The effect of active prophage carriage on the virulence of *Pseudomonas aeruginosa.*

Grace Plahe

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University of Salford, School of Science, Engineering and Environment

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List of Abbreviations

Bp	Base pairs
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CT	Cytotoxic
CTX	C-terminal telopeptide
DNA	Deoxyribose nucleic acid
LB	Luria-Bertani
LES	Liverpool Epidemic Strain
LESB58	Liverpool Epidemic Strain B58
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PAO1	*Pseudomonas aeruginosa* O1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
Phage	Bacteriophage
QS	Quorum sensing
Rcf	Relative centrifugal force
RNA	Ribose nucleic acid
Rpm	Rotations per minute
rt°C	Room temperature °C
Stx	Shiga-toxin
UV	Ultraviolet
WT	Wild type
Φ	Bacteriophage

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Abstract

The Liverpool epidemic strain (LES) of *Pseudomonas aeruginosa* is a key opportunistic pathogen and major cause of death in cystic fibrosis (CF) patients. The bacterium establishes biofilm communities, contributing to immune evasion and adaptation to the CF lung. The success of LES is associated with a set of bacterial viruses called bacteriophages (phages). There are five temperate phages integrated into the LES genome as active prophages, which provide fitness advantages, but can also be induced to their replicative form and destroy their host by lysis. Active phages are very common in the CF lung and infecting *P. aeruginosa* strains commonly carry many prophages, but little is known about the co-operative interactions between the two. This project investigates the relationship between three active LES phages (LES phage 2, 3 and 4) and the model bacterial host strain PAO1.

In vitro culture experiments revealed that the LES prophages affect the growth rate of PAO1 differently depending on the availability of nutrients. Whilst little difference was observed under nutrient rich conditions, prophage carriage significantly hindered growth when nutrients were limited, reaching a maximum optical density 85.8% of that reached by the Wildtype (WT). Biofilm density assays indicated that LES phage carriage significantly enhanced biofilm formation by between 44% and 981% when nutrients were readily available ($P < 0.05$). However, during nutrient starvation, most LES prophages hindered biofilm formation ($P < 0.05$), except for prophage 2 that appeared to promote significantly thicker biofilm growth (P 0.0002).

Infection experiments using the *Galleria mellonella* model showed that prophage carriage affected virulence and survival *in vivo.* The WT strain killed larvae significantly faster than strains carrying prophage ($P < 0.0001$) despite reaching a significantly lower bacterial load than the LES phages 3 and 4 lysogens ($P < 0.05$). These data suggest that LES prophages contribute to adaptation for longer survival *in vivo.*

1.Introduction

1.1 Bacteriophage diversity

Bacteriophages, also known as phages, are viruses that infect bacteria. They are the most common biological entity on the planet, their population estimated at 10^{31} free particles (Pope and Hatfull, 2015, Taylor et al., 2019, Aziz et al., 2015). Phages are present in all environments, with an estimated 10^{9} in every gram of soil and 10^{7} per ml of seawater, killing up to 50% of bacteria produced daily (Ceyssens and Lavigne, 2010, Aziz et al., 2015). As they are so abundant and have such a large effect on bacterial populations, phages are a major factor in driving diversity and geochemical cycles (Ceyssens and Lavigne, 2010).

Tailed phages belong to the order Caudovirales. They consist of a capsid protein head and a tail that connects it to the tail tip, also known as the baseplate (Bragg et al., 2016, Campbell et al., 2020). The phage capsid head can be an equal icosahedral, consisting of 20 equilateral triangles, or can be slightly elongated, and protects the genetic material of the virus in the form of double stranded DNA (Pope and Hatfull, 2015, Campbell et al., 2020). Other forms of genetic material, such as single stranded DNA or RNA genomes have not been identified in the Caudovirales, although it is unclear why (Pope and Hatfull, 2015). In the past, the different morphologies of the tail have been used to classify these phages into three distinct families (Campbell et al., 2020, Ceyssens and Lavigne, 2010). Myoviridae bacteriophage have a long, rigid tail that can be contracted using the sheath that surrounds it, Siphoviridae phage also have long tails although they are flexible and non-contractible. Podoviridae phage do not have tails in the same way the other two families, instead they possess a noncontractile spike to pierce the host cell membrane (Campbell et al., 2020, Ceyssens and Lavigne, 2010). Whilst these terms are still relevant in describing phage particle morphologies, classification systems have more recently moved to more taxonomic strategies.

Though much smaller than their bacterial hosts, bacteriophage genomes (typically between 40 and 45 kilobases in size), still pose a challenge to annotate (Zrelovs et al., 2021).

This is because of their signature mosaic structure, made up of many set modules of code that appear in different orders and genomic contexts (Pope and Hatfull, 2015, Zrelovs et al., 2021, Cresawn et al., 2011). The increase in phage sequencing projects has made it clear that many phage are not related at the genomic level but have a degree of similarity in their overall structure, making it possible to group them into "clusters" (Pope and Hatfull, 2015, Cresawn et al., 2011). The total phage population is still massively underrepresented, due in part to traditional culturing methods being unable to capture phage diversity, and a more structured survey of their genomes is needed to understand their abundance and range (Aziz et al., 2015, Cresawn et al., 2011).

1.2 Therapeutic application of bacteriophages

The first reported therapeutic use of bacteriophages was by Felix d'Herelle, now revered as the father of applied bacteriophage science, who used them to treat dysentery in the 1920's and 30s (Ackermann, 2003, Sulakvelidze et al., 2001). However, the discovery and success of antibiotics, along with unreliable results due to the infection process used by phage not being fully understood, diminished interest and research into phage therapy in the western world (Sulakvelidze et al., 2001). Eastern Europe continued research into applications for phage, with Georgia being the heart of this research (Sulakvelidze et al., 2001). The major advantages of using phage as a treatment for bacterial infections are their specificity and decreased development of bacterial resistance to phage compared to antibiotics. This is hypothesised to be because the phage can mutate and adapt alongside their bacterial hosts (Ackermann, 2003). It has been suggested that bacteria are 10 times less likely to become resistant to phage rather than antibiotics (Bragg et al., 2016, Ackermann, 2003).

A large body of research was performed in Georgia, most of which has been lost as a result of poor documentation and inadequate translation to English (Bragg et al., 2016, Sulakvelidze et al., 2001). However, phage research became increasingly important as the threat of antibiotic resistant bacteria escalated. By the 1960s, strains of resistant bacteria were widespread, some untreatable even with third generation antibiotics and posed a real threat to

worldwide health and wellbeing (Alisky et al., 1998). It took until the 1980s for interest in phage therapy to be rekindled due to the successful treatment of mice infected with a fatal dose of *Escherichia coli* using phages (Bragg et al., 2016, Smith et al., 2017). The use of phage cocktails as pesticide has also attracted a lot of interest over the years (Bragg et al., 2016, Zaczek-Moczydłowska et al., 2020). Using phages in this way allowed more precise targeted clearance of specific pathogens while keeping resistance development to a minimum. All this without effecting the environmental balance as phage are present in large numbers in the surroundings naturally (Zaczek-Moczydłowska et al., 2020). This approach also saves time and money as the phages self-proliferate in the presence of the target bacteria and remain stable in the soil (Bragg et al., 2016).

1.3 Bacteriophage life cycles

All phage infection events begin with the attachment of the phage tail to a specific receptor molecule on the surface of a susceptible bacterial host. The phage genetic material is then transported down the tail and injected into the host cell cytoplasm, where one of two main life cycles is followed (Bragg et al., 2016). One pathway is purely replicative and kills the bacterial host by lysis due to the action of phage-encoded lytic enzymes. The alternative pathway is lysogenic, during which the phage genome integrates into the bacterial genome and can confer advantages that enable the bacterial host to survive and thrive (James et al., 2015, Davies et al., 2016c). Both pathways are illustrated in figure 1.

1.3.1 The lytic cycle

The destructive lytic cycle can be used to control specific bacterial numbers (Zaczek-Moczydłowska et al., 2020). The phage attaches to a specific bacterial host cell surface receptor triggering genetic material in the phage head to be injected into the host cell cytoplasm, hijacking the metabolism and replication machinery (Figure 1) (Bragg et al., 2016, Campbell et al., 2020, Smith et al., 2017). Activation of the lytic cycle genes enables phage replication and production of holins and lysins that create holes in the bacterial cell surface, leading to lysis of

the cell and release of active phage progeny (Argov et al., 2019, Maurice et al., 2013, Feiner et al., 2015). Using virulent phage in their lytic cycle as a treatment option has been proven effective, even against bacteria resistant to third generation antibiotic treatment (Smith et al., 2017). Phage in the lytic cycle continue to use their host for the sole purpose of reproduction whereas temperate phages that follow the lysogenic life cycle can benefit both parties involved (Ackermann, 2003)

1.3.2 The lysogenic cycle

The lysogenic cycle entails integration of phage genetic material into the host cells genome to become a prophage, replicating along with the rest of the bacterial DNA until a change in environment prompts a switch towards the expression of the phages lytic genes (Figure 1) (Argov et al., 2019, Maurice et al., 2013, Bragg et al., 2016, Feiner et al., 2015).

In the lysogenic state the bacteriophage does not kill its host, instead it coexists in the bacterial genome promoting long term survival of the phage as a prophage (Hargreaves et al., 2014a, Feiner et al., 2015). The bacteriophage can stay in this prophage state for long periods of time, some even losing the ability to switch back and becoming cryptic, permanently integrated into the hosts DNA (Wang et al., 2010). Segments of bacterial DNA have been confirmed to be cryptic bacteriophages, some conferring traits such as antibiotic resistance, although others having no known function due to mutations and phage decay (Davies et al., 2016c).

Some temperate phages remain highly active. For example, the prophages present in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa,* commonly found in the Cystic fibrosis lung, have a high rate of spontaneous induction. This means that they readily switch to the lytic life cycle, producing a high titre of active phage in the infected lungs (James et al., 2015). Many different prophages can be present and coexist in any one bacterial host, but the carriage of multiple bacteriophages does not seem to overwhelm the host (Argov et al., 2019). This suggests that the phages communicate to ensure their hosts, and so their own, survival (Argov et al., 2019).

If the phage fails to integrate into the host genome and cannot replicate due to depleted nutrition, they enter the pseudolysogeny cycle and exist as a plasmid in the hosts cytoplasm (Feiner et al., 2015). As soon as there is enough nutrition for the host cell to start DNA replication, the phages will enter one of the more stable cycles (Feiner et al., 2015).

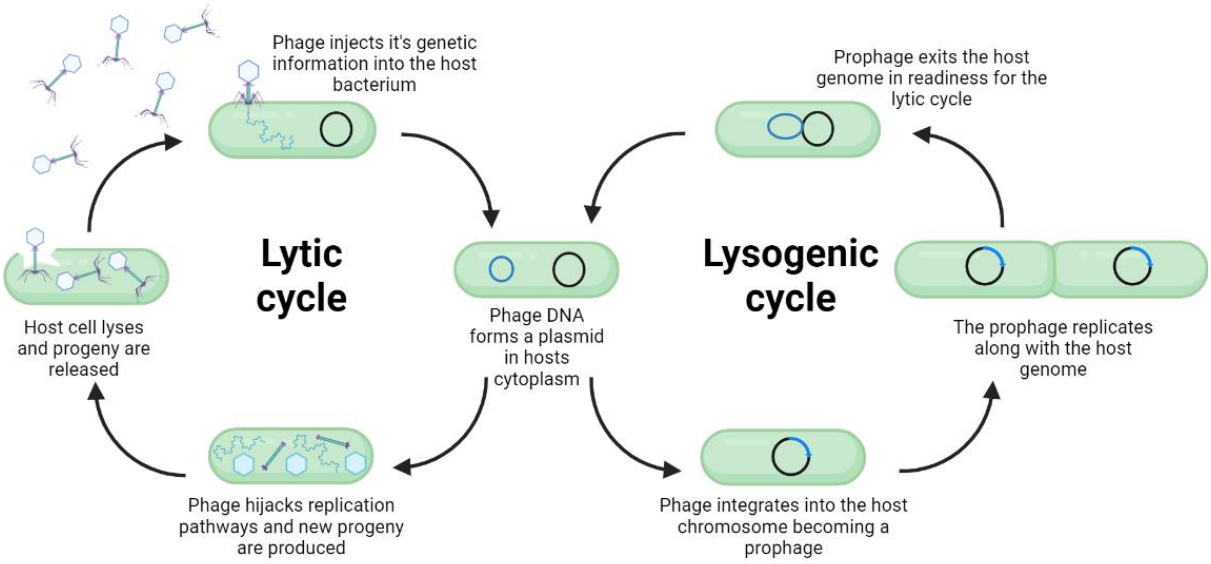


Figure 1. Lytic and lysogenic cycles of Bacteriophage. The two most common life cycles undergone by Bacteriophage. Image created using BioRender.com.

1.4 Role of temperate bacteriophages in evolution of pathogens

The evolution of pathogenic bacteria is thought to have been significantly influenced by temperate phages through lysogenic conversion. This is supported by the observations that i) prophages are more commonly found in the genomes of bacterial pathogens compared to non-pathogens and ii) many prophage sequences include genes associated with pathogenesis (Tsao et al., 2018, Davies et al., 2016b, Bragg et al., 2016). Prophages are known to increase genomic diversification by the introduction of new genetic material, by promoting mutations during the lysogenic cycle and by killing bacteria in their lytic cycle, increasing the selective pressure (Davies et al., 2016b, Hargreaves et al., 2014b, Feiner et al., 2015, Tan et al., 2007).

Several temperate phages have been shown to act as symbionts, integrating into the host genome and activating a subset of genes that can confer advantages (James et al., 2012,

Tsao et al., 2018). Some genes in the phage genome that confer an advantage to the host have been termed morons, or accessory genes, and are thought to have been originally acquired through specialised transduction and horizontal gene transfer (Tsao et al., 2018). For example, the *Vibrio cholerae* prophage, CTXφ, encodes the CT endotoxin operon, which constitutes a major virulence factor (Hargreaves et al., 2014a). Carriage of this prophage enables *V. cholerae* to produce cholera toxin in the human gastrointestinal tract, causing damage to the gut lining and leading to diarrhoea, aiding transmission and spread (Hargreaves et al., 2014a, Hargreaves et al., 2014b). Shiga-toxin (Stx) phages are another group of virulence-associated prophages that convert *Escherichia coli* into Shiga-toxin producers, leading to cytotoxic damage of both human and amoeboid hosts cells (James et al., 2015). This is thought to protect the bacterium from protozoan grazing in the natural environment (Meltz Steinberg and Levin, 2007). Phage have also been shown to benefit the multicellular host of the bacterial lysogens. For example, *Hamiltonella defensa* phage ASPE-2 encodes a toxin, that prevents the aphid host of *H. defensa* from being parasitized by the *Aphidius ervi* wasp larvae (Hargreaves et al., 2014a). These examples demonstrate very obvious virulence traits that can be attributed to specific phage-encoded genes. However, prophages have also been known to influence their bacterial host's growth, survival, immune evasion, motility, biofilm formation and quorum sensing activities through more subtle gene regulation that is less well characterised (Davies et al., 2016c).

Bacterial pathogens are commonly polylysogenic, meaning they carry multiple co-habiting prophages in their genomes (Argov et al., 2019). The *P. aeruginosa* strain LESB58 houses 5 inducible prophages that have been shown to increase its competitiveness compared to the wild type (Argov et al., 2019, James et al., 2012, Tsao et al., 2018). Other temperate phages are also known to protect their hosts from harsh external factors such as damaging oxidative stress in some situations (Hargreaves et al., 2014a).

This Masters thesis focuses on the influence of three LES prophages on the growth and virulence of *P. aeruginosa*.

1.5 *P. aeruginosa*: a diverse opportunistic pathogen

Pseudomonas aeruginosa is a motile, gram-negative bacterium that grows in moist environments such as soil (Stover et al., 2000). It is metabolically diverse, being able to exploit an array of carbon sources, and can grow in the presence or a lack of oxygen (Morita et al., 2014, Stover et al., 2000). *P. aeruginosa* is known to form biofilms on wet surfaces, creating a robust bacterial community with other species to combat harsh environments (Ibberson and Whiteley, 2020, Stover et al., 2000). Being an opportunistic pathogen, *P. aeruginosa* is also known to infect many plants and animals, aided by its ability to adapt to many different niches as well as intrinsic and quick developing antimicrobial resistance (Morita et al., 2014, Mowat et al., 2011). The intrinsic resistance of *P. aeruginosa* is due to its tough outer membrane and its multiple efflux transporters coupled with its ability to deactivate antimicrobial agents (Morita et al., 2014, Smith et al., 2017).

P. aeruginosa has a large bacterial genome (~6.3 million base pairs) due to its increased complexity rather than a difference in organisational structure (Stover et al., 2000). The complexity, size, and number of open reading frames in the *P. aeruginosa* genome suggests that to adapt to a changing environment it develops small gene families. These each contain homologous genes which encode different functions, ensuring that out of the many diverging families some will have the needed adaptations to survive (Stover et al., 2000). The large genome also codes for many different virulence factors and secretory molecules (Ibberson and Whiteley, 2020). This results in populations of *P. aeruginosa* being made up of individuals with different mechanisms of resistance, making treatment challenging (Ashish et al., 2013). The species maintains a core genome, conserving approximately 99.5% of the genomic backbone containing most of the genetic information and virulence factors (Salunkhe et al., 2005, Winstanley et al., 2009). The accessory genome comprises of genomic islands and prophages that encode divergent traits such as antibiotic resistance and biofilm formation (Winstanley et al., 2009). Although the function of some have been established, the functional significance of many of the prophage genes are not well understood.

P. aeruginosa is one of the top 3 opportunistic pathogens in hospitals, causing approximately 10% of infections as it is able to persist on surfaces, even after they have been disinfected (Hancock and Speert, 2000, Morita et al., 2014, Stover et al., 2000). *P. aeruginosa* is a common cause of hospital acquired pneumonia when a ventilator is used, urinary tract infections when a catheter is used and corneal ulceration when contact lenses are used (Stover et al., 2000). This is mainly due to an ability to form robust biofilms on many surfaces, in nature and on medical equipment (Fothergill et al., 2011, Morita et al., 2014, Stover et al., 2000). *P. aeruginosa* is also found readily in burn wounds as many of the hosts secondary defence systems are disrupted, such as neutrophil function, making colonization easier (Hancock and Speert, 2000). In fact, hydrotherapy pools used by burns victims have been highlighted as a major source of infection (Hancock and Speert, 2000).

P. aeruginosa is the most common species of bacteria associated with chronic lung infection in cystic fibrosis (CF) patients, colonizing between 60% and 70% of patients, although there is still no unifying theory explaining why they are so susceptible to infection by this particular microorganism (Aaron et al., 2010, Hancock and Speert, 2000, Workentine and Surette, 2011a). The intrinsic antibiotic resistance, biofilm formation and adaptability make it almost impossible to eradicate, leading to it being the most common cause of death in cystic fibrosis patients (Aaron et al., 2010, James et al., 2015, Mowat et al., 2011, Williams et al., 2015, Workentine and Surette, 2011a).

1.6 Virulence factors of *P. aeruginosa*

P. aeruginosa rarely causes infection in healthy individuals but if an immunocompromised patient is colonized the repercussions can be serious (Salunkhe et al., 2005, Strempel et al., 2013, Hancock and Speert, 2000). It has already been established that *P. aeruginosa* has a high intrinsic resistance to antibiotics due to outer membrane impermeability and multiple efflux systems (Strempel et al., 2013), but its survival is also enhanced by switching phenotype, producing toxins and enzymes, forming biofilms, and through quorum sensing (Strempel et al., 2013, Williams et al., 2015). The production of proteins is intrinsic to each one

of these survival tactics and out of the four protein secretion pathways exhibited in Gram-negative bacteria, *P. aeruginosa* possesses three of them (Stover et al., 2000).

