The impact of synthetic lung sputum media and the presence of clinical *S. maltophilia* on the evolution of imipenem resistance in *P. aeruginosa*

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Declaration

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

Abstract

Bacterial-associated pneumonia of cystic fibrosis (CF) patients is one of the leading causes of death in the under 30 white European population with antibiotic resistant *P. aeruginosa* being primarily responsible. Understanding how antibiotic resistance evolves experimentally has great potential to inform clinical practises. However, there are only a few *in vitro* studies concentrating on antibiotic resistance evolution in simulated *in vivo* growth conditions. We predicted that growth substrate and interspecies interactions can play a more substantial role in shaping the evolution of antibiotic resistance than is usually credited in the current literature. By using Synthetic Cystic Fibrosis Media (SCFM), mucin, and clinical CF isolates we demonstrate both lung sputum and bacterial community members impact the antibiotic susceptibility and resistance evolution of *P. aeruginosa* on both ecological and evolutionary scales. Co-culturing in SCFM increases the antibiotic tolerance of clinical *P. aeruginosa*, *S. aureus* and *S. maltophilia* isolates while simultaneously conferring *S. maltophilia* mediated protection against imipenem to *P. aeruginosa* through antibiotic degradation. Evolving PA01 in SCFM regularly treated with clinical imipenem concentrations led to faster emergence and higher overall levels of imipenem resistance when grown alongside *S. maltophilia* compared to monocultures. Mechanistically, imipenem resistance was associated with loss of function mutations in the OprD porin that likely reduced diffusion of the antibiotic into the bacterial cell. A clear fitness trade-off between imipenem resistance and competitive ability was observed. Our results indicate abiotic and ecological interactions could impact antibiotic resistance evolution during clinical *P. aeruginosa* infection and suggest the presence of *S. maltophilia* may reduce the success of imipenem treatment in individual CF patients.

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Introduction

Clinical antibiotic resistance is defined by a pathogens capacity to survive and progress disease in patients treated with the maximum safe concentration of antibiotics. The World Health Organisation (WHO) reports over 700,000 people die each year from antibiotic resistant infections, predicting the number to rise to 10 million by 2050 [1]. Surviving patients spend tens of millions of extra days recovering from more severe infections [2] imposing a substantial economic and developmental cost that could force up to 24 million people into poverty by 2030 [1]. Antibiotic use has risen by a third since 2000 [3] and a very strong relationship between level of use and rate of resistance has been established [4]. Currently, reducing overall antibiotic use is the main strategy for limiting the global emergence and spread of resistance [5], primarily achieved by reducing their incorrect administration. Between 8.8-23.1% of all antibiotic prescriptions in the UK are classed as inappropriate [6] with this value being considerably higher in the developing world [7, 8]. Most of our current understanding of how antibiotic resistance evolves comes from single species assays in simple laboratory media, not taking into account the impact of host and existing microbial community members. This is a clear shortcoming as other species may shape pathogen responses to antibiotics by creating protective extracellular structures or actively degrading antimicrobials [9, 10]. Incorporating additional bacterial species into clinically representative *in vitro* assays of antibiotic resistance evolution could potentially provide a more comprehensive understanding of infections *in vivo*, and therefore have greater capacity to inform better medical practises and more effective treatments.

Pseudomonas aeruginosa is a resilient opportunistic pathogen

Pseudomonas aeruginosa is an aerobic, gram-negative bacteria that exists in almost every environment, from lakes and soils [11, 12] to floors and sink-networks [13, 14]. Its wide-reaching success is due to several intrinsic biological properties: minimal nutrient requirements [15], innate toxin tolerance [16] and prolific Horizontal Gene Transfer (HGT) [17, 18], which enables it to recombine and acquire genes that are required to thrive in conventionally harsh environments. Its high phenotypic plasticity and rapid capacity to adapt is made possible by a large genome size [19] of 6.3mbp [20] which contains multiple plastic genome regions that can be variably expressed [21]. Over eight percent of *P. aeruginosa*'s gene complement is dedicated entirely to regulation [22] providing unusually high variation in phenotypic expression compared with other bacteria [23]. This physiological flexibility is key to understanding *P. aeruginosa*'s capacity to colonise, adapt and differentiate into a wide range of different environments.

