The Effect of Competitor and Phage on the Evolution and Diversification of *Pseudomonas aeruginosa* in a Synthetic Cystic Fibrosis Nutritional Environment

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Abstract

Lung disease, caused primarily by the bacterium *Pseudomonas aeruginosa*, is the leading cause of death for patients with the genetic disease cystic fibrosis (CF). *P. aeruginosa* is intrinsically antibiotic resistant and chronic lung infections with this bacterium are notoriously intractable to treat. As such, there is much interest in using bacteriophage (phage), viruses that specifically target bacteria, to treat *P. aeruginosa* lung infections of cystic fibrosis patients. However, as with antibiotic resistance evolution, bacteria also evolve resistance to phage. Additionally, there is little understanding about how phage resistance evolution is affected by factors in the CF lung, including bacterial competitors and the nutritional environment.

As such, this project aimed to understand how selection by the lytic pseudomonal phages 14/1 and PNM drives resistance evolution and diversification in the absence and presence of the bacterial competitor, *S. maltophilia*.

In coculture, phage initially reduced the abundance of *P. aeruginosa*, which allowed *S. maltophilia* to increase in density. *P. aeruginosa* evolved resistance to the phages present and increased in density with time. Phage also increased *P. aeruginosa* diversification of non-ancestral morphologies, but this effect was abrogated by the presence of the competitor. Phage resistance evolution was associated with trade-offs in growth after 48 hours and increased susceptibility to colistin. Evolution with competitor was associated with increased susceptibility to tobramycin whilst adaptation to the nutritional environment was linked to increased susceptibility to meropenem.

These findings have important implications for the future of phage therapy. Herein, it is shown that application of phage not only reduces focal pathogen densities but also increases susceptibility to antibiotics. Therefore, phage applied together with antibiotics may be optimal to eliminate *P. aeruginosa* from the lungs of cystic fibrosis patients.

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Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1: Introduction

## 1.1 Introduction to cystic fibrosis and mechanism of pathology

Cystic fibrosis (CF) is a recessively inherited genetic disorder caused by mutations of the gene which codes for the cystic fibrosis transmembrane conductance regulator (CFTR). CF affects 1 in 2,500 live births (Folkesson *et al.*, 2012) and it has been estimated that patients born in 2018 who are homozygous for the most common CFTR mutation, deletion of phenylalanine at position 508 (ΔF508) which affects 50 – 90% of CF patients (Dechecchi, 2018), have a median life expectancy of only 46 and 41 years for males and females, respectively (Keogh *et al.*, 2018). However, this is a considerable improvement in life expectancy compared to the 1950s, when median survival rates for patients with CF was only a few months and has primarily been driven by antibiotic therapy (Elborn, 2016).

The result of the ΔF508 mutation is that the CFTR protein is recognised as misfolded by the quality control mechanisms of the secretory pathway and targeted for degradation (Ameen *et al.*, 2007). Therefore, there is no or significantly reduced, functional CFTR protein on the apical surface of epithelial cells in people with CF, affecting a range of body systems including the pancreas, liver, kidneys, and reproductive tract.

The primary manifestation of this disease and greatest cause of mortality in CF patients, is associated with the epithelium of the lungs and inability to clear bacteria. There are several non-mutually exclusive hypotheses which explain the mechanism of pathology in the CF lung. The 'low-volume' hypothesis explains that in the lungs of healthy patients, CFTR transports chloride ions from the epithelia to the lumen and negatively regulates the absorption of sodium ions by the epithelial sodium channel (ENaC) (Stutts *et al.*, 1995). As such, when CFTR is lost due to the ΔF508 mutation, chloride ion secretion is reduced and sodium ion import is uninhibited, leading to an increased concentration of ions in the epithelia. As a result, the osmotic potential for water to enter the lumen is decreased and the mucus on the apical surface of the lung epithelium becomes dehydrated and viscous, which compresses the cilia on the apical surface (Fig. 1) (Clunes and

Boucher, 2007). Subsequently, mucociliary clearance from the lungs is impeded, which means inhaled microorganisms cannot be cleared, thus resulting in infection.
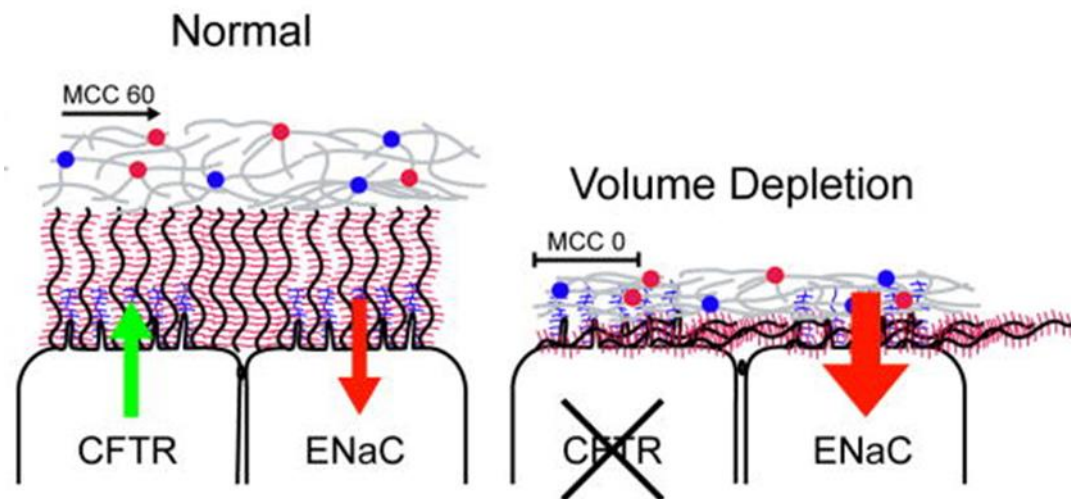
Figure 1: In non-CF airways (left), CFTR regulates the depth of the airway surface liquid (ASL) by exporting chloride ions and inhibiting sodium ion import via ENaC. The ASL is maintained at a depth which facilitates effective mucociliary clearance (MCC). In CF airways (right), the absence of CFTR leads to defective chloride ion secretion and unchecked sodium ion absorption. This results in dehydration of the ASL that collapses the cilia. Adapted from Clunes and Boucher, 2007 Fig. 1.

Another hypothesis explains that in patients with CF, there is dysregulation of the host inflammatory immune response. For example, Tirouvanziam *et al.* (2000) showed that exacerbated inflammation is a fundamental component of CF, rather than a response to persistent infection, by comparing leukocyte accumulation in uninfected *ex-vivo* tracheal samples from foetuses with and without CF. Significantly increased accumulation of leukocytes was observed in the subepithelial layer of naïve CF airways compared to non-CF airways and when challenged with *Pseudomonas aeruginosa,* CF samples had greater epithelial exfoliation which contributed to rapid invasion of *P. aeruginosa* to the lamina propria. Furthermore, Rosenfeld *et al.* (2001) found that the concentration of interleukin-8, a chemoattractant cytokine, in

bronchoalveolar lavage was elevated in CF subjects compared to non-CF controls, independent of whether the CF patients had bacterial lung infections. This suggests a pro-inflammatory state is at the core of CF pathology, independent of infection.

1.2 Evolution and diversification of *Pseudomonas aeruginosa* within the cystic fibrosis lung

The leading cause of mortality in CF patients is chronic bacterial lung infection which causes progressive loss of lung function (Elborn, 2016). *Pseudomonas aeruginosa*, a gram-negative soil bacterium, is an opportunistic pathogen which infects the majority of CF patients by the time they reach 20 years of age and is the main cause of morbidity (Folkesson *et al.* 2012). Environmental reservoirs are likely sources of *P. aeruginosa*, as a longitudinal study of CF patients in their first three years of life found that the genotypes of initial colonising strains were unique between patients (Burns *et al.*, 2001). However, transmissible epidemic strains of *P. aeruginosa* have been found to spread between CF patients, possibly via patient-patient interactions at CF treatment centres and may also be associated with increased mortality (Fothergill *et al.*, 2012).

P. aeruginosa intermittently colonises the lungs of a CF patient before transitioning to a chronic infection (Johansen and Hoiby, 1992, Burns *et al.*, 2001). Transition from an acute to chronic infection is accompanied by a range of characteristic phenotypic and genotypic changes in *P. aeruginosa* as it adapts to the CF lung. Loss of motility occurs, as found by Mahenthiralingam *et al.* (1994) who report that 39% of *P. aeruginosa* isolates from CF patients were non-motile compared to 1.4% of environmental isolates. Additionally, they found that non-motile isolates were resistant to phagocytosis by macrophages and suggest that loss of motility may occur to evade the host immune system and allow *P. aeruginosa* to survive in the CF lung.

Transition to a chronic infection is also frequently accompanied by conversion to a mucoid phenotype, which is due to excessive production of the exopolysaccharide alginate. This forms a protective glycocalyx on the

surface of the bacterium and is associated with biofilm development. The mucoid phenotype protects *P. aeruginosa* from phagocytosis by the host immune system (Cabral *et al.*, 1987), thus facilitating survival in the CF lung and maintaining a chronic infection. Additionally, CF patients whose sputum samples contain culturable mucoid *P. aeruginosa* have a ten-fold worse survival rate compared to CF patients producing sputum samples where mucoid *P. aeruginosa* is not observed (Henry *et al.*, 1992). A potential explanation for this is that mucoid *P. aeruginosa* forms biofilms which are harder to eradicate than planktonic bacteria, due to oxygen limitation and thus low metabolic activity of the biofilm-associated bacteria (Walters *et al.*, 2003).

Furthermore, the growth rate of chronically infecting *P. aeruginosa* slows, as found by Yang *et al.* (2008) who reported that clinical isolates grew two- to threefold slower than the common lab strain PAO1. In LB medium, PAO1 has a generation time of between 24 and 27 minutes, compared to CF isolates which have a generation time of between 50 and 74 minutes. They suggest that slow growth is an evolutionary trade-off when evolving resistance to antibiotics, that are widely used to control bacterial infections in the CF lung. Furthermore, La Rosa *et al.* (2021) reverted the slow growth phenotype of three clinical isolates to high growth rates by culturing in lab medium, thus removing the selective pressure to evolve antibiotic resistance in the CF lung. Following evolution in the lab and a return to high growth, they found that the minimum inhibitory concentrations of six clinically relevant antibiotics were reduced and *P. aeruginosa* became more susceptible. Therefore, growth and antibiotic resistance are genetically linked.

Another, potentially surprising, phenotype that *P. aeruginosa* displays when it adapts to the CF lung and transitions to a chronic infection, is a reduction in the production of virulence factors. Smith *et al.* (2006) genotyped *P. aeruginosa* isolates from early and late infections from 29 patients and found that late isolates of *P. aeruginosa* were frequently mutated in the gene *lasR*, a transcriptional regulator of quorum sensing. Quorum sensing is a system by which bacteria communicate and measure the cell density of their community by detecting the concentration of a secreted small molecule. At

high cell densities, quorum sensing coordinates the expression of virulence factors. As such, late isolates lacked expression of many virulence factors including motility, pyoverdine production, and protease production. Therefore, virulence factors are selected against during chronic infection of the CF lung and the authors suggest the purpose of this is to evade the immune system as virulence factors also act as ligands to activate the host's immune system. Furthermore, virulence factors that may have been important in establishing infection may become unnecessary once the infection has been established, thus leading to their loss over time.

The CF lung causes diversification of *P. aeruginosa* with respect to phenotype and genotype during infection. Factors such as availability of nutrients, spatial structure of the lung, presence of bacterial competitors, and antibiotic use, may contribute to the evolution and diversification of *P. aeruginosa* within the CF lung.

The nutritional complexity of the CF lung likely also causes diversification of *P. aeruginosa*. Schick and Kassen (2018) found that in a defined medium that models the nutritional environment of the CF lung, synthetic cystic fibrosis medium (SCFM) (Palmer *et al.*, 2007), *P. aeruginosa* rapidly diversified with respect to traits seen during chronic infection. These included a reduction in pyoverdine production, swimming motility, twitching motility, and an increase in biofilm production. Schick and Kassen (2018) also found that antibiotic resistance evolves as a pleiotropic effect of adapting to the nutritional environment of the CF lung, with populations evolving in SCFM displaying greater resistance to ciprofloxacin.

The lung is a highly structured environment, composed of millions of alveoli. These separated compartments produce a heterogeneous environment that, when colonised by *P. aeruginosa*, provide isolated niches for independent evolution of sublineages. Jorth *et al.* (2015) dissected the lungs of 10 chronically infected CF patients and studied variations between clonally related isolates of *P. aeruginosa* from the upper, middle, and lower lobes. The authors found that each lung pair contained between 12 and 40 subpopulations. These spatially segregated isolates had variation in

phenotypes including virulence, swimming motility and resistance to antibiotics.

CF lung infections are frequently coinfections by multiple bacterial species. Interactions between these competing species also likely affect diversification of *P. aeruginosa*. Tognon *et al.* (2017) found that co-evolving *P. aeruginosa* with *Staphylococcus aureus* selected for *orfN* mutants of *P. aeruginosa*. The OrfN amino acid sequence shares 60% identity to WbpL of PAO1, which is involved in the initiation of biosynthesis of O-specific antigens of lipopolysaccharide (LPS). As such, they constructed Δwbpl mutants and found that these mutants showed reduced production of O-specific antigens when analysed by SDS-PAGE electrophoresis. Furthermore, these mutants had increased fitness in the presence of *S. aureus*, meaning loss of LPS is an adaptation by *P. aeruginosa* to growing in the presence of *S. aureus*. Interestingly, experimentally evolved and genetically constructed mutants showed increased resistance to beta-lactam antibiotics, suggesting an evolutionary trade-on to evolving in the presence of a competitor.

Antibiotics are used to control bacterial infections of the CF lung. However, the strong selective pressure imposed by the presence of antibiotics in the treated lung selects for *P. aeruginosa* to evolve resistance. Wong *et al.* (2012) experimentally evolved *P. aeruginosa* in SCFM with and without a clinically relevant concentration of ciprofloxacin. Following eight days of evolution, they found that evolved populations showed a 32- to 192-fold increase in minimum inhibitory concentration (MIC) compared to the ancestor. Additionally, the presence of mucin in SCFM mitigated the costs of adaptation to ciprofloxacin and allowed the bacteria to evolve resistance faster. Moreover, they found considerable variation in fitness of single genotypes suggesting substantial genetic diversity in evolving populations. Interestingly, 6/7 of the ciprofloxacin-resistant genotypes carried loss of function mutations in *orfN*, necessary for glycosylation of type A flagellins.

