The effects of subinhibitory antibiotic concentrations on *Pseudomonas aeruginosa* within model Cystic Fibrosis bacterial communities

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Abstract

Chronic pulmonary bacterial infections are a leading cause of morbidity and mortality among Cystic Fibrosis (CF) patients. Despite traditional classification of infections in terms of the most prominent detected pathogen, CF pulmonary infections are highly polymicrobial. The most prevalent of the bacterial pathogens among patients is *Pseudomonas aeruginosa*, infection with which results in greatly decreased lung function and increased risk of mortality. Antibiotic treatment is key to managing bacterial infections, but treatment often fails to clear the target bacteria. A contributing factor is likely failure of treatment to deliver sufficient doses of antibiotic to bacterial populations, resulting in exposure to subinhibitory concentrations which are known to select for high-level resistance. In this thesis, I find that, in addition to selecting for high-level resistance, subinhibitory antibiotic concentrations can drastically impact the coculture dynamics between *P. aeruginosa* and another CF-associated species, *Stenotrophomonas maltophilia*, such that the usually dominant *P. aeruginosa* was made to coexist with- or driven extinct by-*S. maltophilia* in cocultures treated with a subinhibitory concentration of tobramycin. From this observation, I go on to find that increasing the size of the community through the addition of *Staphylococcus aureus*, also a CF-associated species, magnifies this effect of tobramycin. However, when the viscosity of the liquid lab media was increased to more resemble the thick mucus in the CF lung, treatment with tobramycin resulted in a community where all three species were able to coexist. Further investigation found that this coexistence was stable in the absence of viscosity and tobramycin, suggesting coevolution between the three species. Likelihood of coexistence differed between different selection lines and was influenced differently by individual evolved species. Sequencing of coexisting isolates did not reveal a concrete mechanism, but suggested candidate genes in *P. aeruginosa* involved with polyamine synthesis that warrant further investigation. Sequencing also found that mutations were constrained in communities of increasing complexity. Together these findings suggest that the results of ineffective antibiotic treatments can be wide ranging and unexpected, and make the case for further investigation into interspecies interactions within the CF lung, which are currently largely unknown.

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Author's Declaration

I declare that this thesis is a presentation of original work and that 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 History, Pathology, and Prognosis of Cystic Fibrosis

"Woe to the child who tastes salty from a kiss on the forehead, for he is bewitched and will soon die." Recorded in 1857 in a book of children's songs from Switzerland (Rochholz, 1857), this adage warns of the early death of a child that presents with one of the most apparent identifiers of Cystic Fibrosis (CF). Medical descriptions of patients that most likely died from complications of CF have appeared in the 16th and 17th centuries (Navarro, 2016), though it was not until 1936–1938 when the disease was named "Cystic Fibrosis" after descriptions of extensive fibrosis in the pancreas of deceased children (Andersen, 1938; Navarro, 2016).

Cystic Fibrosis is an inherited autosomal recessive disease and the most prevalent inherited disorder amongst Caucasians, with 10 070 cases in the UK in 2019 (UK Cystic Fibrosis Registry, 2020), with a further 39 800 cases in Europe (Zolin *et al.*, 2020) and 31 200 in the USA (Cystic Fibrosis Foundation Patient Registry, 2020); at least 90 000 people worldwide. The genetic basis for CF was identified in 1989 by groups led by Lap-Chee Tsui, Jack Riordan, and Francis S. Collins (Riordan *et al.*, 1989; Rommens *et al.*, 1989). Following the work of Paul Quinton (1983) that implicated a chloride ion channel as the causative agent of the disease, they found both the gene and the mutation—a deletion that resulted in the loss of a phenylalanine residue at position 508 (ΔF508)—responsible for the condition after manually searching through chromosome 7 (Riordan *et al.*, 1989; Rommens *et al.*, 1989). This gene was named the Cystic Fibrosis transmembrane conductance regulator (CFTR) and mutations in this gene—of which ΔF508 is the most common, though over 1000 other causative mutations have been identified (O'Sullivan and Freedman, 2009)—leads to impaired transport of chloride ions across the cell membrane. This imbalance of ions results, primarily, in thicker mucus secretions throughout the body. This mucus affects multiple systems within the body, blocking ducts within the pancreas—resulting in reduced production of digestive enzymes and greatly increased risk of diabetes—and liver—resulting in liver disease—as well as the respiratory system, where uncleared

mucus results in an environment that is suitable for bacterial infection (O'Sullivan and Freedman, 2009).

The genetic cause of CF was found without prior identification of the defective protein, as in the discovery of many other genetic conditions at the time, and this new method was met with much fanfare with the promise of a gene therapy to essentially cure the condition (Pearson, 2009; Cooney, McCray and Sinn, 2018). Though these predictions of a cure have still yet to pass—gene therapy for the condition is still in development (Cooney, McCray and Sinn, 2018)—the treatment over the past 30 years has improved drastically such that half of people born with CF in the UK today will be predicted to live to 49 years old (UK Cystic Fibrosis Registry, 2020), rising from 43 for those born 2007–2011, whilst the median age at death has increased from 23 in 2002 to 31 in 2019 (UK Cystic Fibrosis Registry, 2006). Reductions in patient mortality have come from improvements in the management of the physical impairments relating to the disease, improved diagnosis early in life, and aggressive antibiotic treatment (Davis, 2006). Mucus thinners, bronchodilators, and physiotherapy regimens to clear mucus all aid patients with the difficulties of breathing, whilst pancreatic enzyme supplements and improved awareness of nutritional care aid patients with the digestive complications of the disease (Davis, 2006; Gaskin, 2013). However, the primary cause of mortality remains the chronic bacterial infection within the lungs that leads to inflammation and a severe decline in lung function. Infections are managed with antibiotics (UK Cystic Fibrosis Trust, 2009; Waters *et al.*, 2019), though complete clearance of infections is difficult and antibiotic resistance is a major problem for the long term efficacy of treatments (Waters *et al.*, 2019). Specifically, bacterial responses to antibiotics within the polymicrobial CF lung microbiomes are poorly understood.

1.2 Bacterial Infections in Cystic Fibrosis

1.2.1 The CF lung environment is ideal for bacterial growth

Previously, the lungs of a healthy person were thought to be sterile. However, it has become accepted that, as with perhaps every area of the human body, there is a lung microbiome (Dickson *et al.*, 2015; Li *et al.*, 2016; Feigelman *et al.*, 2017; Einarsson *et al.*, 2019). These are often bacteria microaspirated from the mouth and upper airways (Dickson *et al.*, 2015), and only colonise the lungs transiently and at low abundance (Dickson and Huffnagle, 2015). The problem arises when lung conditions allow chronic colonisation by bacteria, as is the case in CF.

A number of factors make the CF lung environment an ideal place for bacteria to grow. Thick mucus secretions provide a substrate for bacteria to grow in, and impaired mucociliary clearance results in less turnover within the lungs, giving bacteria more time to establish and spread to new areas (O'Sullivan and Freedman, 2009). CF mucus is rich in amino acids and extracellular DNA (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007), providing ample carbon sources on which to grow. Oxygen gradients arise within the mucus, which allows growth of both aerobic and anaerobic bacteria (Conrad *et al.*, 2013). Immune system function is impaired (O'Sullivan and Freedman, 2009), further reducing bacterial clearance, and may cause damage that can further aid bacterial expansion (Conrad *et al.*, 2013; Whelan and Surette, 2015). And the thickness of the mucus and the complex branching structure of the lower airways also poses a physical barrier that can prevent diffusion of antibiotics, increasing difficulties of bacterial clearance during treatment (Moriarty *et al.*, 2007; Bos *et al.*, 2015, 2017).

1.2.2 How to study and sample the lung microbiome

It is very difficult to gain an understanding of the full composition and inside of a live person's lungs. The least invasive and most often employed method of sampling the microbiome of a CF patients' lungs is to analyse the bacteria within an expectorated sample, i.e., sputum from the lungs is coughed up and spit out. This method has the potential downsides of being unrepresentative of the whole of the lungs and possibly being unduly influenced by bacteria in the upper airways, though

there is debate on the matter (O'Toole, 2018). More invasive methods involve patients undergoing a bronchoscopy, in which the lower airways can be directly sampled via bronchoalveolar lavage—washing the airways and sampling the fluid collected—or protected brush—sending a brush down the bronchoscope to directly sample and collect mucus plugs. These more invasive methods enable the sampling of specific regions of the lungs with less risk of cross contamination, at the cost of requiring more specialised equipment and, at the very least, discomfort to the patient.

In much of clinical practice, particularly prior to the advent of affordable DNA-based detection methods, bacteria are identified using classical microbiological techniques through culturing on species-specific selective agar. In the past this led to an underestimation of the numbers of taxa that live within a given patient, and a focus on the easily culturable species that are now termed the "traditional" CF pathogens: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex species—such as *Burkholderia cenocepacia—Haemophilus influenzae,* and *Achromobacter xylosidans*. Infections were, and still are, classified in terms of which of the traditional pathogens have been cultured from the patient's sputum. However, culture-independent detection methods—primarily through amplification and sequencing of the 16S rRNA gene from DNA isolated from the patient samples—have since shown that CF infections can be highly polymicrobial (Zhao *et al.*, 2012; Price *et al.*, 2013; Coburn *et al.*, 2015; Kramer *et al.*, 2015; Mahboubi *et al.*, 2016; Zemanick *et al.*, 2017; Muhlebach *et al.*, 2018; Einarsson *et al.*, 2019; Layeghifard *et al.*, 2019).

1.2.3 Prevalence of "traditional" CF pathogens

Despite the current understanding of a diverse underlying community, focus on the traditional CF pathogens during patient check-ups and treatment still provides some useful information to the clinician in terms of prognosis and treatment focus. As such, detailed information is gathered providing statistics for infection rates.

In the UK in 2019, the most prevalent CF pathogen among children <16 years old was *Staphylococcus aureus*, where 10.2 % of the 3 966 patients were chronically infected—defined here as three or more positive culture tests within a year—and 26.8 % were infected intermittently (UK Cystic Fibrosis Registry, 2020). The two other most prevalent pathogens were *Pseudomonas aeruginosa*, 5.2 % chronic and 18.8 %

intermittent, and *Haemophilus influenzae* with 24.4 % of patients. Important to note is that these figures are not mutually exclusive, in that a patient testing positive for *P. aeruginosa* and *S. aureus* would be counted in both categories, but co-infection proportions were not provided. When split by age group, *H. influenzae* is the most prevalent at ages 0–3 and 4–7, but is overtaken by *P. aeruginosa* at ages 8–11. The prevalence of *P. aeruginosa* infections continues to rises steeply across the age groups, making chronic *P. aeruginosa* infection the most prevalent across all adult patients ≥16 years old in the UK, with 39.4 % of the 6 104 adults chronically infected, and a further 16.7 % intermittently infected (UK Cystic Fibrosis Registry, 2020). The next most prevalent species is *S. aureus*, with 21.0 % chronically infected, and 17.5 % intermittently, followed by species of the fungal genus *Aspergillus* in 19.8 % of adults.

Similar rates of *P. aeruginosa* prevalence are reported in the EU overall (Zolin *et al.*, 2020), where 13.9 % of children and 45.4 % of adults (29.1 % of all patients) were chronically infected in 2018; and in the USA in 2019 (Cystic Fibrosis Foundation Patient Registry, 2020), with 27.7 % of all patients chronically infected and 16.7 % intermittently infected. Generally, prevalence rates of *P. aeruginosa* infection across age groups rise from the late teen years to peak in the late-20s to early–30s where around half of all patients test positive for *P. aeruginosa* (Cystic Fibrosis Foundation Patient Registry, 2020; UK Cystic Fibrosis Registry, 2020; Zolin *et al.*, 2020).

Despite the large prevalence of *P. aeruginosa* infection, prevalence rates have been decreasing year on year: in the EU, both prevalence and incidence (the number of new cases of infection within a year) rates have decreased significantly on average between 2012 and 2016, by -0.73 % per year overall for prevalence and -0.12 % per year overall for incidence (Hatziagorou *et al.*, 2019); and in the USA both rates also significantly decreased between 2006–2012, by -1.7 % per year for prevalence overall and -3.3 % for per year for incidence (Salsgiver *et al.*, 2016). These changes are most pronounced in 18–25 year olds, where prevalence has decreased significantly, and by the largest percentage, across multiple populations (Salsgiver *et al.*, 2016; Acosta *et al.*, 2017; Hatziagorou *et al.*, 2019; UK Cystic Fibrosis Registry, 2020). This is often attributed to the strong focus on anti-*Pseudomonal* antibiotic therapies, where positive cultures are now met with intense antibiotic treatment with the aim of eradicating the infecting *P. aeruginosa* (Castellani *et al.*, 2018; Hatziagorou *et al.*, 2019).

However, though prevalence rates of *P. aeruginosa* infection have been decreasing, prevalence of other CF pathogens has been increasing. In Europe the prevalence of *S. aureus*, *Stenotrophomonas maltophilia* and nontuberculous Mycobacteria (NTM) have increased significantly between 2012–2016, again particularly within adults ≥18 (Hatziagorou *et al.*, 2019); the same is true in the USA between 2006–2012, where prevalence of both methicillin-sensitive and methicillin-resistant *S. aureus* (MSSA; MRSA) has increased significantly—MRSA across all age groups, and MSSA in adults ≥18—as has *S. maltophilia* (Salsgiver *et al.*, 2016).

In 2018, across Europe 35.68 % of all patients were chronically infected with *S. aureus*, while 7.68 % of all patients were infected with *S. maltophilia* (Zolin *et al.*, 2020). In the USA prevalence of *S. aureus* infections is greater than that of *P. aeruginosa*, and has been since 2004, whereby approximately 70 % of patients in 2019 were infected—~50 % of all patients with MSSA, and ~20 % of all patients with MRSA (Cystic Fibrosis Foundation Patient Registry, 2020). As with the decrease in *P. aeruginosa* prevalence, it is possible that the increases in other CF pathogens is due to the focus on *P. aeruginosa* eradication.

The focus on *P. aeruginosa* is warranted, as both children and adults with *P. aeruginosa* infections have decreased lung function and increased risk of mortality (Nixon *et al.*, 2001; Emerson *et al.*, 2002; Courtney *et al.*, 2007; Hubert *et al.*, 2013; Kerem *et al.*, 2014), and as such understanding the ways in which it adapts to the lungs, other bacteria, and antibiotic treatments are crucial in developing improvements to patient care.

1.3 Introduction to Pseudomonas aeruginosa*—a focal CF lung pathogen*

Pseudomonas aeruginosa is a Gram-negative soil bacterium that is highly adaptable to multiple different environments due to its large genome of ~6 Mb (Stover *et al.*, 2000; Winstanley *et al.*, 2009), complex regulatory system (Balasubramanian *et al.*, 2013), multiple redundant metabolic pathways (Oberhardt *et al.*, 2008; Bartell *et al.*, 2017), and suite of virulence factors used to outcompete other bacteria, colonise the lung and evade the host immune system (Balasubramanian *et al.*, 2013; O'Brien and Fothergill, 2017; Limoli and Hoffman, 2019). It is common in nosocomial infections, often in catheters or open wounds, where it poses a particular problem because of its intrinsic resistance to multiple antibiotics and ability to readily develop further resistances (Poole, 2011; López-Causapé, Cabot, *et al.*, 2018). This combination of factors makes *P. aeruginosa* especially suited to infection within the CF lung environment, wherein it is able to readily establish chronic infections. As discussed above, *P. aeruginosa* is the most prevalent of the traditional CF pathogens in Europe, as well as being a model system for investigating social traits in bacteria, and as such a great deal of research has been performed examining different aspects of its behaviour and physiology. Here, the focus will be on providing an overview of its adaptation to chronic infection within the CF lungs, its interactions with other species, and its adaptation towards antibiotic treatments.

1.3.1 *Pseudomonas aeruginosa* adapts rapidly in the CF lungs

Adaptation occurs rapidly within the lungs (Winstanley, O'Brien and Brockhurst, 2016; Rossi *et al.*, 2020). Phenotypic adaptation likely first occurs on initial infection as *P. aeruginosa* is known to differentially express a large number of genes when growing in CF sputum (Palmer *et al.*, 2005). CF sputum is rich in amino acids, and naïve lab strains of *P. aeruginosa* increase expression of genes associated with amino acid uptake and degradation when grown in CF sputum compared with growth in minimal media supplemented with glucose (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007). Biosynthesis of a number of amino acids is concomitantly downregulated (Palmer *et al.*, 2005), and these amino acids are thought to be readily available to *P. aeruginosa in vivo* (Turner *et al.*, 2015). Sputum also increases expression of part of *P. aeruginosa*'s quorum sensing system via the *Pseudomonas*

quinolone signalling pathway, which results in increased expression of virulence factors such as pyocyanin and pyochelin that can influence interactions with other bacteria—see § 1.4 below (Palmer *et al.*, 2005; O'Brien and Fothergill, 2017).

Following the initial phenotypic changes that allow colonisation, phenotypic responses are further altered by genetic mutations that take place early during infection (Jørgensen *et al.*, 2015; Bartell *et al.*, 2019). There are a number of different mutations suggested to be pathoadaptive, in that they are found in isolates from multiple different patients and suggest adaptation to the CF lung environment (Marvig *et al.*, 2013, 2014; Feliziani *et al.*, 2014; Jeukens *et al.*, 2014; Jiricny *et al.*, 2014; Marvig *et al.*, 2015; Williams *et al.*, 2018; Gabrielaite *et al.*, 2020), though genetic diversity is very high between isolates both within the same patient and across different patients (Jeukens *et al.*, 2014; Diaz Caballero *et al.*, 2015; Jorth *et al.*, 2015; Marvig *et al.*, 2015; Sommer, Marvig, *et al.*, 2016; Williams *et al.*, 2018; Bartell *et al.*, 2019; Kordes *et al.*, 2019; Gabrielaite *et al.*, 2020). Phenotypic diversity is also relatively high between isolates (Ashish *et al.*, 2013; Clark *et al.*, 2015; Davies *et al.*, 2017; Rossi *et al.*, 2018; Wu *et al.*, 2019), but there is also a set of phenotypes that *P. aeruginosa* commonly converges upon, such as slow growth, antibiotic resistance, and reduction in virulence (La Rosa *et al.*, 2018; Bartell *et al.*, 2019; Rossi *et al.*, 2020).

Quorum sensing (QS)—a system through which bacteria are able to detect intraspecific population density through production and reception of a diffusible signalling factor to effect density-dependent beneficial changes in phenotype—is essential for *P. aeruginosa* virulence across multiple environments including the CF lung (Winstanley and Fothergill, 2009; Lee and Zhang, 2015; Turkina and Vikström, 2019). *Pseudomonas aeruginosa* possesses a complex and interlinked hierarchical network of multiple QS systems: two acyl-homoserine lactone-based systems, LasRI—the master regulator system at the top of the hierarchy—and RhlRI; and the quinolone-based *Pseudomonas* quinolone signal (PQS) system (Lee and Zhang, 2015). Each system regulates expression of numerous genes and traits—such as virulence factor production, biofilm growth, motility, and metabolism (Diggle *et al.*, 2003; Schuster *et al.*, 2003; Darch *et al.*, 2018)—as well as reciprocally regulating each other system, and so disruption of function at any such level within the hierarchy can have wide-ranging effects (Lee and Zhang, 2015; Turkina and Vikström, 2019). Mutations in the

gene encoding the LasR regulator protein are very common among *P. aeruginosa* CF isolates (Smith *et al.*, 2006; Marvig *et al.*, 2015; Feltner *et al.*, 2016; Williams *et al.*, 2018), though it is unclear what exactly the selective pressure for such mutations may be (Feltner *et al.*, 2016). The presence of mutations in *lasR* is often heterogenous within *P. aeruginosa* populations (D'Argenio *et al.*, 2007), and mutations accumulate over time in the lungs such that late-stage populations often harbour greater proportions of *lasR* mutants (Hoffman *et al.*, 2009). Mutations that result in loss of function of the LasR protein can be beneficial within the CF lung environment, where such isolates have been found to: grow to higher densities when using certain amino acids as a carbon source (D'Argenio *et al.*, 2007); be more tolerant of nitrosative stress in low oxygen concentrations (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2010); and be more resistant to β-lactam antibiotics due to increased β-lactamase production (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2009). Additionally, an *in vitro* study modelling the physical structure of the lungs found that *lasR* mutants grew to a greater density than wild-type *P. aeruginosa* in *ex vivo* pig lung sections, further suggesting that such mutations are beneficial in the lung environment itself (Harrison *et al.*, 2014). Though virulence factor production is under the control of QS, and as such LasR, Feltner *et al.*, (2016) found no correlation between virulence factor production and *lasR* mutants, instead finding that in LasR deficient CF isolates the RhlRI system was able to act independently of LasR regulation to give production of beneficial virulence factors—such as LasA proteases, rhamnolipids, and pyocyanin—and other QS associated traits.

Iron acquisition *in vivo* is essential for growth, and iron concentrations are low in the CF lungs (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007; Turner *et al.*, 2015). During infection *P. aeruginosa* secretes iron chelating siderophores such as pyoverdine and pyochelin to scavenge iron from the environment (Rossi *et al.*, 2018). Increased expression comes as a result of an iron-limiting environment (Palmer *et al.*, 2005) as well as from QS activation (Diggle *et al.*, 2003), and is essential during competition with other bacteria within the CF lung—see § 1.4.1 below. Siderophore-mediated iron uptake is a social trait, as the iron-siderophore complex can be taken up by bacteria other than the siderophore producer, but through the course of infection pyoverdine production is often lost, via mutations in the *pvd* operon, likely as a result of non-producing cheats (Andersen *et al.*, 2015, 2018). Consequently, pyoverdine

production is heterogeneous between patients (Andersen *et al.*, 2015; Kang *et al.*, 2019). Subsequent to this loss *P. aeruginosa* has been observed to switch to iron acquisition directly from haemoglobin using the *Pseudomonas* haem utilisation (Phu) system, whereby pyoverdine inactivating mutations in *pvdS* were followed by mutations in the promoter region of the Phu system regulator *phuR* that resulted in increased growth in the presence of haemoglobin (Marvig *et al.*, 2014). Loss of pyoverdine can also result in a switch to pyochelin production, which possesses a weaker affinity for iron but is more efficient to produce (Dumas, Ross-Gillespie and Kümmerli, 2013; Andersen *et al.*, 2018).

Increased biofilm formation is a very common adaptation to the CF lungs; indeed, environmental factors within the lungs such as the high viscosity of the mucus can trigger the change from planktonic growth to biofilm aggregates (Staudinger *et al.*, 2014; Schick and Kassen, 2018). Beyond a switch in growth mode, *P. aeruginosa* isolates are known to develop a mucoid phenotype that comes as a result of loss of function mutations in *mucA*, the negative regulator of the transcription factor *algU*; without sequestration by MucA, AlgU triggers expression of multiple downstream genes (Folkesson *et al.*, 2012), which leads to, along with downregulation of virulence factors such as rhamnolipids and the type III secretion system, an overproduction of the exopolysaccharide alginate (Rau *et al.*, 2010). Alginate is one of the primary components of the *P. aeruginosa* biofilm matrix (Ryder, Byrd and Wozniak, 2007), and biofilm growth and the mucoidy phenotype play important roles in interspecies interactions and antibiotic tolerance—see § 1.4.1 & 1.5.1.4 below—as well as protecting from oxidative stress from the immune system (Ryder, Byrd and Wozniak, 2007); mucoidy specifically has long been associated with adverse clinical outcomes (Pedersen *et al.*, 1992). The mucoid phenotype and mutations in *mucA* are relatively common (Ashish *et al.*, 2013; Marvig *et al.*, 2015; Williams *et al.*, 2018), though reversion has been observed through further mutation of *algU* suggesting that alginate overproduction is costly to some degree and only provides transient benefits (Marvig *et al.*, 2015). Further to this, Bartell *et al.* (2019) found that the mucoid phenotype did not correlate with the development of the other studied phenotypes that they had mapped to convergent evolutionary trajectories, and propose that mucoidy can arise as a response to temporary pressures like antibiotic treatment.

Pseudomonas aeruginosa metabolism within CF is very complex, and it is clear that there is no one adaptive metabolic strategy that is converged upon (Markussen *et al.*, 2014; Jørgensen *et al.*, 2015; Turner *et al.*, 2015; Rossi *et al.*, 2018); rather, *P. aeruginosa* is able to utilise its varied metabolic capacity to suit the specific nutrient environment within a specific patient (Quinn, Lim, *et al.*, 2016), and competition with both other intraspecific phenotypes (Jørgensen *et al.*, 2015) and other species—see § 1.4.1 below. Some commonalities in phenotypic adaptation exist, however. Amino acid auxotrophy is common among CF isolates—particularly methionine, leucine, and arginine—whereby isolates lose the ability to biosynthesise amino acids that are readily available (Barth and Pitt, 1996; Fothergill *et al.*, 2010; Turner *et al.*, 2015; La Rosa *et al.*, 2018; La Rosa, Johansen and Molin, 2019). Metabolic flux through glycolysis is reduced, particularly the catabolism of pyruvate—*aceE* and *aceF*, encoding pyruvate dehydrogenase, have been identified as pathoadaptive mutations (Feliziani *et al.*, 2014; Marvig *et al.*, 2015)—and the TCA cycle becomes driven via the glyoxyate shunt to use assimilated amino acids (Rossi *et al.*, 2018, 2020). These changes may contribute to reduction in oxidative stress from the immune system (Rossi *et al.*, 2020). Late-stage isolates have also been shown to have a reduced growth rate, increased oxygen consumption, reduced metabolite uptake—of succinate, glucose, and ornithine—and differential secretion of metabolites—early-stage isolates secrete ornithine and acetate, late-stage pyruvate, lactate, and glycine—as well as changes in the order of amino acid assimilation (La Rosa *et al.*, 2018). Unfortunately, the complexity of the *P. aeruginosa* regulatory system and the variety of regulator mutations found makes it difficult to attribute these metabolic changes to specific genetic mutations (Marvig *et al.*, 2015; Turner *et al.*, 2015; La Rosa *et al.*, 2018; Rossi *et al.*, 2018; La Rosa, Johansen and Molin, 2019).

A complicating factor when it comes to understanding evolutionary trajectories over the course of chronic infection is that *P. aeruginosa* diversification within the lungs is highly prevalent (Ashish *et al.*, 2013; Hall *et al.*, 2014; Jeukens *et al.*, 2014; Clark *et al.*, 2015; Diaz Caballero *et al.*, 2015; Jorth *et al.*, 2015; O'Brien *et al.*, 2017; Williams *et al.*, 2018; Kordes *et al.*, 2019). Multiple different lineages of *P. aeruginosa* have been shown to coexist within the lungs (Diaz Caballero *et al.*, 2015; Jorth *et al.*, 2015; O'Brien *et al.*, 2017; Williams *et al.*, 2018; Kordes *et al.*, 2019), and there can

be rapid turnover of lineages within the lung (Fothergill *et al.*, 2010; Williams *et al.*, 2018). Diversification can be driven by spatial separation: sampling of different regions of explanted lungs has shown that regional isolation can result in diversity between regions, as lineages within each region evolve independently (Jorth *et al.*, 2015; Kordes *et al.*, 2019). Diversification can also be driven by hypermutators: mutations in DNA mismatch repair genes *mutS/L* result in the hypermutator phenotype that gives rise to a greater number of genetic mutations in a shorter amount of time (Marvig *et al.*, 2013; Feliziani *et al.*, 2014). When hypermutators arise, the phenotype can come to dominate the population of *P. aeruginosa* in the patient whilst still driving diversity, as hypermutator bacteria are able to more rapidly develop mutations that increase fitness (Feliziani *et al.*, 2014). Maintenance of diversity is highly beneficial as subpopulations will be able to withstand antibiotic treatments or changes in nutrient availability, and in the case of transmissible strains maintaining virulent populations may help establish subsequent infections (O'Brien *et al.*, 2017).

1.4 Pseudomonas aeruginosa *interactions with coinfecting CF species*

The lung microbiome is diverse and highly individualised within each patient (Zhao *et al.*, 2012; Price *et al.*, 2013; Coburn *et al.*, 2015; Kramer *et al.*, 2015; Li *et al.*, 2016; Zemanick *et al.*, 2017; Einarsson *et al.*, 2019). Diversity has been found to decrease as patients age and advance through stages of lung disease (Zhao *et al.*, 2012; Coburn *et al.*, 2015; Li *et al.*, 2016; Zemanick *et al.*, 2017; Carmody *et al.*, 2018), such that within post-mortem and explant lungs *P. aeruginosa* is completely dominant (Bjarnsholt *et al.*, 2009; Goddard *et al.*, 2012; Jorth *et al.*, 2015). This domination of *P. aeruginosa* is driven in part by its interactions with the coinfecting species, which are largely antagonistic (Pompilio *et al.*, 2015; O'Brien and Fothergill, 2017; Limoli and Hoffman, 2019).

1.4.1 *Pseudomonas aeruginosa* is highly antagonistic to other species and an efficient coloniser

Bacterial interactions are complex and wide-ranging, and can be cooperative and facilitative or antagonistic and inhibitory (Mitri and Richard Foster, 2013). "Positive" interactions in CF involving *P. aeruginosa* include: metabolic cross-feeding, whereby one species produces a metabolite that another species is able utilise in its own metabolism—this has been observed in *P. aeruginosa*, which upregulates lactate metabolism genes in response to lactate production by *Rothia mucilaginosa* (Gao *et al.*, 2018); and facilitation of growth of numerous *Streptococcus* species, albeit by an unknown mechanism (Scott *et al.*, 2019). However, *P. aeruginosa* is more usually involved in antagonistic interactions with other species. *Pseudomonas aeruginosa* possesses a suite of antimicrobial factors and expression can be triggered in numerous ways. For example, *P. aeruginosa* responds to the presence of the interspecies bacterial signalling molecule autoinducer 2 by increasing production of its PQS-related virulence factors (Li *et al.*, 2015). *Pseudomonas aeruginosa* can also increase expression of host factors to mediate bacterial killing, such as through inducing production of bactericidal phospholipases by bronchial epithelial cells at concentrations sufficient to kill other bacterial species, but survivable by *P. aeruginosa* (Pernet *et al.*, 2014).

Here though, the focus will be specifically on interactions between *P. aeruginosa* and two commonly cocultured CF pathogens, *Staphylococcus aureus* and *Stenotrophomonas maltophilia*.

1.4.1.1 Interactions with Staphylococcus aureus *are generally antagonistic*

Staphylococcus aureus is a Gram-positive species that, though normally a commensal bacteria colonising the skin and nasal passage (Van Belkum *et al.*, 2009), is capable of causing a very wide range of pathogenic infections, from skin and wound infections to bacteraemia and endocarditis (Tong *et al.*, 2015), as well as of course chronic CF infections. Its widespread success as a pathogen comes as a result of its production of numerous virulence factors that interact with the host to aid infection (Tam and Torres, 2019). Within CF, *S. aureus* is often acquired earlier in life—see § 1.2.3 above—where infection is associated with increased lung inflammation and damage (Sagel *et al.*, 2009; Pillarisetti *et al.*, 2011). Much like *P. aeruginosa*, over the course of infection *S. aureus* adapts to the lung environment and other bacterial species present (Goerke and Wolz, 2010), and shows increased within-patient population heterogeneity when compared to commensal colonisation (Goerke *et al.*, 2007). Reported rates of coinfection with *P. aeruginosa* vary between 10–30 % of patients infected with either species (Limoli and Hoffman, 2019), and coinfections are often associated with worse outcomes than *S. aureus* infection alone (Sagel *et al.*, 2009; Hubert *et al.*, 2013; Limoli *et al.*, 2016; Schwerdt *et al.*, 2018; Cios *et al.*, 2019), though not always (Ahlgren *et al.*, 2015).

The interactions between *P. aeruginosa* and *S. aureus* have been studied extensively due to the prevalence of coinfections. Research so far suggests that the interaction between the two is mostly one-sided, whereby *P. aeruginosa* uses its antimicrobial factors and extensive metabolism to both outcompete and actively supress *S. aureus* (Nguyen and Oglesby-Sherrouse, 2016; Hotterbeekx *et al.*, 2017; Limoli and Hoffman, 2019).

When grown as a coculture *in vitro P. aeruginosa* and *S. aureus* are able to coexist for a short time, though eventually the dynamic shifts such that *P. aeruginosa* essentially fully outcompetes *S. aureus* (Baldan *et al.*, 2014; Filkins *et al.*, 2015; Magalhães, Lopes and Pereira, 2017; Woods *et al.*, 2019). The extent of the

domination by *P. aeruginosa* is partly dependent on the initial ratio of the two species, where *S. aureus* can survive better when at much greater density (Woods *et al.*, 2019), but the main determinant appears to be the extent of the given *P. aeruginosa* strain's virulence factor production (Baldan *et al.*, 2014; Filkins *et al.*, 2015; Nguyen *et al.*, 2015; Frydenlund Michelsen *et al.*, 2016; Limoli *et al.*, 2017; Bernardy *et al.*, 2020; Price *et al.*, 2020).

The primary antimicrobial virulence factors used by *P. aeruginosa* are 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), pyocyanin, rhamnolipids, and proteases, as well as pyoverdine siderophores acting indirectly for iron competition, and are all regulated via QS systems (Diggle *et al.*, 2003; Hoffman *et al.*, 2006; Biswas *et al.*, 2009; Filkins *et al.*, 2015; Nguyen *et al.*, 2015; Limoli *et al.*, 2017; O'Brien and Fothergill, 2017; Radlinski *et al.*, 2017; Limoli and Hoffman, 2019).

HQNO is an alkyl-quinolone that can be produced from the PQS production pathway, and acts as a respiratory chain inhibitor against *S. aureus*, as well as other Gram-positive bacteria (Machan *et al.*, 1992; Hoffman *et al.*, 2006; Nguyen and Oglesby-Sherrouse, 2016). Its inhibition of aerobic respiration in *S. aureus* results in greatly reduced growth (Hoffman *et al.*, 2006), and also results in increased tolerance to both tobramycin (Hoffman *et al.*, 2006) and vancomycin (Orazi and O'Toole, 2017). Iron limited conditions are able to trigger production of HQNO, PQS, and other alkyl-quinolones (Nguyen *et al.*, 2015). In this environment, where *P. aeruginosa* uses siderophores to efficiently outcompete *S. aureus* for iron, as well as limiting aerobic respiration, *S. aureus* switches to fermentative metabolism of glucose that produces lactate (Filkins *et al.*, 2015; Tognon *et al.*, 2019). This switch facilitates *P. aeruginosa* competition further, as it can upregulate lactate uptake in response (Tognon *et al.*, 2019), and ultimately result in *S. aureus* autolysis from which *P. aeruginosa* can acquire more iron (Mashburn *et al.*, 2005; Filkins *et al.*, 2015; Nguyen *et al.*, 2015).

Pyocyanin is a redox active pigment produced by the *phz* operon in *P. aeruginosa*, that has roles in host inflammation and antimicrobial action (Rada and Leto, 2013; O'Brien and Fothergill, 2017). Similarly to HQNO, pyocyanin has a role in *S. aureus* inhibition (Biswas *et al.*, 2009), and *P. aeruginosa* has been shown to upregulate its synthesis in response to the presence of N-acetylglucosamine (GlcNAc),

as well as peptidoglycan—of which GlcNAc is a component—shed by Gram-positive bacteria (Korgaonkar and Whiteley, 2011; Korgaonkar *et al.*, 2013).

Rhamnolipids and proteases, such as LasA protease and elastase encoded by the *lasA* and *lasB* genes, play roles in *S. aureus* inhibition: *lasA* and *lasB* were also upregulated by the presence of GlcNAc (Korgaonkar and Whiteley, 2011), and both factors increase the susceptibility of *S. aureus* to antibiotics (Radlinski *et al.*, 2017). Rhamnolipids form pores in the membrane that allow tobramycin access into the cell, and LasA protease begins the breakdown of peptidoglycan that allows vancomycin to disrupt peptidoglycan synthesis and result in cell lysis (Radlinski *et al.*, 2017).

When *in vitro* coculture results in a stable coexistence between the two species, in which *S. aureus* is not actively killed, this is usually because the *P. aeruginosa* strain used does not produce these antimicrobial factors (Frydenlund Michelsen *et al.*, 2016; Bernardy *et al.*, 2020). Deletion of *pqsA*, which mediates the first step in the PQS production pathway, often results in *S. aureus* survival in coculture experiments, demonstrating the importance of PQS regulated virulence factors such as HQNO, rhamnolipids, and pyoverdine (Mashburn *et al.*, 2005; Hoffman *et al.*, 2006; Xiao *et al.*, 2006; Nguyen *et al.*, 2015). Mucoid *P. aeruginosa*, which overproduces alginate due to *mucA* mutations, often coexists with *S. aureus* (Limoli *et al.*, 2017; Bernardy *et al.*, 2020), which is most likely because alginate production at any level, either modest or overproduction, downregulates production of PQS virulence factors (Limoli *et al.*, 2017); even the addition of exogenous alginate downregulates these factors (Price *et al.*, 2020). As such, it is often observed that chronic *P. aeruginosa* isolates from late in CF infection are less antagonistic towards *S. aureus* both *in vitro* and during *in vivo* acute infection models (Baldan *et al.*, 2014; Frydenlund Michelsen *et al.*, 2016; Cigana *et al.*, 2018; Bernardy *et al.*, 2020). Whether loss of antagonism plays an active role during CF infection, or is a by-product of other adaptations to the lung environment, remains to be seen.

Generally, *P. aeruginosa* is unaffected in its ability to grow by the presence of *S. aureus*. Mutations in *P. aeruginosa* that are specific to coculture with *S. aureus* appear to be rare, though one *in vitro* evolution study found that, with continuous replacement of *S. aureus* populations, *P. aeruginosa* developed a mutation that resulted in loss of the O-antigen that caps LPS and increased fitness in *S. aureus*

coculture (Tognon *et al.*, 2017), though the mechanism for this selection was unclear. However, along with this extensive, one-sided antagonism, there are a few examples of the presence of live *S. aureus* being to the benefit of *P. aeruginosa*. A factor secreted by *S. aureus*, Staphylococcal protein A (SpA) has been shown to bind to one of the *P. aeruginosa* exopolysaccharides, Psl, involved in the biofilm matrix, which can protect *P. aeruginosa* from immunoglobulin G-mediated phagocytosis (Armbruster *et al.*, 2016). In binding to Psl, SpA can also alter the structure of *P. aeruginosa* biofilms to form aggregates, and this aggregate formation decreases *P. aeruginosa* susceptibility to tobramycin and may contribute to failure of early infection *P. aeruginosa* eradication therapy (Beaudoin *et al.*, 2017).

1.4.1.2 Interactions with Stenotrophomonas maltophilia *are not well characterised*

Stenotrophomonas maltophilia, previously classified as *Xanthomonas maltophilia*, is a Gram-negative species that is widespread in aqueous environments, and is an opportunistic pathogen of humans (Brooke, 2012; Chang *et al.*, 2015). Despite low virulence, its intrinsic resistance to multiple classes of antibiotics and affinity for adherence to plastic surfaces can pose significant problems during nosocomial infections (Crossman *et al.*, 2008; Looney, Narita and Mühlemann, 2009; Brooke, 2012; Chang *et al.*, 2015; Yin *et al.*, 2017). Regarded as an emerging pathogen of CF patients (Parkins and Floto, 2015; Hatziagorou *et al.*, 2019), its importance in CF infections is being increasingly recognised as several studies find worse clinical outcomes in those infected with *S. maltophilia* (Talmaciu *et al.*, 2000; Waters *et al.*, 2011; Berdah *et al.*, 2018). Though there is not as much known about adaptation to the lungs as other CF pathogens, observational studies of CF isolates have found that there is high genetic and phenotypic diversity between CF isolates (Vidigal *et al.*, 2014; Pompilio *et al.*, 2016; Esposito *et al.*, 2017; Alcaraz *et al.*, 2020), and that biofilm formation is likely an important factor during CF infection (Pompilio *et al.*, 2010).

Similarly, compared with *S. aureus*, the interactions between *S. maltophilia* and *P. aeruginosa* have not been studied in as much depth; coinfections between the species have been observed, though at varying rates between studies (Talmaciu *et al.*, 2000; Marchac *et al.*, 2004; Spicuzza *et al.*, 2009; Capaldo *et al.*, 2020). During mixed

species biofilm growth *in vitro*, lab strains of *P. aeruginosa* and *S. maltophilia* are not able to reach an equilibrium, and instead *P. aeruginosa* will eventually outcompete *S. maltophilia* (Magalhães, Lopes and Pereira, 2017). And this dynamic is not changed when the two species are derived from the same CF patient: *P. aeruginosa* was found to inhibit planktonic growth, and significantly reduce viability of *S. maltophilia* in mixed biofilms (Pompilio *et al.*, 2015). In the same study, *P. aeruginosa* was also found to inhibit *S. maltophilia* growth in a contact dependent manner, i.e., not via secreted metabolites, but instead likely by the type VI secretion system (Pompilio *et al.*, 2015). Additionally, *P. aeruginosa* gene expression was altered in mixed species biofilm, with a significant increase in *algD* and *aprA* expression—production of an alginate precursor and an alkali protease, respectively—and a significant decrease in *lasI* and *rhlR* expression—both involved in QS regulation; this was coupled with non-significant increases in *mexE* and *mexC*—multi-substrate efflux pumps—and *exoS* and *toxA* toxins (Pompilio *et al.*, 2015).

There is some evidence[1] that *P. aeruginosa* can react to *S. maltophilia* via a group of interbacterial signalling molecules called diffusible signal factor (DSF), which are a group of unsaturated fatty acids (Ryan *et al.*, 2008; Twomey *et al.*, 2012). In response to production of DSF by *S. maltophilia*, lab strains of *P. aeruginosa* were shown to alter the structure of co-species biofilms to become more filamentous, and upregulate the *pmrAB* operon resulting in an increase in cationic antimicrobial protein tolerance (Ryan *et al.*, 2008). Multiple CF isolates of *P. aeruginosa* were also shown to react to DSF, and addition of exogenous DSF to *P. aeruginosa* during acute infection of mice enhanced colonisation (Twomey *et al.*, 2012).

[1] Multiple publications from this group regarding *S. maltophilia* and cell signalling have been retracted due to allegations of image manipulation—Ryan *et al.* (2006) *PNAS.*, Fouhy *et al.* (2007) *J. Bacteriol.*, McCarthy *et al.* (2011) *J. Bacteriol.*, and Ryan *et al.* (2012) *Mol. Microbiol.*—though the cited publications have not been disputed at time of writing.

1.5 Antibiotic treatment of Pseudomonas aeruginosa *and evolution of antibiotic resistance*

Antibiotic treatment is essential for maintaining patient health. Over the course of their lives, patients will be persistently treated with multiple different antibiotics in an effort to manage or clear the bacterial infections (UK Cystic Fibrosis Trust, 2009; Mogayzel *et al.*, 2013; Langton Hewer and Smyth, 2017; Smith, Rowbotham and Regan, 2018). Particular attention is paid to eradication of *P. aeruginosa*, where treatment consists of aggressive courses of the anti-Pseudomonal antibiotics colistin and tobramycin (Langton Hewer and Smyth, 2017; Smith, Rowbotham and Regan, 2018). Antibiotics are also administered as treatment for pulmonary exacerbations—acute periods of increases in respiratory symptoms of the disease—and for regular maintenance of patient bacterial load (UK Cystic Fibrosis Trust, 2009; Mogayzel *et al.*, 2013; Waters *et al.*, 2019).

1.5.1 *Pseudomonas aeruginosa* rapidly evolves resistance to antibiotics

Pseudomonas aeruginosa is regarded as a critical bacterial pathogen by numerous health organisations due to its ability to develop high level antibiotic resistance (Botelho, Grosso and Peixe, 2019); the World Health Organisation have recognised *P. aeruginosa* as an organism for which new antibiotics are urgently needed (Tacconelli *et al.*, 2018), and its antibiotic resistance is a major cause of mortality in nosocomial infections due to treatment failure (Rosenthal *et al.*, 2016). And though in the context of CF *P. aeruginosa* does not represent an acute cause of mortality as in nosocomial infections, its longevity within the lungs is largely attributable to its antibiotic resistance evolution.

Pseudomonas aeruginosa possesses an array of efflux pumps that result in an intrinsic resistance to many antibiotics (Poole, 2011), and is able to develop chromosomal mutations that provide increased tolerance and resistance to essentially all available antipseudomonal antibiotics (López-Causapé, Cabot, *et al.*, 2018). Furthermore, there are often numerous pathways to tolerance of a given antibiotic due to the large mutational resistome, and selection for these mutations can differ between different environments. Due to intense antibiotic use with CF patients, and the widespread presence of mutations in resistance genes of *P. aeruginosa* CF isolates

(Marvig *et al.*, 2015; Greipel *et al.*, 2016; Williams *et al.*, 2018), it is believed that antibiotics pose the largest selection pressure on *P. aeruginosa* evolution within the CF lung (Rossi *et al.*, 2020).

Though many antibiotic resistance related genes are observed to be mutated in CF isolates, such as those resulting in resistance to β-lactams (*ampC*, *ftsI*, *mexAB/mexR*), or carbapenems (*oprD*), here the focus will be on the mutational development of resistance to three critical antipseudomonal antibiotics used in the treatment of CF infections (UK Cystic Fibrosis Trust, 2009; Langton Hewer and Smyth, 2017)—ciprofloxacin, colistin, and tobramycin—as well as the phenotypic resistance provided by biofilm growth and persister cell formation.

1.5.1.1 Mechanisms of tobramycin resistance

Tobramycin is an aminoglycoside antibiotic that targets the ribosome, inhibiting protein synthesis via binding to the 30S ribosomal subunit (Kotra, Haddad and Mobashery, 2000), and is widely administered among CF patients, particularly as eradication therapy when *P. aeruginosa* is initially detected (UK Cystic Fibrosis Trust, 2009; Langton Hewer and Smyth, 2017), as well as for general maintenance of patient bacterial load (Mogayzel *et al.*, 2013). Resistance to tobramycin in CF is mediated by cell impermeability through active efflux via the MexXY-OprM efflux pump, target modifications, and outer membrane modifications (Masuda *et al.*, 2000a; Hocquet *et al.*, 2003; Prickett *et al.*, 2017; Bolard, Plésiat and Jeannot, 2018; Bolard *et al.*, 2019); though environmental *P. aeruginosa* isolates can acquire tobramycin inactivating enzymes via horizontal gene transfer this is rarely observed in CF (MacLeod *et al.*, 2000).

Increased aminoglycoside resistance most often comes as a result of overexpression of *mexXY*. MexXY is an efflux pump that associates with the outer membrane porin OprM to actively remove numerous substrates such as antibiotics from inside the cell, including aminoglycosides (Masuda *et al.*, 2000a, 2000b). Transient adaptive resistance in response to tobramycin—which subsides on cessation of treatment—results from an increased expression of *mexXY* (Hocquet *et al.*, 2003), and overexpression can be cemented by mutations in the negative regulator MexZ (Vogne *et al.*, 2004). Mutations in the genes encoding the efflux pump MexXY and its

regulator MexZ are widely observed in *P. aeruginosa* CF isolates (Marvig *et al.*, 2013; Feliziani *et al.*, 2014; Marvig *et al.*, 2015; Greipel *et al.*, 2016; Sommer, Alanin, *et al.*, 2016; Prickett *et al.*, 2017; Singh *et al.*, 2017; Kordes *et al.*, 2019), and mutations in *mexZ* are perhaps the most commonly observed antibiotic resistance mutations across all isolates (Greipel *et al.*, 2016; Frimodt-Møller *et al.*, 2018). Additionally, *mexXY* overexpression can be caused by repression of *mexZ* via mutations in the *parRS* two-component system involved in colistin resistance (Muller, Plésiat and Jeannot, 2011)—see § 1.5.1.2 below. On its own, overexpression of *mexXY* provides only a modest increase in tobramycin tolerance, but in the heterogeneous lung environment antibiotic diffusion can be impaired (Moriarty *et al.*, 2007; Bos *et al.*, 2017) and as such small increases in tolerance can lead to longer survival and an increased chance of developing further resistance mutations (Prickett *et al.*, 2017; Frimodt-Møller *et al.*, 2018).

More recently identified tobramycin resistance determinants are mutations in *fusA1*, a gene that encodes the highly conserved elongation factor G that promotes movement of tRNA and mRNA in the ribosome complex (Wintermeyer *et al.*, 2011). Mutations in *fusA1* have been observed in numerous CF isolates (Marvig *et al.*, 2013; Feliziani *et al.*, 2014; Markussen *et al.*, 2014; Greipel *et al.*, 2016; Bolard, Plésiat and Jeannot, 2018), as well as during *in vitro* evolution under tobramycin treatment (López-Causapé, Rubio, *et al.*, 2018; Scribner *et al.*, 2020). Much like *mexZ* mutations, the increase in tobramycin tolerance of *fusA1* mutations is not large, but could contribute to a greater tolerance when combined with other mutations (Bolard, Plésiat and Jeannot, 2018).

Outer membrane permeability also contributes to tobramycin tolerance, via gain of function mutations in *pmrB* (Bolard *et al.*, 2019), part of the *pmrAB* two-component system usually associated with colistin resistance—see § 1.5.1.2 below. Genes PA4773, PA4774, and PA4775 are predicted to encode proteins involved in polyamine biosynthesis, and are transcribed during *pmrAB* activation (Johnson *et al.*, 2012). It is suggested that these genes contribute to the production of the polyamines spermidine and norspermidine which can localise at the outer membrane surface and decrease membrane permeability (Johnson *et al.*, 2012; Bolard *et al.*, 2019). This in turn may prevent internalisation of tobramycin and improve the efficacy of MexXY

efflux (Bolard *et al.*, 2019). Selection for *pmrB* mutations has been shown during *in vitro* evolution under tobramycin selection (López-Causapé, Rubio, *et al.*, 2018).

1.5.1.2 Mechanisms of colistin resistance

Colistin is a cationic antimicrobial peptide (CAMP) that displaces Ca^{2+} and Mg^{2+} cations in the outer membrane, resulting in destabilisation and cell lysis (Bialvaei and Samadi Kafil, 2015). Regarded as an antibiotic of last resort in many other clinical settings, colistin is used regularly during antipseudomonal therapy for CF (UK Cystic Fibrosis Trust, 2009; Langton Hewer and Smyth, 2017). Resistance to colistin is mediated by LPS modification via the *arnBCADTEF* operon, whereby 4-amino-L-arabinose is added to the phosphate groups in the lipid A portion of LPS, masking the negative charges and preventing infiltration of colistin and other CAMPs (McPhee, Lewenza and Hancock, 2003; Moskowitz, Ernst and Miller, 2004). This process can be regulated by several two-component systems, *phoPQ* (McPhee *et al.*, 2006; Miller *et al.*, 2011), *parRS* (Fernández *et al.*, 2010; Muller, Plésiat and Jeannot, 2011), and *pmrAB* (Moskowitz, Ernst and Miller, 2004). Among CF *P. aeruginosa* isolates, mutations are most commonly observed in *pmrAB* (Moskowitz *et al.*, 2012; Marvig *et al.*, 2013; Jochumsen *et al.*, 2016; Bricio-Moreno *et al.*, 2018; Williams *et al.*, 2018; Bolard *et al.*, 2019), with *parRS* (Greipel *et al.*, 2016) and *phoPQ* (Miller *et al.*, 2011; Jochumsen *et al.*, 2016) being rarer.

Both PmrAB and PhoPQ are activated by Mg^{2+} limiting conditions (McPhee *et al.*, 2006), and can activate transcription of the *arnBCADTEF* operon independently of each other (McPhee, Lewenza and Hancock, 2003; Moskowitz, Ernst and Miller, 2004; Miller *et al.*, 2011). Gain of function mutations in the sensor kinase *pmrB* that result in constitutive activation of the transcriptional regulator *pmrA* can give high level colistin resistance (Moskowitz *et al.*, 2012; Bolard *et al.*, 2019), and stepwise acquisition of *pmrB* and *phoQ*, in addition to other mutations, can interact epistatically to further increase colistin resistance (Jochumsen *et al.*, 2016). And though *pmrB* gain of function mutations provide increased fitness in the presence of colistin, *pmrB* loss of function mutations have been observed in transmissible CF isolates, where increased susceptibility to tobramycin and ciprofloxacin appear to be

in a trade-off against resistance to lysozyme and inhibition of CFTR function that can aid in early colonisation (Bricio-Moreno *et al.*, 2018).

The *parRS* system is activated by the presence of CAMPs specifically and results in expression of the *arnBCADTEF* operon, the *pmrAB* operon, and, as mentioned in § 1.5.1.1 above, the MexXY efflux pump (Fernández *et al.*, 2010; Muller, Plésiat and Jeannot, 2011). Expression of the *pmrAB* operon and *mexXY* have been shown to both aid in colistin resistance (Johnson *et al.*, 2012; Puja *et al.*, 2020). Spermidine possibly produced by PA4774 in the *pmrAB* operon has been suggested to act in an equivalent manner to Mg^{2+} to stabilise the outer membrane and compete with CAMP binding (Johnson *et al.*, 2012), while deletion of *mexXY* sensitises *P. aeruginosa* to colistin and suggests a complex interplay between MexXY and LPS modification that is not fully understood (Puja *et al.*, 2020).

1.5.1.3 Mechanisms of ciprofloxacin resistance

Ciprofloxacin is a fluoroquinolone antibiotic that inhibits DNA synthesis through binding to DNA topoisomerase II (DNA gyrase) and IV (Hooper and Jacoby, 2016). Ciprofloxacin resistance can be mediated via efflux pump, primarily MexCD-OprJ overexpression from mutations in the *nfxB* transcriptional regulator (Poole *et al.*, 1996)—but also to a lesser extent by both MexXY-OprM and MexAB-OprM (Masuda *et al.*, 2000b)—and by target alteration through mutations in *gyrA*, *gyrB*, *parC*, and *parE*, encoding subunits of DNA gyrase and DNA topoisomerase IV respectively (Bruchmann *et al.*, 2013).

Mutations in both sets of genes have been observed in CF isolates—*mexCD* and *nfxB* (Jalal *et al.*, 2000; Marvig *et al.*, 2015); *gyrAB* and *parCE* (Marvig *et al.*, 2013; Markussen *et al.*, 2014; Marvig *et al.*, 2015; Greipel *et al.*, 2016)—and it is unclear whether mutations in one set of genes are selected for over the other set, or occur in concert. During *in vitro* evolution under selection at low ciprofloxacin concentrations—below the minimum inhibitory concentration (MIC)—*nfxB* mutations developed but did not fix in the populations, whereas *gyrAB* mutations were maintained, suggesting that *nfxB* mutations were too costly in that environment (Jørgensen *et al.*, 2013). Selection with inhibitory concentrations of ciprofloxacin alone, or in combination with ceftazidime evolution both consistently selected for

nfxB mutations, along with *mexR* mutations that resulted in overexpression of *mexAB* (Vestergaard *et al.*, 2016), and selection with increasing concentrations of ciprofloxacin has been shown to result in mutations in both sets of genes (Yen and Papin, 2017). Mode of growth also appears to have some effect under ciprofloxacin selection, whereby populations grown as biofilms resulted in *nfxB* mutations whilst populations grown as planktonic cells resulted in *gyrAB* mutations (Ahmed *et al.*, 2018). However, mutations in *nfxB* are associated with high fitness costs *in vitro* (Stickland *et al.*, 2010), and overexpression of *mexCD* results in less effective action of MexXY and MexAB, possibly due to greater metabolic demands from the increased protonmotive force required, resulting in increased susceptibility to aminoglycosides (Jeannot *et al.*, 2008).