P. aeruginosa also possesses a diverse suite of virulence factors which, due to its ubiquitous nature, enables it to operate as an opportunistic pathogen across plant and animal species, including humans. *P. aeruginosa* infections are a persistent problem throughout hospitals where it regularly infects burns [24], pulmonary-deficient lungs [25] and patients with compromised mucosal immunity [26]. In intensive care units *P. aeruginosa* is one of three bacteria that cause the overwhelming majority of all nosocomial infections [27, 28, 29] and is the number one cause of ventilator associated pneumonia [30]. Its capacity to exist on hospital surfaces [31] and in sink networks for several months [32] can result in high transmission between patients via direct contact with hospital

workers [33, 34] or indirect transmission via intermediate environmental reservoirs [32, 35]. Eliminating these contaminated sites is nearly impossible due to hospital strains possessing high degrees of innate and adapted resistance to most disinfectants [36]. This long-term persistence and extreme durability makes it difficult to prevent transmission between patients.

A large section of the population particularly vulnerable to opportunistic *P. aeruginosa* infection are Cystic Fibrosis (CF) patients. Cystic fibrosis is a recessive genetic disorder that results in the dysfunction or downregulation of the CF Transmembrane Regulator ion channel, responsible for maintaining chloride ion homeostasis at mucosal surfaces [37]. Its dysfunction leads to a characteristic overproduction of thick mucus which interferes with digestion and mucosal immunity leading to regular bouts of respiratory inflammation and repeated chronic bacterial infections [38, 39]. Cystic fibrosis is the most common lethal hereditary disease among white Europeans [40], with incidence rates typically between 1:2000 – 1:4000 depending on country [41]. Due to a 1% yearly mortality rate and a median life expectancy of 29 years [42], it is one of the major causes of death in the under 30's. The vast majority of infections are polymicrobial [43] as lung conditions support an incredibly diverse microbial community [44, 45, 46] consisting of bacterial, viral and fungal species that form a complex network of interspecies and interhost interactions [47, 48]. As a result, nearly 90% of CF-related deaths are caused by respiratory failure directly associated with severe bacterial infections [49, 50], predominantly *Pseudomonas*.

Antibiotic resistance evolution is a major hurdle in the treatment of *P. aeruginosa* infection of the CF lung, resulting in less effective therapy, higher mortality rates and greatly reduced quality of life. In the UK, prolonged lung infections increase the medication cost alone by £3.2k per CF patient per year on average [51, 52]. Although acute infections are often successfully treated in juveniles, long-term chronic *P. aeruginosa* infections are much harder to eradicate due to the establishment of antibiotic resistant biofilms [53, 54]. Historically chronic *P. aeruginosa* infection was present in over 80% of CF patients beyond the age of 20 [55]. Uneven testing regimes makes modern carriage rates hard to predict [42] although some European cohort studies still show that over 80% of CF patients harbour the bacterium [56], while in the USA chronic infection rates are between 65% and 70% [57]. *P. aeruginosa* infection of CF patients remains a major problem for western healthcare and represents an important eco-evolutionary biomedical problem as bacterial adaptation to the lung and the evolution of antibiotic resistance are both intimately linked with disease progression. Treatment against acute and chronic *Pseudomonas* infection is primarily antibiotic-based making the global trend of increasing resistance [58] a major challenge for treating CF patients [59]. Multi-drug resistant (MDR) strains of *P. aeruginosa* are commonplace in the clinical environment [60] and have a significantly higher prevalence in CF patients [61, 62], which substantially reduces the pool of effective antibiotics available. This is especially problematic when treating *P. aeruginosa* whose intrinsic tolerance to toxins confers complete or partial resistance to several classes of antibiotics [63, 64]. Increased drug resistance requires stronger, later stage antibiotics to clear or control infection, which are typically associated with increased toxicity, side effects and a considerable decrease in patient quality of life [65, 66, 67]. Extremely drug resistant strains require the use of 'last line' antibiotics, usually at the cost of potentially life-threatening side-effects [65]. Understanding how *P. aeruginosa* evolves antibiotic resistance under *in vivo* growth conditions can give critical insights into informing future treatments for CF patients.