1.3 Cystic fibrosis lung co-infections with *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is a gram-negative bacterium found ubiquitously in aqueous-associated environments, from endoscopes (Kovaleva *et al.*, 2010) to washed salads (Qureshi *et al.*, 2005) and is frequently isolated from the lungs of CF patients. Salsgiver *et al.* (2016) found that *S. maltophilia* was prevalent in the lungs of 13.4% of 25,530 CF patients. Furthermore, they found that the prevalence of *S. maltophilia* had increased significantly between 2006 and 2012 with a 1.4% increase, compared to *P. aeruginosa* which had a significant decrease of 1.7% during the same timeframe. CF patients are also commonly infected with multiple bacterial species, and *S. maltophilia* is frequently co-isolated with *P. aeruginosa* from the CF lung, as found by Talmaciu *et al.* (2000) who reported that of the patients positive for *S. maltophilia*, 74.5% were coinfected with *P. aeruginosa*. Similarly, Spicuzza *et al.* (2009) found that 61% of subjects in their study were coinfected with *S. maltophilia* and *P. aeruginosa*. These findings indicate that *S. maltophilia* and its association with *P. aeruginosa*, may need to be considered when treating CF lung infections.

Talmaciu *et al.* (2000) and Berdah *et al.* (2018) found that treatment of CF lung infections with antibiotics predisposes patients to colonisation by *S. maltophilia*. For example, Talmaciu *et al.* (2000) reported that 50.9% of patients who became positive for *S. maltophilia* were treated chronically with antibiotics, compared to 25.4% of patients negative for *S. maltophilia*. The findings of Berdah *et al.* (2018) support these, as they report that 34.7% of *S. maltophilia* positive patients received intravenous antibiotics in the two years preceding *S. maltophilia* colonisation compared to 8.6% of *S. maltophilia* negative patients. The risk of being colonised by *S. maltophilia* following antibiotic treatment may be due to *S. maltophilia* having strong antimicrobial resistance (Crossman *et al.*, 2008) and therefore in the antibiotic-treated CF lung, *S. maltophilia* has a competitive advantage over competing bacteria and as such is able to survive and thrive.

Infection of CF patients with *S. maltophilia* is associated with greater disease severity. For example, Talmaciu *et al.* (2000) found that patients positive for *S. maltophilia* had significantly worse CF status and spirometric values than *S. maltophilia* negative CF controls. Furthermore, Berdah *et al.* (2018) found that compared to age, sex and CFTR mutation matched controls, CF patients positive for *S. maltophilia* had significantly lower lung function, more frequent disease exacerbations and hospitalisations. In agreement with these findings, Waters *et al.* (2011) reported that CF patients chronically infected with *S. maltophilia* had significantly increased risk of pulmonary exacerbations requiring hospitalisation compared to CF patients who had never been infected with *S. maltophilia*. Additionally, Yin *et al.* (2017) found that patients co-infected with *S. maltophilia* and *P. aeruginosa* had over a five times greater mortality rate than those infected with *P. aeruginosa* alone. A possible explanation for the increase in disease severity observed when CF patients are co-infected with *P. aeruginosa* and *S. maltophilia* is that the intrinsically antibiotic resistant *S. maltophilia* detoxifies the lung environment of anti-pseudomonal drugs. Bottery *et al.* (2021) found that the PAO1 was able to survive at an imipenem concentration of 16 µg/mL when grown in co-culture with *S. maltophilia,* compared to 4 µg/mL when grown alone. The authors attribute this phenomenon to the ability of *S. maltophilia* to hydrolyse imipenem via expression of *bla*$_{L1}$, a metallo-L1-β-lactamase, which decreases the concentration of imipenem in the environment, thus allowing *P. aeruginosa* to survive. Therefore, *in vivo* co-infections of *P. aeruginosa* and *S. maltophilia* may cause greater disease severity due to increasing the ability of the focal pathogen to survive antibiotic treatment and thus cause disease. In conclusion, *S. maltophilia* is an emerging pathogen for CF patients and particularly for those treated with antibiotics. Colonisation by *S. maltophilia* is also associated with increased pathology, meaning there is a need for greater understanding about this pathogen in the CF context and its interactions with *P. aeruginosa* to inform treatment. Therefore, coculturing these bacteria together in an *in vitro* model of CF may provide insights into CF lung infections.

1.4 Phage treatment of *Pseudomonas aeruginosa* and evolution of phage resistance

With the rise of multiple-drug-resistant bacteria and a discovery gap in a new major class of antibiotic since 1987 (Silver *et al.*, 2011), there is much interest in using bacteriophage (phage) therapeutically to control bacterial infections. Phages are viruses that specifically target bacteria and this specificity is governed by their attachment to bacterial cell surface receptors. Once attached to the host, phage inject their genetic material, exploiting the host's transcription and translation machinery to replicate and assemble new viral progeny. Lytic phages then cause host lysis which releases the viral progeny and kills the bacterial cell. The initial infection is thus propagated and can spread through the whole population of the bacterial host (Labrie *et al.*, 2010).

Phages have many advantages over antibiotics, including high specificity to the target bacteria (Gupta and Prasad, 2011), causing minimal disruption to the patients' microflora. They are also able to self-replicate, meaning their titres increase exponentially to overwhelm the host bacteria (Levin and Bull, 1996). Another promising aspect of phage therapy is that phage have been found to coevolve with bacteria to become more infective and thus able to retain efficacy in controlling bacterial populations (Buckling and Rainey, 2002). Furthermore, Friman *et al*. (2016) evolved phage by culturing with *P. aeruginosa* CF lung isolates, which when isolated and applied to ancestral bacterial strains, caused a greater reduction in bacterial densities than ancestral phages. Additionally, Borin *et al*. (2021) showed that 'trained' (pre-evolved) phage was able to suppress the density of the bacterial host for between 13 and 28 days compared to the 'untrained' phage, that had not been exposed to the host before, which could only suppress the host for 3 days. The trained phage could utilise two receptors to gain entry to the host and therefore resistance to trained phage evolved later due to less common mutations conferring resistance, more mutations required to evolve resistance, and higher costs of resistance. Also, trained phage co-evolved with the host to retain infectivity as resistance arises, as contemporary trained phage was better able to suppress bacterial density and had greater

efficiency of plating, compared to the ancestor trained phage. However, Moulton-Brown and Friman (2018) found weak evidence for coevolutionary dynamics.

Furthermore, phage has proven to be effective in treating a model of a CF lung infection. For example, Waters *et al.* (2017) showed that administration of phage to a murine model of chronic lung infection of *P. aeruginosa* completely cleared the bacteria from the lungs of treated mice when phage was administered at 24- and 36-hours post-infection or 48- and 60-hours post infection (Fig. 2).
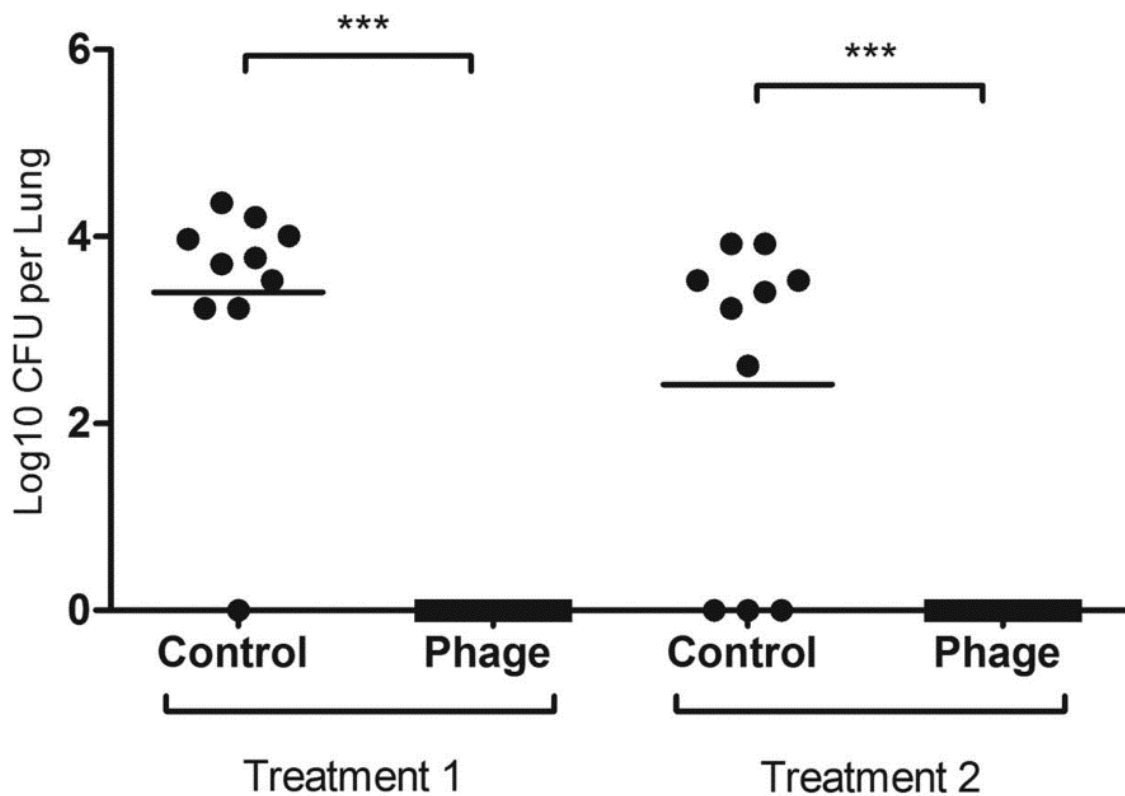
Figure 2: Colony-forming units (log CFU per lung) of *P. aeruginosa* present in the lungs of mice following infection. The mice were treated with phage or phosphate-buffered saline control using different protocols: (Treatment 1) phage administration at 24 and 36 hours post infection (Treatment 2) phage administration at 48 and 60 hours post infection. Adapted from Waters *et al.* 2017 Fig. 2.

However, there are limitations associated with using phage therapeutically. An important constraint is that the strong selective pressure of phage infection selects for bacteria to evolve resistance. Broniewski *et al.* (2020) found that WT *P. aeruginosa* were able to evolve CRISPR- and surface modification-based immunity, which involves mutation of the receptor that the phage uses to attach to the host, in response to genetically diverse phage populations, resulting in phage extinction by no later than day 5 post-inoculation for all replicates.

Another result of phage selection pressure is that *P. aeruginosa* diversifies with respect to colony morphotypes. For example, Hosseinidoust *et al.* (2013) found that *P. aeruginosa* diversified into five distinct colony morphologies due to evolving in the presence of lytic phage. These colony morphotypes were phage resistant and their virulence was significantly increased relative to controls. For example, phage resistant populations had significantly increased production of the virulence factors pyoverdine and pyocyanin. Therefore, applying phage to treat chronic infections of *P. aeruginosa* in CF patients may alter the pathogen's virulence and increase patient morbidity and mortality. Miller and Rubero (1984) were able to convert non-mucoid strains of *P. aeruginosa,* along with PAO1, to a mucoid phenotype by growing with lytic phage isolated from sputum samples of CF patients. Additionally, Scanlan and Buckling (2012) report that co-evolving lytic phage with *P. fluorescens* selects for the bacteria to evolve a mucoid phenotype and found that this phenotype conferred resistance to phage. These findings taken together with those of Henry *et al.* (1992), that the mucoid phenotype of *P. aeruginosa* was associated with poorer clinical outcomes for CF patients, are not promising for the future of phage therapy. However, Friman *et al.* (2013) found that *P. aeruginosa* isolates from chronic CF infections were less phage resistant than *P. aeruginosa* isolates from intermittently colonising infections, suggesting that phage therapy may still be useful in treating CF lung infections. Also, Alemayehu *et al.* (2012) found that a two-phage mixture produced a 100-fold reduction in bioluminescence of a mucoid strain of *P. aeruginosa* growing as a biofilm. It is obvious that we do not yet fully understand the effects of phage on the diversification of *P.*

aeruginosa. Additionally, this phenomenon may be even more complex within the CF lung and affected by further factors such as nutrients and commonly co-occurring species such as *S. maltophilia*.

Another foreseeable problem with phage therapy in the CF lung is that whilst using pseudomonal phages to kill *P. aeruginosa* seems the obvious treatment rationale, as *P. aeruginosa* causes the most morbidity and mortality (Elborn, 2016), this may open a niche for colonisation by other competing bacteria, such as *S. maltophilia* (Talmaciu et al., 2000; Berdah et al., 2018). Given that *S. maltophilia* is associated with increased pathology (Talmaciu et al., 2000; Berdah et al., 2018; Waters et al., 2011; Yin et al., 2017) focussing only on the eradication of *P. aeruginosa*, may be a weakness in treatment strategy.

1.5 Phage interactions with factors in the cystic fibrosis lung

As set out above, factors including nutrients, lung structure, competitors, and antibiotics influence the evolution of *P. aeruginosa*. Together with the diversifying effect of phage itself, phage interact with these variables in the CF lung meaning the evolutionary outcomes are likely to be different and more nuanced *in vivo*.

Phage interacts with the polymicrobial community present in the CF lung and together this may have important effects on the evolution and diversification of *P. aeruginosa*. Mumford and Friman (2017) found that PAO1 that had evolved in the presence of *S. aureus*, *S. maltophilia*, or both, had significantly decreased resistance to phage compared to the monoculture control. Furthermore, evolving PAO1 in the presence of competitors led to reduced growth in the absence of competitors and this effect was compounded by also evolving in the presence of phage. Together, these results suggest competitors constrained phage-resistance evolution of *P. aeruginosa* and lead to increased costs of adaptation. Additionally, Alseth *et al.* (2019) found that co-culturing *P. aeruginosa* with a bacterial community consisting of *Staphylococcus aureus*, *Burkholderia cenocepacia*, and *Acinetobacter baumannii*, in the presence of the phage DMS3vir caused a significantly greater proportion of *P. aeruginosa* to evolve CRISPR-based phage

resistance relative to monoculture controls. The authors suggest that the reason for this phenomenon is that there are greater levels of interspecific competition in co-cultures which favours evolution of CRISPR-based resistance, rather than surface modification resistance, as mutation of the receptor the phage uses to bind confers greater fitness costs than CRISPR-based resistance. Furthermore, *P. aeruginosa* clones with CRISPR-based resistance had greater capacity to kill the *Galleria mellonella* infection model, relative to surface modification clones, suggesting that the mechanism by which *P. aeruginosa* evolves resistance has important implications for its virulence.

Phage and antibiotics interact to influence the evolution of *P. aeruginosa*. Gurney *et al*. (2020) found that co-culturing PAO1 with lytic phage OMKO1 in the presence of antibiotic decreased MICs relative to antibiotic alone evolutionary controls. Also, combined antibiotic-phage treatments reduced MICs relative to untreated controls. Together, these results suggest that phage prevented evolution of antibiotic resistance in *P. aeruginosa* and increased the susceptibility of the bacteria to antibiotics.