1.5.1.4 Phenotypic resistance

In addition to the mutational pathways listed above, antibiotic resistance in *P. aeruginosa* can also develop through phenotypic resistance resulting from the biofilm mode of growth (Ciofu and Tolker-Nielsen, 2019), or the formation of persister cells (Harms, Maisonneuve and Gerdes, 2016).

Growth as a biofilm is the primary mode of growth for bacteria across all environments, and especially within the CF lung where the high viscosity of the sputum results in formation of aggregates (Bjarnsholt *et al.*, 2013; Staudinger *et al.*, 2014; Ciofu and Tolker-Nielsen, 2019). Biofilm aggregates can be formed as a result of production or overproduction of the exopolysaccharide components that make up the *P. aeruginosa* biofilm matrix—alginate, Psl, and Pel (Ryder, Byrd and Wozniak, 2007)—but can also be driven in exopolysaccharide non-producers entirely by physical restriction due to the concentrations of host polymers, such as mucin and extracellular DNA, that form the densely packed structure of CF mucus, termed depletion aggregation (Goltermann and Tolker-Nielsen, 2017; Secor *et al.*, 2018).

Both aggregate formations, with or without exopolysaccharides, display increased tolerance to tobramycin, colistin, and ciprofloxacin, though perhaps through different mechanisms. Overexpression of alginate in *mucA* mutants can result in increases of up to 50-fold in tobramycin tolerance (Goltermann and Tolker-Nielsen, 2017), and deletion of Pel synthesis results in an increased susceptibility to tobramycin

(Colvin *et al.*, 2011). During initial formation, Psl can sequester positively charged molecules, such as tobramycin and colistin, and increase tolerance; indeed, increased concentration of Psl produced directly correlated with increased tolerance of colistin (Billings *et al.*, 2013; Tseng *et al.*, 2013). The interior of biofilm aggregates is often anaerobic, due to hypoxia within the CF mucus (Worlitzsch *et al.*, 2002), and heterogeneity of bacterial growth throughout aggregates can provide antibiotic tolerance via slow growth and decreased activity of antibiotic targets (Nguyen *et al.*, 2011; Stewart *et al.*, 2016; Ciofu and Tolker-Nielsen, 2019). Additionally, extracellular DNA can itself sequester positively charged antibiotics, as well as cause upregulation of *pmrAB*, resulting in increased colistin and tobramycin tolerance (Mulcahy, Charron-Mazenod and Lewenza, 2008).

Persister cells are a widespread bacterial phenotype that is characterised by a physically dormant state that results in minimal-to-no growth and the ability to withstand prolonged antibiotic treatment independently of specific antibiotic resistance mechanisms (Lewis, 2010; Harms, Maisonneuve and Gerdes, 2016; Vogwill *et al.*, 2016). Persisters constitute a small proportion of a given bacterial population, as part of standing phenotypic heterogeneity, but formation can also be triggered by stress responses (Harms, Maisonneuve and Gerdes, 2016). The relevance of these persister fractions to CF is that they can provide a further cause of treatment failure, whereby populations rebound after treatment due to persistence, and late stage isolates of *P. aeruginosa* from CF patients have been shown to produce higher proportions of persister cells suggesting its importance as a chronic adaptation phenotype (Mulcahy *et al.*, 2010).

1.6 Selective pressures for evolution of antibiotic resistance

1.6.1 Antibiotic treatment and exacerbations

During their lives CF patients will likely undergo acute periods of increased respiratory symptoms of the disease, termed pulmonary exacerbations. Though there is no firm definition of what constitutes an exacerbation, they are typically characterised by increases in coughing, sputum production, inflammation, and acute worsening of lung function often measured as forced expiratory volume in 1 second (FEV_1) (Flume *et al.*, 2009). It is not clear what exactly causes exacerbations, particularly because, as mentioned previously, the lung microbiome is highly individualised to a given patient and as such there are no single, specific taxa that have been identified as the causative agent of exacerbations (Price *et al.*, 2013). However, studies have found many different associations between the bacterial community and exacerbations.

It is frequently observed that the lung community remains relatively stable when comparing before and after an exacerbation event, in terms of total bacterial load, community diversity, and composition (Tunney *et al.*, 2011; Fodor *et al.*, 2012; Zhao *et al.*, 2012; Price *et al.*, 2013; Li *et al.*, 2016; Zemanick *et al.*, 2017; Carmody *et al.*, 2018; Layeghifard *et al.*, 2019). When changes in abundance of species are observed this is as a result of the antibiotic treatment patients undergo for the exacerbation (Zhao *et al.*, 2012; Carmody *et al.*, 2018), and during this treatment it is the more antibiotic tolerant species such as *P. aeruginosa* and other traditional CF taxa that are detected, often increasing in relative abundance (Daniels *et al.*, 2013), whereas anaerobic species decline in relative abundance during this period (Carmody *et al.*, 2018). Because the community composition remains relatively stable following an exacerbation, it has been suggested that a probable cause of exacerbations is growth in a previously uninfected region of the lungs (Fodor *et al.*, 2012; Conrad *et al.*, 2013). Whelan and Surette (2015) further suggest that the difficulty in detecting the changes in community composition that result in an exacerbation is that only a small fraction of the total lung community expands into a new region and causes damage, and this small expansion is quickly cleared by antibiotic treatment, to be replaced by the persistent antibiotic tolerant species.

Anaerobic bacteria are likely to play a greater in role in CF lung infections than previously thought from culture-dependent studies, due to the difficulty of detection from their fastidious nature. Sequencing based studies have shown that anaerobes make up a large proportion of the lung microbiome and are very prevalent amongst patients. Some of the genera that are most frequently found include *Streptococcus*, *Prevotella*, *Veillonella*, *Fusobacterium*, *Gemella*, and *Granulicatella* (Zhao *et al.*, 2012; Price *et al.*, 2013; Coburn *et al.*, 2015; Kramer *et al.*, 2015; Mahboubi *et al.*, 2016; Zemanick *et al.*, 2017; Muhlebach *et al.*, 2018; Einarsson *et al.*, 2019; Layeghifard *et al.*, 2019). Though the specific species identities may differ from patient to patient, these anaerobic taxa form a core community that is shared across healthy lungs and CF lungs (Li *et al.*, 2016; Einarsson *et al.*, 2019), and paediatric and adult CF patients (Zemanick *et al.*, 2017; Einarsson *et al.*, 2019). The most likely source for these anaerobes, in both healthy and CF lungs, is repeated micro-aspiration from the upper airways and oral microbiome.

Addressing the difficulty in detecting small changes in community composition, more recent studies, with larger sample sizes of patients and over longer periods of time, have suggested that in the period leading up to an exacerbation there is a small increase in the relative abundance of rare anaerobic taxa within the patient microbiome, and that this can result in an exacerbation (Quinn, Whiteson, *et al.*, 2016; Carmody *et al.*, 2018; Layeghifard *et al.*, 2019). These studies fit within the climax-attack model for CF community dynamics (Conrad *et al.*, 2013), that suggests that the CF microbiome can be partitioned into two functionally distinct communities: the transient "attack" community that drives damage to the lungs via increased inflammation; and the stable "climax" community that goes on to colonise the damaged areas and persist over time due to slower growth phenotypes and greater adaptation to the lungs. Network cooccurrence analysis (Quinn, Whiteson, *et al.*, 2016) on communities sampled over 10 years (Zhao *et al.*, 2012) suggests that distinct metabolic functions are performed by: anaerobic bacteria undergoing fermentation on sugars in the "attack" community, resulting in decreased pH and production of short-chain fatty acids that have been shown to cause inflammation in the CF lung (Mirković *et al.*, 2015); and more traditional CF pathogens such as *P. aeruginosa*, *S. aureus*, and *S. maltophilia* that metabolise amino acids, resulting in increased pH, in the "climax"

community. Altogether, this can provide a potential model to explain the steady decline in lung function that patients experience as they age, and how the lungs end up as a monoculture of *P. aeruginosa* (Goddard *et al.*, 2012; Jorth *et al.*, 2015; Whelan and Surette, 2015).

1.7 Thesis overview

This thesis includes the following chapters, presented in the form of research papers:

Chapter 2. The effects of antibiotic combination treatments on *Pseudomonas aeruginosa* tolerance evolution and coexistence with *Stenotrophomonas maltophilia*.

In this chapter I investigated how the evolution of antibiotic tolerance in *P. aeruginosa* was affected by sub-MIC concentrations of the antibiotics ciprofloxacin, colistin, and tobramycin, administered alone and in combination over the course of a 21-day selection experiment. I also investigated whether the evolution of tolerance in *P. aeruginosa* was affected by the presence of a competitor species in the form of *S. maltophilia*, and how the coculture dynamics between the two were affected by the antibiotics. I found that greater numbers of antibiotics selected for greater increases in both tolerance and minimum inhibitory concentration and found that the combination of ciprofloxacin and tobramycin selected for high levels of tolerance and minimum inhibitory concentration of all antibiotics. I found that there was no effect of competitor on evolution of antibiotic tolerance, but found that subinhibitory concentrations of tobramycin, and combinations containing tobramycin, altered the coculture composition outcomes between the two species such that cocultures normally completely dominated by *P. aeruginosa* instead consisted of a mixture of the two species or dominance of *S. maltophilia*. This suggested that despite having little effect on *P. aeruginosa* grown individually, low doses of antibiotic, in this case tobramycin, could act as mechanisms that increase diversity between species.

Chapter 3. Evolution of coexistence in a simple Cystic Fibrosis-like community.

Having observed that a sub-MIC concentration of tobramycin altered the dynamic between *P. aeruginosa* and *S. maltophilia*, I expanded upon the system used in Chapter 2 to investigate how the individual species abundance within a three-species community, consisting of *P. aeruginosa*, *S. maltophilia*, and *Staphylococcus aureus,* was affected by the same sub-MIC concentration of tobramycin. In this chapter I also investigated the effect of another potential diversity maintaining mechanism, the spatial structure of the environment effected via increased media viscosity, as this factor is common to the CF lung environment but often absent from *in vitro* experiments. I found that there was little effect of increased viscosity alone on the abundance of species, but that the subinhibitory concentration of tobramycin again altered the community composition from one dominated by *P. aeruginosa* whilst *S. maltophilia* was undetectable, to one in which *P. aeruginosa* was undetectable and both *S. aureus* and *S. maltophilia* were able to grow to high abundance. The combination of increased viscosity and tobramycin resulted in stable coexistence of the three species. We then investigated whether stable coexistence of the final timepoint communities had evolved to be possible in the absence of the two environmental diversity maintaining mechanisms, and found that evolved communities could coexist in the baseline media whilst ancestral communities could not. However, the likelihood of coexistence within each evolved community replicate varied to a high degree, and the relative contribution of each species in the evolved communities also varied, suggesting that the replicate selection lines had evolved along different trajectories.

Chapter 4. Identification of mutations resulting from selection imposed by bacterial interactions, antibiotics, and viscosity.

In this chapter I extended the work from the previous chapter by investigating the genetic mutations that evolved during the selection experiment performed in Chapter 3. I sequenced 193 isolates in total, derived from each replicate community and treatment combination. The factorial nature of the original selection experiment allowed investigation into the differences in selection across community and treatment

gradients. I also investigated the coexisting isolates in detail to identify whether any mutations may contribute in a known way to mediating coexistence. I found that parallel mutations were selected across media treatments rather than community composition, but that in *P. aeruginosa* fewer mutations were selected in the more complex communities, suggesting that communities can constrain adaptation. I was not able to concretely identify genetic mechanisms that contributed to coexistence, but found potential candidate genes in *P. aeruginosa* involved in polyamine synthesis that warrant further study.

Chapter 2. The effects of antibiotic combination treatments on *Pseudomonas aeruginosa* tolerance evolution and coexistence with *Stenotrophomonas maltophilia*

2.1 Abstract

Pseudomonas aeruginosa is the most common bacterial pathogen of Cystic Fibrosis (CF) patients, who typically undergo aggressive antibiotic treatment regimens to attempt to eradicate *P. aeruginosa* and control pulmonary exacerbations. The physical complexity of the CF lung can result in some bacterial populations experiencing sub-minimum inhibitory concentration (MIC) antibiotic doses. Though the response of *P. aeruginosa* to sub-MIC antibiotics is well characterised in monocultures, they are less well understood in the presence of other bacteria, which is often the case with polymicrobial CF infections. Here we used a short-term *in vitro* selection experiment to investigate how the evolution of tolerance to three different antibiotics, administered at sub-MIC doses alone and in combination, was affected by the presence of a competitor, *Stenotrophomonas maltophilia*, in two different *P. aeruginosa* strains—the lab strain PAO1, and a clinical CF isolate LESB58. Increases in tolerance towards- and the MIC of- a given antibiotic were primarily driven by the presence of that antibiotic in the treatment. We observed a reciprocal cross tolerance between ciprofloxacin and tobramycin, and the combination of these two antibiotics resulted in high-level MICs for all three of the antibiotics used. Whilst neither the tolerance nor the MIC evolution was affected by the presence of *S. maltophilia*, extinctions of *P. aeruginosa* were more frequent in the presence of the competitor when treated with antibiotic treatments containing tobramycin, to which *S. maltophilia* was innately the most tolerant. Furthermore, the lab adapted *P. aeruginosa* PAO1 strain was driven extinct more frequently than the lung-adapted clinical strain. Together our findings suggest that sub-MIC antibiotic exposure remains a concern for MIC evolution, and that treatment with low concentrations of antibiotics can have unexpected ecological outcomes in a simple two-species coculture, which could be further magnified in a polymicrobial community such as CF.

2.2 Introduction

Cystic Fibrosis (CF) is a genetic condition that is characterised by impaired chloride ion channel function, resulting in thick mucus secretions in the lungs that are susceptible to chronic bacterial infection (Hart and Winstanley, 2002). Of the bacterial species that infect CF patients, *Pseudomonas aeruginosa* is the most common pathogen (UK Cystic Fibrosis Registry, 2020; Zolin *et al.*, 2020) and is very difficult to treat due to its intrinsic resistance to many antibiotics and its ability to readily evolve resistance to new antibiotics (Poole, 2011; López-Causapé, Cabot, *et al.*, 2018). Though usually dominated by one bacterial pathogen, such as *P. aeruginosa*, CF infections are often polymicrobial (Zhao *et al.*, 2012; Coburn *et al.*, 2015; Kramer *et al.*, 2015; Zemanick *et al.*, 2017; Einarsson *et al.*, 2019). Many different bacterial species can co-occur with *P. aeruginosa* in the CF lungs, and though the microbiome is highly individualised within each patient (Zhao *et al.*, 2012; Coburn *et al.*, 2015) there are many commonly co-isolated species such as the obligate anaerobic taxa *Prevotella*, *Veillonella*, and Streptococcal groups *milleri* and *mitis*, along with more traditional CF species such as *Staphylococcus aureus* and *Stenotrophomonas maltophilia* (Zhao *et al.*, 2012; Price *et al.*, 2013; Li *et al.*, 2016; Einarsson *et al.*, 2019). To what extent the presence of other species affects the antibiotic resistance development of *P. aeruginosa* remains unknown.

Over the course of their lives patients with CF will be treated with a number of different antibiotics; for example, during treatment to eradicate *P. aeruginosa* or to help resolve pulmonary exacerbations (UK Cystic Fibrosis Trust, 2009). These antibiotics are often administered via inhalation as a nebulised solution—as in the case of tobramycin and colistin—to deliver the treatment directly to the site of infection in the lungs, but can also be administered as intravenous solution or orally (UK Cystic Fibrosis Trust, 2009). Though the doses administered are very high, and are often given multiple times per day to maintain a therapeutic dose—i.e., a concentration greater than the minimum inhibitory concentration (MIC) required to kill the bacteria—the thick mucus secretions and the complex branching structure of the lungs themselves will likely result in a gradient of antibiotic concentrations (Moriarty *et al.*, 2007; Bos *et al.*, 2017). Thus, despite the best efforts of the treatment

regimens, many pockets of bacteria within the lungs are likely to experience antibiotic concentrations below that required to kill those bacteria, which could potentially promote antibiotic resistance evolution.

Selection for antibiotic resistance differs between antibiotics administered at or above MIC and below MIC. At concentrations greater than MIC the driver of selection is whether the bacteria can survive the antibiotic challenge, and so any mutations in the bacterial population that increase MIC, regardless of the impact on other competitive growth traits, would be selected for. This strong selection pressure under high antibiotic concentrations can lead to selection for single-step high-level resistance mutations, as mutations conferring lower levels of tolerance would either not survive the challenge or be outcompeted by higher-level resistance mutants, and there would not be time for low-level mutations to accumulate to reach higher resistance due to the all-or-nothing nature of the >MIC selection. This has been shown by Wistrand-Yuen *et al.*, (2018) to lead to repeatable mutations in specific genes that confer the required resistance, whereby in their study *Salmonella enterica* serovar Typhimurium replicate isolates treated with >MIC concentrations of streptomycin consistently developed mutations in the gene *rpsL*, which conferred an increase in MIC by modifying the antibiotic target. Below MIC the selective pressure differs, such that any mutation that confers an increase in growth, and thus a competitive advantage, relative to other members of the population would be selected regardless of whether this mutation would increase the MIC. The weak pressure results in a higher likelihood that a given mutation could increase fitness via alternative mechanisms, and the lack of antibiotic-mediated killing also results in a longer selective window during which more mutations can accumulate and either ameliorate the costs of higher-level resistance (Bottery, Wood and Brockhurst, 2017), or together confer high-level resistance via epistatic interactions (Jochumsen *et al.*, 2016). This too is demonstrated in the study by Wistrand-Yuen *et al.*, (2018), where they found that exposure to streptomycin at a concentration ¼ of the MIC of the susceptible WT strain resulted in isolates developing a high-level resistance, similar to >MIC selection, but via mutations in multiple different genes: individually each gene conferred only low-level increases in MIC, but together gave an epistatic effect that increased MIC by 256-fold. This series of mutations was likely only able to arise during the timeframe of their

experiment due to the presence of mutator phenotypes in these isolates (Wistrand-Yuen *et al.*, 2018), though this study and prior work by the same researchers demonstrated that the concentrations required to exert sufficient selection pressure to both enrich for resistant mutants in the population and generate *de novo* mutations (the minimal selective pressure, MSC) can be far lower than the MIC (Gullberg *et al.*, 2014). Furthermore, it has also been shown that subinhibitory antibiotic concentrations—i.e., concentrations that result in little-to-no growth impairment for the bacteria—drive phenotypic diversification across many traits in *P. aeruginosa* strain LESB58, in addition to antibiotic resistance (Wright *et al.*, 2013), and alter gene expression to modulate quorum sensing or biofilm formation (Linares *et al.*, 2006; Cummins *et al.*, 2009). How these sub-MIC effects of antibiotics act in polymicrobial communities, typical of CF patients, has been relatively unexplored.

Due to the chronic nature of bacterial infections in CF and the huge impact of infection upon a patient's health (Emerson *et al.*, 2002), it is essential that the antibiotic treatments administered are able to control the bacterial populations. To maximise efficacy, and attempt to diminish the development of resistance, antibiotic treatments are often administered either sequentially or in combination. For example, *P. aeruginosa* eradication therapy consists of either nebulised tobramycin, or a combination of oral ciprofloxacin and nebulised colistin (UK Cystic Fibrosis Trust, 2009; Langton Hewer and Smyth, 2017), and there is evidence for effective combination of colistin and tobramycin (Herrmann *et al.*, 2010). Antibiotic cycling can be an effective method of preventing the development of long-lasting resistance by using a new, unused antibiotic on any remaining bacteria that have developed resistance to the first antibiotic, as these populations should possess less standing genetic or phenotypic variation and be less able to withstand the novel antibiotic (Imamovic and Sommer, 2013). Antibiotic cycling could also be used to exploit collateral sensitivity that can arise as a result of antibiotic resistance whereby increased resistance to one antibiotic leads to increased sensitivity to another, and cycling between these antibiotics could slow or negate overall resistance evolution (Imamovic and Sommer, 2013; Kim, Lieberman and Kishony, 2014). Combinations of antibiotics are also administered depending upon the different bacterial species of concern in the patient's lungs—drugs effective against *P. aeruginosa* are not necessarily effective

against *S. aureus*. Combinations can therefore target different species simultaneously; they can also act synergistically to kill an individual species (Barbosa *et al.*, 2018). The effects of lethal concentrations (>MIC) of antibiotic combinations on individual bacterial species have been explored in the literature, particularly in terms of the ability for a bacterial species to develop resistance (Vestergaard *et al.*, 2016; Barbosa *et al.*, 2021); less known are the effects of sub-MIC concentrations of these combinations.

Finally, competition with other bacterial species could also change the trajectory of antibiotic resistance evolution of a focal pathogen species in various ways (Letten, Hall and Levine, 2021). First, the presence of competitors more tolerant of an antibiotic treatment than the susceptible pathogen species could increase the strength of competition between the two and lead to a decrease in relative pathogen abundance, potentially even triggering extinctions (Bottery, Pitchford and Friman, 2020). Competitor-mediated reduction in the population density of the focal pathogen could further slow resistance evolution by reducing the mutation supply rate and the emergence of *de novo* resistance (De Visser and Rozen, 2006). If antibiotic resistance evolves, it is often associated with metabolic costs, such as production of additional molecular machinery like efflux pumps or modification of the antibiotic target, and these costs could reduce pathogen competitiveness in the presence of non-resistant mutants, or species unaffected by a given antibiotic, when antibiotic concentrations are low (Stickland *et al.*, 2010; Jørgensen *et al.*, 2013; Klümper *et al.*, 2019). While it has been suggested that bacterial interactions are predominantly competitive (Foster and Bell, 2012), it is also possible that other interacting bacteria could facilitate antibiotic resistance of the focal species via horizontal gene transfer of resistance genes (Botelho, Grosso and Peixe, 2019). Alternatively, other species could provide protection from antibiotics via secretions that break down antibiotics (Bottery, Wood and Brockhurst, 2016, 2017) or create protective microenvironments via production of biofilms (Beaudoin *et al.*, 2017; Klümper *et al.*, 2019). Despite being a ubiquitous selective force in nature, there are relatively few studies directly testing the effect of bacterial inter-species interactions on the evolution of antibiotic resistance.

Here we focused on studying how the evolution of antibiotic resistance of *P. aeruginosa* is affected by the presence of *S. maltophilia*, which is increasing in

prevalence among CF patients (Salsgiver *et al.*, 2016; Hatziagorou *et al.*, 2019). *Stenotrophomonas maltophilia* infection has been associated with an increased number of pulmonary exacerbations (Waters *et al.*, 2011; Berdah *et al.*, 2018), and decreased lung function (Berdah *et al.*, 2018), and has been cocultured with *P. aeruginosa*, albeit at varying rates (Talmaciu *et al.*, 2000; Marchac *et al.*, 2004; Spicuzza *et al.*, 2009; Capaldo *et al.*, 2020). In order to investigate this, we performed a short-term *in vitro* serial transfer experiment in which we grew both of the lung-naïve laboratory *P. aeruginosa* strain PAO1 and the lung-adapted Liverpool Epidemic Strain B58 (LESB58; Winstanley *et al.*, 2009) either alone in monoculture or in the presence of *S. maltophilia* (resulting in four different cultures: PAO1, LESB58, PAO1 & *S. maltophilia*, and LESB58 & *S. maltophilia*). Two strains of *P. aeruginosa* were chosen to compare the potential effect of previous exposure to antibiotic treatments and other infecting bacteria on the evolution of antibiotic resistance. Each of these cultures were treated with one of the eight combinations (see Methods) of the anti-Pseudomonal antibiotics ciprofloxacin, colistin, and tobramycin. These antibiotics were selected because of their use in either *P. aeruginosa* eradication therapy or for treatment of pulmonary exacerbations (UK Cystic Fibrosis Trust, 2009; Mogayzel *et al.*, 2013; Langton Hewer and Smyth, 2017; Smith, Rowbotham and Regan, 2018), and for their differing modes of action: ciprofloxacin inhibits DNA replication, and thus bacterial replication, by binding to DNA topoisomerases II (DNA-gyrase) and IV (Hooper and Jacoby, 2016); colistin destabilises the outer membrane by displacing Ca^{2+} and Mg^{2+} ions, permeabilising the cell (Bialvaei and Samadi Kafil, 2015); and tobramycin inhibits RNA translation by binding to the 30S ribosomal subunit (Kotra, Haddad and Mobashery, 2000). Each of the antibiotics were applied at a sub-MIC concentration that had small but contrasting effects on the growth of all three bacterial strains (Appendix Figure A.1), which could have triggered changes in competitive interactions. During the serial transfer experiment, which took place over 21 days, we tracked the presence of *P. aeruginosa* and *S. maltophilia* for any extinctions and monitored changes in total population densities across the 192 selection lines. Following the experiment, we measured the ability of the evolved focal *P. aeruginosa* isolates to grow in the treatment concentrations of the individual antibiotics relative

to ancestral stock strains. Moreover, the MICs of each antibiotic were determined for all evolved *P. aeruginosa* isolates.

We hypothesised that: i) competitive interactions between *P. aeruginosa* and the competitor could be affected by differences between the two in their susceptibility to the antibiotics and antibiotic combinations; ii) competition with another species would constrain the evolution of antibiotic resistance in *P. aeruginosa*—as resource competition, for example, could result in reduced population size—and that this effect would be less pronounced in the case of the clinical strain LESB58 due to prior adaptation to the CF lung bacterial community; and iii) antibiotic combination treatments could promote resistance evolution, due to generalised resistance mechanisms such as upregulation of multiple efflux pumps resulting in a simultaneous increase in resistance to multiple antibiotics.

We found increases in antibiotic tolerance or MIC were not generally enhanced by antibiotic combinations; rather increases in tolerance or MIC to a given antibiotic were driven by the presence of that antibiotic in the treatment combination. There was little effect of the presence of the competitor on the ability of either *P. aeruginosa* strain to increase antibiotic tolerance or MIC. However, while both *P. aeruginosa* strains were able to dominate the "No Antibiotic" control treatments when in coculture, tobramycin-containing antibiotic treatments triggered *P. aeruginosa* extinctions in 15 % of co-culture populations, which was more common in PAO1 than LESB58. An explanation for this result could be that *S. maltophilia* had the greater innate tolerance to tobramycin, which was especially clear in comparison with lung-naïve PAO1 strain. Together, these results suggest that the efficacy of antibiotics could be magnified in polymicrobial communities due to competition and asymmetry in innate antibiotic tolerances.

2.3 Materials and Methods

2.3.1 Bacterial strains and culture conditions

Two strains of *Pseudomonas aeruginosa* were used as the focal pathogen species: PAO1, a lab adapted reference strain (ATCC 15692), and LESB58, a transmissible CF lung isolated strain (Winstanley *et al.*, 2009). *Stenotrophomonas maltophilia* type strain ATCC 13637—isolated from the oropharyngeal tract of a cancer patient (Hugh and Leifson, 1963)—was used as the coculture competitor. The base media used throughout was a 50:50 mix of nutrient broth without NaCl (Sigma; 5 g/l peptic digest of animal tissue, 3 g/l beef extract, pH 6.9) and PBS (8 g/l NaCl, 2 g/l KCl, 1.42 g/l Na_2HPO_4, 2.4 g/l KH_2PO_4), hereafter 'NB', that allowed stable coexistence of both the focal pathogen (*P. aeruginosa*) and competitor (*S. maltophilia*) species over a single 72 h growth period. All cultures, unless otherwise stated, were grown at 37ºC with shaking at 180 rpm.

2.3.2 Selection experiment

During the selection experiment, a focal bacterium (either *P. aeruginosa* strain PAO1 or LESB58) was grown in a culture either alone (monoculture) or with *S. maltophilia* (coculture), and treated with subinhibitory concentrations of ciprofloxacin (CIP), colistin (CST), and tobramycin (TOB) antibiotics in all one-, two- and three-way combinations ("No Antibiotic", CIP, CST, TOB, CIP+CST, CIP+TOB, CST+TOB, CIP+CST+TOB). Each treatment was replicated 6 times for both focal pathogens in the absence and presence of *S. maltophilia* resulting in a total of 192 selection lines. During the initial setup overnight cultures, from frozen stocks, of PAO1, LESB58, and *S. maltophilia*, were diluted down to the same optical density at 600 nm (OD_{600}; approximately 0.17 at 600 nm), corresponding to cell densities of $7.4x10^6$, $2.2x10^7$, and $4.6x10^6$ CFU/ml respectively. Monocultures consisted of 20 µl of the *P. aeruginosa* strain, whilst cocultures mixed 10 µl of the *P. aeruginosa* strain with 10 µl of *S. maltophilia*, each in 200 µl of NB supplemented with one of the eight antibiotic treatments for a total volume of 220 µl. The concentrations of the antibiotics (0.03215 µg/ml ciprofloxacin (Sigma Aldrich), 2 µg/ml colistin (Acros Organics), and 0.5 µg/ml tobramycin (Acros Organics)) were chosen to be below the minimum

inhibitory concentration of all three species (as determined below; Table 2.1, Appendix Figure A.1), and were kept constant across each combination. Selection lines were grown in 96-well plates. To measure the dynamics of the bacterial densities, all replicates were homogenised by mixing and OD_{600} of each replicate was measured every 72 hours (Tecan Infinite 200). After measurement, each replicate was again mixed and 20 µl of the culture was transferred to 200 µl of fresh media with the same antibiotic treatment, which were incubated for 72 hours until the next serial transfer. Presence or absence of each species in each culture was determined following each transfer by growing subsamples of the 72 hour cultures on different selective agar; *Pseudomonas* selective agar (Oxoid; *Pseudomonas* agar base: 16 g/l gelatin peptone, 10 g/l casein hydrolysate, 10 g/l potassium sulphate, 1.4 g/l magnesium chloride, 11 g/l agar, 1 % vol/vol glycerol; *Pseudomonas* CN selective supplement: 200 µg/ml centrimide, 15 µg/ml sodium nalidixate), and *S. maltophilia* selective agar: LB agar (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) supplemented with 64 µg/ml tobramycin incubated at 30ºC rather than 37ºC—*S. maltophilia* is innately resistant towards tobramycin at 30ºC (Mooney, Kerr and Denton, 2001). Some monoculture replicates were contaminated with *S. maltophilia* (14 PAO1 selection lines, and 9 LESB58 selection lines), and these were excluded from the analyses. Bacteria from each replicate were picked from the agar plates, grown overnight in NB, and cryopreserved in 20 % glycerol to be frozen at -80ºC. The selection experiment was carried out for 21 days, equalling 6 serial transfers. Assuming a growth period lasting ~8 hours and a doubling rate of ~1 hour, approximately 50 *P. aeruginosa* generations, though this does not take into account growth at later stages in the 72-hour period between transfers or differences in growth rate between antibiotic treatments.

2.3.3 Determination of MIC and antibiotic tolerance

Both prior to and following the selection experiment, the minimum inhibitory concentration (MIC) of each of the three antibiotics—ciprofloxacin, colistin, and tobramycin—was determined by broth microdilution for the three bacteria strains. Briefly, overnight cultures, from frozen stocks, were diluted 1 in 10 in PBS and further diluted 1 in 10 into NB with antibiotic concentrations ranging from 32 µg/ml to

0.015625 μg/ml (2^5 to 2^{-6}) and grown in static conditions, in triplicate. OD_{600} was measured after 24 hours (Tecan Sunrise). MIC was defined as the lowest concentration of antibiotic at which there was no growth. For the evolved strains, the MIC_{50} of a bacterial population was defined as the MIC required to inhibit half of the replicates of that population.

Difference in tolerance of the antibiotics, at the treatment concentrations used during the selection experiments, for each individual replicate of the evolved bacteria was calculated as:

$$\text{endpoint } OD_{600} \text{ (growth) of evolved replicate bacteria} - \text{endpoint } OD_{600} \text{ (growth) of ancestral bacteria}$$

When considering the difference in tolerance of bacteria from an environment as a whole:

$$\text{mean growth of all evolved replicates} - \text{growth of ancestral bacteria}$$

with the minimum tolerance being:

$$(\text{mean evolved growth} - \text{S. E. M. of evolved growth}) - (\text{growth of ancestral bacteria})$$

and maximum:

$$(\text{mean evolved growth} + \text{S. E. M. of evolved growth}) - (\text{growth of ancestral bacteria})$$

All bacteria (ancestral and evolved) were also grown without antibiotic in NB for 24 hours, and difference in growth without antibiotic was calculated as above.

2.3.4 Statistical analyses

All data were analysed in R version 3.6.3 (R Core Team, 2019). Data manipulation and graphing were performed using the *tidyverse* suite of packages (Wickham, 2017), along with *egg* for figure assembly (Auguie, 2019), and *ggbeeswarm* for point plotting (Clarke and Sherrill-Mix, 2017).

We fit the following models: i) To the population density dataset, two linear regression models were fit, one to each *P. aeruginosa* strain. The response variable was natural logarithm transformed OD_{600} values, and antibiotic treatment and competitor as the predictor variables. Two-way type II ANOVA was performed using the *car* package (Fox, Weisberg and Price, 2019). Post-hoc pairwise comparisons were computed from estimated marginal means using Tukey's honest significance test, through the `emmeans` and `contrast` functions of the *emmeans* package (Lenth, 2019). Full pairwise comparisons can be found in Additional File 1.1.

ii) To the tolerance dataset, separate linear regression models, following the same general procedure as in i), were fit for each response (i.e., growth difference relative to the ancestor during CIP exposure, CST exposure, TOB exposure, and "No Antibiotic" exposure) for the two *P. aeruginosa* strains, eight in total. Here, the response variable was difference in growth relative to the ancestor, and post-hoc pairwise comparisons were computed between treatments alone, after observing no effect of competitor nor interaction. Full pairwise comparisons can be found in Additional File 1.2.

iii) To the MIC dataset, individual Pearson Chi-squared tests of independence were performed for each *P. aeruginosa* strain in each antibiotic, six in total. MIC values were represented as ordered nominal variables, and frequency of observed MIC for each replicate in each treatment was tabulated. Cultures were grouped in this tabulation, as pairwise tests of independence between cultures within each treatment showed no difference between cultures, giving 2x2 contingency tables of MIC by treatment. Chi-squared tests were computed using the `chisq_test` function from the *coin* package (Hothorn *et al.*, 2008). Pairwise tests of independence with Benjamini-Hochberg false discovery rate corrections were performed between each treatment using the `pairwiseOrdinalIndependence` function from the *rcompanion* package (Mangiafico, 2020). Full pairwise comparisons can be found in Additional File 1.3.

Table 2.1: Minimum inhibitory concentrations for the three ancestral strains, along with the experimental concentrations used of each antibiotic and the EUCAST breakpoint concentrations for these antibiotics (EUCAST: European Committee on Antimicrobial Susceptibility Testing, 2019).

Antibiotic	**Treatment concentration (µg/ml)**	**Minimum inhibitory concentration (µg/ml) [treatment concentration as proportion of MIC]**			**EUCAST Breakpoint concentration (µg/ml) for *P. aeruginosa***
		PAO1	LESB58	*S. maltophilia*	
Ciprofloxacin	0.03125	0.0625 [1/2]	1 [1/32]	0.125 [1/4]	0.5
Colistin	2	4 [1/2]	4 [1/2]	8 [1/4]	2
Tobramycin	0.5	1 [1/2]	2 [1/4]	8 [1/16]	4

2.4 Results

2.4.1 The effect of antibiotic treatments on bacterial densities and coculture composition

At the final timepoint of the selection experiment we measured a proxy of total population density, as optical density at 600 nm, of each bacterial culture to determine the extent to which the low concentrations of antibiotics used had inhibited bacterial growth; here the measurements were of the entire populations, i.e., the total bacterial density of species in the cocultures that were alive at the final timepoint, not just *P. aeruginosa*.

In the case of PAO1, there was no effect of evolving in coculture vs monoculture on population density (Table 2.2, $p > 0.05$), and in LESB58 the significant effect of culture on total population density (Table 2.2, $p = 0.022$) was likely driven by the colistin (CST) mono-treatment where the total population densities of LESB58 cocultures were significantly higher than the monocultures (Pairwise Tukey post-hoc, $t(71) = 3.62$, $p = 0.024$; Figure 2.1B, D). Overall, total population densities were similar between the monocultures and the cocultures within a given antibiotic treatment. For both genotypes there was a significant effect of antibiotic treatment on total population density (Table 2.2, $p < 0.001$). Figure 2.1A–D shows that, with the exception of the CST mono-treatment, antibiotic treatment generally decreased the total population density relative to the "No Antibiotic" control treatment. However, pairwise comparisons showed that none of the monocultures differed significantly between treatments in either strain (Figure 2.1A, B); of the cocultures, the total population density of the CST+tobramycin (TOB) treatment was significantly lower than the "No Antibiotic" control treatment for both strains (Pairwise Tukey post-hoc, PAO1: $t(65) = 4.21$, $p = 0.0041$; LESB58: $t(71) = 3.98$, $p = 0.0081$; Figure 2.1C, D), and in LESB58 alone the total population densities of the triple treatment were also significantly lower than the "No Antibiotic" control treatment (Pairwise Tukey post-hoc, $t(71) = 3.98$, $p = 0.0081$; Figure 2.1D). Taken together these results show that though the antibiotic treatments somewhat reduced population density compared to the "No Antibiotic" control treatment there was little increased reduction from

combination treatments vs mono-treatments, and the combination of CST+TOB was universally effective at reducing population density.

In addition to measuring the effects on bacterial densities of the antibiotic treatments, we compared the composition of *P. aeruginosa* and *S. maltophilia* cocultures at the end of the experiment to examine the effects of antibiotics on the species coexistence. We found that *P. aeruginosa* survived in all monocultures across the different treatments (with the exception of a single PAO1 replicate going into extinction under CST+TOB treatment). This suggests that the low concentrations of the antibiotic were not sufficient to kill *P. aeruginosa*, even in combination. However, in the presence of a competitor, *S. maltophilia*, extinctions of *P. aeruginosa* were more common (Figure 2.1E, F). In the absence of antibiotics, both *P. aeruginosa* genotypes were able to dominate the cocultures, driving *S. maltophilia* extinct in all replicates; the same was true in the ciprofloxacin (CIP) and CST treatments. In contrast, the TOB mono-treatment allowed prolonged coexistence between the two bacteria: *S. maltophilia* was able to, with both *P. aeruginosa* genotypes, survive with *P. aeruginosa* in two of the six TOB mono-treatment replicates, and fully outcompete *P. aeruginosa* in a further three replicates (Figure 2.1E, F). The two *P. aeruginosa* genotypes differed in their capacity to coexist with *S. maltophilia* across the antibiotic combination treatments, with the laboratory strain PAO1 driven extinct more often than the clinical strain LESB58; PAO1 was only able to survive in two CIP+TOB treated replicates and was otherwise driven extinct in the remaining 22 replicates across the other tobramycin-containing combination treatments (Figure 2.1E). In contrast, the LESB58 strain dominated *S. maltophilia* in all of the combination treatments except for the CST+TOB treatment, in which *P. aeruginosa* was driven extinct in two replicates (Figure 2.1F). A likely explanation for these results is that *S. maltophilia* had a greater innate tolerance of tobramycin than *P. aeruginosa* (Appendix Figure A.1), which in turn led to a relative increase in competitive ability in tobramycin-containing treatments. In the case of PAO1, this effect was magnified in the tobramycin-containing combination treatments, whereas only one of these combinations resulted in LESB58 extinctions (Figure 2.1E, F). Together, these results suggest that tobramycin selection led to competitive exclusion of *P. aeruginosa*, or the coexistence of the two

species, whilst both the other antibiotics and the absence of antibiotics led to the extinction of *S. maltophilia*.

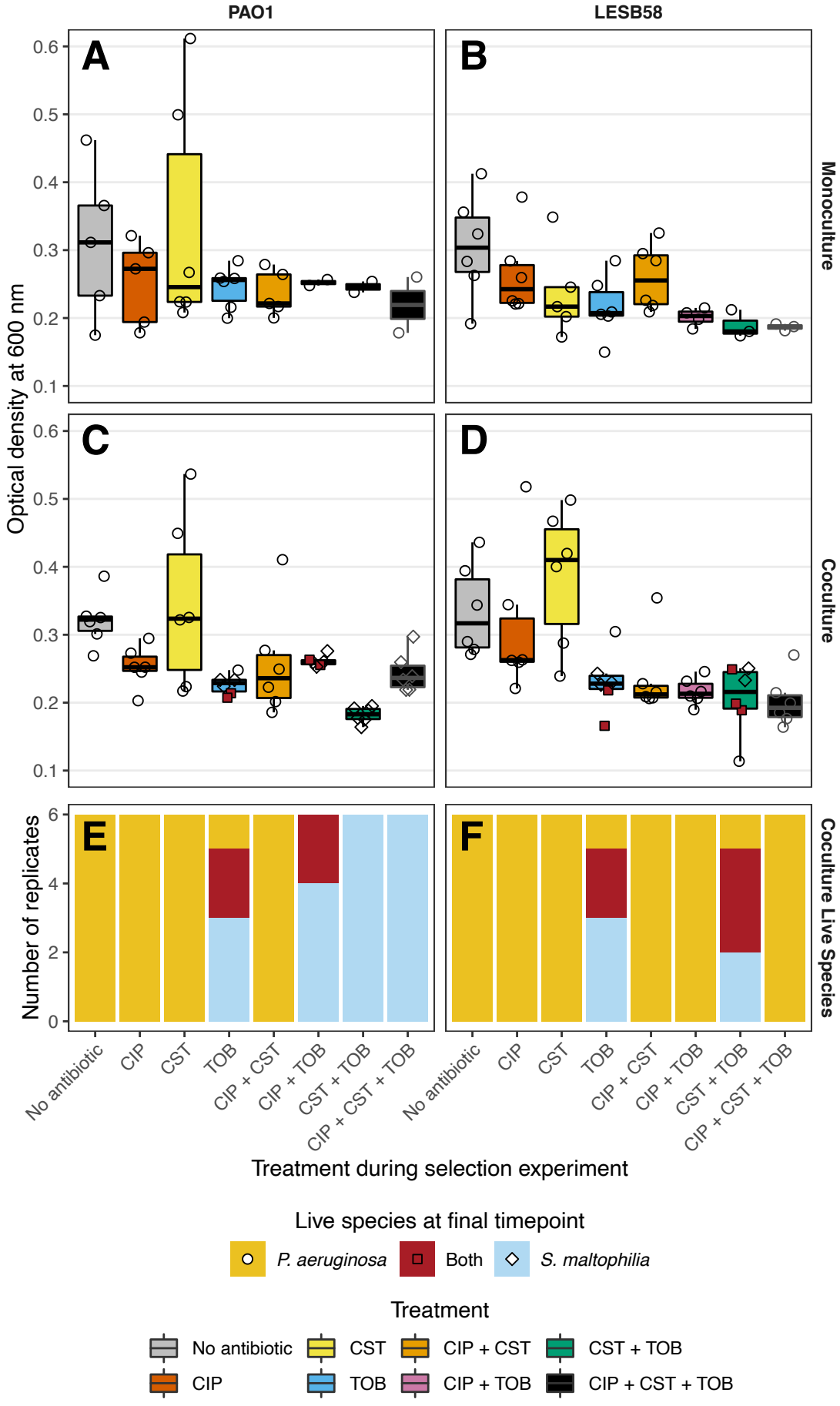

PAO1
LESB58
A
B
C
D
E
F
Monoculture
Coculture
Coculture Live Species
Optical density at 600 nm
Number of replicates
0.6
0.5
0.4
0.3
0.2
0.1
6
4
2
0
No antibiotic
CIP
CST
TOB
CIP + CST
CIP + TOB
CST + TOB
CIP + CST + TOB
Treatment during selection experiment
Live species at final timepoint
P. aeruginosa
Both
S. maltophilia
Treatment
No antibiotic
CIP
CST
TOB
CIP + CST
CIP + TOB
CST + TOB
CIP + CST + TOB

Figure 2.1: Total population density and composition of cocultures at the final timepoint of the selection experiment.

A–D: Boxplots of optical density at 600 nm of each replicate population from each of the eight treatments. Panel columns show the *P. aeruginosa* genotype, rows monocultures or cocultures. Points represent individual replicates; N = 6. Shapes show live species at the final timepoint: *P. aeruginosa* as circles, *S. maltophilia* as diamonds, and both as red squares. Boxplot fill colours show treatment. **E–F:** The surviving species from each treatment replicate of the cocultures (N = 6). Colours represent the surviving species: *P. aeruginosa* in orange, *S. maltophilia* in blue, and both in red.

Table 2.2: ANOVA tables of natural logarithm transformed total population density (as OD_{600}) at the final timepoint of the selection experiment.

Strain	ANOVA term	D.F.	Sum Sq.	F value	P value
PAO1	Treatment	7	1.67	4.42	0.00045
	Culture	1	0.0024	0.044	0.83
	Interaction	7	0.22	0.58	0.77
	Residuals	65	3.50		
LESB58	Treatment	7	2.81	8.30	2.29×10^{-7}
	Culture	1	0.28	5.51	0.022
	Interaction	7	0.53	1.57	0.16
	Residuals	71	3.44		

2.4.2 Effects of antibiotic treatments on *P. aeruginosa* antibiotic tolerance and relative cost of tolerance

To test whether there had been adaptation by the bacteria to better tolerate the antibiotics, we measured the ability of each of the surviving evolved *P. aeruginosa* to grow individually in the treatment concentrations of each of the antibiotics and compared these to their respective ancestral isolates. Some *P. aeruginosa* monoculture replicates were removed from the analyses due to contamination with *S. maltophilia* (see Methods § 2.3.2). Additionally, as *P. aeruginosa* went extinct in some of the tobramycin-containing treatments (24/143 selection lines), the evolution of tolerance was compared using only surviving treatment replicates.

The ability of either *P. aeruginosa* strain to tolerate the treatment concentrations of antibiotic was not affected by previous exposure to the competitor species *S. maltophilia* (Figure 2.2, Table 2.3). However, for both *P. aeruginosa* strains there was a significant effect of treatment on the tolerance to each antibiotic (Table 2.3). To investigate this effect further we compared levels of antibiotic tolerance evolution between different antibiotic treatments and looked at the possibility of cross-tolerance or sensitivity. For both *P. aeruginosa* strains, post-hoc analysis showed that there was a significant increase in ciprofloxacin tolerance when treated with the CIP mono-, CIP+CST and CIP+TOB treatments compared to the "No Antibiotic" control treatment (Pairwise Tukey post-hoc, PAO1: $t(48) = 10.46$ (CIP); 9.72 (CIP+CST); 10.27 (CIP+TOB), $p < 0.001$; LESB58: $t(66) = 4.57$ (CIP); 4.47 (CIP+CST); 3.47 (CIP+TOB), $p < 0.001$; Figure 2.2A, B). There were similar differences in tobramycin tolerance, whereby the TOB and CIP+TOB treatments significantly increased tolerance in PAO1 (Pairwise Tukey post-hoc, $t(48) = 6.48$ (TOB); 6.38 (CIP + TOB), $p < 0.001$; Figure 2.2E), and all tobramycin containing treatments significantly increased tolerance in LESB58 (Pairwise Tukey post-hoc, $t(66) = 5.61$ (TOB); 5.69 (CIP+TOB); 5.66 (CST+TOB); 5.56 (CIP+CST+TOB), $p < 0.001$; Figure 2.2F). However, colistin-containing treatments did not generally result in increased colistin tolerance: for both strains no colistin-containing treatments resulted in any statistical change in colistin tolerance compared to the "No Antibiotic" control treatments (Pairwise Tukey post-hoc, $p > 0.05$; Figure 2.2C, D).

We observed cross-tolerance between ciprofloxacin and tobramycin, i.e., the CIP mono-treatment provided tobramycin tolerance, and vice versa (Figure 2.2). In PAO1, the CIP mono-treatment trended towards increased tobramycin tolerance (Pairwise Tukey post-hoc, $t(48) = 3.03$, $p = 0.07$; Figure 2.2E), whilst TOB mono-treatment gave a significant increase in ciprofloxacin tolerance compared to the "No Antibiotic" control treatment (Pairwise Tukey post-hoc, $t(48) = 3.94$, $p = 0.006$); however, the increase in ciprofloxacin cross tolerance from the TOB mono-treatment was not as great an increase as resulted from the CIP mono-treatment, as the two groups differed significantly to each other (Pairwise Tukey post-hoc, $t(48) = 5.69$, $p < 0.001$; Figure 2.2A). A similar pattern emerged in LESB58, where the CIP mono-treatment gave a significantly higher tobramycin tolerance than the "No Antibiotic" control (Pairwise Tukey post-hoc, $t(66) = 5.61$, $p < 0.001$; Figure 2.2F), and, though not significant, a majority of replicates treated with TOB or CST+TOB had a greater ciprofloxacin tolerance than the mean "No Antibiotic" control (Figure 2.2B). Additionally, the CIP+TOB combination treatment resulted in cross-tolerance to colistin in both strains (Pairwise Tukey post-hoc, PAO1: $t(48) = 5.01$, $p < 0.001$; LESB58: $t(66) = 3.32$, $p = 0.03$; Figure 2.2B, F). Colistin-containing treatments did not provide any collateral tolerance towards the other antibiotics; and in the case of tobramycin tolerance, the combination of CIP+CST did not give cross-tolerance as in the CIP mono-treatment (Figure 2.2C, G). These results suggest that, generally, development of tolerance to a given antibiotic was driven by prior exposure, and that tolerance was not sensitive to the presence of other antibiotics, which in most cases neither enhanced nor restricted by combinations of antibiotics.

We also found that tolerance of the three antibiotics changed from their initial ancestral values differently between the two *P. aeruginosa* strains. Tolerance of both ciprofloxacin and tobramycin showed the same patterns in both strains: in the case of the clinical isolate LESB58, the "No Antibiotic" control treatment resulted in a loss of tolerance to these antibiotics relative to the ancestor, whilst the antibiotic treatments maintained the ancestral-level tolerances of ciprofloxacin and tobramycin. In contrast, the "No Antibiotic" control treatment of the lab strain PAO1 maintained ancestral-level tolerance, whilst the antibiotic treatments increased the tolerance of the evolved isolates by as much as 9-fold relative to the ancestor. Tolerance of colistin showed an

inverse pattern, whereby PAO1 generally lost tolerance relative to its ancestor while LESB58 maintained and increased its tolerance. The differences in tolerance evolution with respect to their ancestors highlights the disparity between the naïve lab strain PAO1 and the lung-adapted clinical strain LESB58 and suggests that previous exposure to antibiotics in the lungs plays a role in further tolerance evolution.

To test if the selection in different antibiotic treatments led to a cost of tolerance, we also grew each of the surviving evolved replicates in media without antibiotic and compared this to the growth of their respective ancestors (Figure 2.2G, H). This measure acts as a proxy indicator of the cost of antibiotic tolerance and the bacteria's relative ability to compete for resources. The majority of both *P. aeruginosa* genotype replicates across all treatments evolved to grow better in the growth media relative to their ancestors (Figure 2.2G, H), and the increase in growth, relative to the ancestor, was greater in the lung-adapted LESB58 than the lab-adapted PAO1. However, this increase clearly varied between the antibiotic treatments. In the case of both genotypes, the TOB mono-treatment constrained adaptation, resulting in a significantly reduced growth compared to the "No Antibiotic" control treatment (Pairwise Tukey post-hoc, PAO1: $t(44) = 4.47$, $p = 0.001$; LESB58: $t(66) = 3.40$, $p = 0.02$). Moreover, the growth of evolved LESB58 populations treated with any tobramycin-containing treatment were significantly below the "No Antibiotic" control treatment (Pairwise Tukey post-hoc, $t(66) = 3.58$, $p = 0.01$ (CIP+TOB); 4.81, $p < 0.001$ (CST+TOB); 3.89, $p = 0.005$ (CIP+CST+TOB); Figure 2.2H). Together these results suggest that adapting to tolerate tobramycin was costly to the pathogen strains.

In summary, both pathogen strains were readily able to develop tolerance to ciprofloxacin and tobramycin, primarily driven by the presence of those antibiotics in the treatments, and exposure to these two antibiotics promoted some reciprocal cross-tolerance. Adaptation to the growth media was also constrained in the presence of tobramycin, and this could explain the coexistence observed between *P. aeruginosa* and *S. maltophilia*: though tobramycin tolerance increased, the cost associated with this tolerance could prevent sufficient growth for *P. aeruginosa* to exclude *S. maltophilia*, as in the "No Antibiotic" control treatment for example.

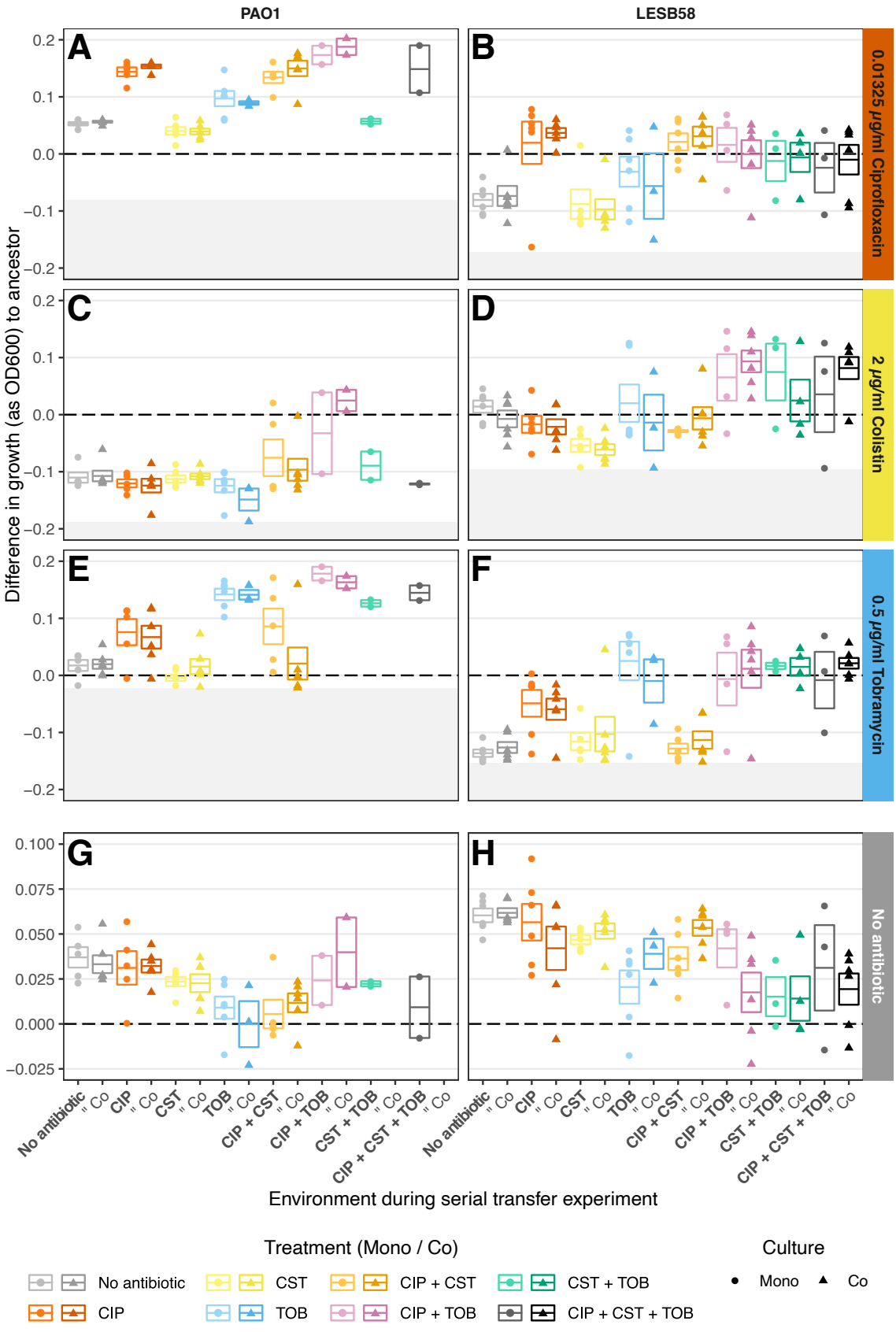

PAO1
LESB58
A
B
C
D
E
F
G
H
0.01325 µg/ml Ciprofloxacin
2 µg/ml Colistin
0.5 µg/ml Tobramycin
No antibiotic
Difference in growth (as OD600) to ancestor
No antibiotic
CIP
CST
TOB
CIP + CST
CIP + TOB
CST + TOB
CIP + CST + TOB
Co
Environment during serial transfer experiment
Treatment (Mono / Co)
Culture
Mono
Co

Figure 2.2: Growth of each evolved replicate population, relative to their respective ancestor, in the same treatment concentrations of each antibiotic (A–F) or without antibiotic (G–H), measured in separate growth assays at the end of the selection experiment.