The evolution of antibiotic resistance in *P. aeruginosa*

High levels of innate resistance and considerable adaptive potential makes *P. aeruginosa* one of the most challenging pathogens to treat with antibiotics. In the WHO's priority list of pathogens with the highest global need for novel antibiotic development, carbapenem resistant *P. aeruginosa* was ranked in the top three at 'Priority 1: Critical' [68]. Innate or intrinsic resistance refers to the underlying capacity of a bacteria to resist antibiotics, typically conferred through inherent structural or metabolic features shared by all members of the species. In *Pseudomonas*, innate resistance against most antibiotic groups is particularly high [69, 70] due to heavily restricted porin sizes [71, 72] leading to substantial outer membrane impermeability [70], alongside the constitutive expression of multiple resistance associated efflux pumps and degradative/inhibitory enzymes [69, 70, 73]. In contrast to innate, adaptive or acquired resistance emerges as a result of evolution in environments containing antibiotics and can be loosely grouped into four main mechanisms [74]: reduced antibiotic uptake via porins [75], increased antibiotic removal via efflux pumps [75, 76], modification of antibiotic targets and modification of the antibiotics themselves [77, 78]. Like many bacterial pathogens *P. aeruginosa* can develop antibiotic resistance phenotypes at the population level, protecting the whole community and enabling long term persistence. Extracellular biofilms are formed by a mesh of secreted proteins, polysaccharides, lipids and free-DNA [79]. They act as a semi-solid physical barrier to antibiotic penetration [80] and create structural heterogeneity in the environment, reducing diffusion and supporting the formation of tightly controlled micro-niches which can enhance antibiotic tolerance [81, 82, 83]. Once established, infective *P. aeruginosa* populations contain a small proportion of slow growing 'persister cells' whose reduced metabolism lowers their sensitivity to antibiotics, enabling reseeding of the environment when most of the population has been eradicated [84, 85, 86]. Both biofilm and persister cell formation are highly advantageous mechanisms to resist antibiotic treatment and are regularly selected in clinical isolates [86, 87].

Ultimately, adapted antibiotic resistance is caused by either gain or loss of function mutations which evolve through two distinct pathways: *de novo* mutation or HGT. *De novo* mutations are randomly incurred changes in nucleotide sequence generated during errors in DNA replication or from exposure to mutagenic chemicals [88]. In gene coding regions, Single Nucleotide Polymorphisms (SNPs) can introduce stop codons or alter amino acid translation which increases, decreases or completely removes the functionality or efficiency of a protein [89]. More complex insertion and deletion SNPs can cause reading frameshifts which de-aligns all subsequent codons leading to incorrect translation and usually protein misfolding and dysfunction [90]. Sometimes loss of function mutations do not directly cause changes in phenotype, but instead induce the reorganisation of cellular metabolism which indirectly causes the observed phenotype [89]. Less common *de novo* mutations include the deletion or duplication of gene regions which can alter transcriptional output and enable gene-pairs to neo- or sub-functionalise [91]. Furthermore, mutations in non-coding regions can also induce resistance phenotypes through alterations in non-coding RNA's [92] and by reducing the capacity for regulatory sequences to bind to upstream gene regions [93]. Alternatively, HGT is the acquisition of novel genetic material from other populations typically via mobile genetic elements such as integrons, plasmids and phages [69, 94]. Although relatedness between species constrains the rate of HGT [95, 96] species in different phylogenetic kingdoms have been shown to share genetic material [97]. The introduction of novel antibiotic resistance genes through HGT is a

common mechanism of resistance evolution in clinical *P. aeruginosa* [98, 99, 100]. Typically, acquired resistance to individual antibiotics does not incur a fitness cost because compensatory mutations are regularly evolved [101, 102, 103], which alleviate the reduction in fitness usually associated with gain or loss of function mutations. However, the use of combination therapies and alternating antibiotic regimes means MDR is widespread in clinical *P. aeruginosa* and usually does come with a distinct fitness cost [104].