In conclusion, the CF lung is a highly complex environment that harbours a diverse range of bacteria. The web of interactions that exists between the lung, competitors, antibiotics, and phage all contribute to the evolution and diversification of the *P. aeruginosa* and are summarised in Fig 3. Therefore, to better understand this intricate system, this project will examine the effect of administering pseudomonal phage to a coculture of *P. aeruginosa* and *S. maltophilia* in a synthetic cystic fibrosis nutritional model.
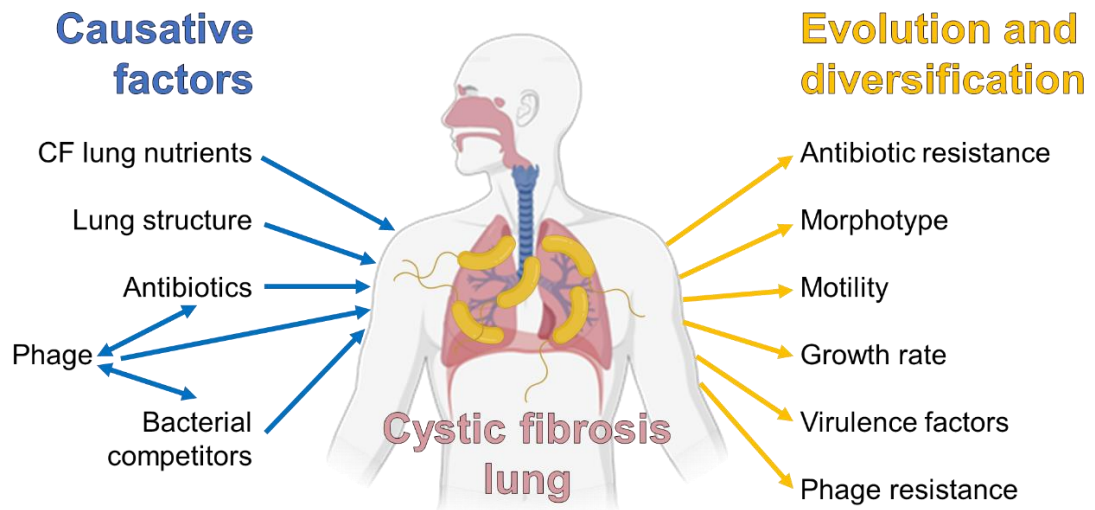
Figure 3: A summary of the factors influencing the evolution and diversification of *P. aeruginosa* within the CF lung.

1.6 Project Aims

This project aims to understand how the presence of *S. maltophilia* affects the ecological and evolutionary interactions between *P. aeruginosa* and its lytic phages, 14/1 and PNM, which will inform potential therapeutic considerations for phage therapy.

Hypotheses on ecological outcomes:

- The presence of a competitor could magnify the negative effect of phages, leading to lower *P. aeruginosa* abundances.
- In turn, this could also lead to a reduction in phage abundances and less clear bottom-up density regulation by the phage over time.

Hypotheses on evolutionary outcomes:

- Negative density effects could constrain phage-bacteria evolution and coevolution, leading to lower levels of resistance and phage infectivity in the presence of competitor.
- The presence of competitor could also affect the evolution of *P. aeruginosa* phage resistance by magnifying the relative importance of potential resistance-associated growth costs.

Chapter 2: Materials and Methods

2.1 Bacterial and phage strains, and growth media

Pseudomonas aeruginosa PA01:rfp strain was used, which was previously constructed by inserting the red fluorescent dTomato gene into the attTn7 site of wild type PAO1 by electroporation using *E. coli* str-DH5α pUC18T-mini-Tn7T-dsRedExpress plasmid (Choi and Schweizer, 2006).

Stenotrophomonas maltophilia-518951 strain was isolated from a sputum sample taken from the respiratory tract of a Danish adult with Cystic Fibrosis suffering from chronic infection. The sputum sample was provided by Sören Molin and Helle Krogh Johansen at the Rigshospitalet Copenhagen (Jelsbak et al., 2007).

A frozen glycerol coculture of PAO1:rfp and *S. maltophilia* 518951 was provided and following 24 hours culture at 37 °C with shaking at 180 rpm in 15 mL LB broth (10 g/L tryptone, 10 gL NaCl, 5 g/L yeast extract), the density of *P. aeruginosa* was found to be ~5 × 10^6 using Pseudomonas Selective Agar and that of *S. maltophilia* to be ~5 x 10^4 using LB agar + 10 mg/L meropenem. To prepare *P. aeruginosa* monoculture stocks, single colonies of *P. aeruginosa* were picked, grown as above to a density of ~5 × 10^6 and cryopreserved at -80 °C in 20% glycerol.

Mono- and cocultures were prepared by scraping a small amount of frozen glycerol stock with a p200 tip and inoculating 15 mL LB broth (10 g/L tryptone, 10 gL NaCl, 5 g/L yeast extract). Cultures were incubated for 24 hours at 37 °C with shaking at 180 rpm.

SCFM was made as per Palmer et al. (2007).

14/1 and PNM bacteriophage (phage) (Hall *et al.*, 2016) cultures were prepared by inoculating 15 mL LB broth with a small amount of PAO1 frozen glycerol stock. 10 µL of phage was added and cultures were incubated for 24 hours at 37 °C with shaking at 180 rpm. Phage was isolated by adding 100 µL of chloroform to 900 µL of culture, followed by vigorous shaking and vortexing. Samples were then centrifuged at 13000 rpm for three minutes and stored at 4 °C.

2.2 Experimental evolution of *Pseudomonas aeruginosa* in the absence and presence of competitor and phage

Control (no phage), 14/1, PNM, 14/1 + PNM, +/- *S. maltophilia* were separated across individual 24-well plates to avoid cross-contamination, with 6 replicates per treatment. 1 mL of SCFM was inoculated with 10 µL of overnight mono- or co-culture and 10 µL of corresponding phage. 10 µL of sterile distilled water was added to control populations. Plates were placed inside humidified zip-lock bags and incubated statically at 37 °C. Every three days, OD 600 nm of evolved populations was measured. 20 µL of each evolved population was serially transferred into 1 mL of fresh SCFM. The experiment was run for 21 days, resulting in a total of seven transfers. At each timepoint, 600 µL of each population was cryopreserved at -80 °C in 20% glycerol for future analysis.

Phage was isolated at each timepoint as per section 2.1 for use in coevolution assays, with the exception that 300 µL of evolved populations were diluted in 600 µL of sterile distilled water prior to the addition of chloroform.

2.3 Measuring phage densities during the evolution experiment

Phage population densities were measured at every transfer during the evolution experiment. First, tenfold serial dilutions to 10^{-6} were made in sterile distilled water for each phage replicate. 100µL of PAO1:rfp overnight culture was then added to 10mL of molten LB soft agar and spread over LB agar plates. The top agar was allowed to set before 5 µL of phage from each dilution was spotted onto the agar and were allowed to dry. The plates were incubated at 37 °C for 24 hours before plaques in the bacterial lawn were counted and phage density estimated as plaque forming units (PFU) per mL.

2.4 Isolation of clones from evolved populations

Evolved populations from the final timepoint (day 21) were thawed and vortexed. Tenfold serial dilutions to 10^{-6} were made in sterile distilled water for each population. 10 µL of each population from dilutions 10^{-3} – 10^{-6} was plated onto LB agar plates and spread using sterile glass beads. Plates were

allowed to dry before the glass beads were removed and the plates incubated at 37 °C for 48 hours. Following incubation, the number of colonies of each morphotype were counted. Single colonies of *P. aeruginosa* from each evolved population were picked to reflect the diversity observed, with a total of four clones per individual morphotype and inoculated into 96-well plates containing 200 µL of SCFM per well. Plates were placed inside humidified zip-lock bags and incubated at 37 °C for 48 hours. Following incubation, clones were cryopreserved at -80 °C in 20% glycerol on plates for future analysis.

2.5 Measuring resistance against ancestral phage

Resistance was quantified by comparing the growth of evolved *P. aeruginosa* clones in the absence and presence of ancestral phage 14/1 or PNM. A sterile pin replicator was used to inoculate (~0.2 µL) each clone from different evolutionary treatments into 96-well plates containing 200 µL SCFM per well. Ancestral 14/1 and PNM phage stocks were diluted 1:10 and 10 µL of phage was added per well to the corresponding 96-well plate. 10 µL sterile distilled water was added to the control plates. Plates were placed inside humidified zip-lock bags and incubated statically at 37 °C for 48 hours before OD 600 nm was measured. Phage resistance was quantified as relative bacterial growth (RBG). This was calculated by dividing the growth of the bacterial isolates when phage was applied by their growth in negative control (absence of phage). As such, an RBG value of 1 indicates complete resistance and a lower value indicates greater susceptibility.

2.6 Phage-bacteria co-evolution

Coevolution of phage was determined using time-shift assays and exposing evolved *P. aeruginosa* to either ancestral phage or evolved phage from different timepoints, as isolated in section 2.2. A sterile pin replicator was used to inoculate (~0.2 µL) the following evolutionary treatments; *P. aeruginosa* + *S. maltophilia* + 14/1; *P. aeruginosa* + *S. maltophilia* + PNM; *P. aeruginosa* + 14/1; *P. aeruginosa* + PNM into 96-well plates containing 200 µL SCFM per well. 10 µL of either ancestral, day 12, or day 21 phage isolates were added to their co-evolving bacterial population. Plates were

placed inside humidified zip-lock bags and incubated statically at 37 °C for 48 hours before OD 600 nm was measured.

2.7 Measuring changes in evolved *Pseudomonas aeruginosa* growth

Maximum growth following 48 hours growth was measured. A sterile pin replicator to inoculate (~0.2 µL) each evolutionary treatment into 96-well plates containing 200 µL SCFM per well. Plates were incubated in a SPECTROstar Nano (BMG LABTECH) spectrophotometer at 37 °C. OD 600 nm was measured every 15 minutes for 72 hours.

2.8 Quantifying changes in the antibiotic resistance of evolved *Pseudomonas aeruginosa*

Colistin, tobramycin and meropenem 1000 µg/mL stocks were made in distilled water and filter sterilised then stored at -20 °C. Each antibiotic was added to aliquots of SCFM to give concentrations of; 0.25, 0.6 and 1.7 µg/mL for colistin; 1.0, 1.5, and 32 µg/mL for tobramycin; 0.4, 0.56, and 32 µg/mL for meropenem. 200 µL of antibiotic-supplemented SCFM was added per well to 96-well plates. A sterile pin replicator was used to inoculate (~0.2 µL) each evolutionary treatment into the corresponding antibiotic. Plates were placed inside humidified zip-lock bags and incubated statically at 37 °C for 24 hours before OD 600 nm was measured.

2.9 Statistical analysis

Analysis was conducted in R 4.0.4 and figures were produced using the package ggplot2 (Wickham, 2009).

For all analysis where linear mixed effects models were constructed, the replicate population the clones were isolated from was included as a random effect to control for pseudoreplication in the experimental design. Additionally, the well ID was nested within the replicate population random effect to control for repeated measurements of the same clones in different environments. Tukey comparison of group means with Bonferroni correction was used for subsequent analysis.

Chapter 3: Results and Discussion

3.1 Population dynamics in coculture

It is important to understand how the densities of the focal bacteria, *P. aeruginosa*, and its competitor change over the course of the experiment and how these are influenced by interactions with the phages present.

It was hypothesised that because phages 14/1 and PNM are specific to *P. aeruginosa* and do not bind or lyse *S. maltophilia*, that the abundance of *S. maltophilia* may increase when both bacteria are cocultured with phage. To test for significant effects and interactions between bacterial species, time, and phage, a linear mixed effects model was constructed. The model found a significant effect of bacterial species (ANOVA: $F_{1,115} = 67.56$, $p < 0.001$), time (ANOVA: $F_{2,115} = 23.95$, $p < 0.001$), and phage (ANOVA: $F_{3,115} = 2.99$, $p = 0.0337$) on CFU, with significant interactions between all three of these variables ($p < 0.001$). Therefore, the data was subset into different phage treatments to allow a greater understanding of the effects of species and time.
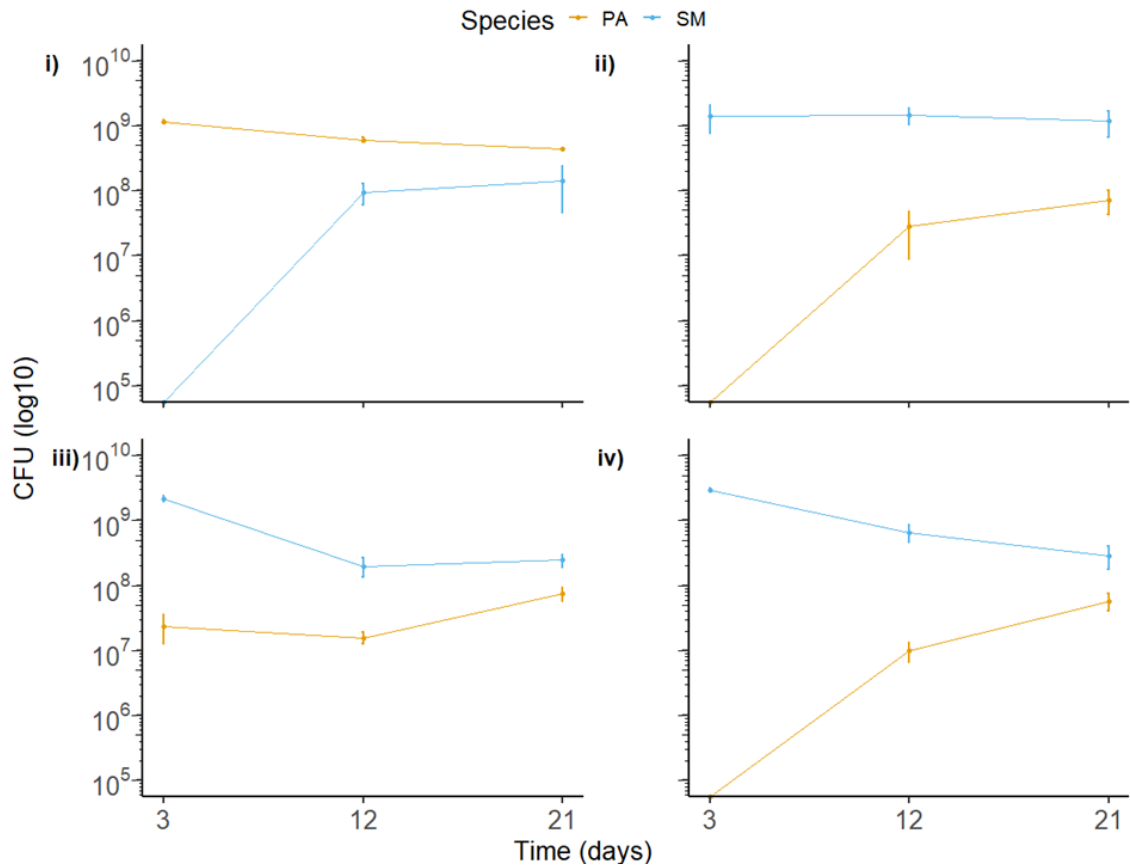
Figure 4: Log10 colony forming units (CFU) of *P. aeruginosa* (PA) and *S. maltophilia* (SM) in each coculture replicate population, sampled at days 3, 12 and 21 during the evolution experiment. i) Control (no phage) ii) 14/1 iii) PNM iv) 14/1 + PNM. Mean ± SEM, n = 6 biological replicates.