Panel columns show the two *P. aeruginosa* genotypes, whilst panel rows show growth in the presence and absence of different antibiotics. The horizontal dashed line represents growth equal to that of the ancestor (i.e., relative change in OD_{600} = 0). Each point represents the mean growth for three technical replicates of one replicate population, minus the growth to a similarly replicated ancestral growth. Boxes show mean of all populations (centre line), and upper and lower limits represent the maxima and minima as defined in the methods. Shapes show mono- (circle; 'Mono') and cocultures (triangle; 'Co'); colours show antibiotic treatment, with lighter and darker shades representing the absence and presence of the *S. maltophilia* competitor. Shaded grey portions of the panels represents no growth.

Table 2.3: ANOVA tables for growth of each evolved *P. aeruginosa* population when exposed to each antibiotic in growth assays, as difference in growth relative to ancestor.

Strain	Environment	ANOVA term	D.F.	Sum Sq.	F value	P value
PAO1	Ciprofloxacin	Treatment	7	0.15	47.64	$<2x10^{-16}$
		Culture	1	$3.9x10^{-4}$	0.89	0.35
		Interaction	5	$9.0x10^{-4}$	0.41	0.84
		Residuals	48	0.02		
PAO1	Colistin	Treatment	7	0.06	6.25	$3.15x10^{-5}$
		Culture	1	$9.8x10^{-5}$	0.08	0.78
		Interaction	5	$5.7x10^{-3}$	0.89	0.50
		Residuals	48	0.06		
PAO1	Tobramycin	Treatment	7	0.18	15.08	$3.14x10^{-10}$
		Culture	1	$1.6x10^{-3}$	0.99	0.32
		Interaction	5	0.01	1.38	0.25
		Residuals	48	0.08		
PAO1	No Antibiotic	Treatment	7	$7.7x10^{-3}$	5.06	$2.3x10^{-4}$
		Culture	1	$1.1x10^{-6}$	$5.0x10^{-3}$	0.94
		Interaction	5	$5.7x10^{-4}$	0.52	0.76
		Residuals	48	0.01		
LESB58	Ciprofloxacin	Treatment	7	0.16	7.25	$1.96x10^{-6}$
		Culture	1	$3.8x10^{-5}$	0.01	0.91
		Interaction	7	$3.9x10^{-3}$	0.17	0.99
		Residuals	66	0.21		
LESB58	Colistin	Treatment	7	0.16	8.14	$4.08x10^{-7}$
		Culture	1	$3.1x10^{-5}$	0.01	0.92
		Interaction	7	0.02	0.81	0.59
		Residuals	66	0.19		

LESB58	Tobramycin	Treatment	7	0.31	14.75	$2.07x10^{-11}$
		Culture	1	$5.9x10^{-4}$	0.20	0.66
		Interaction	7	$6.2x10^{-3}$	0.30	0.95
		Residuals	66	0.20		
LESB58	No Antibiotic	Treatment	7	0.02	6.25	$1.24x10^{-5}$
		Culture	1	$2.5x10^{-5}$	0.06	0.81
		Interaction	7	$3.9x10^{-3}$	1.39	0.23
		Residuals	66	0.03		

2.4.3 Changes in pathogen MIC of antibiotics

As well as measuring the ability of the different *P. aeruginosa* genotypes to tolerate the treatment concentrations of the three antibiotics, we measured the MIC of each antibiotic for each surviving replicate and calculated the MIC_{50}—the MIC capable of inhibiting 50 % of replicates—for each evolved replicate population. To gauge the potential significance of the changes in MIC value, we have used the EUCAST breakpoint values (EUCAST: European Committee on Antimicrobial Susceptibility Testing, 2019; Table 2.1)—concentrations of antibiotics that are used as the cut-off at which bacteria are deemed clinically "resistant" to said antibiotic—as a reference point. The MIC values are not directly comparable as we did not perform the MIC assays according to the EUCAST guidelines, and as such we can make no judgement on whether any of our strains were clinically resistant; rather, the EUCAST values are included to contextualise the changes in MIC that have arisen as a result of the relatively low, sub-MIC antibiotic concentrations used in our experiment. For both *P. aeruginosa* strains, across all three antibiotics, there was no effect of competition on the MICs observed within treatment groups, though there was a consistent significant association between antibiotic treatments and MIC of the evolved isolates for both strains in each antibiotic (Table 2.4).

2.4.3.1 Changes in ciprofloxacin MIC

Both of the *P. aeruginosa* strains were able to develop large increases in MIC to ciprofloxacin (Figure 2.3A, B). The MIC of ciprofloxacin for PAO1 replicates from

the "No Antibiotic" control treatment remained mostly unchanged relative to the ancestor, at 0.125 μg/ml, though a pair of individual replicates increased their MIC by three-fold (Figure 2.3A); whereas in LESB58 the baseline effect of the "No Antibiotic" control was a decrease in MIC of three-fold compared to the ancestor, from 1 μg/ml to 0.125 μg/ml—to the same concentration as the laboratory PAO1 strain (Figure 2.3B). Pairwise Chi-squared tests of independence showed that the CIP, CIP+CST, and CIP+TOB treatments all resulted in significantly greater MIC values amongst isolates of both strains than the "No Antibiotic" control treatment (Pairwise independence; PAO1—CIP: $X^2(1, N = 22) = 15.61$, $p = 0.002$; CIP+CST: $X^2(1, N = 22) = 10.77$, $p = 0.010$; CIP+TOB: $X^2(1, N = 15) = 8.34$, $p = 0.018$; Figure 2.3A; LESB58—CIP: $X^2(1, N = 24) = 19.03$, $p > 0.001$; CIP+CST: $X^2(1, N = 24) = 13.71$, $p = 0.0019$; CIP+TOB: $X^2(1, N = 22) = 13.31$, $p = 0.0019$; Figure 2.3B). The MIC_{50} in each of these treatments was at or above the EUCAST breakpoint value of 0.5 μg/ml, and in LESB58 this was also true of the CST+TOB and triple treatments that, unlike in PAO1, also resulted in significantly increased MIC values (Pairwise independence; CST+TOB: $X^2(1, N = 19) = 10.03$, $p = 0.0064$; CIP+CST+TOB: $X^2(1, N = 21) = 9.95$, $p = 0.0064$; Figure 2.3B). Furthermore, many of the TOB mono-treated isolates from both strains had MIC values at or above the EUCAST breakpoint, and in LESB58 these were significantly different to the "No Antibiotic" control treatment (Pairwise independence; TOB: $X^2(1, N = 21) = 7.34$, $p = 0.019$), suggesting that there is some collateral tolerance provided by tobramycin as also seen in the growth measurements. The MIC values for evolved LESB58 isolates reached higher levels than in PAO1, with 18 LESB58 isolates reaching 4 or 8 μg/ml compared with one PAO1 isolate, and there was also greater variation in MIC value among the LESB58 isolates of a given treatment than PAO1. Overall, both *P. aeruginosa* strains were readily able to develop an increase in ciprofloxacin MIC, with the clinical LESB58 strain able to reach marginally higher MIC values than the laboratory PAO1 strain, and this was driven mostly by the presence of ciprofloxacin, though the presence of tobramycin also resulted in some increase in MIC.

2.4.3.2 Changes in colistin MIC

As opposed to ciprofloxacin, the MICs of colistin did not generally increase as a result of the antibiotic selection (Figure 2.3C, D). In the case of PAO1, though there was a significant association between treatment and MIC of the evolved isolates (Table 2.4), the majority of treatments resulted in no change to the ancestral MIC of 4 μg/ml (Figure 2.3C); pairwise comparisons showed that the CIP+TOB treatment was the driver of this association as the MIC differed significantly from the "No Antibiotic" control treatment (Pairwise independence; $X^2(1, N = 15) = 8.32$, $p = 0.037$; Figure 2.3C). There was slightly more variation amongst the LESB58 isolates treated with CIP, CST, or TOB mono-treatments, though no changes greater than one-fold for more than a single replicate were found. The MICs of isolates treated with the combinations CIP+TOB and CST+TOB, however, were both significantly different to the "No Antibiotic" control treatment (Pairwise independence; CIP+TOB: $X^2(1, N = 22) = 6.13$, $p = 0.037$; CST+TOB: $X^2(1, N = 19) = 6.21$, $p = 0.037$; Figure 2.3C). Overall, there was a greater increase in MIC for the clinical strain LESB58 than PAO1, though neither showed much change, and in both strains any increase was driven primarily by the CIP+TOB treatment rather than the presence of colistin.

2.4.3.3 Changes in tobramycin MIC

The MIC changes for tobramycin were similar between both genotypes (Figure 2.3E, F). The "No Antibiotic" control treated isolates of PAO1 maintained the ancestral MIC of 1 μg/ml (Figure 2.3E), whereas again, as was the case with ciprofloxacin MIC, the "No Antibiotic" control treated LESB58 isolates decreased in MIC relative to their ancestor, from 2 μg/ml down to 0.5 and 1 μg/ml (Figure 2.3F). For both strains the TOB and CIP+TOB treatments gave a significant increase in MIC compared with the "No Antibiotic" control treatment (Pairwise independence; PAO1—TOB: $X^2(1, N = 20) = 8.54$, $p = 0.0035$; CIP+TOB: $X^2(1, N = 15) = 12.00$, $p < 0.001$; Figure 2.3E; LESB58—TOB: $X^2(1, N = 21) = 12.67$, $p = 0.0035$; CIP+TOB: $X^2(1, N = 22) = 11.35$, $p = 0.0053$; Figure 2.3F), and for LESB58 this was the case for the CST+TOB and triple treatments as well (Pairwise independence; CST+TOB: $X^2(1, N = 19) = 13.77$, $p = 0.0035$; CIP+CST+TOB: $X^2(1, N = 21) = 13.22$, $p = 0.0035$). Many LESB58 isolates reached MIC values equal to or greater than the EUCAST breakpoint value of

4 μg/ml, whilst this was much rarer in PAO1 (Figure 2.3E, F). Both strains also had a significant increase in MIC compared to the "No Antibiotic" control treatment as a result of the CIP mono-treatment (Pairwise independence; PAO1: $X^2(1, N = 22) = 7.98$, $p = 0.019$); LESB58: $X^2(1, N = 24) = 10.58$, $p = 0.0064$), though this increase did not reach the clinical breakpoint as the tobramycin containing treatments did; indeed, in LESB58 the MIC of the CIP mono-treated isolates was significantly lower than the TOB, CST+TOB, and CIP+CST+TOB treatments (Pairwise independence; TOB: $X^2(1, N = 21) = 7$, $p = 0.021$; CST+TOB: $X^2(1, N = 19) = 8.05$, $p = 0.014$; CIP+CST+TOB: $X^2(1, N = 21) = 6.64$, $p = 0.023$) suggesting that the collateral tolerance provided by ciprofloxacin is weak compared to that provided by tobramycin. Overall, though fewer isolates reached the clinical breakpoint value than did when considering ciprofloxacin MIC, both strains were similarly able to develop increases in tobramycin MIC, which was again primarily driven by the presence of tobramycin in the treatments.

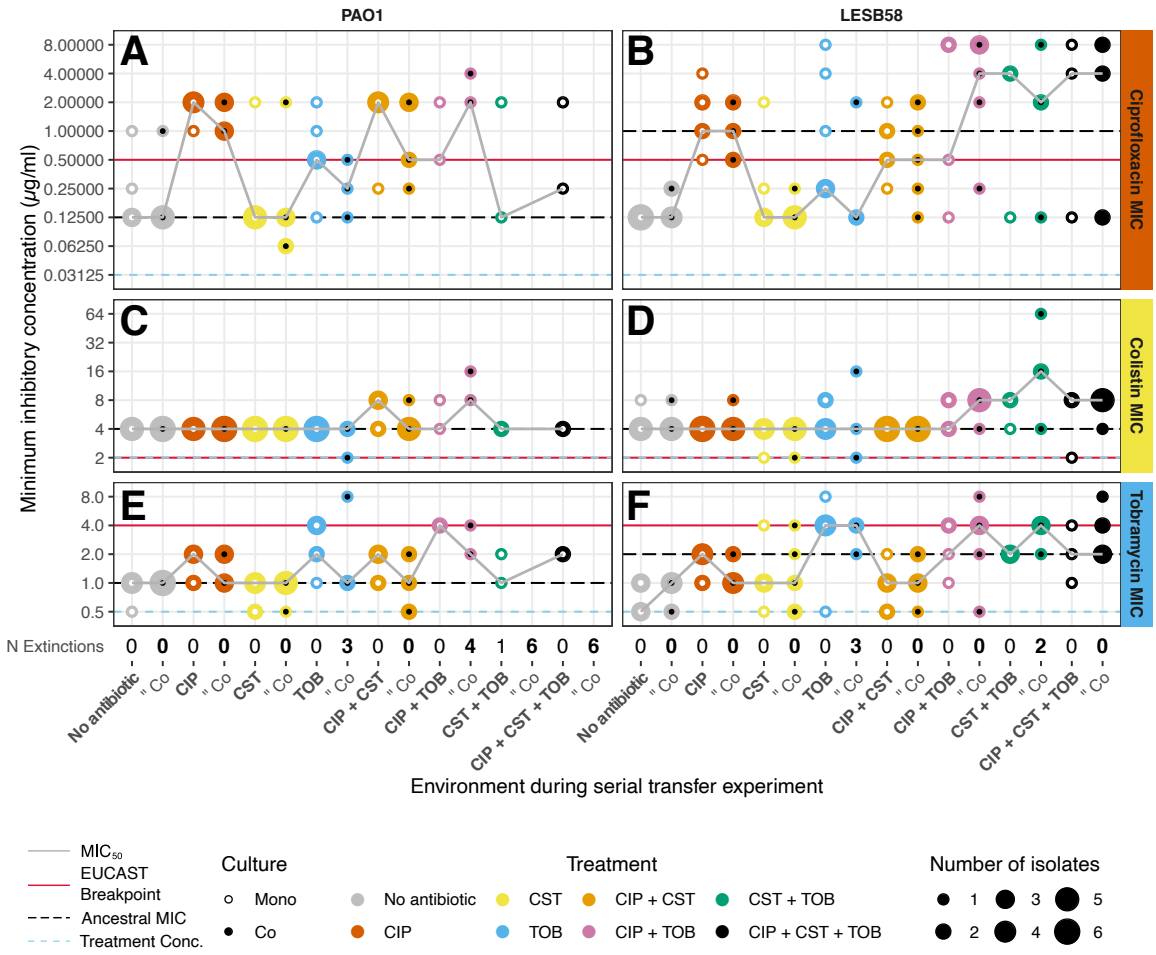


Figure 2.3: The MIC of the three individual antibiotics for each evolved replicate population of the focal pathogen strains.

Panel columns show *P. aeruginosa* genotype and rows the MIC of each antibiotic. The dashed line represents the MIC of the respective ancestors; the red line represents the EUCAST breakpoint value for *P. aeruginosa*; the grey line shows the MIC_{50} of each treatment across replicate populations, as defined in the methods. The size of each point represents the number of replicates with the specified MIC. Colour of the points represents treatment; white centre dot represents monoculture and black represents coculture. Number of extinctions in each treatment and coculture shown beneath the X-axis. MIC was measured in triplicate for each replicate.

Table 2.4: Pearson Chi-Squared Test of Independence for the MIC of each antibiotic for both *P. aeruginosa* strains.

Strain	Antibiotic	X^2	D.F.	P value
PAO1	Ciprofloxacin	32.13	7	3.84×10^{-5}
	Colistin	29.00	7	1.44×10^{-4}
	Tobramycin	30.55	7	7.52×10^{-5}
LESB58	Ciprofloxacin	33.47	7	2.16×10^{-5}
	Colistin	29.89	7	9.97×10^{-5}
	Tobramycin	37.86	7	3.22×10^{-6}

2.5 Discussion

The presence of *P. aeruginosa* within the polymicrobial communities in CF patient lungs, and the clinical exacerbations of symptoms that can result from community changes (Carmody *et al.*, 2018; Layeghifard *et al.*, 2019), result in aggressive antibiotic treatments to manage or clear the bacteria from the lungs. Antibiotics are given in high doses, and often in combination, but the physical structure of the CF lung can result in antibiotic concentration gradients and subpopulations of bacteria that experience sub-MIC antibiotic doses (Moriarty *et al.*, 2007; Bos *et al.*, 2017). Antibiotic selection can act differently when experienced at sub-MIC concentrations (Andersson and Hughes, 2012), when antibiotics are used in combination (Vestergaard *et al.*, 2016; Barbosa *et al.*, 2018), and when bacteria are in polymicrobial cultures (Klümper *et al.*, 2019). We sought to investigate these factors by examining the antibiotic tolerances of *P. aeruginosa* evolved during an *in vitro* selection experiment, whereby *P. aeruginosa* was grown alone or in coculture with *S. maltophilia* and treated with every combination of the anti-Pseudomonal antibiotics ciprofloxacin, colistin, and tobramycin, each administered at sub-MIC doses.

2.5.1 *Pseudomonas aeruginosa* was driven extinct only in tobramycin treated cocultures

At the final timepoint of the selection experiment, we found that when in monoculture *P. aeruginosa* went extinct in only one replicate as a result of the antibiotic treatments (PAO1 treated with CST+TOB). In coculture without antibiotics, *P. aeruginosa* was the only species detectable, but when treated with an antibiotic combination that contained tobramycin (i.e., TOB, CIP+TOB, CST+TOB, or the triple treatment) *S. maltophilia* survival and *P. aeruginosa* extinction were both more common. A possible explanation for this observation is that *S. maltophilia* ancestor had a greater tolerance of tobramycin than either *P. aeruginosa* strain, which could have driven its dominance in these cocultures. Indeed, *S. maltophilia* possesses a temperature dependent resistance towards tobramycin that manifests when grown at 30ºC (Mooney, Kerr and Denton, 2001), which could further explain the greater effect of tobramycin on the cocultures than the other two antibiotics; for example,

S. maltophilia had a greater initial tolerance of colistin than *P. aeruginosa* strain LESB58, but only *P. aeruginosa* survived in the CST mono-treatment.

The competitive ability of the two *P. aeruginosa* strains was affected differently by the tobramycin-containing combination treatments. The laboratory strain PAO1 was driven extinct in each tobramycin combination except for two CIP+TOB replicates, whereas the clinical strain LESB58 was only affected in the CST+TOB treatment. This can, to some degree, be explained by differences in initial antibiotic tolerance, in that LESB58 was more tolerant of ciprofloxacin than PAO1 and was unaffected in the CIP+TOB and triple treatments where PAO1 was mostly driven extinct. Less clear is the effect of colistin, as LESB58 was less tolerant than PAO1 yet survived in the CST+TOB treatment where PAO1 was again driven extinct.

The differences in survivability of the two strains reflects the differences in evolutionary history between the two. As PAO1 was first isolated in 1955 (Holloway, 1955), its long use in the lab as a model strain has likely resulted in adaptations to grow in lab media after repeated culturing. On the other hand LESB58, a transmissible strain isolated in 1988 from CF patients (Winstanley *et al.*, 2009), has adapted to the CF lungs; for example, LESB58 produces more biofilm than PAO1 (Kukavica-Ibrulj *et al.*, 2008), and, as is often typical of CF isolates, lacks motility (Winstanley *et al.*, 2009). However, more uncommon to CF isolates is that LESB58 has retained its bacterial competition factors, where continued production is likely beneficial during infection of new patients for clearing competitors (O'Brien *et al.*, 2017), and expresses these factors earlier within the growth cycle than PAO1 (Salunkhe *et al.*, 2005; Fothergill *et al.*, 2007). As LESB58 produces a greater amount of such competition factors than PAO1 (Salunkhe *et al.*, 2005), this may have contributed to LESB58 survival in treatments where PAO1 was unable to survive, such as CIP+TOB and the triple treatment. An alternative possibility in these ciprofloxacin containing combinations is that LESB58 contains several prophage elements (Winstanley *et al.*, 2009) that are inducible in low concentrations of ciprofloxacin (Fothergill *et al.*, 2011) and have been shown to result in lysis of PAO1 (James *et al.*, 2012). Bacteriophages are usually very host specific (Koskella and Meaden, 2013), though *S. maltophilia*-specific bacteriophages have been identified that were capable of infecting *P. aeruginosa* via the type IV pilus (Peters *et al.*, 2015; McCutcheon, Peters and Dennis, 2018). It may

be possible that LESB58 bacteriophages are capable of infecting *S. maltophilia*, and so phage induction that resulted in lysis of *S. maltophilia* may have contributed to LESB58 survival in the CIP+TOB and triple treatments, and it would be of interest to test such hypotheses in the future.

The baseline interaction between these two species appears to be antagonistic whereby *P. aeruginosa* will competitively exclude *S. maltophilia*. A study by Pompilio *et al.*, (2015) has investigated some of the interactions between CF isolated *P. aeruginosa* and *S. maltophilia* and found evidence that during planktonic growth *P. aeruginosa* killed *S. maltophilia* via a contact dependent mechanism, likely a type VI secretion system. Similar results have been found during mixed species biofilm growth, whereby the two species were not able to reach an equilibrium; *P. aeruginosa* would always eventually outcompete *S. maltophilia* (Pompilio *et al.*, 2015; Magalhães, Lopes and Pereira, 2017). Ultimately though, further work is required to understand the interactions between these species, as there is little research currently, but our observation of the strong effect of a low dose of tobramycin is interesting particularly in the context of treatment for CF polymicrobial infections.

2.5.2 Cost of tobramycin tolerance may contribute to competitive outcomes with *S. maltophilia*

We investigated the ability of the surviving *P. aeruginosa* replicates to tolerate the treatment concentrations of the individual antibiotics, and found that generally *P. aeruginosa* was able to develop an increase in tolerance to the antibiotics, and that increased tolerance of an antibiotic was driven by the presence of that antibiotic in the treatment, i.e., *P. aeruginosa* treated with a ciprofloxacin-containing treatment developed an increase, relative to the "No Antibiotic" control treatment, in ciprofloxacin tolerance.

When treated with tobramycin-containing treatments, both *P. aeruginosa* strains increased their tolerance to tobramycin, including the surviving coculture replicates (Figure 2.2). One might expect that the evolution of increased tobramycin tolerance would restore the competitive dominance of *P. aeruginosa* observed in the "No Antibiotic" control treatment (Figure 2.1E, F). However, we found that TOB treatment resulted in a relative decrease in growth compared to the ancestor in the

absence of tobramycin, suggesting a cost to the increased tolerance (Figure 2.2G, H). We thus suggest that this costly tolerance mechanism likely promoted coexistence in the TOB mono-treatments, as growth of *P. aeruginosa* was constrained despite increased tolerance. Evolution of antibiotic tolerance increase in polymicrobial communities has been rarely studied (Bottery, Pitchford and Friman, 2020). However, Klümper *et al.*, (2019) have studied the effects of community on intraspecific competition between gentamicin susceptible and resistant *E. coli*. They found that in the absence of community the resistant strain was able to outcompete the susceptible strain over multiple antibiotic concentrations, whereas the presence of the community selected for the susceptible strain as only in the highest antibiotic concentration (100 µg/ml, 10-fold greater than the susceptible strain MIC) were the benefits of resistance outweighed by the metabolic cost of resistance. It is difficult to draw comparisons between our study and theirs, as the relationships between the bacteria involved were very different: their community was much larger than a simple two-species coculture, and *E. coli* strain MG1655 does not appear to be as antagonistic towards the other species as *P. aeruginosa* is to *S. maltophilia*; however, the cost of tobramycin tolerance was magnified in the presence of *S. maltophilia* such that both species could coexist, and were more species to be added to our consortium then we might expect the costs of tolerance to be further magnified with greater resource competition and to further impact *P. aeruginosa* viability. This, coupled with the observation by Scheuerl *et al.*, (2020) showing that bacterial adaptation is generally constrained in larger communities, suggests that in our experimental setup addition of further species might lead to more consistent extinctions of *P. aeruginosa* as the chances of tolerance evolution would be further limited due to associated costs.

2.5.3 Sublethal antibiotic concentrations select for high level MICs, which is magnified with increasing combinations

We observed increases in *P. aeruginosa* MIC of the three antibiotics that were consistent with the increases in tolerance observed, in that generally increases in MIC of a given antibiotic were driven by the presence of that antibiotic in the treatment. However, we observed some notable exceptions to this generalisation, such as a cross-tolerance between ciprofloxacin and tobramycin, and in LESB58 large and moderate

increases in ciprofloxacin and colistin MIC when treated with tobramycin-containing combinations (Figure 2.3). Although no genomic analyses were performed on the evolved isolates here, we can speculate as to the likely mutational causes of the observed increases in MIC.

Ciprofloxacin and tobramycin share tolerance mechanisms in *P. aeruginosa*, in that increased tolerance of both antibiotics can be mediated by increased production of the MexXY-OprM efflux pump (Masuda *et al.*, 2000b; Hocquet *et al.*, 2003). Indeed, *P. aeruginosa* strain LESB58 may constitutively express *mexXY* as the negative regulator *mexZ* was found to be a pseudogene (Winstanley *et al.*, 2009), and could explain the greater initial tolerance in both ciprofloxacin and tobramycin compared to PAO1. However, despite the prevalence among CF isolates of mutations resulting in increased *mexXY* expression (Marvig *et al.*, 2015; Greipel *et al.*, 2016), previous studies performed using higher antibiotic concentrations have suggested that *in vitro* selection for mutations such as these are rare (Yen and Papin, 2017; López-Causapé, Rubio, *et al.*, 2018). In the case of the CIP+TOB and triple combination treatments, it is possible that PAO1 mutants overexpressing *mexXY* could be selected due to the greater inhibition of growth observed in these treatments (Figure 2.1A–D), and increased adaptation rate has been observed in *P. aeruginosa* populations treated with this combination (Barbosa *et al.*, 2018). The adaptations from to the individual treatments are not as clear: low concentrations of tobramycin can select for mutations in *fusA1* (López-Causapé, Rubio, *et al.*, 2018; Scribner *et al.*, 2020), encoding elongation factor G that facilitates movement of mRNA and tRNA through the ribosome, which can provide modest increases in MIC and is also associated with a growth cost (Bolard, Plésiat and Jeannot, 2018); and sublethal concentrations of ciprofloxacin can select for mutations in *gyrAB* (Jørgensen *et al.*, 2013), encoding the drug's target DNA gyrase, particularly when grown as planktonic cells rather than biofilms (Ahmed *et al.*, 2018). However, there is little evidence of these mutations providing cross-tolerance, and though collateral resistance has been observed between evolved *P. aeruginosa* isolates with different mutational profiles (Barbosa *et al.*, 2017), this result is contrary to a similar experiment performed by Wright *et al.*, (2013) that showed that LESB58 evolved during selection with a low concentration of

tobramycin resulted in reduced tolerance of ciprofloxacin, and so it would be of interest to investigate the true mechanisms here further.

Colistin and tobramycin also share resistance mechanisms in the form of activation of the *pmrAB* operon (Moskowitz, Ernst and Miller, 2004; Johnson *et al.*, 2012; Bolard *et al.*, 2019) and the ParRS two-component system (Fernández *et al.*, 2010; Muller, Plésiat and Jeannot, 2011; Puja *et al.*, 2020). Colistin tolerance is primarily mediated by activation of the *arnBCADTEF* operon, resulting in modification of lipid A to mask negatively charged phosphate and inhibit colistin infiltration (McPhee, Lewenza and Hancock, 2003; Moskowitz, Ernst and Miller, 2004). Activation of *arnBCADTEF* can come as a result of both the ParRS and PmrAB two-component systems. ParRS responds to presence of cationic antimicrobial peptides, such as colistin, and activates expression of both the *arnBCADTEF* and *pmrAB* operon, as well as the efflux pump *mexXY*, all of which have been shown to contribute to colistin tolerance (Fernández *et al.*, 2010; Muller, Plésiat and Jeannot, 2011; Puja *et al.*, 2020). The *pmrAB* operon encodes genes involved in the synthesis of the polyamines spermidine and norspermidine, which can localise on the surface of the outer membrane and decrease permeability to aminoglycosides, as well as mask negatively charged regions on the outer membrane, resulting in increased tobramycin tolerance (Johnson *et al.*, 2012; Bolard *et al.*, 2019). Due to the complex gene regulation by these two systems, gain of function mutations in either *pmrB* or *parS* can result in increased tolerance to both antibiotics, and indeed have been observed in *P. aeruginosa* treated with aminoglycosides *in vitro* (Barbosa *et al.*, 2017; López-Causapé, Rubio, *et al.*, 2018) and in clinical isolates (Jochumsen *et al.*, 2016; Bolard *et al.*, 2019).

The efficacy of these multiple systems suggests an explanation for the lack of increased tolerance of *P. aeruginosa* replicates treated with CST, in that the inhibition of the low concentration of colistin used was likely small enough to be easily overcome by the mechanisms outlined above resulting in a low selection pressure for mutational overactivation of either system. The combination of both colistin and tobramycin resulted in greater inhibition of all *P. aeruginosa* populations (Figure 2.1A–D) and the observed increase in MIC of surviving CST+TOB treated replicates (Figure

2.3D) suggests that the selection pressure there was perhaps sufficient to result in *pmrB* or *parS* activating mutations.

2.5.4 Caveats and improvements

Interpretation of the effects of antibiotic treatments on MIC values is complicated by the variable sample sizes of the pairwise comparisons between treatments that come as a result of extinctions, and significance tests could be overinfluenced by high level outliers, for example in the ciprofloxacin MIC values for TOB treated LESB58 replicates. There was also greater variation among ciprofloxacin MIC values than colistin or tobramycin, and this could be due to the nature of using a two-fold dilution series in that the majority of variation for ciprofloxacin was between 0.125 μg/ml and 2 μg/ml—a change of 4-fold but an absolute difference of 1.875 μg/ml—while colistin and tobramycin MIC values varied generally by two- or three-fold but with absolute differences between 3–12 μg/ml. Were there to be the same gradation between 1–4 μg/ml as between 0.125–2 μg/ml it is possible that the same degree of variation would be observed among tobramycin MIC values as ciprofloxacin. Greater resolution of MIC could be obtained by measuring inhibition on agar plates using, for example, Etest strips or other disk diffusion assays that result in continuous zones of inhibition.

This study system has allowed us to observe the simple eco-evolutionary dynamics between *P. aeruginosa* and *S. maltophilia* under low antibiotic concentrations; but the system could be improved in a number of ways, particularly by better emulating the natural conditions of the lungs. Firstly, growth in shaken liquid conditions results in planktonic growth that can give different evolutionary trajectories compared to the aggregate biofilm lifestyle common within the CF lung (Ahmed *et al.*, 2018; Ciofu and Tolker-Nielsen, 2019). Aggregate formation can be triggered by increased viscosity (Staudinger *et al.*, 2014; Secor *et al.*, 2018), and a greater degree of biofilm formation could alter the interspecies interactions, particularly between antagonistic species (Pompilio *et al.*, 2015; Magalhães, Lopes and Pereira, 2017; McNally *et al.*, 2017). Nutrient composition has also been shown to alter bacterial gene expression, particularly between lab media and CF sputum (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007), and so better mimicking the nutrient composition

of sputum would likely alter the interspecies interactions to a degree. Antibiotic gradients are likely common in the lungs (Moriarty *et al.*, 2007; Bos *et al.*, 2017), and so expanding the number of concentrations used would allow exploration of the different possible evolutionary trajectories within simple communities (Klümper *et al.*, 2019). Finally, the addition of a different species, either to replace *S. maltophilia* or to expand the community, would likely result in different ecological outcomes as the observed effects of tobramycin between *P. aeruginosa* and *S. maltophilia* would not hold for a different species with differing tobramycin susceptibility. It would therefore be of interest to determine whether a similar effect would be observed between *P. aeruginosa* and *S. aureus* in the presence of colistin, to which *S. aureus* is innately immune as a Gram-positive species, or whether a larger community inhibits *P. aeruginosa* antibiotic resistance evolution.

2.5.5 Significance and Conclusion

Evolution of *P. aeruginosa* within the CF lung is very complex, where different antibiotic treatments, bacterial community, and host factors result in widespread genotypic and phenotypic heterogeneity, both within *P. aeruginosa* in a single patient and between different patients (Zhao *et al.*, 2012; Diaz Caballero *et al.*, 2015; Marvig *et al.*, 2015; Williams *et al.*, 2018). Given this complexity we can only provide interpretations within the specific set of circumstances explored here. These suggest that the ability of *P. aeruginosa* to evolve antibiotic tolerance within the lung communities exposed to low antibiotic concentrations may depend on the species identity of the other members of these communities. Here we see that, in the specific context of the interaction between *P. aeruginosa* and *S. maltophilia*, low concentrations of tobramycin can exert an inhibitory effect on *P. aeruginosa* that results in more effective competition by *S. maltophilia* that can drive extinction of both naïve and adapted *P. aeruginosa* strains, likely when unable to develop tolerance. And in the event of developing tolerance, *P. aeruginosa* growth may still be suppressed due to the cost of that tolerance, as suggested by coexistence with *S. maltophilia*. Combination treatments containing tobramycin were more effective at driving competitive exclusion in the naïve PAO1 strain, suggesting that during early infection combination antibiotic treatments may be more effective at clearing *P. aeruginosa*

than individual treatments. In particular, the combination of colistin and tobramycin was also effective in this manner against the lung adapted strain LESB58, and has also been shown to be effective at killing during biofilm growth (Herrmann *et al.*, 2010). Antibiotic treatment of CF patients more often has the aim of managing infections and symptoms rather than total bacterial clearance (Waters *et al.*, 2019) and so our results provide further insight into the large range of potential outcomes of this treatment, in that we reinforce the dangers of sublethal antibiotic exposure when it comes to development of resistance (Gullberg *et al.*, 2011; Wistrand-Yuen *et al.*, 2018), and show that there may be unexpected ecological effects that alter community composition and perhaps the course of patient disease.

In summary, we found that the negative effects of sublethal antibiotic concentrations can be magnified in the presence of more tolerant competitors, leading to more frequent extinctions, and that the negative effects can persist even with evolution of antibiotic tolerance due to the associated growth costs. Crucially, these patterns were antibiotic-specific—here only under tobramycin exposure—and affected the clinical LESB58 to a lesser degree than the lab strain PAO1. Even though unaffected by the presence of a competitor, development of antibiotic tolerance in response to sublethal antibiotic concentrations, administered both individually and in combination, selected for high level MIC—particularly with ciprofloxacin. These results suggest that differences in antibiotic susceptibility can magnify competition in bacterial communities, and that failure to administer sufficient antibiotic doses when used in combination present a possible cause for concern during treatment.

Chapter 3. Evolution of coexistence in a simple Cystic Fibrosis-like community

3.1 Abstract

Cystic Fibrosis polymicrobial bacterial communities are highly diverse, and increased diversity is associated with lesser clinical symptoms. It is not known which ecological mechanisms maintain bacterial diversity within the lungs, particularly in communities containing *Pseudomonas aeruginosa*, as numerous *in vitro* experiments show *P. aeruginosa* to be highly antagonistic to other bacterial species. Here, we used a short-term *in vitro* selection experiment to investigate how species abundance within a simple three-species CF-like community, consisting of *P. aeruginosa*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia*, was affected by the potential diversity maintaining mechanisms of increased media viscosity and a subinhibitory concentration of tobramycin antibiotic, previously demonstrated to increase diversity. We found: that increased viscosity alone did not increase coexistence, as communities were dominated by *P. aeruginosa* whilst *S. maltophilia* was undetectable; that the effect of tobramycin on *P. aeruginosa* was exacerbated in a larger community, as *P. aeruginosa* became undetectable within communities; and that the combination of the two factors resulted in stable coexistence of the three species for the duration of the experiment. Following this observation, we investigated the stability of evolved community coexistence in the absence of diversity maintaining mechanisms and found that evolved communities could coexist without viscosity or tobramycin, whereas ancestral communities could not. However, the likelihood of coexistence in evolved communities was highly variable between selection lineages, and the contribution of each evolved species to the coexistence outcome was dependent upon different phenotypic traits between selection lineages. Together, these results show that coexistence between antagonistic CF-associated species can evolve under certain selective pressures, and this may play a role in the short-term maintenance of diversity within the lungs. However, questions remain as to the effects of antibiotics within larger communities and across other environmental gradients,

and highlight that further research is required to understand the interaction between communities and antibiotic selection within the lungs.

3.2 Introduction

Bacteria rarely live alone in nature, but rather are found in polymicrobial communities, and despite the former ubiquity of classifying infection by presence of single pathogen species, Cystic Fibrosis-associated bacterial communities are similarly polymicrobial. Both within and between patient diversity is high (Zhao *et al.*, 2012; Price *et al.*, 2013; Coburn *et al.*, 2015; Li *et al.*, 2016; Einarsson *et al.*, 2019; Layeghifard *et al.*, 2019) despite the general predominance of antagonistic interspecies interactions between bacteria (Mitri and Richard Foster, 2013; Limoli and Hoffman, 2019). *Pseudomonas aeruginosa* is thought to be highly antagonistic to the growth of other bacterial species, producing a large range of secreted factors that enhance *P. aeruginosa* competition for resources or actively inhibit other bacterial species (O'Brien and Fothergill, 2017; Limoli and Hoffman, 2019). The ability of *P. aeruginosa* to outcompete cocultured bacteria is frequently demonstrated *in vitro* (Filkins *et al.*, 2015; Nguyen *et al.*, 2015; Pompilio *et al.*, 2015; Magalhães, Lopes and Pereira, 2017), yet diversity still persists among patients, and as such it is unclear which ecological mechanisms maintain this diversity.

Many diversity maintaining mechanisms are likely acting upon *in vivo* CF bacterial communities, such as spatial separation, asymmetries in antibiotic susceptibility, and niche differentiation through nutrient utilisation. Spatial structure can act to increase diversity by dampening the strength of competitive interactions between species through both physical separation and reduced diffusion. Spatial structure in the CF lungs is mediated through both the high viscosity of the CF sputum (Tomaiuolo *et al.*, 2014; Goltermann and Tolker-Nielsen, 2017; Secor *et al.*, 2018) and the physical distance within the complex branching structure of the lungs (Conrad *et al.*, 2013; Jorth *et al.*, 2015; Kordes *et al.*, 2019), as well as by biofilm formation (Bjarnsholt *et al.*, 2013). Growth as a biofilm is the primary mode of growth for bacteria across many different environments, including in CF (Bjarnsholt *et al.*, 2013; Ciofu and Tolker-Nielsen, 2019). Typically, biofilms form from bacterial cells that have adhered to a surface prior to production of the biofilm matrix (Bjarnsholt *et al.*, 2013);

however, in CF formation of biofilm-like cell aggregates by *P. aeruginosa* is common (Alhede *et al.*, 2011; Darch *et al.*, 2018; Secor *et al.*, 2018), whereby the cells self-adhere and produce matrix components to form free-floating aggregates. The formation of such aggregates is facilitated in CF by the high viscosity environment through both active bacterial processes—production of matrix exopolysaccharides—and physical processes—depletion aggregation due to the physical density of molecules in the surrounding sputum (Bjarnsholt *et al.*, 2013; Staudinger *et al.*, 2014; Goltermann and Tolker-Nielsen, 2017; Secor *et al.*, 2018). Indeed, some clinical isolates of *P. aeruginosa* such as the Liverpool Epidemic Strain have adapted to primarily form free-floating aggregates, even *in vitro* without a viscous environment (Gagné-Thivierge, Barbeau, *et al.*, 2018; Gagné-Thivierge, Kukavica-Ibrulj, *et al.*, 2018). Importantly, both adherent and non-adherent biofilms—i.e., surface attached biofilms and free-floating aggregates—result in the same benefits and side-effects for the bacteria residing within, such as increased antimicrobial tolerance, slower growth, and spatial structure (Bjarnsholt *et al.*, 2013; Ciofu and Tolker-Nielsen, 2019). High viscosity can also limit diffusion of secreted competition factors such as iron chelating siderophores (Kümmerli *et al.*, 2009; Granato *et al.*, 2018), which are critical for growth in the iron-limited conditions within the lungs (Turner *et al.*, 2015; Rossi *et al.*, 2018). Siderophores contribute to *P. aeruginosa* domination within *in vitro* cocultures (Filkins *et al.*, 2015; Nguyen *et al.*, 2015), and so reduced diffusion could limit their competitive advantage. Physical separation of bacterial populations growing within different lung compartments results in evolution of distinct lineages (Jorth *et al.*, 2015; Kordes *et al.*, 2019) suggesting that mixing within the lung may be limited, increasing diversity (Conrad *et al.*, 2013).

Antibiotics are a powerful selective force within the CF lungs (Rossi *et al.*, 2020), due to the ubiquity of treatment and the long-term nature of treatment regimens (UK Cystic Fibrosis Trust, 2009; Waters *et al.*, 2019). Bacterial species may differ in their susceptibility to different antibiotics, which could alter the competitive dynamics between species and mediate coexistence, and the fluctuation of antibiotic concentrations during treatments could further affect species diversity (Letten, Hall and Levine, 2021). And though over longer timescales evolution of resistance can be essential for surviving repeated antibiotic exposure (Daniels *et al.*, 2013; Marvig *et al.*,

2015), fitness trade-offs of resistance mutations could serve to maintain diversity by decreasing competitive fitness in the absence of antibiotics (Letten, Hall and Levine, 2021). Such fitness trade-offs could be particularly acute within communities that are exposed to sublethal antibiotic treatments (Klümper *et al.*, 2019), which is likely to occur within CF lungs due to the difficulty of delivering sufficient drug doses across the entire lungs (Moriarty *et al.*, 2007; Bos *et al.*, 2017).

Adaptation to the lung environment and the different niches therein could also drive diversity, as species utilise different nutrients (Conrad *et al.*, 2013; Quinn *et al.*, 2014; La Rosa *et al.*, 2018; La Rosa, Johansen and Molin, 2019). Early- and late-stage clinical *P. aeruginosa* isolates have been shown to metabolise different nutrients at different points within the growth cycle as they adapt to the nutrient composition of the lungs (La Rosa *et al.*, 2018), the knock-on effects of which can cause nutrient usage overlap with other species and change the degree of competition. Cross-feeding between species could also facilitate diversity, as one species produces metabolites that the other can utilise (Flynn *et al.*, 2016; Gao *et al.*, 2018; Tognon *et al.*, 2019). Increased diversity may come as a by-product of adaptation to the lungs, as *P. aeruginosa* has been observed to lose or reduce production of a number of competition factors during evolution within the lungs (Andersen *et al.*, 2015; Marvig *et al.*, 2015; Frydenlund Michelsen *et al.*, 2016; Bernardy *et al.*, 2020). This can be as a result of mutations that provide other benefits within the lungs, such as the switch to the mucoid phenotype that can protect *P. aeruginosa* from antibiotic and immune stress (Ryder, Byrd and Wozniak, 2007; Goltermann and Tolker-Nielsen, 2017; Bartell *et al.*, 2019). The overproduction of alginate that comes as a result of mutations in the *mucA* gene results in concomitant downregulation of quorum-sensing (QS) related secreted competition factors such as siderophores and phenazines (Folkesson *et al.*, 2012; Limoli *et al.*, 2017). Siderophore production can be lost during infection, often as a result of the breakdown of intraspecies cooperation (Andersen *et al.*, 2015, 2018), and mutations in the QS systems that facilitate such cooperation, such as in the *lasR* gene, are also common during infection, and also result in reduced production of competition factors (Jiricny *et al.*, 2014; Marvig *et al.*, 2015; Azimi *et al.*, 2020).

Ultimately, diversity cannot be maintained indefinitely, and, as patients age and advance through disease stages, the diversity of the lung microbiome decreases

(Zhao *et al.*, 2012; Coburn *et al.*, 2015; Li *et al.*, 2016; Zemanick *et al.*, 2017; Carmody *et al.*, 2018). This association presents a chicken-and-egg problem, as it is not known whether increased lung damage during later disease stages results in reduced diversity, or whether reduced diversity results in increased lung damage. An emerging hypothesis is that the intensity of antibiotic regimens, particularly during pulmonary exacerbations, is a strong driver of decreased diversity (Zhao *et al.*, 2012; Daniels *et al.*, 2013; Carmody *et al.*, 2018), as high concentrations select for bacterial species that are able to survive the treatment whilst clearing species that cannot; this could then allow expansion of the more tolerant species to the cleared area, termed competitive release (Whelan and Surette, 2015). Often, *P. aeruginosa* is the dominant species within post-mortem and explant lung samples (Bjarnsholt *et al.*, 2009; Goddard *et al.*, 2012; Jorth *et al.*, 2015), though it is unclear whether this is due to its competitive ability, its capacity to withstand and evolve resistance to antibiotic treatments (López-Causapé, Cabot, *et al.*, 2018), or a combination of the two factors.

Previously, we found that treatment with a low concentration of tobramycin (TOB) was able to drive coexistence between two competitive CF-associated species *P. aeruginosa* and *Stenotrophomonas maltophilia*—see Chapter 2. We hypothesised that this was due to differences in susceptibility to the antibiotic that substantially reduced the competitive ability of *P. aeruginosa* relative to *S. maltophilia*. From this observation we then decided to investigate whether this outcome during treatment with a low concentration of tobramycin would hold with the addition of anther species to the community. In addition, we asked whether increased viscosity could act as a diversity maintaining mechanism *in vitro*, as high viscosity is a prominent feature of CF mucus that is rarely captured during *in vitro* experiments. Furthermore, we sought to investigate whether coexistence could evolve to occur stably across different media environments, and in this case whether changes would occur in the same species to drive this coexistence. Finally, we explored the phenotypic and genotypic—see Chapter 4—mechanisms that could facilitate coexistence.

To this end, we continued the use of our model coculture system of *P. aeruginosa* and *S. maltophilia*, to which we chose to add another common CF pathogen, *Staphylococcus aureus*, to create a three-species community. Interactions between *P. aeruginosa* and *S. aureus* are frequently observed to be antagonistic *in vitro*

(Palmer *et al.*, 2005; Hoffman *et al.*, 2006; Filkins *et al.*, 2015; Nguyen *et al.*, 2015), though *P. aeruginosa* and *S. aureus* have been observed together as a coinfection within patients at varying rates (Hubert *et al.*, 2013; Limoli *et al.*, 2016; Limoli and Hoffman, 2019; Cystic Fibrosis Foundation Patient Registry, 2020). We chose to use the clinical CF Liverpool Epidemic Strain B58 (LESB58; Winstanley *et al.*, 2009) of *P. aeruginosa* to examine how a transmissible CF adapted strain would be affected during coculture with these species. Whether all three species have been cultured from a patient is unclear, particularly as coinfection rates are less widely available than detection of individual species (Salsgiver *et al.*, 2016; Hatziagorou *et al.*, 2019). However, they are at least hypothesised to occupy a similar ecological niche to each other due to shared nutrient and pH requirements (Quinn *et al.*, 2015; Quinn, Whiteson, *et al.*, 2016).

We performed a short-term, fully factorial, *in vitro* serial transfer experiment, wherein we grew all seven combinations of the three species in media supplemented with increased viscosity and/or 0.5 μg/ml of tobramycin over the course of 22 days. Each community–treatment combination was replicated four times, for a total of 112 independent selection lines. We tracked species abundance over time by enumerating each species on selective agar plates.

Based on work in the previous chapter and evidence from the literature, we predicted that *P. aeruginosa* would dominate the three-species community within the Liquid media. However, we hypothesised that: i) increasing the viscosity of the environment would promote coexistence of the three-species, as it would enable greater spatial separation between the species and limit direct competition; ii) that treatment with the low concentration of tobramycin would again favour *S. maltophilia* due to differences in susceptibility to tobramycin between the three species; and iii) that when increased viscosity and tobramycin treatment were combined, the effect of tobramycin on the communities would be diminished due to spatial separation reducing any competitive advantages of the tobramycin selection. Were coexistence to occur, we sought to further study whether the coexistence was purely as a result of the environmental factors, the removal of which would cease the coexistence, or whether coexistence could evolve between the species to result in more stable interactions in the absence of environment coexistence maintaining mechanisms.

We found that within the three-species communities *P. aeruginosa* was able to dominate both the baseline "Liquid" treatment and the "Viscous" treatment, whilst the addition of tobramycin within the "Liquid + TOB" treatments promoted coexistence between *S. aureus* and *S. maltophilia* and the exclusion of *P. aeruginosa*. Stable coexistence between all three species was only observed in the combination of "Viscous + TOB" treatment, where all three species grew to high abundance over the course of the selection experiment. To investigate the stability of coexistence within the evolved Viscous + TOB treated communities, we isolated the individual species from the replicate evolved communities and used individual isolates to reconstitute each replicate community in a repeatable manner. We grew the evolved replicate communities in all four media conditions—thus removing the environmental factors in which they originally coexisted—and found that coexistence continued in the Liquid treatment without environmental diversity maintaining mechanisms. We further investigated the adaptation and relative contribution of each evolved species to this outcome by replacing each evolved species within the communities with their ancestral strain, and found that each of the evolved species mediated coexistence differently within their replicate communities. We also found that coexistence was highly variable between replicate evolved communities, suggesting that there were independent evolutionary trajectories between replicate communities and that outcomes were dependent on complex interactions between the three species. Finally, we investigated the individual growth characteristics of clones of each species to try and ascertain mechanisms involved that may contribute to the observed coexistence. We found that reductions in growth of *P. aeruginosa* relative to the other species may increase the likelihood of coexistence, whilst increased adherent biofilm formation may decrease that likelihood, and that changes in indirect interactions via supernatants may also contribute to increased likelihood of coexistence.

3.3 Materials and Methods

3.3.1 Bacterial strains and culture conditions

The constituent species of our community were the *Pseudomonas aeruginosa* clinical CF isolate Liverpool Epidemic Strain B58 (LESB58; Winstanley *et al*. 2009), *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884—isolated from human pleural fluid (ATCC 12600; Cowan, Shaw and Williams, 1954)—and *Stenotrophomonas maltophilia* type strain ATCC 13637—isolated from the oropharyngeal tract of a cancer patient (Hugh and Leifson, 1963). The base media used throughout was nutrient broth without NaCl (Sigma, 5 g/l peptic digest of animal tissue, 3 g/l beef extract, pH 6.9) supplemented with PBS salts (68.5 mmol/l NaCl, 1.35 mmol/l KCl, 5 mmol/l Na_2HPO_4, 0.9 mmol/l KH_2PO_4), hereafter 'NB'. Overnight cultures were grown at 37ºC with shaking (180 rpm) in NB.

3.3.2 Community selection experiment

The selection experiment was a full factorial design, wherein communities consisting of every combination of *P. aeruginosa*, *S. aureus*, and *S. maltophilia*—single-species, two-species, and three-species communities, seven in total—were grown in media supplemented with either increased viscosity or a low dose of tobramycin, or both, and each combination of community and media treatment was replicated 4 times, for a total of 112 independent selection lines.

For initial setup, overnight cultures of *P. aeruginosa*, *S. aureus* and *S. maltophilia* were diluted in PBS to the same optical density at 600 nm (OD, Tecan Infinite 200), corresponding to approximately $6.1x10^6$ CFU/ml, $4.2x10^6$ CFU/ml, and $1.6x10^8$ CFU/ml respectively. 180 µl of total diluted bacteria was mixed with 2 ml of media to make up each community, whereby the total volume of bacteria added was divided equally between the number of species in the community, e.g., for two-species communities the volume of bacteria added consisted of 90 µl each of the two species in the community, etc. In the three-species community, this resulted in starting densities of ~10^5 CFU/ml each of *P. aeruginosa* and *S. aureus*, and ~10^6 CFU/ml *S. maltophilia*. Selection lines were grown in 24-well plates.

Each community was grown in one of four media treatment conditions: NB, "Liquid"; NB supplemented with 20 % w/vol ficoll PM400 (GE Healthcare), "Viscous"; NB supplemented with 0.5 µg/ml tobramycin (Alfa Aesar), "Liquid + TOB"; and NB supplemented with both ficoll and tobramycin, "Viscous + TOB". The concentration of tobramycin was chosen to be below the minimum inhibitory concentration of all three species: 4 µg/ml for *P. aeruginosa*, 32 µg/ml for *S. aureus*, and 8 µg/ml for *S. maltophilia* (as determined in Chapter 2 § 2.3.3 and Appendix Figure B.12). Ficoll is a neutrally charged, highly branched polymer of sucrose that is commonly used to create density gradients for separation of cells. Preliminary assays determined that ficoll increased the viscosity of the media without forming a gel in the manner of agar, which enabled subsamples to be taken for a serial transfer experiment.

24-well plates were incubated at 37ºC without shaking for 48 hours, after which each well was thoroughly mixed—including scraping the sides and bottom—and 180 µl of homogenised culture was transferred to 2 ml of fresh media of the same treatment. The selection experiment was carried out for 22 days, equalling 10 transfers. To track the dynamics of each population, at every third transfer subsamples of each replicate community were serially diluted in PBS and 10 µl of the serial dilutions between 10^{-1} and 10^{-7} were spotted onto selective agar plates. These were: *Pseudomonas* selective agar (Oxoid; *Pseudomonas* agar base: 16 g/l gelatin peptone, 10 g/l casein hydrolysate, 10 g/l potassium sulphate, 1.4 g/l magnesium chloride, 11 g/l agar, 1 % vol/vol glycerol; *Pseudomonas* CN selective supplement: 200 µg/ml centrimide, 15 µg/ml sodium nalidixate); *S. aureus* selective plates, containing LB agar (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) supplemented with 64 µg/ml colistin (Acros Organics)—as Gram-positive bacteria are innately resistant to colistin; and *S. maltophilia* selective plates, containing LB agar supplemented with 64 µg/ml tobramycin (Acros Organics) and incubated at 30ºC rather than 37ºC, as *S. maltophilia* is innately resistant towards tobramycin at 30ºC (Mooney *et al*. 2001). During the experiment, communities were only plated on agar that would select for an expected constituent species, e.g., the *P. aeruginosa* & *S. aureus* population was not plated onto *S. maltophilia* selective agar. Only at the final timepoint were all communities grown on each selective agar. CFU/ml was calculated from these counts. Finally, at every

count timepoint, 200 µl of each replicate community was cryopreserved in 20 % glycerol and frozen at -80ºC.