1.6.1 Quorum sensing

The two main quorum sensing systems in *P. aeruginosa*, LasI/R and RhlI/R, as well as the third more recently described PQS system, are responsible for global biofilm and virulence factor regulation (Ibberson and Whiteley, 2020, O'Loughlin et al., 2013). Quorum sensing (QS) is a density-dependent mechanism of bacterial communication. The bacteria produce QS signals (auto-inducers) as they grow. Once a certain concentration of auto-inducer is reached in the surrounding environment, it is detected by bacterial response-regulators and the expression of different traits can be triggered or inhibited (Høiby et al., 2010, Ibberson and Whiteley, 2020, James, 2014, Strempel et al., 2013). Quorum sensing can increase tolerance to host immune defences and antibiotics, promote biofilm formation, or increase the expression of a wide range of other virulence factors (Høiby et al., 2010, Ibberson and Whiteley, 2020). QS signals can also be used to either co-operate with or antagonise other species, conferring an advantage to the bacterium producing the signals. For example, *P. aeruginosa* produces quinolones that cause *Staphylococcus aureus* to lyse, releasing its iron into the environment when *P. aeruginosa* needs it (Ibberson and Whiteley, 2020). Interestingly, the Liverpool Epidemic Strains have been shown to develop mutations in QS systems during chronic infection. In certain cases, the QS response regulators are inactivated, freeing up energy for other processes that are of more use in a chronic infection and decreasing virulence to evade the host immune system (Ashish et al., 2013, Ibberson and Whiteley, 2020, Smith et al., 2017, Workentine and Surette, 2011a).

1.6.2 Toxins and other secreted virulence factors

The wide array of secreted molecules that are produced by *P. aeruginosa* all have different roles to play, many of which are based in the promotion of pathogenicity (Woods and Iglewski, 1983). Among these products, toxin A is known to be one of the more highly produced and studied, probably due to it being very toxic towards mammalian cells by inhibiting their protein synthesis (Woods and Iglewski, 1983). The production of this toxin, and

many other virulence factors, is dependent on the iron availability in the surrounding environment. This is also a main factor that controls the production of elastase (Woods and Iglewski, 1983), one of three proteases that are produced by *P. aeruginosa*, the other two being protease I and alkaline protease (Wretlind and Pavlovskis, 1983, Morihara et al., 1965). Elastase degrades casein, elastin and fibrinogen, among other proteins, and has been shown to cause haemorrhaging and necrosis when injected into an experimental lung model (Wretlind and Pavlovskis, 1983, Liu, 1966). The enzyme is secreted as an inactive proenzyme and only becomes active when broken down by other *P. aeruginosa* cells or by itself (Wretlind and Pavlovskis, 1983). Virulence factors such as elastase and pyocyanin have been implicated in the formation of robust biofilms, aiding in the protection and survival of their host (Diggle et al., 2003). Pyocyanin has also been indicated in other virulence factor pathways that make *P. aeruginosa* hard to eradicate, such as the inhibition of human cilia when infecting the lung in the case of the Liverpool Epidemic Strain (LES) (Fothergill et al., 2007). This, along with the induction of host neutrophil apoptosis, means the production of this phenazine makes the bacterium far more virulent (Fothergill et al., 2007).

1.6.3 Biofilms

Biofilms are a community of cooperating bacteria, protected by a thick, hard to penetrate coat of extracellular matrix (James, 2014, Fothergill et al., 2007). The biofilm is initiated when planktonic bacteria attach to a surface (Høiby et al., 2010). The bacteria then bind irreversibly, multiply and begin to produce the extracellular matrix which builds resistance and structure (Høiby et al., 2010). This extracellular matrix is made up of polysaccharides, proteins, DNA and lipids, creating a strong, dynamic and well-organised structure (Høiby et al., 2010, James, 2014). Diverse species work together, utilizing micro-environments with different properties created by nutrient, pH and oxygen gradients (James, 2014). Once the biofilm is mature and fully formed, sections dissipate and release bacterial cells into the environment, spreading the infection (Figure 2) (Høiby et al., 2010). *P. aeruginosa* can produce a mature biofilm and, in doing so, adopts a more sedate phenotype, losing its flagella and switching to a mucoid phenotype (Høiby et al., 2010, Sonawane et al., 2006, Workentine and Surette, 2011b).

The mucoid variants over-produce alginate polysaccharide which aids the formation of a robust barrier while the loss of the now redundant flagella stops them being a stimulus to the hosts innate immune system (Hancock and Speert, 2000, Filho, 2013, Sonawane et al., 2006). The switch to a more sedentary lifestyle is thought to be in response to selective pressure from the host immune system, in particular neutrophils (Sonawane et al., 2006). Bacteria within a biofilm exhibit increased antimicrobial resistance and a stronger defence against the hosts immune response (James, 2014). This, combined with the extracellular matrix slowing antibiotic penetration, protects the central structure (Høiby et al., 2010). Rates of mutation and horizontal gene transfer are also observed at a higher rate in biofilms (Høiby et al., 2010). The host immune system produces antimicrobial peptides like LL-37 to combat biofilm formation by triggering dispersal. However, this is achieved by the upregulation of quorum sensing genes to increase production of PQS, a signalling molecule that upregulates genes involved in virulence, antibiotic resistance, and swarming motility (Strempel et al., 2013). Thus, LL-37 encourages biofilm dispersal but also upregulates virulence and antibiotic resistance (Strempel et al., 2013).

Siryaporn et al. (2014) have found that production of virulence factors such as pyocyanin and elastase can also be triggered by non- specific adhesion to host cells, via the exposed cell surface protein PiY1. The PiY1 regulator controls a variety of surface associated behaviours and reacts to both chemically and mechanically distinct surfaces (Siryaporn et al., 2014). Adhesion being one of the initial steps in biofilm formation, activation of these virulence factors through binding could be the initial step in creating an environment suitable for a chronic infection (Siryaporn et al., 2014).

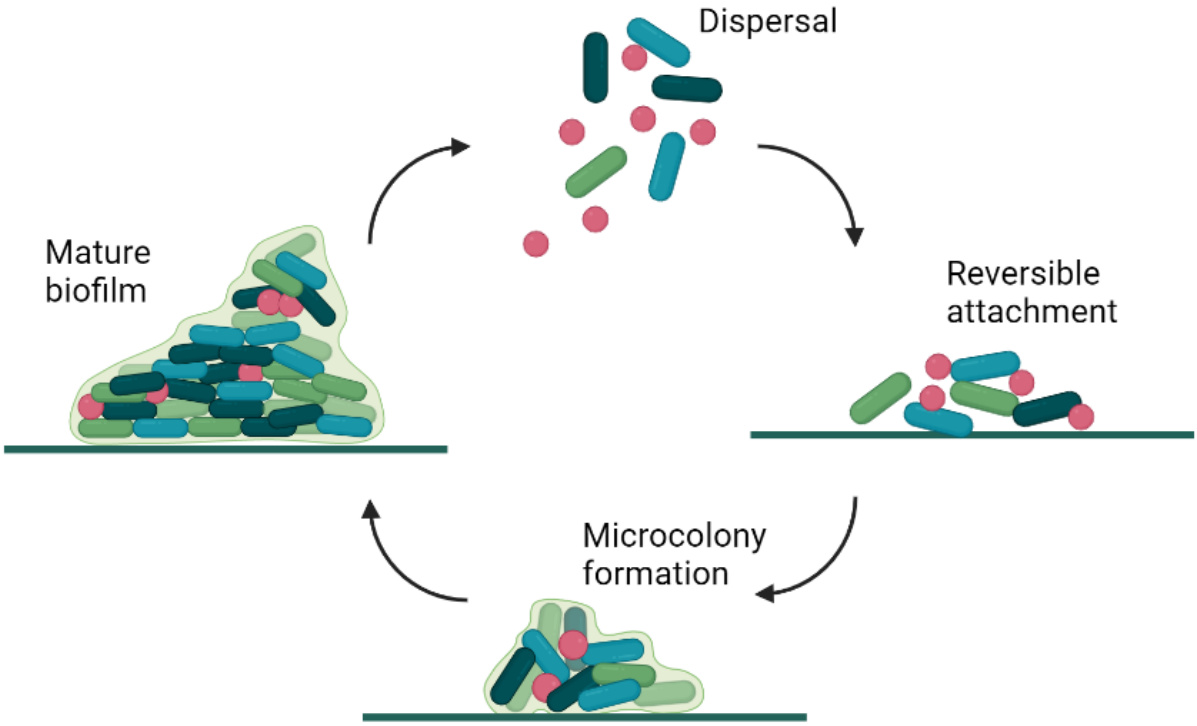


Figure 2. Formation of a biofilm. Planktonic cells adhere reversibly to a surface before producing an extracellular matrix and becoming a microcolony. Once the biofilm has become established and mature, planktonic cells are released and seed new surfaces. Image created using BioRender.com.

1.6.4 Inter-species co-operation

P. aeruginosa is a common member of synergistic bacterial communities, identified in at least 80% of polymicrobial chronic wound communities (Korgaonkar et al., 2013). Bacteria working together is a common survival mechanism that increases the persistence, severity, and resistance of an infection (Korgaonkar et al., 2013). *P. aeruginosa* commonly coinfects with *Staphylococcus aureus* and there have been reports that virulence is boosted when the two species grow together (Korgaonkar et al., 2013). Even though many mechanisms and virulence factors of individual bacterial species are well understood, very little is known about the molecular changes and processes caused by synergy (Korgaonkar et al., 2013). Korgaonkar et al. established that *P. aeruginosa* experiences a boost in virulence when in contact with N-acetylglucosamine, found in Gram-positive cell walls, explaining why *P. aeruginosa* is highly lytic towards Gram-positive bacteria such as *S. aureus* (Ibberson and Whiteley, 2020). Lysing the

Gram-positive bacteria releases cell wall fragments, and so more N-acetylglucosamine, into the surroundings, boosting *P. aeruginosa* virulence (Korgaonkar et al., 2013).

1.7 *P. aeruginosa* is a major multi-drug-resistant threat

P. aeruginosa is becoming increasingly common in hospitals due to its ability to persist in disinfectants and its fast-developing antibiotic resistance (Morita et al., 2014, Stover et al., 2000). It is described as the number one cause of airway infection in patients suffering from cystic fibrosis, being present in up to 80% of adult cystic fibrosis sufferers as a chronic infection (McCallum et al., 2001, Williams et al., 2015). Inappropriate antibiotic treatment selects for resistant strains of bacteria, creating new and harder to treat strains with a built-in immunity for these certain antibiotics (Morita et al., 2014). Adaptation to antibiotics has progressed so far that some treatments promote traits in bacteria that protect them against multiple threats. For example, it has been shown that treatment with carbapenems and fluroquinolones promote enhanced biofilm production, protecting *P. aeruginosa* strains by producing thicker, stronger biofilms (Morita et al., 2014). One antibiotic can also trigger a response that provides protection against other treatments, like ciprofloxacin promoting mutations that protect against the carbapenem class of antibiotics (Morita et al., 2014). These effects could be due to antibiotics being used as signalling molecules in natural bacterial communities (Morita et al., 2014). The reactions to ineffective treatments make diagnosis and identification of bacteria increasingly important.

Changes in environment, such as the heterologous conditions in the cystic fibrosis lung, also decrease antibiotic diffusion efficiency, creating areas with lower concentrations of antibiotics and allowing communities of bacteria to become accustomed to sub-lethal levels (Jorth et al., 2015). This promotes further development of antibiotic resistance via spontaneous mutation and horizontal gene transfer, particularly the acquisition of plasmids, mainly conferring aminoglycoside and β-lactam resistance (Smith et al., 2017, Jorth et al., 2015).

The success of *P. aeruginosa* could also be due to the high volume of regulatory genes (8.4% of the genome) which control functions such as hydrolysis of large molecules, transport of organic compounds across the outer membrane and chemotaxis systems (Stover et al.,

2000). All of these systems have an intrinsic role in the development of antimicrobial resistance (Stover et al., 2000). Permeability of the *P. aeruginosa* outer membrane is 10 to 100-fold lower than other Gram-negative bacteria (Hancock and Speert, 2000, Stover et al., 2000). The lack of large porins, most notably OprF, paired with the increase in smaller porins, slow the rate of antibiotic uptake and allow secondary resistance mechanisms to work more efficiently (Hancock and Speert, 2000). Efflux systems, such as the MexAB-OprM system, also actively expel antibiotics (Hancock and Speert, 2000, Smith et al., 2017).

1.8 Chronic infection of the cystic fibrosis lung

Cystic fibrosis (CF) is an autosomal recessive condition caused by a variety of mutations in the cystic fibrosis transmembrane conductance regulator gene (*cftr*), resulting in defective chlorine transport across a range of cell types, most notably the airway epithelial cells (Ibberson and Whiteley, 2020, Filho, 2013). The prevalence of mutations in the *cftr* gene is very low, under 1,500 have been described, the most common being the deletion of phenylalanine reside gene ΔF508 (Filho, 2013). This reduces transport of chloride and bicarbonate ions across the cell membranes and increases sodium ion absorption through sodium channels with implications on the function of multiple organs (Ibberson and Whiteley, 2020, Smith et al., 2017). This decreases the surface liquid that lines the airways in the lungs and leads to production of particularly thick mucus (Ibberson and Whiteley, 2020, Smith et al., 2017). The defects in the CFTR proteins and the imbalance of ions also lead to a reduction in mucociliary clearance, leading to the build-up of mucus, providing ample nutrients and protection for many different bacterial species (Ibberson and Whiteley, 2020, Smith et al., 2017). Most CF patients therefore show increased susceptibility to chronic infection by a range of microbial species varying with age, treatment, exposure to other patients and other environmental factors (Filho, 2013).

The most common infections tend to be by non-lactose fermenting Gram-negative bacteria such as *P. aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia* (Filho, 2013). Once in the lung, these bacteria produce virulence factors, most regulated by quorum sensing molecules, including a range of toxins and enzymes such as elastase (Smith et al., 2017). Further reduction in mucociliary clearance is caused by *P. aeruginosa* production of pyocyanin

and hydrogen cyanide (Sonawane et al., 2006). The pathogen associated molecular patterns (PAMP) are recognized by pattern recognition receptors of the host immune system, triggering an innate immune response (Smith et al., 2017, Sonawane et al., 2006). Neutrophils swarm to the infection site and promote a massive inflammatory response, producing antibacterial substances such as defensins and more elastase (Smith et al., 2017). However, these responses also increase tissue damage around the infection and further hinder mucociliary clearance (Smith et al., 2017, Sonawane et al., 2006). Bacteria such as *P. aeruginosa* can evade detection by downregulating their PAMPs and repressing flagella expression (Sonawane et al., 2006). Once CF lung infections have become established, they are usually life-long and polymicrobial, with shifts in diversity over time (Filho, 2013). Intermittent periods of stability and exacerbation are commonly experienced with repeated damage to lungs each time, leading to progressively decreased lung function (Fothergill et al., 2007).

Staphylococcus aureus and *Haemophilus influenzae* are amongst the earlier colonizers, usually appearing in the first few months of life (Filho, 2013). *P. aeruginosa* is known to colonise the cystic fibrosis lung later in the patient's lifetime and leads to an inflammatory response, hard to eradicate biofilms and a poor prognosis resulting in an increase in morbidity and mortality (McCallum et al., 2001, Mowat et al., 2011).

Most of the *P. aeruginosa* strains that infect the lungs of cystic fibrosis patients are from the environment which then subsequently adapt to the lung, although there have been transmissible strains identified, such as the Liverpool, Midlands and Australian Epidemic Strains (Williams et al., 2015). *P. aeruginosa* effects patients in different ways, some experiencing a rapid decline in lung function while others only experience mild symptoms (Aaron et al., 2010). This could be due to a variety of host and microbial factors including *cftr* mutations, the lung microbiome and individual infecting strains (Aaron et al., 2010). Once established in the cystic fibrosis lung, regional isolation promotes evolution and development of many distinct *P. aeruginosa* phenotypes (Jorth et al., 2015). Different strains of *P. aeruginosa* colonise different areas of the lung, the Liverpool Epidemic Strain (LES) forming biofilms in the bronchial lumen and strains such as PAO1 and PA14 persisting in the lower alveolar regions in a chronic lung infection rat model (Davies et al., 2016a, Winstanley et al., 2009). As the environments in the

lungs affected by cystic fibrosis differ so widely in nutrient availability, oxygen availability and other conditions, many strains develop, each suited to its own specialised niche in the lung (Jorth et al., 2015). The production of thick mucus and airway obstructions prevents strains from mixing and different treatments put the bacteria under stress, forcing them to adapt to their surroundings (Jorth et al., 2015). These adaptations lead the bacteria to a more stationary existence, turning certain strains into biofilm hyperproducers (James et al., 2012, Mowat et al., 2011, Williams et al., 2015).

1.9 The Liverpool Epidemic Strain of *P. aeruginosa*

The Liverpool Epidemic Strain (LES) has been associated with increased morbidity and mortality despite sharing 95% of its genome with the standard lab strain PAO1 (Stover et al., 2000, Holloway, 1955, Strempel et al., 2013). LESB58 was the first sequenced isolate, cultured in 1988 from the sputum of a cystic fibrosis patient exhibiting ceftazidime resistance, 8 years before it was described in a report written on an outbreak of the strain at Alder Hey Children's Hospital in Liverpool (Cheng et al., 1996, Winstanley et al., 2009).

Although other epidemic strains have been identified, such as pulsotype I in Australia (Armstrong et al., 2003) and the Manchester epidemic strain in the UK (Tsao et al., 2018), LES is the most common strain of *P. aeruginosa* found in cystic fibrosis patients in the UK, also being found in patients in north America (Scott and Pitt, 2004, Aaron et al., 2010). The LES is also the only strain to ever reportedly jump from a cystic fibrosis patient to a healthy individual (McCallum, 2002).

The LES is highly competitive and capable of super-infection (McCallum et al., 2001). Its success could be due to the fact it is able to survive on dry surfaces more successfully than other *P. aeruginosa* strains, or due to the presence of multiple genomic islands and prophages in its chromosome (Panagea et al., 2005). Strains with disrupted prophage genes do not survive as successfully as those with undamaged prophage genes, highlighting the advantage they confer (Winstanley et al., 2009). Colonisation with the LES has been shown to worsen prognosis and makes treating the infection more difficult due to the increased variation and resistance (Williams et al., 2015).

The LES develops antibiotic resistance quickly due to common mutations in efflux pump associated genes such as mexZ (Mowat et al., 2011). In fact, a widespread ceftazidime resistance in cystic fibrosis patients was one of the first warning signs of the LES outbreak (Cheng et al., 1996).