In the absence of phage (Fig. 4i), there was a significant effect of bacterial species (ANOVA: $F_{1,25}$ = 228.89, p < 0.001) and time (ANOVA: $F_{2,25}$ = 16.58, p < 0.001) on CFU. Additionally, there was a significant interaction between species and time (ANOVA: $F_{2,25}$ = 35.78, p < 0.001). To investigate the direction and magnitude of these interactions, a Tukey test was used to conduct pairwise comparisons between groups. Firstly, the CFU of *P. aeruginosa* decreased over the course of the evolution experiment with significant pairwise comparisons between the level of *P. aeruginosa* at day 3 contrasted against that of day 12 (Tukey: p < 0.001) and day 21 (Tukey: p < 0.001). The model failed to identify a significant change is *S. maltophilia* density over the course of the evolution experiment, however there are

significant pairwise comparisons between the CFU of *P. aeruginosa* and *S. maltophilia* at day 3 (Tukey: p < 0.001), 12 (Tukey: p < 0.001), and 21 (Tukey: p < 0.01). To summarise, when *P. aeruginosa* and *S. maltophilia* are evolved in the absence of phage, *P. aeruginosa* dominated each population at day 3, as in this environment, phage susceptibility confers no competitive disadvantage. However, *P. aeruginosa* densities decrease over the course of the evolution experiment, whilst there is a trend of *S. maltophilia* density increasing. This may be due to *P. aeruginosa* adapting to the growth media and evolving a slow growth rate, such as that seen during evolution in vivo (Yang *et al.*, 2008), meaning *S. maltophilia* has a greater competitive advantage and is able to compete better against *P. aeruginosa* for nutrients.

For 14/1 treatment (Fig. 4ii) there was a significant effect of species (ANOVA: $F_{1,25}$ = 18.15, p = 0.003) but not time on CFU and a non-significant interaction between these variables. Therefore, *S. maltophilia* densities were significantly greater than that of *P. aeruginosa* densities and the density of either species did not change significantly over the course of the evolution experiment. For PNM treatment (Fig. 4iii) there was a significant effect of species (ANOVA: $F_{1,25}$ = 125.45, p < 0.001) and time (ANOVA: $F_{2,25}$ = 74.84, p < 0.001) on CFU, with a significant interaction between these variables (ANOVA: $F_{2,25}$ = 77.96, p < 0.001). Post-hoc Tukey tests revealed significant pairwise comparisons between the density of *S. maltophilia* at day 3 contrasted against that of day 12 (Tukey: p < 0.001) and 21 (Tukey: p < 0.001). Therefore, the density of *S. maltophilia* at the start of the evolution experiment was significantly greater than at later sampling timepoints. Furthermore, there were significant contrasts between the density of *S. maltophilia* at day 3 compared to the density of *P. aeruginosa* at day 3 (Tukey: p < 0.001). Therefore, initially the density of *S. maltophilia* is significantly greater than that of *P. aeruginosa*, however this effect is lost as the density of *S. maltophilia* decreases over time and at day 21 there is a non-significant difference between species.

Similar effects were seen when *S. maltophilia* and *P. aeruginosa* were cocultured with phages 14/1 and PNM (Fig. 4iv). In this treatment, there was also a significant effect of bacterial species (ANOVA: $F_{1,25}$ = 178.85, p <

0.001) and time (ANOVA: $F_{2,25} = 73.77$, $p < 0.001$) on CFU, with a significant interaction between these variables (ANOVA: $F_{2,25} = 80.13$, $p < 0.001$). Post-hoc tests revealed significant contrasts between the density of *S. maltophilia* on day 3 against day 12 (Tukey: $p < 0.001$) and 21 (Tukey: $p < 0.001$), meaning *S. maltophilia* significantly decreases in density over the course of the evolution experiment. Additionally, there were significant contrasts between the CFU of *S. maltophilia* and *P. aeruginosa* at day 3 (Tukey: $p < 0.001$) 12 (Tukey: $p = 0.0012$) but not 21. Therefore, the density of *P. aeruginosa* relative to *S. maltophilia* significantly increases until the final timepoint, when the difference in CFU between the species is non-significant.

A potential explanation for these findings is that *P. aeruginosa* is susceptible to infection and lysis by 14/1 and PNM and therefore has less competitive advantage versus *S. maltophilia* in phage environments. However, at later sampling timepoints (day 12 and day 21), there is a trend of *P. aeruginosa* density increasing and that of *S. maltophilia* decreasing. This is likely due to *P. aeruginosa* evolving resistance to the phages in coculture, meaning it has greater competitive advantage and can increase in abundance versus *S. maltophilia*. The result that *P. aeruginosa* density in phage cultures is reduced relative to non-phage culture is supported by Mumford and Friman (2017) who found similar effects.

In summary, although the dynamics of *P. aeruginosa* and *S. maltophilia* densities change over the course of the evolution experiment dependent of phage absence/presence, the overall trends remain the same. Briefly, *S. maltophilia* dominates in the presence of phage and *P. aeruginosa* dominates in the absence of phage. This is an important finding as it suggests that the composition of the bacterial community in the CF lung can be completely changed by targeting the focal pathogen with a specific phage.

The implications of this finding suggest both positive and negative consequences for phage therapy. As application of phage can initially reduce the focal pathogen load, this may suggest that pseudomonal lung infections could be controlled by therapeutically administering phage. However, even over a short evolution period employed here, an increase in density of *P.*

aeruginosa is observed in phage cultures which suggests the evolution of phage resistance. As such, in the CF lung, whilst phage may initially reduce *P. aeruginosa* densities to the benefit of the patient, this effect may not be permanent as resistance quickly evolves. Evolution of phage resistance could be slowed by administering 'cocktails' of phage - a combination of phages with different host-range specificities. The necessity for the host to acquire multiple resistance mutations, which may have associated pleiotropic costs, means that phage cocktails decrease the probability of a resistant mutant emerging. Yang *et al.* (2020) found that a five-phage cocktail could inhibit the development of phage-resistant *P. aeruginosa* for up to five days, whereas resistance to single phage applications developed in less than 48 hours.

When phage was applied to a focal pathogen/competitor coculture, an increase in density of *S. maltophilia* was observed (Fig. 4). Infection with *S. maltophilia* has been linked to increased CF severity (Talmaciu *et al.*, 2000; Berdah *et al.*, 2018) potentially through the ability of *S. maltophilia* to detoxify commonly employed anti-pseudomonal drugs, such as imipenem (Bottery *et al.*, 2021). In this context, *P. aeruginosa* can survive antibiotic therapy and may explain why patients positive for *S. maltophilia* have a worse disease status. Therefore, if application of phage reduces *P. aeruginosa* densities, leading to an increase in density of *S. maltophilia*, then this may cause increased disease as *S. maltophilia* protects *P. aeruginosa* against the effects of beta-lactam antibiotics.

3.2 The effect of phage and competitor on diversification of *Pseudomonas aeruginosa*

Phage has a diversifying effect on *Pseudomonas* species with respect to colony morphology. Therefore, before altering the eco-evolutionary dynamics of the CF lung by therapeutically applying phage, we must understand the potential consequences this may have on the diversification of *P. aeruginosa*. Additionally, these dynamics may be different *in vivo* due to the presence of a competitor.

As such, the morphotype diversification of *P. aeruginosa* was examined following 21 days evolution in SCFM with phage and competitor, by plating the evolved populations onto LB agar. Fig. 6 shows representative images of the diverse range of morphotypes observed. Fig. 5 indicates that, generally, phage increased the presence of non-ancestral *P. aeruginosa* morphotypes, with most phage-cultured populations showing a greater proportion of non-ancestral morphologies and greater number of diverse morphologies. Additionally, there may also be an effect of presence of a competitor on the diversification of *P. aeruginosa*, with increased number and proportion of diverse morphologies evolved in the absence of a competitor.
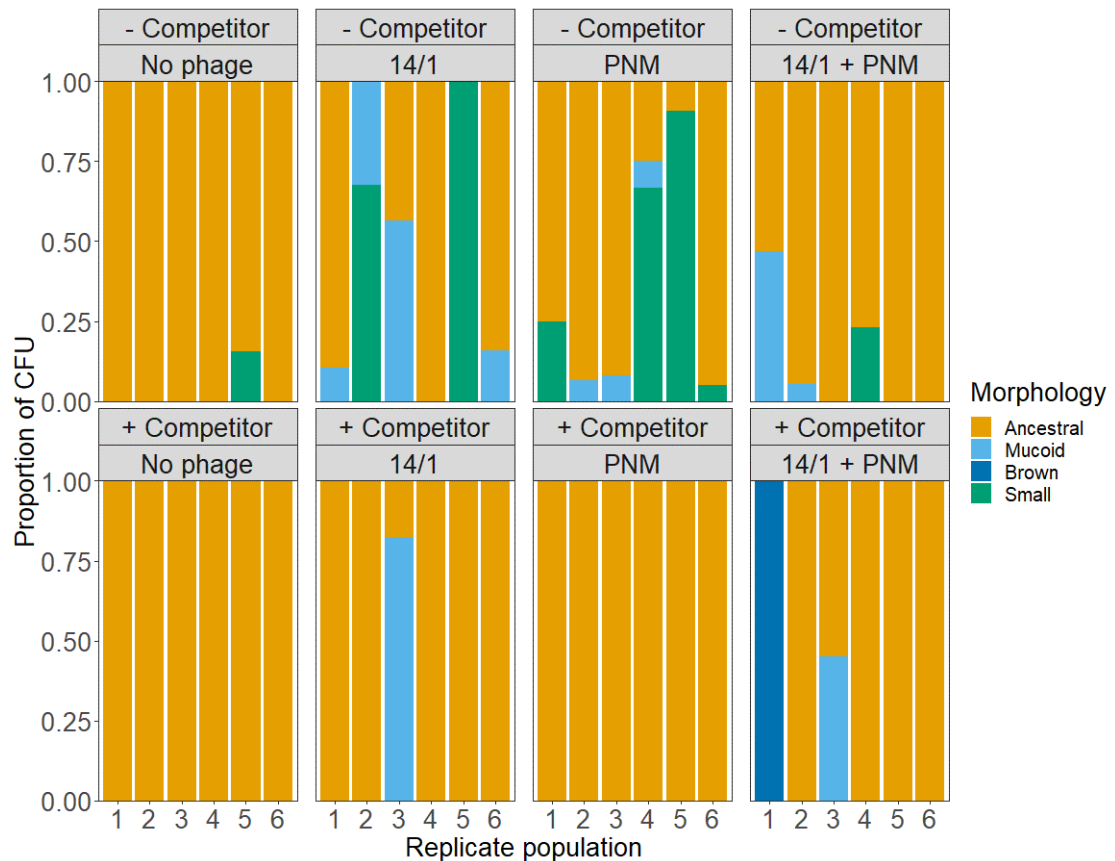
Figure 5: Proportion of *P. aeruginosa* colony forming units (CFU) with different morphotypes following 21 days evolution in SCFM. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath.
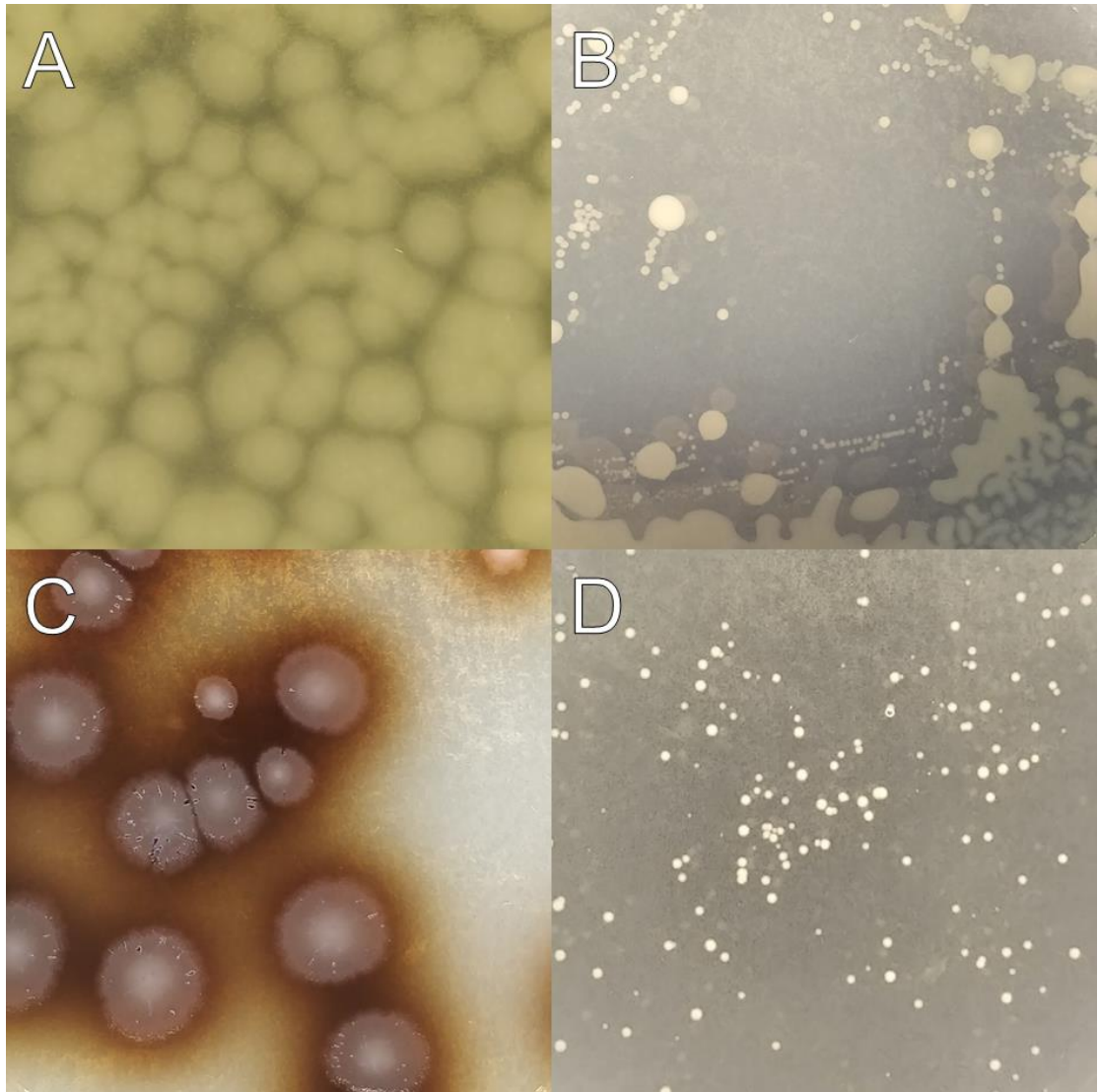
Figure 6: Representative images of colony morphotypes grown on LB agar following the evolution experiment. A) Ancestral B) Mucoid C) Brown D) Small.

