Tryptone 15 g (Oxoid)

Sodium chloride 10 g (Sigma)

Yeast extract 5 g (Oxoid)

For plates, 15 g bacteriological agar (Oxoid) was added prior to autoclaving.

Artificial sputum medium

To make 1 litre the following components (Sigma) are required:

Mucin (whole porcine) 5 g

DNA (herring sperm) 4 g

Diethylenetriaminepentaacetic acid (DTPA) 5.9 mg

NaCl 5 g

KCl 2.2 g

Egg yolk emulsion 5 ml

20 amino acids 5 g (250 mg of each)

Method:

Cysteine and tyrosine were dissolved in 25 ml water and 0.5 M KOH, respectively. The remaining 18 amino acids were dissolved in 100 ml SDW. DTPA, NaCl and KCl were added, and SDW added to a final volume of 250 ml. When fully dissolved, the solutions of cysteine and tyrosine were added.

DNA and mucin were each dissolved separately into 250 ml SDW. This was done slowly, at room temperature, over an 8 hour period with constant stirring. The solutions were stirred overnight at 4˚C to ensure complete dissolution.

The DNA, mucin, aminoacid/ salt solution and egg yolk emulsion were combined and SDW added to a volume of 850 ml.

The pH was adjusted to 6.9 by titration with a sterile 0.5 M Tris solution. Water was added to a final volume of 1 L.

The ASM was sterilised by vacuum-assisted filtration through a 0.2 μm filter (Millipore). Filters were rinsed with SDW a maximum of 3 times before discarding. One filter was used to sterilise ~250 ml ASM. The ASM was stored at 4˚C.

Pseudomonas isolation agar

Pseudomonas isolation agar base (Oxoid) was dissolved in 500 ml ddH_2O and autoclaved. The media was cooled to 50˚C and a vial of C-N supplement (Oxoid) added, prior to pouring.

M9 Minimal media

56.4 g M9 minimal salts (Sigma) were dissolved in 1 L SDW and autoclaved to give a 5X stock. 200 ml 5X stock was added to 780 ml ddH_20 and autoclaved. The media was allowed to cool and supplemented with 2 ml 1 M $MgSO_4$, 0.1 ml 1 M $CaCl_2$ and 20 ml 20% (w/v) glucose solution (all filter sterilised). For solid media, 15 g of bacteriological agar was added prior to autoclaving. For M9Ca-Glu broth (fluorescence studies), the agar was omitted and the media supplemented with **100** ml 20% (w/v) glucose solution and 100 ml 10% (w/v) casein hydrolysate (Sigma) solution. The volume of water was adjusted accordingly.

Swarming medium contains (L^{-1}):

NH_4Cl 1.07 g

Na_2HPO_4 1.70 g

KH_2PO_4 3.0 g

NaCl 0.5 g

Dextrose 1.98 g

Casein hydrolysate 5 g

Bacteriological agar 5 g (all Sigma)

980 ml ddH_2O was added and the mixture autoclaved. After autoclaving, the media was cooled to 50 ˚C, supplemented with 10 ml 0.1 M $MgSO_4$ and 10 ml 0.1 M $CaCl_2$ and poured immediately. The plates were dried for 30 minutes in a laminar flow hood and used immediately. Consistent drying of plates is necessary as bacterial swarming is highly affected by surface moisture levels.

Swimming media contains (L^{-1}):

Tryptone 10 g

NaCl 5 g

Bacteriological agar 3 g

After autoclaving and pouring, plates were dried on the bench for 18 hours.

B. Solutions

Preparation of 5X TBE (for agarose gel electrophoresis)

54 g Tris base (Fisher) and 27.5 g boric acid (Fisher) were dissolved in 980 ml ddH_2O and autoclaved. 20 ml sterile 0.5 M Ethylenediaminetetraacetic acid (EDTA) at pH 8.0 was added aseptically. The stock was stored at room and diluted 10-fold in ddH_2O to obtain the required working concentration of 0.5X for agarose gel electrophoresis.

4X PEG/ NaCl (for phage precipitation)

25 g polyethylene glycol (PEG) 8000 and 5.8 g NaCl were dissolved in 50 ml water by stirring for approximately 30 minutes. The mixture was autoclaved and stirred continuously during cooling to prevent phase separation. When diluted to the required 1X working concentration, PEG is at a concentration of 10% (w/v) and NaCl at a concentration of 0.5M.