At the final timepoint, up to six clones of each species from each replicated community—where detectable—were randomly picked from their respective selective agar and grown in NB in 96-well plates overnight before freezing in 20 % glycerol at -80ºC.

3.3.3 Calculation of difference in CFU counts between selective agar and LB agar

The selective agars used to enumerate species within the communities resulted in reduced yield when compared to growth on LB agar. To estimate these differences, varying dilutions—undiluted, 1 in 20, and 1 in 100—of single-species overnight cultures were counted using both LB agar and the respective selective agar using the spotting method detailed in Methods § 3.3.2 above. Simple linear regression models were fit to $\log_{10}$-transformed paired counts, i.e., $\log_{10}$ abundance on LB agar was the dependent variable and $\log_{10}$ abundance on selective agar was the independent variable (Appendix Figure B.1), and these models were used to predict the limit of detection of each agar. Model details and predicted limits of detection are included in Table 3.1. Different suppliers of *Pseudomonas* selective agar supplements were used during the selection experiment (Oxoid) compared to the media replacement and ancestor replacement assays (EO Labs), which resulted in different yields despite the same formulation, and as such separate models were fit for the two supplements.

We initially sought to use these models to adjust the CFU values taken during the selection experiment and follow-up assays to account for the reduced yield; this was performed on $\log_{10}$-transformed counts using the `predict` function in R, and then exponentiated back to CFU/ml, such that:

$$\text{Predicted CFU/ml} = 10^{(\log_{10}\text{ Selective agar CFU/ml} \bullet \text{Regression Coefficient}) + \text{Intercept}}$$

Where regression coefficient and intercept are the model values as shown in Table 3.1.

However, the CFU counts taken in these experiments exceeded the counts used to fit the models, and as these models do not have an upper limit the predicted CFU counts were in the range of 10^{13}–10^{16} CFU/ml, which is likely inaccurate. Due to these limitations, in figures we present the abundance as the CFU/ml from the selective agar plates without adjusting the values, but figures showing the predicted CFU values can be found in Appendix B.

3.3.4 Isolation of evolved clones from stably coexisting "Viscous + TOB" treated three-species communities

To investigate the evolution of coexistence in three-species communities, we focused on the communities treated with Viscous + TOB that showed stable coexistence throughout the selection experiment—see Results § 3.4.1. At the end of the selection experiment, six clones of each species in each replicate evolved community were isolated—see Methods § 3.3.2—and from this pool we randomly selected one representative clone for each species in each replicate "Viscous + TOB" community (Table 3.2) after discounting outlying clones based on growth relative to their ancestor—as calculated in Methods § 3.3.7.1 below. When performing the remaining assays evolved communities were considered as being a single entity, each constituted of bacteria isolated from the same evolved replicate.

3.3.5 Media replacement assay

To assess if the species coadaptation within the evolved "Viscous + TOB" communities was dependent on the presence of viscosity or tobramycin, each evolved replicate community was grown in triplicate in each of the four original media treatments, following the same setup protocol as in the selection experiment except for the initial densities of the species, where ~10^3 CFU of each species, from dilutions of ~10^5 CFU/ml, were added to each community. Communities were grown in triplicate and cultured for 6 days, after which species abundances were measured using selective agar and adjusted as described in Methods § 3.3.3 above. Ancestral communities were grown at the same time in triplicate as a control.

3.3.6 Ancestor replacement assay

To investigate the relative contribution of the identity of each evolved species for maintaining coexistence in the evolved "Viscous + TOB" communities, we performed a fully factorial "time-shift" assay whereby each member of the evolved communities was swapped for its respective ancestor. These swaps were performed for each permutation of ancestor and evolved species, resulting in 7 replacements—replacement of each single community member, or two community members at a time. These communities were then grown in triplicate in Liquid media (no viscosity or tobramycin) on two separate occasions, for a total N = 6, following the same protocol as described in Methods § 3.3.5 above, although here ~10^4 CFU of each species were added to the communities.

3.3.7 Quantifying species trait changes underlying evolution of coexistence

3.3.7.1 Evolutionary changes in species relative growth

To quantify evolutionary changes in coexisting species competitive ability and tobramycin tolerance, difference in growth of all evolved species clones was compared to their respective ancestor. Growth was measured in Liquid NB media (competitive ability estimated as relative growth) and in the presence of 0.5 µg/ml tobramycin (tobramycin tolerance). Briefly, overnight cultures of all clones, from frozen stocks, were diluted 1 in 10 in PBS. 20 µl each of each diluted isolate were then grown in 180 µl NB, or NB supplemented with 0.5 µg/ml tobramycin, for 48 hours. Evolved clones were grown in triplicate; ancestral clones were grown in triplicate on 6 occasions, for N = 18. Optical density at 600 nm (OD_{600}) was measured at 24 and 48 hours (Tecan Sunrise).

3.3.7.2 Quantifying changes in adherent biofilm formation

In addition to measuring evolutionary changes in relative growth, we assessed the ability of each species to form adherent biofilms, as changes in degree of adherence could allow species to exploit a different spatial niche within the wells. Here, crystal violet stains the bacteria that have adhered to the wells, while free-floating bacteria are washed away. Ethanol is used to dissolve the crystal violet, and

optical density of dissolved crystal violet provides a proxy for adherent biofilm formation. As floating bacteria are washed away, this assay does not account for biofilm formation in floating aggregates.

These assays were performed using the same cultures in which changes in relative growth in both NB and NB + 0.5 μg/ml tobramycin were taken. After taking the 48-hour growth measurement, 20 μl of 50 % vol/vol crystal violet, diluted with distilled water, were added to each well. Plates were washed in tap water after 15 mins and left to dry overnight. 230 μl ethanol was added to each well and after 15 mins shaking OD_{600} was measured (Tecan Sunrise).

Per capita adherence was calculated as:

$$\frac{\text{Evolved adherence (Crystal Violet } OD_{600})}{\text{Evolved growth } (OD_{600})}$$

with minimum being:

$$\frac{\text{Evolved adherence} - \text{S. E. M.}}{\text{Evolved growth} + \text{S. E. M.}}$$

and maximum being:

$$\frac{\text{Evolved adherence} + \text{S. E. M.}}{\text{Evolved growth} - \text{S. E. M.}}$$

3.3.7.3 Evolutionary changes in supernatant-mediated species interactions

To investigate potential changes in facilitation and antagonism between the evolved species in coexisting communities, we grew evolved species clones (Table 3.2), and their ancestors, in the cell-free supernatants of the other species clones from the same evolved replicate community. In other words, within an evolved community, each species was grown in each other species' supernatant: e.g., *P. aeruginosa* from evolved community 1 was grown in the supernatants of *S. aureus* and *S. maltophilia* from evolved community 1. In addition, each evolved species was grown in the

supernatant of the other ancestral species, and vice versa. Finally, the ancestral isolates were grown in each other species' supernatants. Growth in cell-free supernatant measures the degree of contact-independent interaction between species, encompassing nutrient usage overlap, potential cross-feeding, and active inhibition by secreted secondary metabolites.

To prepare cell-free supernatants, evolved and ancestral isolates were grown individually from frozen stocks as overnight cultures, and then inoculated into falcon tubes containing 10 ml of NB, and grown for 48 hours, after which they were centrifuged at 4000 rpm, 4ºC for 10 minutes. The supernatant of each isolate was collected, leaving the pellet behind, filter sterilised using 0.22 µm pore width filters, and mixed with fresh NB at a ratio of 4 parts supernatant to 1 part NB (80 % vol/vol supernatant) to provide a fresh supply of nutrients. Each isolate was concurrently grown overnight and diluted to ~10^5 CFU/ml in PBS. 20 µl of diluted cultures were added to 180 µl of supernatant and grown in 96-well plates over 48 hours. Each species was also grown in 20 % vol/vol NB diluted in PBS—corresponding to the fresh nutrient concentration added to supernatants. OD_{600} measurements were taken at 24 and 48 hours to quantify bacterial densities (Tecan Infinite 200).

Relative growth indices (RGI) were calculated for each species clone by comparing growth of species *i* in the supernatant of species *j* to growth of species *i* in 20 % NB:

$$\frac{\text{Growth } (OD_{600}) \text{ of species } i \text{ in supernatant } j}{\text{Growth } (OD_{600}) \text{ of species } i \text{ in 20 \% NB}} = \text{RGI of species } i$$

Thus, an RGI of 1 indicates equal growth in supernatant as in 20 % NB, <1 indicates lower growth in supernatant, and >1 indicates higher growth in supernatant. RGI plotted on a $\log_{10}$ scale such that increases and decreases appear symmetric about the baseline value of 1.

3.3.7.4 Quantifying production of secreted pyocyanin in P. aeruginosa

Pseudomonas aeruginosa secretes a large number of inhibitory secondary metabolites, and to investigate how production of one such metabolite, pyocyanin,

may influence supernatant interactions we measured pyocyanin production of evolved *P. aeruginosa* relative to the ancestor. Overnight cultures from frozen stocks were diluted 1 in 10 in PBS, and 20 µl each of dilute cultures were grown in 180 µl NB in 96-well plates. After 48 hours, OD_{600} was measured (Tecan Sunrise), contents of each well were transferred to a round bottom well 96-well plate, and plates were centrifuged at 4000 rpm, 4ºC for 10 minutes. 100 µl of cell-free supernatant was transferred to flat bottom well 96-well plates and OD_{695} was measured as a proxy for pyocyanin concentration (O'Brien *et al.*, 2017). Per capita pyocyanin production relative to the ancestor was calculated as:

$$\frac{\left(\frac{\text{Evolved pyocyanin production (as } OD_{695})}{\text{Evolved growth (as } OD_{600})}\right)}{\left(\frac{\text{Ancestor pyocyanin production}}{\text{Ancestor growth}}\right)}$$

with minimum being:

$$\frac{\left(\frac{\text{Evolved pyocyanin production} - \text{S. E. M. of technical replicates}}{\text{Evolved growth} + \text{S. E. M. of technical replicates}}\right)}{\left(\frac{\text{Ancestor pyocyanin production}}{\text{Ancestor growth}}\right)}$$

and maximum being:

$$\frac{\left(\frac{\text{Evolved pyocyanin production} + \text{S. E. M.}}{\text{Evolved growth} - \text{S. E. M.}}\right)}{\left(\frac{\text{Ancestor pyocyanin production}}{\text{Ancestor growth}}\right)}$$

3.3.8 Statistical analyses

All data were analysed in R version 3.6.3 (R Core Team, 2019). Data manipulation and graphing were performed using the *tidyverse* suite of packages (Wickham, 2017), along with *patchwork* for figure assembly (Pedersen, 2020), *ggbeeswarm* for point plotting (Clarke and Sherrill-Mix, 2017), and *rcartocolor* and *scico* for colour palettes (Nowosad, 2018; Pedersen and Crameri, 2020).

3.3.8.1 Selection experiment analyses

Selection experiment analyses were carried out using the *vegan* package (Oksanen *et al.*, 2019). For each community, Bray-Curtis dissimilarity indices were calculated from species abundances in each replicate using the `vegdist` function. PERMANOVA was performed on dissimilarity indices grouped by the presence/absence of viscosity and tobramycin using the `adonis` function to investigate the effects of these factors on community composition; `adonis` settings used were permutations = free, number of permutations = 10 000. Pairwise PERMANOVA with false discovery rate adjustment was performed using the `pairwise.adonis` function from the *pairwiseAdonis* package (Martinez Arbizu, 2020). Full pairwise comparisons in Additional File 2.2. To address the uncertainty in species abundance due to growth on selective plates, models were also performed on selective plate adjusted abundance counts, and similar results were found between the two datasets in terms of significant effects (Additional File 2.1).

3.3.8.2 Media replacement analyses

Due to low sample size and an increase in uncertainty over species abundance, due to different selective media supplements, communities were analysed using detected species outcome rather than abundance. The outcome of each community replicate was determined by noting which of three species were detected at the final timepoint. Outcome frequencies were analysed with pairwise Fisher's exact tests with false discovery rate adjustments using the `pairwiseNominalIndependence` function from the *rcompanion* package (Mangiafico, 2020). Comparisons were performed between media treatments using counts pooled across all evolved communities, and within media treatments comparing ancestor counts to pooled evolved community counts, as low sample size prevented comparisons between individual communities. Full table of pairwise comparisons can be found in Additional File 2.3.

3.3.8.3 Ancestor replacement analyses

High replicate number allowed analysis of community outcomes, determined in the same manner as above, using multinomial logistic regression, which extends logistic regression by allowing a multi-level outcome rather than a binary outcome.

The model was computed using the `multinom` function from the *nnet* package (Venables and Ripley, 2002), and was fit using outcome as the response variable, and predictor variables of ancestor vs evolved identity of each species and evolved community replicate (Model call: Outcome ~ (*P. aeruginosa* + *S. aureus* + *S. maltophilia*) x Community). Model coefficients can be found in Additional File 2.4. Type-III ANOVA on the model was performed using the `Anova` function from the *car* package (Fox, Weisberg and Price, 2019) to determine effects of each predictor. Predicted probabilities of the regression model were computed using the `emmeans` function from the *emmeans* package (Lenth, 2019), and Tukey pairwise comparisons between predicted probabilities of outcomes were computed using the `contrast` function from the same package. Full table of pairwise comparisons can be found in Additional File 2.5.

3.3.8.4 Growth measurement, biofilm, and pyocyanin analyses

Separate repeated measures ANOVA models were fit for each species in each growth condition—both with and without tobramycin, six in total—using the `anova_test` function from the *rstatix* package (Kassambara, 2020). The response variable in each case was growth (as OD_{600}), the between-subject factor was evolved replicate community, and the within-subject factor was time. In the case of a significant interaction between time and community, ANOVA tests were performed on each individual level of time to establish the time(s) at which the community effects were significant. Pairwise comparisons were computed using the `pairwise_t_test` function from *rstatix*, and *p* values were adjusted for false discovery rate. Pairwise comparisons were performed between communities at each level of time in the case of significant interactions, and between levels of community averaged by time without significant interactions. Full table of pairwise comparisons can be found in Additional File 2.6.

Simple one-way linear regression models were fit to the biofilm and pyocyanin datasets, one for each species, with biofilm/pyocyanin production per capita as the response and evolved community as the predictor.

3.3.8.5 Supernatant growth analyses

To analyse effect of supernatant on species growth, linear regression models were fit for the supernatant of each species. Due to low replication, no interaction terms were included in the models. The predictor was $\log_{10}$-transformed relative growth index, and the predictor factors were supernatant identity (with levels for ancestor and each evolved replicate community) and species identity (with levels for each ancestor species and species from each evolved replicate community). To determine significance of each factor overall, Type-II two-way ANOVA were computed for each model using the `Anova` function from the *car* package (Fox, Weisberg and Price, 2019), and familywise Tukey honest significance difference tests were performed using the `tukey_hsd` function from the *rstatix* package. Full table of familywise comparisons can be found in Additional File 2.7.

Table 3.1: Selective agar CFU/ml vs. LB agar CFU/ml linear regression model coefficients, and predicted limits of detection in CFU/ml, with minimum and maximum predicted using 95 % C.I.

Selective agar	Regression term	Coefficient estimates	2.5 % C.I.	97.5 % C.I.	Model R^2
Pseudomonas, Oxoid	(Intercept)	-1.318	-4.427	1.791	0.666
	Log_{10}(Selective agar)	1.698	1.089	2.308	
Pseudomonas, EO Labs	(Intercept)	1.776	-0.0568	3.608	0.862
	Log_{10}(Selective agar)	1.499	1.001	1.996	
LB & 64 µg/ml colistin (*S. aureus*)	(Intercept)	0.235	-0.0595	0.529	0.986
	Log_{10}(Selective agar)	0.978	0.9313	1.025	
LB & 64 µg/ml tobramycin (*S. maltophilia*)	(Intercept)	3.641	3.046	4.235	0.905
	Log_{10}(Selective agar)	0.997	0.867	1.127	

Selective agar	Predicted LOD	Minimum LOD[†]	Maximum LOD
Pseudomonas, Oxoid	5 979 ($5.9x10^3$)	1 000 ($1.0x10^3$)	397 858 ($3.9x10^5$)
Pseudomonas, EO Labs	1 869 878 ($1.9x10^6$)	211 331 ($2.1x10^5$)	16 544 805 ($1.7x10^7$)
LB & 64 µg/ml colistin (*S. aureus*)	1 478 ($1.5x10^3$)	1 000 ($1.0x10^3$)	2 839 ($2.8x10^3$)
LB & 64 µg/ml tobramycin (*S. maltophilia*)	4 278 567 ($4.3x10^6$)	1 101 800 ($1.1x10^6$)	16 614 754 ($1.7x10^7$)

[†] Adjusted minimum LOD capped at true LOD of 1 000 CFU/ml

Table 3.2: Clones selected from the "Viscous + TOB" evolved three-species communities for follow up experiments

(media replacement, ancestor replacement, supernatant growth).

Species	Evolved Community replicate			
	1	**2**	**3**	**4**
P. aeruginosa	1	5	2	2
S. aureus	2	3	4	3
S. maltophilia	4	6	2	2

3.4 Results

3.4.1 Dynamics of coculture compositions during the selection experiment

At the final timepoint of the selection experiment we measured the abundance of each species within each of the seven communities and compared how the composition differed between media treatments. All single species cultures were able to grow to high abundance regardless of media treatment. Within the two-species communities, the *P. aeruginosa* containing communities were typically dominated by *P. aeruginosa*, with little effect of viscosity, though the addition of 0.5 μg/ml tobramycin (TOB) promoted coexistence between *P. aeruginosa* and *S. maltophilia*. Coexistence was consistently observed within the *S. aureus* & *S. maltophilia* community. In the three-species community, we found that viscosity alone again had little effect and *P. aeruginosa* dominated these communities, whilst the "Liquid + TOB" treatment promoted coexistence of *S. aureus* and *S. maltophilia* with *P. aeruginosa* being excluded. However, the "Viscous + TOB" treatment promoted coexistence of all three-species at high abundance, and this was consistent throughout the whole selection experiment.

3.4.1.1 Bacterial abundances in single-species cultures and two-species communities

The singles species cultures were all largely unaffected by the different media treatments and able to grow to high densities, with the exception of *S. aureus* within the Liquid media which was declining in abundance after the second count at 12 days (Appendix Figures B.4–6). However, different dynamics were observed in the pairwise two-species communities. The *S. aureus* & *S. maltophilia* community was unaffected by media treatment, and both species were able to coexist with each other throughout the experiment (Appendix Figure B.9). However, the pairwise communities containing *P. aeruginosa* were affected by the media treatments (Figure 3.1B, C). At the final timepoint within the *P. aeruginosa* & *S. aureus* communities, we found that *P. aeruginosa* was able to survive to a high abundance in all media treatments, and *S. aureus* was able to coexist with *P. aeruginosa* at a greater abundance in the Viscous + TOB treatment, at ~10^5 CFU/ml, compared with in the Liquid treatment at

~10^4 CFU/ml, whereas the abundance of *S. aureus* dropped below the limit of detection consistently in the Viscous and Liquid + TOB treatments (Figure 3.1B). There was a significant effect of viscosity on community composition (PERMANOVA: $F_{(1, 14)} = 9.95$, $p < 0.001$; Table 3.3) though pairwise comparisons did not demonstrate a significant difference between any two media conditions (PERMANOVA post-hoc: FDR-adjusted $p > 0.05$; Additional File 2.2) likely due to low replicate numbers. Within the Liquid + TOB treated communities, the drop below the detection limit occurred at the beginning of the experiment, before the first timepoint at day 6, whereas within the Viscous communities there was a steady decline in *S. aureus* abundance over the course of the experiment such that abundance was around the limit of detection and likely dropped to just below ~10^3 CFU/ml at the final timepoint (Figure 3.2).

The pairwise communities of *P. aeruginosa* and *S. maltophilia* were affected by the media treatments differently, whereby there was a significant effect of tobramycin on species abundance (PERMANOVA: $F_{(1, 14)} = 8.55$, $p < 0.001$; Table 3.3) and a significant effect of the interaction between tobramycin and viscosity (PERMANOVA: $F_{(1, 14)} = 3.77$, $p = 0.029$; Table 3.3). Within the Liquid and the Viscous treatments, *P. aeruginosa* again grew to a high abundance, whilst *S. maltophilia* was driven below the detection limit—after the first timepoint at 6 days in both treatments (Appendix Figure B.8)—whereas the addition of 0.5 µg/ml tobramycin to the Liquid media allowed both species to grow to similar abundances, ~10^7 CFU/ml (Figure 3.1C). Pairwise comparisons between both the Liquid and Viscous treatments and Liquid + TOB media were near significant (Pairwise PERMANOVA post-hoc: Liquid: $F_{(1, 6)} = 13.38$, FDR-adjusted $p = 0.084$; Viscous: $F_{(1, 6)} = 11.36$, FDR-adjusted $p = 0.084$; Figure 3.1C). Similar to *S. aureus* pairwise communities, *S. maltophilia* could coexist with *P. aeruginosa* within the Viscous + TOB media treatment, though not as equally as in the Liquid + TOB treatment. Overall, there seemed to be little effect of viscosity, but the addition of tobramycin changed the dynamic between *P. aeruginosa* and *S. maltophilia*.

3.4.1.2 Bacterial abundances within three-species community

Within the three-species community, the dynamic between the species was altered in similar ways to both pairwise communities across the different treatments. Contrary to our hypothesis, there was little effect of viscosity alone on community composition (PERMANOVA: $F_{(1, 15)} = 2.12$, $p = 0.11$; Table 3.3): in the Liquid and Viscous media treatments, *P. aeruginosa* was able to grow to a high abundance, whereas *S. aureus* was undetectable more often in the Viscous media and grew to a lower abundance of ~10^3 CFU/ml, compared with ~10^5 CFU/ml in the Liquid media (Figure 3.1A). In both the Liquid and Viscous media, *S. maltophilia* was undetectable (Figure 3.1A), and this occurred after the first timepoint at 6 days in both media conditions (Figure 3.2A, B). This suggests a baseline dynamic of a community dominated by *P. aeruginosa*, which also mirrored that within both pairwise cocultures, whereby *S. aureus* grew to lower abundance in Viscous than in Liquid media (Figure 3.1B) and *S. maltophilia* was undetectable (Figure 3.1B).

There was a significant effect of tobramycin on the species abundance (PERMANOVA: $F_{(1, 15)} = 22.55$, $p < 0.001$; Table 3.3), whereby the addition of 0.5 μg/ml of tobramycin to the Liquid media changed the three species community from one dominated by *P. aeruginosa* to one where *S. maltophilia* was able to coexist with *S. aureus*, with both species at high abundance, whilst *P. aeruginosa* became undetectable in three of four replicates (Figure 3.1A). Indeed, the species abundance within the Liquid + TOB treatment differed significantly to both the Liquid and Viscous treatments (Pairwise PERMANOVA post-hoc: Liquid: $F_{(1, 6)} = 20.72$, FDR-adjusted $p = 0.048$; Viscous: $F_{(1, 6)} = 36.53$, FDR-adjusted $p = 0.048$; Figure 3.1A). *Pseudomonas aeruginosa* from these three replicates decreased in abundance steadily across the experiment, becoming undetectable after the second timepoint at 12 days, whereas both *S. aureus* and *S. maltophilia* remained at high abundances throughout in all replicates (Figure 3.2C). This effect of tobramycin on the community composition supports our hypothesis that the effects of a low dose of antibiotic would be magnified in a larger community, as in the *P. aeruginosa* & *S. maltophilia* pairwise community both species consistently coexisted whereas the addition of one other species led to exclusion of *P. aeruginosa*. However, as *P. aeruginosa* from replicate 2 of the three-species community coexisted with *S. aureus* and *S. maltophilia* to the final timepoint,

this suggests that the ecological dynamic between these species is complex and subject to variation.

The combination of increased viscosity and tobramycin treatment resulted in a community where all three species were able to coexist across all four replicates (Figure 3.1A). There was a near significant interaction effect between viscosity and tobramycin (PERMANOVA: $F_{(1, 15)} = 2.57$, $p = 0.069$; Table 3.3), and though not significantly different in composition to the Liquid + TOB treatment in pairwise comparison (Pairwise PERMANOVA post-hoc: $F_{(1, 6)} = 2.77$, FDR-adjusted $p = 0.071$), the detection of all three species suggests some support for our hypothesis that the addition of viscosity would mitigate the effects of tobramycin treatment on community composition. This coexistence was largely consistent across the selection experiment, whereby *S. aureus* and *S. maltophilia* abundances at each timepoint were similar to that observed in the Liquid + TOB treatment (Figure 3.2C, D), and *P. aeruginosa* in replicates 1 and 4 grew to a similar abundance to *S. aureus* throughout (Figure 3.2D). As with the Liquid + TOB treatment, however, there was variability in ecological trajectory as *P. aeruginosa* in replicate 2 decreased in abundance between timepoint 1 at 3 days and timepoint 3 at 18 days—though rebounded at the final timepoint—and *P. aeruginosa* in replicate 3 steadily increased in abundance from timepoint 2 at 12 days to the final timepoint whilst *S. maltophilia* abundance in the same replicate decreased over that time (Figure 3.2D), which suggests a complex dynamic between the three species in this treatment environment.

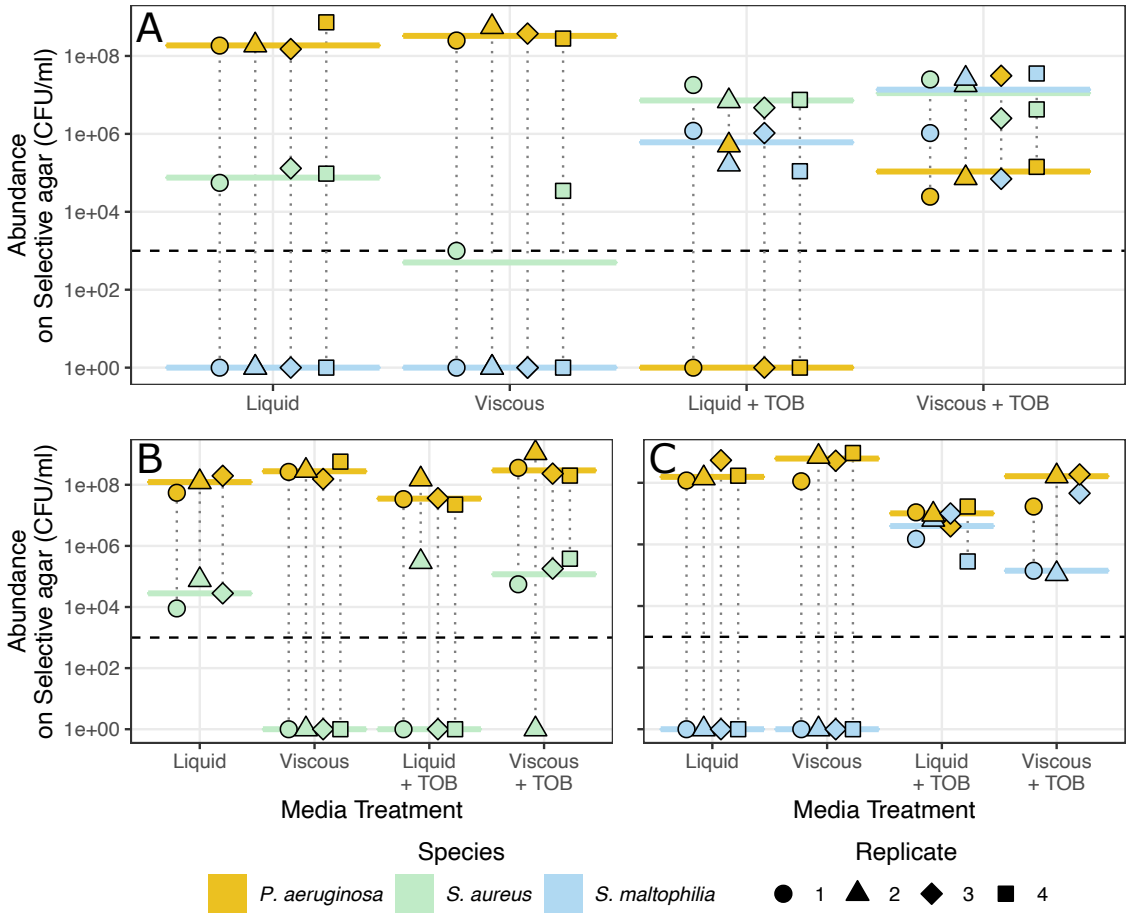


Figure 3.1: The abundance of each species at the final timepoint of the selection experiment, within the different media treatments and communities.

A: Three-species community. **B:** *P. aeruginosa* & *S. aureus* community. **C:** *P. aeruginosa* & *S. maltophilia* community. The *S. aureus* & *S. maltophilia* pairwise community that showed stable coexistence across treatments is shown in Appendix Figure B.9. Points represent CFU/ml for individual replicates. Coloured horizontal lines represent median abundance of the species. Colours represent species, shapes represent replicates. Vertical dotted lines link isolates from the same replicate. Horizontal dotted lines represent the limit of detection—i.e., 1000 CFU/ml. Replicates in pairwise communities < 4 due to removal because of contamination. See Appendix Figure B.2 for predicted CFU values, as detailed in Methods § 3.3.3.

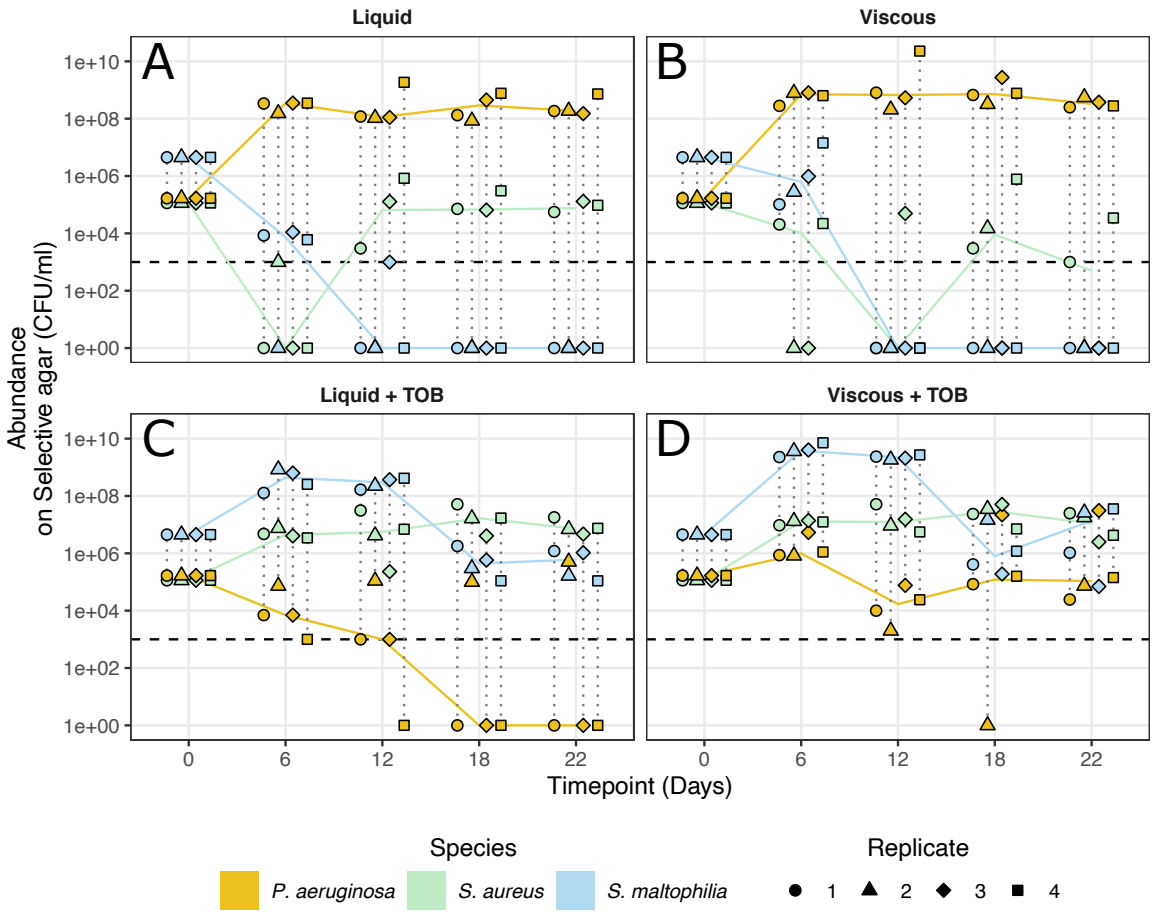


Figure 3.2: The abundance of each species within the three-species communities at each sampling point during the selection experiment.

Panels represent each media condition. Points represent the CFU/ml of a species from an individual replicate. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate. Horizontal dotted lines represent the limit of detection of 1000 CFU/ml. See Appendix Figure B.3 for predicted CFU values, as detailed in Methods § 3.3.3.

Table 3.3: PERMANOVA tables of species abundance across the two media factors viscosity and tobramycin. Calculated from Bray-Curtis dissimilarity indices of each replicate community. Permutation: free. Number of permutations: 10 000

Community	PERMANOVA term	D.F.	Sum Sq.	Mean Sq.	F value	R2	P value
Triple	Viscosity	1	0.26	0.26	2.12	0.05	0.11
	Tobramycin	1	2.78	2.78	22.55	0.57	9.99×10^{-5}
	Visc:TOB Interaction	1	0.32	0.32	2.57	0.066	0.069
	Residuals	12	1.48	0.12		0.31	
	Total	15	4.84			1	
P. aeruginosa & *S. aureus*	Viscosity	1	0.94	0.94	9.95	0.43	0.0010
	Tobramycin	1	0.12	0.12	1.26	0.054	0.28
	Visc:TOB Interaction	1	0.11	0.11	1.13	0.048	0.32
	Residuals	11	1.04	0.09		0.47	
	Total	14	2.20			1	
P. aeruginosa & *S. maltophilia*	Viscosity	1	0.39	0.39	2.92	0.11	0.055
	Tobramycin	1	1.15	1.15	8.55	0.33	0.00080
	Visc:TOB Interaction	1	0.51	0.51	3.77	0.14	0.029
	Residuals	11	1.48	0.13		0.42	
	Total	14	3.54			1	

3.4.2 Investigating the stability of coexistence within evolved communities

Following on from the observation that the Viscous + TOB treatment resulted in continued coexistence of the three species, we sought to investigate whether this was purely as a result of the treatment environment, i.e., ecological mechanisms. Were this the case, in the absence of coexistence maintaining mechanisms—here the Viscous + TOB treatment—no coexistence should be observed between the final timepoint species isolates from these communities. However, coevolution of the three species may result in adaptations that increase the likelihood of coexistence in the absence of such mechanisms, i.e., the absence of viscosity of tobramycin.

To investigate this further, we isolated and selected individual clones of each species from each replicate of the final timepoint three-species community in the Viscous + TOB treatment (Methods § 3.3.4, Table 3.2). We then tested if evolved community coexistence was dependent on the presence of viscosity or tobramycin, or the presence of specific evolved species in each community. In all of these experiments, each evolved replicate community was treated as an individual entity, in that the three species isolated from evolved community 1 were always grown together, as were the three species from evolved community 2, and so on. Similarly, technical replicates of each evolved community were performed as though the evolved communities were singular entities, each time using the same mixture of the three species that constitute a given evolved community.

3.4.2.1 The role of viscosity and tobramycin in evolved community coexistence

To test whether the evolved communities were able to continue to coexist outside of the Viscous + TOB media treatment in which they had been selected, we grew each evolved community in triplicate in each of the four media treatments over 6 days—to replicate the period up to the first timepoint of the selection experiment—and compared the compositions to ancestral communities. We did so by classifying each technical replicate community in terms of which of the three species were detectable at the end of the assay (Figure 3.3). Full abundances are presented in Figure 3.4. When viscosity was removed and communities were grown in the Liquid + TOB

media, coexistence of evolved communities was diminished compared with growth in the Viscous + TOB treatment. When tobramycin was removed, evolved community coexistence was reduced to a lesser extent, and showed comparable coexistence to the ancestral community. When both factors were removed, and communities were grown in the Liquid treatment, evolved coexistence of evolved communities remained at the same level as in the Viscous treatment, but ancestral communities did not coexist, suggesting adaptation between the evolved species.

As in the selection experiment, both the ancestral community and the four evolved communities were able to coexist across every replicate in the Viscous + TOB treatment (Figure 3.3D, Figure 3.4D). However, the coexistence was more varied in other media environments.

Removal of viscosity did not change the outcome of the ancestral community, and all species were able to coexist similar to the original selection experiment (Figure 3.3C, Figure 3.4C). However, the dynamic within the evolved communities changed such that *P. aeruginosa* became undetectable in two replicates each of evolved communities 1, 2, and 4 (Figure 3.3C). Though the low replicate numbers did not enable between-replicate community comparisons, across all evolved communities the outcomes observed in the Liquid + TOB treatment significantly differed to the Viscous + TOB treatment (Pairwise Fisher's Exact test: FDR-adjusted $p = 0.027$). And compared with the ancestral community, the outcomes of the evolved communities as a whole did not differ significantly (Pairwise Fisher's Exact test: FDR-adjusted $p > 0.05$). This further suggests that the effects of tobramycin are diminished in the viscous environment, as the outcomes of the "Viscous + TOB" evolved communities resembled that of the ancestor despite selection in the presence of tobramycin.

The removal of tobramycin did not result in significantly different outcomes compared with the Viscous + TOB treatment (Pairwise Fisher's Exact test: FDR-adjusted $p > 0.05$), though there were differing outcome frequencies between the two treatments (Figure 3.3B, D). With the removal of tobramycin relative to the Viscous + TOB treatment, the ancestral community was able to coexist in 2 of three replicates. Among the evolved communities *S. aureus* and/or *S. maltophilia* became undetectable in several replicates (Figure 3.3B) whilst *P. aeruginosa* was able to grow to a high abundance (Figure 3.4B). However, as a whole, the evolved community

outcomes were not significantly different to the ancestral community (Pairwise Fisher's Exact test: FDR-adjusted $p > 0.05$). The difference relative to the Viscous + TOB treatment, though small, suggests that tobramycin plays a role within the Viscous environment in mediating coexistence.

When both viscosity and tobramycin were removed from the media, there was a significant difference between ancestral and evolved communities (Pairwise Fisher's Exact test: FDR-adjusted $p = 0.018$), whereby in the ancestral community only *P. aeruginosa* was detectable whilst across most of the evolved communities all three species were able to coexist (Figure 3.3A). Though there was increased variability in outcome in two of the evolved communities when compared to the Viscous + TOB treatment, considered as a whole the outcomes of the evolved communities between these two treatments did not differ (Pairwise Fisher's Exact test: FDR-adjust $p = 0.26$). Taken together, this suggests that there had been adaptation between the three species within these evolved communities to increase the likelihood of coexistence without ecological coexistence maintaining mechanisms.

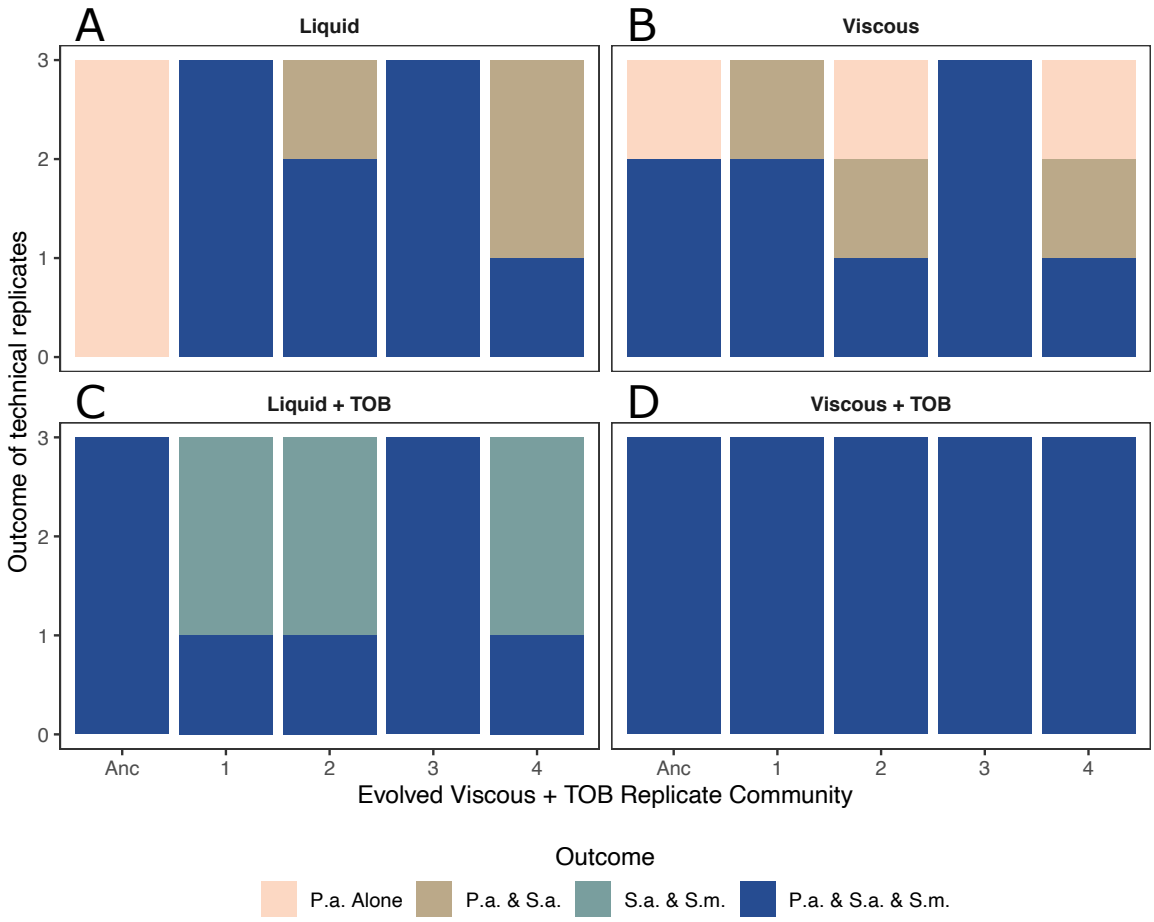


Figure 3.3: Outcome of ancestral vs evolved communities grown in the four media treatments classified by species detected.

Panels show the observed outcome of the technical replicates of each community, shown on the X-axis, following growth in the four media treatments. Communities consisted of either ancestral species isolates (Anc) or isolates from evolved communities 1–4. The community compositions were classified into outcomes determined by which species were detected—i.e., an abundance greater than the limit of detection—in the abundance counts in Figure 3.4 for each technical replicate (N =μ3); e.g., if all three species were detected the outcome was classified as P.a. & S.a. & S.m., etc. Colours represent the four observed outcomes. Abbreviations: P.a. = *P. aeruginosa*; S.a. = *S. aureus*; S.m. = *S. maltophilia*.

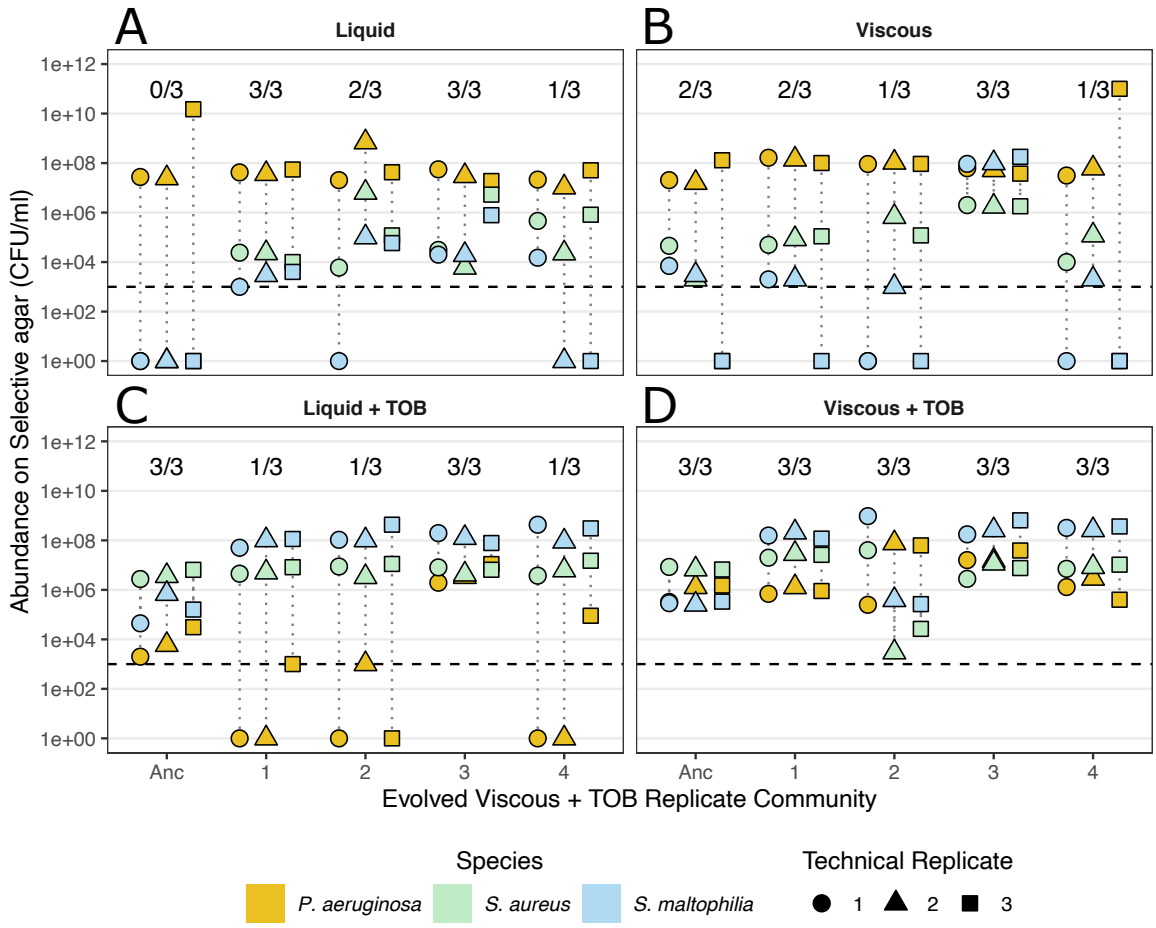


Figure 3.4: Species abundances within ancestral and evolved communities grown in the four media treatments.

Panels show the growth of the technical replicates of each community, shown on the X-axis, in the four media treatments. Communities consisted of either ancestral species isolates (Anc) or isolates from evolved communities 1–4. Points represent abundance of species in individual technical replicates (N = 3). Numbers above represent the number of technical replicates out of 3 with coexistence of all species. Colours show species, shapes show technical replicates. Vertical dotted lines join species in the same technical replicate. Horizontal dotted line represents the limit of detection of 1000 CFU/ml. See Appendix Figure B.10 for predicted CFU values, as detailed in Methods § 3.3.3.

3.4.2.2 The role of evolved species identity in coexistence within Liquid media

To further investigate the ability of the evolved communities to coexist without the facilitative effect of the Viscous + TOB treatment, we conducted ancestor replacement assays, where we tested whether swapping one or two of the evolved species with their respective ancestors affected the species coexistence. Such experiments could reveal if the coexistence in evolved communities was determined by the evolution of one or more community members. We grew these 'replaced' communities in Liquid media and assessed the coexistence outcomes of each community after 6 days in a manner similar to the media replacement assays.

Overall, we found that replacement of evolved species reduced the likelihood of three-species coexistence (Figure 3.5, Figure 3.6). Replacement of *S. maltophilia* with its ancestor generally had the greatest effect on coexistence, reducing its likelihood, whilst replacement of *S. aureus* had the least. However, there was large variation in likelihood of coexistence between evolved replicate communities: coexistence in evolved community 3 was the most robust across replacement of different species, and evolved community 1 was also able to coexist well, whereas evolved communities 2 and 4 were not able to coexist in this assay. The variability of coexistence between replicates suggests that each community evolved along different trajectories despite the same starting conditions.

Due to the stochastic nature of the outcomes, we used multinominal logistic regression to model the effects of ancestor replacements on the likelihood of each outcome. Normal logistic regression models the likelihood of a binary outcome, here one community composition outcome vs another, across a range of predictor variables, here whether each species in the community was either evolved or ancestral. Multinomial logistic regression extends this model from a binary outcome to incorporate multiple outcomes, and so is able to model the likelihood of each of the four of the possible community composition outcomes that we observed. In addition to the identity of each species, we also included replicate community as a predictor due to the high variation between replicates. Using this model, we obtained the predicted probabilities of each outcome in each community, shown in Figure 3.7. Full coefficients of the model can be found in Additional File 2.4. Performing ANOVA

on the model we found that overall there was a significant effect of ancestral vs evolved *S. maltophilia* on outcome (Table 3.4, $p = 0.0042$), and that this effect differed between evolved communities (Table 3.4, $p = 0.039$). The effect of ancestral vs evolved *P. aeruginosa* also differed significantly between communities (Table 3.4, $p < 0.001$).

Evolved replicate community 1 fully coexisted consistently in Liquid media during the prior experiment (Figure 3.3A), and here all three species were detectable in three of 6 replicates of the fully evolved community (Figure 3.5A). Replacing *P. aeruginosa* or *S. aureus* with their ancestors did not change the outcomes of evolved replicate community 1, but when *S. maltophilia* was replaced, no replicate gave coexistence of all three species (Figure 3.5A); here, the outcome of *P. aeruginosa* & *S. aureus* was significantly more likely than the three species outcome (Pairwise Tukey post-hoc: $t(48) = 5.01$, $p = 0.0031$; Figure 3.7A), and the *P. aeruginosa* & *S. aureus* outcome was significantly more likely than with no ancestral species (Pairwise Tukey post-hoc: $t(48) = 4.16$, $p = 0.039$; Figure 3.7A). When two species were replaced with their ancestors, we found no coexistence among the replicates (Figure 3.5B). When *P. aeruginosa* was replaced in combination with *S. aureus*, *S. aureus* was not detected in any replicate community, and the same occurred with *S. maltophilia* when both *P. aeruginosa* and *S. maltophilia* were replaced (Figure 3.5B). Additionally, in both of these combinations the probability of the *P. aeruginosa* alone outcome increased (Figure 3.7A). When both *S. aureus* and *S. maltophilia* were replaced there were no replicates in which only *P. aeruginosa* was detected, though *S. maltophilia* was undetectable across a majority of replicates (Figure 3.5B) and the *P. aeruginosa* & *S. aureus* outcome was significantly more likely than the three-species outcome (Pairwise Tukey post-hoc: $t(48) = 5.29$, $p = 0.0012$; Figure 3.7A). Together, this suggests that in evolved replicate community 1 *S. maltophilia* played a large role in coexistence, as replacement significantly changed the outcome probabilities; and that *P. aeruginosa* may have become less competitive, as when *P. aeruginosa* was the only evolved species it was unable to exclude *S. aureus* and *S. maltophilia*.

Previously, evolved replicate community 2 had a variable outcome of either all three species or only *P. aeruginosa* & *S. aureus* (Figure 3.3A), whereas here the evolved community consistently resulted in the *P. aeruginosa* & *S. aureus* coexistence

outcome (Figure 3.5C). Replacing *S. maltophilia* did not affect coexistence as in evolved community 1 and suggests that *S. maltophilia* may not have adapted to grow to a high abundance in this replicate. On the other hand, replacing *P. aeruginosa* resulted in decreased detection of *S. aureus*, as the *P. aeruginosa* & *S. aureus* outcome was significantly less likely than in the evolved community (Pairwise Tukey post-hoc: $t(48) = 5.52$, $p < 0.001$; Figure 3.7B), and in the majority of replicates *P. aeruginosa* & *S. maltophilia* coexisted (Figure 3.5C). In pairwise replacements the *P. aeruginosa* & *S. maltophilia* outcome was observed more often, particularly when *P. aeruginosa* was one of the ancestral species (Figure 3.5D). Overall, this suggests that *P. aeruginosa* may have become more competitive towards *S. maltophilia* and less competitive towards *S. aureus*, as replacement with the ancestor led to more competitive exclusions of *S. aureus*.

Evolved replicate community 3 showed the most stable coexistence across all media treatments (Figure 3.3) and had the most three-species outcomes across all replacement combinations (Figure 3.5E, F). Replacing *P. aeruginosa* within this community reduced *S. maltophilia* detection frequency, whereas replacing *S. maltophilia* resulted in a greater degree of coexistence (Figure 3.5E), such that three-species coexistence was significantly more likely than either *P. aeruginosa* "monoculture" or *P. aeruginosa* & *S. aureus* coexistence (Pairwise Tukey post-hoc: $t(48) = 5.82$, $p < 0.001$; Figure 3.7C). This suggests that *P. aeruginosa* became less competitive than its ancestor, whilst *S. maltophilia* became more competitive. Additionally, replacing *S. aureus* led to more frequent coexistence of *P. aeruginosa* & *S. maltophilia* (Figure 3.5C) making this outcome significantly more likely than *P. aeruginosa* "monoculture" or *P. aeruginosa* & *S. aureus* coexistence (Pairwise Tukey post-hoc: $t(48) = 5.82$, $p < 0.001$; Figure 3.7C) and suggesting that *S. aureus* also became more competitive as its ancestor could not survive as often with the evolved species (Figure 3.5C). Evolved replicate community 3 was the only community in which replacement of both *S. aureus* and *S. maltophilia* still resulted in coexistence (Figure 3.5F, Figure 3.7C), further suggesting that the reduction of competitive ability in the evolved *P. aeruginosa* may have strongly affected coexistence in this community.

Full coexistence was rare in evolved replicate community 4 across all the different media treatments (Figure 3.3). The most common outcomes in Liquid media were either coexistence of *P. aeruginosa* & *S. aureus*, or *P. aeruginosa* "monoculture" (Figure 3.5G). Replacing *S. aureus* increased the frequency of the *P. aeruginosa* "monoculture" outcome (Figure 3.5G), while replacing *P. aeruginosa* marginally increased the predicted probability of coexistence within the model (Figure 3.7D). Replacing *S. maltophilia* alone or in combination with *P. aeruginosa* resulted in *S. maltophilia* becoming undetectable more often (Figure 3.5G, H), and the *P. aeruginosa* & *S. aureus* outcome became significantly more likely than the *P. aeruginosa* & *S. maltophilia* outcome in both communities (Pairwise Tukey post-hoc: *S.m.* Anc: $t(48) = 4.91$, $p = 0.0041$; *P.a.* & *S.m.* Anc: $t(48) = 4.12$, $p = 0.043$; Figure 3.7D), and significantly more likely than the three-species outcome in the *S. maltophilia* ancestor community (Pairwise Tukey post-hoc: $t(48) = 4.49$, $p = 0.015$; Figure 3.7D). This suggests that there was a complex interplay between the three evolved species in this community, as the changes in competitive ability of each evolved species appears to depend upon the competitive ability of the other species present.