To summarise the effect of phage and competitor on the evolution of non-ancestral *P.* aeruginosa morphotypes, a binary code was applied to each replicate to indicate whether that population contained a divergent morphotype (Fig. 7). Greater numbers of non-ancestral morphotypes were observed when *P. aeruginosa* was evolved in the absence of competitor (Mann-Whitney: $W = 432$, $n_1 = 24$, $n_2 = 24$, $p = 0.0004188$). Additionally, when *P. aeruginosa* evolved in the absence of a competitor, phage had a significant effect on the diversification of non-ancestral morphotype (Kruskal-Wallis: $\chi^2 = 10.052$, *d.f.* $= 3$, *p* $= 0.01813$), but not in the presence of the

competitor (Kruskal-Wallis: χ^2 = 4.0159, *d.f.* = 3, *p* = 0.2598). Therefore, phage has a significant effect on the diversification of *P. aeruginosa* morphotype, but this effect is abrogated when *P. aeruginosa* is evolved in the presence of its competitor, *S. maltophilia*. Competitor presence may constrain the evolution of *P. aeruginosa* morphotype due to the clear negative effect *S. maltophilia* has on *P. aeruginosa* population density in coculture, as found previously (Fig. 4). Therefore, a smaller bacterial population has less opportunity to evolve and may explain why less diversity is observed when *P. aeruginosa* is cultured with a competitor and phage.
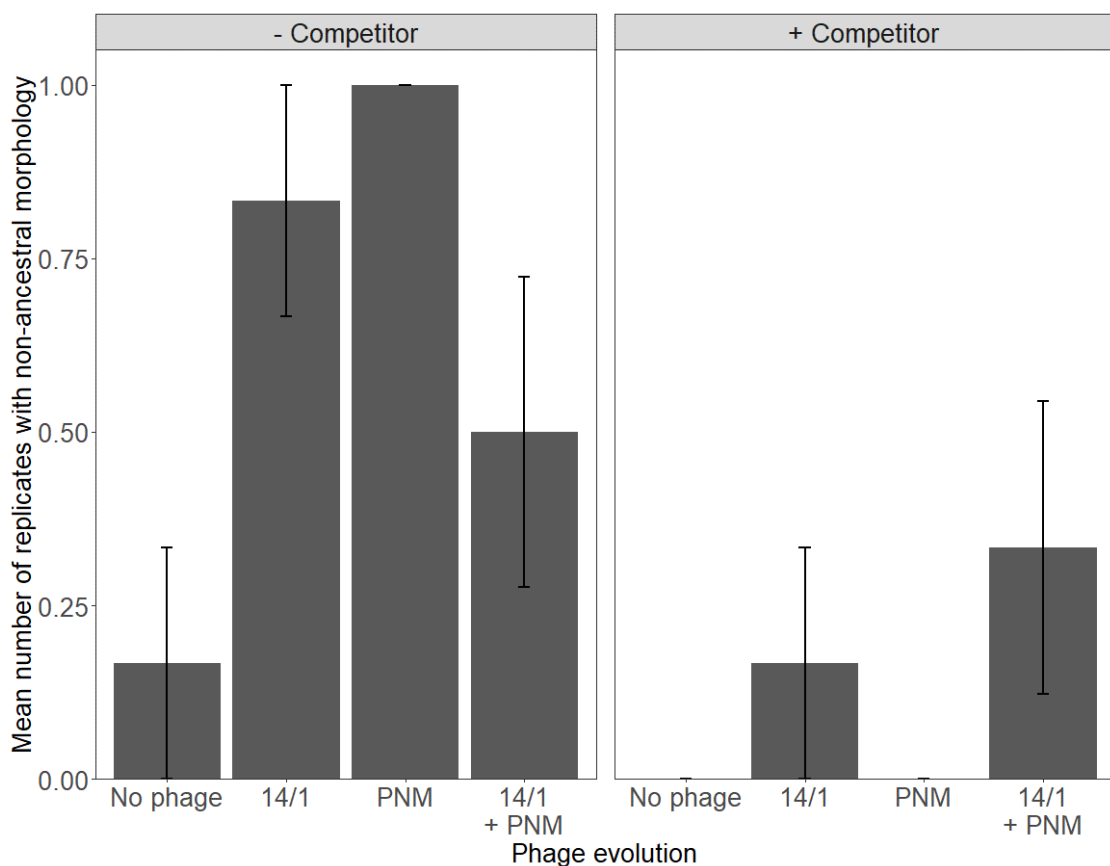
Figure 7: Mean number of replicate populations in which *P. aeruginosa* evolved a non-ancestral morphology. Mean ± SEM, n = 6 biological replicates.

Although phage promotes the evolution of diverse *P. aeruginosa* morphotypes, as found herein, this effect is mitigated by the presence of a competitor. As such, *in vivo*, phage may not have the diversifying effect it is

predicted to have by *in vitro* studies (Hosseinidoust *et al.*, 2013), due to the frequent presence of bacterial competitors. Therefore, therapeutic application of phage may reduce *P. aeruginosa* densities and may not have such a significant effect on diversification of morphotypes, as predicted *in vitro*. Additionally, if these morphotypes are no more virulent than the ancestral type, then the diversifying effect of phage may be even less relevant to clinical outcomes. This will be examined later with respect to antibiotic susceptibility and phage resistance, however more work on these isolated clones would be ideal to understand any further changes to their virulence such as production of virulence factors pyoverdine and pyocyanin, swimming, twitching and swarming motility, and their capacity to cause disease in the *Galleria mellonella* model.

Morphotypes of clones were stable and retained their growth style when restreaked onto new agar plates (data not shown). Therefore, it is likely that there are genetic determinants of colony morphotype. A future direction of this work could include sequencing these clones to link morphotype to genetic mutations.

3.3 Evolution of phage resistance

It was hypothesised that *P. aeruginosa* evolved resistance to phages 14/1 and PNM in coculture, due to the observed increase in density of *P. aeruginosa* over the course of the evolution experiment (Fig. 4). Additionally, evolution of phage resistance over a short period of time has been observed previously (Yang *et al.*, 2020). Phage resistance was quantified as bacterial growth in the presence of ancestral phages relative to bacteria that had evolved in the absence of phage during the selection experiment (Fig. 8)
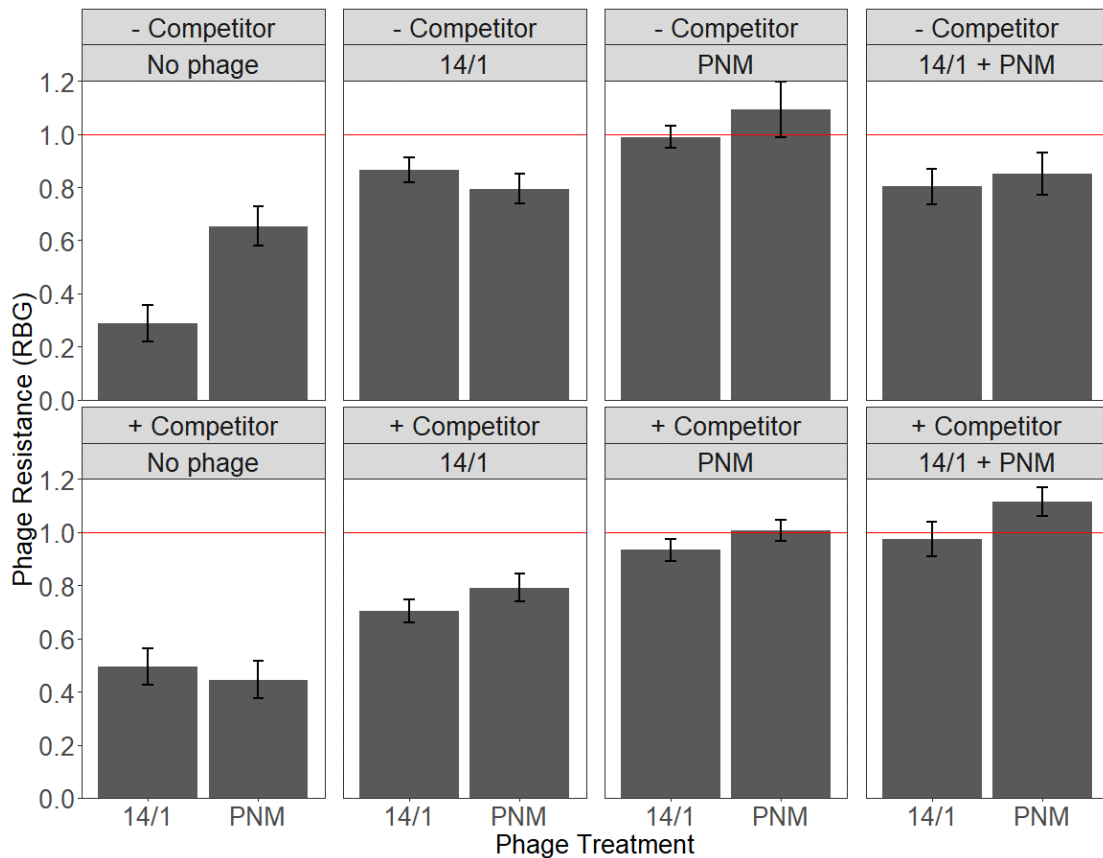
Figure 8: Resistance of evolved *P. aeruginosa* to phage 14/1 and PNM. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. The red line indicates complete resistance to phage. RBG = relative bacterial growth.

The model found a non-significant main effect of competitor presence during the evolution experiment (hereafter referred to as 'competitor evolution') on phage resistance (ANOVA: $F_{1,487} = 1.73$, $p = 0.1888$). However, there was a significant effect of phage presence (hereafter referred to as 'phage evolution') during the evolution experiment on phage resistance (ANOVA: $F_{3,487} = 56.23$, $p < 0.001$). Additionally, the type of phage that clones were subsequently exposed to (phage treatment) had a significant effect on phage resistance (ANOVA: $F_{1,487} = 4.98$, $p = 0.0261$). There were significant interactions between competitor evolution and phage evolution on phage resistance (ANOVA: $F_{3,487} = 3.64$, $p = 0.0128$). Therefore, the data was subset into -competitor and +competitor categories to allow greater understanding of the direction of these effects. As there was a significant

effect of phage treatment on resistance, further mixed effects models were constructed whereby the resistance to each phage treatment were set as response variables and phage presence set as explanatory variables.

For clones evolved in the absence of competitor, previous exposure to 14/1 or PNM phage during the evolution experiment resulted in increased phage resistance against ancestral 14/1 phage relative to no-phage control treatment (ANOVA: $F_{3,99} = 29.12$, $p < 0.001$). Therefore, *P. aeruginosa* evolved resistance to 14/1, independent of whether it had been exposed to 14/1 or PNM phage, indicative of cross-resistance evolution.

For clones evolved in the absence of competitor, there was a significant effect of phage evolution on resistance to PNM (ANOVA: $F_{3,99} = 4.45$, $p = 0.0056$). However, post-hoc pairwise comparison reveals that only *P. aeruginosa* isolates that have evolved in the presence of PNM alone have significantly increased resistance to PNM relative to control (Tukey: $p = 0.00436$). *P. aeruginosa* that evolved with 14/1 or 14/1 + PNM have non-significant differences in resistance to PNM relative to control. Therefore, the dynamics of phage cross-resistance evolution for PNM are different to that of 14/1, meaning there is a unidirectional evolution of cross-resistance whereby *P. aeruginosa* evolved with PNM evolved resistance to 14/1, but *P. aeruginosa* evolved with 14/1 does not evolve resistance to PNM.

Altered dynamics of phage resistance evolution are found when clones evolved in the presence of the competitor. There was a significant effect of phage evolution on resistance against 14/1 (ANOVA: $F_{3,76} = 14.26$, $p < 0.001$). However, post-hoc comparison of means reveals that *P. aeruginosa* evolved with 14/1 has non-significantly different resistance to 14/1, relative to control evolution populations (Tukey: $p = 0.079$). Therefore, the presence of a competitor abrogates the evolution of 14/1 resistance in populations that have evolved with 14/1. However, the presence of a competitor does not block the evolution of resistance to 14/1 in populations that have evolved with PNM or 14/1 + PNM, with their means differing significantly to control populations (Tukey: $p < 0.001$)

For clones evolved in the presence of competitor, previous exposure to 14/1 or PNM phage during the evolution experiment resulted in increased phage resistance against ancestral PNM phage relative to no-phage control treatment (ANOVA: $F_{3,99}$ = 29.12, $p < 0.001$). Therefore, *P. aeruginosa* evolved resistance to PNM, independent of if it had previously been exposed to 14/1 or PNM phage, indicative of cross-resistance evolution. This dynamic is different to that observed when *P. aeruginosa* is evolved in the absence of competitor, as previously PNM cross-resistance did not evolve in -competitor treatments. Therefore, the presence of a competitor allows for the evolution of cross-resistance.

It is unexpected that evolution of cross resistance occurs because the phages co-evolved here infect *P. aeruginosa* via different receptors. Phage 14/1 infects the host by binding to an LPS receptor (Betts *et al*., 2014) and PNM gains entry via type IV pili (Ceyssens *et al*. 2011). Therefore, the mechanism by which resistance evolved may not be associated with mutation of the receptor that the phage uses to enter the host (Broniewski *et al*. (2020), but mutations of global regulator genes that may control the expression of multiple phage receptors (Wright *et al*., 2019).

3.3.1 Effect of colony morphology on phage resistance

The mucoid phenotype is governed by overproduction of exopolysaccharides and is thought to grant resistance to phage by blocking access to the phage receptor (Darch et al., 2017). Therefore, it was hypothesised that different observed morphologies (Fig. 6) may have differential susceptibility to phage. Due to the stochastic appearance of morphologies in each of the evolution lines, it was not possible to analyse the data as a full data set, given that not all evolution lines produced the same morphologies and indeed sometimes only one morphology was observed. As such, for evolution lines that produced multiple morphologies, the effect of morphology on phage resistance was compared within each of these evolutions. However, there was no effect of morphology on phage resistance, regardless of which evolution was analysed (Appendices Fig. 1). The reason why the present study might have found that the mucoid colony phenotype does not grant

resistance to phage is due to the experimental design – mucoid colonies were inoculated into SCFM concurrently with phage. As such, the mucoid phenotype was not allowed to develop and thus provide protection to phage, before being exposed to infection.

3.4 Trade-offs in antibiotic susceptibility associated with phage resistance

It was hypothesised that *P. aeruginosa* that has evolved in the presence of phage and developed resistance (Fig. 8) may have associated fitness trade-offs. Antibiotics selected for *P. aeruginosa* susceptibility testing were colistin, tobramycin and meropenem, as the former two are the most commonly used antibiotics to treat CF in the UK (Hewer, 2012). Concentrations of antibiotic tested were selected from preliminary ETEST strip data (data not shown) according to growth/no growth of ancestral colonies. It was necessary to drop the 14/1 + PNM phage evolutions from experimental analysis due to shortened time available for laboratory work, due to the impact of COVID-19.