Some of the LES isolates also exhibit a reduction in virulence over time (Winstanley et al., 2009). The quorum sensing regulator LasR is responsible for controlling many of the virulence factors that are of interest in cystic fibrosis infections (Mowat et al., 2011). Mutations in the quorum-sensing regulator gene *lasR* are common in the LES, aiding survival by initiating quorum sensing earlier in the growth curve and so triggering an earlier release of virulence factors (Mowat et al., 2011, Winstanley et al., 2009). This confers a clear advantage to the LES (Winstanley et al., 2009). Once the quorum sensing regulatory factors have remodelled the LES environment the genes are downregulated in chronic infections, decreasing exacerbations and allowing more energy to be directed to other, more useful systems(Ibberson and Whiteley, 2020). A reduction in exacerbations is also achieved by the LES losing its flagella which are known to be major toll receptor used by other *P. aeruginosa* strains to induce an inflammatory response in their host (Winstanley et al., 2009). The reduction in virulence and inflammatory response allows the LES to remain local and decreases the chance of eradication by the immune system, helping to explain how the LES is more successful in the cystic fibrosis lung than other strains (Pope and Hatfull, 2015, Winstanley et al., 2009, Mowat et al., 2011). Increased biofilm production and strength, along with a faster response to antibiotics makes the LES a formidable infection.

1.10 Bacteriophages of *P. aeruginosa*

Approximately 6000 different phages have been visualized using electron microscopy and around 10% of them infected the *Pseudomonas* species (Ceyssens and Lavigne, 2010). The majority of phages infecting *P. aeruginosa* belong to the *Caudovirales* family, tailed bacteriophages with a capsid head containing double stranded DNA (Sepúlveda-Robles et al., 2012). Less than 3% of described *P. aeruginosa* phages are filamentous and contain different forms of genetic material such as single stranded DNA and RNA (Sepúlveda-Robles et al., 2012,

Ceyssens and Lavigne, 2010). *Pseudomonas* phages are widespread and present in practically every biological niche, making them a powerful tool in epidemiological tracing (Ceyssens and Lavigne, 2010). Studies of co-evolution between *Pseudomonas fluorescens* bacteriophages and their host showed a 10 to 100 fold increase in mutation rates in a quarter of observed bacterial cultures when grown in the presence of phages SDW25 and phi2, indicating a sharp rise in the chance of overcoming subsequent phage infection (Ceyssens and Lavigne, 2010).

47 of known *Pseudomonas* phages had been sequenced by 2010 and many are well described (Ceyssens and Lavigne, 2010). *Siphoviridae* phages make up around 47% of *Pseudomonas* phages that have been described; all so far have been in a temperate form and have the typical mosaic genetic formation (Ceyssens and Lavigne, 2010). The largest *Pseudomonas* specific phages sequenced to date are the phiKZ-likes phages, virulent phages made up of between 210Kb and 316Kb that encode between 201 and 460 genes (Ceyssens and Lavigne, 2010). Although horizontal gene transfer is common, the diversity between phages is large but clearly limited, demonstrated by closely related viruses being isolated from many different ecological niches around the world (Ceyssens and Lavigne, 2010). B3-like phages are the most studied temperate *Pseudomonas* phage group and gene product homologues are found in many other bacterial species such as *E.coli, Salmonella enterica* and *Vibrio cholerae*, hinting at phages having a close evolutionary link (Ceyssens and Lavigne, 2010).

The bacteriophages that infect the *Pseudomonas* species have long promoted interest due to their diversity, potential therapeutic applications, and the effect they have on bacterial evolution and fitness (Ceyssens and Lavigne, 2010). Accessory genes are known to influence the way the bacterial host interacts with its environment, but the range of interactions promoted by *Pseudomonas* phages make them a point of increasing interest (Hargreaves et al., 2014a). Some phages are known to help their host, providing them with genes needed to survive in hostile environments or to fight off immune cells and treatments. Phage phiCTX is known to enable its host, strain PAS10, to produce CTX, a cytotoxin that forms pores in the membranes of microbial competitors and increases the host's virulence (Ceyssens and Lavigne, 2010). Phage Pf4 aids biofilm formation, strengthening bacterial host defences and decreasing the chance of removal (Zaczek-Moczydlowska et al., 2020). Conversely, phage F116 has biofilm degrading

properties, allowing it to penetrate and infect its host regardless of its extracellular matrix (Ceyssens and Lavigne, 2010). Yu-A like phages have been reported to encode a photolyase enzyme that repairs damage done by ultraviolet (UV) light to the DNA, a major cause of viral decay in marine environments, ensuring its own survival and replication but not that of its host (Ceyssens and Lavigne, 2010).

1.10.1 LES prophages

There are multiple active prophages present in the LES as part of the accessory genome along with several other genomic islands (James et al., 2012). These constitute the only major difference between LES and the lab strain PAO1, the two strains otherwise sharing 95% of their genetic material (James et al., 2012). LESB58 was the first reported LES isolate and it harbours 5 active prophages, numbered 2 to 6, in its genome (Winstanley et al., 2009). Although no classic virulence genes have been identified on the LES phage genomes, they are currently undergoing re-annotation, using new tools, and there are several lines of evidence to suggest that they confer a specific advantage to the LES in the cystic fibrosis lung. For example: It has been shown that 4 of the LES phages confer a positive advantage to the LES host when competed against transposon mutants of LES phage genes or other virulent strains in a rat model of chronic infection (Davies et al., 2016a, James et al., 2012, Winstanley et al., 2009). Disruption of the LES prophage genes decreases the competitive index by 10 to 100 fold (Lemieux et al., 2015). The LES is thought to outcompete other *P. aeruginosa* strains in the cystic fibrosis lung due to its enhanced survival abilities and by lysing the other strains (Davies et al., 2016a). This is theoretically achieved by the active LES phages infecting and lysing other bacteria (Davies et al., 2016a). Further analysis of the LES lysogens in PAO1 suggests that the LES prophages may confer increased antibiotic resistance and virulence, along with enhancing biofilm production and being linked to mucoidal changes (Davies et al., 2016b, James et al., 2015).

Although all LES phages have been detected in abundance in the sputum of CF patients, the LES phage 2 has been observed to be the most prolific of the LES phages, present at the highest concentration of free phage particles in the sputum of most LES infected cystic fibrosis patients (James et al., 2015). The high abundance may be explained by high rates of

spontaneous induction, stressful conditions in the CF lung such as H_2O_2 from oxidative bursts, and the likely presence of inducing antibiotics. In particular, ciprofloxacin has been shown to trigger induction of the LES phages (Fothergill et al., 2011). Individual lysogens of LES prophages 2, 3 and 4 have been created by infecting susceptible lab strain PAO1 with purified suspensions of each (James et al., 2012). The PAO1 lysogens are much less stable than the original LES host, exhibiting an increased rate of spontaneous induction although this is not thought to be due to random integration into less stable sites (James et al., 2012). Both LES phages 2 and 3 have been shown to integrate within or near genes associated with the type IV pilus and quorum sensing regulators suggesting a degree of adaptation (Davies et al., 2016b). Disrupting the type IV pilus could be a strategy for preventing superinfection by other phages as they are a common phage receptor for *Pseudomonas* phages (Ceyssens and Lavigne, 2010, Davies et al., 2016b). There may be other implications of integration at other sites, such as QS genes, which may affect expression of virulence and biofilm-associated genes, influencing the wider adaptation of the bacterial host. Although there is evidence that the LES phage 5 is active (James et al., 2015), it is only present in a small number of the LES isolates and has not yet been successfully isolated (personal communication, Prof C James).

Each LES phage has been shown to infect a different range of *P. aeruginosa* hosts from other sources (James et al., 2012). They have been shown to use the type IV pilus to infect the PAO1 host. When these surface structures are not available or don't have their normal structure due to mutation, the bacteria are resistant to LES phage infection (James et al., 2012, Davies et al., 2016b). Although all 5 LES prophages can co-infect the same host, there is evidence of phage immunity systems at play. For example, the LES prophage 2 has been found to block infection of PAO1 by LES phage 3 and greatly reduces the frequency of the LES phage 4 infections (James et al., 2012). This may be partially explained by the large amount of shared sequence between LES phages 2 and 3 (over 30%). Their genome organisation bears a similarity to lambdoid phages, suggesting a mechanism of phage-exclusion via the Cl repressor gene (Winstanley et al., 2009, Ptashne, 1992). The LES phage 3 also has a large region of DNA sequence in common with the LES phage 5 which bears a striking resemblance to that of the O-antigen converting phage D3 (Winstanley et al., 2009). There is evidence that the three LES

phages, 2,3 and 4, complement each other and a strain containing all three may be more successful than a strain containing any single lysogen (Davies et al., 2016a).

Norfloxacin and Ciprofloxacin, among other antibiotics, induce the prophages by putting them under stress and forcing them to switch to their lytic cycle, promoting free phage production and host destruction (James et al., 2012, Fothergill et al., 2011). Although this seems like a great treatment option, some antibiotics are known to promote toxin production such as with the Shiga toxin in *E. coli* (James et al., 2015). Even though phage in the lytic cycle reduce bacterial numbers, they are not eradicated by phage treatment alone (James et al., 2015). A combination therapy of specific phage and targeted antibiotics would be the best strategy for eradication (James et al., 2015). Despite clear evidence for the role of LES phages in the pathogenicity of the LES in the CF lung, the mechanisms are not fully understood.

1.11 Aims and objectives

The literature shows that bacteriophages have a considerable effect on the survival of their bacterial hosts, be it through the lytic or lysogenic cycles, and they drive evolution and adaptation in bacterial communities. This is abundantly clear in *P. aeruginosa* and, in particular, the LES. This project explores the effect that prophages 2, 3 and 4 have on their host. Specifically, the project investigates the effects each of the prophages have on biofilm formation, virulence, and motility. Through this the project aims to pinpoint some key measurable phenotypes that are altered as a direct effect of prophages carriage. A set of four defined experiments were designed to aid our understanding of this relationship.

Specific objectives:

1. Growth profiling: The rate of lysogen growth was compared to WT growth in both nutrient rich and depleted conditions to determine if the prophages provided an advantage (Davies et al., 2016b).
2. Biofilm production: Microtiter crystal violet plate assays were used to determine whether prophage carriage affected biofilm density (Tompsett, 2021, Davies et al., 2016b).

3. Motility: The lysogens were grown in conjunction with WT on swimming, swarming and twitching agar to observe the effect prophage carriage had on the hosts motility (Davies et al., 2016a).
4. Virulence: The *Galleria mellonella* model was used to establish whether the addition of prophage increased or decreased the virulence of its host in the presence of a primitive immune system (Tsai et al., 2016).

2. Materials and methods

2.1 Bacterial strains, phage, and growth conditions

The lysogen strains used in this project were first created by James et al. (2012) to explore the different infection properties of each of the individual phages had on their host, PAO1. These same lysogens were used to establish the effects each of the LES phages have on the virulence of PAO1.

2.1.1 Bacterial strains, lysogens and phage suspensions

The well-characterised lab strain of *P. aeruginosa,* PAO1 (Holloway et al., 1955) was used as a model strain to investigate the relative effects of different combinations of the LES (Liverpool Epidemic Strain) prophage carriage in an empirical manner. Table 2.1 details the lysogens and phage suspensions used.

Table 2.1: PAO1 lysogens and LES phages. Name and reference of each bacterial and phage filtrate used in the project.

Strain / Phage type	**Reference**
PAO1 Wildtype (WT)	Holloway et al., 1955
PAO1(LESΦ2) single lysogen	James et al., 2012
PAO1(LESΦ3) single lysogen	James et al., 2012
PAO1(LESΦ4) single lysogen	James et al., 2012
PAO1(LESΦ2,3,4) triple lysogen	James et al., 2012
Purified LES phage 2 in LB	James et al., 2012
Purified LES phage 3 in LB	James et al., 2012
Purified LES phage 4 in LB	James et al., 2012

2.1.2 Setting up bacterial cultures

P. aeruginosa strain PAO1 was recovered from stocks kept at -80 °C at The University of Salford, streaked out onto a fresh Luria Bertani (LB) agar (Neogen) and incubated at 37 °C for 24 h. One

colony from the stock LB plate was used to seed 10 ml fresh LB broth (Neogen) and incubated in a rotator incubator (Stuart, orbital incubator SI500) at 37 °C and 100 rpm for 24 h. Using a sterile, filter pipette tip (Starlab), 500 µl of the culture was diluted with 500 µl sterile LB broth in a 1 ml cuvette (Fisherbrand) and the optical density (OD) was measured at a wavelength of 600 nm using a spectrophotometer (Geneflow, Jenway 6300). The proportions to achieve an OD_{600} of 0.5 were calculated using the formula

$$C1 \times V1 = C2 \times V2$$

Once an OD_{600} of 0.5 was achieved, the culture (5 ml) was added to 50 ml of sterile LB broth to achieve a further 1:10 dilution (OD_{600} 0.05). This was repeated for all cultures to start each growth experiment or set up cultures for other assays.

2.2 Growth profiling experiments

To determine the effects of prophage carriage on growth, cultures of WT PAO1 and each of the four lysogens (Table 2.1) were prepared in triplicate according to section 2.1.2. The 50 ml cultures were incubated at 37 °C with shaking (130 rpm) for 12 h. OD_{600} was measured, and viable counts were performed at 2 h intervals.

Each growth experiment was repeated using M9 minimal media (Table 2.2) supplemented with 0.4% succinate (M9+S) (Sigma-Aldrich).

Table 2.2: Composition of M9 salts solution (2022). Once autoclaved, 200ml of the M9 salts solution was added to 700ml sterile distilled water with 40ml sterile 10% Succinic acid (Sigma-Aldrich), 100µl sterile 1M $CaCl_2$ and 2ml sterile 1M $MgSO_4$ (Sigma-Aldrich). The volume was then made up to 1L using sterile distilled water resulting in M9+S.

Chemical name	**Formular**	**Molar conc. (mol^{-1})**	**Manufacturer**
Disodium phosphate	Na_2HPO_4-$7H_2O$	0.564	Sigma-Aldrich
Monopotassium phosphate	KH_2PO_4	0.138	Fisher Chemical
Sodium chloride	NaCl	0.053	Sigma-Aldrich
Ammonium chloride	NH_4Cl	0.117	Sigma-Aldrich

2.2.1 Viable cell quantification using the Miles Misra technique

A serial 10-fold dilution of each culture was performed each hour by diluting 100 µl in 900 µl sterile LB broth. Each dilution was then spotted (10 µl) in triplicate on LB agar and allowed to dry at room temperature (rt °C). Once dry, the plates were incubated at 37 °C for 24 h and once visible colonies had formed the most countable dilutions were quantified. From this the mean colony forming units ($CFUml^{-1}$) were determined for each time point and a growth curve was plotted.

$$\text{Mean } CFUml^{-1} = ((\text{count 1} + \text{count 2} + \text{count}) \ 3 \ /3) * \text{dilution factor} * 100$$

2.2.2 Comparing growth in an automated growth profiler

Since the standard growth profiling method was labour intensive and susceptible to contamination at each time-point, growth profiles of each lysogen were also compared to WT using the CR9001 growth profiler (Enzyscreen) at The University of Liverpool. In brief, cultures were set up as detailed in 2.1.2 in both LB and M9+S, 150 µl of each was distributed per well into a 96 well plate (System Duetz) in replicates of 6. Since *P. aeruginosa* auto-fluoresces, the growth profiler measured the increase in fluorescent pixels over time to provide a "density value". Measurements were read at 10 min intervals, with shaking in between, but only 2 h intervals were plotted for 12 h. This method provided a more accurate comparison of growth profiles.

2.3 Resazurin assay

The effect of prophage carriage on metabolic activity was investigated using the redox indicator, resazurin (BDH Chemicals LTD). Alongside taking samples for the miles misra growth curve protocol (2.2.1), 100 µl of the ongoing culture was added to an equal volume of 0.02 % (v/v) resazurin in the well of a 96 well plate in triplicate. The plate was then incubated for 45 min at 37 °C and 150 rpm. The absorbance of the well was subsequently recorded at 544/590nm. This was repeated at each time point for 12 h. This methodology was based on the methodology used by Barber (2019).

2.4 Measurement of phage production by spot plaque assay

Spontaneous induction of each LES prophages was measured during growth profiling experiments. Each hour a 1 ml sample was taken from each replicate, filtered using a sterile 0.2 µm syringe filter (Starlab) and the filtrates were stored at 4 ºC for up to 3 months. Phage numbers were quantified from each filtrate using a spot plaque assay technique as follows: The WT PAO1 strain was grown overnight in LB broth at 37 ºC and 130 rpm. It was then diluted down 1:1000 (10 µl to 10 ml LB broth) and grown to exponential phase (OD_{600} 0.5 -0.9) at 37 ºC and 130 rpm. The required volume of pre-prepared top agar (LB + 0.4% bacteriological agar (Neogen)) was melted in a microwave at 15 s intervals until completely molten and then cooled to approximately 40 °C. The exponential phase WT PAO1 culture was added to the cooled top agar at a ratio of 1:50 (100 µl of culture to 5 ml molten top agar) and 5 ml of the mixture was poured onto the required amount of set, sterile LB agar plates and allowed to set at room temperature (rt) °C. 10-fold serial dilutions were prepared in sterile Phosphate-Buffered Saline (PBS) (VWR life science) for each phage filtrate from the growth profiling experiments. Depending on the time points the filtrate was taken from, the filtrates were diluted by up to a factor of 10^9. Once the top agar had set, the petri dish was divided into 4 segments and 20 µl of each diluted phage filtrate was spotted in triplicate (three spot per segment). Spots were allowed to dry completely at rt °C before incubation at 37 ºC for 24 h. Once a lawn of PAO1 had grown and plaques were visible, the dilution with the most countable plaques was quantified in each case and the plaque forming units ($PFUml^{-1}$) were calculated for each time point.

$$\text{Mean p.f.u ml}^{-1} = ((\text{count 1} + \text{count 2} + \text{count}) 3 /3) * \text{dilution factor} *50$$

2.5 Confirmation of LES lysogens and plaque production by Polymerase Chain Reaction

To ensure that each filtrate collected during the growth profiling experiments were pure and contained the expected phage, a Polymerase chain reaction (PCR) was performed. Templates included each of the filtrates collected at the final time point (t=12) from growth profiling

experiments. This ensured that the culture had remained pure throughout the experiment and that the phages isolated and enumerated using plaque assays were the correct ones.

The PCR reaction was set up using a master mix consisting of 12 µl MyTaq Red Mix (Bioline), 2µl of primer diluted to 10 $pmol^{-1}$ (Table 2.3, Eurofins) and 9 µl PCR grade water. Once the master mix was prepared, 2 µl of the template DNA was added producing a final volume of 25 µl per sample. These samples were subjected to a thermal cycle of 95 °C for 5 min before amplification using cycling conditions: 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min for 30 cycles followed by 72 °C for 5 min and 12 °C for 10 min (Verbal communication, Revathy Krishnamurthi)

Table 2.3 Primers targeting each phage. The primers were procured from eurofins and were used at a concentration of 10 $pmol^{-1}$.

Primer pair name	**Sequence 5'-3'**	**Expected amplicon size**
Phage 2 primer	Forward TGCGACGAAGACGAACTGAA Reverse CGTCCTGCGCAGATTGATTG	**235bp**
Phage 3 primer	Forward TAAGCGCGTTGTACTCAGCA Reverse ACCTCTACCTCGGTGCTCTT	**785bp**
Phage 4 primer	Forward TCCATGGGCGTTTAATGGCT Reverse CTCGCTCAGGTAATAGCGCA	**435bp**

2.5.1 Gel electrophoresis

Once the PCR cycles had been completed the products were run on a gel alongside a 1Kb hyperladder (Bioline) to ensure the desired fragments had been isolated. The products were run on a 1% agarose gel with a 1:1000 dilution of GelRed (Thermos Fisher) at 100 volts. The gel was visualised using the GBox Chemi XX6 (Syngene).