Overall, these experiments suggest that though coexistence can evolve between these competing species, this coexistence is still dependent on the specific combination of environmental factors that the communities are grown in (Figure 3.3, Figure 3.4), and that coexistence can likely arise by different mechanisms as each evolved species can contribute to the coexistence to different degrees (Figure 3.5).

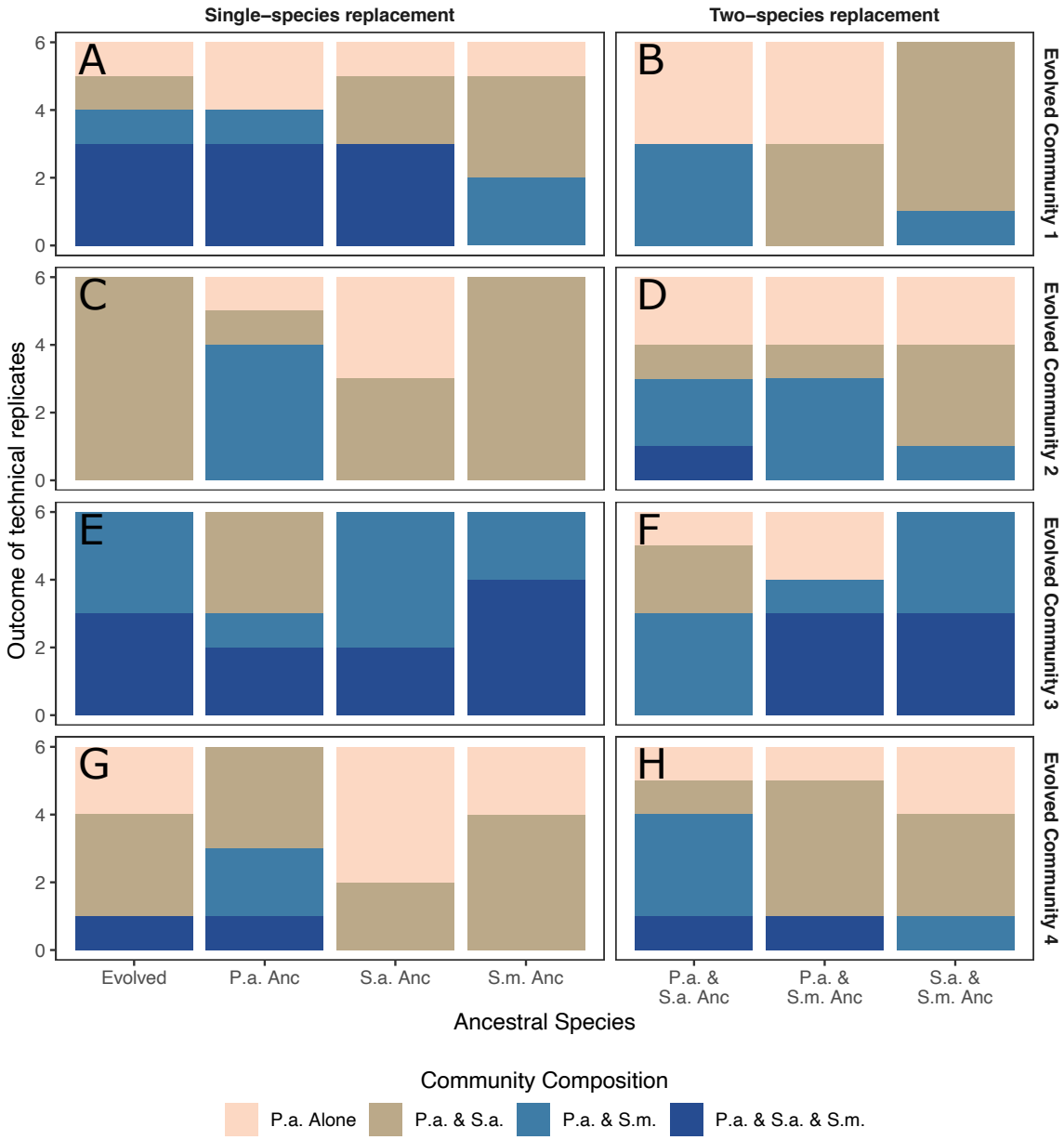


Figure 3.5: Community composition outcomes in evolved three-species communities with single-species or two-species ancestor replacements.

The X-axis shows the ancestral species (Anc) within the evolved communities. The panel columns show single-species and two-species ancestor replacement, and panel rows show each of the four evolved communities. Colours represent the four observed community composition outcomes. Each community was grown in triplicate on two separate occasions, total N = 6 for each community. Abbreviations: P.a. = *P. aeruginosa*; S.a. = *S. aureus*; S.m. = *S. maltophilia*.

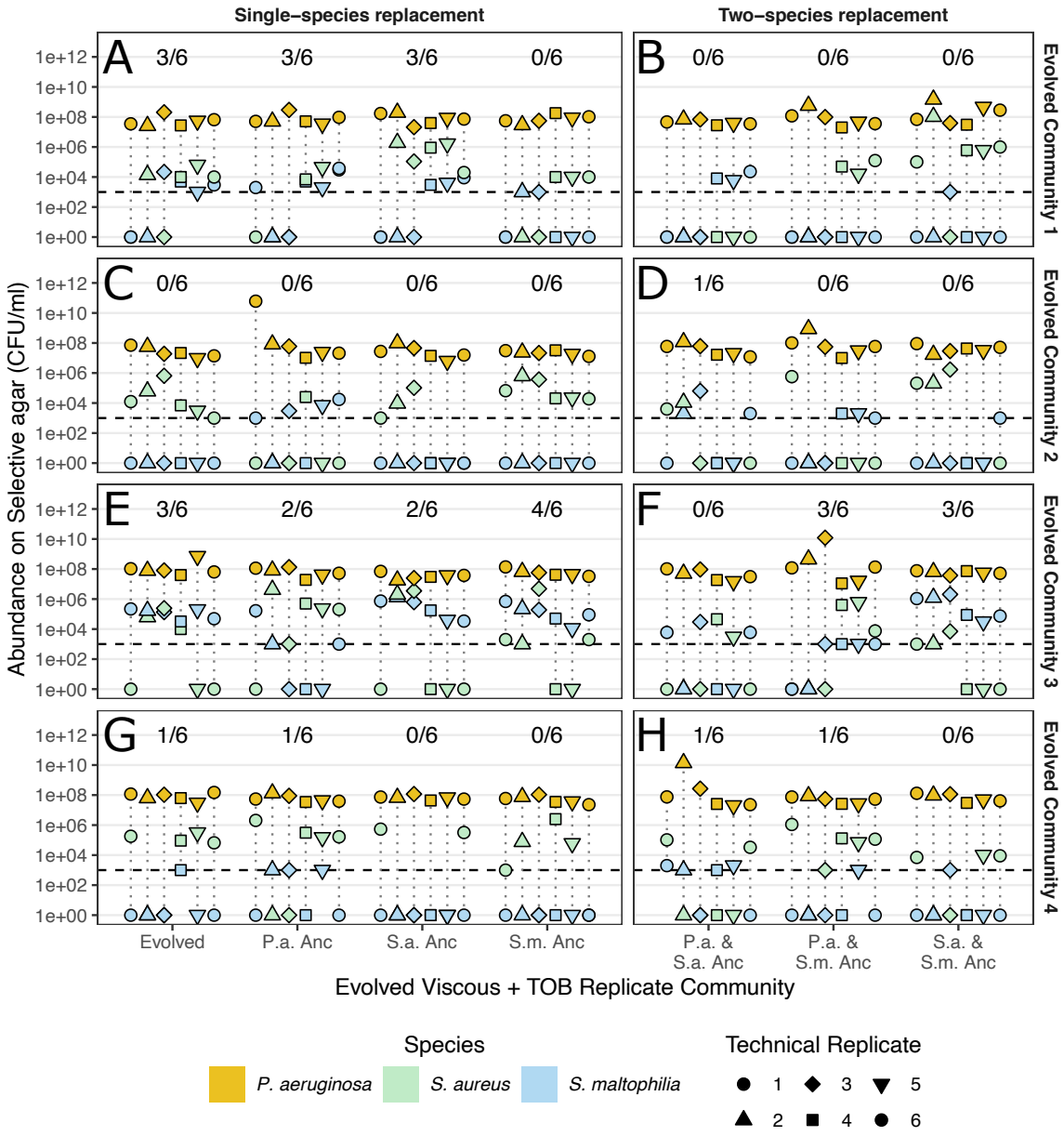


Figure 3.6: Species abundance within evolved communities with single-species or two species ancestor replacements.

The X-axis shows the ancestral species (Anc) within the evolved communities. The panel columns show single-species and two-species ancestor replacement, and panel rows show each of the four evolved communities. Points represent abundance of species in individual technical replicates (N = 6). Numbers above represent the number of technical replicates out of 6 with coexistence of all species. Colours show species, and shapes show technical replicates. Vertical dotted lines join species in the same technical replicate. Horizontal dotted line represents the limit of detection of

1000 CFU/ml. See Supplementary Figure B.11 for predicted CFU values, as detailed in Methods § 3.3.3.

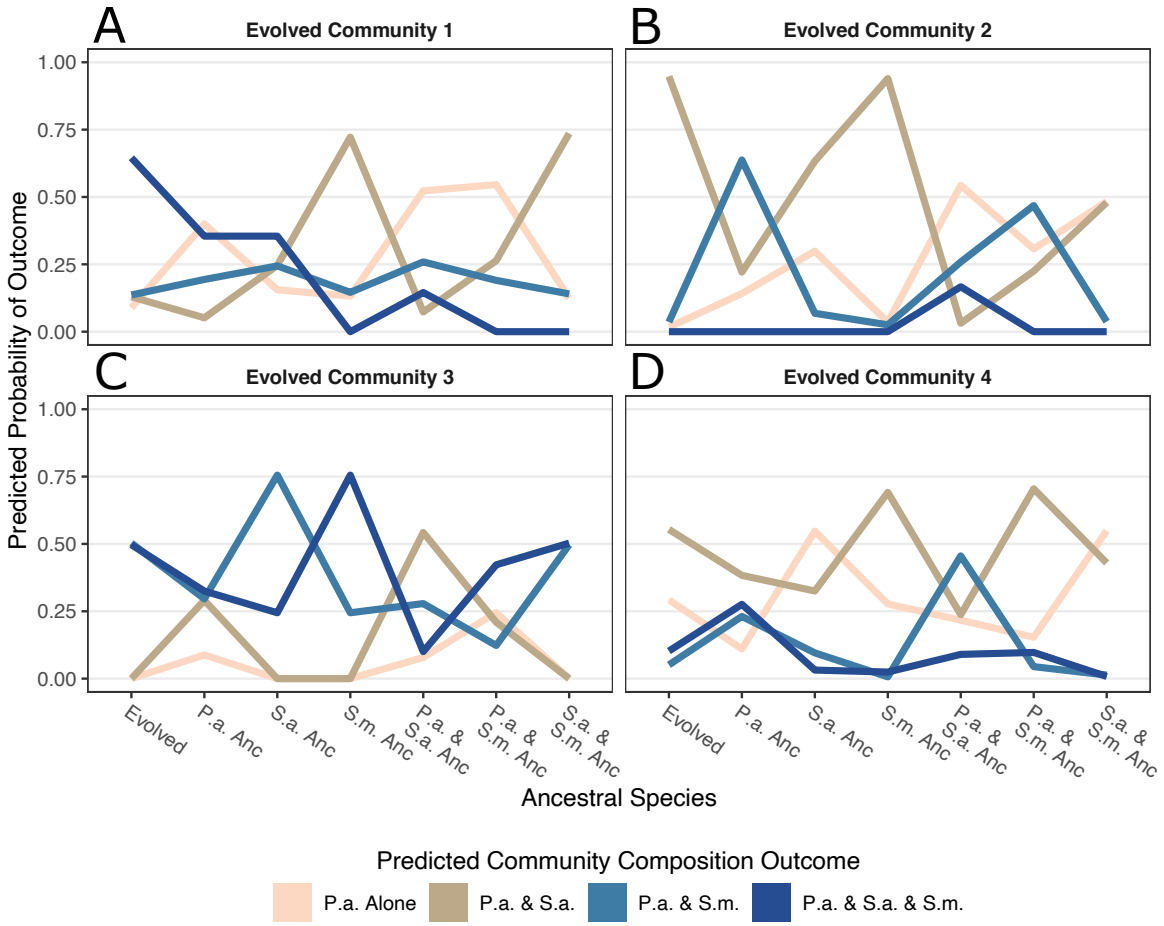


Figure 3.7: Predicted probabilities of the community composition outcomes in evolved three-species communities for each ancestor replacement.

Predicted probabilities derived from the multinomial logistic regression model, where the response variable was observed outcome, and the predictor variables were Ancestor vs Evolved *P. aeruginosa*, *S. aureus*, and *S. maltophilia*, evolved community identity, and the interactions between community identity and each species. Model call: Outcome ~ (*P. aeruginosa* + *S. aureus* + *S. maltophilia*) x Community. The X-axis shows the ancestral species (Anc) within the evolved communities. The panels show each of the four evolved communities. Colours represent the four predicted community composition outcomes. Abbreviations: P.a. = *P. aeruginosa*; S.a. = *S. aureus*; S.m. = *S. maltophilia*.

Table 3.4: Type-III ANOVA table of multinomial logistic regression model.

Where response was outcome in evolved communities grown in Liquid media and the predictor variables were ancestor vs evolved *P. aeruginosa*, *S. aureus*, and *S. maltophilia*, evolved community identity, and the interactions between community identity and each species.

Model call: Outcome ~ (*P. aeruginosa* + *S. aureus* + *S. maltophilia*) x Community.

ANOVA term	**D.F.**	**Likelihood ratio (Chi sq.)**	***P* value**
P. aeruginosa (Ancestor vs Evolved)	3	1.10	0.78
S. aureus (Ancestor vs Evolved)	3	3.23	0.36
S. maltophilia (Ancestor vs Evolved)	3	13.22	0.0042
Community	9	51.76	5.02×10^{-8}
P. aeruginosa:Community interaction	9	55.86	8.35×10^{-9}
S. aureus:Community interaction	9	13.42	0.14
S. maltophilia:Community interaction	9	17.69	0.039

3.4.3 Investigating potential evolutionary mechanisms that may contribute to species coexistence

As each evolved community was able to coexist to a different degree, and each was affected differently when the constituent species were replaced with their respective ancestors, there were likely numerous different mechanisms involved to mediate the evolved coexistence. We investigated a small number of these to begin to identify how the coexistence may have arisen during the selection experiment, and whether these mechanisms may play a role in the Liquid media. To this end, we: measured growth in the Liquid and Liquid + TOB treatments over 48 hours, to get a measure of competitive ability of the isolates; measured adherent biofilm formation, as increased adherence could allow evolved isolates to grow in distinct niches within the wells and possibly increase coexistence; pyocyanin production in *P. aeruginosa*, as one of the secreted inhibitory factors that may influence growth of the other species; and the growth of each species in other species' supernatant, to examine the extent to which indirect interactions play a role in growth.

3.4.3.1 Individual growth in Liquid media

We grew each evolved clone isolated from the evolved coexisting communities in the Liquid media treatment to examine differences in growth, and thus competitive ability, and after this growth period we also adherence of each isolate, which acts as a proxy measurement for adherent biofilm formation.

Repeated measures ANOVA on *P. aeruginosa* growth showed there was a significant interaction effect of evolved community replicate and time (Repeated Measures ANOVA: $p < 0.001$; Table 3.5), and ANOVA within timepoint measurements showed a significant effect of evolved community replicate at 24 hours (ANOVA: $F_{(3, 19)} = 11.13$, FDR-adjusted $p = 0.00039$) though not at 48 (ANOVA: $F_{(3, 19)} = 0.408$, FDR-adjusted $p = 0.75$). Pairwise comparisons found that evolved community 3 grew to a significantly lower density at 24 hours than the other three communities (Pairwise Tukey post-hoc, $p < 0.001$, Figure 3.8A), which suggests that isolates from evolved replicate community 3 grow at a slower rate than the other isolates.

There was a significant effect of evolved community replicate on adherent biofilm formation (ANOVA: $F_{(3, 19)} = 79.098$, $p < 0.001$; Figure 3.8B), whereby isolates from evolved replicate 2 produced significantly more adherent biofilm than the other communities (Pairwise Tukey post-hoc; FDR-adjusted $p < 0.001$; Figure 3.8B), and isolates from evolved replicate 3 produced significantly more than isolates from community 1 (Pairwise Tukey post-hoc; FDR-adjusted $p = 0.016$; Figure 3.8B). The clone from community 2 used in the media change and ancestor replacement experiments had ~6 times the adherence of the ancestor (Figure 3.8B), which may have contributed to its comparative domination of community 2 (Figure 3.5B).

Staphylococcus aureus consistently grew to a higher density than its ancestor across communities (Figure 3.8C), and there was a significant effect of time on *S. aureus* growth (Repeated Measures ANOVA: $p = 0.007$, Table 3.5), likely driven by evolved replicate 2 whereby density increased from 24- to 48 hours. *Stenotrophomonas maltophilia* grew to around the same degree as its ancestor over both timepoints (Figure 3.8E). Despite a significant interaction of evolved community replicate and time (Repeated Measures ANOVA: $p = 0.016$, Table 3.5), there were no within timepoint differences (ANOVA: $p > 0.05$). However, there was a significant effect of time on growth (Repeated Measures ANOVA: $p < 0.001$, Table 3.5), whereby density decreased at 48 hours. There were no significant effects of evolved replicate community on adherent biofilm formation (ANOVA: *S. aureus*: $F_{(3, 20)} = 2.28$, $p = 0.11$; *S. maltophilia*: $F_{(3, 20)} = 0.72$, $p = 0.55$; Figure 3.8D, F), though both species produced more adherent biofilm than their ancestors. Together these results suggest that all species adapted to some degree regarding their growth and biofilm formation relative to their ancestors.

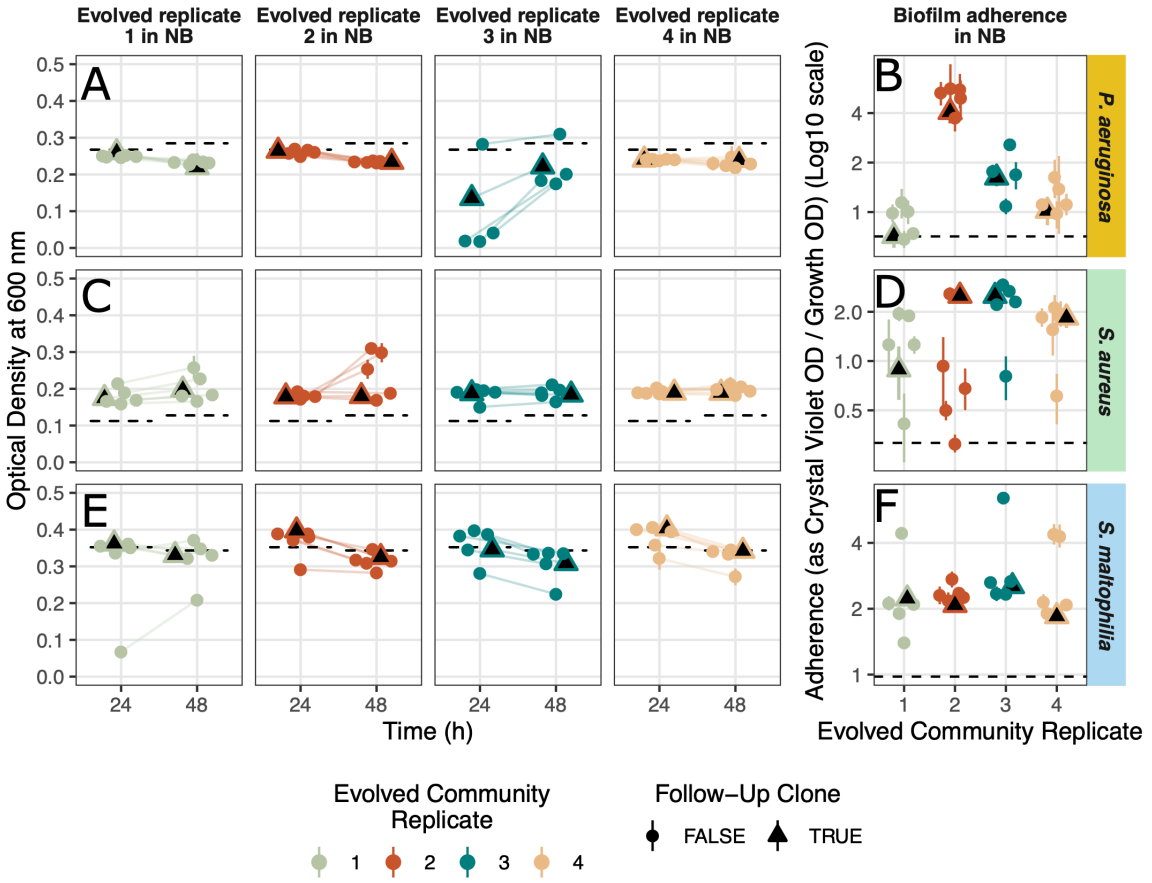


Figure 3.8: Growth in Liquid media and biofilm adherence of individual clones of each species from the evolved communities.

Panel rows show each species, and panel columns are grouped by: **(A, C, E)** growth in Liquid media (NB), where each individual panel shows an evolved replicate community; and **(B, D, F)** biofilm adherence following said growth. Within the panel groups A-C-E, time is along the X-axis, whilst within panels B-D-F evolved community replicate is along the X-axis. Each point represents one of the 6 clones isolated from each evolved community. Triangles indicate the clone used during the follow up experiments (Table 3.2). Error bars in panels A-C-E represent ± S.E.M. for three technical replicates, whereas error bars in panels B-D-F show maxima and minima as defined in Methods § 3.3.7.2. Dashed lines represent the growth or adherence of the ancestor.

Table 3.5: Repeated measures ANOVA table for growth of each species in Liquid (NB) media.

Response = Relative growth; Between-subject factor = Evolved community; Within-subject factor = Time.

Species	ANOVA term	D.F.	Sum. Sq.	F value	P value
P. aeruginosa	*Between Subject:*				
	Community	3	0.055	6.20	0.004
	Errors	19	0.056		
	Within Subject:				
	Time	1	0.003	6.07	0.023
	Community:Time interaction	3	0.038	27.2	4.35×10^{-7}
	Errors	19	0.009		
S. aureus	*Between Subject:*				
	Community	3	0.003	0.93	0.45
	Errors	20	0.019		
	Within Subject:				
	Time	1	0.005	9.10	0.007
	Community:Time interaction	3	0.005	2.73	0.071
	Errors	20	0.012		
S. maltophilia	*Between Subject:*				
	Community	3	0.012	0.72	0.55
	Errors	20	0.11		
	Within Subject:				
	Time	1	0.015	21.1	0.00018
	Community:Time interaction	3	0.009	4.35	0.016
	Errors	20	0.014		

3.4.3.2 Individual tobramycin tolerance

In addition to measuring growth in the Liquid media, we also measured growth in 0.5 μg/ml tobramycin—the concentration used in the Liquid + TOB treatment. This measure acts an indication of evolution of tolerance of tobramycin, and changes in tolerance between species can affect community outcomes—see Chapter 2.

The evolved *P. aeruginosa* isolates had greatly altered tolerance compared to their ancestor (Figure 3.9A). There was a significant interaction effect of evolved community replicate and time (Repeated Measures ANOVA: $p < 0.001$; Table 3.6), and ANOVA within timepoint measurements showed a significant effect of evolved community replicate at 48 hours (ANOVA: $F_{(3, 19)} = 12.41$, FDR-adjusted $p < 0.001$) though not at 24 (ANOVA: $F_{(3, 19)} = 1.65$, FDR-adjusted $p = 0.21$). Density at 24 hours did not differ significantly between evolved community replicates (Pairwise Tukey post-hoc, FDR-adjusted $p > 0.05$) but was much lower than that of the ancestor (Figure 3.9A), suggesting a greatly reduced growth rate in tobramycin. The density of isolates from evolved replicate community 2 at 48 hours differed significantly to the other communities (Pairwise Tukey post-hoc, FDR-adjusted $p < 0.05$), suggesting a reduced maximum growth during tobramycin treatment. There was also a significant effect of evolved community replicate on adherent biofilm formation (ANOVA: $F_{(3, 19)} = 5.37$, $p = 0.008$; Figure 3.9B), such that isolates from replicate 2 formed more adherent biofilm than the other communities (Pairwise Tukey post-hoc, FDR-adjusted $p < 0.05$), as they did without antibiotic (Figure 3.8B). However, there were some unusually large values for adherent biofilm per unit growth, likely due to the low growth of replicate 2 isolates.

Isolates of *S. aureus* all increased their tolerance to tobramycin relative to the ancestor (Figure 3.9C). There was a significant interaction between evolved community replicate and time (Repeated Measures ANOVA: $p < 0.001$, Table 3.6), and ANOVA within timepoint measurements showed a significant effect of evolved community replicate at both 24- (ANOVA: $F_{(3, 19)} = 4.79$, FDR-adjusted $p = 0.011$) and 48 hours (ANOVA: $F_{(3, 19)} = 6.88$, FDR-adjusted $p = 0.004$). At 24 hours, though pairwise comparisons showed that isolates from evolved replicate community 1 grew to a significantly lower density than communities 2 and 4 (Pairwise Tukey post-hoc;

FDR-adjusted $p < 0.05$), the size of this effect was very low, and all communities grew to a similar density. At 48 hours, however, pairwise comparisons showed that isolates from evolved community 2 grew to a significantly greater density than communities 3 and 4 (Pairwise Tukey post-hoc, FDR-adjusted $p < 0.05$), suggesting a greater improvement in maximum growth in tobramycin.

Much like growth without antibiotic, *S. maltophilia* tobramycin tolerance remained largely unchanged relative to its ancestor (Figure 3.9E). And again, though there was a significant interaction of evolved community replicate and time (Repeated Measures ANOVA: $p = 0.006$, Table 3.6), there were no within timepoint differences (ANOVA: $p > 0.05$). There was again a significant effect of time on growth (Repeated Measures ANOVA: $p < 0.001$, Table 3.6), whereby density decreased at 48 hours.

There were again no significant effects of evolved replicate community on adherent biofilm formation for *S. aureus* nor *S. maltophilia* (ANOVA: *S. aureus*: $F_{(3, 20)} = 3.02$, $p = 0.054$; *S. maltophilia*: $F_{(3, 20)} = 0.27$, $p = 0.85$; Figure 3.9D, F), though adherent biofilm formation remained similar to the ancestor in *S. aureus*.

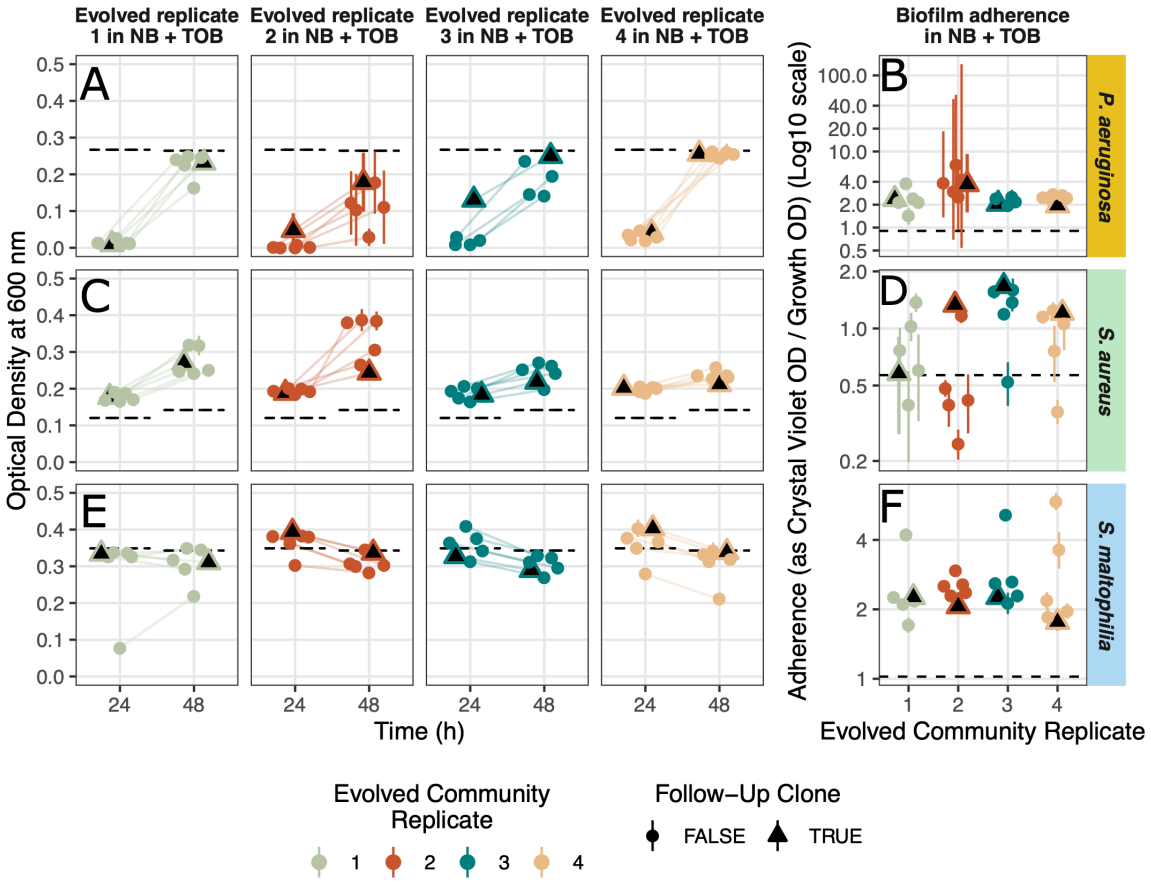


Figure 3.9: Growth in Liquid + TOB media and biofilm adherence of individual clones of each species from the evolved communities.

Panel rows show each species, and panel columns are grouped by: **(A, C, E)** growth in Liquid + TOB media (NB + 0.5 µg/ml TOB), where each individual panel shows an evolved replicate community; and **(B, D, F)** biofilm adherence following said growth. Within the panel groups A-C-E, time is along the X-axis, whilst within panels B-D-F evolved community replicate is along the X-axis. Each point represents one of the 6 clones isolated from each evolved community. Triangles indicate the clone used during the follow up experiments (Table 3.2). Error bars in panels A-C-E represent ± S.E.M. for three technical replicates, whereas error bars in panels B-D-F show maxima and minima as defined in Methods § 3.3.7.2. Dashed lines represent the growth or adherence of the ancestor.

Table 3.6: Repeated measures ANOVA table for growth of each species in Liquid + TOB (NB + 0.5 µg/ml TOB) media.

Response = Relative growth; Between-subject factor = Evolved community; Within-subject factor = Time.

Species	ANOVA term	D.F.	Sum. Sq.	F value	P value
P. aeruginosa	*Between Subject:*				
	Community	3	0.039	7.41	0.002
	Errors	19	0.033		
	Within Subject:				
	Time	1	0.35	608	6.82×10^{-16}
	Community:Time interaction	3	0.025	14.2	4.20×10^{-5}
	Errors	19	0.011		
S. aureus	*Between Subject:*				
	Community	3	0.017	5.00	0.01
	Errors	20	0.022		
	Within Subject:				
	Time	1	0.077	127	4.09×10^{-10}
	Community:Time interaction	3	0.018	10	0.000309
	Errors	20	0.012		
S. maltophilia	*Between Subject:*				
	Community	3	0.013	0.96	0.43
	Errors	20	0.092		
	Within Subject:				
	Time	1	0.016	24.3	8.15×10^{-5}
	Community:Time interaction	3	0.011	5.60	0.006
	Errors	20	0.013		

3.4.3.3 Evolution of supernatant-mediated interactions between coexisting species

The final aspect that we investigated was contact-independent competition through nutrient usage and secreted secondary metabolites. Here, we grew each species in the cell-free supernatant of each other species from the same evolved community and compared that growth to the growth of the species in diluted NB. Leftover in the supernatant of a bacterial culture will be a mix of nutrients that that bacteria did not utilise, the by-products of metabolised nutrients, and any secreted secondary metabolites produced by that bacteria. By growing another species in this supernatant one can indirectly test a combination of factors such as nutrient usage overlap—do the two species metabolise the same nutrients in the media—cross-feeding—does one species produce a by-product that the other species can metabolise—and inhibition by secondary metabolites—does a species produce factors that actively inhibit the growth of the other species. Though this assay cannot distinguish between these factors, whether a species' supernatant is facilitative or antagonistic and how this changed between the ancestor and evolved communities could help inform the mechanisms of coexistence.

Pseudomonas aeruginosa supernatant, from both ancestral and evolved clones, was generally antagonistic to the other two species, as *S. aureus* and *S. maltophilia* grew worse than in 20 % NB (Figure 3.10A–D). There was a significant effect of supernatant identity (i.e., supernatant from the ancestor, evolved community 1, etc.) on relative growth index (Table 3.7, $p < 0.001$), whereby the supernatant of each evolved community was significantly more inhibitory than that of the ancestor (Familywise Tukey post-hoc, $p < 0.05$; Figure 3.10A–D). Supernatant from evolved communities 1 and 4 were particularly inhibitory—both were significantly different to supernatant from evolved community 3 (Familywise Tukey post-hoc, $p < 0.05$). *Pseudomonas aeruginosa* is known to produce numerous secreted competitive factors that inhibit bacterial growth, and we measured the production of one such factor, pyocyanin (Figure 3.10E). There was a significant effect of evolved community on pyocyanin production per capita (ANOVA; $F_{(2, 20)} = 5.438$, $p = 0.007$), and the clones from evolved communities 1 and 4 that were used in the supernatant assay produced

more pyocyanin per capita than the clones used from communities 2 and 3, which could explain some of the observed increase in inhibition. However, as the supernatants of all evolved communities became more inhibitory relative to the ancestor it is likely that other secreted factors are playing a role as well as pyocyanin. That *P. aeruginosa* supernatant became more inhibitory in coexisting communities was surprising. Evolved *S. aureus* in communities 1 and 3 were less inhibited by evolved *P. aeruginosa* supernatant, which suggests adaptation that may have contributed to the observed coexistence in these communities (Figure 3.3, Figure 3.5), though evolved and ancestor *S. maltophilia* were often equally inhibited by *P. aeruginosa* supernatant and so the role of this increased inhibition within evolved communities remains unclear.

The supernatant of *S. aureus* was consistently facilitatory, increasing growth of both other species by around 2-fold (Figure 3.10A–D). There was no significant effect of supernatant identity on growth of the other species (Table 3.7, $p = 0.33$), suggesting that *S. aureus* metabolism and secretion were largely unchanged in these communities. However, there was a significant effect of growing species identity on growth in *S. aureus* supernatant (Table 3.7, $p < 0.001$), whereby each evolved *P. aeruginosa* clone grew significantly worse than the *P. aeruginosa* ancestor (Familywise Tukey post-hoc, $p \leq 0.05$). And though not significant, evolved *S. maltophilia* grew marginally worse than ancestral *S. maltophilia* in *S. aureus* supernatant. This could suggest that, as *P. aeruginosa* or *S. maltophilia* adapted to the nutrient composition of the media, resource usage overlap between either of the species and *S. aureus* increased resulting in reduced growth. However, the lack of change in the facilitation of growth by *S. aureus* supernatant more generally suggests that *S. aureus* played a negligible role in community coexistence.

As with *S. aureus*, the supernatant of *S. maltophilia* was generally facilitative to the growth of other species (Figure 3.10A–D), though not to the same extent, and did not differ significantly between ancestral and evolved *S. maltophilia* clones (Table 3.7, $p = 0.13$). However, there was a significant effect of species identity on growth within *S. maltophilia* supernatant (Table 3.7, $p < 0.001$). Evolved *P. aeruginosa* in community 3 grew significantly worse than all other *P. aeruginosa* clones (Familywise Tukey post-hoc, $p < 0.05$), while *S. aureus* in this community grew significantly better than all

other *S. aureus* clones except for evolved community 1 (Familywise Tukey post-hoc, $p < 0.05$). These combined changes in community 3, coupled with the increased growth of *S. aureus* in this community within evolved *P. aeruginosa* supernatant, suggest that adaptation by *S. aureus* and increased nutrient usage overlap between *P. aeruginosa* and *S. maltophilia* could contribute to coexistence. Evolved *S. aureus* in community 1 also grew significantly better in *S. maltophilia* supernatant than all other *S. aureus* clones excluding community 3 (Familywise Tukey post-hoc, $p < 0.05$), and the degree of increased growth in communities 1 and 3 suggests a possible increase or development of cross-feeding, though evolved *S. aureus* more generally grew better than the ancestor (Figure 3.10A–D).

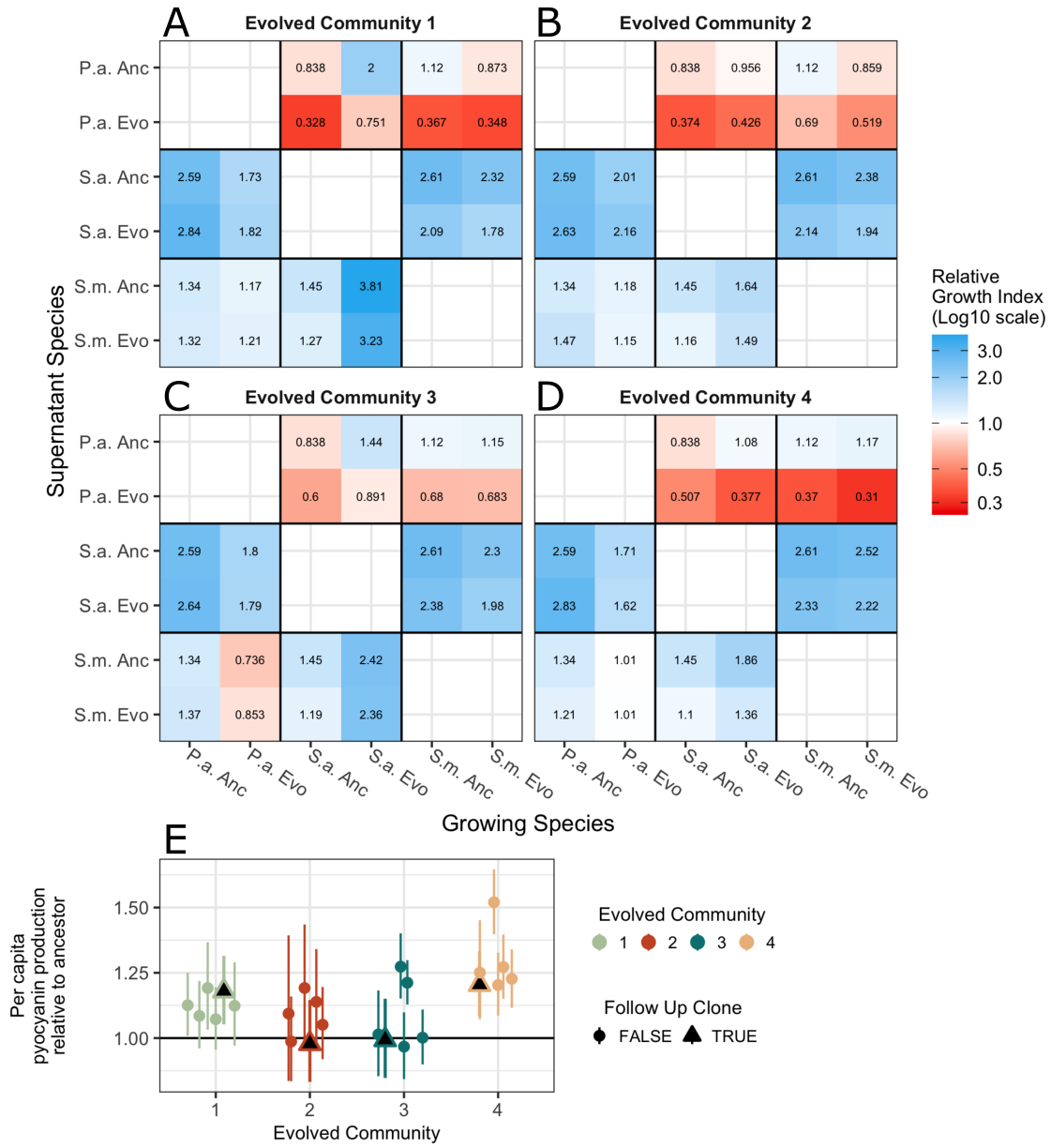


Figure 3.10: Relative growth indices of evolved and ancestral species from evolved communities grown in other species' supernatant (A–D), and pyocyanin production of evolved *P. aeruginosa* (E).

A–D: Heatmap of growth in supernatant relative to growth in diluted NB. X-axis indicates the species that was grown, and Y-axis indicates the supernatant that species was grown in. Panels show the evolved communities. Relative growth index value is shown on the heatmap squares, and colours represent the relative growth index transformed to a $\log_{10}$ scale. Ancestor by ancestor data is repeated across all four panels. **E:** Pyocyanin production per capita in *P. aeruginosa* relative to the ancestor.

Each point represents one of the 6 clones isolated from each evolved community. Error bars show maxima and minima as defined in Methods § 3.3.7.4. Triangles indicate the clone used during the follow up experiments (Table 3.2).

Table 3.7: Two-way Type-II ANOVA tables for each species' supernatant.

Response = Log_{10}-transformed Relative growth index; Predictors = Supernatant species identity (i.e., ancestor, evolved replicate community 1, etc.), Growing species identity (i.e., *P. aeruginosa* ancestor, *S. aureus* evolved replicate 1, *S. maltophilia* evolved replicate 2, etc.).

Supernatant species	**ANOVA term**	**Sum Sq.**	**D.F.**	***F* value**	***P* value**
P. aeruginosa	Supernatant identity	0.78	4	34.46	1.72×10^{-6}
	Species identity	0.22	9	4.27	0.011
	Residuals	0.068	12		
S. aureus	Supernatant identity	0.0069	4	1.29	0.33
	Species identity	0.12	9	9.57	0.00031
	Residuals	0.016	12		
S. maltophilia	Supernatant identity	0.011	4	2.20	0.13
	Species identity	0.60	9	51.55	3.22×10^{-8}
	Residuals	0.015	12		

3.5 Discussion

The importance of diversity within the CF lung microbiome poses an interesting problem. Patients with greater microbial diversity tend to have better lung function than patients with lower diversity (Li *et al.*, 2016), but whether greater lung function comes as a result of high diversity, or vice versa, is unclear. Here, we investigated the influence of two possible diversity maintaining factors, increased media viscosity and a low dose of antibiotic, on the species abundance within a simple CF-associated community, consisting of the species *P. aeruginosa*, *S. aureus*, and *S. maltophilia*.

3.5.1 Three-species coexistence was only consistently observed in the presence of both high viscosity and tobramycin

At the final timepoint of the selection experiment, the only media treatment in which we observed consistent coexistence of all three species was in the Viscous + TOB treatment (Figure 3.1A). This was contrary to our hypothesis, in which we had expected that the Viscous media treatment would also drive species coexistence; we found that the composition of the Viscous media treatment was no different to the baseline Liquid media, wherein *P. aeruginosa* dominated the communities at high abundance whilst *S. maltophilia* was not detected. That viscosity alone did not drive coexistence suggests that further diversity maintaining mechanisms are in play within the CF lung. One important factor that was not included within our experimental system was the nutrient composition of the media. The nutrient composition of CF sputum is rich in amino acids and extracellular DNA and poor in iron availability, and has been shown to result in different gene expression in *P. aeruginosa* (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007; Turner *et al.*, 2015; Rossi *et al.*, 2018). Among the changes in expression are a reduction in production of QS related competition factors, which could alter the interactions between species (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007). However, *P. aeruginosa* also upregulates production of competition factors, particularly Staphylococcal inhibitory factors, in the presence of *S. aureus* (Mashburn *et al.*, 2005; Korgaonkar and Whiteley, 2011; Korgaonkar *et al.*, 2013), and so it would be of interest to determine to what extent nutrient composition would alter our observations and whether increased viscosity would have a greater effect in this environment.

There was, however, a strong effect of the addition of tobramycin on the composition of the communities, such that *P. aeruginosa* was excluded from the community (Figure 3.1A). Previously, we had found that this concentration of tobramycin enabled coexistence between *P. aeruginosa* and *S. maltophilia*—see Chapter 2—and we observed the same effect within the *P. aeruginosa* & *S. maltophilia* two-species community treated with Liquid + TOB in this experiment as well (Figure 3.1C). The addition of *S. aureus* resulted in a change of dynamics. Within the *S. aureus* & *S. maltophilia* two-species community, both species were able to coexist across all media conditions (Appendix Figure B.9), and the supernatant assays showed that the two ancestors facilitated each other's growth (Figure 3.10A–D). Additionally, the minimum inhibitory concentration of tobramycin for the ancestral *S. aureus* was much greater than the minimum inhibitory concentration for the ancestral *P. aeruginosa* (Methods § 3.3.2; Appendix Figures A.1 & B.12), suggesting greater tobramycin tolerance, and though this advantage was not enough to overcome the antagonism likely exerted by *P. aeruginosa* (Hotterbeekx *et al.*, 2017; Limoli and Hoffman, 2019) in the pairwise two-species community, taken together this suggests a mechanism by which *P. aeruginosa* was excluded within the three-species community: the presence of tobramycin increased the competitive fitness of *S. maltophilia* relative to *P. aeruginosa*, and the facilitative interaction between *S. aureus* and *S. maltophilia* allowed both to grow to high abundance, which likely exacerbated the fitness defect experienced by *P. aeruginosa*. Over time *P. aeruginosa* abundance decreased, and with decreasing abundance *P. aeruginosa* became less likely to develop ameliorating mutations that might increase fitness, consistent with the hypothesis that increased community diversity constrains evolution (Scheuerl *et al.*, 2020). This was the case in three of the four replicate communities, and that *P. aeruginosa* was able to survive and coexist within the remaining replicate suggests that there may have been an adaptation within this community; or alternatively reinforces our later observation that the outcome within each community is subject to complex processes due to interactions between the species and the experimental procedure.

That we observed coexistence between these three species in the Viscous + TOB media treatment suggests that viscosity was able to act as a driver of coexistence, but that within our system the existing competitive dynamics needed to

be overcome first, here with the addition of tobramycin. Though treated with tobramycin, the evolved *P. aeruginosa* isolates from these coexisting communities showed reduced tolerance of tobramycin relative to the ancestor (Figure 3.9A, D). One possibility to explain why tolerance was not selected for in these communities is that the presence of the other community members acted as a greater selective pressure on *P. aeruginosa* to adapt to the increased competition within the environment and/or a different niche space within the microcosms. This would in line with observations by Klümper *et al.*, (2019), who showed that selection of antibiotic resistant strains within communities can be constrained, as the minimum selective pressure of the antibiotic increased significantly for bacteria embedded in a community compared to without the community. Another possibility is that the effective concentration of tobramycin was further reduced by limited diffusion or sequestration by the ficoll within the viscous media, reducing the selective pressure for tolerance. In CF, it is known that negatively charged macromolecules within the sputum, DNA and mucin, are capable of binding and sequestering the positively charged tobramycin (Ramphal *et al.*, 1988). Ficoll is neutrally charged, and so is unlikely to bind tobramycin in this way, but its highly branched structure may still be capable of sequestering small amounts of antibiotic.

3.5.2 Continued coexistence in the "Viscous + TOB" treatment resulted in evolution of coexistence in the absence of diversity-maintaining mechanisms

Transplantation of evolved coexisting "Viscous + TOB" communities to other media treatments showed that coexistence could be maintained when both diversity maintaining factors were removed (Figure 3.3). However, although the likelihood of coexistence had increased, it was not guaranteed; over multiple replicates of the same evolved communities the community composition outcomes were shown to be variable (Figure 3.3, Figure 3.5). This suggests that even with highly similar initial starting conditions, complex interactions within the communities can result in different outcomes. Additionally, the likelihood of coexistence was different between evolved replicate communities and depended upon different species within each (Figure 3.5), suggesting that evolutionary trajectories were distinct between replicates.

The four coexisting evolved replicate communities were able to coexist within the baseline Liquid media treatment where the ancestral community could not (Figure 3.3A, Figure 3.4A). It is worth noting here that the outcome within the ancestral community was different to that observed in the selection experiment after 6 days (Figure 3.2A), which we ascribe to a less equal starting density of the three species during the selection experiment—see Methods § 3.3.2 & 3.3.5. Indeed, as the initial densities were more even between species in the media change experiment, this reinforces the changes we have observed among the evolved isolates as the likelihood of coexistence was greatly increased relative to the ancestor.

The evolved replicate communities were affected by the Liquid + TOB treatment differently to the ancestor, such that *P. aeruginosa* became undetected within the evolved communities (Figure 3.3C, Figure 3.4C). As previously discussed, *P. aeruginosa* was already at a competitive disadvantage within this treatment, and we found that the evolved isolates had greatly reduced growth at 24 hours compared to the ancestor (Figure 3.9A) whereas the other species maintained or increased their growth (Figure 3.9C, E) and as such it is likely that during this time *P. aeruginosa* was outcompeted within the evolved communities. That *P. aeruginosa* was not outcompeted in the Viscous + TOB treatment (Figure 3.3D) despite this greatly reduced tolerance further suggests that viscosity protects species in competition from the negative effects of antibiotic treatment.

Though outcomes were similar between evolved communities grown in the Liquid treatment and Viscous + TOB treatment (Figure 3.3), the abundances were not as even between the species in the Liquid treatment (Figure 3.4). This suggests that coexistence would likely not be stable over a longer time period. Indeed, given enough time coexistence cannot continue indefinitely due to evolutionary processes that act in concert with the ecological processes (Pinsky, 2019), and so whether *P. aeruginosa* would eventually dominate as in the CF lung would be an interesting question to investigate.

3.5.3 Despite coexistence, *P. aeruginosa* became more inhibitory towards the other species

Each evolved replicate community appeared to follow different evolutionary trajectories, as we found that the likelihood of coexistence was variable between replicate communities and affected differently by replacement of each species with its ancestor (Figure 3.5). Evolved communities 1 and 3 had the greatest likelihood of coexistence (Figure 3.5, Figure 3.7), though community 3 was the most stable to ancestor replacements. In contrast, evolved communities 2 and 4 were less likely to coexist—indeed coexistence was only observed once in community 2 during the ancestor replacement assay—and again the effects of species replacement differed between the two.

Coexistence within evolved replicate community 1 appeared to be mediated largely by evolved *S. maltophilia*, as its replacement, both individually and with other species, diminished both its own survival and the coexistence of the community (Figure 3.5A, B). Of the phenotypes that we measured there were none that could explain this contribution of *S. maltophilia* within community 1: growth of the follow-up isolate was largely similar to the ancestor (Figure 3.8E); though adherent biofilm formation had increased, this increase was consistent across replicates (Figure 3.8F); and evolved *S. maltophilia* grew similarly in other species' supernatant, and produced similar supernatant, to its ancestor (Figure 3.10A). There were observable changes in the other species, however. Evolved *S. aureus* was able to grow to much higher densities in the supernatant of both *P. aeruginosa* and *S. maltophilia* than its ancestor (Figure 3.10A), which may have stabilised the community somewhat despite increased inhibition from *P. aeruginosa*, and the follow-up clone of *P. aeruginosa* produced the least adherent biofilm of the four replicates (Figure 3.8B), which may also have contributed.

On the other hand, coexistence within evolved replicate community 3 appeared to be mediated by reduced competitive ability of *P. aeruginosa*, as replacement with the ancestor decreased the likelihood of coexistence (Figure 3.5E, F). Here, the phenotypes provided some evidence for this, as the growth of *P. aeruginosa* the follow-up clone from evolved replicate community 3 was greatly diminished relative to the ancestor and the other communities (Figure 3.8A), and grew

to a much lower density in *S. maltophilia* supernatant than its ancestor (Figure 3.10C). Here too, evolved *S. aureus* was able to grow much better in both *P. aeruginosa* and *S. maltophilia* supernatant (Figure 3.10C), suggesting the possibility that *S. aureus* plays a greater role in mediating coexistence than suggested by the ancestor replacement assay.

Where coexistence was less likely, in communities 2 and 4, the phenotypic data provide some potential mechanisms for this too. Unlike in other evolved community replicates, *P. aeruginosa* isolates from community 2 greatly increased their production of adherent biofilm (Figure 3.8A), and as such all three species had increased adherence which may have resulted in increased competition between species to occupy the same physical space around the surface of the well, providing *P. aeruginosa* with increased opportunity to inhibit growth of the other species. *Pseudomonas aeruginosa* has been observed to grow on top of *S. aureus* in mixed species biofilms (Chew *et al.*, 2014), which would limit *S. aureus* access to nutrients, and has been shown to inhibit *S. maltophilia* growth in a contact-dependent manner (Pompilio *et al.*, 2015).

In community 4, *P. aeruginosa* isolates produced a greater amount of pyocyanin than the ancestor (Figure 3.10E) and evolved *P. aeruginosa* supernatant was more inhibitory to the other species than in communities 2 and 3 (Figure 3.10D). Though the same increase in pyocyanin production and supernatant inhibition was seen in *P. aeruginosa* from evolved replicate community 1, *S. aureus* from community 4 had not evolved to grow to higher densities within the supernatant as it had in community 1, suggesting that lack of co-adaptation between species contributed to the reduced coexistence of evolved replicate community 4.

Overall, the indirect interactions between evolved species appear complex, as *P. aeruginosa* supernatant becomes more inhibitory to the other species, whilst *S. aureus* and *S. maltophilia* develop an increase in facilitative interactions; this facilitation between *S. aureus* and *S. maltophilia* may counteract the inhibition by *P. aeruginosa* to drive coexistence. Of the four combinations of evolved and ancestral *S. aureus* in evolved and ancestral *S. maltophilia* supernatant, the greatest facilitation of growth was for evolved *S. aureus* in ancestral *S. maltophilia* supernatant, the least growth was ancestral *S. aureus* in evolved *S. maltophilia* supernatant, whilst the

remaining combinations were somewhere between. This suggests a coadaptation between the two species such that as *S. maltophilia* adapts to the media, and alters nutrient usage or by-product production, *S. aureus* adapts to the changes in *S. maltophilia* nutrient usage, by shifting its own nutrient usage or developing a degree of cross-feeding, though whether *S. maltophilia* or *S. aureus* changes first is unclear with the current data.

3.5.4 Caveats and improvements

The method that we used to measure abundance within the communities limited the certainty with which we could investigate coexistence, as we found that growth on the selective agar plates resulted in a reduced yield of *P. aeruginosa* and *S. maltophilia* when compared with LB agar (Table 3.1). Accounting for this reduction did not prove reliable, as the range of densities that we measured during the preliminary comparisons did not encompass the range of densities observed in the above experiments, and so predicted species abundance did not fully account for density dependent effects. An alternative method to measure species abundance in a community would be through qPCR of 16S rRNA sequences to establish relative abundance using read counts. However, this method is more expensive, and is not without its own limitations as biases present in both the DNA extraction and amplification steps can influence the observed abundances in ways that can be difficult to correct for (Brooks *et al.*, 2015). Another alternative could be to fluorescently label the species, which would have the added benefit of facilitating calculation of competitive fitness, rather than the indirect measures of growth we have used. However, were we to investigate communities larger than we have used here, the qPCR method would likely be the most feasible due to the difficulty of establishing sufficient numbers of selective plates.