C. Reannotation of published LES phage genomes

Table C.i Reannotation of the LES ϕ2 genome. Original annotation as detailed on the Pseudomonas database is given. For ORFs encoding hypothetical proteins, a PSI-BLAST search was conducted using default parameters, and the highest scoring significant hit to a protein with a known function is reported. Alignments were inspected manually and putative function of protein updated where confident.

Gene number	Locus ID	Gene name	Previous annotation	Sub-cellular localisation	Highest significant match to protein with annotated function	Putative function	Expect (*E*) value (cut off = *E*-06)	Score	Query cover (%)	% Identity
1	PLES_07891		Integrase	Cytoplasmic		Integrase				
2	PLES_07901		hypothetical protein	Cytoplasmic	None	Unknown				
3	PLES_07911		hypothetical protein	Cytoplasmic	None	Unknown				
4	PLES_07921		hypothetical protein	Unknown	None	Unknown				
5	PLES_07931		unknown	Cytoplasmic	None	Unknown				
6	PLES_07941		conserved hypothetical protein	Unknown	None	Unknown				
7	PLES_07951		transcriptional regulator, LuxR family	Cytoplasmic		Transcriptional regulator, LuxR family				
8	PLES_07961		transcriptional regulator	Unknown		Transcriptional regulator, LuxR family				
9	PLES_07971	endY	EndY	Unknown		EndY				
10	PLES_07981		hypothetical protein	Cytoplasmic	None	Unknown				
11	PLES_07991		hypothetical protein	Unknown	None	Unknown				
12	PLES_08001		putative phage-related protein	Unknown	WP_023875736.1; DNA-binding protein (*Pseudomonas aeruginosa*)	DNA-binding protein	2.00E-166	472	99	94

13	PLES_08011		hypothetical protein	Cytoplasmic	WP_023517982; Primosomal protein (*Pseudomonas aeruginosa*)	Unknown	3.00E-114	343	100	59
14	PLES_08021		DNA replication protein DnaC	Cytoplasmic		DNA replication protein DnaC				
15	PLES_08031		Putative DnaB-like replicative helicase	Cytoplasmic		Putative DnaB-like replicative helicase				
16	PLES_08041		Putative metallophosphoesterase	Unknown		Serine/ threonine protein phosphatase				
17	PLES_08051		hypothetical protein	Unknown	None	Unknown				
18	PLES_08061		hypothetical protein	Cytoplasmic	WP_019486505.1; Antiterminator protein Q (*Pseudomonas aeruginosa*)	Antiterminator protein Q	6.00E-50	164	100	100
19	PLES_08071		DNA-binding protein	Unknown		DNA-binding protein				
20	PLES_08081		Holin, phage lambda	Unknown		Holin				
21	PLES_08091		Predicted chitinase; lytic protein	Unknown		Chitinase (lytic protein)				
22	PLES_08101		Putative lysis protein Rz	Unknown		Rz lysis protein				
23	PLES_08111		hypothetical protein	Unknown	Conserved hypothetical protein	Unknown				
24	PLES_08121		Putative small subunit (Nu1 homolog) of DNA packaging dimer	Cytoplasmic		Small subunit (Nu1 homolog) of DNA packaging dimer				
25	PLES_08131		Putative large subunit (GpA homolog) of DNA packaging dimer	Cytoplasmic		Large subunit (GpA homolog) of DNA packaging dimer				
26	PLES_08141		Putative portal protein	Cytoplasmic		Portal protein				
27	PLES_08151		chaperone with DnaK; heat shock protein	Unknown		Chaperone with DnaK; heat shock				