2.6 Comparison of biofilm densities

The effect of LES prophage carriage on biofilm density was assessed using the methodology described by Tompsett (2021). The PAO1 WT and LES lysogens were grown for 18-20 h in either LB broth of M9+S medium at 37 °C, with shaking at 130 rpm. The resultant optical densities of the cultures were measured using a spectrophotometer and subsequently diluted to an OD_{600} of 0.005. Diluted cultures (100 µl) were added to a flat-bottomed 96 well microtiter plate (Greiner bio-one, CELLSTAR®) in replicates of 6 with un-inoculated fresh media as a negative control and incubated for 18 h at 37 °C. The planktonic growth was measured using a FLUOstar omega microplate reader (BMG) to ensure the validity of negative controls. The contents of each well was removed by aspiration and discarded as bio-waste. The wells were washed using distilled water 2-4 times and then dried thoroughly by incubation at 50 °C without a lid. The remaining biofilms that had formed on the micro-well plastic surfaces were quantified by adding 125 µl of 0.1% crystal violet solution (Pro-lab diagnostics) to each well and incubating at rt °C for 10 min. Stained biofilms were washed 2 to 4 times using distilled water and dried thoroughly as previously. Remaining dye was solubilised by adding 200 µl of 95 % ethanol for 15 min. The biofilm density was then reported by colour intensity that was quantified using a FLUOstar omega microplate reader at OD_{570}. The process was repeated using M9+S as the growth medium. The mean blank/negative control readings were subtracted from the other readings to adjust for background staining.

2.7 Swimming, swarming and twitching motility assays

The effect of prophage carriage on *P. aeruginosa* motility was assessed using three simple agar plate assays previously described by Davies et al. (2016a). Overnight cultures of PAO1 WT and the LES lysogens were prepared in LB and incubated at 37 °C rotating at 100 rpm. To establish the extent of swimming motility, a loopful of the overnight culture was deposited onto the surface of swimming motility agar consisting of 10 g tryptone (Neogen), 0.093 mol^{-1} NaCl (Sigma-Aldrich) and 3 g of bacterial agar n° 2 (Neogen) to 1L dH_2O. Swimming assay plates were incubated at 37 °C for 18 - 20 h and the diameter of growth was measured in cm. The extent of

swarming motility was established in the same way except using swarming motility agar consisting of 0.02 mol^{-1} NH_4Cl (Sigma-Aldrich), 0.294 mol^{-1} Na_2HPO_4 (Alfea Aesar), 0.02 mol^{-1} KH_2PO_4 (Fisher chemical), 0.009 mol^{-1} NaCl (Sigma-Aldrich), 0.01 mol^{-1} Dextrose (Oxoid), 5 g casein hydrolysate (Oxoid), and 5 g bacterial agar nº 2 to 1 L dH_2O, adding 10 ml of 0.1 M $MgSO_4$ (BDH Limited) and 0.1 M $CaCl_2$ (Honeywell Fluka) once cooled.

The extent of twitching motility was established using LB agar. A sterile toothpick was inoculated with a small amount of the PAO1 culture and stabbed through the centre of the agar. It was ensured that the toothpick was applied all the way through the agar. It was then incubated at 37 ºC for 24 h. The agar was then completely removed from the petri-dish using sterile tweezers and discarded as bio-hazard waste. The petri dish was flooded with crystal violet solution (1%) and incubated at rt °C for 15 min. The crystal violet was then discarded, the plate was washed briefly with sterile water and the diameter of the stained area left by the culture was measured. All motility assays were repeated in triplicate for all 3 separate lysogens and the triple lysogen and compared to WT.

2.8 *In vivo* virulence assays using *Galleria mellonella*

Since previous evidence suggested a possible role for LES phages in virulence, a simple *in vivo* assay was performed to compare the virulence of WT vs LES lysogens using the *Galleria mellonella* infection model that includes a rudimentary immune system (Tsai et al., 2016). The methodology is based on that described by Latimer et al. (2012) and that described by Cowley (2016).

2.8.1 Preparation of bacterial inoculant

A colony of PAO1 Wild type was inoculated into LB (5 ml) and incubated at 37 ºC with shaking at 100 rpm for 18-20 h. *G. mellonella,* purchased from Live Foods Direct (https://www.livefoodsdirect.co.uk/Category/Waxworms) and stored at 4 ºC in the dark for up to 2 weeks prior to use, were pre-incubated at 37 ºC for 30 min before use. Bacterial cells were harvested from 1 ml of the overnight culture by centrifugation at 5,000 rcf for 10 min in a table-

top microfuge (Eppendorf centrifuge 5430). The supernatant was discarded, and the bacterial pellet was washed by re-suspension in sterile PBS (1 ml) and vortexed to homogenise the sample. This wash step was repeated 2 more times to ensure that all secreted products and metabolites were removed. The washed bacterial cells were diluted to OD_{600} 0.01 in sterile PBS.

2.8.2 Inoculation and monitoring of *G. mellonella*

16 healthy *G. mellonella* larvae (pale colour, no darkening, and movement when touched) were selected for each condition (detailed in table 2.4) and placed into sterile petri dishes. It was ensured that all the *G. mellonella* were of a healthy colouration (pale), lively and between 2 cm and 3 cm long. Each larva belonging to test groups 2-7 was inoculated with PBS or bacterial culture (5µl) as prepared in 2.8.1 by injection into the hemocele of the *G. mellonella* via the second to last leg stump on the left side using a 10 µl glass syringe (Hamilton). This was repeated for all 16 *G. mellonella* (16 technical replicates for each culture), all being placed into the same petri dish once injected. Group 1 did not require injection and acted as a control for morbidity or mortality due to the act of injection alone. Once all three experimental replicates for both the WT PAO1 and control groups had been injected, they were incubated at 37 °C along with both the uninfected control group that had not been injected and those that had been injected with sterile PBS. Every 20 min the larvae were stimulated with a sterile pipette tip to assess their health status. If they did not react, they were rolled onto their backs and if they failed to wright themselves or if there was no movement they were classified as dead, removed, and frozen. A further indication of morbidity or death was the appearance of dark patches and eventually complete black colouration throughout. The experiment was continued until two individuals from the control groups, or all the individuals inoculated with the bacterial strain were observed to be dead. This was repeated for all bacterial strains being tested.

Table 2.4 *G. mellonella* test groups. Each larva was injected with 10 µl of culture or, in the case of the control group, sterile PBS. There was also a control group that was not injected.

Group	Condition	Number of larvae
1	No injection	16 x 3
2	PBS	16 x 3
3	PAO1 WT	16 x 3 repeat cultures
4	PAO1(LESΦ2) single lysogen	16 x 3 repeat cultures
5	PAO1(LESΦ3) single lysogen	16 x 3 repeat cultures
6	PAO1(LESΦ4) single lysogen	16 x 3 repeat cultures
7	PAO1(LESΦ2,3,4) triple lysogen	16 x 3 repeat cultures
	Total	384

2.8.3 *G. mellonella* bacterial load count

Each frozen *G. mellonella* was homogenised in sterile PBS using a tissue processor (Qiagen, Tissue Lyser II Tissue Processor). Each larva was processed in a sterile 1.5 ml microfuge tube with 1 ml of sterile PBS and a 5 mm stainless steel bead (Qiagen). The tissue processor was set at 25Hz for 30 min. The *G. mellonella* homogenates were diluted to a factor of 10^{-8} using sterile PBS and 10 µl of each dilution was spotted onto LB agar in triplicate. These were left to dry at rt°C before incubating at 37 °C for 24 h until countable colonies became visible. It was ensured that the colony morphologies were similar to that of PAO1 and then counted. The colony forming units (CFU ml^{-1}) were determined and the data was plotted as a boxplot.

3. Results

3.1 Effect of LES prophage carriage on bacterial host density

The effect of LES prophage carriage on the growth of bacterial hosts was assessed using the LES lysogens of the well-characterised *P. aeruginosa* reference strain PAO1. Growth profiles under nutrient rich conditions were measured in Luria Bertani (LB) broth using a growth profiler that measured the growth by imaging density of pixels over time. Since the LES prophages were isolated from LESB58 and have been suggested to confer an advantage in the CF lung, it would be appropriate to assess the effects of prophage carriage on growth in conditions that more accurately reflect the CF lung environment. As a prelude to more sophisticated infection models, growth profiles of each LES lysogen were also compared to WT under nutrient limiting conditions to mimic the CF lung. This was first attempted using glucose to supplement M9 minimal media. However, the culture failed to grow under these conditions and so growth was achieved by supplementing M9 minimal medium with 0.4% succinate in accordance with Paliy and Gunasekera (2007).

3.1.1 LES prophage carriage mainly enhances growth in nutrient rich conditions

Figure 3 shows the growth of PAO1 lysogens in LB over a 12 h period. The density of each lysogen was compared to wild-type (WT) PAO1 at each time point using Mann Whitney U analysis. The density of WT increased at the fastest rate for the first five hours of the experiment. However, just before the 6^{th} hour of growth, the density of the PAO1 lysogens carrying LES prophages 3 and 4 as well as the triple lysogen overtook the growth of the naïve WT. Between hour 6 and 8 the LES prophage 3, LES prophage 4 and the triple lysogen exhibited almost identical density whilst the naïve WT continued to grow at a slower rate. Between 9 and 12 h there was no further increase in growth. PAO1 lysogens carrying all 3 prophages remained at the highest density, with LES prophage 3 and 4 lysogens showing a marginally lower density. During this time, the WT density was consistently lower (17.8 – 20.8%) than the triple lysogen at every timepoint ($P < 0.05$).

The growth profile of the LES prophage 2 lysogen was significantly different to the WT and all other lysogens at every timepoint (P <0.05). Initially (0-3 h), the lysogen grew at a similar rate to the other LES lysogens, reaching 0.75 units within 3 h. However, growth between 3 – 8 h continued at a much slower rate than the WT and the other 3 lysogens. All the lysogens and WT showed the same general trend between 8 and 12 h with stalled or decreasing densities. The phage 3 and 4 lysogens showed a decrease in density of 16.1% and 16.9% respectively during this time (phage 3 P-value>0.05, phage 4 P-value>0.05). By contrast, the triple lysogen density continued to increase until 8.67 h and then began to drop from a maximum of 5.6, almost mirroring the phages 3 and 4 profiles (P-value>0.05).

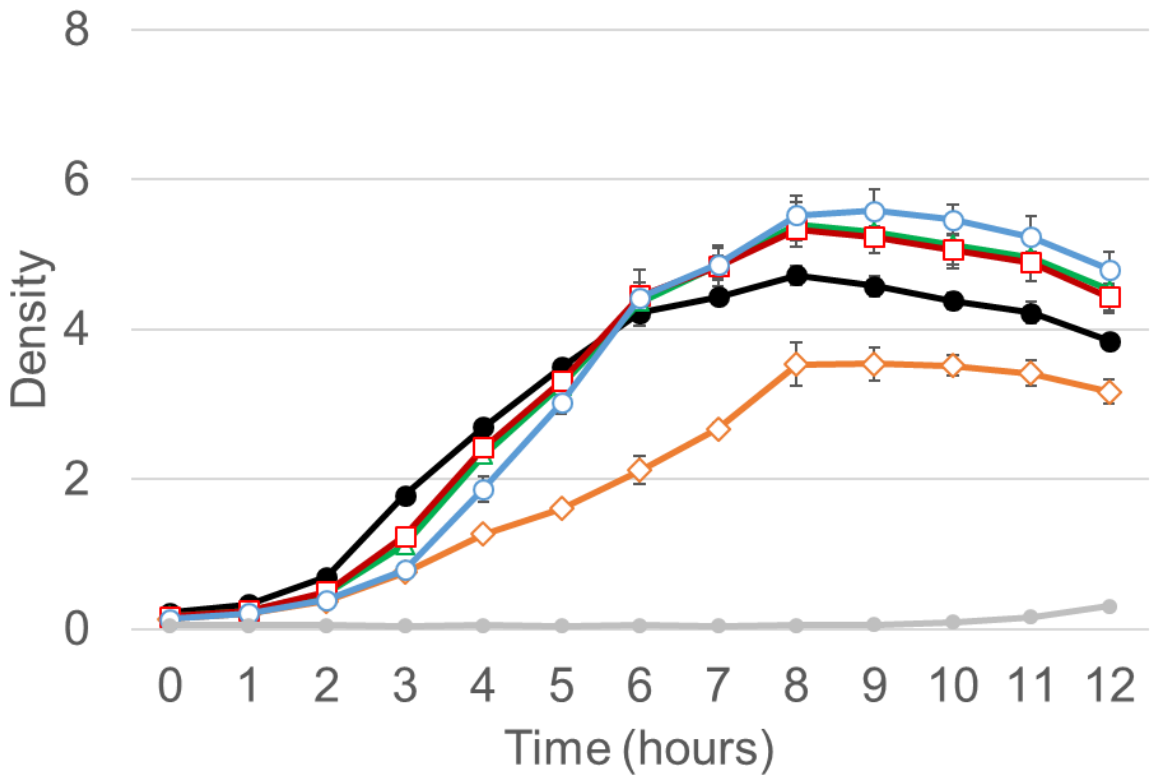


Figure 3. *P. aeruginosa* PAO1 vs lysogen growth in LB media. ● WT (Naïve), ◊ Phage 2 Lysogen, Δ Phage 3 Lysogen, □ Phage 4 Lysogen, ○ Phage 2,3,4 triple Lysogen, ● Negative control (sterile LB broth). Data points show mean of 6 technical replicates and error bars represent standard deviation.

These data suggest that some instances of prophage carriage enhance host growth rate. The lysogens carrying phage 3 and phage 4 grew more successfully than the WT although, as the phage 2 lysogen demonstrates, carriage of a prophage can also hinder growth rate. This could be due to higher rates of spontaneous induction of prophage 2. There is also potential evidence of a synergistic relationship in the triple lysogen when the presence of prophages 3 and 4 may have a stabilizing effect. This would provide an explanation as to why the lysogen containing all three bacteriophages had the highest growth rate. Further investigation compared phage production rates to test this hypothesis (see section 3.3).

3.1.2 Prophage carriage is costly in nutrient limiting conditions

Overall, the WT and LES lysogens grew to much lower densities in the supplemented minimal medium, and very different growth profiles were observed. Figure 4 shows that the lysogens were less fit than WT overall in this setting and that the lysogen carrying prophage 2 was the least fit. For the initial 3 h the single lysogens carrying either LES prophage 2, 3 or 4 had the fastest increasing densities ranging from a growth rate of 0.21 pixels/hr to 0.23 pixels/hr. After 3 h the lysogens carrying the LES prophages 3 or 4 continued to increase whereas the LES prophage 2 lysogen grew considerably more slowly, only reaching 0.48 pixels after 12 h. The final density of the LES prophage 2 lysogen was 49.5% lower than that of the LES prophage 4 lysogen. The difference in final density between lysogens carrying the LES prophage 2 and those carrying the LES prophage 3 was even more pronounced, lysogen 2 having reached a density 57.9% lower than lysogen 3 (Mann Whitney U, $P < 0.05$). Single lysogens carrying the LES prophage 3 and 4 shared very similar growth profiles until 7 h where the phage 3 lysogen showed a sharper increase in density, although growth of these two lysogens showed the same general trend between 9 – 12 h, separated by an average of 15.1% ($P > 0.05$). Whilst the single lysogens showed a similar trajectory of growth, the profiles for WT and triple lysogen exhibited a different pattern. The triple lysogen showed a longer lag period, but then cell density grew exponentially between the 3 h and 8 h, followed by a levelling off at a reading of 1.41 pixels, dropping slightly by 2.8% by 12h ($P < 0.05$).

The naïve WT strain showed a very similar growth profile to the triple lysogen in M9+S, but grew more rapidly between 4h – 6h, reaching a maximum density of 1.69 pixels at 8h. For the remaining 4 hours the cell density of the WT dropped by 10.7%, maintaining a considerably higher cell density than any of the lysogens carrying the LES prophages. The final cell densities of WT, the triple lysogen, phage 3 and phage 4 lysogens all had a difference of 0.23 pixels or less between each of them. The negative control stayed at a density of 0 for 11 hours of the study but showed a slight increase in the last hour.

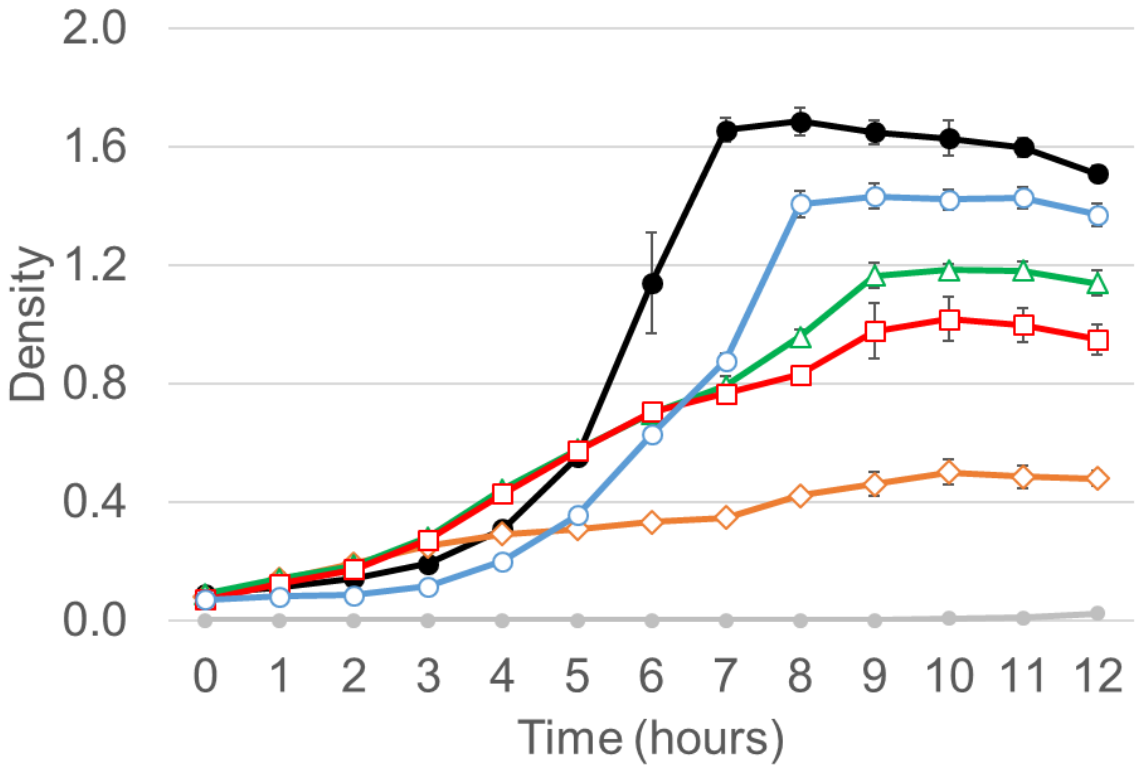


Figure 4. *P. aeruginosa* PAO1 vs lysogen growth in minimal M9 media supplemented with 0.4% succinate. ● WT (Naïve), ◊ Phage 2 Lysogen, Δ Phage 3 Lysogen, □ Phage 4 Lysogen, ○ Phage 2,3,4 triple Lysogen, ● Negative control. Data points show mean of 6 replicates and standard deviation.

These data suggest that the carriage of prophages in minimal media is damaging to the hosts survival. Each lysogen carrying a single prophage had a lower growth rate than that of the WT. The triple lysogen also had a lower growth rate than that of the WT but it was fitter than the

single lysogens. This suggests that the bacteriophage cooperate and confer more of an advantage to their host when coinfecting. There could also be unseen benefits to carrying prophages in minimal media that make the reduction in growth rate worth it.