It is important to note that not all resistance phenotypes that emerge are due to genetically evolved traits and can often result from environmentally induced changes in gene expression. *P. aeruginosa* demonstrates considerable behavioural plasticity when responding to stress, utilising a wide array of transcription factors and methylation-based epigenetic modifications to augment protein production based on the environment [105, 106, 107]. Quorum sensing (QS) is a cell-cell signalling mechanism used for controlling gene expression, ubiquitous throughout bacterial species, [108]. It utilises small soluble peptides with relatively low production costs, to coordinate gene expression at the populational level based on cell density. This type of regulated plasticity is very useful for responding to environmental change and as a result QS regulated traits are typically involved in counteracting both biotic and abiotic stress [109]. Furthermore, growth conditions considerably impact gene regulation and biofilm formation [46, 110], simply growing *P. aeruginosa* on isobutyrate in place of L-glutamate carbon sources results in a 487-fold increase in colistin resistance [111]. Transcriptome analysis has demonstrated a substantial difference in the relative expression of 1000's of genes in clinical *P. aeruginosa* CF isolates when grown in different laboratory models [112]. This is a persistent problem for clinical antibiotic sensitivity tests where pathogenic samples that record as sensitive *in vitro* are often not successfully controlled during subsequent patient treatment [113]. This issue is further amplified during polymicrobial infections where antibiotic treatment against a focal pathogen rarely takes into account the patient's microbiota [48]. Incorporating ecological elements into *in vitro* antibiotic resistance evolution assays is the first step required to give clinicians a robust understanding of how the microbial ecology of individual patients may impact treatment outcomes.

The impact of inter-species interactions on antibiotic tolerance and evolution

Bacterial communities consist of a wide range of interspecies interactions, many of which have the potential to alter the antibiotic sensitivity of individual members. The production of inhibitory or degradative enzymes is a common resistance mechanism across genera [114] and can potentially provide community level protection by reducing local concentrations, enabling the survival of sensitive strains [115]. This effect can emerge from secreted enzymes in the environment [116, 117] or intracellular enzymes that internally break down antibiotics [9]. Alternatively, the secretion of toxic compounds and signalling molecules can induce favourable transcription profiles and metabolic rates which increase tolerance [118, 119, 120]. Multi-species biofilms are also common [121] with their benefits extending to all inhabitants [122]. This protection can be enhanced when different species contribute novel compounds which augment the structural properties of the matrix [10, 122]. Furthermore, local microenvironments created by biofilms can intensify otherwise weak interspecies interactions [52]. Conversely, ecological interactions can actively decrease tolerance to antimicrobials in communities with cross-feeding networks where bacteria rely on products from other species to perform specific metabolic functions [123, 124]. Antibiotic treatment can remove sensitive community members, disrupt feeding networks, and lead to the loss of tolerance in

otherwise resistant species [124]. Many of these ecological interactions are density dependent, requiring moderately high concentrations of cells to have a substantial effect. Therefore, the emergence of these interactions are dependent on both the complexity of the community in question and the capacity of the environment to support high growth rates. More diverse communities decrease the likelihood that individual species exist at sufficiently high numbers for an interaction to emerge. This means that community composition and environmental growth conditions need to be considered when validating observed interactions *in vitro*.

Importantly the effect ecologically derived antibiotic tolerance has on the evolution of antimicrobial resistance is mostly unknown. The development of community level protection may theoretically raise the Minimum Selective Concentration (MSC), the antibiotic concentration required to positively select for adaptive resistance. Thus, potentially costly resistance evolution in sensitive species provides a reduced fitness advantage in the presence of antibiotics, which could drive inter-species co-dependence. Alternatively, since competitive interactions tend to dominate in bacterial communities [125], tolerant individuals may drive the evolution of resistance in sensitive species as resistant species can grow and acquire nutrients during antibiotic treatment, imposing a competitive advantage. There is some evidence that bacterial adaptation and resistance evolution can be constrained in multi-species communities which reduces the rate of resistance emergence [125, 126]. However, this was in the absence of any clear ecologically conferred resistance. Microbial communities consist of a complex network of interspecies interactions with substantial capacity to impact antibiotic tolerance. Despite this the inclusion of microbial community members into *in vitro* studies investigating the evolution of clinical antibiotic resistance is rare [127], representing one of the major gaps in the current literature.