						protein				
28	PLES_08161		hypothetical protein	Unknown	WP_014232304.1; Recombinase RecA (*Vibrio* sp. EJY3)	Putative recombinase	2.00E-09	60.1	71	44
29	PLES_08171		hypothetical protein	Cytoplasmic	YP_006561142.1; Minor tail protein (*Burkholderia* phage AH2)	Putative minor tail protein	5.00E-06 (3 iterations)	53.5	59	24
30	PLES_08181		hypothetical protein	Unknown	None	Unknown				
31	PLES_08191		hypothetical protein	Unknown	CCD58454.1; Structural protein (*Pseudomonas* phage LPB1)	Putative structural protein	2.00E-35 (2 iterations)	137	98	16
32	PLES_08201		hypothetical protein	Unknown	AHK93583.1; Pre-pilin like leader sequence (*Pseudomonas aeruginosa* LES400)	Unknown	6.00E-51	171	39	100
33	PLES_08211		Putative tail length tape measure protein	Unknown		Tail length tape measure protein				
34	PLES_08221		hypothetical protein	Unknown	None	Unknown				
35	PLES_08231		hypothetical protein	Unknown	KDF04243.1; Phage tail fibre protein (*Raoultella planticola* ATCC 33531)	Putative tail fibre protein	1.00E-26 (2 iterations)	114	83	37
36	PLES_08241		hypothetical protein	Unknown	None	Unknown				
37	PLES_08251		hypothetical protein	Unknown	None	Unknown				
38	PLES_08261		hypothetical protein	Outer Membrane	None	Unknown				
39	PLES_08271		hypothetical protein	Unknown	YP_009030620.1; Structural protein (*Pseudomonas* phage KPP25)	Putative structural protein	4.00E-17	84.7	96	34
40	PLES_08281		hypothetical protein	Unknown	None	Unknown				
41	PLES_08291		hypothetical protein	Unknown	None	Unknown				
42	PLES_08301		hypothetical protein	Cytoplasmic	YP_009030615.1; Structural protein (*Pseudomonas* phage KPP25)	Structural protein	2.00E-48	160	97	80

43	PLES_08311		hypothetical protein	Unknown	None	Unknown				
44	PLES_08321		hypothetical protein	Cytoplasmic	None	Unknown				

Table C.ii. Reannotation of the LES ϕ3 genome. Original annotation as detailed on the Pseudomonas database is given. For ORFs encoding hypothetical proteins, a PSI-BLAST search was conducted using default parameters, and the highest scoring significant hit to a protein with a known function is reported. Alignments were inspected manually and putative function of protein updated where confident.

Gene number	Locus ID	Gene name	Previous annotation	Sub-cellular localisation	Highest significant match to protein with annotated function	Putative function	Expect (*E*) value (cut off = *E*-06)	Score	Query cover (%)	% Identity
1	PLES_13201		putative arsenate reductase, glutaredoxin family	Unknown		Arsenate reductase				
2	PLES_13211		phage integrase, putative	Cytoplasmic		Integrase				
3	PLES_13221		excisionase, putative	Unknown		Excisionase				
4	PLES_13231		hypothetical protein	Unknown	None	Unknown				
5	PLES_13241		hypothetical protein	Cytoplasmic	None	Unknown				
6	PLES_13251		hypothetical protein	Unknown	None	Unknown				
7	PLES_13261		hypothetical protein	Unknown	None	Unknown				
8	PLES_13271		hypothetical protein	Cytoplasmic	None	Unknown				
9	PLES_13281		hypothetical protein	Unknown	None	Unknown				

10	PLES_13291		hypothetical protein	Unknown	None	Unknown				
11	PLES_13301		hypothetical protein	Cytoplasmic	None	Unknown				
12	PLES_13311		DNA methylase N-4/N-6	Unknown		DNA methyltransferase				
13	PLES_13321		hypothetical protein	Unknown	None	Unknown				
14	PLES_13322		hypothetical protein	Unknown	None	Unknown				
15	PLES_13331		transcriptional regulator, LuxR family	Cytoplasmic		Unknown				
16	PLES_13341		hypothetical protein	Cytoplasmic	YP_001039693.1; ParB-like nuclease domain (*Burkholderia* phage BcepF1)	Putative parB-like nuclease	9.00E-62	210	84	45
17	PLES_13351		hypothetical protein	Cytoplasmic	None	Unknown				
18	PLES_13361		protein of unknown function DUF1654	Unknown		Unknown				
19	PLES_13371		hypothetical protein	Unknown	None	Unknown				
20	PLES_13381		hypothetical protein	Cytoplasmic	WP_023118143.1; Transcriptional regulator PrtR (*Pseudomonas aeruginosa*)	Transcriptional regulator; LexA like	2.00E-180	508	99	99
21	PLES_13391		hypothetical protein	Cytoplasmic	WP_012613696.1; DNA-binding protein (*Pseudomonas aeruginosa*)	DNA-binding protein	4.00E-169	478	100	100
22	PLES_13401		hypothetical protein	Unknown	WP_029885823.1; Replication protein (*Pseudomonas putida*)	Putative replication protein	1.00E-58	200	100	42
23	PLES_13411		unknown	Unknown	None	Unknown				
24	PLES_13421		ninG protein	Cytoplasmic		ninG protein				