3.4.1 Quantifying phage resistance-growth trade-offs

To understand what effect evolving in the presence of a competitor and phage has on antibiotic susceptibility, we must first understand whether growth in the absence of antibiotics is affected by these variables. This is because reduced growth in the absence of antibiotics may be a compounding factor when trying to understand how growth is affected by antibiotics. As such, pairwise comparisons between *P. aeruginosa* that was evolved with or without competitor and in the absence of phage (Fig. 9, 10, 11), reveal that evolution with of *S. maltophilia* has a significant effect on the growth of *P. aeruginosa* at 0 µg/mL antibiotic ($p = 0.0484$), whereby competitor-evolved *P. aeruginosa* has increased growth relative to *P. aeruginosa* that has evolved alone. Additionally, for *P. aeruginosa* that evolved in the absence of competitor, contrasts between no phage and 14/1 or PNM evolution reveal that evolution with PNM, but not 14/1, has a significant negative effect on the growth of *P. aeruginosa* ($p = 0.0043$ and $p = 0.1312$, respectively). However, for clones that evolved in the presence of *S. maltophilia*, both phage 14/1 and PNM significantly reduced the growth of *P. aeruginosa* ($p < 0.001$) relative to no phage evolution. Therefore, the effect of

evolving with phage on growth of *P. aeruginosa* is dependent on the presence of a competitor, suggesting interaction between these variables. To investigate these interactions further, 14/1 and PNM evolutions were contrasted against their counterparts in each competitor evolution. This revealed that there was an interaction between competitor presence and 14/1 ($p = 0.0045$) whereby the presence of a competitor and phage reduces the growth of *P. aeruginosa* below that of 14/1 alone, but not for competitor and PNM ($p = 0.8092$). To summarise, phage resistance evolution causes trade-offs with maximum growth, and for 14/1 there are additive negative effects of evolution with *S. maltophilia*. As such, the growth of *P. aeruginosa* is reduced when evolved in the presence of competitor or phage.

3.4.2 Colistin susceptibility

To test for significant effects of phage and competitor evolution on the susceptibility of evolved clones to colistin (Fig. 9) a linear mixed effects model was constructed. The model found significant effects of competitor evolution (ANOVA: $F_{1,835} = 144.37$, $p < 0.001$), phage evolution (ANOVA: $F_{2,835} = 367.66$, $p < 0.001$) and concentration of colistin used in growth measurements (ANOVA: $F_{3,835} = 25.46$, $p < 0.001$) on bacterial density, with significant interactions between all three of these variables ($p < 0.001$). Therefore, to allow greater understanding of the interaction of these variables, post-hoc pairwise comparisons of means via Tukey tests were conducted.
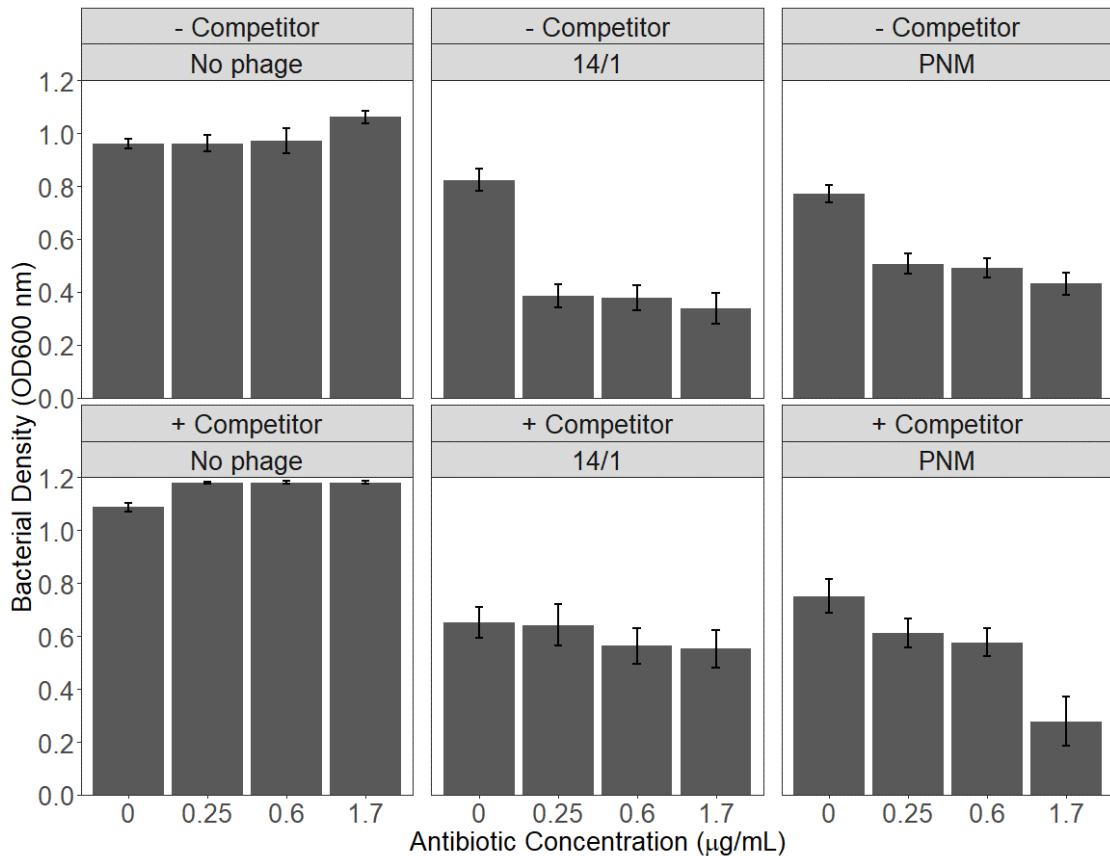
Figure 9: Susceptibility of evolved *P. aeruginosa* to colistin. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment*,* with which phage was present indicated underneath. Mean ± SEM.

For *P. aeruginosa* that was evolved in the absence of competitor and phage (Fig. 9 top-left), there was no effect of increasing concentrations of colistin on growth (all pairwise comparisons to 0 µg/mL colistin $p > 0.05$). However, when *P. aeruginosa* evolved with 14/1 (Fig. 9 top-centre), concentration of colistin had a significant negative effect on growth (all pairwise comparisons to 0 µg/mL colistin $p < 0.001$). Additionally, when *P. aeruginosa* evolved with PNM (Fig. 9 top-right), the same effects were observed (all pairwise comparisons to 0 µg/mL colistin $p < 0.001$). Therefore, evolution with phage in the absence of a competitor and thus developing resistance to phage (Fig. 8), caused trade-offs in colistin susceptibility whereby phage-resistant *P. aeruginosa* becomes more susceptible to colistin.

For *P. aeruginosa* that was evolved in the presence of competitor, in the absence of phage (Fig. 9 bottom-left), there was no effect of increasing concentrations of colistin on growth (all pairwise comparisons to 0 µg/mL colistin p > 0.05). Similarly, when *P. aeruginosa* evolved with 14/1 (Fig. 9 bottom-centre) there was no effect of increasing concentration of colistin on growth (all pairwise comparisons to 0 µg/mL colistin p > 0.05). However, the negative effects of colistin concentration may not be seen because the poor growth of *P. aeruginosa* in the absence of antibiotics compounded this result. Additionally, when *P. aeruginosa* evolved with PNM (Fig. 9 bottom-right), there were non-significant comparisons between 0 µg/mL and 0.25 µg/mL, or 0.60 µg/mL. However, there was a significant difference between 0 µg/mL and 1.70 µg/mL (p < 0.001). Therefore, in this evolution, only high concentrations of colistin reduced the growth of *P. aeruginosa* below that seen in the absence of antibiotic. However, as reported previously, because the growth in the absence of antibiotic of *P. aeruginosa* that has evolved with competitor and phage is significantly lower than that in the control evolution, this was a compounding effect when investigating the effect of concentration of colistin on growth. However, if the growth in each concentration of colistin was contrasted against its counterpart in the no-phage control (compare Fig. 9 bottom-centre and bottom-right to bottom-left), then significant reduction of growth was observed (all pairwise comparisons p < 0.001).

To further investigate how evolution with competitor interacts with phage in influencing colistin susceptibility, contrasts between competitor evolutions at the same concentration of colistin were made. For *P. aeruginosa* evolved with 14/1, this revealed that evolution in the absence of competitor increased trade-offs in colistin susceptibility above that of evolution in the presence of competitor (Fig. 9 compare top-centre to bottom-centre) (contrasts against each level of colistin P < 0.05). However, for PNM evolution, the only significant contrast in growth is seen at 1.7 µg/mL colistin (Fig. 9 compare top-right to bottom-right). Therefore, PNM and competitor do not interact to alter trade-offs in colistin susceptibility, above the effect of evolution with phage.

In conclusion, phage resistance evolution increased colistin susceptibility and the presence of a competitor marginally decreased this effect for 14/1. This has an important therapeutic implication, as it suggests that following phage exposure, *P. aeruginosa* could be eradicated by subsequent application of colistin. Indeed, synergy has been shown between specific combinations of phage and antibiotics (Chaudry *et al.*, 2017), whereby antibiotic-phage treatment reduces *P. aeruginosa* densities below the level expected if both factors killed independently. However, rapid resistance evolution to antibiotic-phage treatment has been reported (Moulton-Brown and Friman, 2018).

The findings herein are supported by the work of Gurney *et al.* (2020), who found that coculturing *P. aeruginosa* with lytic phage decreased MICs of ciprofloxacin and tetracycline relative to *P. aeruginosa* that was evolved alone. The present findings extend our understanding of the interactions between phage and different antibiotics in influencing the evolution of *P. aeruginosa*. Additionally, as Gurney *et al.* (2020) used traditional lab media, we can conclude that evolution with phage in SCFM does not alter the expected trade-offs in antibiotic susceptibility. As such, similar trade-offs may be expected *in vivo*.

These findings are in part supported by the work of Bottery *et al.* (2021) who found that *S. maltophilia* can increase the MIC of imipenem against *P. aeruginosa*. However, where the present work differs to that of Bottery *et al.* (2021) is that following coculture, *P. aeruginosa* was isolated from *S. maltophilia* before antibiotic susceptibility testing, whereas Bottery *et al.* (2021) measured antibiotic susceptibility in coculture. Therefore, the mechanism to explain why evolving in the presence of 14/1 and *S. maltophilia* decreased the susceptibility of *P. aeruginosa* to colistin is dependent on the interactions between *S. maltophilia* and *P. aeruginosa* during coculture i.e., how *S. maltophilia* has influenced the evolution of *P. aeruginosa*, and not caused by the presence of *S. maltophilia* directly.

3.4.3 Tobramycin susceptibility

To test for significant effects of phage and competitor evolution on the susceptibility of evolved clones to tobramycin (Fig. 10) a linear mixed effects

model was constructed. The model found significant effects of competitor evolution (ANOVA: $F_{1,835} = 33.74$, $p < 0.001$), phage evolution (ANOVA: $F_{2,835} = 281.39$, $p < 0.001$) and concentration of tobramycin (ANOVA: $F_{3,835} = 1306.31$, $p < 0.001$) on bacterial density, with significant interactions between all three of these variables ($p < 0.001$). Therefore, to allow greater understanding of the interaction of these variables, post-hoc pairwise comparisons of means via Tukey tests were conducted.
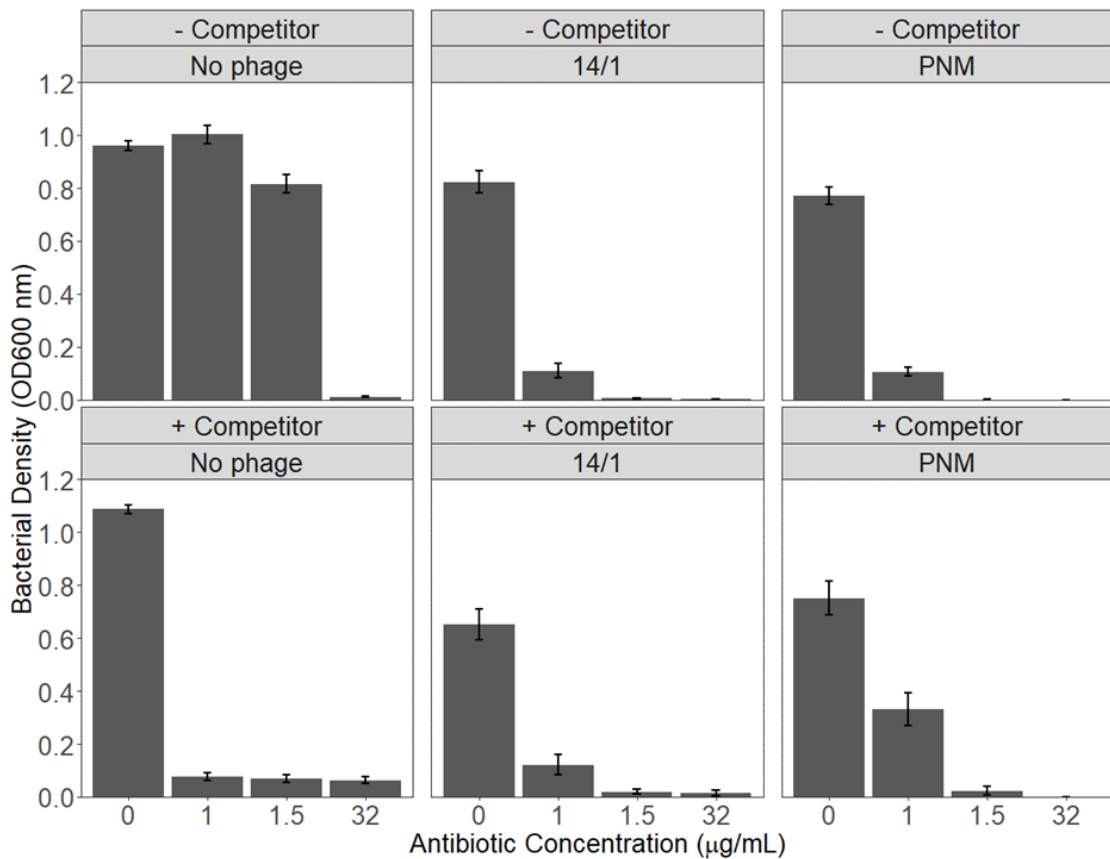
Figure 10: Susceptibility of evolved *P. aeruginosa* to tobramycin. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. Mean ± SEM.

The pattern of trade-offs with tobramycin susceptibility differs from that of colistin susceptibility, however some overall effects remain the same. For *P. aeruginosa* that has evolved in the absence of competitor and phage (Fig. 10 top-left), there was a significant negative effect of increasing concentration of

tobramycin on growth. When the growth of different concentrations of tobramycin were contrasted against growth in 0 µg/mL, there was a significant difference in growth at 1.5 µg/mL (p = 0.0022), and 32 µg/mL (p < 0.001), but not 1 µg/mL. However, when *P. aeruginosa* evolved with either 14/1 or PNM, all concentrations of tobramycin significantly reduce growth (all pairwise comparisons to 0 µg/mL tobramycin p < 0.001).