The CF lung microbiome is incredibly diverse containing around 30 microbes on average [128], and as a result infections are polymicrobial [129, 130] consisting of several disease causing pathogens including *P. aeruginosa*. The lung conditions provide the optimum environment for high density, high diversity growth: the sputum itself is relatively nutrient rich [131] and excess mucous provides a physical scaffold for growth [132, 133] while protecting against the host immune system [55]. CF microbial community composition varies throughout time and emerging evidence suggests that periods of ecological instability may be the primary driver of pulmonary exacerbation in patients [48]. Several ecological interactions between *P. aeruginosa* and CF community members have been linked to increased antibiotic tolerance through a variety of proposed mechanisms. Most observed interactions have been identified between *S. aureus* and *P. aeruginosa*, the main focal pathogens of CF infection [134]. Evolving *P. aeruginosa* in the presence of *S. aureus* alters its lipopolysaccharide composition conferring increased beta-lactam resistance [135], while 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) secretion by *P. aeruginosa* increases *S. aureus*'s tolerance to tobramycin and vancomycin by suppressing aerobic respiration and inducing small colony formation [120, 136]. However, HQNO has also been shown to increase *S. aureus*'s tolerance to chloroxylenol and several other antibiotics [137], demonstrating positive and negative effects. Alterations in biofilm architecture has similarly been observed as *S. aureus* contributes staphylococcal protein-A which interacts with *P. aeruginosa*'s psl-polysaccharide to increase biofilm tobramycin resistance [10]. These interactions are not limited to *S. aureus*: *B. cenocepacia* also interacts with *P. aeruginosa* inducing the development of firmer, more resistant biofilms [138]. Enzyme mediated community level antibiotic protection has also been recorded: *Prevotella* produced beta-lactamase can protect *P. aeruginosa* against ceftazidime when grown at high cell densities [139]. There is also some

evidence the production of Diffusible Signal Factors (DSF's) by *S. maltophilia* increases *P. aeruginosa*'s tolerance to polymyxins [118], 15/40 clinical strains produce DSF's and when *P. aeruginosa* was grown in 200 times the mean DSF concentration of clinical sputum [140] it lead to marginally increased tolerance [118]. Most theoretical mechanisms for ecologically derived antibiotic resistance have been identified as likely taking place in the CF lung yet no study has investigated if these interactions impact the evolution of antibiotic resistance. However, if we want experimental results to be able to inform clinical practise, *in vitro* assays must be performed under clinically representative growth conditions.

Choosing an experimental model for investigating CF infection

Despite the advantages that come from using simple laboratory media they suffer from the major drawback of being unrepresentative of *in vivo* growth conditions. Although antibiotic treatment is a major selection pressure during infection it is simply one of many environmental factors. Therefore any investigation into antibiotic resistance evolution during CF lung infection requires using the most accurate and reliable model system available. Animal models are one option, despite CF not existing in mice a partial CF-like phenotype can be induced by the knock-out of a CFTR homologue gene with 78% Amino Acid (AA) sequence homology to its human equivalent [141]. CF mice are incredibly inefficient as models because they do not establish chronic airway infections and pathogenesis is completely different compared to humans [142]. In contrast, CFTR-homologue knockouts in pigs and ferrets are much more accurate as models of human infection [143], although considerably more expensive and unethical to test on. A common alternative to the use of animals are human epithelial cells which work well for studying virulence, disease progression and immune factor interactions [144, 145]. A more complex variant is the *ex vivo* pig lung model which closely mimics *P. aeruginosa* virulence factor production, QS and tissue damage in humans. Both models work well however they introduce a lot of uncontrollable variation, making it hard to elucidate the impact of individual factors alongside still being quite costly. Organoid models, three-dimensional tissue structures grown from stem cells, offer a more complex CF model as each cell can express its entire genetic complement [146, 147]. While they excel in studying how specific genetic factors correlate with treatment outcomes [146], their high specificity to individual patients severely limits the wider application of any observed trends.