25	PLES_13431		hypothetical protein	Unknown	YP_009007130.1; Putative antitermination protein Q (*Pseudomonas* phage vB_PaeP_Tr60_A)	Putative antitermination protein Q	3.00E-12	68.6	94	33
26	PLES_13441		hypothetical protein	Cytoplasmic Membrane	WP_023875730.1; Transcriptional regulator (*Pseudomonas aeruginosa*)	Putative transcriptional regulator	1.00E-96	286	99	99
27	PLES_13451		Putative uncharacterized protein PflO1_0304	Unknown	WP_003098540.1; mRNA interferase (*Pseudomonas aeruginosa*)	mRNA interferase	2.00E-34	121	98	100
28	PLES_13461		hypothetical protein	Unknown	WP_004351282.1; Holin (*Pseudomonas aeruginosa*)	Holin	6.00E-86	258	100	100
29	PLES_13471		COG3179: Predicted chitinase	Unknown		Lytic enzyme; putative chitinase				
30	PLES_13481		hypothetical protein	Unknown	None	Unknown				
31	PLES_13491		hypothetical protein	Unknown	None	Unknown				
32	PLES_13501		Putative small subunit (Nu1 homolog) of DNA packaging dimer	Cytoplasmic		DNA packaging protein				
33	PLES_13511		Putative large subunit (GpA homolog) of DNA packaging dimer	Cytoplasmic		Putative large subunit of DNA packaging dimer				
34	PLES_13521		Putative portal protein	Cytoplasmic	WP_003129698.1; Plasmid partitioning protein ParB (*Pseudomonas aeruginosa*)	Putative portal protein	0	1168	100	100
35	PLES_13531		Putative Clp protease	Cytoplasmic		Caseinolytic protease (ClpP)				
36	PLES_13541		hypothetical protein	Unknown	WP_009467351.1; Putative RecA/RadA recombinase (*Roseibium* sp. TrichSKD4)	Unknown	1.00E-17 (2 iterations)	81.9	90	33
37	PLES_13551		hypothetical protein	Cytoplasmic	None	Unknown				

38	PLES_13561		hypothetical protein	Unknown	CCD58454.1; Structural protein (*Pseudomonas* phage LPB1)	Putative structural protein	3.00E-58 (3 iterations)	197	98	15
39	PLES_13571		hypothetical protein	Unknown	AHK93583.1; Pre-pilin like leader sequence (*Pseudomonas aeruginosa* LES400)	Unknown	6.00E-51	171	39	100
40	PLES_13581		Putative tail length tape measure protein	Unknown		Tail-length tape measure protein				
41	PLES_13591		hypothetical protein	Unknown	None	Unknown				
42	PLES_13601		hypothetical protein	Unknown	None	Unknown				
43	PLES_13611		hypothetical protein	Unknown	KFD04243.1; Phage tail fibre (*Raoutella planticola* ATCC 33531)	Unknown	3.00E-27 (2 iterations)	116	85	36
44	PLES_13621		hypothetical protein	Unknown	None	Unknown				
45	PLES_13631		hypothetical protein	Unknown	None	Unknown				
46	PLES_13641		hypothetical protein	Unknown	KCW18267.1; Bacterial Ig-like domain family protein (*Acinetobacter baumannii* 42057 4)	Unknown	3.00E-53 (4 iterations)	208	74	16
47	PLES_13651		hypothetical protein	Unknown	YP_009030620.1; Structural protein (*Pseudomonas* phage KPP25)	Putative structural protein	2.00E-20	94.4	94	35
48	PLES_13661		hypothetical protein	Unknown	None	Unknown				
49	PLES_13671		hypothetical protein	Unknown	WP_003150136.1; Tail fiber protein (*Pseudomonas aeruginosa*)	Putative tail fibre protein	5.00E-117	353	78	68
50	PLES_13681		hypothetical protein	Unknown	YP_009030615.1; Structural protein (*Pseudomoas* phage KPP25)	Putative structural protein	8.00E-42	143	100	58
51	PLES_13691		hypothetical	Unknown	WP_001175088.1; Phage tail	Unknown	7.00E-16	77.7	97	21

			protein		protein (*Vibrio cholerae*)		(4 iterations)			
52	PLES_13701		hypothetical protein	Unknown	None	Unknown				
53	PLES_13711		hypothetical protein	Unknown	None	Unknown				

Table C.iii Reannotation of the LES ϕ4 genome. Original annotation as detailed on the Pseudomonas database is given. For ORFs encoding hypothetical proteins, a PSI-BLAST search was conducted using default parameters, and the highest scoring significant hit to a protein with a known function is reported. Alignments were inspected manually and putative function of protein updated where confident. Putative function was assigned based on BLASTP searches of published anti-CRISPR gene protein sequences. [a] - reported in the form Genbank accession number; Protein name (Organism). [b] - no matches to proteins with an annotated function in the NCBI database.