3.2 Viable cell count in different media

Quantification of viable cells is an alternative method for assessing growth rates. The miles misra technique allows the number of colony forming units (CFU) to be established at each time point by establishing how many viable bacterial cells are present as opposed to other techniques that quantify how many cells, living or dead, are in a sample. The $CFUml^{-1}$ of each lysogen was measured every 2 h in both rich LB media and minimal M9+S media as with the previous experiment.

3.2.1 Viable cell counts were not affected by prophage carriage in nutrient rich conditions

Figure 5 illustrates that all lysogens grew at similar rates to WT in rich LB media. Over the first 6 h the lysogen containing the LES phage 4 had the highest proportion of viable cells, apart from at 4 h where the triple lysogen density was higher ($1.7x10^{12}$ $CFUml^{-1}$), but this was not significant (Mann Whitney U, P=0.71). After 6 h the WT strain densities overtook phage 4 lysogens and reached $1.7x10^{14}$ $CFUml^{-1}$. Every other strain grown in rich LB media reached its highest proportion of viable bacterial cells at the final time point of 12 h and no significant difference between them was determined (Mann-Whitney U tests performed to compare each lysogen to WT, P>0.05).

The lowest viable cell counts were consistently observed for the phage 3 lysogen.

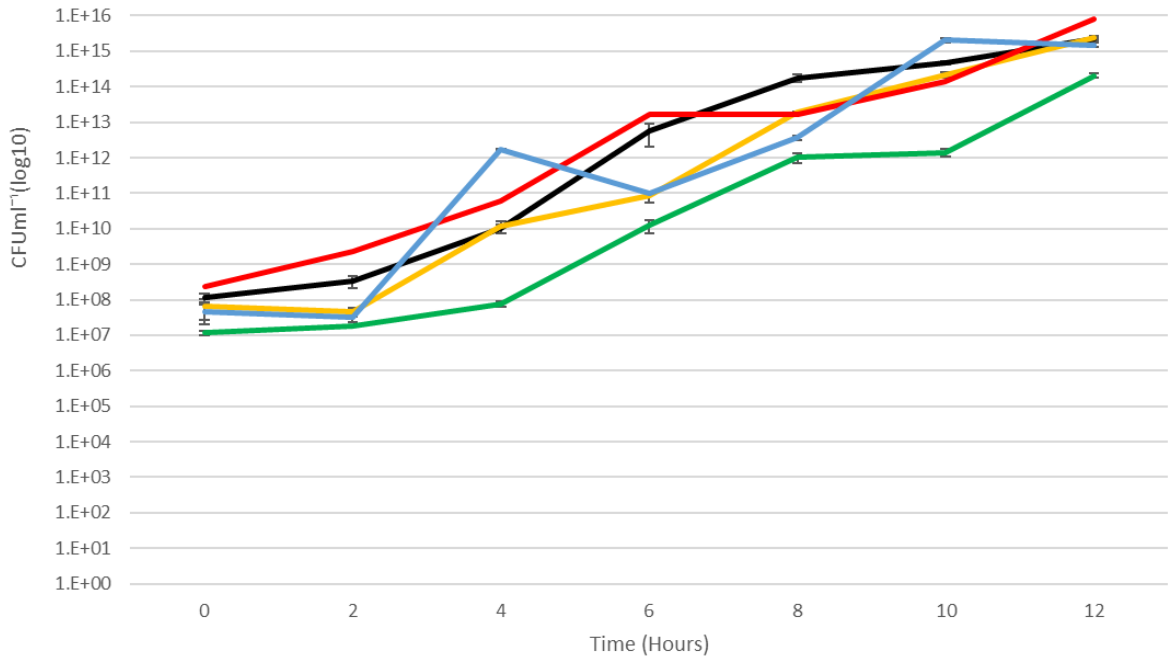


Figure 5: Proportion of viable bacterial cells at 2-hour intervals over 12 hours being grown in LB media. ─ WT (naïve), ─ Phage 2 Lysogen, ─ Phage 3 Lysogen, ─ Phage 4 Lysogen, ─ Triple lysogen**,** ─ Negative control. Each data point shows the mean of 3 technical repeats and the error bars represent the standard deviation.

3.2.2 Viable cell counts were not affected by prophage carriage when nutrients were limited

All the growth profiles measured by viable cell counts in the minimal M9 media were similar. Although WT grew more rapidly for the first few hours, viable cell numbers were surpassed by phage 4 lysogens after 4 h and by the other lysogens after 6 h. After 6h no obvious differences in viable cell counts were observed (Figure 6).

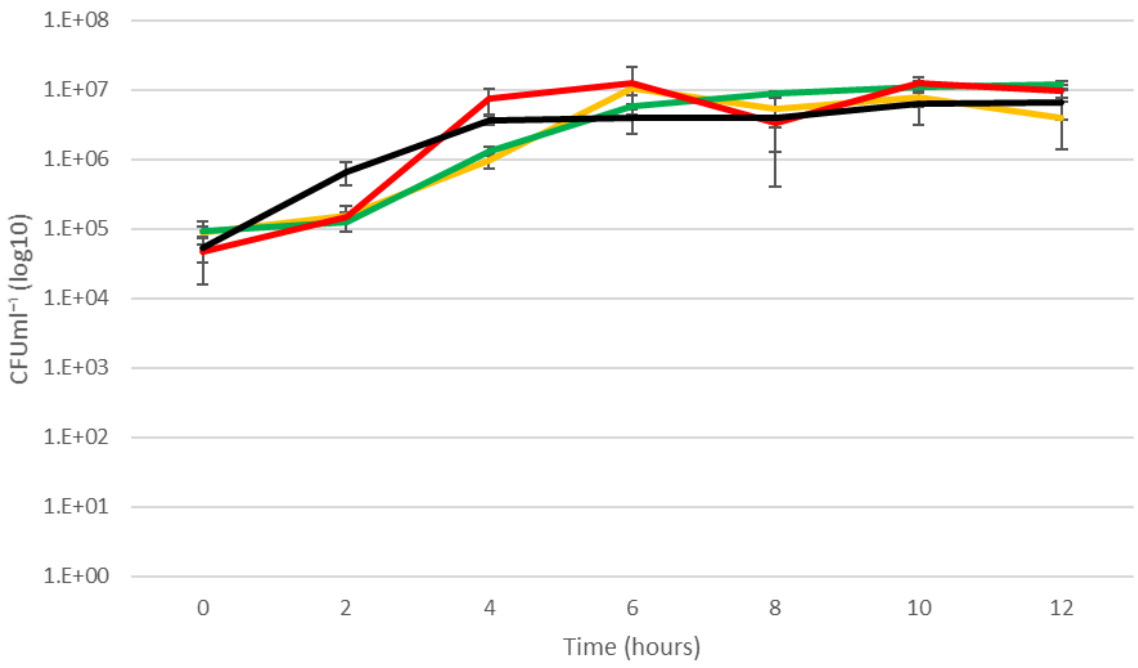


Figure 6: Viable cell counts present in M9 minimal media supplemented with 0.4% succinic acid over 12 hours. ─ WT (naïve), ─ Phage 2 Lysogen, ─ Phage 3 Lysogen, ─ Phage 4 Lysogen**,** ─ Negative control. Each data point shows the mean of 3 technical repeats and the error bars represent the standard deviation.

3.3 Active LES phage production during lysogen growth

Since the LES prophages are known to be active, the growth profiles of the LES lysogens are likely to be affected by a proportion of the bacterial population undergoing phage mediated lysis. To assess this, plaque assays were performed on filtered supernatants from growth profiling experiments to quantify the level of phage production every hour.

3.3.1 Bacteriophage production in rich LB media

It was speculated that spontaneous induction of free LES phages could affect viable cell count measurements. Free phage densities were thus measured at each time point using a spot plaque assay (Figure 7). All phage densities were high at time 0 h ($2.54x10^5$– $2.59x10^6$ $PFUml^{-1}$) due to carry over from overnight cultures. The densities increased with similar trends over the 12 h period reaching between $3.04x10^7$ and $4.73x10^8$ $PFUml^{-1}$. The LES phage 3 was produced in

the highest quantities in rich LB media, increasing rapidly after 4 h of growth (Figure 7). Production of the LES phages 2 and 4 both remained at lower densities compared to the LES phage 3. Mann Whitney U tests were used to compare densities of phage 3 lysogens to each of the other lysogens at every time point, all suggesting significant difference ($P<0.05$).

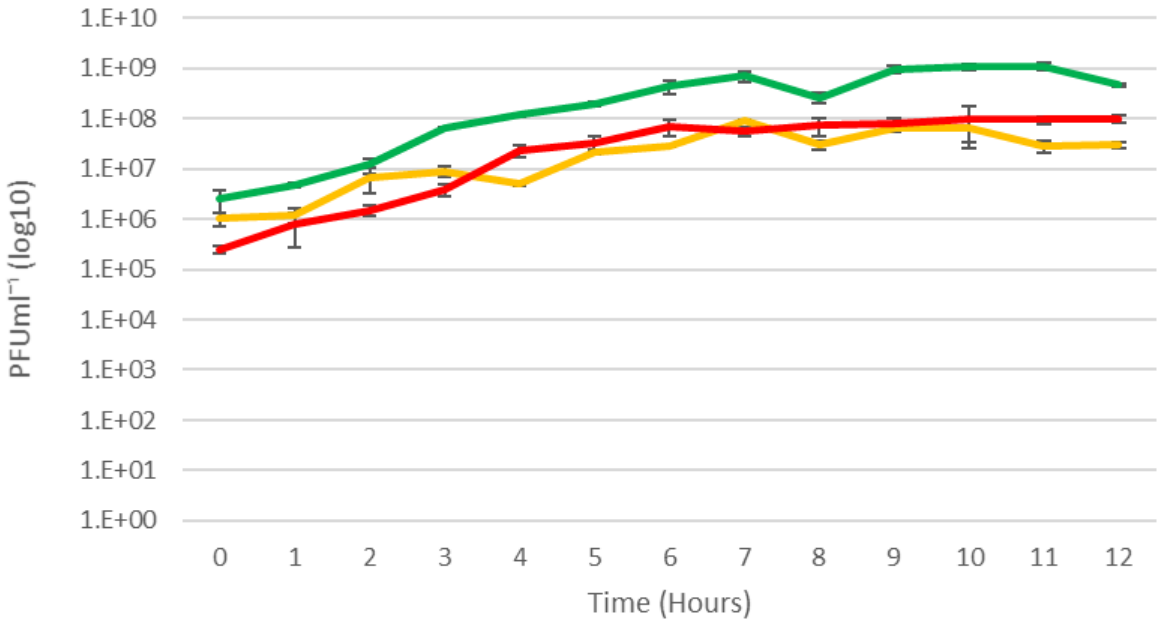


Figure 7: Phage production of lysogen 2, 3 and 4 grown in LB medium for 12 hours. — Phage 2 Lysogen, — Phage 3 Lysogen, — Phage 4 Lysogen**,** — Negative control. Each data point shows the mean of 3 technical repeats and the error bars represent the standard deviation.

3.3.2 Bacteriophage production in minimal M9 media

Phage production in M9+S was detected at a lower level than when the LES lysogens were grown in LB media (Figure 8). This could be due to the host cells growing at a slower rate but may also indicate reduced rates of spontaneous induction. The lysogen containing the LES phage 3 produced the highest proportion of active phage for the majority of the experiment, maintaining this until 9h where production of the LES phage 2 overtook it. The LES phage 2 and 3 followed a similar production pattern, mirroring each other's peaks and troughs. No significant differences were detected (Mann Whitney U, $P=0.608$). They both reached their highest phage concentration at 10 h, 3.2×10^7 PFU ml^{-1} and 2.9×10^7 PFU ml^{-1} respectively. The

LES phage 4 also reached its highest concentration of $4.1x10^6$ PFU ml^{-1} at 10 h although its production was much lower throughout.

The LES phage 2 was the only lysogen to exhibit an increase in phage production when grown in M9+S media as opposed to in LB although this difference was not significant. A maximum density of 3.2×10^7 PFU ml^{-1} was reached when grown in M9+S as opposed to 9.3×10^6 PFU ml^{-1} when grown in LB media (Mann-Whitney U, P=0.09). The other two lysogens had a markedly higher phage production rate when grown in LB media, but again the difference was not significant (Mann Whitney U, P >0.05).

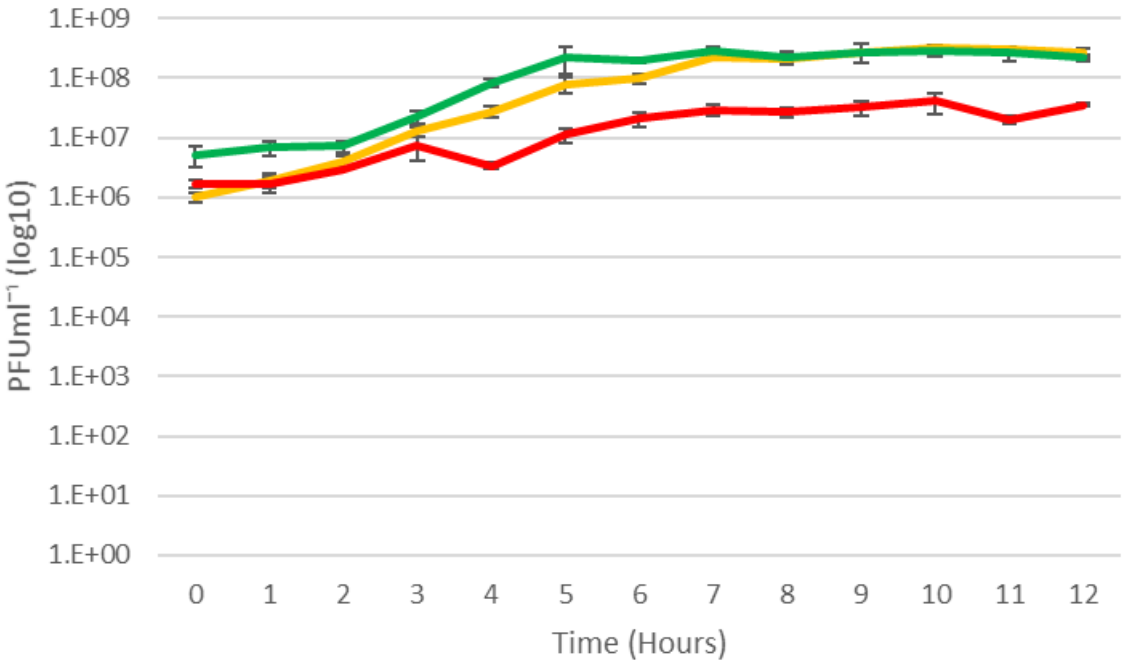


Figure 8: Phage production of lysogen 2, 3 and 4 grown in M9 minimal media supplemented with 0.4% succinic acid for 12 hours. — Phage 2 Lysogen, — Phage 3 Lysogen, — Phage 4 Lysogen**,** — Negative control. Each data point shows the mean of 3 technical repeats and the error bars represent the standard deviation.

3.4 PCR confirmation of phage 2, 3 and 4

After optimizing the PCR reaction mix and cycling times it was confirmed that the phage filtrates obtained during the growth profiling assays were pure and contained only the expected

bacteriophages in each case. A 235bp product was amplified from LES phage 2 samples and products of 785bp and 435bp were amplified from LES phage 3 and LES phage 4 samples respectively.

3.5 Metabolic activity

Resazurin, 7-Hydroxy-3H-phenoxazin-3-one 10-oxide, is used to determine the metabolic rate of cultures due to it being broken down into resorufin by cells and this being directly proportional to the number of metabolically active cells present (Lescat et al., 2019). The desired outcome of this protocol was to determine whether the LES prophages affected the metabolic rate of the bacterium or whether the lytic cycle was contributing to the turbidity of the sample but culling viable cells.

The WT strain appeared to have the highest metabolic activity throughout the assay, initially fluorescing at an absorbance of 218538.5 at T=0 and remaining fairly constant apart from a dip in fluorescence at 3h (136199.2) and ending the assay at a fluorescence of 212415.8 (Figure 9).

The activity of the three LES lysogens all increased slightly during the assay but remained at a considerably lower fluorescence (ranging from 186.69 to 65702.03) than the WT strain. Mann Whitney U tests were performed to compare the fluorescence of each lysogen to WT at each timepoint, all P values <0.05. The LES phage 4 lysogen started at the lowest fluorescence (186.7) and was metabolizing at the highest rate observed for the LES lysogens at 11h (65702.03).

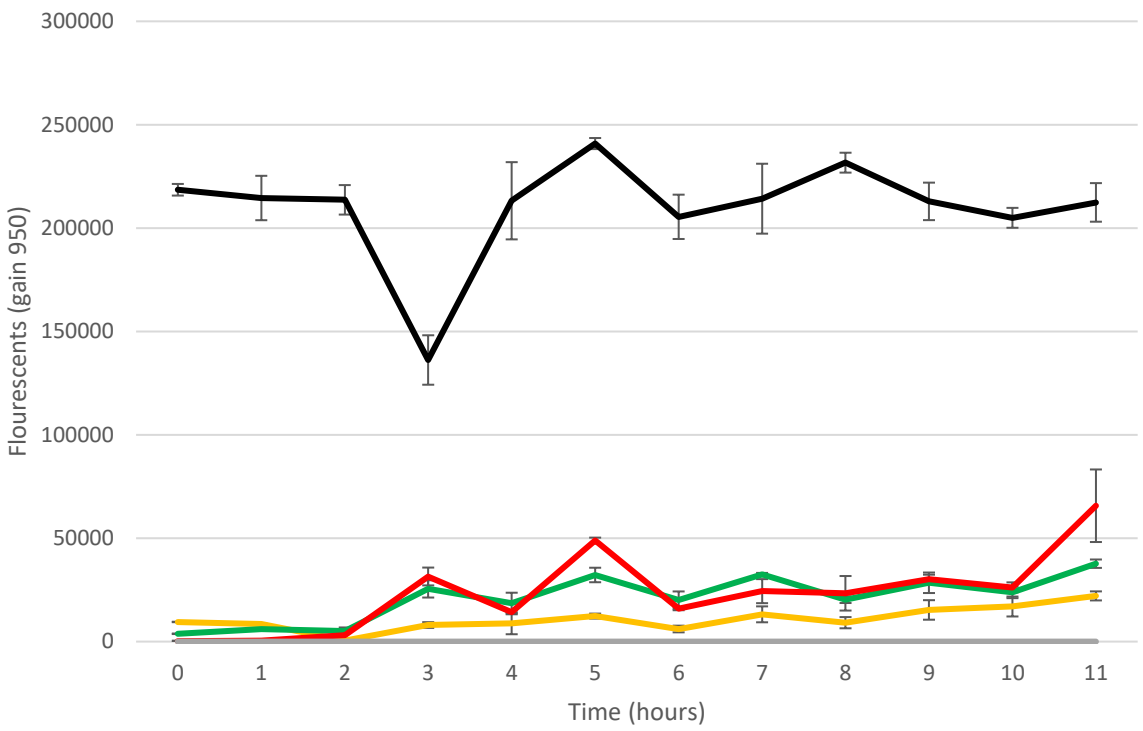


Figure 9: Metabolic activity of WT and LES lysogens in LB media determined by the fluorescents of Resazurin. ─ WT (naïve), ─ Phage 2 Lysogen, ─ Phage 3 Lysogen, ─ Phage 4 Lysogen, ─ Negative control. Each data point shows the mean of 3 technical repeats and the error bars represent the standard deviation.