A contributing factor to the stochasticity of coexistence outcomes was likely the experimental system that we employed. We made efforts to ensure that homogenous mixes of bacteria were transferred to fresh media every 48 hours, though to achieve this the biofilm structures within each well were broken up and bacteria were made to repeatedly re-establish growth. These methods do not fully protect against sampling bias, as it would still be possible to simply miss rare species, and

repeated periods of exponential growth phases may increase the competition between species for resources and increase the likelihood of exclusions. In the CF lungs there are likely fewer instances of rapid population growth and expansion as is found within our system, which could result in a different degree of interspecies interactions. A possible method to address this difference could be to perform experiments within larger batch culture devices that can maintain the growing mass of bacteria and as such do not break down the physical structure of the communities.

It would be of interest to investigate the community structure within our community microcosms, to establish the extent to which the species associate within physical space. This could be performed using fluorescent *in-situ* hybridisation on our existing isolates to visualise the locations of each species, or through use of fluorescently labelled isolates. It would also be beneficial to further phenotype the isolates to establish other possible interactions, such as nutrient utilisation or secretion of other factors such as siderophores.

Another aspect of the ecology of these species to investigate could be the ability of each species to invade established communities of the other species and whether this varies across environmental gradients, similar to investigations by Grainger *et al.*, (2019) on bacterial strains grown in different pH and sugar concentrations. It is likely that *P. aeruginosa*, particularly strain LESB58 (Fothergill *et al.*, 2007), is very effective at invading communities, evidenced by the general dominance we and others have observed and its acquisition in later life among patients (UK Cystic Fibrosis Registry, 2020), and if this is affected by environmental factors, as we have found coexistence to be, then this might be translatable to clinical practice to help reduce *P. aeruginosa* infection.

3.5.5 Significance and conclusion

Whether the mechanisms that have evolved here to mediate coexistence would play a role within the CF lung is unclear. Late-stage clinical *P. aeruginosa* isolates have been shown to possess a number of mutations and adaptations that could increase coexistence, such as reduced production of virulence factors in mucoid or quorum sensing deficient isolates (Schuster *et al.*, 2003; Folkesson *et al.*, 2012), and altered nutrient usage profiles (La Rosa *et al.*, 2018). However, in the later stages of CF

pulmonary disease, *P. aeruginosa* is often the sole dominant species within patient lungs (Goddard *et al.*, 2012; Jorth *et al.*, 2015), suggesting that these adaptations facilitate dominance. An alternative hypothesis put forth by Whelan and Surette, (2015) is that persistent antibiotic exposure merely selects for the species with the greatest capacity to withstand the treatment, which is often *P. aeruginosa*. This is in line with one of the big questions of interactions within CF, the possibility that, due to the size and complex structure of the lungs, species do not mix and instead exist within nearby but disparate locations (Conrad *et al.*, 2013), and that following antibiotic treatment the surviving bacteria expand to occupy the vacated regions.

However, as we have observed that certain antibiotic types and concentrations can result in coexistence between certain species, this raises the possibility that infections could be managed in other ways to maintain diversity whilst reducing overall bacterial load. The feasibility of such management is unclear, though likely low. The changes in interaction between usually antagonistic species also raises the point about how little is known about the interactions between species within the lungs, and the compositions of communities. Further investigation into interspecies interactions may highlight other methods for managing infections.

In summary, we found that a combination of potential diversity maintaining factors, increased viscosity and treatment with a low dose of tobramycin, resulted in stable coexistence between three CF-associated species, *P. aeruginosa*, *S. aureus*, and *S. maltophilia*. During this coexistence, the species evolved such that the likelihood of coexistence in the absence of diversity maintaining factors increased. However, the likelihood of evolved coexistence varied substantially between evolved replicates and was also dependent upon different species and growth traits between evolved replicates. These results suggest that coexistence can arise as a result of environmental factors present within the lungs and evolve to become stable—though whether these mechanisms contribute to diversity within patients requires further investigation—and that there is much still to investigate regarding the interactions between species within larger communities.

Chapter 4. Identification of mutations resulting from selection imposed by bacterial interactions, antibiotics, and viscosity

4.1 Abstract

Bacterial pathogens that infect Cystic Fibrosis (CF) patients are known to adapt to the lung environment over the course of chronic infections and do so via multiple evolutionary trajectories. How the different species present within the communities adapt to each other, and how selection for adaptive mutations to the environment might be altered during growth in a community, have been explored to a lesser degree. Previously, we performed a fully factorial *in vitro* experiment, in which three CF-associated bacterial species, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia*, were grown in communities consisting of each combination of the three species and treated with each combination of increased media viscosity and a subinhibitory tobramycin concentration. Here, we have sequenced a total of 193 isolates from two timepoints within the selection experiment and performed variant calling analysis to identify single nucleotide polymorphisms and small insertions and deletions. The isolates were sampled evenly from each of the surviving species within each combination of community and treatment. The factorial design allowed us to interrogate how selected mutations differ across the different environments and communities, and how these mutations may affect the phenotypic measurements we performed previously. We also further investigated the evolved three-species isolates found to coexist and sequenced a greater number of species clones within these coexisting replicates to gain greater confidence regarding the identity of any mutations. We found that within each species selection for parallel mutations was observed within different media treatments rather than different communities. Within *P. aeruginosa*, the number of observed mutations decreased in more complex environments, i.e., with a greater number of added treatment factors or species, suggesting that adaptation to the media factors may have been constrained by interspecies competition. Though we were unable to identify the mechanisms

influencing coexistence, we found candidate *P. aeruginosa* genes in polyamine synthesis that warrant further investigation.

4.2 Introduction

Like any environmental niche, the Cystic Fibrosis (CF) lung possesses a distinct set of characteristics to which any infecting bacteria must adapt. Within the lung there is a unique nutrient composition available, consisting of high concentrations of amino acids (Palmer, Aye and Whiteley, 2007); there is a spatial structure imposed by the high viscosity mucus (Tomaiuolo *et al.*, 2014; Secor *et al.*, 2018); there are oxygen concentration gradients, ranging from the anaerobic within the mucus and blocked airways (Worlitzsch *et al.*, 2002), through the microaerobic and the aerobic (Alvarez-Ortega and Harwood, 2007); and there are antibiotic concentration gradients, as patients undergo treatment with the aim of clearing any bacterial infections (Bos *et al.*, 2017).

In addition to these abiotic factors, infecting bacteria must compete with and adapt to the other coinfecting or co-occurring species. The CF lung is highly polymicrobial (Zhao *et al.*, 2012), and interactions between bacteria, both positive and negative, can shape these communities (Quinn, Whiteson, *et al.*, 2016) and likely the course of disease progression for the patient (Layeghifard *et al.*, 2019). Bacteria interact directly, through production and secretion of inhibitory factors (Limoli and Hoffman, 2019) and via contact dependent mechanisms such as type VI secretion (Pompilio *et al.*, 2015); and indirectly, though competition for resources such as iron (Filkins *et al.*, 2015; Nguyen *et al.*, 2015) or cross-feeding on metabolic by-products (Flynn *et al.*, 2016; Gao *et al.*, 2018; Tognon *et al.*, 2019).

Adaptation within the CF lungs rarely follows one consistent trajectory, where phenotypic and genetic diversity are the norm (Jorth *et al.*, 2015; Marvig *et al.*, 2015; Bartell *et al.*, 2019; Kordes *et al.*, 2019; Alcaraz *et al.*, 2020; Bernardy *et al.*, 2020). In *Pseudomonas aeruginosa*, numerous adaptive genes have been identified among CF isolates (Marvig *et al.*, 2015; Gabrielaite *et al.*, 2020). However, the exact selective pressures driving these adaptations are often unclear.

Previously in Chapter 3, we performed a selection experiment that investigated how three CF-associated bacterial species, *Pseudomonas aeruginosa*, *Staphylococcus*

aureus, and *Stenotrophomonas maltophilia*, adapted to the abiotic factors of increased viscosity and a low, sub-minimum inhibitory concentration dose of tobramycin, within differing community compositions. We found that treatment of the three-species community with the combination Viscous + TOB treatment facilitated prolonged coexistence between the species that was not observed in the other treatment environments, and that coexistence of the evolved communities was more stable outside of the Viscous + TOB treatment than the ancestor community.

To further investigate the genetic mechanisms that might underpin this increased coexistence we sequenced a subset of the coexisting isolates. In addition, as the selection experiment was designed in a fully factorial manner, we also sequenced isolates from each of the community and treatment combinations to attempt to identify whether any observed mutations can be attributed to individual factors we manipulated during the experiment.

We sequenced the genomes of a total of 193 individual isolates from the selection experiment, along with the three ancestral species using the Illumina sequencing platform, and performed variant calling analysis to identify single nucleotide polymorphisms (SNPs) and other variants (insertions and deletions; INDELs). We found that parallel mutations between isolates were most common in *P. aeruginosa*; and across species parallel mutations were generally either observed in a majority of all isolates or in response to the different media treatments. In all three species, mutations in regulatory genes were the most common, suggesting that altered expression of a number of genes were beneficial for adaptation to the specific environmental factors that we manipulated. Though we were unable to identify the specific mechanisms governing coexistence, we identified candidate genes in *P. aeruginosa* involved with polyamine synthesis that warrant further investigation. The large number of different variants overall suggests that the strength of selection within our experimental system was likely relatively low, and that, as in CF, there may be multiple evolutionary trajectories to adapt to these environments.

4.3 Materials and Methods

4.3.1 Bacterial isolation and sequencing

Bacteria from the selection experiment performed in Chapter 3 were isolated for cloning. Following the abundance count at the final timepoint of the selection experiment (up to) 6 clones of each surviving bacterial species were isolated from each uncontaminated replicate community and were cryopreserved at -80ºC. One clone per species per replicate community was selected randomly for sequencing after excluding any outlying clones based upon growth measurements. To further investigate the potential mechanisms of coexistence in Viscous + TOB treated three-species communities, all 6 clones of *Pseudomonas aeruginosa* were sequenced, alongside 3 clones of both *Staphylococcus aureus* and *Stenotrophomonas maltophilia*, to provide a greater sequencing depth in this treatment of interest. Isolates of the species from intermediate timepoint counts of the three-species populations were sequenced to explore a potential order in the accumulation of mutations. The counts were chosen as the last count in their respective treatments in which all three species were still observed: for the treatments without tobramycin this was after the first count (timepoint 3); for the treatments with tobramycin, this was after the second count (timepoint 6). No bacteria were isolated from the intermediate counts at the time they were performed, and so cryopreserved whole population samples were streaked onto selective agar at a separate time to isolate the individual clones for sequencing. Difficulty with isolating from the frozen population samples resulted in some species replicates being excluded from sequencing despite being counted at the time of performing the selection experiment. A full list of the sequenced isolates can be found in Appendix Table C.1.

DNA isolation and genome sequencing was performed by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1), using an Illumina platform, from whole bacterial isolates prepared and sent according to their instructions. Briefly, overnight cultures of each isolate were streaked on LB agar and, following incubation for 24 h at 37ºC, streak plates were prepared from a single colony of each isolate. After another incubation the total

growth from each streak plate was scraped into a supplied Microbank (Pro Lab Diagnostics) freezer tube, and subsequently posted by first class mail to MicrobesNG the next day.

4.3.2 Variant calling

MicrobesNG supplied trimmed 2x250 bp paired-end reads, which were aligned to the respective species' reference genomes using *BWA-MEM* version 0.7.17 (Li, 2013). Reference genomes were downloaded from RefSeq (O'Leary *et al.*, 2016) selected based on the strain used during the selection experiment: *P. aeruginosa* strain LESB58 (RefSeq accession NC_011770.1; Winstanley *et al.*, 2009), and *S. aureus* subsp. *aureus* Rosenbach 1884 (DSM 20231; RefSeq accessions NZ_CP011526.1 and NZ_CP011527.1 for the chromosome and plasmid respectively; Shiroma *et al.*, 2015). Two assemblies exist on RefSeq for the *S. maltophilia* strain ATCC13637 / NCTC10257, accessions NZ_CP008838.1 (Davenport *et al.*, 2014) and NZ_LT906480.1 (Wellcome Sanger Institute, 2014). Alignment of the reads from our ancestral *S. maltophilia* isolate to reference NZ_CP008838.1 resulted in a large number of variants, five of which were insertions of 100-200 bp, whereas alignment to NZ_LT906480.1 resulted in a small number of variants. The two references share a percentage identity of 99.8257 %, and pairwise alignment using *Mauve* (Darling, Mau and Perna, 2010) showed that assembly NZ_CP008838.1 had several genomic rearrangements compared with NZ_LT906480.1, likely explaining the mismatches. As such, assembly NZ_LT906480.1 was used as the reference sequence for *S. maltophilia*.

Alignments were filtered (to remove unmapped reads: reads giving not-primary and supplementary alignments), sorted, and indexed using *samtools* version 1.9 (Li *et al.*, 2009), and duplicates were marked using *Picard* `MarkDuplicates` version 2.22.3 (Broad Institute, 2019). Genome coverage was computed from alignments using *bedtools* version 2.29.2 (Quinlan and Hall, 2010). *GATK* `HaplotypeCaller` version 4.1.6.0 (Poplin *et al.*, 2017) was used to identify single-nucleotide polymorphisms (SNPs) and INDEL variants, and *SnpEff* version 4.3.1t (Cingolani *et al.*, 2012) was used to annotate and predict the impact of those variants. Custom databases were built for *SnpEff*, according to their instructions, to incorporate the updated annotations performed by RefSeq in 2020 (Li *et al.*, 2021).

Ancestral genome alignments and evolved three-species community Viscous + TOB isolates were visually inspected using IGV version 2.4.17 (Thorvaldsdóttir, Robinson and Mesirov, 2013) to identify possible larger deletions or structural variations.

The *Pseudomonas* Genome Database (Winsor *et al.*, 2016), UniProt (Bateman *et al.*, 2021), and SMART (Letunic, Khedkar and Bork, 2021) were used to identify structure and function of individual genes and proteins.

4.3.3 Variant analysis

Downstream analyses were performed using R version 3.6.3 (R Core Team, 2019). Data manipulation and graphing were performed using the *tidyverse* suite of packages (Wickham, 2017), along with *patchwork* for figure assembly (Pedersen, 2020), *ggbeeswarm* for point plotting (Clarke and Sherrill-Mix, 2017), *ggrepel* for label positioning (Slowikowski, 2021), and *rcartocolor* and *wesanderson* for colour palettes (Nowosad, 2018; Ram and Wickham, 2018).

Annotated variant files were imported using the *vcfR* package (Knaus and Grünwald, 2017). Variants present in the ancestral genomes of the three species were removed from the evolved isolates to give a set of variants that developed during the selection experiment. Each isolate was checked for reversion of any of the ancestral variants, of which there were none.

A full table of observed variants in each isolate, and summaries of variant loci found across isolates grouped by community composition, media treatment, and timepoint, can be found in Additional File 3.

Phenotypic data—i.e., growth, antibiotic tolerance, adherence, etc.—for each isolate was collected as detailed in the Methods of Chapter 3.

4.3.4 Statistical analyses

The following models were fit to the data: i) To investigate number of mutations per species, the `anova_test` function from the *rstatix* package (Kassambara, 2020) was used to perform a simple one-way ANOVA, with number of mutations as the dependent variable, and species the independent variable. This was fit for each

timepoint, and post-hoc comparisons were performed using the `tukey_hsd` function from the same package.

ii) To investigate number of mutations per isolate between different communities and treatments, the `anova_test` function was used to perform a two-way ANOVA, where square-root transformed number of mutations per isolate—to approximate a normal distribution—was the dependent variable, and community and treatment were independent variables. No interaction term was fit due to unequal sample sizes between groups. Separate models were fit for each species, and only isolates from the final timepoint were used. Post-hoc pairwise Tukey-adjusted comparisons were computed using the `emmeans` and `contrast` functions from the *emmeans* package (Lenth, 2019), to get back-transformed estimates.

iii) To investigate the effects of PmrAB and WalKR/YycHI mutations on growth of *P. aeruginosa* and *S. aureus* respectively, the `anova_test` function was used to perform a one-way ANOVA, where growth as optical density was the dependent variable and presence of mutations in the given system was the independent variable. Separate models were fit for each growth environment, i.e., NB and NB + 0.5 μg/ml TOB, and for each measurement timepoint as repeated measures ANOVA was not feasible with the unequal group sizes of isolates with each mutation. Post-hoc Tukey-adjusted pairwise comparisons between mutation identity were performed using the `tukey_hsd` function.

No statistical models were fit to the remaining mutation comparisons due to the vastly different sample sizes of mutations across each isolate, e.g., valid comparison between three isolates with *fusA* mutations and 70 without would not be robust.

Table 4.1: Glossary for the shorthand used to name the isolates.

e.g., PA5C30 is the only sequenced *P. aeruginosa* isolate at the final timepoint from replicate 3 of the *P. aeruginosa & S. maltophilia* community, treated with Liquid + TOB.

Identifier	Meaning
Species	
PA	*Pseudomonas aeruginosa*
SA	*Staphylococcus aureus*
SM	*Stenotrophomonas maltophilia*
Community	
1	*P. aeruginosa* monoculture
2	*S. aureus* monoculture
3	*S. maltophilia* monoculture
4	*P. aeruginosa* & *S. aureus* coculture
5	*P. aeruginosa* & *S. maltophilia* coculture
6	*S. aureus* & *S. maltophilia* coculture
7	Three-species coculture
Treatment	
A	Liquid media
B	Viscous media
C	Liquid media with 0.5 µg/ml Tobramycin
D	Viscous media with 0.5 µg/ml Tobramycin
Replicate	
1–4	From replicate population 1–4
Clone	
0	The only clone sequenced from a final timepoint replicate
I	The only clone sequenced from an intermediate timepoint replicate
1–6	The specific clone sequenced from a replicate

4.4 Results and Discussion

4.4.1 Summary of mutations observed

We sequenced the genomes of 193 bacterial isolates from our selection experiment investigating the effects of abiotic factors on the eco-evolutionary dynamics of a model CF-like bacterial community, to attempt to understand the genetics underpinning the changes observed in the experiment. Three of these isolates were the ancestral bacteria used to begin the selection experiment, one each of *P. aeruginosa*, *S. aureus* and *S. maltophilia*; any variants observed in each of these isolates (Appendix Figure C.1) were filtered from the variant lists for all other isolates, and no revertant mutations were identified among these ancestral variants. The other 190 isolates were taken from the evolved populations: one isolated clone per surviving replicate bacterial population at the final timepoint; one clone per replicate from each media treatment of the three species communities was taken from intermediate timepoints; and for the coexisting three-species Viscous + TOB treated communities 6 clones per replicate of *P. aeruginosa* were sequenced, as our focal species, along with 3 clones per replicate of *S. aureus* and *S. maltophilia*.

4.4.1.1 Stenotrophomonas maltophilia *had more mutations per isolate than the other species*

Across all of the isolates of the three different species, we found that there was a significant difference between the species in the number of mutations per isolate (ANOVA: $F_{(2,\ 152)} = 11.16$, $p < 0.001$; Table 4.2) with *S. maltophilia* harbouring significantly more mutations than both *P. aeruginosa* and *S. aureus* (Tukey post-hoc, $p < 0.001$); *P. aeruginosa* and *S. aureus* did not differ with respect to each other, though *S. aureus* developed more mutations per isolate than *P. aeruginosa* (Table 4.4). Among the *S. maltophilia* isolates, two had mutations in the DNA mismatch repair protein coding sequence *mutL* (isolates SM5C40 and SM7D11), and these isolates had a total of 17 and 27 variants respectively. The DNA mismatch repair system, comprised of the proteins MutL and MutS, is responsible for correcting errors that occur during DNA replication, and malfunction of the system results in a hypermutator

phenotype characterised by a greater number of mutations (Polosina and Cupples, 2010). These two isolates clearly contribute to the observed difference, particularly the large standard deviation among *S. maltophilia* isolates, but their removal from the dataset still resulted in a significant effect of species on mutations per isolate (ANOVA; $F_{(2, 150)} = 12.33$, $p < 0.001$). When in a community with *P. aeruginosa*, *S. maltophilia* was driven extinct in a majority of populations, and so the sequenced *S. maltophilia* isolates represent those isolates that were able to survive and compete with *P. aeruginosa*, potentially suggesting selection imposed by other species could have driven the relatively higher mutation rates, as has been observed previously by bacteriophage selection (Pal *et al.*, 2007). Alternatively, this strain of *S. maltophilia* may simply possess a greater mutation rate than *P. aeruginosa* strain LESB58.

4.4.1.2 Parallel mutations were largely observed across media treatments

We observed varying degrees of parallel mutations at genetic loci across the different species. In *P. aeruginosa* (Figure 4.1), we found that the PmrAB two-component regulatory system was mutated across nearly every community and treatment combination—see § 4.4.3 below. Other parallel mutations were largely governed by media treatment rather than community composition: in the Liquid treatment we found parallel mutations in the transcriptional regulator MvfR, and several genes likely involved in cyclic di-GMP (c-di-GMP) signalling, the oligoribonuclease *orn* and the putative diguanylate cylase PLES_RS21350, as well as others. Across multiple Viscous treated isolates, the gene encoding the quorum sensing transcriptional regulator LasR was mutated—see § 4.4.5 below. And tobramycin treatment resulted in mutations of *fusA*, encoding elongation factor G, in three isolates—see § 4.4.4 below—and the putative polyamine synthesis gene *speD2* in three other isolates, including a coexisting Viscous + TOB treated isolate (Figure 4.4A)—see § 4.4.2.1 below.

In *S. aureus*, we found there were fewer parallel mutated loci than in *P. aeruginosa* (Figure 4.2). The WalKR two-component system, involved in cell wall synthesis, was mutated across isolates from two- and three-species communities, including coexisting Viscous + TOB treated isolates (Figure 4.4B), suggesting involvement with interspecies interactions—see § 4.4.2.3 below—whereas another

cell wall synthesis-related gene, *fmtA*, was mutated across monoculture isolates. The elongation factor *fusA* was mutated across multiple tobramycin treated isolates. The remaining mutations were in a hypothetical protein, AA076_RS15250, and 16S rRNA, AA076_RS09700.

There were still fewer parallel mutations across *S. maltophilia* isolates (Figure 4.3) than both other species, though there were also fewer sequenced *S. maltophilia* isolates in total. The *S. maltophilia* genome is largely uncharacterised, and many genes are annotated on predicted homology alone. As such, three of the four parallel mutations were uncharacterised: CKW06_RS1185, encoding a putative two-component histidine kinase, was mutated in 8 isolates from liquid conditions, i.e., Liquid and Liquid + TOB treatments; CKW06_RS13105, also encoding a putative histidine kinase, was mutated in 3 isolates, as was CKW06_RS20000, encoding a putative response regulator. As each of these loci are predicted to be part of regulatory systems the impacts of mutations may be widespread, and as such difficult to interpret without greater characterisation. The remaining, non-intergenic parallel loci was the global transcription regulator catabolite repressor protein (CRP), which was mutated in isolates treated with tobramycin, including coexisting Viscous + TOB treated isolates though none that were used as representative isolates in Chapter 3. The CRP regulon is not well characterised in *S. maltophilia*, and the system has been shown to control the regulation of numerous different genes that differ across the numerous different species within which the system is found (Soberón-Chávez *et al.*, 2017).

Across the three species, mutations in regulatory systems were the most common, and such mutations would be expected to have wider ranging and possibly more subtle effects on phenotype than mutations in individual pathways due to the likely large numbers of genes under regulatory control of such systems. We also find that, at least in *P. aeruginosa*, there was a significant effect of both community composition and media treatment on the number of variants observed in each isolate (ANOVA: $p < 0.05$, Table 4.3), and pairwise comparisons showed that the Viscous + TOB treatment accumulated significantly fewer mutations per isolate than the Liquid treatment (Pairwise Tukey post-hoc, $p = 0.0032$, Table 4.5, Additional File 4.2), whilst the three-species community accumulated significantly fewer mutations than the *P. aeruginosa* & *S. aureus* community (Pairwise Tukey post-hoc, $p = 0.043$,

Table 4.5, Additional File 4.2), with a trend across both factors to there being fewer mutations in more complex environments. This suggests that within the Liquid treatment environment there were more dispensable traits, the loss or alteration of which were beneficial, compared with the Viscous + TOB treatment environment. And constraint of mutations within more complex communities is somewhat in line with observations by Scheuerl *et al.*, (2020), who found that adaption of focal species, in their case to metabolise different substrates, became more limited in more diverse communities.

It is difficult here to make such estimations about mutations in *S. aureus* and *S. maltophilia*, as unlike *P. aeruginosa* they were often undetectable at the final timepoint of the selection experiment, due to abundance below the limit of detection. As such, it would be difficult to ascertain whether the number and identity of mutations we observed contributed to the survival of these isolates, or whether they remained abundant in their respective communities by chance.

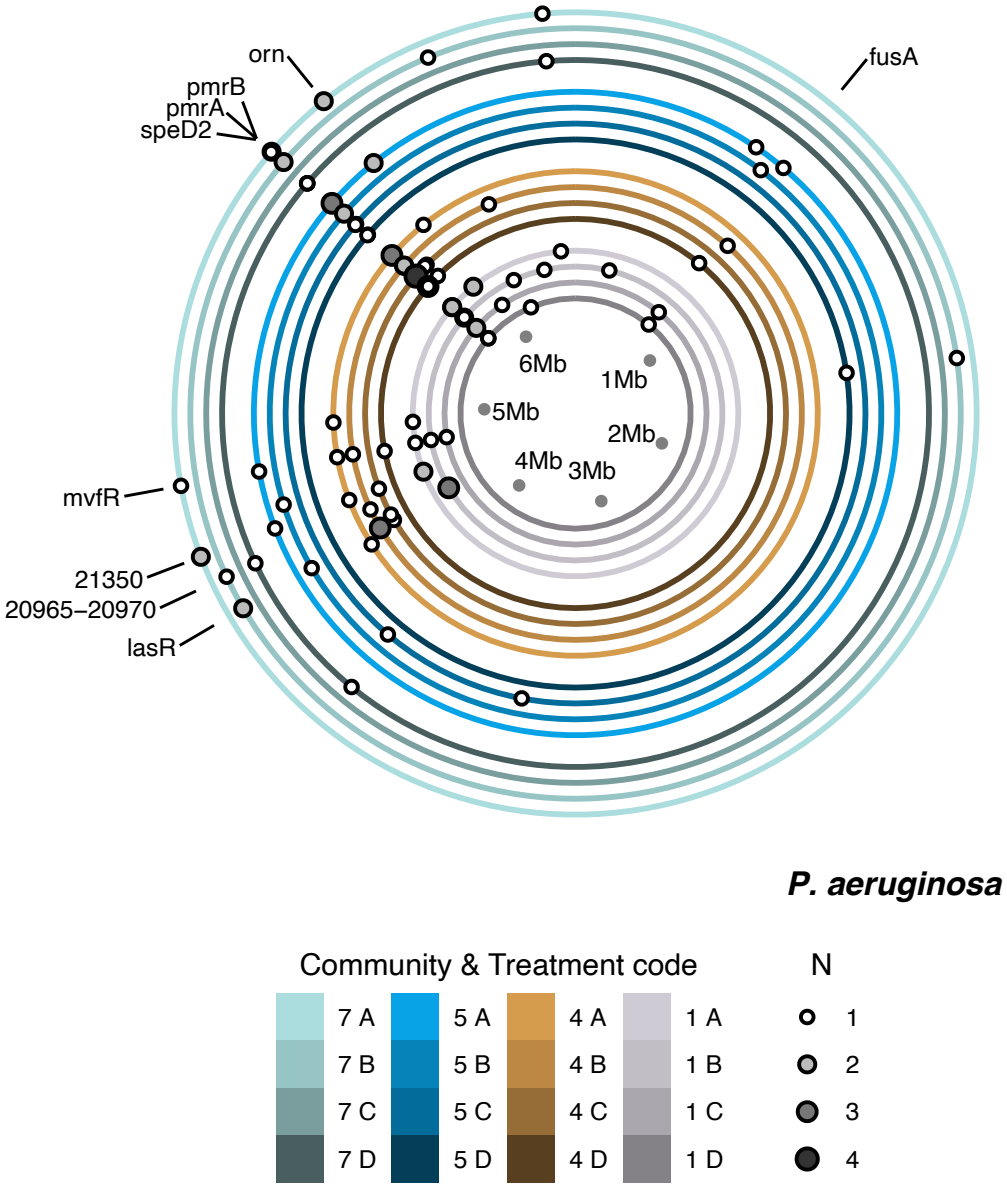


Figure 4.1: Overview of observed SNPs and INDELs of all final timepoint *P. aeruginosa* isolates.

Each ring represents the *P. aeruginosa* chromosome. Rings are grouped by community composition and coloured by the combination of community composition and media treatment. Each ring represents all replicates from a given community (1: *P. aeruginosa* alone, 4: *P. aeruginosa* & *S. aureus,* 5: *P. aeruginosa* & *S. maltophilia*, 7: Three-species) and treatment (A: Liquid, B: Viscous, C: Liquid + TOB, D: Viscous + TOB),

and dots represent observed variants at a locus on the genome. Dots are sized and coloured by the number of replicates that had mutations in the indicated locus. Dots are labelled when a locus is mutated ≥3 times across all individual isolates. Labels show the gene name, when named, or the numbered locus tag with the PLES_RS prefix omitted; intergenic mutations are labelled as the flanking loci separated by a dash. Within each set of rings are distance markers in Mb.

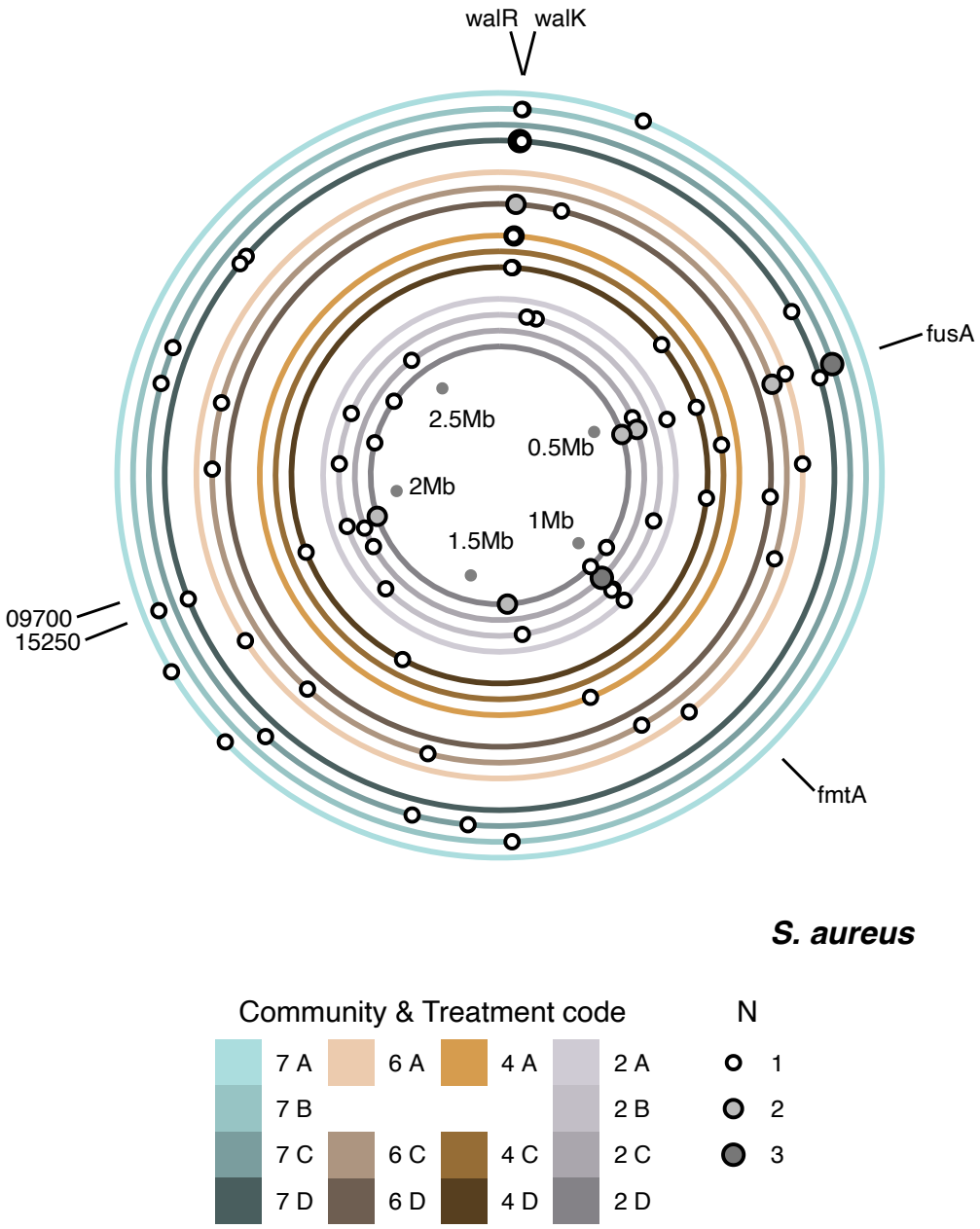


Figure 4.2: Overview of observed SNPs and INDELs of all final timepoint *S. aureus* isolates.

Each ring represents the *S. aureus* chromosome. Rings are grouped by community composition and coloured by the combination of community composition and media treatment. Each ring represents all replicates from a given community (2: *S. aureus* alone, 4: *P. aeruginosa* & *S. aureus,* 6: *S. aureus* & *S. maltophilia*, 7: Three-species) and treatment (A: Liquid, B: Viscous, C: Liquid + TOB, D: Viscous + TOB), and dots

represent observed variants at a locus on the genome. Dots are sized and coloured by the number of replicates that had mutations in the indicated locus. Dots are labelled when a locus is mutated ≥3 times across all individual isolates. Labels show the gene name, when named, or the numbered locus tag with the AA076_RS prefix omitted. Within each set of rings are distance markers in Mb.

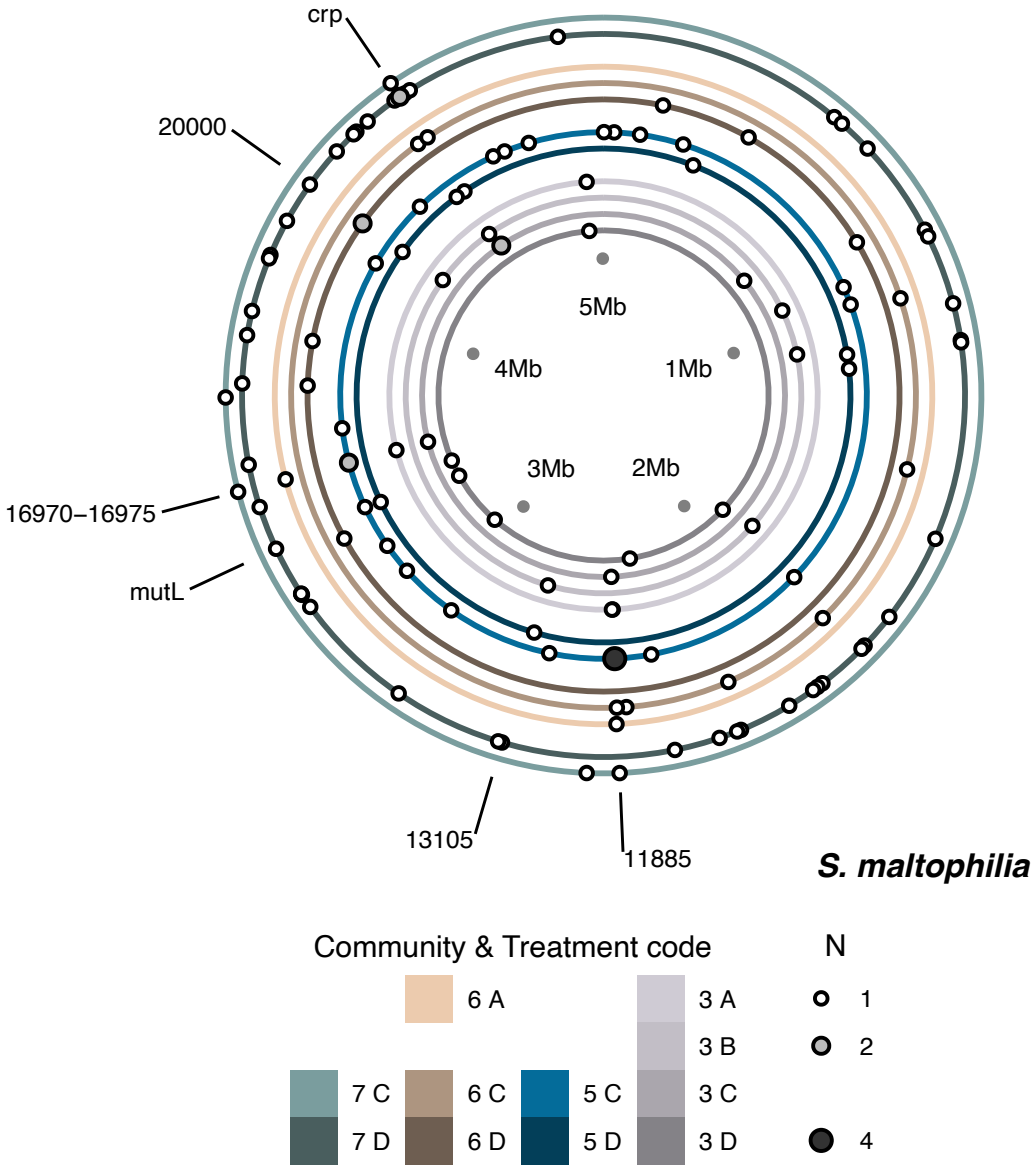


Figure 4.3: Overview of observed SNPs and INDELs of all final timepoint *S. maltophilia* isolates.

Each ring represents the *S. aureus* chromosome. Rings are grouped by community composition and coloured by the combination of community composition and media treatment. Each ring represents all replicates from a given community (1: *S. maltophilia* alone, 5: *P. aeruginosa* & *S. maltophilia*, 6: *S. aureus* & *S. maltophilia*, 7: Three-species) and treatment (A: Liquid, B: Viscous, C: Liquid + TOB, D: Viscous + TOB),

and dots represent observed variants at a locus on the genome. Dots are sized and coloured by the number of replicates that had mutations in the indicated locus. Dots are labelled when a locus is mutated ≥3 times across all individual isolates. Also labelled is the location of *mutL*, which was mutated in isolates SM5C40 and SM7D11. Labels show the gene name, when named, or the numbered locus tag with the CKW06_RS prefix omitted; intergenic mutations are labelled as the flanking loci separated by a dash. Within each set of rings are distance markers in Mb.

Table 4.2: ANOVA tables of number of variants across species, split by timepoints.

Community	ANOVA Term	D.F.	Sum Sq.	F value	P value
Intermediate	Species	2	20.2	2.68	0.084
	Residuals	32	120.7		
Final	Species	2	140.9	11.12	$3x10^{-5}$
	Residuals	155	959.8		

Table 4.3: ANOVA tables of square-root transformed number of observed variants in each isolate across community composition and media treatment, split by species.

Species	ANOVA Term	D.F.	Sum Sq.	F value	P value
P. aeruginosa	Community	3	1.37	3.71	0.016
	Treatment	3	1.97	5.34	0.002
	Residuals	66	8.10		
S. aureus	Community	3	1.32	1.96	0.14
	Treatment	3	0.49	0.73	0.54
	Residuals	39	8.74		
S. maltophilia	Community	3	0.52	0.85	0.48
	Treatment	3	0.12	0.20	0.89
	Residuals	27	5.49		

Species	Timepoint	Isolates (n)	Total variants	Mean variants per isolate	Standard deviation	Standard Error of the Mean	Of which SNPs	Of which INDELs	Isolates with no variants
P. aeruginosa		12	13	1.08	0.9	0.26	10	3	3
S. aureus	Intermediate	12	19	1.58	0.9	0.26	18	1	0
S. maltophilia		11	32	2.91	3.21	0.97	29	3	1
P. aeruginosa		73	138	1.89	0.99	0.12	98	40	3
S. aureus	Final	46	97	2.11	1.34	0.2	78	16	2
S. maltophilia		36	152	4.22	4.81	0.8	101	51	0

Species	Timepoint	Total variants	Of which SNPs	Synonymous	Non-synonymous	Intergenic	Coding Sequence	Resulting in: Stop codon	… Missense variant
P. aeruginosa		13	10	1	9	0	9	1	8
S. aureus	Intermediate	19	18	2	16	2	14	2	12
S. maltophilia		32	29	11	18	4	14	1	12
P. aeruginosa		138	98	0	98	5	93	11	81
S. aureus	Final	94	78	4	74	10	64	6	54
S. maltophilia		152	101	9	92	19	73	1	69

Table 4.4: Summary of observed SNP and INDEL variants, found using GATK HaplotypeCaller, across all sequenced isolates. (Starts previous page)

Species	Timepoint	Total variants	Of which INDELs	Intergenic	Coding Sequence	Resulting in: Frameshift
P. aeruginosa	Intermediate	13	3	0	3	3
S. aureus		19	1	0	1	1
S. maltophilia		32	3	2	1	0
P. aeruginosa	Final	138	40	7	33	25
S. aureus		94	16	0	16	10
S. maltophilia		152	51	26	25	18

Table 4.5: Number of variants and number of isolates within each community and treatment, for each species.

Species	Community	Treatment	Isolates (N)	Total variants (N)	Mean	Std. Dev.	S.E.M.
P. aeruginosa	*P. a.* Mono	A	4	11	2.75	0.50	0.25
		B	4	10	2.50	1.00	0.50
		C	3	6	2.00	1.00	0.58
		D	2	4	2.00	1.41	1.00
	P.a. & *S.a.*	A	3	8	2.67	0.58	0.33
		B	4	11	2.75	0.50	0.25
		C	4	12	3.00	2.00	1.00
		D	4	7	1.75	0.96	0.48
	P.a. & *S.m.*	A	4	10	2.50	0.58	0.29
		B	4	7	1.75	0.96	0.48
		C	4	6	1.50	0.58	0.29
		D	3	3	1.00	1.00	0.58
	P.a. & *S.a.* & *S.m.*	A	4	10	2.50	0.58	0.29
		B	4	7	1.75	0.50	0.25
		C	1	0	0.00		
		D	21	26	1.24	0.54	0.12
S. aureus	*S.a.* Mono	A	1	2	2.00		
		B	3	10	3.33	1.15	0.67
		C	3	10	3.33	1.53	0.88
		D	4	10	2.50	0.58	0.29
	P.a. & *S.a.*	A	3	5	1.67	1.15	0.67
		C	1	1	1.00		
		D	3	8	2.67	3.06	1.76

	S.a. & S.m.	A	1	5	5.00		
		C	3	8	2.67	1.15	0.67
		D	3	4	1.33	0.58	0.33
	P.a. & S.a. & S.m.	A	3	4	1.33	1.53	0.88
		B	2	4	2.00	1.41	1.00
		C	4	9	2.25	1.26	0.63
		D	12	17	1.42	0.67	0.19
S. maltophilia	*S.m.* Mono	A	2	6	3.00	1.41	1.00
		B	2	6	3.00	1.41	1.00
		C	2	5	2.50	0.71	0.50
		D	2	6	3.00	1.41	1.00
	P.a. & S.m.	C	3	12	4.00	3.61	2.08
		D	3	14	4.67	2.31	1.33
	S.a. & S.m.	A	1	2	2.00		
		C	3	8	2.67	0.58	0.33
		D	3	8	2.67	0.58	0.33
	P.a. & S.a. & S.m.	C	2	5	2.50	0.71	0.50
		D	11	36	3.27	1.74	0.52

4.4.2 Mutations in coexisting isolates

In Chapter 3, having observed continued coexistence between the three species when treated with Viscous + TOB, we investigated the ability of evolved isolates from each replicate to coexist outside of that treatment. Though we found that coexistence in Liquid media was possible for these evolved communities, the frequency with which this occurred varied between replicates. To investigate the genetic mechanisms mediating this varied coexistence, we sequenced the replicates from the three-species Viscous + TOB treated communities to a greater depth than the

replicates from other communities and treatments, to determine what might be the differences between isolates used during the follow-up experiments in Chapter 3, and whether these were representative of the species populations within each replicate.

We found that the follow-up isolates of both *P. aeruginosa* and *S. aureus* were largely representative of all of the isolates sequenced from their respective replicates, in that the same mutated loci were observed across clones from a given replicate, but that *S. maltophilia* isolates were more heterogeneous both within and between replicates (Figure 4.4).

4.4.2.1 Pseudomonas aeruginosa *isolates possessed mutations in genes involved with polyamine synthesis*

Evolved replicates 1, 2, and 4 had mutations in or between two genes encoding proteins predicted to be involved with polyamine synthesis, annotated as *speD2* (PLES_RS26565) and *speE2* (PLES_RS26570). The mutation in isolate PA7D25 resulted in a stop codon at position 44 of *speE2*, the mutation in isolate PA7D42 resulted in a frameshift from a 22 bp deletion in *speD2*, whilst isolate PA7D11 had a single bp deletion in the intergenic region between the two genes, which has been suggested to contain a promoter region for *speE2* (Johnson *et al.*, 2012). Genes *speD2* and *speE2* are annotated as such as the encoded proteins contain domains present in the S-adenosylmethionine decarboxylase SpeD (encoded by PLES_RS03240) and the spermidine synthase SpeE (encoded by PLES_RS18830); SpeD2 shares a sequence identity of 28.2 % with SpeD, and SpeE2 shares a sequence identity of 38.3 % with SpeE2. The homologous genes in *P. aeruginosa* strain PAO1 are PA4773 and PA4774 respectively, and are part of the PmrAB operon (Johnson *et al.*, 2012).

Polyamines are polycationic molecules that are ubiquitous across nature, which in bacteria are involved in stabilising both intracellular RNA structures and extracellular outer membrane structures (Shah and Swiatlo, 2008). *Pseudomonas aeruginosa* can undergo polyamine biosynthesis utilising SpeD and SpeE via the well characterised arginine and ornithine metabolic pathways (Lu *et al.*, 2002; Nakada and Itoh, 2003; Shah and Swiatlo, 2008). In addition, *P. aeruginosa* possesses the inducible polyamine synthesis genes, PA4773, PA4774, and the uncharacterised PA4775, which has no known homologues (Johnson *et al.*, 2012; Bolard *et al.*, 2019). These

PmrAB operon polyamine genes have been shown to result in production of the polyamines spermidine and norspermidine: PA4774 KO mutants have been observed with reduced amounts of surface-localised spermidine (Johnson *et al.*, 2012), and individual knock outs each of the three genes have been shown to reduce surface-localised norspermidine (Bolard *et al.*, 2019). Production of surface-localised polyamines can stabilise the outer membrane by functioning in an equivalent manner to Mg^{2+} cations, and can act in place of such cations when conditions are limited, for example in the presence of cation chelating extracellular DNA (Johnson *et al.*, 2012). This membrane stabilisation can decrease membrane permeability and increase tolerance of polymyxin and aminoglycoside antibiotics, including tobramycin (Bolard *et al.*, 2019; Puja *et al.*, 2020).

In light of this, why, what are likely to be, disabling mutations in these genes were selected for in the three-species Viscous + TOB treatment is unclear. We also found similar frameshift mutations in *speD2* in isolates PA5C40 and PA5D20, and in isolate PA7A20 we found the same stop codon gain in *speE2*. All of these isolates had low tolerance of tobramycin after 24 hours, which may be explained by increased membrane permeability due to reduced polyamine production. Common among these isolates is coculture with *S. maltophilia*, suggesting that these mutations may be involved in interactions between the two species. However, further work into characterising these isolates would be required.

4.4.2.2 Pseudomonas aeruginosa *from evolved replicate community 3 had no identifiable SNPs*

The evolved replicate community that showed the greatest degree of coexistence was replicate 3, wherein it appeared that changes in *P. aeruginosa* growth where a likely contributor to this increased coexistence Chapter 3. However, the only nucleotide variant we found in *P. aeruginosa* isolate PA7D32 was in PLES_RS18780 that is predicted to encode an ATPase involved in type III secretion, a system not usually involved with interbacterial competition. Further investigation revealed a genomic region, flanked by 16S rRNA sequences, that had double the sequencing read coverage of the rest of the genome (Appendix Figure C.3). This double coverage was unique to PA7D32 and encompassed bases $5.13x10^6$ to $5.60x10^6$, comprising

~420 coding sequences. Re-aligning the reads to the 16S rRNA sequences and inspecting the alignments revealed no evidence of tandem duplication, nor could we find other structural variants. As such, we were not able to find any conclusive genomic evidence that could explain the observed decreases in growth of this isolate, nor that could contribute to the increased coexistence, though there may have been movement of prophage elements or insertion sequences within the genome that affected gene expression.

4.4.2.3 Staphylococcus aureus *isolates possessed mutations in the essential WalKR two-component system*

All four *S. aureus* isolates from the coexisting Viscous + TOB treated communities either had SNPs in *walK* or deletion of a genome segment spanning the end of *yycH* through the first half of *yycI* (Appendix Figure C.2). The WalKR two-component system, and its associated accessory proteins YycHI, form a highly conserved, essential regulatory system in Gram-positive bacteria that is involved with cell wall metabolism (Dubrac *et al.*, 2007; Villanueva *et al.*, 2018; Gajdiss *et al.*, 2020). Among the genes upregulated by WalR are those that encode autolysins that facilitate the breakdown of peptidoglycan to allow cell growth and division (Dubrac *et al.*, 2007). The WalKR system is the only essential two-component system in *S. aureus*, deletion of which results in loss of viability (Villanueva *et al.*, 2018). However, mutations that result in reduced activity of WalKR lead to reduced peptidoglycan turnover (Dubrac *et al.*, 2007; Villanueva *et al.*, 2018) and a thickening of the cell wall that imparts resistance to vancomycin and other antibiotics that target the cell wall (Howden *et al.*, 2011).

We found that, across all *S. aureus* isolates, those with mutations in this system had significantly greater tolerance of tobramycin than isolates without such mutations at both the 24- and 48-hour measurement timepoints (ANOVA: $p < 0.001$, Table 4.6), suggesting that these isolates may have gained tolerance as a result of decreased permeability via increased cell wall thickness. Pairwise comparisons found that isolates with mutations in either *walK* or *walR* were both able to grow to significantly greater densities at both timepoints than isolates without mutations in this system, (Pairwise Tukey post-hoc: $p < 0.05$, Additional File 4.3), and the same was true at

48 hours of isolates with *yycHI* deletions (Pairwise Tukey post-hoc: $p < 0.05$, Additional File 4.3). However, these statistics should be interpreted with caution, as because the tolerance measurement was obtained via optical density there was the possibility that increased cell wall thickness may increase the degree to which light was occluded by the cells. Though there was not a significant effect on growth without antibiotic at 24 hours, there was a significant effect at 48 hours (ANOVA: $p = 0.005$, Table 4.6), and as such we cannot exclude this possibility. Additionally, these tests were not able to capture the contribution of other genes to this phenotype, and so it is possible that these mutations interact with others in a manner that we have not explored; nor were these tests able to account for the biased and unbalanced population structure due to differences in sequencing depth and number of surviving isolates.

The accessory proteins YycHI form a complex with the histidine kinase WalK that localises at the cell membrane, and though neither YycH or YycI are essential, deletion of either results in reduced activity of WalK (Cameron *et al.*, 2016; Poupel *et al.*, 2016; Gajdiss *et al.*, 2020). In the case of *S. aureus* isolate SA7D12, there was strong evidence for the truncation of YycH and deletion of YycI from the insert sizes of the aligned paired-end reads (Appendix Figure C.2), which would likely result in impaired WalK function and the observed increased tobramycin tolerance. It is also possible that this deletion may have contributed to the increased tolerance of *P. aeruginosa* supernatant that we observed in this isolate Chapter 3 (Figure 3.10). However, we found a similar increased tolerance in isolate SA7D34 that did not share the *yycHI* deletion; and although isolate SA7D34 had a SNP in *walK*, the same SNP—resulting in amino acid change Lys262Glu—was shared with isolate SA7D43 which did not show increased tolerance of *P. aeruginosa* supernatant, suggesting the possibility of other undetected structural variants.

Mutations in the WalKR-YycHI system were the most common among all *S. aureus* isolates, though were not found in the single-species community isolates. They were selected for in communities containing *P. aeruginosa* regardless of media treatment, and *walR* mutants were also found in Viscous + TOB treated isolates from the *S. aureus* & *S. maltophilia* community, suggesting that selection was driven by interaction with other species, particularly *P. aeruginosa*. We previously found that

P. aeruginosa supernatant-mediated inhibition of *S. aureus* increased among evolved coexisting isolates, suggesting that WalKR-YycHI mutations may arise in response to inhibition, though, as mentioned above, the mutations we found did not map exactly to observed changes in tolerance of *P. aeruginosa* supernatant.