Gene number	Locus ID	Gene name	Previous annotation	Sub-cellular localisation	Highest significant match to protein with annotated function	Putative function	Expect (*E*) value (cut off = *E*-06)	Score	Query cover (%)	% Identity
1	PLES_15491		Putative c repressor	Unknown		c repressor				
2	PLES_15501	orf2	Ner-like protein	Unknown		Ner-like protein (transcriptional regulator)				
3	PLES_15511		hypothetical protein	Cytoplasmic	None	Unknown				
4	PLES_15521		hypothetical protein	Unknown	None	Unknown				
5	PLES_15531	orf6	transposase A	Cytoplasmic		Transposase A and DNA binding protein (dual function)				
6	PLES_15541	orf7	DNA transposition protein	Cytoplasmic		DNA transposition protein				
7	PLES_15551	orf8	hypothetical protein	Cytoplasmic	YP_004934660.1; TroR domain transcriptional regulator (*Rhodobacter* phage RcapMu)	Unknown	4.00E-09	61.1	78	24

8	PLES_15561		hypothetical protein	Unknown	gi|501646228; LacI transcriptional regulator (*Desulfovibrio vulgaris*)	Transcriptional regulator	6.00E-16	78.6	71	38
9	PLES_15571		hypothetical protein	Unknown	None	Unknown				
10	PLES_15581		COG4396: Mu-like prophage host-nuclease inhibitor protein Gam	Cytoplasmic		Host-nuclease inhibitor protein Gam				
11	PLES_15591		hypothetical protein	Unknown	YP_002332437.1; Host nuclease inhibitor protein (*Pseudomonas* phage MP29)	Unknown	1.00E-75	237	99	95
12	PLES_15601		hypothetical protein	Unknown	YP_950438; Host nuclease inhibitor protein (*Pseudomonas extremaustralis*)	Unknown	2.00E-06	51.2	98	40
13	PLES_15611		Hypothetical protein	Unknown	None	Unknown				
14	PLES_15621		Hypothetical protein	Unknown	None	Unknown				
15	PLES_15631		hypothetical protein	Cytoplasmic	None	Unknown				
16	PLES_15641		putative integral membrane protein	Unknown	gi|371779623; Putative Mor protein (*Pseudomonas* phage LPB1)	Integral membrane and Mor protein (dual function)	4.00E-80	231	100	98
17	PLES_15651	orf20	hypothetical protein	Cytoplasmic Membrane	gi|620709885; Putative N-acetyl transferase YedL (*Bordetella bronchiseptica* OSU054)	Membrane protein				
18	PLES_15661	orf21	putative structural protein	Unknown		Structural protein				
19	PLES_15671	orf21a	hypothetical protein	Unknown	None	Unknown				
20	PLES_15681	orf22	hypothetical protein	Unknown	gi|371779627; Rz protein (*Pseudomonas* phage LPB1)	Rz protein	8E-85	255	100	94
21	PLES_15691	orf23	hypothetical	Unknown	gi|492020221; Phage	Unknown	8e -13 (4	68.4	93	19