However, there are also two standardised laboratory medias designed to simulate CF sputum: the less complex Artificial Sputum Media (ASM) utilises 20 amino acids, two salts, egg yolk emulsion, fish sperm DNA and Tris buffer [148], while the more accurate SCFM contains nine salts, 19 amino acids, glucose, iron sulphate, four vitamins and MOPs buffer. SCFM was originally designed by Palmer et al. (2007) [106] to replicate the average chemical composition of CF lung sputum samples and has undergone several iterations, notably the addition of mucin, the primary chemical component of the CF lung. Other variants also incorporate salmon sperm DNA, however the large-scale phenol extraction required comes at considerable extra cost and preparation time [52], while providing little additional biological accuracy. SCFM's main benefit is that its exact content and concentration is known, creating the most accurate growth media from a chemical perspective. RNA-sequence profiling demonstrates SCFM induces expression profiles most similar to *in vivo* growth conditions for genes involved in bacterial metabolism, infection and antibiotic resistance, compared with simple laboratory media, ASM, murine models and human epithelial cells [112]. Although time consuming to make, SCFM is considerably easier to use over long periods enabling more replication and is the

most chemically similar to actual CF lung sputum, which is important as this is the main source of nutrients for respiratory pathogens.

Study overview

There are several key gaps in the literature this study is attempting to address. Firstly, to help fix the overwhelming lack of knowledge in how *P. aeruginosa* adapts to SCFM, which is currently addressed in only one study [149]. Secondly, to understand how the common respiratory pathogen *S. maltophilia* can interact with *P. aeruginosa* in the CF lung, as there has not yet been a study investigating how ecological interactions impact the long-term evolution and adaptation of antibiotic resistance in *P. aeruginosa*. Lastly, despite the clinical importance of imipenem, there remains limited understanding of how resistance evolves in *P. aeruginosa* at clinically relevant concentrations of antibiotics. To begin to address these areas we divided our study into two conceptual sections: Ecological, focusing on the short-term effects of substrate and species interactions, and Evolutionary, looking at how these features impacted the phenotype and genotypes of *P. aeruginosa* over time. We first screened a selection of clinical *S. aureus, S. maltophilia* and *P. aeruginosa* isolates in six clinically relevant antibiotics in LB and SCFM. These were grown in both monocultures and co-cultures to identify any potential ecological interactions. We then developed a growth model based on clinical and theoretical data of *P. aeruginosa*'s growth and antibiotic pharmacokinetics in the CF lung to more closely mimic the selective pressures operating upon *P. aeruginosa.* Finally, a long-term selection experiment in the presence of regular imipenem treatment and a resistant strain of clinical *S. maltophilia* was performed before populations were analysed at both phenotypic and genetic levels.

Ultimately, the aim of this study was to help bridge the current disparity between *in vitro* models and *in vivo* clinical data by including often overlooked complexities of CF infection. We found that lung sputum in general, and *S. maltophilia* in the context of imipenem, significantly increased *P. aeruginosa*'s capacity to survive individual antibiotic treatments. Both factors altered *Pseudomonas'* evolution during simulated imipenem therapy, with *S. maltophilia* leading to faster resistance emergence and increased overall tolerance in *P. aeruginosa*. These results have significant potential to both advance the field of evolutionary ecology and inform medical professionals on the best therapeutic option for individual patients.