3.6 Effect of LES prophage carriage on biofilm density

Increased biofilm formation is known to be one of the adaptations to the CF lung that makes the LES strain so difficult to eradicate (Davies et al., 2016b).

When comparing biofilm production, all three LES lysogens formed denser biofilms than the naïve WT in rich LB media after 18 h (Figure 10). Conversely, the WT biofilms reached a higher density than single lysogens carrying phage 3 (Mann Whitney U, P=0.0086) and phage 4 (Mann Whitney U, P=0.0379) in M9+S media. However, lysogens carrying prophage 2 formed by far the thickest biofilms in both LB and M9+S media compared to WT, achieving an OD_{570} of 0.7 in M9+S compared to 0.09 by the WT (Mann Whitney U, P=0.0002) and one of 0.59 compared to 0.05 by WT in LB (Mann Whitney U, P=0.0022).

There was a clear advantage to carrying phage 2 for biofilm formation in both media.

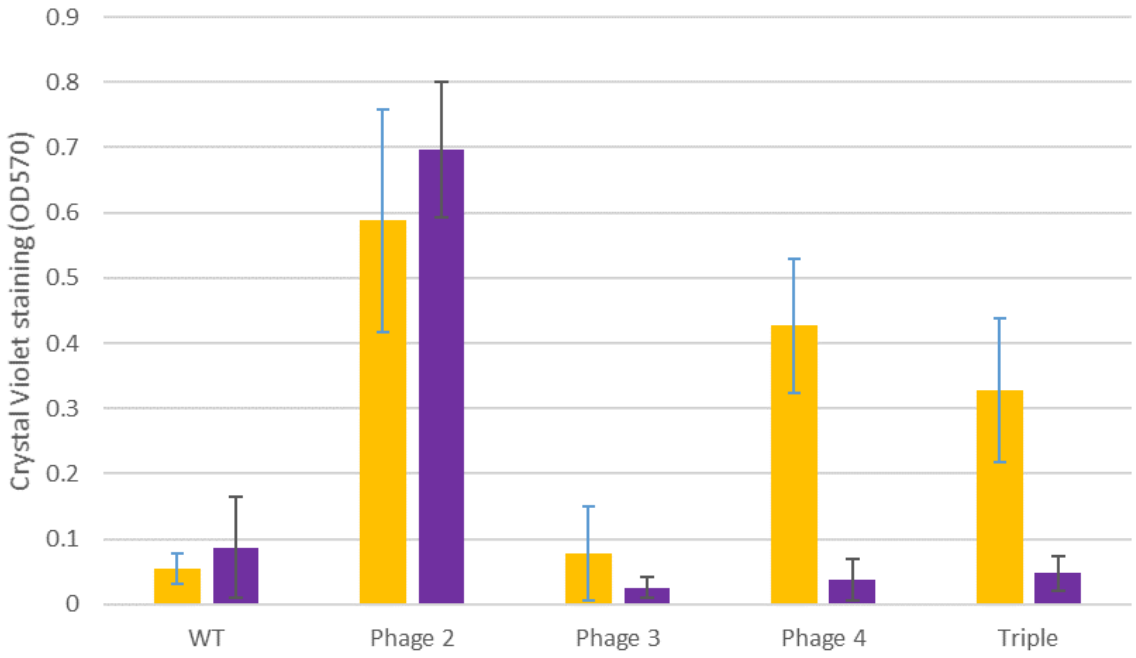


Figure 10: Effect of LES Prophage Carriage on Biofilm Density: Biofilm formation in • LB and • M9+S over 18 h. Each data point shows the mean of six replicates and standard deviation. Mann Whitney U tests were performed to establish the significance of WT biofilm forming capacities compared to the lysogens.

3.7 Effect of prophage carriage on motility of PAO1

Motility of the host bacterium is known to be affected in other relationships between bacteriophage and their hosts (Davies et al., 2016c). The LES strain has been observed to lack type-IV pilus and flagella expression, adopting a more sedentary phenotype and this could have been influenced by the prophages carried in its genome (Davies et al., 2016c, Davies et al., 2016a). Each of the LES lysogens were plated out onto specific agar designed to measure swimming, swarming and twitching motility. These were compared to the growth of the naïve WT grown on the same agars.

Each of the LES lysogens and the naïve WT PAO1 exhibited all three types of motilities (figure 11). A growth diameter between 5 cm and 7 cm was observed on the swimming specific agar although the naïve WT travelled the shortest distance (mean diameter 5cm). The lysogen carrying the LES prophage 2 and the triple lysogens both travelled 6 cm (± 0.01 cm) on the

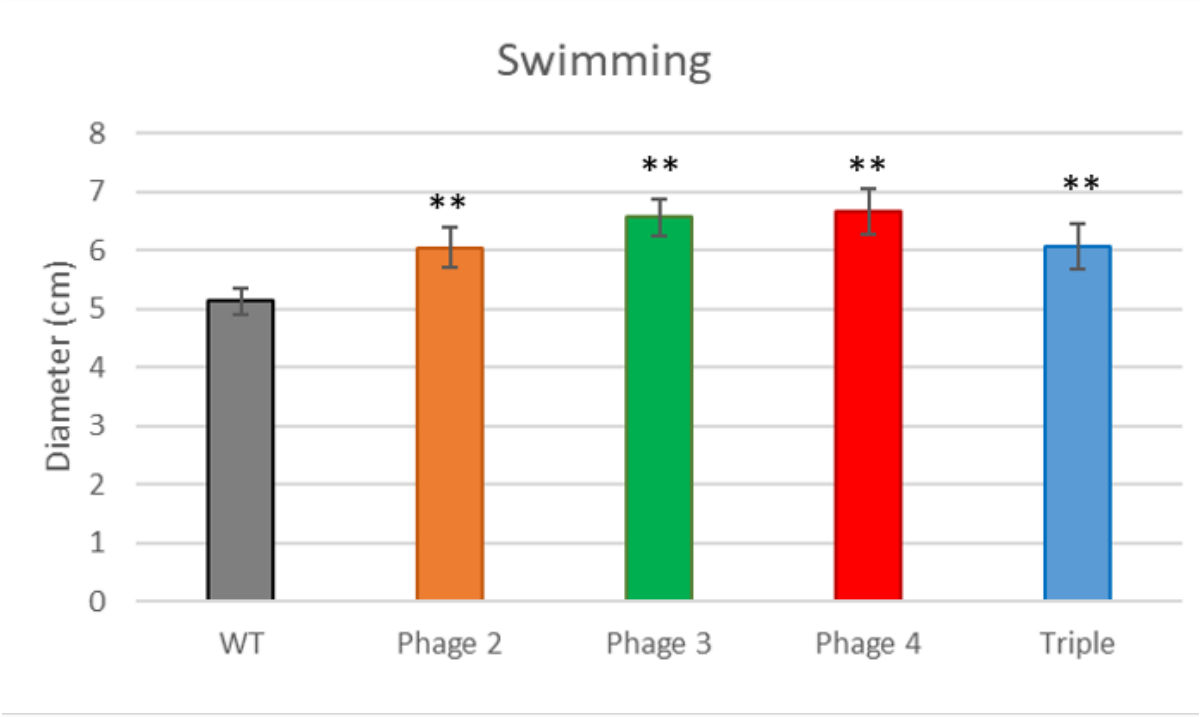
swimming agar. Single lysogens carrying the LES prophage 3 or LES prophage 4 had similar swimming diameters, traveling 6.57 cm and 6.67 cm respectively. A two sample T-test was performed to compare the motility of each lysogen to the naïve WT. All returned a P-value of 0.01 or less confirming that the motility of lysogens were significantly greater than the naïve PAO1 WT.

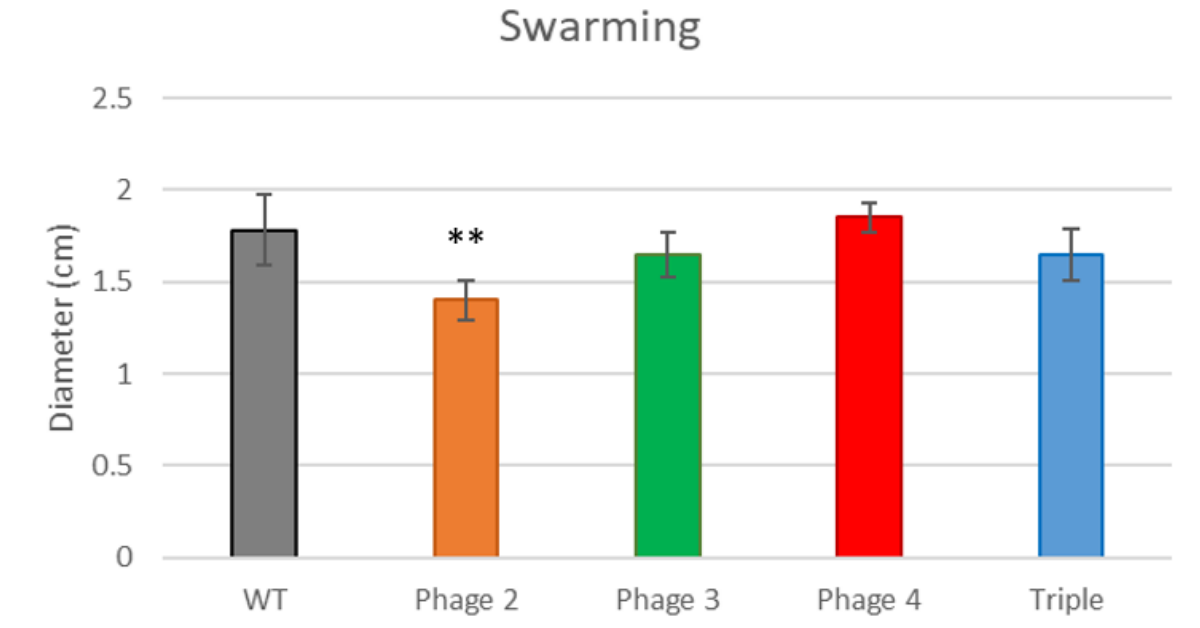
Both swarming and twitching motility by WT and the LES lysogen variants were less prominent than swimming motility. The LES lysogen carrying prophage 2 showed the smallest mean diameter on the swarming agar (1.4 cm) and both of the LES lysogens carrying prophage 3 and the triple lysogens exhibited the same level of swarming motility (mean diameter 1.65 cm ± 0.15 cm and 0.25 cm respectively). The LES lysogens carrying the prophage 4 swarmed the farthest distance (mean diameter 1.85 cm ±0.08 cm), only slightly more than the naïve WT (mean diameter 1.78 cm ± 0.19 cm). Thus, prophage carriage did not appear to have a major effect on swarming motility, as the LES lysogens and WT travelled within 0.5 cm of each other. However, a 2-sample t-tests suggested that the small reduction in swarming by lysogens carrying the LES prophage 2 compared to WT was significant (P = 0.004). Small differences between WT and lysogens carrying the LES prophage 3, prophage 4, and the triple lysogen were not significant (P = 0.193, 0.469, and 0.203 respectively).

The strains grown on the twitching agar travelled the shortest distance. The single LES lysogens carrying prophage 3 and 4 produced the smallest diameters, measuring 0.92 cm ±0.32 and 0.98cm ± 0.37 respectively. The naïve WT and the triple LES lysogen produced very similar diameters, 1.05cm ±0.3 and 1.07cm ±0.4 respectively. A 2-sample T-Test revealed that these differences in motility were not significant, comparing the naïve WT to lysogens carrying LES prophage 3 (P = 0.086), LES prophage 4 (P = 0.694) and all 3 LES prophages (P = 0.864). Single lysogens carrying the LES prophage 2 travelled significantly further on the twitching agar (mean diameter 1.25cm± 0.05, P = 0.011).

In summary, the LES prophage carriage may affect swimming motility, but the LES prophages 3 and 4 did not appear to significantly affect swarming or twitching motility (P>0.05). Though the LES lysogens carrying prophage 2 showed small but significant decreases in swarming, and

increases in twitching motility, these traits were not preserved in the LES triple lysogen. These data suggest either that the LES prophages 3 and 4 mask the effects of the LES prophage 2, or that the single LES lysogen carrying prophage 2 harboured other mutations to account for differences in phenotype.





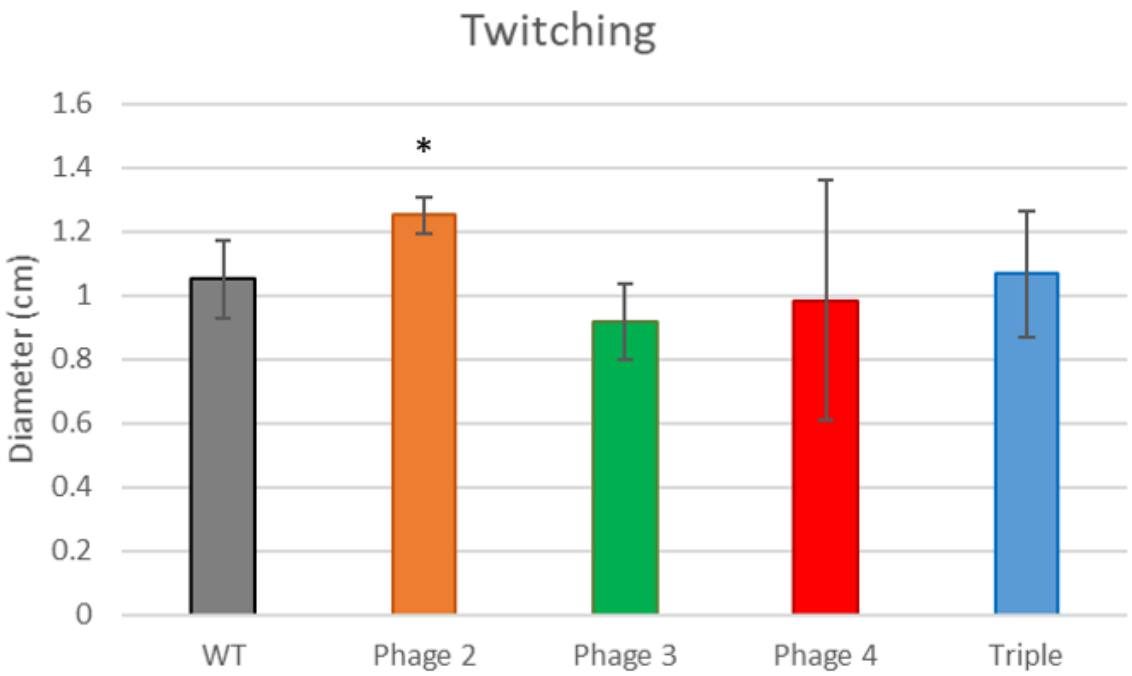


Figure 11: Swimming, swarming and twitching motility of WT PAO1 vs lysogens. Using LB agar (twitching) and specific swimming and swarming agar, each data point shows the mean of 6 repeats and standard deviation. Motility of lysogens was compared to WT using the student t-test. Significant differences are indicated with * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.0001$).

3.8 Effect of LES prophage carriage on bacterial virulence *in vivo*

It is known that the presence of prophages in the bacterial host genome can affect virulence (Davies et al., 2016b). The ability of the host bacterium to in turn kill its host was explored using the model organism *Galleria mellonella*. Each lysogen was grown to mid-exponential phase and adjusted to an OD_{600} of 0.01 before inoculation of *G. mellonella* larvae. Survival of the larvae (16 per group) was monitored for a period of 24 h. On death (indicated by no response to touching, and black colouration) larvae were frozen and later homogenised using a tissue processor to assess the bacterial load.

3.8.1 Prophage carriage reduces rate of killing of *G. mellonella.*

The first *G. mellonella* larvae deaths were observed 10 h post inoculation for all WT and LES lysogen treatments (figure 12). The naïve WT clearly exhibited the most virulence, causing the death of 38% of the *G. mellonella* larvae within 10 h and 100% in each of the repeats (n = 16) within 15 h. Lysogen carriage appeared to reduce virulence in all cases (Log-rank test, P<0.0001).

The lysogen carrying the LES prophage 2 was observed to be the least virulent overall, killing between 1 and 2 larvae every hour over a 9 h period and taking 19.5 h to kill all 16 larvae.

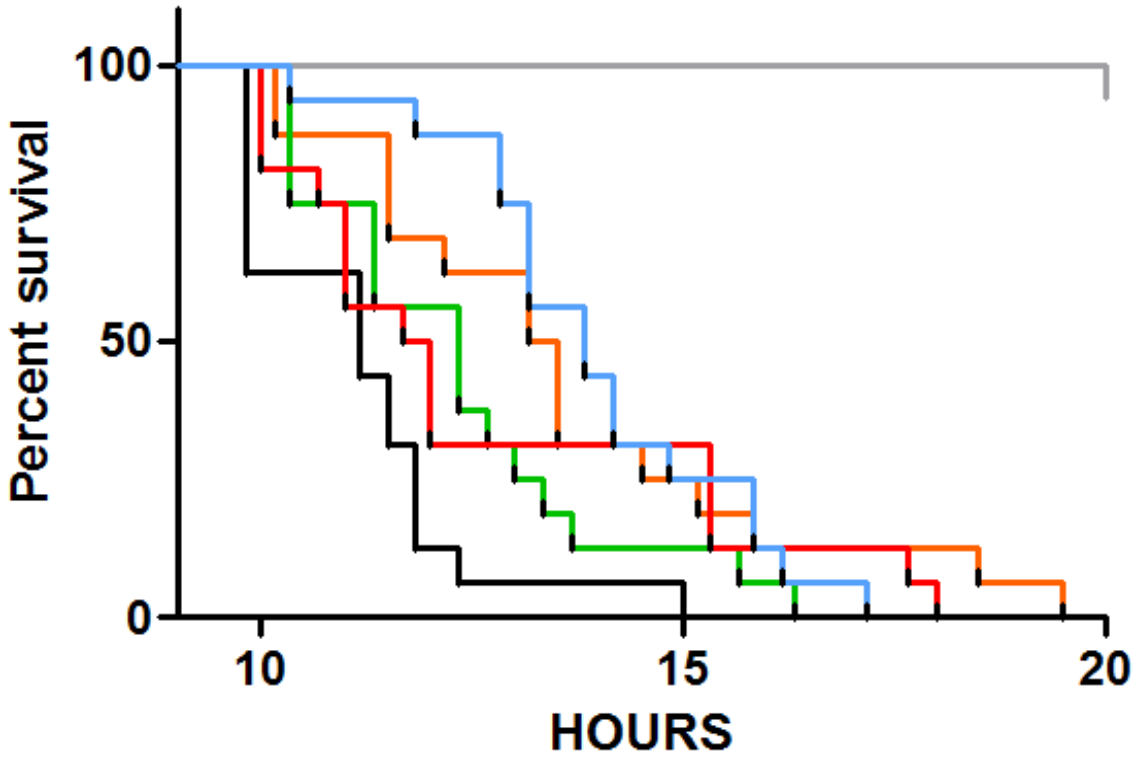


Figure 12: Time taken for WT PAO1 vs lysogens to kill *Galleria mellonella.* Data points show mean of 3 repeats, each containing 12 larvae, to die over 20 hours. ─ WT (naïve), ─ Phage 2 Lysogen, ─ Phage 3 Lysogen, ─ Phage 4 Lysogen, ─ Triple lysogen**,** ─ Negative control. A log-rank (Mantel-cox) test was performed on the results to determine their significance.