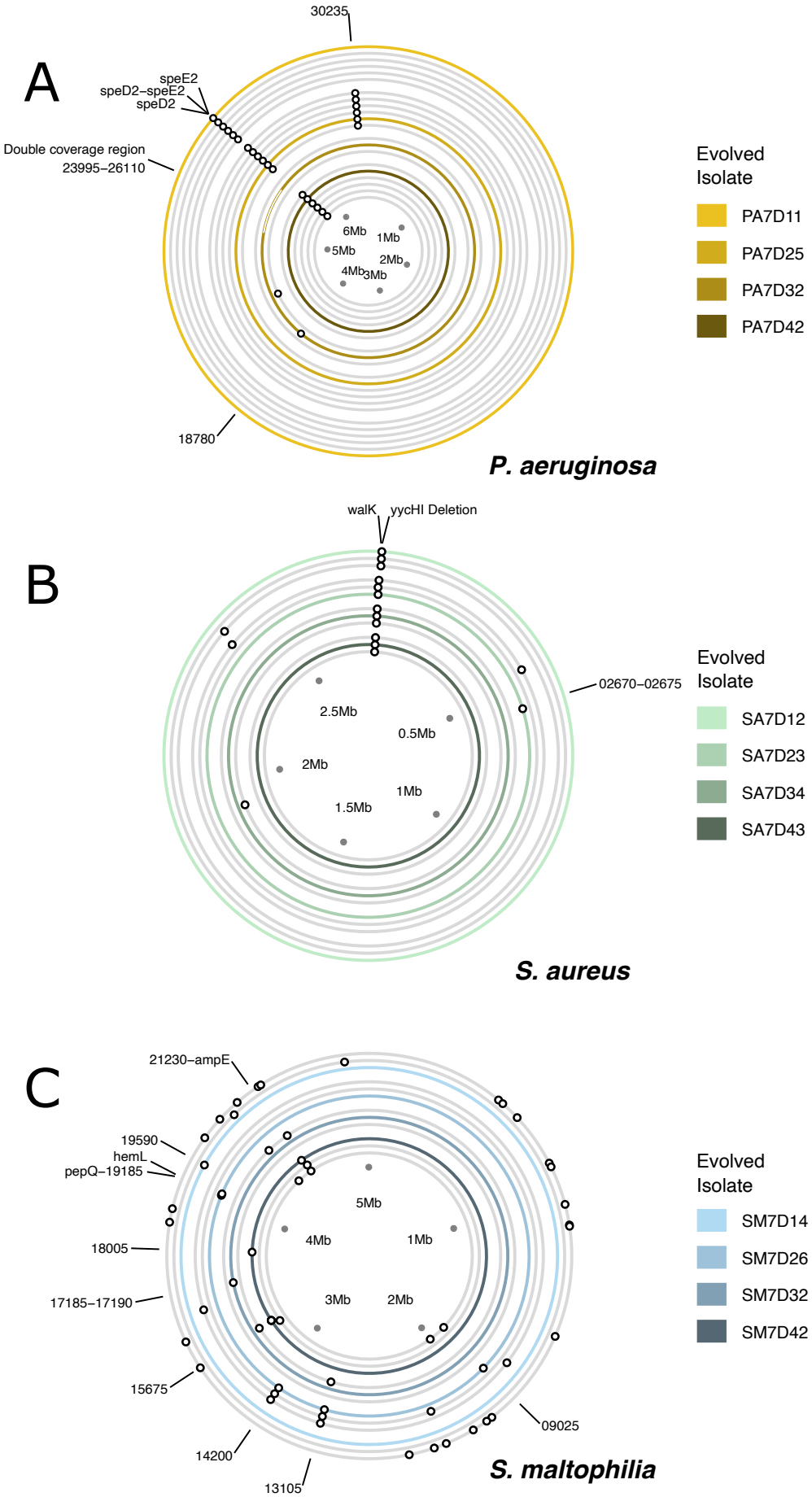

A
30235
speE2
speD2–speE2
speD2
Double coverage region
23995–26110
6Mb
1Mb
5Mb
2Mb
4Mb
3Mb
18780
Evolved Isolate
PA7D11
PA7D25
PA7D32
PA7D42
P. aeruginosa
B
walK
yycHI Deletion
02670–02675
2.5Mb
0.5Mb
2Mb
1Mb
1.5Mb
Evolved Isolate
SA7D12
SA7D23
SA7D34
SA7D43
S. aureus
C
21230–ampE
19590
hemL
pepQ–19185
18005
17185–17190
15675
14200
13105
09025
5Mb
4Mb
1Mb
3Mb
2Mb
Evolved Isolate
SM7D14
SM7D26
SM7D32
SM7D42
S. maltophilia

Figure 4.4: Mutations observed in final timepoint isolates from the three-species "Viscous + TOB" (7D) communities.

Each ring represents the chromosome of an individual isolate of the respective species. Highlighted rings represent the isolates that were used during the evolved community investigations in Chapter 3. Rings are grouped by replicate community, with 1 the outermost and 4 the innermost; individual rings within each group represent the numbered clones from each replicate, again sorted numerically with the lowest numbered clone the outermost and the highest numbered the innermost. Dots represent observed variants at a given genomic locus in each clone, and variants present in the highlighted clones are labelled. Labels show the gene name, when named, or the numbered locus tag with the prefix omitted—*P. aeruginosa* prefix is PLES_RS, *S. aureus* prefix is AA076_RS, and *S. maltophilia* prefix is CKW06_RS; intergenic loci are labelled as the flanking loci separated by a dash. Within each set of rings are distance markers in Mb.

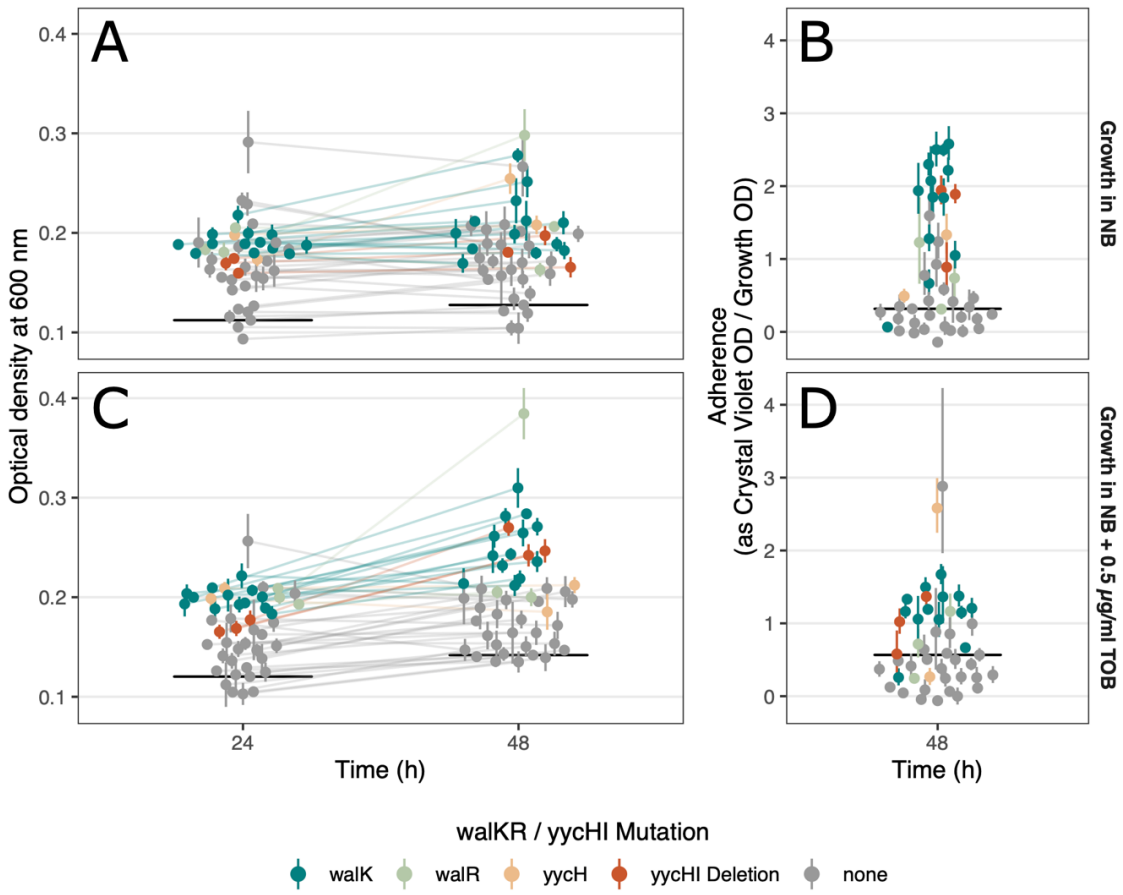


Figure 4.5: Growth and adherence of *S. aureus* isolates with mutations in the WalKR two-component system or *yycHI* accessory genes.

(A–B): Growth, as OD_{600}, of *S. aureus* isolates at 24- and 48 hours in (A) NB or (B) NB + 0.5 µg/ml tobramycin. **(C–D):** Adherence, as Crystal Violet assay OD_{600} / Growth OD_{600}, after 48 hours in (A) NB or (B) NB + 0.5 µg/ml tobramycin. Colours show the presence/absence of mutations in *walKR/yycHI*. Horizontal black lines represent the value of the ancestor. Error bars represent ± S.E.M. of three technical replicates.

Table 4.6: ANOVA table of growth (as OD_{600}) of *S. aureus* isolates by presence of WalKR or YycHI mutation, split by media condition and time.

Condition	Time	ANOVA Term	D.F.	Sum Sq.	F value	P value
NB	24	WalKR/YycHI mutation	4	0.008	1.57	0.2
		Residuals	42	0.051		
	48	WalKR/YycHI mutation	4	0.024	4.38	0.005
		Residuals	42	0.057		
NB + 0.5 µg/ml TOB	24	WalKR/YycHI mutation	4	0.026	8.27	$5.15x10^{-5}$
		Residuals	42	0.033		
	48	WalKR/YycHI mutation	4	0.079	16.8	$2.60x10^{-8}$
		Residuals	42	0.05		

4.4.3 Mutations in *pmrAB* were the most common among *P. aeruginosa* isolates, and may aid growth in the media composition

The most prevalent mutated loci in *P. aeruginosa* were the two component regulatory system genes *pmrAB*: 42 of the 73 *P. aeruginosa* isolates had a mutation in either *pmrA* or *pmrB* (26 and 16 respectively), though no isolates had both. The PmrAB regulatory system is one of many two-component regulatory systems in *P. aeruginosa* (Stover *et al.*, 2000). The sensor kinase PmrB is induced by low Mg^{2+} concentrations (McPhee, Lewenza and Hancock, 2003), and activates the transcriptional regulator PmrA, which is known to increase the transcription of at least 36 genes, including its own operon discussed above (McPhee *et al.*, 2006). Best characterised among these genes is the LPS modification operon *arnBCADTEF-ugd* that increases tolerance of cationic antimicrobial peptides such as colistin (Moskowitz, Ernst and Miller, 2004), though the number of other genes regulated by PmrA suggest that mutations in this system could have wide ranging effects.

We found mutations in the PmrAB system in isolates from every community and treatment combination, except for in the three-species community when treated with Liquid + TOB or Viscous + TOB, suggesting that selection for these mutations was driven by adaptation to the NB media and/or the laboratory growth system itself. Indeed, we found that there was a significant effect of presence of mutations in PmrAB

on growth in NB media at both 24- and 48 hours (ANOVA: $p < 0.001$, Table 4.7), as isolates with either *pmrA* or *pmrB* mutations both grew to a significantly higher optical density in NB media than those without after both 24 and 48 hours (Pairwise Tukey post-hoc: $p < 0.05$, Additional File 4.4). This suggests that these mutations may be associated with an increased growth rate and/or maximum density. There was also a significant effect of mutations in PmrAB on growth in NB + 0.5 μg/ml TOB after 24 hours (ANOVA: $p < 0.001$, Table 4.7), but the unusual distribution of isolates at this time (Figure 4.6C), split between high and low growth, and the inconsistent distribution of mutations between those two groups suggest that other genetic mechanisms were influencing growth in tobramycin. Mutations in *pmrAB* were gained early in the selection experiment, as intermediate three-species community isolates from the liquid and viscous treatments, sampled at the first count after 6 days, had mutations that were maintained in their respective replicates until the final timepoint (Additional File 4.4), which further suggests that these mutations were beneficial to the isolates.

Across all isolates we found 22 unique *pmrA* mutations, and 15 unique *pmrB* mutations. To investigate whether mutations in a particular domain of either protein had an effect on growth we mapped each amino acid change within the proteins to growth of the respective isolate (Figure 4.7, Figure 4.8). This showed that isolates with mutations predicted to be highly disruptive to the proteins—i.e., truncations through gain of a stop codon, or INDELs that resulted in frameshifts—grew to a similar optical density to isolates with changes to a single amino acid, suggesting that deactivation of this system may have been beneficial to growth in the media. However, as PmrA regulates the transcription of many different genes, and we measured relatively few phenotypes, it is also likely that we have yet to identify the true phenotypic effects of these mutations.

That there was such a variety of mutations in these genes was possibly to be expected, as Mehta *et al.*, (2019) have demonstrated that every domain within PmrB can be a potential target for mutation: they collated observed mutations in *pmrB* from multiple studies of experimentally-evolved and clinically-derived isolates with increased colistin tolerance and showed that mutations along the length of the gene were capable of effecting sufficient change in PmrB function to increase activity of the

system. Although these mutations resulted in increases in colistin tolerance, implying a gain-of-function for PmrB as opposed to the likely loss-of-function we have observed, both gain- and loss-of-function mutations in *pmrB* have been observed among clinical isolates (Moskowitz *et al.*, 2012; Jochumsen *et al.*, 2016; Bricio-Moreno *et al.*, 2018; Mehta *et al.*, 2019), suggesting that different changes within this system are beneficial in different environments.

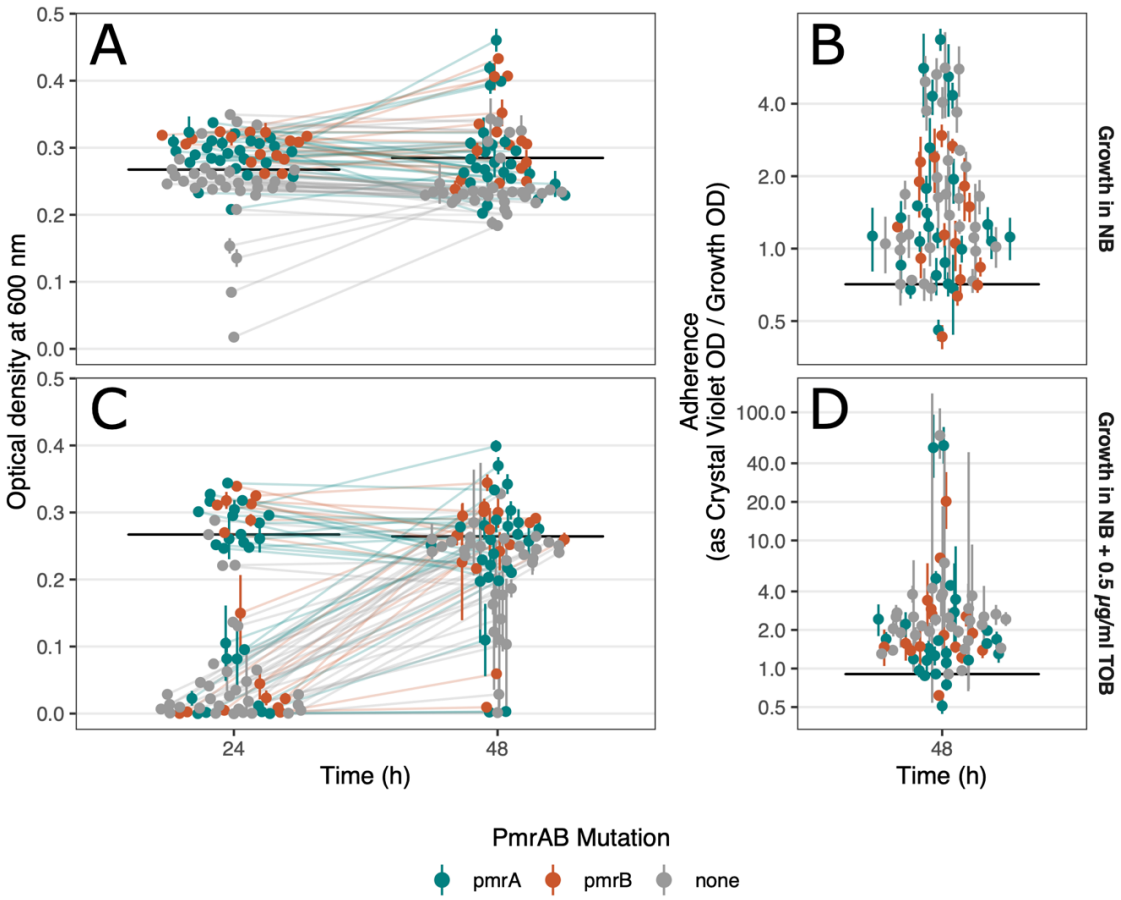


Figure 4.6: Growth and adherence of *P. aeruginosa* isolates with mutations in the PmrAB system.

(A–B): Growth, as OD_{600}, of *P. aeruginosa* isolates at 24- and 48 hours in (A) NB or (B) NB + 0.5 µg/ml tobramycin. **(C–D):** Adherence, as Crystal Violet assay OD_{600} / Growth OD_{600}, after 48 hours in (A) NB or (B) NB + 0.5 µg/ml tobramycin, plotted on a Log10 scale. Colours show the presence/absence of mutations in *pmrAB*. Horizontal black lines represent the value of the ancestor. Error bars represent ± S.E.M. of three technical replicates.

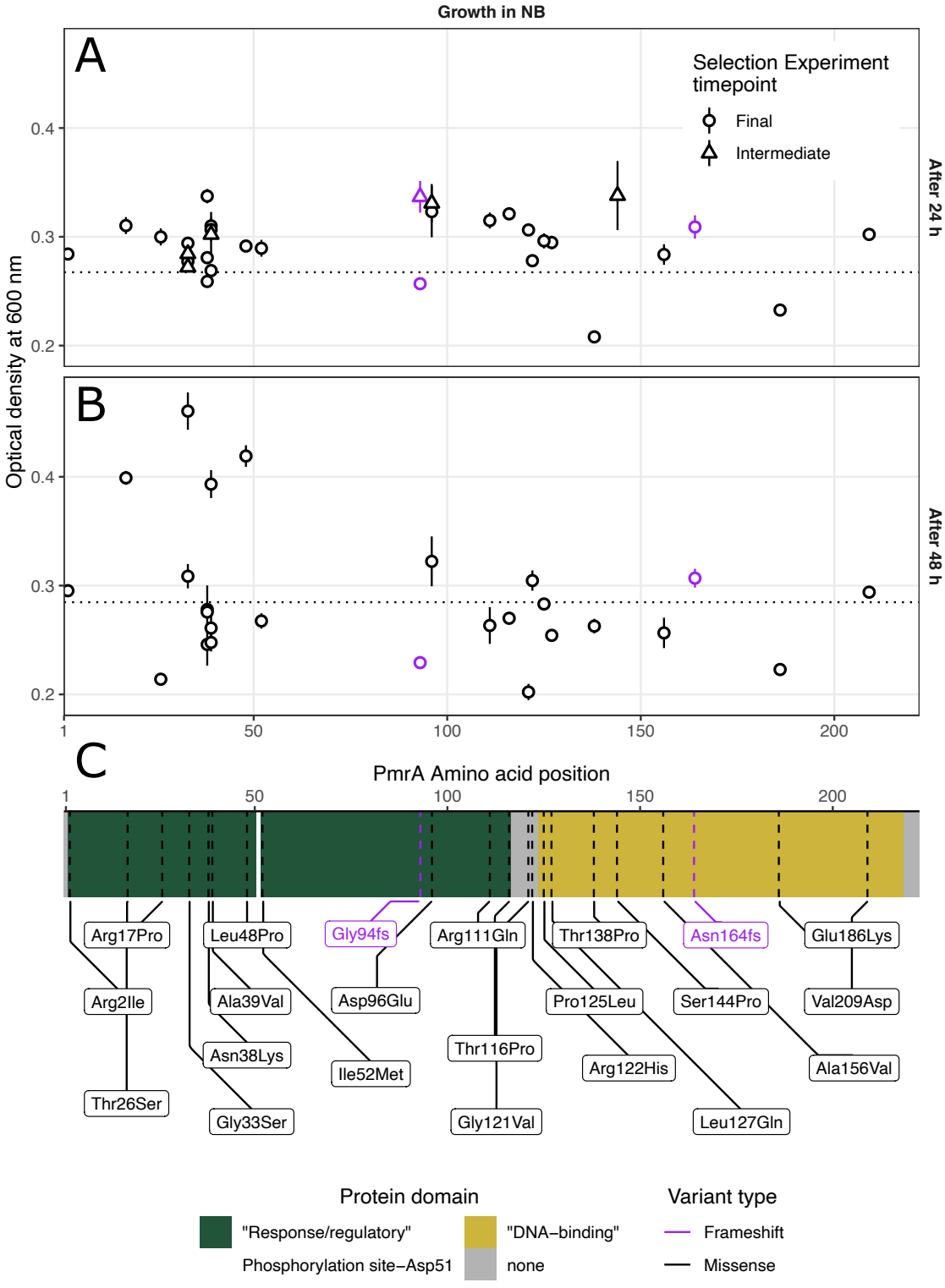


Figure 4.7: Growth of *P. aeruginosa* in NB media ordered by mutations in PmrA.

(A–B): Growth, as OD_{600}, of *P. aeruginosa* isolates in NB media after (A) 24- and (B) 48 hours. The X-axis shows the amino acid position of the isolate's PmrA mutation.

Shapes represent timepoint at which a clone was isolated: circles = Final; triangles = Intermediate. Colour represents the type of variant the mutation results in: purple = frameshift; black = Missense. The dashed line represents the growth of the ancestor. Error bars represent ± S.E.M. of three technical replicates. **(C):** Graphical representation of the domains of PmrA and the location of the variant mutations. Predicted protein domain annotations were retrieved from UniProt (Bateman *et al.*, 2021). Dashed lines show the location of a variant, which are labelled below. Colour of lines represents the type of variant.

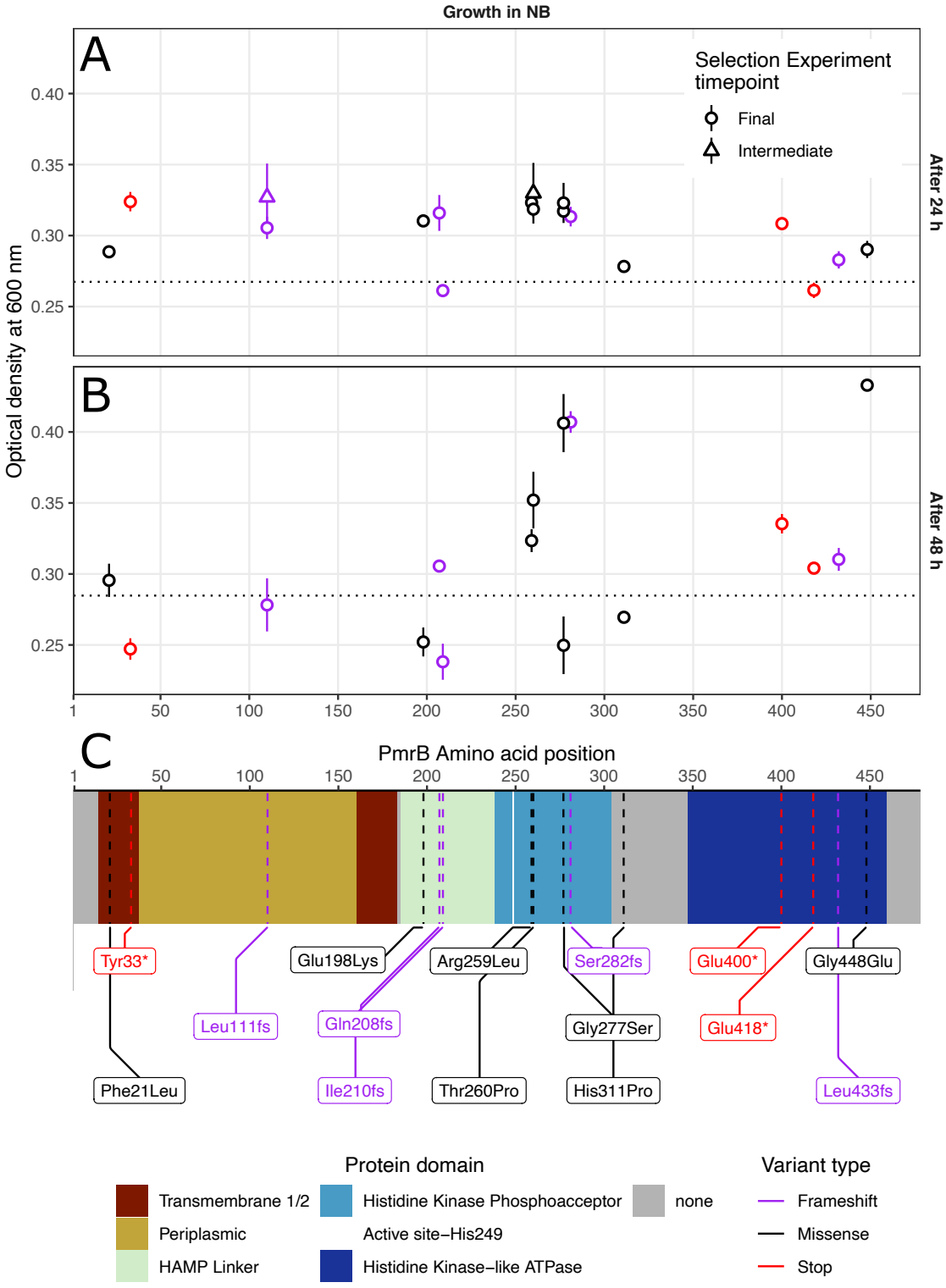


Figure 4.8: Growth of *P. aeruginosa* in NB media ordered by mutations in PmrB.

(A–B): Growth, as OD_{600}, of *P. aeruginosa* isolates in NB media after (A) 24- and (B) 48 hours. The X-axis shows the amino acid position of the isolate's PmrB mutation.

Shapes represent timepoint at which a clone was isolated: circles = Final; triangles = Intermediate. Colour represents the type of variant the mutation results in: red = gain of stop codon; purple = frameshift; black = Missense. The dashed line represents the growth of the ancestor. Error bars represent ± S.E.M. of three technical replicates. **(C):** Graphical representation of the domains of PmrB and the location of the variant mutations. Predicted protein domain annotations were retrieved from SMART (Letunic, Khedkar and Bork, 2021). Dashed lines show the location of a variant, which are labelled below. Colour of lines represents the type of variant.

Table 4.7: ANOVA table of growth (as OD_{600}) of *P. aeruginosa* isolates by presence of PmrAB mutation, split by media condition and time.

Condition	Time	ANOVA Term	D.F.	Sum Sq.	F value	P value
NB	24	PmrAB mutation	2	0.052	11.30	5.48×10^{-5}
		Residuals	71	0.16		
	48	PmrAB mutation	2	0.061	10.39	0.00011
		Residuals	71	0.21		
NB + 0.5 µg/ml TOB	24	PmrAB mutation	2	0.27	10.25	0.00012
		Residuals	71	0.94		
	48	PmrAB mutation	2	0.019	1.35	0.27
		Residuals	71	0.50		

4.4.4 Mutations in elongation factor G, FusA, found in all three species under tobramycin treatments

A striking observation among the sequenced isolates was that we found mutations in the conserved gene *fusA*, encoding elongation factor G involved in protein synthesis, in all three species when treated with tobramycin (Figure 4.1, Figure 4.2, Additional File 3.7). Elongation factor G binds to the ribosome complex and facilitates movement of tRNA and mRNA during translation (Wintermeyer *et al.*, 2011). Mutations in *fusA* are often found in *S. aureus* due to its role in resistance to fusidic acid, an anti-Staphylococcal antibiotic that inhibits the action of elongation factor G (Norström, Lannergård and Hughes, 2007; Chen *et al.*, 2010), though *fusA*

mutations have also been observed in *S. aureus* treated with the aminoglycoside neomycin (Kim, Lieberman and Kishony, 2014). Recently, mutations of *fusA* in *P. aeruginosa* have been identified as a mechanism for tolerance of tobramycin (Bolard, Plésiat and Jeannot, 2018; López-Causapé, Rubio, *et al.*, 2018; Scribner *et al.*, 2020).

We found that the three *P. aeruginosa* isolates with *fusA* mutations (PA1C30, PA1D10, and PA4D30) had increased tolerance of tobramycin, with among the highest growth after 24 h (Figure 4.9A). These isolates all possessed the same mutation, which resulted in an amino acid change of Tyr552Cys, which had previously been found in *P. aeruginosa* evolved under tobramycin selection by López-Causapé *et al.*, (2018). The selection regimen that they used increased the tobramycin concentration over time, whereas we have used a constant concentration of 0.5 µg/ml, and that this low concentration of tobramycin can select for *fusA* mutations is also consistent with work by Scribner *et al.*, (2020), who used the same concentration and found similar mutations in *fusA*. Interestingly, *S. aureus* isolates with mutations in *fusA* did not display an increased tolerance of tobramycin (Figure 4.9B). However, though we did not measure it, it is possible that there was an increase in tobramycin minimum inhibitory concentration for these isolates, and it is likely that these isolates would also be resistant to fusidic acid.

This finding of parallel mutations in *fusA* across species is in line with the work by Scribner *et al.*, (2020), who found that these mutations arise in a somewhat predictable manner in not only *P. aeruginosa* and *S. aureus*, but also *Escherichia coli*, *Acinetobacter baumanii*, and *Salmonella enterica*. Though only one of our isolates had a *fusA* mutation, it is possible that *S. maltophilia* may be added to this list. In the context of CF, tobramycin is used as an anti-Pseudomonal antibiotic (Langton Hewer and Smyth, 2017), but together these data suggest that there can be collateral effects on untargeted bacteria, in this case *S. aureus*, that may make those bacteria more difficult to treat.

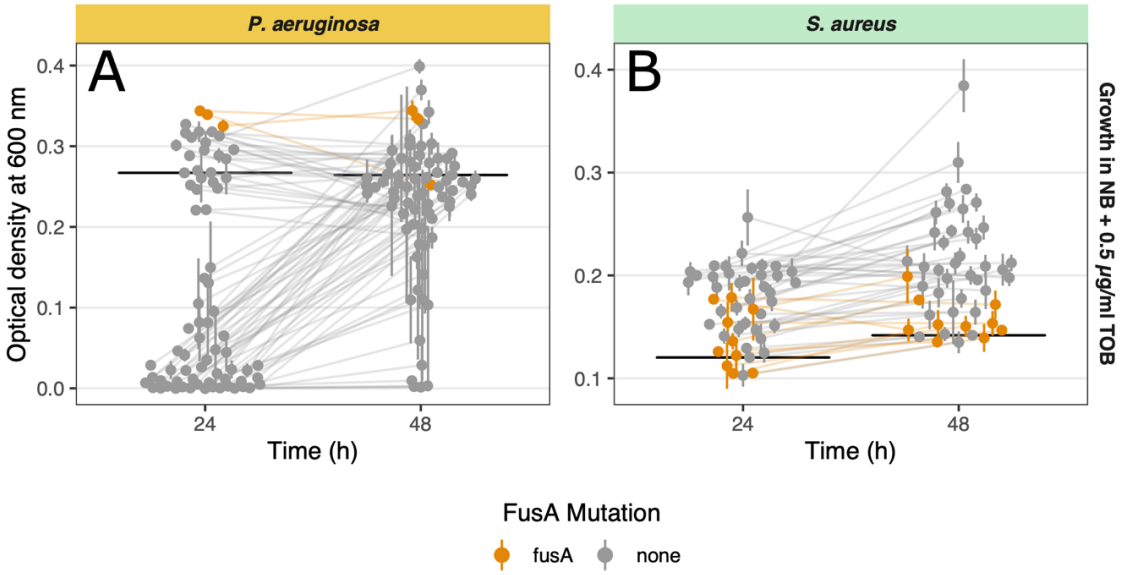


Figure 4.9: Tobramycin tolerance of *P. aeruginosa* and *S. aureus* isolates with *fusA* mutations.

(A–B): Growth, as OD_{600}, of (A) *P. aeruginosa* isolates or (B) *S. aureus* isolates at 24- and 48 hours NB + 0.5 μg/ml tobramycin. Colours show the presence/absence of mutations in *fusA*. Horizontal black lines represent the value of the ancestor. Error bars represent ± S.E.M. of three technical replicates.

4.4.5 Quorum sensing-related mutations observed in *P. aeruginosa* isolates from both Liquid and Viscous treated communities

4.4.5.1 Mutations in LasR were found among Viscous treated isolates

We found mutations in the quorum sensing regulator LasR in *P. aeruginosa* isolates from at least one replicate of each community under the viscous media treatment (Figure 4.1), and 6 of the total 10 were INDELs resulting in frameshifts that likely deactivated the protein. In *P. aeruginosa*, the LasRI two-component system is the master regulator of one of three interlinked quorum sensing systems, and as such controls the regulation of a large number of genes (Schuster *et al.*, 2003; Balasubramanian *et al.*, 2013). As quorum sensing regulates many cooperative behaviours within *P. aeruginosa*, deactivating mutations in LasR, as we have found here, result in strains with reduced, or that lack, production of virulence factors and public goods such as siderophores (Schuster *et al.*, 2003; D'Argenio *et al.*, 2007), and

produce less biofilm (Sakuragi and Kolter, 2007), though still benefit from the production by other members of the population without the associated cost of that production. Indeed, we see that the production of pyocyanin, one such virulence trait under the control of LasR, is greatly reduced in *lasR* mutants (Figure 4.10A), and though adherence is broadly similar to the ancestor (Figure 4.10B), *P. aeruginosa* strain LESB58 primarily produces non-adherent biofilm aggregates (Gagné-Thivierge, Barbeau, *et al.*, 2018) and as such our adherence assay may not detect true differences in total biofilm formation.

Viscous or structured environments have been shown to select for cooperative traits rather than against, as when diffusion is limited by viscosity the benefit of secreted cooperative goods is limited to those that produce those goods and their immediate neighbours rather than the whole population (Kümmerli *et al.*, 2009; Granato *et al.*, 2018), and so it would be expected that the Liquid treatment should select for LasR mutations rather than the Viscous. One possible explanation here could be that within the Viscous treatment aggregate biofilm formation was mediated by the viscous environment itself rather than production of exopolysaccharides to form the biofilm matrix, termed depletion aggregation (Secor *et al.*, 2018). As a result, *P. aeruginosa* was able to gain the benefit of biofilm growth without the cost of matrix production; indeed, Staudinger *et al.*, (2014) have shown that QS negative *P. aeruginosa* were able to form aggregates in a viscous environment. Additionally, the lack of LasR mutations in the Viscous + TOB treatment may be explained by the increase in interspecies competition which resulted in *P. aeruginosa* maintaining production of pyocyanin and other virulence and competition factors under the control of LasR.

4.4.5.2 Mutations in MvfR were found primarily among Liquid treated isolates

We found mutations in the transcriptional regulator MvfR in 7 isolates from across the different communities, four of which in Liquid media treatments (Figure 4.1). Itself under the control of LasR, MvfR (also known as PqsR) is part of the QS regulatory system that directly regulates production of the *Pseudomonas* quinolone signal (PQS) and other alkyl-quinolones (Déziel *et al.*, 2005; Wade *et al.*, 2005). The PQS signal controls expression of virulence factors such as pyocyanin and

rhamnolipids (Xiao *et al.*, 2006), and as such the *P. aeruginosa* isolates that we have found with mutations in MvfR had greatly reduced pyocyanin production (Figure 4.10A). Interestingly, these mutants also had among the greatest growth in NB of all isolates after 48 hours (Figure 4.10B), suggesting that this increase drove selection for these mutants in the Liquid media. That they were not selected to the same degree in other media conditions suggests that the loss of other PQS associated traits, or expression of other genes under the control of MvfR, was more costly than the growth benefit. Three of the seven isolates were from the *P. aeruginosa* monoculture, and two from the *P. aeruginosa* & *S. aureus*, within both of which *P. aeruginosa* experienced a lesser degree of competition, suggesting that maintenance of the PQS controlled virulence factors may have been important in competition with the other bacterial species.

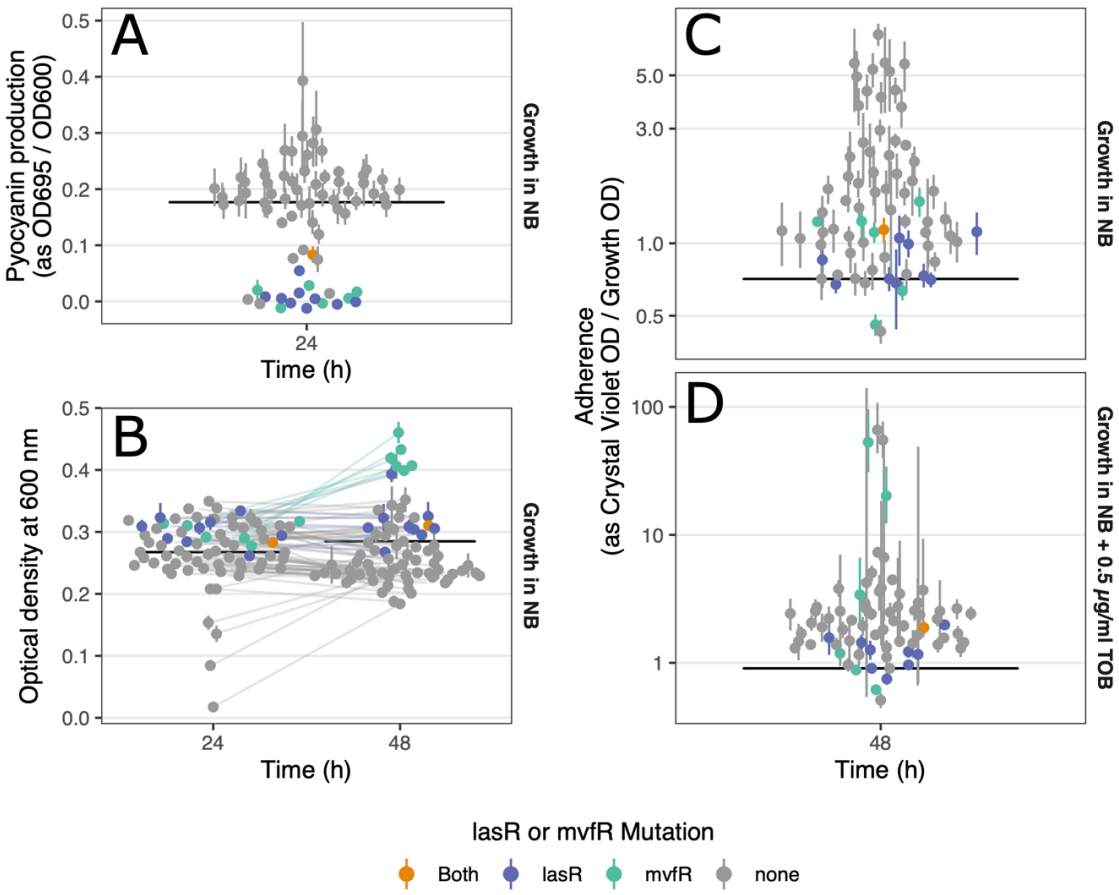


Figure 4.10: Pyocyanin production, growth, and adherence of *P. aeruginosa* isolates with mutations in quorum sensing regulation.

(A): Pyocyanin production, as OD_{695} / OD_{600}, after 24 hours growth in NB. **(B):** Growth, as OD_{600}, of *P. aeruginosa* isolates at 24 and 48 hours in NB. **(C–D):** Adherence, as Crystal Violet assay OD_{600} / Growth OD_{600}, after 48 hours in (A) NB or (B) NB + 0.5 µg/ml tobramycin, plotted on a Log10 scale. Colours show the presence/absence of mutations in *lasR* or *mvfR*. Horizontal black lines represent the value of the ancestor. Error bars represent ± S.E.M. of three technical replicates.

4.4.6 Liquid media treatment selected c-di-GMP signalling mutants in *P. aeruginosa*

We found several mutations in genes involved in synthesis and breakdown of the intracellular secondary messenger molecule cyclic di-GMP (c-di-GMP). In *P. aeruginosa*, intracellular levels of c-di-GMP control the switch between planktonic and biofilm modes of growth (Ha and O'Toole, 2015). Concentrations of c-di-GMP are increased by the action of diguanylate cyclases, of which *P. aeruginosa* possesses around 40 (Wei *et al.*, 2019), and decreased by phosphodiesterases. We found both of these classes of enzyme mutated in our dataset: PLES_RS21350 and PLES_RS30235 encode diguanylate cyclases (Wei *et al.*, 2019), whilst *orn* encodes an oligoribonuclease that has been shown to break down c-di-GMP (Orr *et al.*, 2015). Both PLES_RS21350 and *orn* were mutated in in at least one replicate of each community under the Liquid treatment, though never the same isolate, whereas PLES_RS30235 was mutated in PA7A10 and replicate 2 of the Viscous + TOB three-species community (Figure 4.4A).

We found that isolates with mutations in a diguanylate cyclase produced more adherent biofilm than isolates without (Figure 4.11), suggesting that the mutations in these genes resulted in a gain-of-function. Consistent with this, the amino acid changes of these mutations were in or around PAS sensor domains rather than the GGDEF domains that facilitate c-di-GMP production, which may have resulted in constitutive activation of these enzymes. Of course, further characterisation of these proteins would be required to investigate whether this was the true mechanism. That such mutations were selected for in the Liquid treatment suggests a benefit to growth as a biofilm within our study system.

Deactivation of *orn* has been shown to result in increased biofilm formation (Orr *et al.*, 2015), and two isolates (PA1A40 and PA5A20) showed evidence for this when grown without antibiotic (Figure 4.11A), whereas the other isolates with *orn* mutations showed a modest increase in adherent biofilm formation compared to the ancestor that was similar to the majority of isolates. This suggests that either the majority of mutations were neutral in terms of protein function, or that our biofilm measure has not captured the true variation in exopolysaccharide production.

Alternatively, other mutations were interacting with *orn* to produce different degrees of biofilm formation.

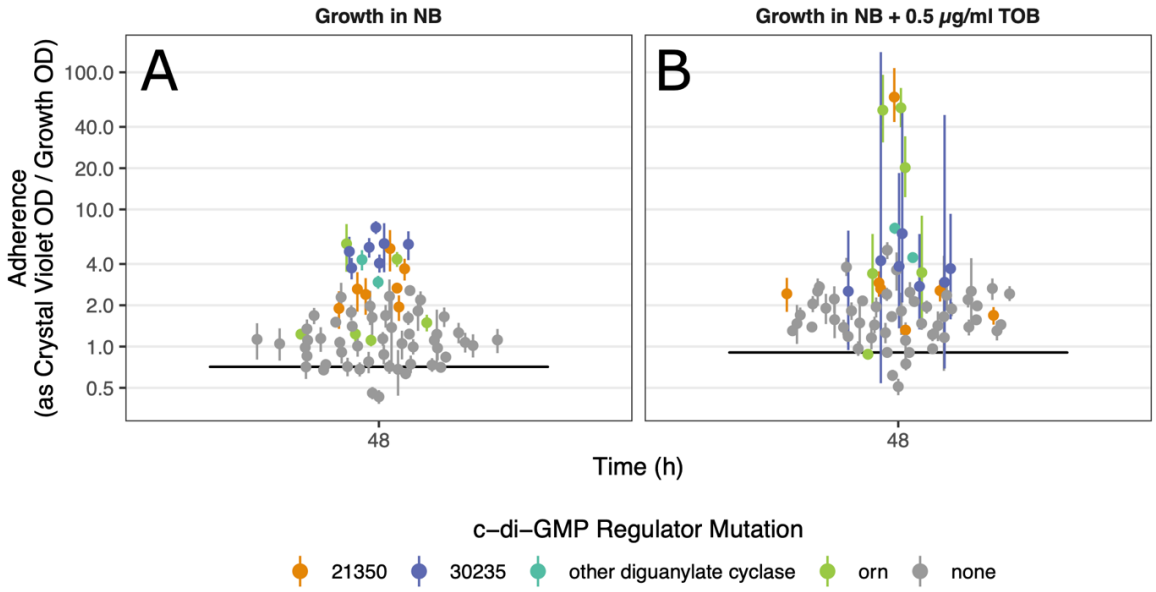


Figure 4.11: Adherence of *P. aeruginosa* isolates with mutations in c-di-GMP regulation.

Panels show adherence, as Crystal Violet assay OD_{600} / Growth OD_{600}, after 48 hours in (A) NB or (B) NB + 0.5 µg/ml tobramycin, plotted on a Log10 scale. Colours show the presence/absence of mutations in c-di-GMP regulation. Horizontal black lines represent the value of the ancestor. Error bars represent ± S.E.M. of three technical replicates.

4.5 Conclusions

Overall, we found that there was a relatively low degree of parallel mutation across isolates of each species, consistent with the relatively weak selective pressure of the antibiotic treatment (Wistrand-Yuen *et al.*, 2018). Of the few parallel mutations we identified, these often appeared as a result of media treatments rather than community composition, suggesting that these posed a greater selective pressure than the other bacterial species. However, there were generally fewer variants among *P. aeruginosa* from the three-species community, suggesting that the presence of the other species may have constrained adaptation somewhat.

We observed phenotypic differences in isolates that were difficult to attribute to the genomic variants that we identified, for example in *P. aeruginosa* isolate PA7D32. One possibility is that these differences were driven by structural variations that were difficult to detect with a single sequencing run using a short-read sequencing platform. As such, it would be of interest to complement the short-read data with long-read sequencing—e.g., PacBio or Nanopore—that would be able to identify any duplications or rearrangements and provide a higher quality genome assembly. The other possibility is that we did not measure enough different phenotypic traits to identify the effects of the mutations that were observed. Phenotypes that could provide further insights include nutrient utilisation, antibiotic minimum inhibitory concentration, or production of a wider range of secreted factors such as siderophores. With a greater range of phenotypes, it would be possible to perform a more in-depth analysis capable of grouping isolates on shared traits and perhaps infer evolutionary trajectories as in Bartell *et al.*, (2019). And coupled with a higher quality genome assembly, genome wide association of traits could also be possible.

We identified candidate mutations in polyamine synthesis genes in *P. aeruginosa* that may mediate coexistence between the three species, and future work could seek to identify the effects that these mutations have in pairwise cultures between *P. aeruginosa* and the other two species.

Chapter 5. General discussion

Management and treatment of Cystic Fibrosis (CF) chronic pulmonary infections is essential for maintaining the health and wellbeing of patients. Though often considered from the perspective of prominent infecting pathogens, chronic infections are highly polymicrobial. Whilst the effects of antibiotics on pathogen species grown individually have been investigated, much less is known about the effects of antibiotic treatments on polymicrobial communities.

In this thesis I have demonstrated that selection with low concentrations of antibiotics can have large effects on community composition. In Chapter 2, I show that despite a lack of effect on growth within monocultures, low concentrations of antibiotics can result in extinction of normally dominant species. In Chapter 3, I show that this effect of antibiotics is further magnified by increased community size. I also show that environmental factors that increase coexistence can result in evolution of coexistence between normally antagonistic species in the absence of these factors. And in Chapter 4 I demonstrate that parallel mutations primarily arose across media treatments rather than communities, but that growth in more complex communities constrained the observed number of mutations.

Selection with low concentrations of antibiotic is well known to result in a greater number of mutations, which in turn increases the likelihood that these mutations may interact to result in high level antibiotic resistance (Andersson and Hughes, 2012; Wistrand-Yuen *et al.*, 2018). We extend this finding to show that selection with combinations of antibiotics administered at low doses can result in greater tolerance than observed under single antibiotic treatments, as shown in Chapter 2. We also show that these low concentrations of antibiotics can result in extinctions of species relatively less tolerant to the antibiotic, despite dominance in the absence of that antibiotic (Chapter 2), and that this effect is greater in larger communities (Chapter 3). Antibiotic treatments in patients often fail to clear the infecting bacteria (Waters *et al.*, 2019). The combination of these effects of low antibiotic doses may contribute to this treatment failure, as within subpopulations that experience less than optimal treatment high level antibiotic resistance would be selected for, and the composition of bacterial species present within the subpopulation

could result in protection of the one or more species from extinction. That efficacy of treatment can be dependent on the composition of the community makes the argument that increased focus should be put on characterising the communities within the lungs of patients so that treatment can be tailored to the individual communities.

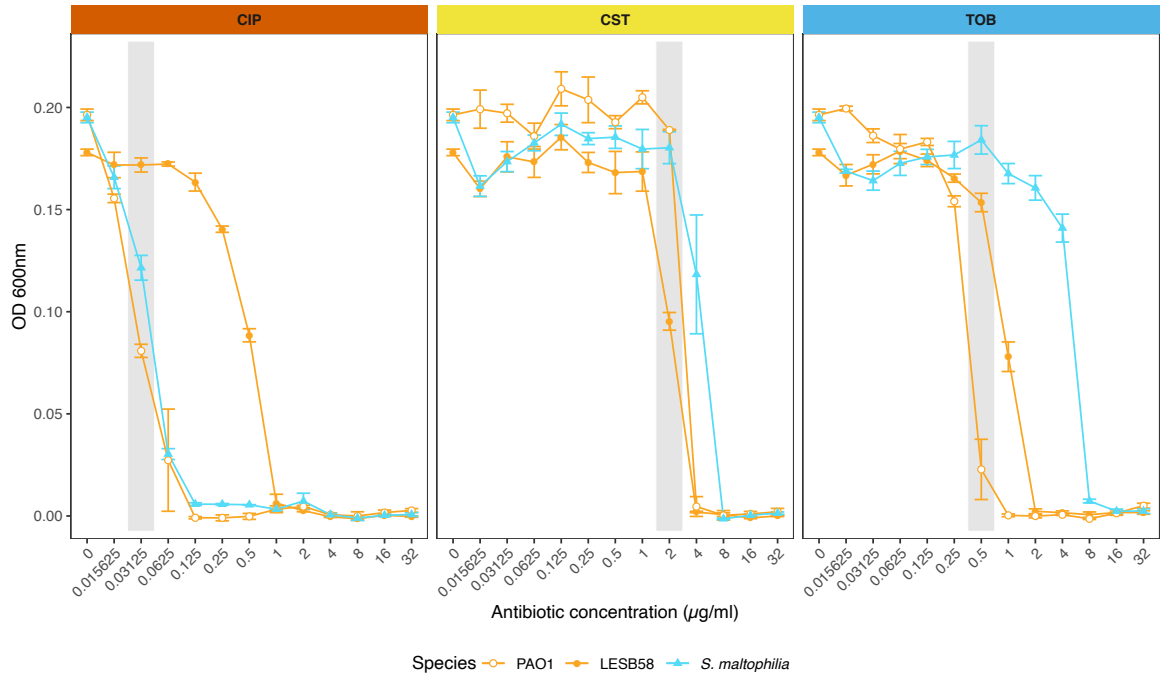
Future work should focus on characterising the nature of interactions between species within bacterial communities. Construction of *in vitro* model communities could facilitate this research, and likely candidates for community members would be oral bacteria and anaerobic taxa that form a core community between patients (Einarsson *et al.*, 2019). With establishment of stable communities, experiments could be performed that investigate the effects of invasion of species such as *P. aeruginosa* (Grainger *et al.*, 2019) and how antibiotic treatments affect community compositions and vulnerability to invasion.

Increasing the accuracy of the *in vitro* environment to match the CF lung would provide greater insights. This could be achieved through the usage of synthetic CF mucus media, which has been developed to mimic the nutrient and macromolecular composition of CF mucus (Palmer *et al.*, 2005; Palmer, Aye and Whiteley, 2007), and may better support establishment of communities. The mimicry of the lungs could be further increased through the usage of the *ex vivo* porcine lung model (Harrison *et al.*, 2014; Harrison and Diggle, 2016), which uses sections of pig lungs to encapsulate the physical structure that is lacking in lab microcosms.

5.1 Conclusions

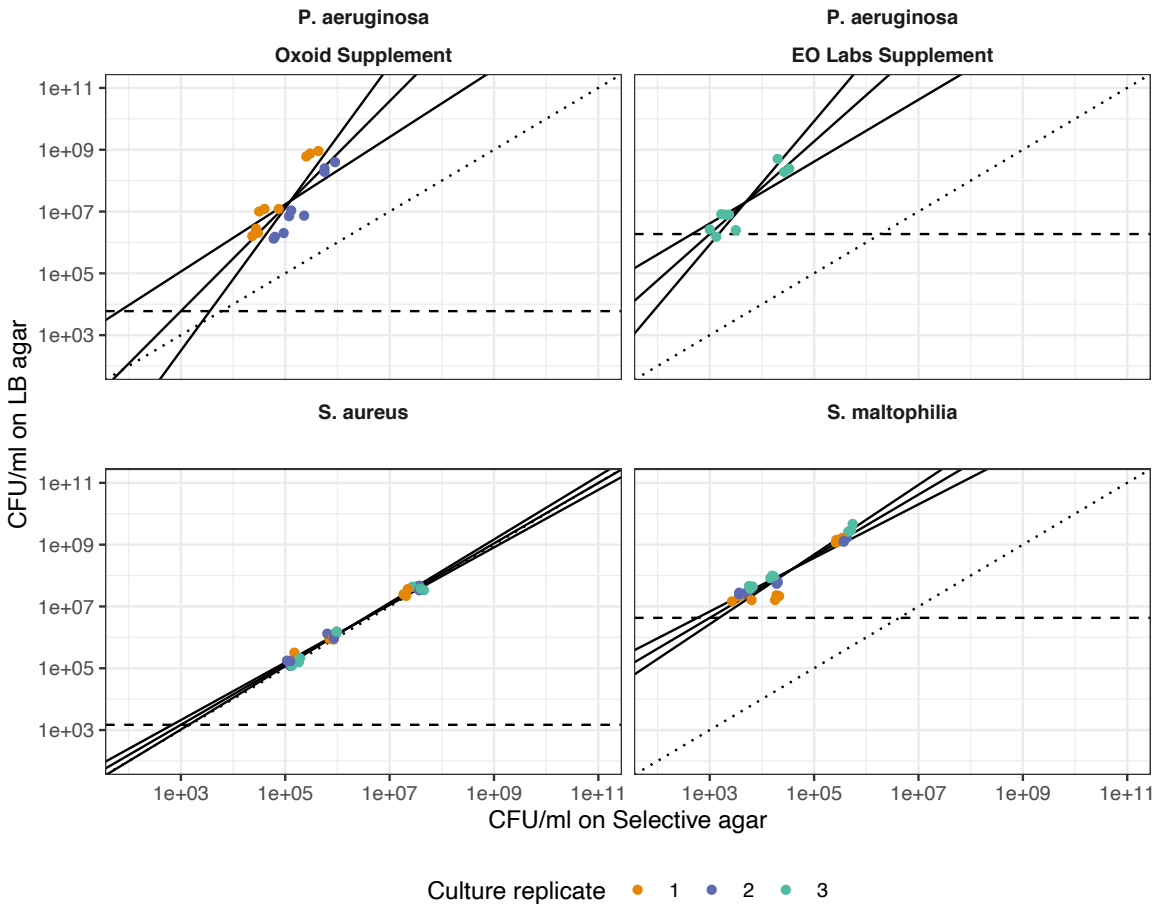
The dynamics within CF pulmonary infections are a black box, as though we can observe the outcomes of the interactions between different species and antibiotic selection, due to its inaccessibility we cannot directly observe what effects these processes have on shaping the community and the course of disease. I believe that what this thesis has shown above all is how little is still known about how the interactions within communities are affected by diverse selective pressures. Greater research into investigating these interactions within communities could aid in identifying methods that are able to better manage CF bacterial infections and increase patient quality of life.