Methods

Bacterial Strains

Eight strains of bacteria were used throughout this study. Two were variants of the laboratory lineage *P. aeruginosa* 01: 'PA01' and 'PA01:rfp' which possessed the tomato-red fluorescent marker. The remaining six were clinical isolates: *S. aureus*-521307, *S. maltophilia*-518951, -521307 and -515698, and *P. aeruginosa*-519857 and -515968. Clinical strains were previously isolated from sputum samples that originated from the respiratory tracts of Danish adults with cystic fibrosis suffering from chronic infection, provided by Sören Molin and Helle Krogh Johansen at the Rigshospitalet Copenhagen [150]. Isolates with the same ID-number originate from the same patients and all were previously been confirmed by 16S and whole genome sequencing. PA01:rfp was previously constructed by the insertion of the red fluorescent dTomato, alongside gentamicin resistance, into the attTn7 site of wild-type PA01 by electroporation via a plasmid originally found in the *E. coli* strain 'DH5α pUC18T-mini-Tn7T-dsRedExpress' [151]. This insertion was also previously confirmed via PCR.

Glycerol stocks of all bacteria were prepared by vortexing 100 µl of cultures grown in 200 µl media for 24 hours with 100 µl of 50% sterile glycerol solution, before being stored at -80^{o}C. Inoculation cultures were prepared by streaking frozen glycerol stocks onto agar plates. Single colonies were randomly selected, mixed into 200 µl media and grown overnight for 16 hours at 37^{o}C with constant shaking. Diluted inoculation cultures were made by combing 10 µl overnight bacterial culture with 190 µl of the appropriate media unless otherwise stated. All shaking performed throughout this study was performed at 220 rpm with an orbital diameter of 12.5 mm. Glycerol stocks of populations evolved in SCFM were cultured in SCFM in place of LB media. Assays involving SCFM used inoculation cultures grown and diluted in SCFM and SCFM agar. Nunc 96-well plates were used for culturing unless otherwise stated, with bacterial growth confined to the internal wells as external wells are more prone to evaporation. External wells were filled with 200 µl media to gauge contamination with the exception of antibiotic broth microdilutions where the left and right columns contained the upper and lower concentration boundaries.

Laboratory media

Luria-Bertani (LB) media and LB reduced salt media (0.5 vs 5 g/L NaCl) were prepared according to the manufacturer's instructions and sterilised via autoclaving at 121^{o}C for 30 minutes. *Pseudomonas* isolation agar was made according to the manufacturer's instructions by dissolving one vial of C-N *Pseudomonas* supplement (100.0 mg cetrimide, 7.5 mg nalidixic acid) in 100 ml sterile media before aseptically adding to 400 ml autoclaved liquid agar media allowed to cool below 60^{o}C. SCFM was prepared fresh on the day of use from sterilised stock solutions of its individual components following concentrations previously establish in Palmer et al. (2007) [106]. To enable *S. aureus* growth minor amounts of vitamin B were added at the following concentrations: Thiamine 1 mg/L, Niacin 1.2 mg/L, Calcium pantothenate 0.25 mg/L and Biotin 0.0005 mg/L. The pH of prepared SCFM was adjusted to between 6.95 and 7.05 before being vacuum filter sterilised through 0.22 nm filters into autoclaved jars. Stock solutions were stored at either 4^{o}C or -4^{o}C and regularly replenished based on their relative stability and biochemical properties. 5g/L of porcine mucin was added to simulate mucous concentrations in the CF lung, based on previous studies on SCFM [152], ASM [153]

[154] and medical reports [155]. To avoid the high loss of mucin experienced during filter sterilisation, mucin was pre-sterilised in a 70% ethanol wash and left for 24 hours in a 70°C water bath to let ethanol to evaporate. Sterilised mucin was then combined with 100 ml of sterile water and left at 4°C for 24 hours with rolling-shaking to fully dissolve, then combined with sterile SCFM. Mucin markedly raises the optical density of pure media and increases filtration time preventing its inclusion in assays that require sensitive measurements of growth or involve the sterilisation of culture media. To solve this problem two media variants were used: SCFM with pre-sterilised mucin added after filtration, referred to as 'SCFM(m+)', and SCFM with mucin dissolved before filter sterilisation, referred to as 'SCFM(m-)'. SCFM(m-) reflects a roughly 90% loss of mucin during filtration, giving an Optical Density (OD) reading (0.060) equivalent to LB media (0.050).