3.8.2 Prophage carriage promotes bacterial survival of *G. mellonella*

At the end of the *in vivo* experiment, the bacterial load was quantified from each frozen *G. mellonella* larva (figure 13). Interestingly, the lowest bacterial loads (ranging 2.4 x 10^6 - 2.8 x 10^7 CFU/ larva) were recovered from the WT treated group, suggesting that killing was not a direct function of bacterial load *in vivo*.

Final bacterial loads of each lysogen were compared to WT using the Mann Whitney U test. Bacterial cell numbers recovered from larvae treated with the LES prophage 2 and triple lysogens were slightly, but not significantly higher than those from WT treated larvae (mean 2x10^6 CFU, P 0.146 and 4x10^7CFU, P 0.207 respectively). Conversely, significantly higher bacterial loads were recovered from larvae that were treated with lysogens carrying the LES prophage 3 (mean 1x10^8 CFU) and prophage 4 (mean 7x10^7CFU) compared to WT (P <0.0001). Bacterial loads from larvae treated with cultures carrying the LES prophage 3, 4, or the combination of all 3 were quite variable, with significant outlying data points when individual larvae were found to harbour much higher bacterial loads, up to 3 x 10^8 CFU (figure 13).

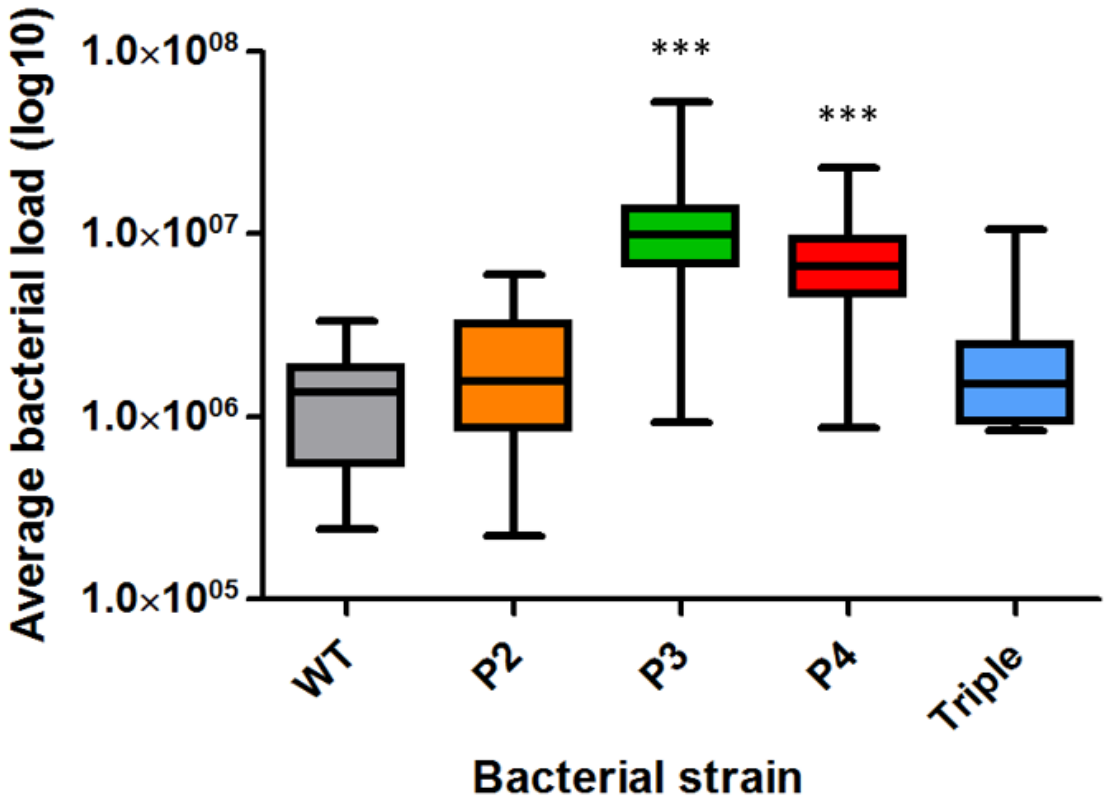


Figure 13: Bacterial load of PAO1 WT and LES lysogens in killed *Galleria mellonella.* The mean bacterial load found in *G. mellonella* after they had died from the infection. Each data point shows the mean of three repeats (mean, median and top and bottom quartile in the box and whisker plot). Bacterial loads from larvae treated with each lysogen were compared to those treated with WT using a Mann-Whitney test. Significant differences are indicated with * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.0001$).

Together, the data displayed in figures 10 and 11 suggest that PAO1 lysogens carrying the LES prophages exhibit reduced virulence and increased survival rates *in vivo*. Overall, the findings of this investigation suggest that despite decreased growth in minimal media, and little effect on either biofilm formation or motility *in vitro*, prophage carriage promotes decreased virulence and increased survival *in vivo.* An exception was observed in the case of biofilm formation by single lysogens carrying the LES prophage 2, that warrants further investigation.

4.Discussion

The most striking findings of this project were the effects that prophage carriage had on bacterial growth under different nutrient conditions. The growth profiler data highlighted the advantage given to lysogens carrying the LES prophages 3 and 4 compared to the naïve WT strain and the lysogen carrying the LES prophage 2 when grown in rich LB media. There was also a distinct advantage to PAO1 when carrying all three prophages, exhibiting a higher growth rate than any of the single lysogens, suggesting cooperation between the cohabiting bacteriophages. Cooperation between phages and their hosts has been observed in many species of bacteria, including *P. aeruginosa* (Borges et al., 2018, O'Brien et al., 2019). This has been shown to affect a range of traits besides growth rate, including virulence factors and immune evasion (Hargreaves et al., 2014a, O'Brien et al., 2019, James et al., 2015).

When carried alone, the LES phage 2 hindered growth considerably in both rich LB media and in minimal M9+S media. Some growth inhibition could be expected due to spontaneous induction of the lytic cycle. However, this assumption was not backed up by the phage production measurements as lysogens containing LES phage 2 did not produce the highest proportion of free phage particles over the 12 h growth curve assay. In fact, phage 2 was produced at the lowest level in LB media, contrary to reports of phage 2 being detected at the highest density in CF sputa (James et al., 2015). Furthermore, bacterial density has previously been demonstrated to remain high despite considerable levels of spontaneous LES phage induction *in vitro* (Davies et al., 2016b) and in the CF lung (James et al., 2015).

Since the completion of lab work on this project, the whole genome sequence of each PAO1 LES phage lysogen has been determined. These data revealed a substantial inversion mutation in the single LES prophage 2 lysogen that inverted over half of the bacterial chromosome and removed a whole ribosomal cluster (Personal communication, Dr Enrique Gonzalez Tortuero, 2022). It is most likely that such a large deletion in the bacterial chromosome is responsible for the reduced growth of the LES phage 2 lysogen. Fresh LES phage 2 lysogens have since been created with no inversion, confirmed by whole genome sequencing (Revathy Krishnamuthy, 2022), but further phenotypic profiling has not yet been performed.

This means that the direct effect of prophage 2 cannot be determined from the data presented in this dissertation. However, *P. aeruginosa* is notorious for its capacity to acquire mutations and diversify in the CF lung, developing highly heterologous populations during chronic infection enabling adaptation to different micro-niches and resilience during intensive antibiotic treatment (Cabot et al., 2016). Others have shown that prophage integration can cause dramatic changes to the bacterial host genome in nature and that the phenomenon is not un-common (Davies et al., 2016c). Such events that are not fatal to the bacterial host have the potential to trigger shifts in bacterial evolution and adaptation. It is thus of interest to compare the fitness and virulence traits of the LES prophage 2 mutant variant to both WT and other PAO1 lysogens.

The idea of phage cooperation would serve as a potential explanation as to why the triple LES lysogen exhibited a higher growth rate than any of the single lysogens in minimal M9+S media (Azulay et al., 2022). The use of succinic acid was not the original proposition but once it was found the strains would not grow in M9 minimal media supplemented with 0.2% glucose as the literature had originally suggested, the assay was revaluated, and 0.4% succinic acid was used as a substitute. This was due to the success had by Paliy and Gunasekera (2007), among others, in growing *Escherichia coli* in M9 media supplemented with succinic acid (Paliy and Gunasekera, 2007). Although the growth rate was lower in M9+S than in LB media, the carriage of the LES phage seemed to be more of a hinderance as all the lysogens exhibited a lower growth rate than that of the naïve WT. This could be explained by the lack of nutrients triggering the lytic cycle, culling high numbers of their hosts. This is known to happen in other bacterial strains, including *P. aeruginosa*, and serves to produce as many progenies as possible under stressful conditions (Argov et al., 2019). Conversely, there is also evidence of the lytic cycle being more common in nutrient rich conditions when there are plentiful susceptible hosts and resources (McDaniel and Paul, 2005). The theory is that when susceptible hosts are plentiful the phage increase their numbers and in nutrient poor conditions they conserve, or "farm", their hosts. This involves the phages slowing the growth rate of their hosts and working to conserve nutrients and so aid survival (McDaniel and Paul, 2005).

Another well-established method to aid survival is the formation of biofilms, which is especially prevalent in the cystic fibrosis lung (Strempel et al., 2013). The carriage of the LES prophages had a clear advantage over the naïve WT in the formation of biofilms when grown in LB media. This has been demonstrated in the literature multiple times, with a variety of bacterial species such as *Escherichia coli* (Hargreaves et al., 2014a) and the *P. aeruginosa* LES strain forming robust biofilms, providing LES with an advantage over other strains in the CF lung (Davies et al., 2016b). This, however, was not reflected when the lysogens were grown in M9+S media. This was unexpected as it was thought that the reduction in nutrients was one of the driving factors in the LES forming hard to remove biofilms and only the LES phage 2 variant lysogen exhibited more robust biofilms than the naïve WT in M9+S (Hargreaves et al., 2014a). This could be due to the inversion mutation detected in the LES phage 2 genome. Others have shown that *Campylobacter jejuni* can form more robust biofilms when an rRNA mutation occurs (Sałamaszyńska-Guz et al., 2017). As the increase in biofilm formation was so stark when LES phage 2 was carried, and there was no other noticeable advantage to carrying any of the other LES prophages, alone or together, the mutation is the most likely explanation.

LES phages have previously been suggested to influence loss of motility by the LES (Davies et al., 2016a). However, direct effects of the LES prophage carriage on swimming, swarming and twitching motility in PAO1 were not evident in this study. A significant difference in swimming and twitching was observed between the WT and the LES phage 2 lysogen, suggesting that the LES phage 2 lysogen variant travelled significantly further, directly contradicting Davies et al. (2016a) who suggested that LES phages caused a reduction in motility. This difference is likely due to the inversion mutation and deletion of the rRNA cluster rather than prophage carriage. It would be interesting to determine whether such a mutated variant, with increased swimming and twitching motility would out-compete the rest of the population *in vivo*.

The *G. mellonella* infection model is an established way to investigate the virulence of a bacterial strain in an organism with a rudimentary immune system (Ménard et al., 2021). Initial observations suggested that the naïve WT was more virulent than the LES lysogens, being the first and the fastest to cause *G. mellonella* larvae death. The remaining larvae, inoculated with

each of the four LES lysogen variants, began to die around the same time as those inoculated with the WT but then took more time to reach lethal levels in all replicates. These findings are corroborated by previous research investigating the virulence of the LES stain, supporting findings that the carriage of LES prophages reduces virulence (Winstanley et al., 2009). This is thought to aid evasion of the immune system when infecting the CF lung and so makes the strain harder to detect and eradicate. Lysogens carrying LES prophage 3 reached the highest density *in vivo* and produced the highest proportion of free phage *in vitro,* but didn't kill the larvae as fast as the WT. This is also true of the other lysogens as the LES phage 4 reached the second highest density before killing the larvae and was only more virulent than the variant PAO1 lysogen carrying LES prophage 2.

Final bacterial density did not correlate with *G. mellonella* survival rates, indicating that delayed killing was due to a reduction in virulence as opposed to a reduction in growth rate. The variant lysogen carrying LES prophage 2 was the least virulent strain *in vivo* but reached higher viable cell densities than the WT before larval death, which had higher growth rates *in vitro*. This suggests a reduction in virulence of the mutated prophage 2 lysogen as well as slower growth. Phage production *in vivo* was not measured, so lower bacterial densities cannot be directly attributed to the lysing effect of the phage, but previous experiments have suggested LES phage 3 to be more abundantly produced than LES phage 2 *in vitro*, supporting the hypothesis that reduced cell numbers of the variant were due to a combination of slower growth and reduced virulence *in vivo* rather than increased lysis. The most likely explanation for this reduction in virulence is the inversion mutation found by Enrique Gonzalez-Tortuero (personal communication, 2022). This inversion was found to cause a deletion of a large number of genes including those encoding type II secretion systems, which are key mediators of virulence in *P. aeruginosa* (Korotkov et al., 2012).

The starkest findings of this project are thought to be predominantly due to the mutation found in the LES phage 2 lysogen. Direct comparison of WT with the newly created LES phage 2 lysogen that does not include the inversion and rRNA cluster deletion is required to gain better understanding of the effects of prophage 2. However, it would be interesting to determine whether prophage 2 could drive such mutations *in vivo* and perform long term co-

evolution experiments to determine whether such drastic mutants could represent phage-driven adaptations to the CF Lung (Davies et al., 2016b).

This report presents evidence that suggests a role for LES prophages in reducing the virulence of *P. aeruginosa in vivo*. However, the assays are limited in that they do not accurately represent the conditions found in the CF lung. In order to explore the reduced virulence hypothesis more thoroughly, use of antibiotics and artificial sputum media (Kirchner et al., 2012) would create a closer simulation of the CF lung environment. It is well known that the antibiotics given to CF patients influences their lung microbiota and is a contributing factor to the development of antibiotic resistant strains (James et al., 2015). DNA synthesis inhibiting antibiotics such as ciprofloxacin also act as inducers of the LES phage lytic cycle (Fothergill et al., 2011) so could fundamentally affect the relationship between phage and bacterial host and alter the dynamics of lysogen survival, growth, and virulence. The environment created by the build-up of mucus in the lungs is also known to concentrate phage densities (Almeida et al., 2019) as well as promote bacterial colonisation and resistance development (Davies et al., 2016a). The formation of biofilms is another important virulence factor that aids in the LES survival (Høiby et al., 2010). The formation of more robust biofilms by lysogens could impact the efficacy and delivery of treatments. Further investigation is needed to determine how prophages enhance biofilm formation. Live dead fluorescence staining along with exopolysaccharide staining such as that described in Jin et al. (2005) could be used to compare WT and lysogen biofilm structure and whether active phage lysis plays a role in promoting increased density, which has been shown for other phages such as pf1-like phages (Ismail et al., 2021). Exploring these factors would give further insight into how the LES is so successful in the CF lung and inform new ways to curb it.

References

2022. *Quest Calculate™ M9 Minimal Salts Preparation and Recipe.* [Online]. AAT Bioquest, Inc. Available: https://www.aatbio.com/resources/buffer-preparations-and-recipes/m9-salts [Accessed 2019].

AARON, S. D., VANDEMHEEN, K. L., RAMOTAR, K., GIESBRECHT-LEWIS, T., TULLIS, E., FREITAG, A., PATERSON, N., JACKSON, M., LOUGHEED, M. D., DOWSON, C., KUMAR, V., FERRIS, W., CHAN, F., DOUCETTE, S. & FERGUSSON, D. 2010. Infection With Transmissible Strains of Pseudomonas aeruginosa and Clinical Outcomes in Adults With Cystic Fibrosis. 304**,** 2145.

ACKERMANN, H. W. 2003. Bacteriophage observations and evolution. *Res Microbiol,* 154**,** 245-51.

ALISKY, J., ICZKOWSKI, K., RAPOPORT, A. & TROITSKY, N. 1998. Bacteriophages show promise as antimicrobial agents. *J Infect,* 36**,** 5-15.

ALMEIDA, G. M. F., LAANTO, E., ASHRAFI, R. & SUNDBERG, L.-R. 2019. Bacteriophage Adherence to Mucus Mediates Preventive Protection against Pathogenic Bacteria. *mBio,* 10**,** e01984-19.

ARGOV, T., SAPIR, S. R., PASECHNEK, A., AZULAY, G., STADNYUK, O., RABINOVICH, L., SIGAL, N., BOROVOK, I. & HERSKOVITS, A. A. 2019. Coordination of cohabiting phage elements supports bacteria-phage cooperation. *Nat Commun,* 10**,** 5288.

ARMSTRONG, D., BELL, S., ROBINSON, M., BYE, P., ROSE, B., HARBOUR, C., LEE, C., SERVICE, H., NISSEN, M., SYRMIS, M. & WAINWRIGHT, C. 2003. Evidence for spread of a clonal strain of Pseudomonas aeruginosa among cystic fibrosis clinics. *J Clin Microbiol,* 41**,** 2266-7.

ASHISH, A., PATERSON, S., MOWAT, E., FOTHERGILL, J. L., WALSHAW, M. J. & WINSTANLEY, C. 2013. Extensive diversification is a common feature of Pseudomonas aeruginosa populations during respiratory infections in cystic fibrosis. *J Cyst Fibros,* 12**,** 790-3.

AZIZ, R. K., DWIVEDI, B., AKHTER, S., BREITBART, M. & EDWARDS, R. A. 2015. Multidimensional metrics for estimating phage abundance, distribution, gene density, and sequence coverage in metagenomes. *Frontiers in Microbiology,* 6.

AZULAY, G., PASECHNEK, A., STADNYUK, O., RAN-SAPIR, S., FLEISACHER, A. M., BOROVOK, I., SIGAL, N. & HERSKOVITS, A. A. 2022. A dual-function phage regulator controls the response of cohabiting phage elements via regulation of the bacterial SOS response. *Cell Reports,* 39**,** 110723.

BORGES, A. L., ZHANG, J. Y., ROLLINS, M. F., OSUNA, B. A., WIEDENHEFT, B. & BONDY-DENOMY, J. 2018. Bacteriophage Cooperation Suppresses CRISPR-Cas3 and Cas9 Immunity. *Cell,* 174**,** 917-925.e10.

BRAGG, R. R., BOUCHER, C. E., VAN DER WESTHUIZEN, W. A., LEE, J. Y., COETSEE, E., THERON, C. & MEYBURGH, L. 2016. The Potential Use of Bacteriophage Therapy as a Treatment Option in a Post-Antibiotic Era. *Antibiotic Resistance.*

CABOT, G., ZAMORANO, L., MOYÀ, B., JUAN, C., NAVAS, A., BLÁZQUEZ, J. & OLIVER, A. 2016. Evolution of Pseudomonas aeruginosa Antimicrobial Resistance and Fitness under Low and High Mutation Rates. *Antimicrobial Agents and Chemotherapy,* 60**,** 1767-1778.

CAMPBELL, P. L., DUDA, R. L., NASSUR, J., CONWAY, J. F. & HUET, A. 2020. Mobile Loops and Electrostatic Interactions Maintain the Flexible Tail Tube of Bacteriophage Lambda. *Journal of Molecular Biology,* 432**,** 384-395.

CEYSSENS, P.-J. & LAVIGNE, R. 2010. Bacteriophages of Pseudomonas. *Future Microbiology,* 5**,** 1041-55.

CHENG, K., SMYTH, R. L., GOVAN, J. R., DOHERTY, C., WINSTANLEY, C., DENNING, N., HEAF, D. P., VAN SAENE, H. & HART, C. A. 1996. Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic. *Lancet,* 348**,** 639-42.

COWLEY, N. L. 2016. *Realism-based Approaches for Evaluating Bacterial Susceptibility to Antimicrobials used in Home and Personal Care Products.* Doctor of Philosophy, Univeristy of Manchester.