As increased concentrations of tobramycin (1.5 and 32 µg/mL) significantly reduce the growth of *P. aeruginosa* that has not evolved with competitor or phage, it is important to contrast the growth of *P. aeruginosa* at each concentration of tobramycin against its counterpart in the phage treatment, to provide a full understanding of how evolving with phage affects growth of *P. aeruginosa*. Thus, there were significant differences in growth between no phage and 14/1 or PNM at 1 µg/mL (p < 0.001) and 1.5 µg/mL (p < 0.001) tobramycin, but not 32 µg/mL (compare Fig. 10 top-centre and top-right to top-left), as a tobramycin concentration of 32 µg/mL significantly reduces the growth of *P. aeruginosa* that has evolved without phage. As such, evolving with phage increases tobramycin susceptibility of *P. aeruginosa*, independent of which phage the focal bacteria have evolved with. However, this effect depends on concentration of tobramycin and is not seen at very high concentrations of tobramycin, as *P. aeruginosa* that has not evolved without competitor and phage is susceptible to 32 µg/mL tobramycin.

For *P. aeruginosa* that evolved in the presence of competitor, in the absence of phage, all concentrations of tobramycin significantly reduce its growth (Fig 6. bottom-left) (all pairwise comparisons to 0 µg/mL colistin p < 0.001). Therefore, the presence of *S. maltophilia* alone during the evolution experiment increased the tobramycin susceptibility of *P. aeruginosa*. Moreover, when *P. aeruginosa* evolved with competitor and with either 14/1 (bottom-centre) or PNM (bottom-right), there was a significant effect of concentration of tobramycin on growth (all pairwise comparisons to 0 µg/mL tobramycin p < 0.001). However, as the +competitor, no phage control was susceptible to all concentrations of tobramycin, due to evolution with competitor altering tobramycin sensitivity, it is important to contrast the growth of *P. aeruginosa* at each concentration of tobramycin against its

counterpart in the phage treatment. Thus, there are no significant negative differences in growth between no phage and 14/1 or PNM at all concentrations of tobramycin (compare Fig. 10 bottom-centre and bottom-right to bottom-left). Therefore, there is a greater effect of evolving with competitor compared to the effect of evolving with phage on increasing tobramycin susceptibility.

3.4.4 Meropenem susceptibility

To test for significant effects of phage and competitor evolution on the susceptibility of evolved clones to meropenem (Fig. 11) a linear mixed effects model was constructed. The model found significant effects of competitor evolution (ANOVA: $F_{1,835}$ = 32.44, p < 0.001), phage evolution (ANOVA: $F_{2,835}$ = 54.22, p < 0.001) and concentration of meropenem (ANOVA: $F_{3,835}$ = 1593.38, p < 0.001) on bacterial density, with significant interactions between competitor and phage evolution (p < 0.001), phage evolution and concentration of meropenem (p < 0.001), but not competitor and concentration of meropenem. Therefore, to allow greater understanding of the interaction of these variables, post-hoc pairwise comparisons of means via Tukey tests were conducted.
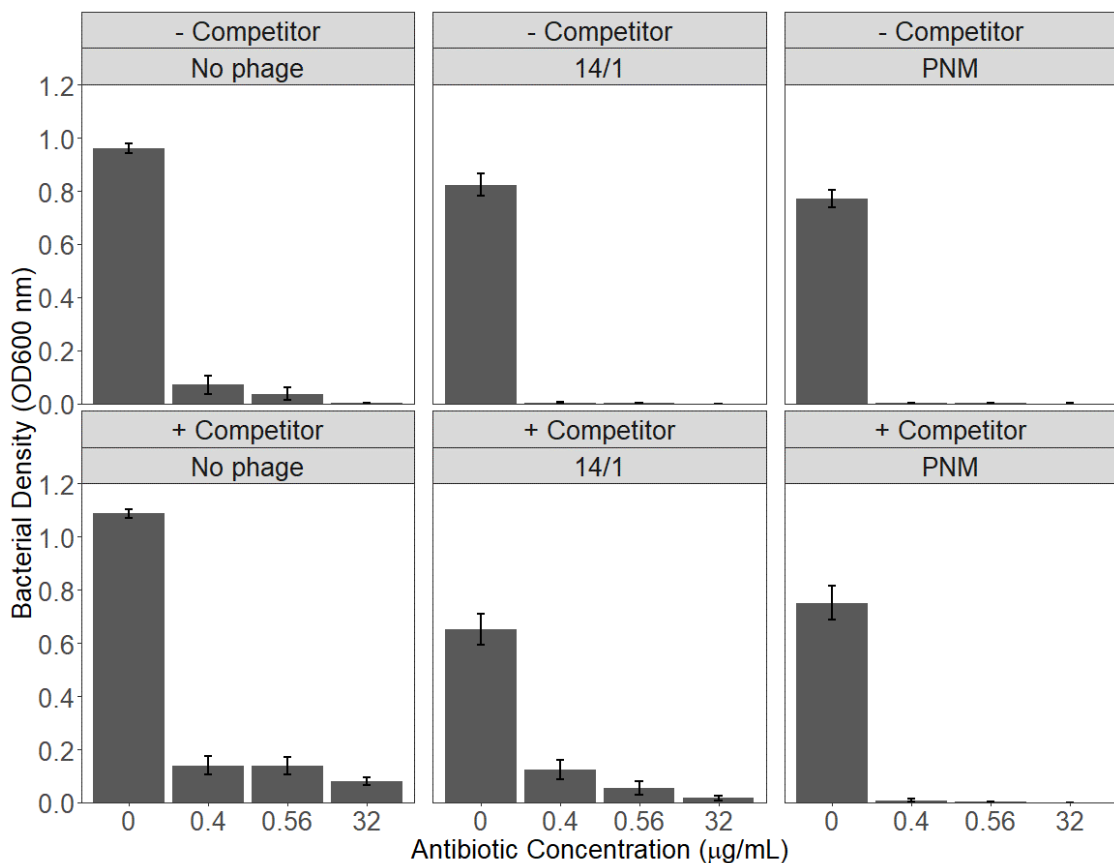
Figure 11: Susceptibility of evolved *P. aeruginosa* to meropenem. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. Mean ± SEM.

For *P. aeruginosa* that evolved in the absence of competitor and phage (Fig. 11 top-left), there was a significant effect of increasing concentration of meropenem on growth, with all pairwise comparisons to 0 µg/mL significantly reducing growth ($p < 0.001$). When *P. aeruginosa* evolved in the absence of competitor and with either 14/1 or PNM, there was a significant effect of concentration of meropenem on growth (all pairwise comparisons to 0 µg/mL meropenem $p < 0.001$). However, because the growth of the no phage control was significantly reduced by all concentrations of meropenem, then there is no increase in susceptibility following evolution with phage. Indeed, there are no significant differences in growth when the same concentrations of meropenem in 14/1 and PNM are contrasted against that in +competitor, no phage (compare Fig. 11 top-centre and top-right to top-left).

For *P. aeruginosa* evolved in the absence of competitor, in the absence of phage (top-left), all concentrations of meropenem significantly reduced growth. The MIC_{90} of *P. aeruginosa* is 8 µg/mL (Sader *et al.*, 2017), as such, *P. aeruginosa* evolves meropenem susceptibility, not due to the presence of competitor or phage, but due to the presence of a factor in all treatments. This is likely due to evolving in synthetic cystic fibrosis medium (Palmer *et al.*, 2007) as Schick and Kassen (2018) found that evolving *P. aeruginosa* in SCFM influenced the diversification of *P. aeruginosa* across a range of metrics, including evolution of ciprofloxacin resistance. It may be expected that *P. aeruginosa* could evolve meropenem sensitivity due to the evolutionary pressure of adapting to the complex synthetic nutritional environment of the CF lung. It is interesting that SCFM may have caused the evolution of meropenem susceptibility, but this effect was not seen with regards to colistin and tobramycin susceptibility. Indeed, Schick and Kassen (2018) also found no effect of evolution in SCFM on colistin or tobramycin resistance.

Likewise, for *P. aeruginosa* that evolved with competitor and with either 14/1 (bottom-centre) or PNM (bottom-left), there was a significant effect of concentration of meropenem on growth (all pairwise comparisons to 0 µg/mL tobramycin $p < 0.001$). However, because the growth of the +competitor, no phage control was significantly reduced by all concentrations of meropenem, then there is no increase in susceptibility following evolution with phage and no interaction between evolution with competitor and phage. Although there are small differences in growth between +competitor, PNM and +competitor, no phage, these are not strong enough to be convincing.

3.4.5 Effect of colony morphology on antibiotic susceptibility

It was hypothesised that different observed morphologies (Fig. 6) may have differential susceptibility to antibiotics. Due to the stochastic appearance of morphologies in each of the evolution lines, it was not possible to analyse the data as a full data set, given that not all evolution lines produced the same morphologies and indeed sometimes only one morphology was observed. As such, for evolution lines that produced multiple morphologies, the effect of

morphology on antibiotic susceptibility was compared within each of these evolutions. However, there was no effect of morphology on bacterial density, regardless of which evolution and which antibiotic was analysed (Appendices Fig. 2 – 4). Therefore, the effect of evolution with phage is more important than the diversifying effect of that phage on bacterial morphology, in influencing antibiotic susceptibility. As such, the *in vivo* effect of phage on *P. aeruginosa* morphology may not be relevant to therapeutic outcomes in terms of antibiotic susceptibility.

3.5 Phage coevolution

A primary advantage of phage therapy versus antibiotics is that phages are hypothesised to co-evolve with bacteria to retain infectivity. As such, as the bacteria evolve resistance against the phage and are better able to resist infection, phage also evolves mechanisms to evade the bacterial immunity and retains its ability to infect. Several studies have shown the increased effectiveness of evolved phage versus ancestral phage at reducing host pathogen densities (Buckling and Rainey, 2002; Friman *et al.*, 2016; Borin *et al.*, 2021). However, other studies found weak evidence for these so-called 'arms race dynamics' (Moulton-Brown and Friman, 2018). As such, additional data is needed to understand this phenomenon and studying the coevolution of phage in an environment with and without *S. maltophilia* will provide a greater understanding of whether these dynamics could be expected to occur *in vivo*.

We were interested in whether phage from prior to, during, or after evolution had differential ability to infect evolved clones (Fig. 12). It was hypothesised that phage from day 21 of the evolution experiment would be better able to reduce the density of *P. aeruginosa* than phage from day 12 or ancestral phage. We were interested in whether the presence of a competitor influenced the evolution of phage and whether different phage had different capacity to coevolve. Therefore, a linear mixed effects model was constructed which found a significant effect of applied phage on bacterial density (ANOVA: $F_{2,559} = 8.06$, p = 0.0004). However, there were significant interactions between applied phage and competitor evolution (ANOVA: $F_{2,559}$

= 4.26, p = 0.0146), which suggests that phage coevolution only occurs in one of the levels of competitor evolution. As such, post-hoc Tukey tests were conducted, which revealed that there was no effect of applied phage on bacterial density for phage evolved in the absence of competitor, independent of which phage *P. aeruginosa* was evolved with. For *P. aeruginosa* that evolved with competitor, there was a significant effect of applied phage on bacterial density, however this was dependent on which phage the bacteria were evolved with. Only for PNM were there significant differences in bacterial density between ancestral phage and day 12 and 21 phage. Therefore, PNM can coevolve with *P. aeruginosa*, however this was only observed in the presence of a competitor.
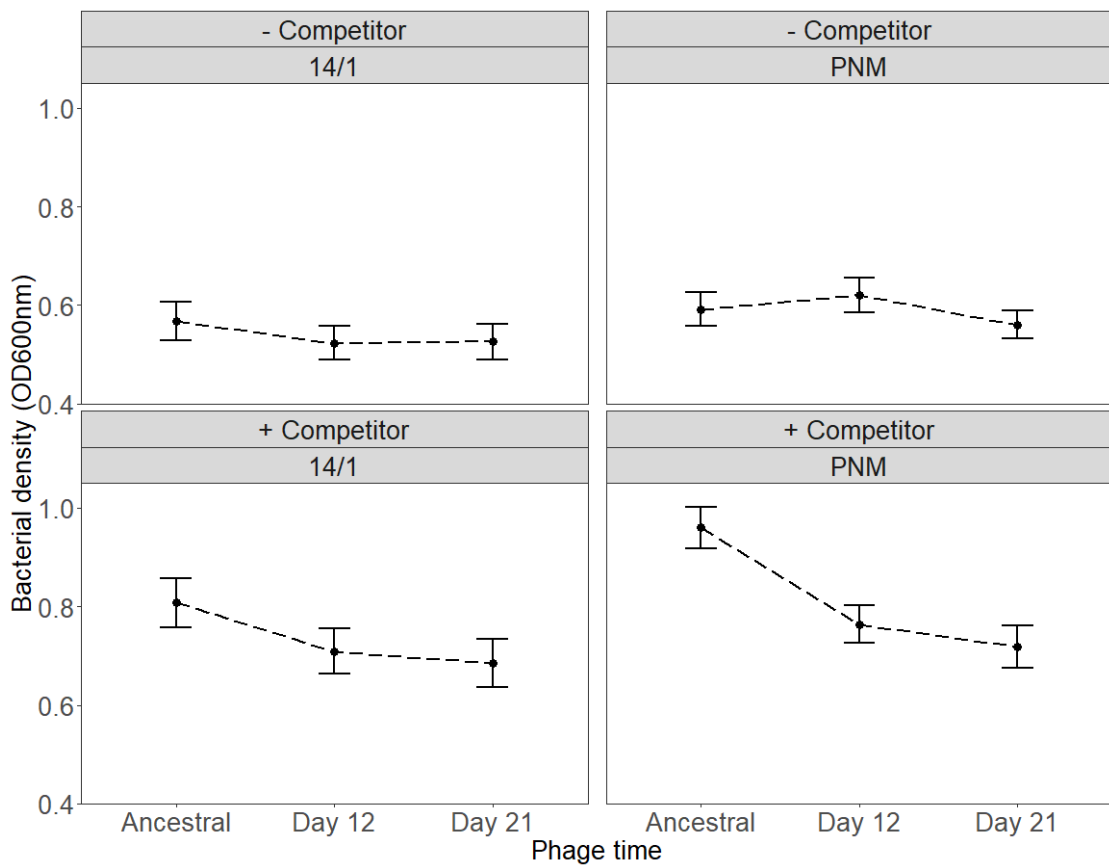
**Figure 12:** Ability of evolved phage to reduce bacterial densities of ancestral *P. aeruginosa*. Mean ± SEM

3.6 Conclusion

In conclusion, phage negatively regulated *P. aeruginosa* density, which allowed the population size of *S. maltophilia* to increase in coculture (Fig. 4). Phage caused colony morphotype diversification of *P. aeruginosa* and this effect was abrogated by the presence of *S. maltophilia* (Fig. 5), likely due to the negative density effects of the competitor. Although interesting, colony morphotype did not have significant effects on phage resistance or antibiotic susceptibility, suggesting limited clinical relevance. Phage resistance evolution arose during the evolution experiment (Fig. 8) and unidirectional cross-resistance was observed. Phage resistance evolution was found to be genetically linked to trade-offs in growth after 48 hours and colistin susceptibility (Fig. 9).The implications of this work suggest a range of implications for the future of phage therapy. As application of phage reduced the density of the focal pathogen, this suggests that phage therapy may be useful to reduce disease in patients with CF. However, phage predation created a niche for colonisation by *S. maltophilia*, which has been linked to increased disease in CF patients. This, taken together with the finding that *P. aeruginosa* evolved resistance to phage and increased in density towards the end of the evolution experiment, mean that *in vivo*, phage may cause increased disease severity over the long term as the population of *P. aeruginosa* recovers and is protected from beta-lactam antibiotics by the detoxifying presence of *S. maltophilia*.