			protein		transposase (*Pasteurella multocida*)		iterations)			
22	PLES_15701		conserved hypothetical DNA-binding protein	Cytoplasmic		DNA-binding protein				
23	PLES_15711		hypothetical protein	Cytoplasmic	None	Unknown				
24	PLES_15721		phage uncharacterized protein	Cytoplasmic	None	Unknown				
25	PLES_15731		portal protein (DNA packing)	Unknown		Portal protein				
26	PLES_15741		virion morphogenesis protein	Unknown		Virion morphogenesis protein				
27	PLES_15751		hypothetical protein	Cytoplasmic	None	Anti-CRISPR protein[b] ACR3112-31	8.00E-25	96.7	98	94
28	PLES_15761	orf29b	hypothetical protein	Unknown	None	Anti-CRISPR protein[b] (similar to gp33 and gp35)	8.00E-84	253	98	88
29	PLES_15771		hypothetical protein	Unknown	None	Anti-CRISPR protein[b] gp36	2.00E-16	76.6	64	92
30	PLES_15781		hypothetical protein	Unknown	None	Unknown				
31	PLES_15791		protease (I) and scaffold (Z) proteins	Unknown		Protease (I) and Scaffold (Z) proteins				
32	PLES_15801		hypothetical protein	Unknown	None	Unknown				
33	PLES_15811		putative major head subunit protein	Unknown		Major head subunit protein				
34	PLES_15821		hypothetical protein	Unknown	None	Unknown				

35	PLES_15831		hypothetical protein	Cytoplasmic	None	Unknown				
36	PLES_15841		hypothetical protein	Unknown	None	Unknown				
37	PLES_15851		hypothetical protein	Unknown	CCD58454.1; Phage structural protein (*Pseudomonas* phage LPB1)	Structural protein	1.00E-174	493	100	96
38	PLES_15861		hypothetical protein	Cytoplasmic	None	Unknown				
39	PLES_15871		hypothetical protein	Outer Membrane	YP_009042226; Tail-length tape measure protein (*Pseudomonas* phage JD024)	Tail-length tape measure protein	0	2248	100	99
40	PLES_15881	orf46e	hypothetical protein	Unknown	YP_006560542.1; Structural protein (*Pseudomonas* phage LPB1)	Structural protein	2.00E-94	294	100	96
41	PLES_15891	orf48	hypothetical protein	Unknown	YP_001595894.1; Structural protein (*Pseudomonas* phage YuA)	Structural protein	7.00E-14	79.7	73	28
42	PLES_15901	orf49	hypothetical protein	Unknown	CCD58460; Structural protein (*Pseudomonas* phage LPB1)	Structural protein	0.00E+00	1075	99	96
43	PLES_15911		hypothetical protein	Unknown	gi|491717003; Phage-tail protein (*Aggregatibacter actinomycetemcomitans*)	Tail protein	3.00E-41	153	99	34
44	PLES_15921		hypothetical protein	Unknown	gi|34610153; Tail assembly protein (*Burkholderia* phage BcepNazgul)	Tail assembly protein	9.00E-18	80.5	91	49
45	PLES_15931		hypothetical protein	Unknown	gi|371779657; Tail protein (*Pseudomonas* phage LPB1)	Tail protein	0.00E+00	1441	100	97
46	PLES_15941		hypothetical protein	Unknown	gi|371779658; Structural protein (*Pseudomonas* phage LPB1)	Structural protein	0.00E+00	725	100	90
47	PLES_15951		hypothetical protein	Cytoplasmic	None	Unknown				
48	PLES_15961		hypothetical protein	Cytoplasmic	None	Unknown				

D. LESφ4 integration sites in PAO1

Table D.i LESφ4 integration site frequencies in PAO1 populations cultured for 120 days in ASM in the presence of LESφ2-4.

Gene function	Gene name	Frequency of isolates with LES ϕ4 prophage integrated into gene											
		Population and integration site location (nt) in PAO1											
		7		8		9		10		11		12	
Motility	*fimU*	0.92	5098546	0.00		0.00		0.00		0.00		0.00	
	fimU/T (intergenic)	0.00		0.00		0.00		0.00		0.00		0.49	5098163
	pilV	0.00		0.00		0.00		0.35	5098936	0.90	5099014	0.46	5098868
	pilW	0.00		0.38	5100022	0.00		0.00		0.00		0.00	
	pilY1	0.00		0.10	5100846	0.00		0.00		0.00		0.05	5101059
Quorum sensing	*lasR*	0.00		0.00		0.00		0.32	1558410	0.00		0.00	
	mvfR	0.08	1086791	0.00		0.00		0.17	1086270	0.00		0.00	
	pqsA	0.00		0.45	1079138	0.08	1079047	0.00		0.05	1078619	0.00	
Other	*bifA*	0.00		0.00		0.10	4895094	0.00		0.00		0.00	
	PA0827 (probable transcriptional regulator)	0.00		0.00		0.00		0.00		0.04	902610	0.00	
	Unassigned	0.00		0.05		0.00		0.00		0.00		0.00	

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