Appendix A. Chapter 2

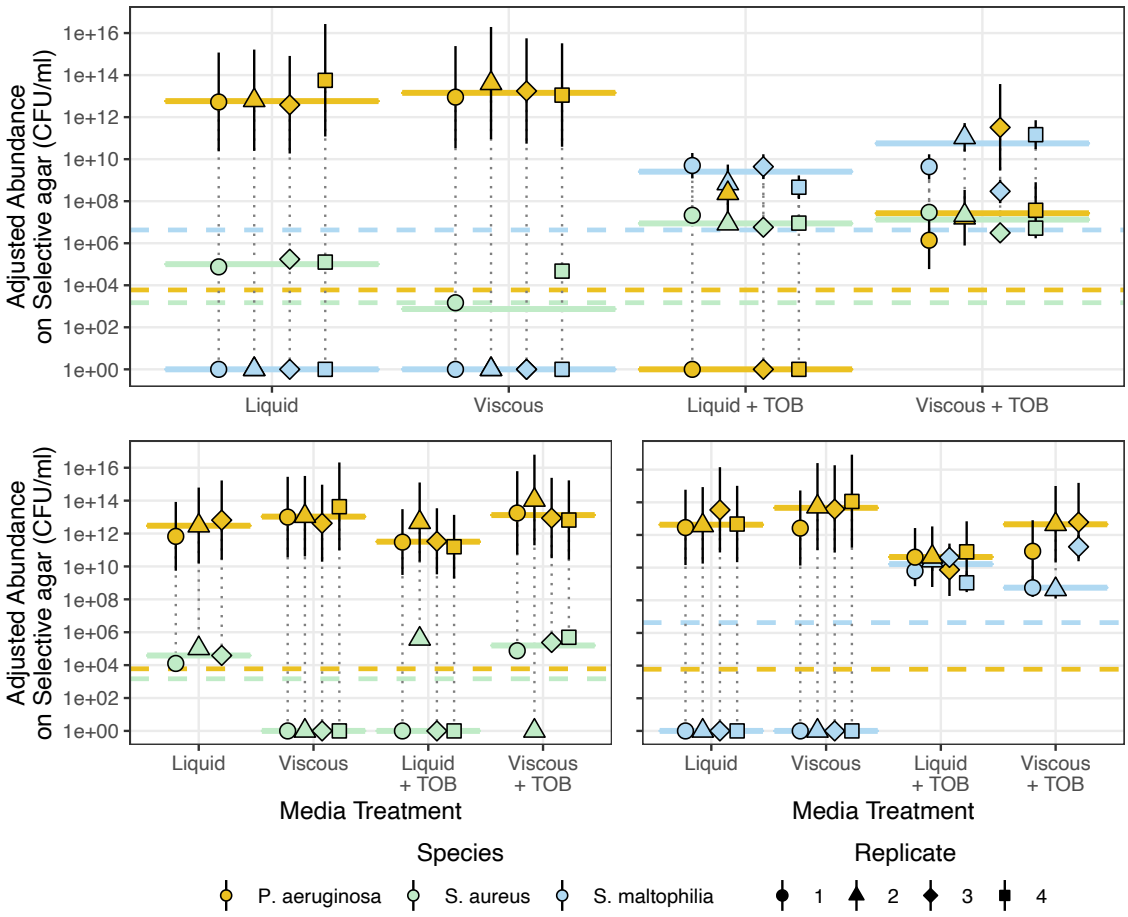


Appendix Figure A.1: MIC curves of ciprofloxacin (CIP), colistin (CST), and tobramycin (TOB) for *P. aeruginosa* strains PAO1 and LESB58, and *S. maltophilia*. Highlighted concentration shows the concentration used in the selection experiment. Points show means of triplicate assays, error bars ± S.E.M.

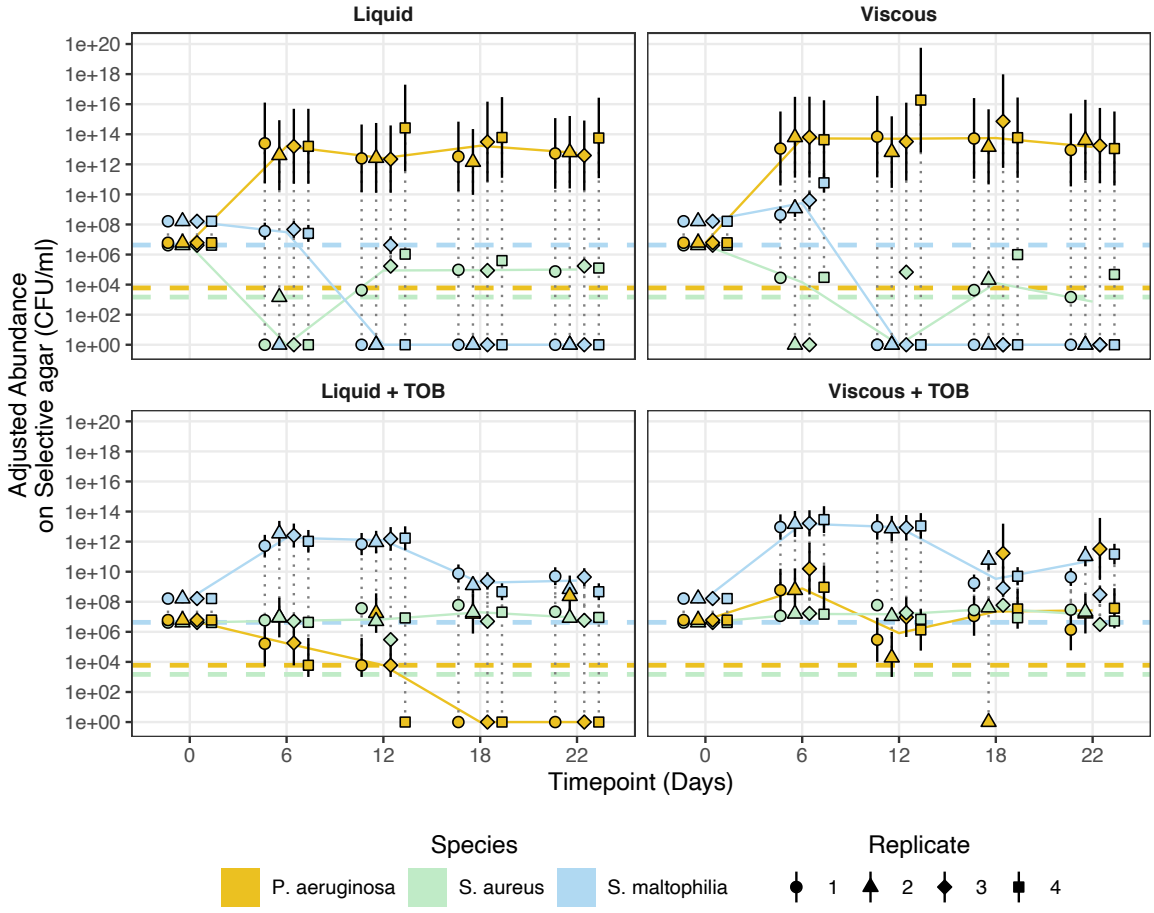
Appendix B. Chapter 3



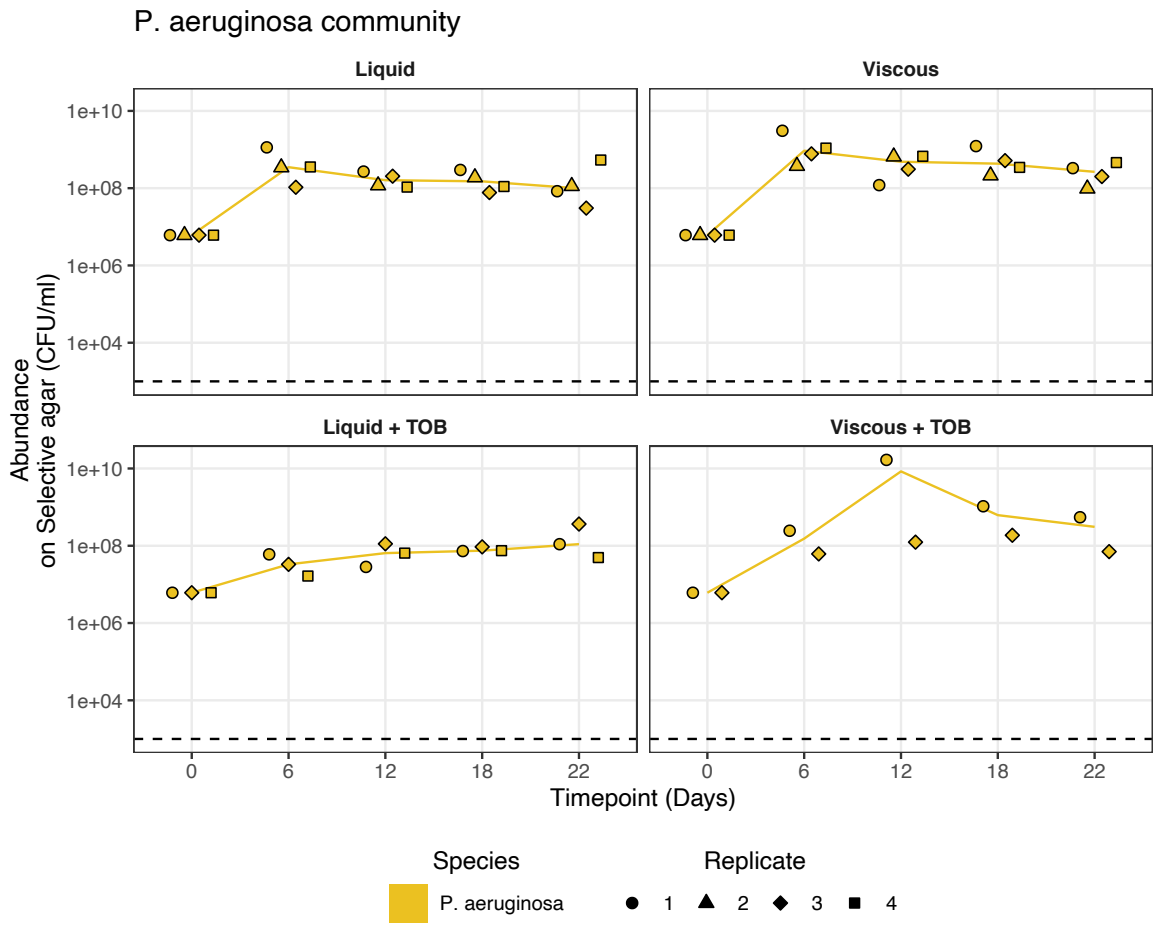
Appendix Figure B.1: Abundance on selective agar vs LB agar for each species. Panels show the abundance of each species on their respective selective agar plates against their abundance on LB agar. The supplier of the supplement for *P. aeruginosa* agar changed over the course of the experiments, and this is reflected in the panels. Dots represent the abundance of cultures, averaged over three technical replicates. Colours represent the overnight culture replicate of the bacterial cultures. Black lines show the regression lines ± 95 % CI fit from the model detailed in Methods § 3.3.3. Horizontal dashed line shows the limit of detection on LB agar of 1000 CFU/ml. Diagonal dotted line shows the gradient of 1.



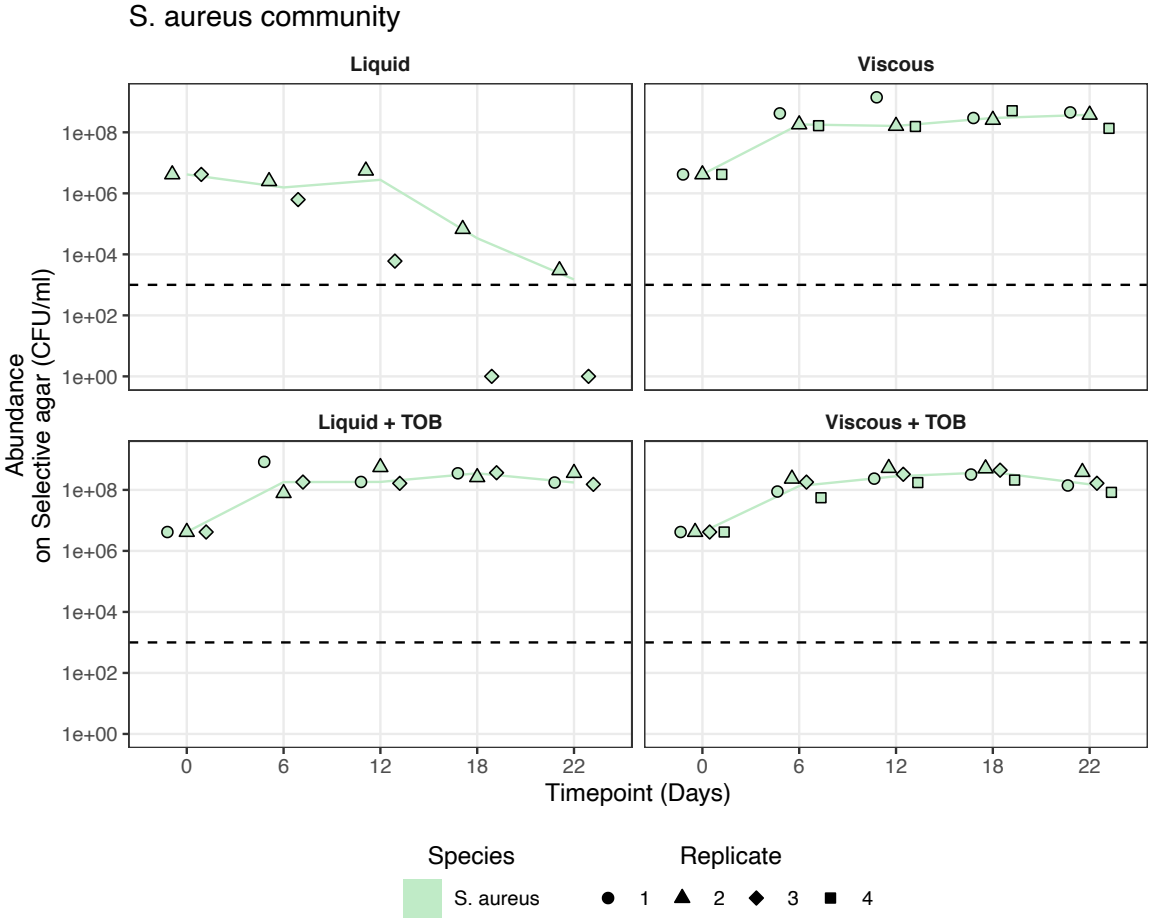
Appendix Figure B.2: The abundance of each species at the final timepoint of the selection experiment, within the different media treatments and communities. **A:** Three-species community. **B:** *P. aeruginosa* & *S. aureus* community. **C:** *P. aeruginosa* & *S. maltophilia* community. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Coloured horizontal lines represent median abundance of the species. Colours represent species, shapes represent replicates. Vertical dotted lines link isolates from the same replicate. Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in pairwise communities < 4 due to removal because of contamination.



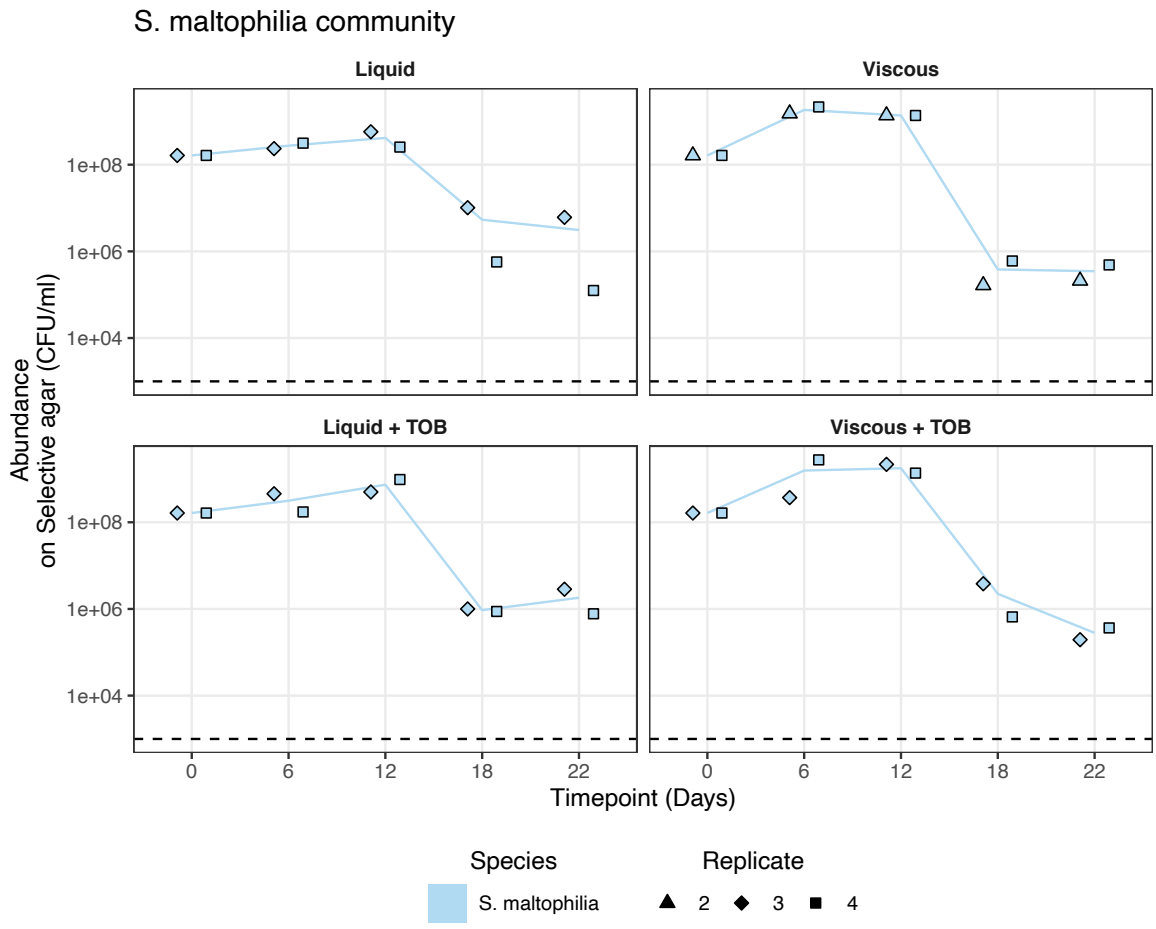
Appendix Figure B.3: The median adjusted abundance of each species within the three-species communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates.



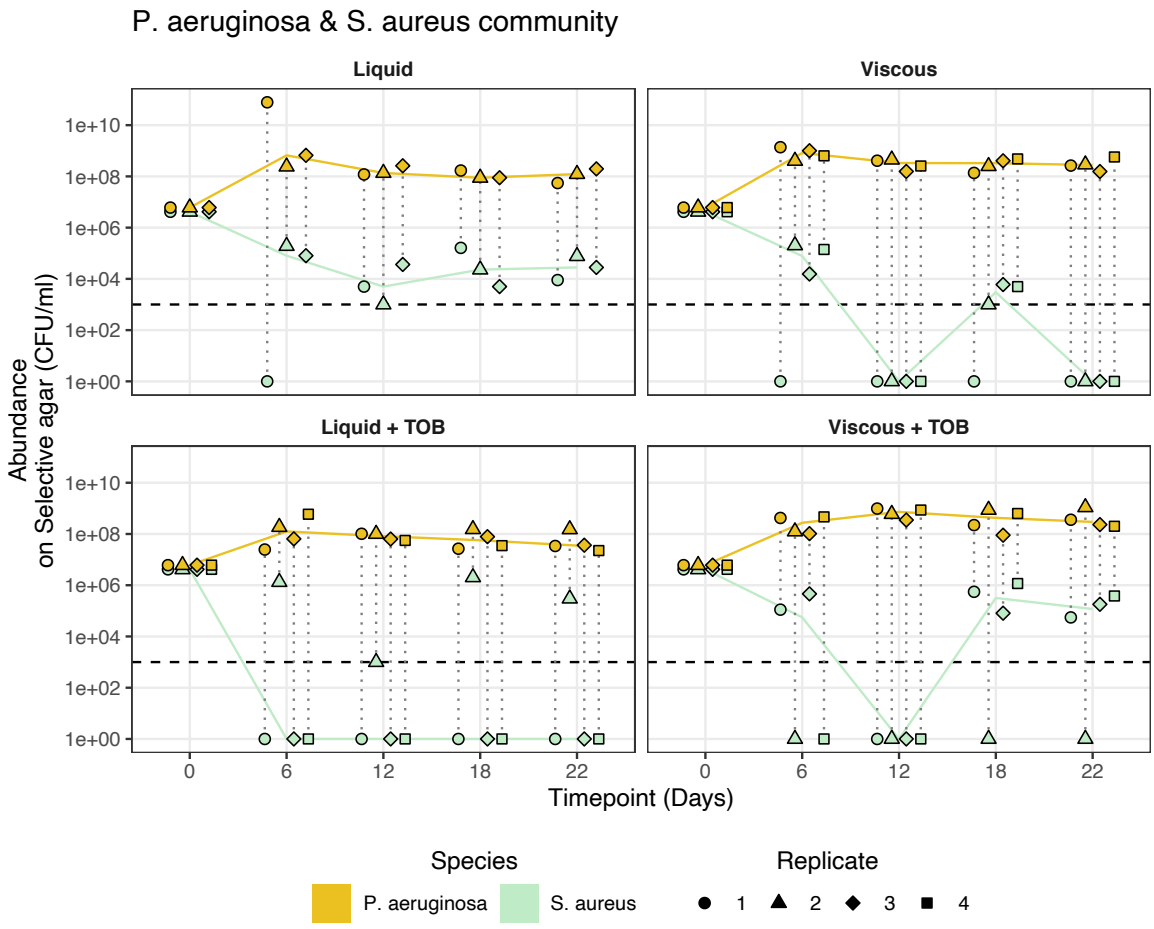
Appendix Figure B.4: The median adjusted abundance of each species within the *P. aeruginosa* mono communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in communities < 4 due to removal because of contamination.



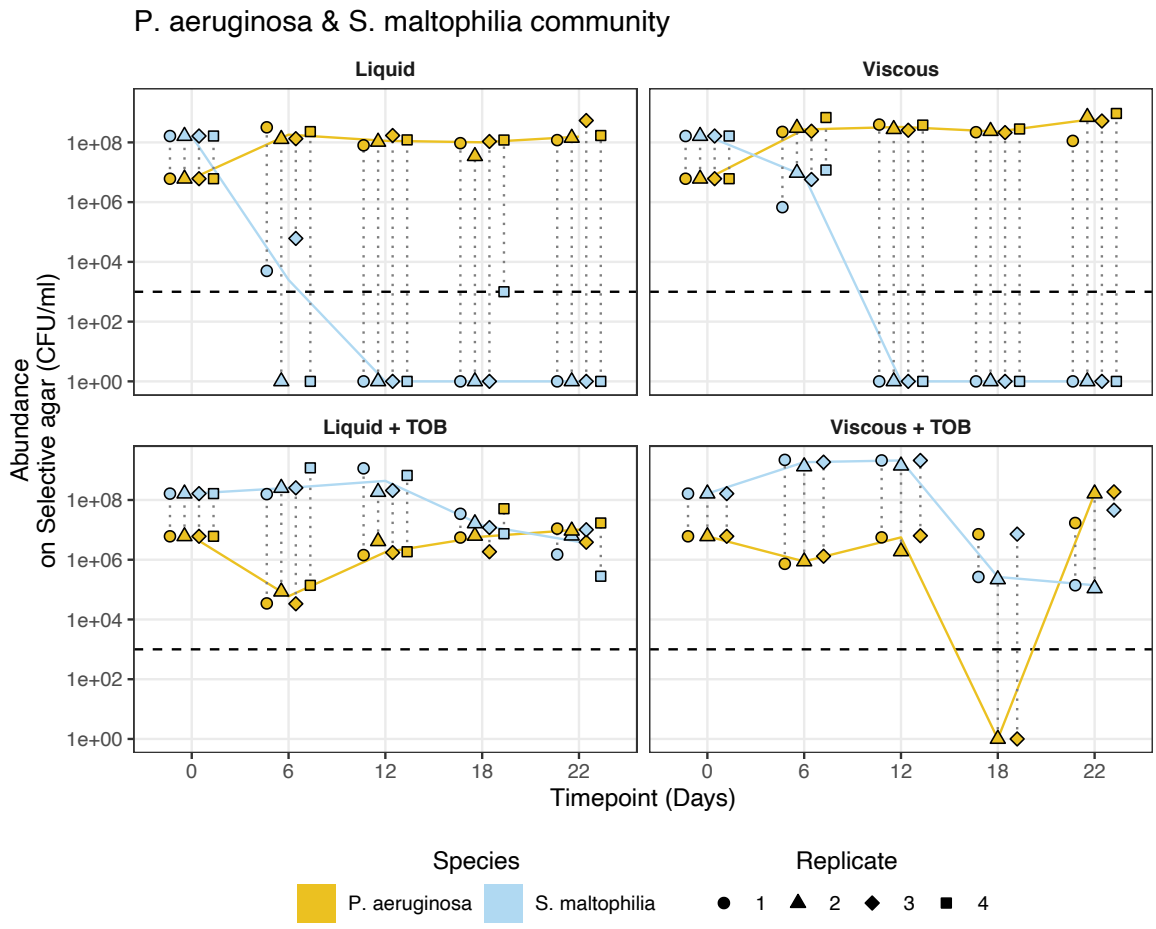
Appendix Figure B.5: The median adjusted abundance of each species within the *S. aureus* mono communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in communities < 4 due to removal because of contamination.



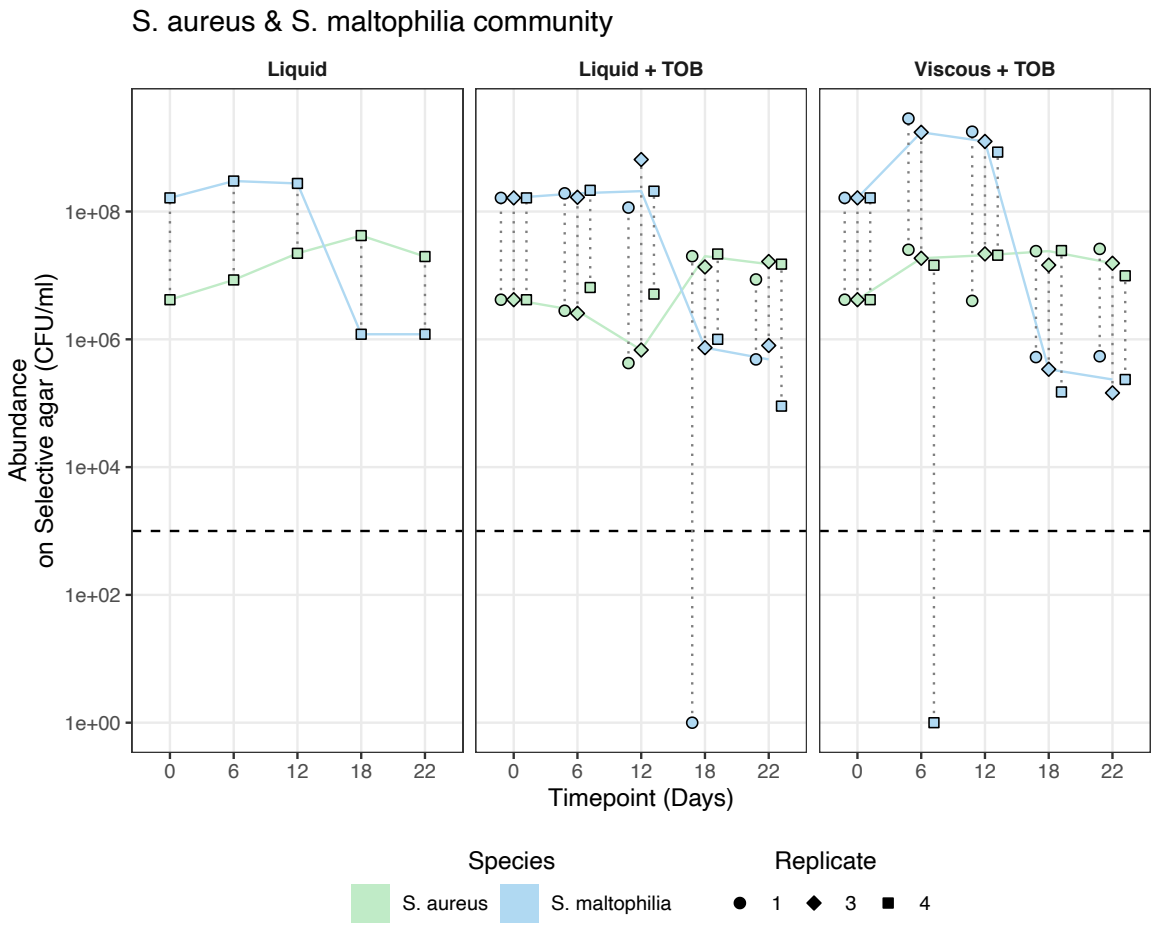
Appendix Figure B.6: The median adjusted abundance of each species within the *S. maltophilia* mono communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in communities < 4 due to removal because of contamination.



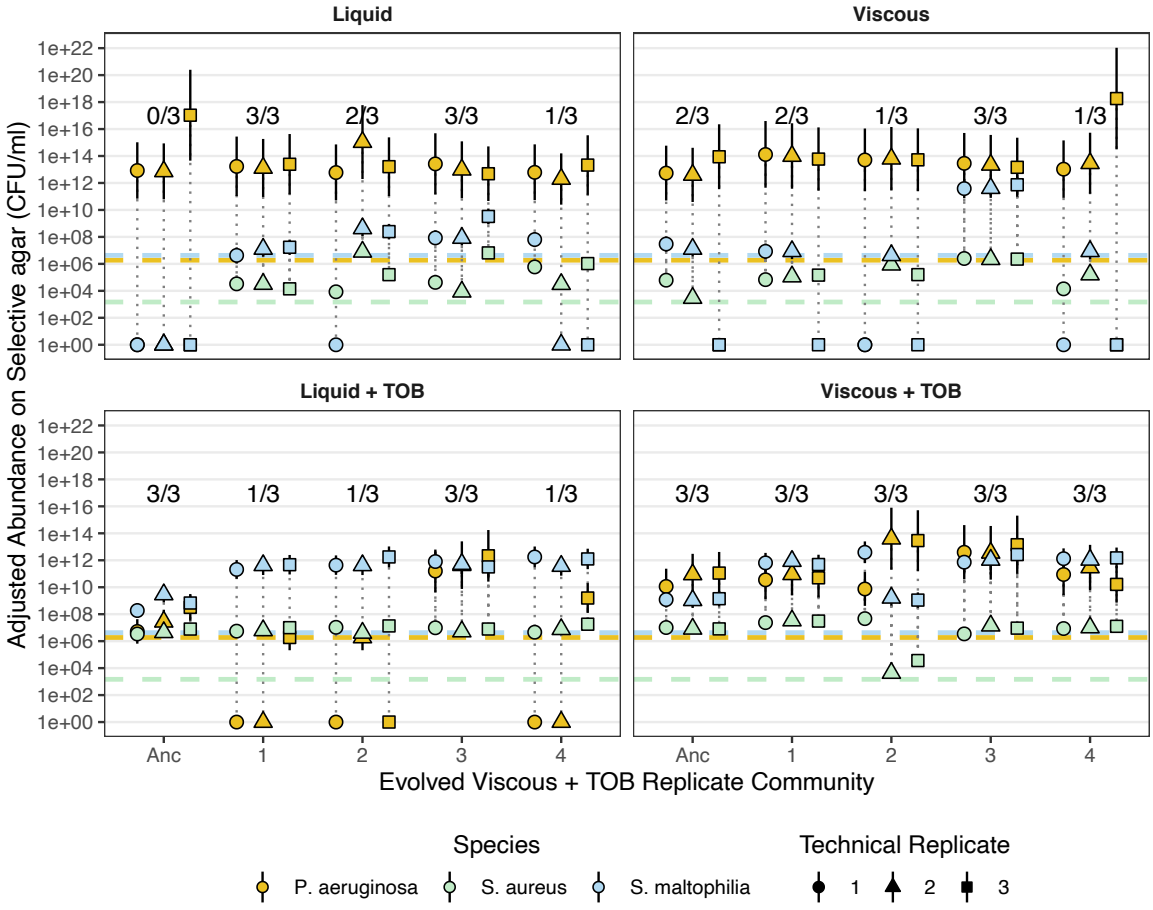
Appendix Figure B.7: The median adjusted abundance of each species within the *P. aeruginosa* & *S. aureus* communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in communities < 4 due to removal because of contamination.



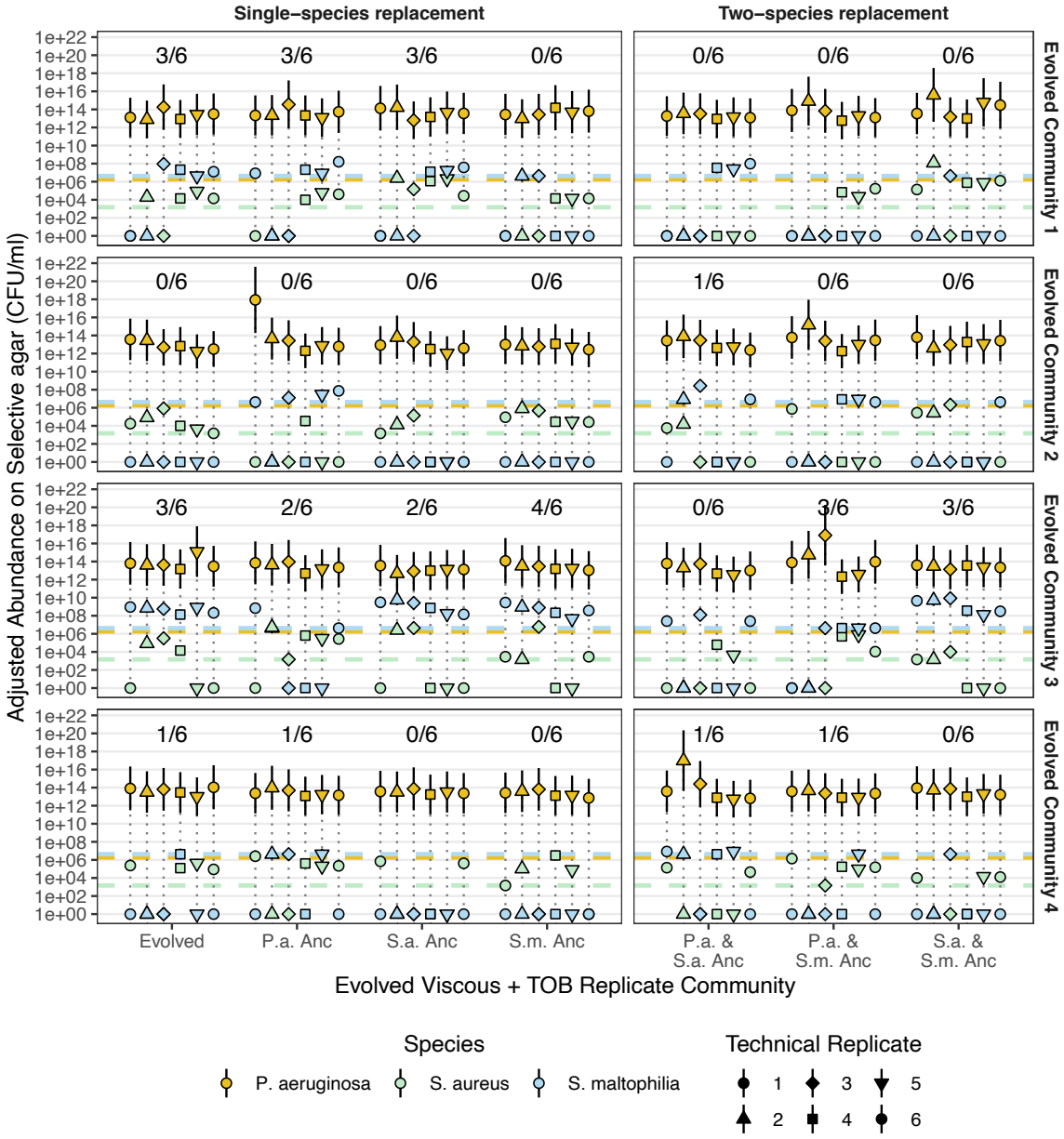
Appendix Figure B.8: The median adjusted abundance of each species within the *P. aeruginosa* & *S. maltophilia* communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in communities < 4 due to removal because of contamination.



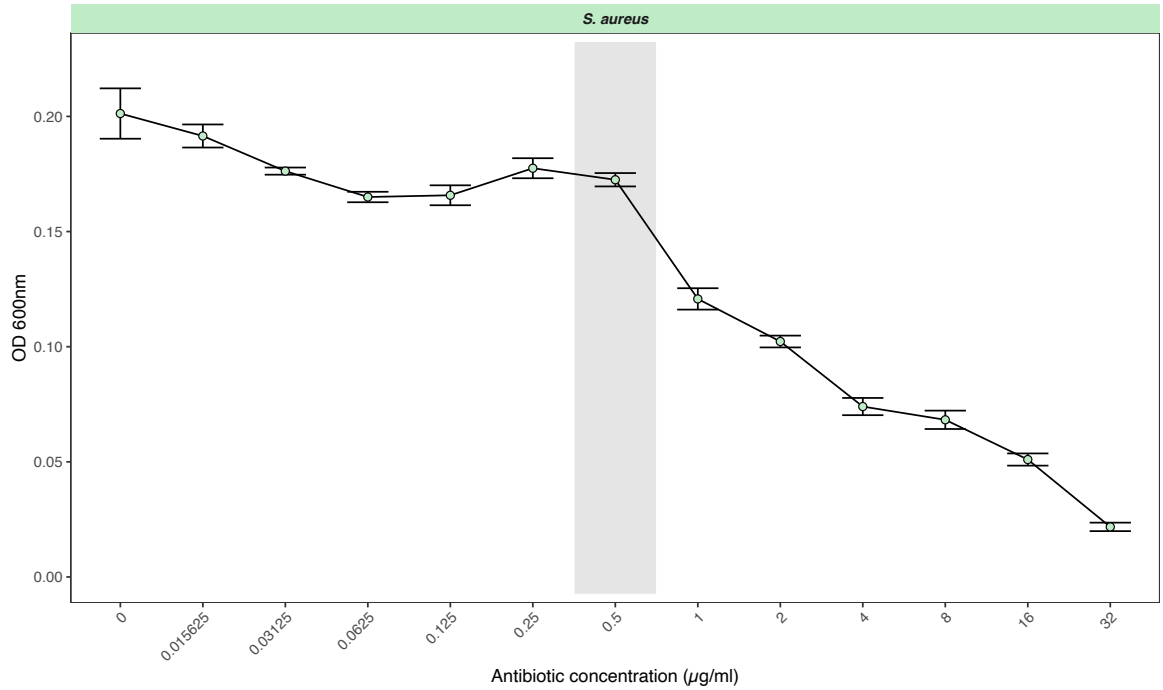
Appendix Figure B.9: The median adjusted abundance of each species within the *S. aureus* & *S. maltophilia* communities at each sampling point during the selection experiment. Panels represent each media condition. Points represent CFU/ml of individual replicates ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3. Solid coloured lines represent median CFU/ml across replicates at each timepoint. Colours represent species, shapes represent replicates. Vertical dotted lines join species from the same replicate Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates. Replicates in communities < 4 due to removal because of contamination.



Appendix Figure B.10: Adjusted Species abundances within ancestral and evolved communities grown in the four media treatments. Panels show the growth of the technical replicates of each community, shown on the X-axis, in the four media treatments. Communities consisted of either ancestral species isolates (Anc) or isolates from evolved communities 1–4. Points represent CFU/ml of individual species ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3, for technical replicates (N = 3). Numbers above represent the number of technical replicates out of 3 with coexistence of all species. Colours show species, and shapes show technical replicates. Vertical dotted lines join species in the same technical replicate. Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates.

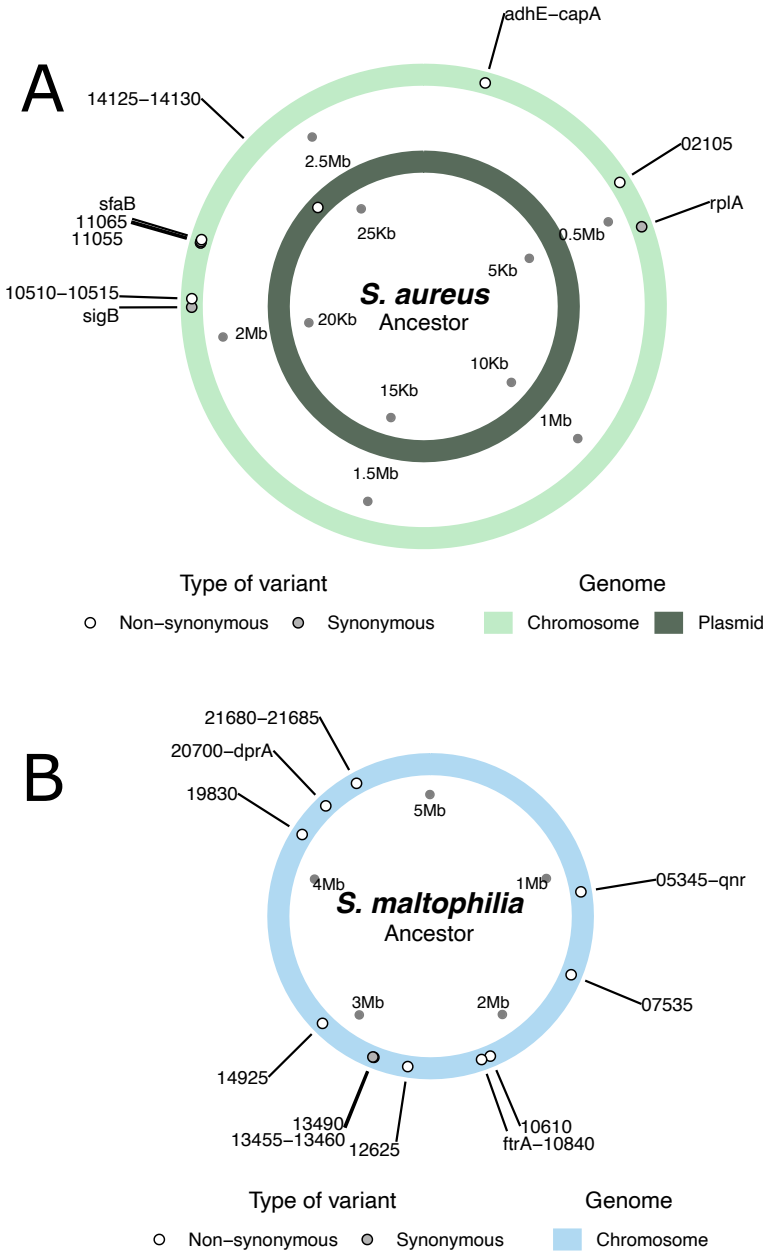


Appendix Figure B.11: Species abundance within evolved communities with single-species or two species ancestor replacements. The X-axis shows the ancestral species (Anc) within the evolved communities. The panel columns show single-species and two-species ancestor replacement, and panel rows show each of the four evolved communities Points represent CFU/ml of individual species ± 95 % CI, adjusted for growth on selective plates as detailed in Methods § 3.3.3, for technical replicates (N = 6). Numbers above represent the number of technical replicates out of 6 with coexistence of all species. Colours show species, and shapes show technical replicates. Vertical dotted lines join species in the same technical replicate. Horizontal dotted lines the adjusted limit of detection—i.e., 1000 CFU/ml adjusted for growth on selective plates.

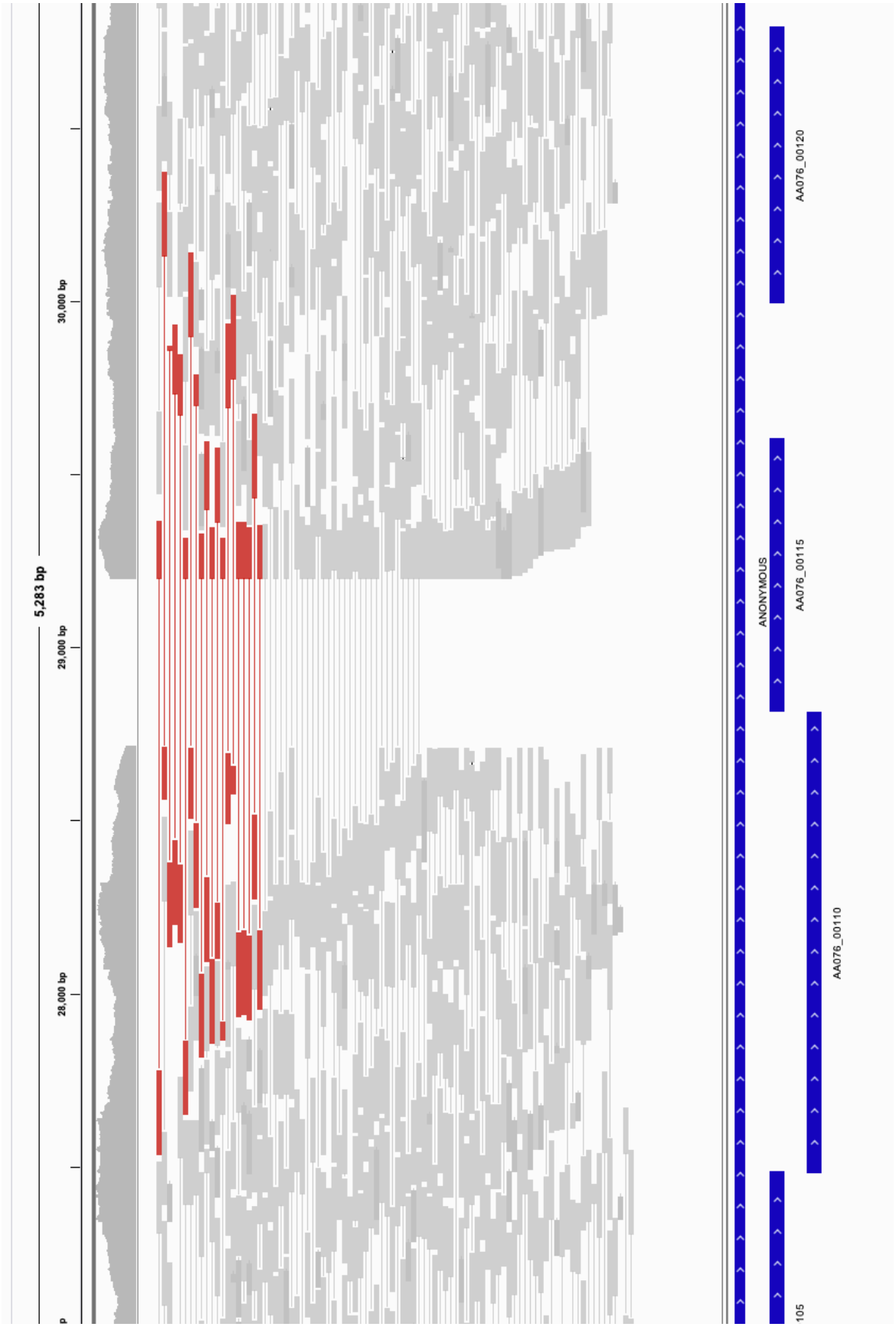


Appendix Figure B.12: MIC curve of tobramycin for *S. aureus*. Performed using the method detailed in Chapter 2 § 2.3.3. Highlighted concentration shows the concentration used in the selection experiment. Points show means of triplicate assays, error bars ± S.E.M.

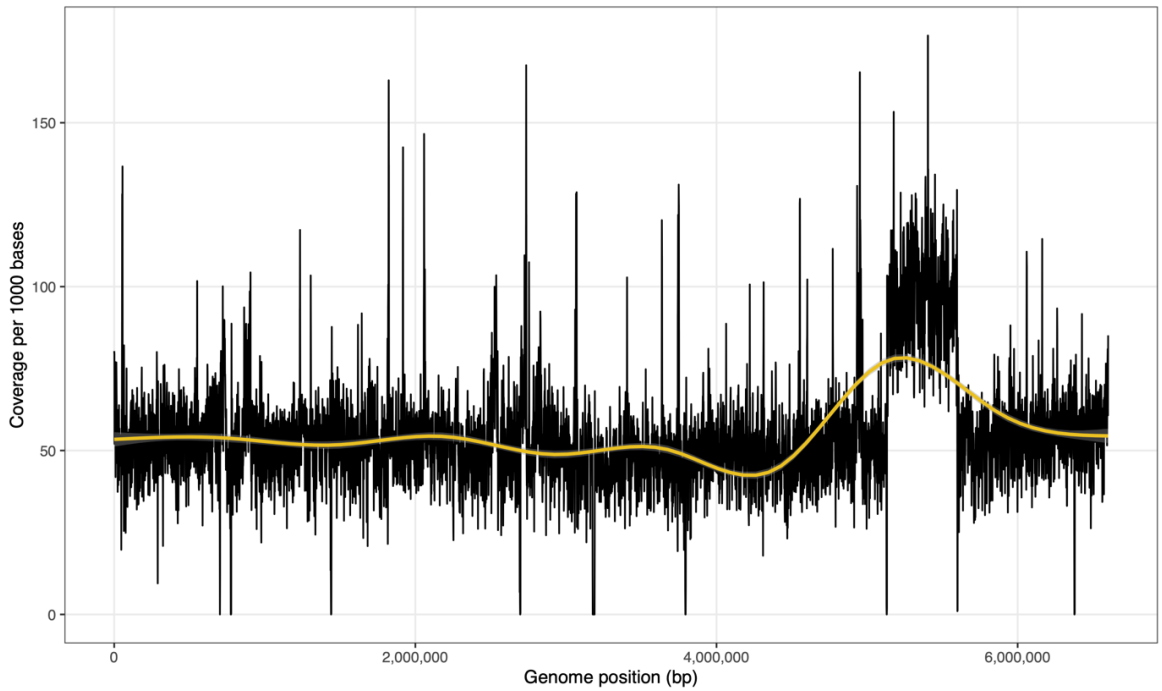
Appendix C. Chapter 4



Appendix Figure C.1: Overview of observed SNPs and INDELs of (A) *S. aureus* and (B) *S. maltophilia* ancestral isolates. Each ring represents a portion of the genome, either chromosome or plasmid. Dots represent observed variants at a locus on the genome. Dots are coloured by whether the mutation at the indicated locus was synonymous (grey) or non-synonymous (white). Labels show the gene name, when named, or the numbered locus tag with the (A) AA076_RS prefix, or (B) CKW06_RS prefix omitted; intergenic mutations are labelled as the flanking loci separated by a dash. Within each set of rings are distance markers in Mb.


5,283 bp
30,000 bp
29,000 bp
28,000 bp
ANONYMOUS
AA076_00120
AA076_00115
AA076_00110
105

Appendix Figure C.2: IGV screenshot showing evidence for deletion of portions of *yycH* and *yycI* (AA076_RS00110, AA076_RS00115). Red reads show read-pairs with larger than expected insert size, indicative of deletion. Grey reads show read-pairs with expected insert size.



Appendix Figure C.3: Read coverage depth per 1000 bases over the length of the chromosome in *P. aeruginosa* isolate PA7D32. Black lines show the coverage per 1000 bases at each genomic position. Yellow line shows the rolling average coverage.

Appendix Table C.1: Origin of sequenced isolates. The number and identity of isolates sequenced of each species, from each replicate community and treatment.

Species	Timepoint	Community	Media treatment	Total sequenced isolates	Replicates sequenced
P. aeruginosa	Final	P.a. mono	Liquid	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. mono	Viscous	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. mono	Liquid + TOB	3	1, 3, 4
P. aeruginosa	Final	P.a. mono	Viscous + TOB	2	1, 3
P. aeruginosa	Final	P.a. & S.a.	Liquid	3	1, 2, 3
P. aeruginosa	Final	P.a. & S.a.	Viscous	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.a.	Liquid + TOB	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.a.	Viscous + TOB	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.m.	Liquid	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.m.	Viscous	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.m.	Liquid + TOB	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.m.	Viscous + TOB	3	1, 2, 3
P. aeruginosa	Final	P.a. & S.a. & S.m.	Liquid	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.a. & S.m.	Viscous	4	1, 2, 3, 4
P. aeruginosa	Final	P.a. & S.a. & S.m.	Liquid + TOB	1	2
P. aeruginosa	Final	P.a. & S.a. & S.m.	Viscous + TOB	21	1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 3.1, 3.2, 3.6, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6
P. aeruginosa	Inter	P.a. & S.a. & S.m.	Liquid	4	1, 2, 3, 4
P. aeruginosa	Inter	P.a. & S.a. & S.m.	Viscous	4	1, 2, 3, 4
P. aeruginosa	Inter	P.a. & S.a. & S.m.	Liquid + TOB	2	1, 2
P. aeruginosa	Inter	P.a. & S.a. & S.m.	Viscous + TOB	2	1, 2

S. aureus	Final	S.a. mono	Liquid	1	2
S. aureus	Final	S.a. mono	Viscous	3	1, 2, 4
S. aureus	Final	S.a. mono	Liquid + TOB	3	1, 2, 3
S. aureus	Final	S.a. mono	Viscous + TOB	4	1, 2, 3, 4
S. aureus	Final	P.a. & S.a.	Liquid	3	1, 2, 3
S. aureus	Final	P.a. & S.a.	Liquid + TOB	1	2
S. aureus	Final	P.a. & S.a.	Viscous + TOB	3	1, 3, 4
S. aureus	Final	S.a. & S.m.	Liquid	1	4
S. aureus	Final	S.a. & S.m.	Liquid + TOB	3	1, 3, 4
S. aureus	Final	S.a. & S.m.	Viscous + TOB	3	1, 3, 4
S. aureus	Final	P.a. & S.a. & S.m.	Liquid	3	1, 3, 4
S. aureus	Final	P.a. & S.a. & S.m.	Viscous	2	1, 4
S. aureus	Final	P.a. & S.a. & S.m.	Liquid + TOB	4	1, 2, 3, 4
S. aureus	Final	P.a. & S.a. & S.m.	Viscous + TOB	12	1.2, 1.5, 1.6, 2.1, 2.2, 2.3, 3.1, 3.4, 3.5, 4.1, 4.3, 4.4
S. aureus	Inter	P.a. & S.a. & S.m.	Liquid	2	2, 4
S. aureus	Inter	P.a. & S.a. & S.m.	Viscous	2	1, 2
S. aureus	Inter	P.a. & S.a. & S.m.	Liquid + TOB	4	1, 2, 3, 4
S. aureus	Inter	P.a. & S.a. & S.m.	Viscous + TOB	4	1, 2, 3, 4
S. maltophilia	Final	S.m mono	Liquid	2	3, 4
S. maltophilia	Final	S.m mono	Viscous	2	2, 4
S. maltophilia	Final	S.m mono	Liquid + TOB	2	3, 4
S. maltophilia	Final	S.m mono	Viscous + TOB	2	3, 4
S. maltophilia	Final	P.a. & S.m.	Liquid + TOB	4	1, 2, 3, 4
S. maltophilia	Final	P.a. & S.m.	Viscous + TOB	3	1, 2, 3
S. maltophilia	Final	S.a. & S.m.	Liquid	1	4

S. maltophilia	Final	S.a. & S.m.	Liquid + TOB	3	1, 3, 4
S. maltophilia	Final	S.a. & S.m.	Viscous + TOB	3	1, 3, 4
S. maltophilia	Final	P.a. & S.a. & S.m.	Liquid + TOB	2	2, 3
S. maltophilia	Final	P.a. & S.a. & S.m.	Viscous + TOB	12	1.1, 1.2, 1.4, 2.1, 2.5, 2.6, 3.1, 3.2, 3.4, 4.2, 4.3, 4.4
S. maltophilia	Inter	P.a. & S.a. & S.m.	Liquid	3	1, 3, 4
S. maltophilia	Inter	P.a. & S.a. & S.m.	Viscous	4	1, 2, 3, 4
S. maltophilia	Inter	P.a. & S.a. & S.m.	Liquid + TOB	2	1, 2
S. maltophilia	Inter	P.a. & S.a. & S.m.	Viscous + TOB	2	2, 4

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