On the other hand, resistance of *P. aeruginosa* to phage was found to be genetically linked to maximum growth after 48 hours and antibiotic susceptibility, meaning *P. aeruginosa* simultaneously grew less well and was more sensitive to antibiotics than control evolution populations. These evolutionary trade-offs could be exploited to the benefit of CF patients by using a combination therapy of phage and antibiotics to eliminate *P. aeruginosa* from patients' lungs. Based on the data herein, antibiotics administered following phage exposure may be more successful than administering antibiotics and phage simultaneously. Indeed, there is work that suggests the latter may be counterproductive due to resistance evolving quickly (Moulton-Brown and Friman, 2018). Future directions of this work

include sequencing *P. aeruginosa* populations evolved in the presence of competitor and phage to understand the genetic determinants of phage resistance evolution and to elucidate the mechanism by which this is linked to trade-offs in antibiotic susceptibility and maximal growth. Additionally, further changes in virulence could be investigated including production of pyoverdine and pyocyanin, and swimming, twitching and swarming motility. By quantifying these changes, a greater understanding of how evolution with competitor and phage affects the virulence of *P. aeruginosa* is captured, thus granting greater clinical relevance. Further to these data, evolved *P. aeruginosa* populations could be administered to the *Galleria mellonella* infection model to understand if observed changes in virulence *in vitro* have a real-world impact on survival of the greater wax moth larvae.
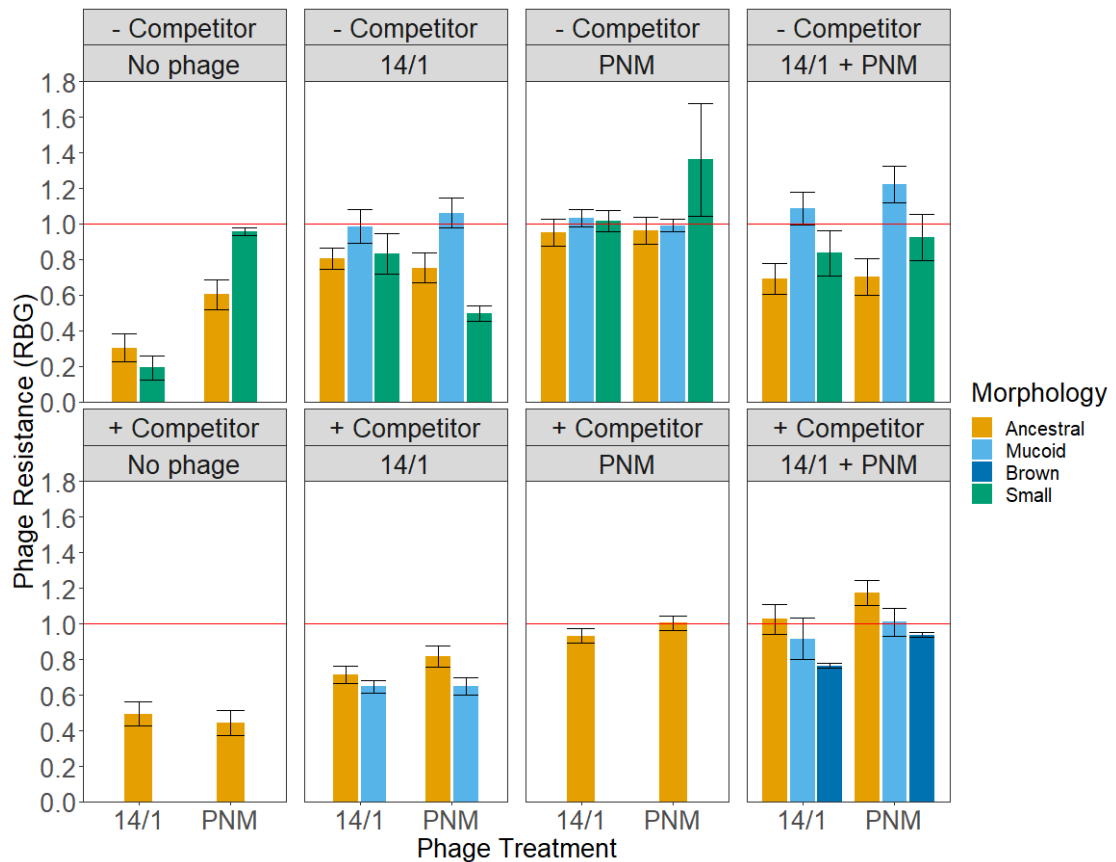
Appendices figure 1: Resistance of evolved *P. aeruginosa* colony morphotypes to phage 14/1 and PNM. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. The red line indicates complete resistance to phage. RBG = relative bacterial growth.

Appendices figure 2: Susceptibility of evolved *P. aeruginosa* colony morphotypes to colistin. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. Mean ± SEM
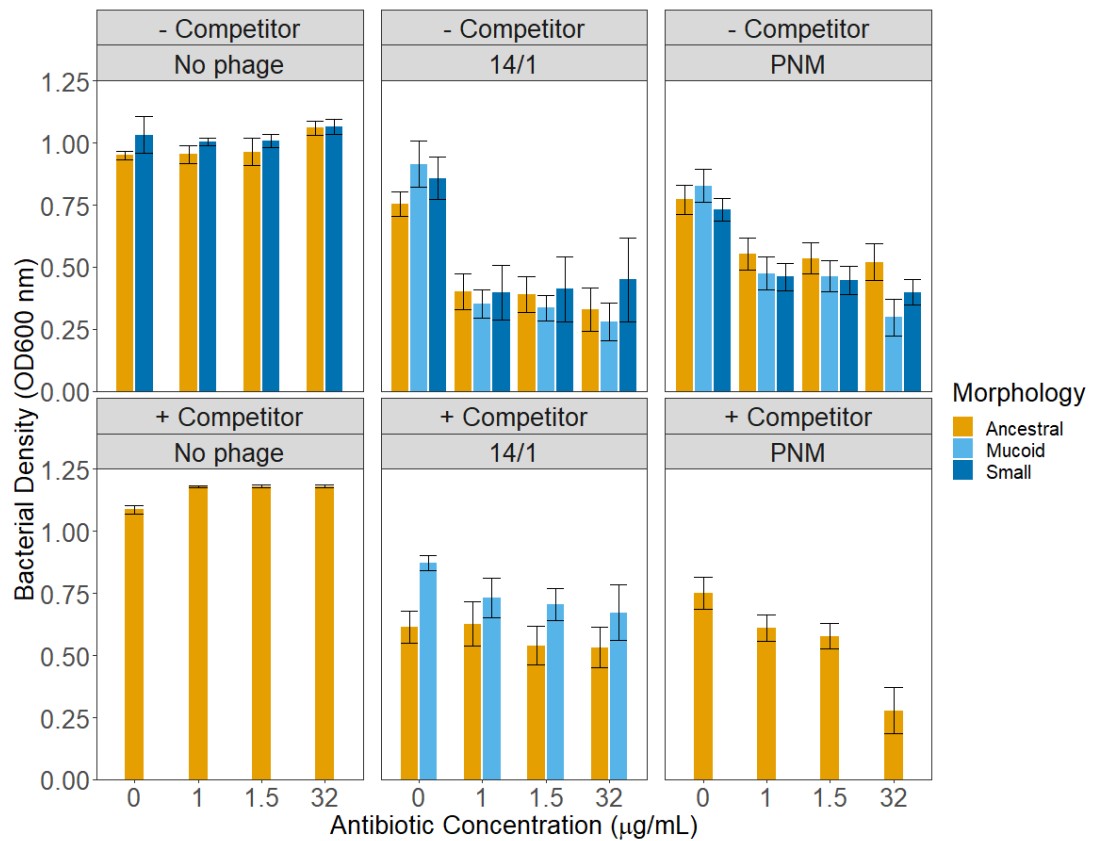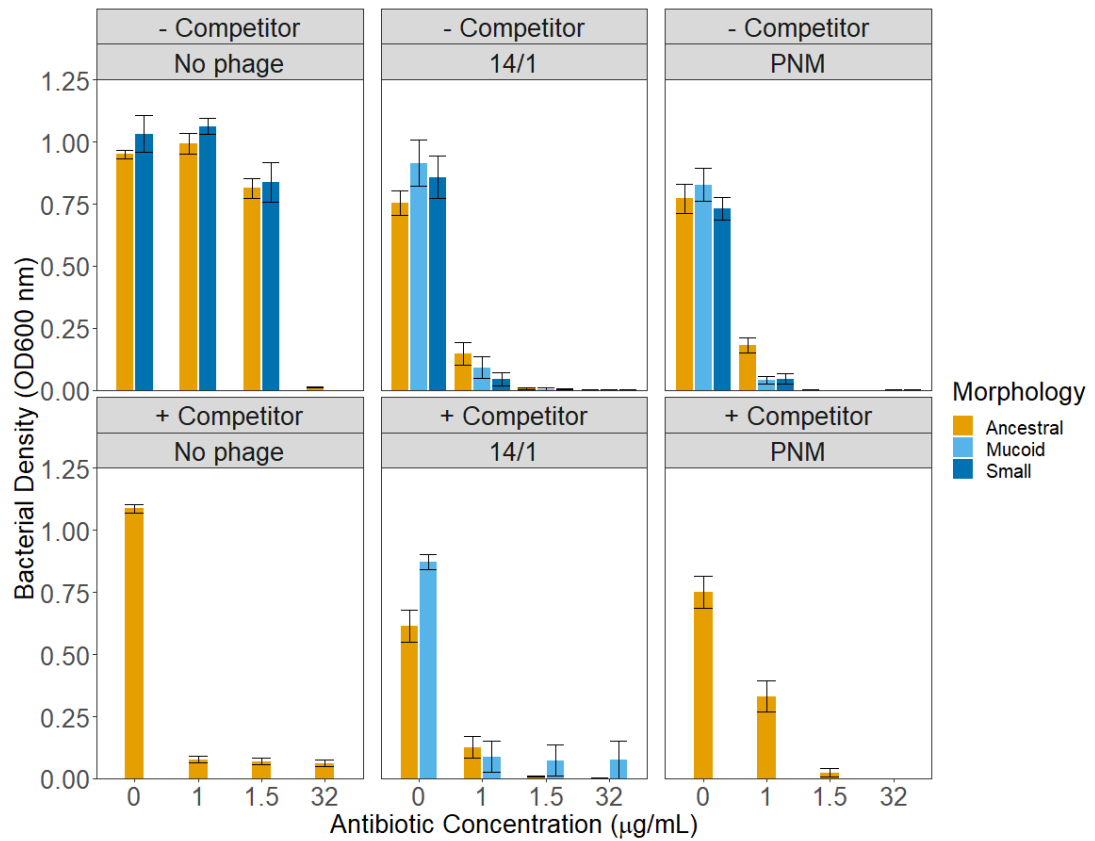
Appendices figure 3: Susceptibility of evolved *P. aeruginosa* colony morphotypes to tobramycin. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. Mean ± SEM
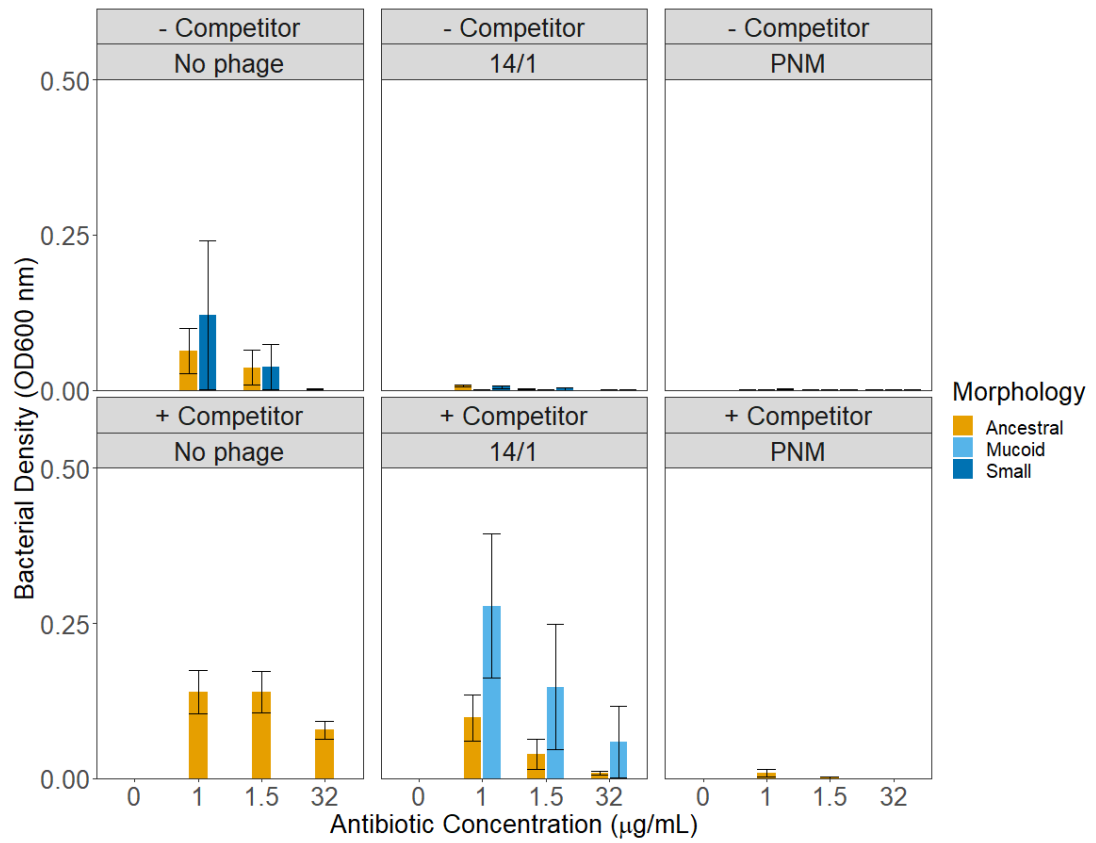
Appendices figure 4: Susceptibility of evolved *P. aeruginosa* colony morphotypes to meropenem. +/- indicates presence/absence of *S. maltophilia* during the evolution experiment, with which phage was present indicated underneath. Mean ± SEM

List of abbreviations

ASL: Airway surface liquid

CF: Cystic fibrosis

CFTR: Cystic fibrosis transmembrane conductance regulator

CFU: Colony forming units

ENaC: Epithelial sodium channel

LB: Luria Bertani broth

LPS: Lipopolysaccharide

MCC: Mucociliary clearance

MIC: Minimum inhibitory concentration

PA: *Pseudomonas aeruginosa*

PFU: Plaque forming units

RBG: Relative bacterial growth

SCFM: Synthetic cystic fibrosis media

SM: *Stenotrophomonas maltophilia*

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