CRESAWN, S. G., BOGEL, M., DAY, N., JACOBS-SERA, D., HENDRIX, R. W. & HATFULL, G. F. 2011. Phamerator: a bioinformatic tool for comparative bacteriophage genomics. *BMC Bioinformatics,* 12**,** 395.

DAVIES, E. V., JAMES, C. E., KUKAVICA-IBRULJ, I., LEVESQUE, R. C., BROCKHURST, M. A. & WINSTANLEY, C. 2016a. Temperate phages enhance pathogen fitness in chronic lung infection. *ISME J,* 10**,** 2553-5.

DAVIES, E. V., JAMES, C. E., WILLIAMS, D., O'BRIEN, S., FOTHERGILL, J. L., HALDENBY, S., PATERSON, S., WINSTANLEY, C. & BROCKHURST, M. A. 2016b. Temperate phages both mediate and drive adaptive evolution in pathogen biofilms. *Proc Natl Acad Sci U S A,* 113**,** 8266-71.

DAVIES, E. V., WINSTANLEY, C., FOTHERGILL, J. L. & JAMES, C. E. 2016c. The role of temperate bacteriophages in bacterial infection. *FEMS Microbiology Letters,* 363**,** fnw015.

DIGGLE, S. P., WINZER, K., CHHABRA, S. R., WORRALL, K. E., CÁMARA, M. & WILLIAMS, P. 2003. The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Molecular Microbiology,* 50**,** 29-43.

FEINER, R., ARGOV, T., RABINOVICH, L., SIGAL, N., BOROVOK, I. & HERSKOVITS, A. A. 2015. A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat Rev Microbiol,* 13**,** 641-650.

FILHO, S. 2013. Pseudomonas aeruginosa infection in patients with Cystic fibrosis: scientific evidence regarding clinical impact, diagnosis and treatment.

FOTHERGILL, J. L., MOWAT, E., WALSHAW, M. J., LEDSON, M. J., JAMES, C. E. & WINSTANLEY, C. 2011. Effect of antibiotic treatment on bacteriophage production by a cystic fibrosis epidemic strain of Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 55**,** 426-8.

FOTHERGILL, J. L., PANAGEA, S., HART, C. A., WALSHAW, M. J., PITT, T. L. & WINSTANLEY, C. 2007. Widespread pyocyanin over-production among isolates of a cystic fibrosis epidemic strain. *BMC Microbiology,* 7**,** 45.

HANCOCK, R. E. & SPEERT, D. P. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Drug Resist Updat,* 3**,** 247-255.

HARGREAVES, K. R., KROPINSKI, A. M. & CLOKIE, M. R. J. 2014a. Bacteriophage behavioral ecology: How phages alter their bacterial host's habits. *Bacteriophage,* 4**,** e29866.

HARGREAVES, K. R., KROPINSKI, A. M. & CLOKIE, M. R. J. 2014b. What Does the Talking?: Quorum Sensing Signalling Genes Discovered in a Bacteriophage Genome. *PLoS ONE,* 9**,** e85131.

HØIBY, N., BJARNSHOLT, T., GIVSKOV, M., MOLIN, S. & CIOFU, O. 2010. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents,* 35**,** 322-332.

HOLLOWAY, B. W. 1955. Genetic recombination in Pseudomonas aeruginosa. *Journal of general microbiology,* 13**,** 572-581.

IBBERSON, C. B. & WHITELEY, M. 2020. The social life of microbes in chronic infection. *Curr Opin Microbiol,* 53**,** 44-50.

ISMAIL, M. H., MICHIE, K. A., GOH, Y. F., NOORIAN, P., KJELLEBERG, S., DUGGIN, I. G., MCDOUGALD, D. & RICE, S. A. 2021. The Repressor C Protein, Pf4r, Controls Superinfection of Pseudomonas aeruginosa PAO1 by the Pf4 Filamentous Phage and Regulates Host Gene Expression. *Viruses,* 13**,** 1614.

JAMES, C. E. 2014. Recent Advances in Studies of Polymicrobial Interactions in Oral Biofilms. *Current Oral Health Reports,* 1**,** 59-69.

JAMES, C. E., DAVIES, E. V., FOTHERGILL, J. L., WALSHAW, M. J., BEALE, C. M., BROCKHURST, M. A. & WINSTANLEY, C. 2015. Lytic activity by temperate phages of Pseudomonas aeruginosa in long-term cystic fibrosis chronic lung infections. *ISME J,* 9**,** 1391-8.

JAMES, C. E., FOTHERGILL, J. L., KALWIJ, H., HALL, A. J., COTTELL, J., BROCKHURST, M. A. & WINSTANLEY, C. 2012. Differential infection properties of three inducible prophages from an epidemic strain of Pseudomonas aeruginosa. *BMC Microbiology,* 12**,** 216.

JIN, Y., ZHANG, T., SAMARANAYAKE, Y. H., FANG, H. H. P., YIP, H. K. & SAMARANAYAKE, L. P. 2005. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in Candida albicans biofilms. *Mycopathologia,* 159**,** 353-360.

JORTH, P., STAUDINGER, B. J., WU, X., HISERT, K. B., HAYDEN, H., GARUDATHRI, J., HARDING, C. L., RADEY, M. C., REZAYAT, A., BAUTISTA, G., BERRINGTON, W. R., GODDARD, A. F., ZHENG, C., ANGERMEYER, A., BRITTNACHER, M. J., KITZMAN, J., SHENDURE, J., FLIGNER, C. L., MITTLER, J., AITKEN, M. L., MANOIL, C., BRUCE, J. E., YAHR, T. L. & SINGH, P. K. 2015. Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs. *Cell Host Microbe,* 18**,** 307-19.

KIRCHNER, S., FOTHERGILL, J. L., WRIGHT, E. A., JAMES, C. E., MOWAT, E. & WINSTANLEY, C. 2012. Use of Artificial Sputum Medium to Test Antibiotic Efficacy Against Pseudomonas aeruginosa in Conditions More Relevant to the Cystic Fibrosis Lung. *Journal of Visualized Experiments*.

KORGAONKAR, A., TRIVEDI, U., RUMBAUGH, K. P. & WHITELEY, M. 2013. Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection. *Proc Natl Acad Sci U S A,* 110**,** 1059-64.

KOROTKOV, K. V., SANDKVIST, M. & HOL, W. G. J. 2012. The type II secretion system: biogenesis, molecular architecture and mechanism. *Nature Reviews Microbiology,* 10**,** 336-351.

LATIMER, J., FORBES, S. & MCBAIN, A. J. 2012. Attenuated virulence and biofilm formation in Staphylococcus aureus following sublethal exposure to triclosan. *Antimicrob Agents Chemother,* 56**,** 3092-100.

LEMIEUX, A.-A., JEUKENS, J., KUKAVICA-IBRULJ, I., FOTHERGILL, J. L., BOYLE, B., LAROCHE, J., TUCKER, N. P., WINSTANLEY, C. & LEVESQUE, R. C. 2015. Genes Required for Free Phage Production are Essential for Pseudomonas aeruginosa Chronic Lung Infections. *The Journal of Infectious Diseases,* 213**,** 395-402.

LESCAT, M., POIREL, L., TINGUELY, C. & NORDMANN, P. 2019. A Resazurin Reduction-Based Assay for Rapid Detection of Polymyxin Resistance in Acinetobacter baumannii and Pseudomonas aeruginosa. *Journal of Clinical Microbiology,* 57.

LIU, P. V. 1966. The Roles of Various Fractions of Pseudomonas Aeruginosa in Its Pathogenesis: II. Effects of Lecithinase and Protease. *Journal of Infectious Diseases,* 116**,** 112-116.

MAURICE, C. F., BOUVIER, C., DE WIT, R. & BOUVIER, T. 2013. Linking the lytic and lysogenic bacteriophage cycles to environmental conditions, host physiology and their variability in coastal lagoons. *Environ Microbiol,* 15**,** 2463-75.

MCCALLUM, S. J. 2002. Spread of an epidemic Pseudomonas aeruginosa strain from a patient with cystic fibrosis (CF) to non-CF relatives. *Thorax,* 57**,** 559-560.

MCCALLUM, S. J., CORKILL, J., GALLAGHER, M., LEDSON, M. J., ANTHONY HART, C. & WALSHAW, M. J. 2001. Superinfection with a transmissible strain of Pseudomonas aeruginosa in adults with cystic fibrosis chronically colonised by P aeruginosa. *The Lancet,* 358**,** 558-560.

MCDANIEL, L. & PAUL, J. H. 2005. Effect of Nutrient Addition and Environmental Factors on Prophage Induction in Natural Populations of Marine Synechococcus Species. *Applied and Environmental Microbiology,* 71**,** 842-850.

MELTZ STEINBERG, K. & LEVIN, B. R. 2007. Grazing protozoa and the evolution of the Escherichia coli O157:H7 Shiga toxin-encoding prophage. *Proceedings of the Royal Society B: Biological Sciences,* 274**,** 1921-1929.

MÉNARD, G., ROUILLON, A., CATTOIR, V. & DONNIO, P. Y. 2021. Galleria mellonella as a Suitable Model of Bacterial Infection: Past, Present and Future. *Front Cell Infect Microbiol,* 11**,** 782733.

MORIHARA, K., TSUZUKI, H., OKA, T., INOUE, H. & EBATA, M. 1965. PSEUDOMONAS AERUGINOSA ELASTASE. ISOLATION, CRYSTALLIZATION, AND PRELIMINARY CHARACTERIZATION. *J Biol Chem,* 240**,** 3295-304.

MORITA, Y., TOMIDA, J. & KAWAMURA, Y. 2014. Responses of Pseudomonas aeruginosa to antimicrobials. *Front Microbiol,* 4**,** 422.

MOWAT, E., PATERSON, S., FOTHERGILL, J. L., WRIGHT, E. A., LEDSON, M. J., WALSHAW, M. J., BROCKHURST, M. A. & WINSTANLEY, C. 2011. Pseudomonas aeruginosa population diversity and turnover in cystic fibrosis chronic infections. *Am J Respir Crit Care Med,* 183**,** 1674-9.

O'BRIEN, S., KÜMMERLI, R., PATERSON, S., WINSTANLEY, C. & BROCKHURST, M. A. 2019. Transposable temperate phages promote the evolution of divergent social strategies in Pseudomonas aeruginosa populations. *Proceedings of the Royal Society B: Biological Sciences,* 286**,** 20191794.

O'LOUGHLIN, C. T., MILLER, L. C., SIRYAPORN, A., DRESCHER, K., SEMMELHACK, M. F. & BASSLER, B. L. 2013. A quorum-sensing inhibitor blocks Pseudomonas aeruginosa virulence and biofilm formation. *Proc Natl Acad Sci U S A,* 110**,** 17981-6.

PALIY, O. & GUNASEKERA, T. S. 2007. Growth of E. coli BL21 in minimal media with different gluconeogenic carbon sources and salt contents. *Applied Microbiology and Biotechnology,* 73**,** 1169-1172.

PANAGEA, S., WINSTANLEY, C., WALSHAW, M. J., LEDSON, M. J. & HART, C. A. 2005. Environmental contamination with an epidemic strain of Pseudomonas aeruginosa in a Liverpool cystic fibrosis centre, and study of its survival on dry surfaces. *J Hosp Infect,* 59**,** 102-7.

POPE, W. H. & HATFULL, G. F. 2015. Adding pieces to the puzzle: New insights into bacteriophage diversity from integrated research-education programs. *Bacteriophage,* 5**,** e1084073.

PTASHNE, M. 1992. *A Genetic Switch: Phage [lambda] and Higher Organisms*, Cell Press.

SAŁAMASZYŃSKA-GUZ, A., ROSE, S., LYKKEBO, C. A., TACIAK, B., BĄCAL, P., UŚPIEŃSKI, T. & DOUTHWAITE, S. 2017. Biofilm Formation and Motility Are Promoted by Cj0588-Directed Methylation of rRNA in Campylobacter jejuni. *Front Cell Infect Microbiol,* 7**,** 533.

SALUNKHE, P., SMART, C. H., MORGAN, J. A., PANAGEA, S., WALSHAW, M. J., HART, C. A., GEFFERS, R., TUMMLER, B. & WINSTANLEY, C. 2005. A cystic fibrosis epidemic strain of Pseudomonas aeruginosa displays enhanced virulence and antimicrobial resistance. *J Bacteriol,* 187**,** 4908-20.

SCOTT, F. W. & PITT, T. L. 2004. Identification and characterization of transmissible Pseudomonas aeruginosa strains in cystic fibrosis patients in England and Wales. *Journal of Medical Microbiology,* 53**,** 609-615.

SEPÚLVEDA-ROBLES, O., KAMEYAMA, L. & GUARNEROS, G. 2012. High Diversity and Novel Species of Pseudomonas aeruginosa Bacteriophages. *Appl Environ Microbiol,* 78**,** 4510-4515.

SIRYAPORN, A., KUCHMA, S. L., O'TOOLE, G. A. & GITAI, Z. 2014. Surface attachment induces Pseudomonas aeruginosa virulence. *Proc Natl Acad Sci U S A,* 111**,** 16860-5.

SMITH, W. D., BARDIN, E., CAMERON, L., EDMONDSON, C. L., FARRANT, K. V., MARTIN, I., MURPHY, R. A., SOREN, O., TURNBULL, A. R., WIERRE-GORE, N., ALTON, E. W., BUNDY, J. G., BUSH, A., CONNETT, G. J., FAUST, S. N., FILLOUX, A., FREEMONT, P. S., JONES, A. L., TAKATS, Z., WEBB, J. S., WILLIAMS, H. D. & DAVIES, J. C. 2017. Current and future therapies for Pseudomonas aeruginosa infection in patients with cystic fibrosis. *FEMS Microbiol Lett,* 364.

SONAWANE, A., JYOT, J., DURING, R. & RAMPHAL, R. 2006. Neutrophil elastase, an innate immunity effector molecule, represses flagellin transcription in Pseudomonas aeruginosa. *Infect Immun,* 74**,** 6682-9.

STOVER, C. K., PHAM, X. Q., ERWIN, A. L., MIZOGUCHI, S. D., WARRENER, P., HICKEY, M. J., BRINKMAN, F. S. L., HUFNAGLE, W. O., KOWALIK, D. J., LAGROU, M., GARBER, R. L., GOLTRY, L., TOLENTINO, E., WESTBROCK-WADMAN, S., YUAN, Y., BRODY, L. L., COULTER, S. N. & FOLGER, K. R. 2000.

Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature,* 406**,** 959-64.

STREMPEL, N., NEIDIG, A., NUSSER, M., GEFFERS, R., VIEILLARD, J., LESOUHAITIER, O., BRENNER-WEISS, G. & OVERHAGE, J. 2013. Human host defense peptide LL-37 stimulates virulence factor production and adaptive resistance in Pseudomonas aeruginosa. *PLoS One,* 8**,** e82240.

SULAKVELIDZE, A., ALAVIDZE, Z. & MORRIS, J. G. 2001. Bacteriophage Therapy. *Antimicrobial Agents and Chemotherapy,* 45**,** 649-659.

TAN, Y., ZHANG, K., RAO, X., JIN, X., HUANG, J., ZHU, J., CHEN, Z., HU, X., SHEN, X., WANG, L. & HU, F. 2007. Whole genome sequencing of a novel temperate bacteriophage of P. aeruginosa: evidence of tRNA gene mediating integration of the phage genome into the host bacterial chromosome. *Cell Microbiol,* 9**,** 479-491.

TAYLOR, V. L., FITZPATRICK, A. D., ZAFRIN, I. & MAXWELL, K. L. 2019. The Diverse Impacts of Phage Morons on Bacterial Fitness and Virulence. *Advances in Virus Research,* 103**,** 1-31.

TOMPSETT, A. 2021. *Proinflammatory cytokines as mediators of phenotypic change in bacteria implicated in ventilator-associated pneumonia.* PhD, University of Salford.

TSAI, C. J.-Y., LOH, J. M. S. & PROFT, T. 2016. Galleria mellonellainfection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence,* 7**,** 214-229.

TSAO, Y. F., TAYLOR, V. L., KALA, S., BONDY-DENOMY, J., KHAN, A. N., BONA, D., CATTOIR, V., LORY, S., DAVIDSON, A. R. & MAXWELL, K. L. 2018. Phage Morons Play an Important Role in Pseudomonas aeruginosa Phenotypes. *J Bacteriol,* 200.

WANG, X., KIM, Y., MA, Q., HONG, S. H., POKUSAEVA, K., STURINO, J. M. & WOOD, T. K. 2010. Cryptic prophages help bacteria cope with adverse environments. *Nature Communications,* 1**,** 147.

WILLIAMS, D., EVANS, B., HALDENBY, S., WALSHAW, M. J., BROCKHURST, M. A., WINSTANLEY, C. & PATERSON, S. 2015. Divergent, coexisting Pseudomonas aeruginosa lineages in chronic cystic fibrosis lung infections. *Am J Respir Crit Care Med,* 191**,** 775-85.

WINSTANLEY, C., LANGILLE, M. G. I., FOTHERGILL, J. L., KUKAVICA-IBRULJ, I., PARADIS-BLEAU, C., SANSCHAGRIN, F., THOMSON, N. R., WINSOR, G. L., QUAIL, M. A., LENNARD, N., CLARKE, L., BIGNELL, A., SEEGER, K., SAUNDERS, D., HARRIS, D., PARKHILL, J., HANCOCK, R. E. W., BRINKMAN, F. S. L. & LEVESQUE, R. C. 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the liverpool epidemic strain of Pseudomonas aeruginosa.(Report). *Genome Research,* 19**,** 12-23.

WOODS, D. E. & IGLEWSKI, B. H. 1983. Toxins of Pseudomonas aeruginosa: New Perspectives. *Clinical Infectious Diseases,* 5**,** S715-S722.

WORKENTINE, M. & SURETTE, M. G. 2011a. ComplexPseudomonasPopulation Structure in Cystic Fibrosis Airway Infections. *American Journal of Respiratory and Critical Care Medicine,* 183**,** 1581-1583.

WORKENTINE, M. P. H. D. & SURETTE, M. G. P. H. D. 2011b. Complex Pseudomonas Population Structure in Cystic Fibrosis Airway Infections. *American Journal of Respiratory and Critical Care Medicine,* 183**,** 1581-3.

WRETLIND, B. & PAVLOVSKIS, O. R. 1983. Pseudomonas aeruginosa Elastase and Its Role in Pseudomonas Infections. *Clinical Infectious Diseases,* 5**,** S998-S1004.

ZACZEK-MOCZYDŁOWSKA, M. A., YOUNG, G. K., TRUDGETT, J., PLAHE, C., FLEMING, C. C., CAMPBELL, K. & O' HANLON, R. 2020. Phage cocktail containing Podoviridae and Myoviridae bacteriophages inhibits the growth of Pectobacterium spp. under in vitro and in vivo conditions. *PLOS ONE,* 15**,** e0230842.

ZACZEK-MOCZYDLOWSKA, M. A., YOUNG, G. K., TRUDGETT, J., PLAHE, C., FLEMING, C. C., CAMPBELL, K. & R, O. H. 2020. Phage cocktail containing Podoviridae and Myoviridae bacteriophages inhibits the growth of Pectobacterium spp. under in vitro and in vivo conditions. *PLoS One,* 15**,** e0230842.

ZRELOVS, N., DISLERS, A. & KAZAKS, A. 2021. Motley Crew: Overview of the Currently Available Phage Diversity. *Frontiers in Microbiology,* 11.