Optical density measurements

Optical density readings at the 595 nm wavelength (OD595) and red fluorescence intensity at excitation:532 and emission:588 nm were measured as an estimate of bacterial growth in liquid culture using the Tecan Infinite Pro plate reader. To account for heterogeneity of growth within individual wells, readings were calculated from an average of five measurement taken in a filled square pattern, with each point 500 μm apart. The cell densities of starting inoculation cultures was calculated by measuring the number of Colony formation Units (CFU) in serially diluted liquid culture on agar. We did not evaluate how increasing OD595 scales with colony formation because the relationship in *P. aeruginosa* has been well documented as being linear [156].

Antibiotic sensitivity assays

Antibiotic sensitivity of *S. aureus*-521307, *S. maltophilia*-518951, -521307 and -515698, *P. aeruginosa*-519857 and -515968, PA01 and PA01:rfp strains were tested against six antibiotics commonly used to treat respiratory infections in CF patients [157]: imipenem, vancomycin, ciprofloxacin, tobramycin, colistin and ceftazidime. Microdilution assays were performed using the rows of a 96-well plate, consisting of eleven 200 μl wells of serially diluted concentrations with one 200 μl well left antibiotic free. The middle concentration was determined by the clinical breakpoint for *P. aeruginosa* as stated by EUCAST (2019) [158], except vancomycin for which the *S. aureus* clinical breakpoint was used due to it targeting gram +ve bacteria. Plate positions were randomised within replicates and measurements were independently repeated three times for each antibiotic-bacteria pairing. All randomisation performed throughout this study used the Mersenne Twister algorithm to assign a random value to each treatment, sorting from largest to smallest, and sequentially assigning plate number and well positions based on that randomised order.

Resistance was defined as the Minimum Inhibitory Concentration (MIC), when the OD595 dropped below double that of starting inoculated media (<0.130). If more than one MIC score was observed another three replicates were performed and the mean score was used. Antibiotic stock solution was made fresh according to the manufacturer's instructions on the morning of the assay and dissolved in LB media or SCFM(m-), before being filter sterilised through 0.22 nm syringe filters. 100 μl of antibiotic stock was added to the first two columns of a 96 well plate, leaving the top and bottom rows empty. 85 μl of media was then added to wells in column one and 100 μl to all wells of the remaining 11 columns. Serial dilutions were then performed from column 2 to column 11: taking

100 µl over, thoroughly mixing then repeating. Finally, columns 2-12 were filled with 85 µl media. Leaving six rows per plate, of 11 wells containing 185 µl serially diluted antibiotic media and one well of 185 µl antibiotic free media. Wells were inoculated with 15 µl of overnight culture diluted by 1:20 (10 µl into 190 µl) in the appropriate media (approximately 3.75 x 10^5 CFU/ml), bringing the starting well OD595 to below 0.065 for LB and SCFM(m-) or 0.6 for SCFM(m+). MIC Plates were then incubated at 37°C for 24 hours with constant shaking before lids were removed and OD595 and Red Fluorescence Intensity (RFI) measurements taken. Every plate had one row inoculated with PA01 to control for variation in antibiotic concentration, if its MIC was less than the clinical breakpoint and matched other PA01 controls the antibiotic dilutions were considered sufficiently accurate.

Antibiotic sensitivity was also measured in paired and triple cultures of PA01:rfp, SM-518951 & SA-521307, alongside a co-culture of PA01:rfp and SM-521307. Polycultures were made by mixing separately grown monocultures in equal proportions, diluting by 1:20, then adding 30 µl diluted inoculation culture to 170 µl antibiotic containing wells. This kept the relative cell proportion for each species equal to the monoculture assays. The MIC of *P. aeruginosa* in mixed culture was determined by measuring the level of RFI. Antibiotic inhibition was considered significant when red fluorescence was less than triple that of starting inoculated media (<3,000). This MIC measurement was consistent with 100% of OD595 MICs determined during single species assays, enabling the two to be used interchangeably. The MICs of polycultures was measured in triplicate for all antibiotics screened except for imipenem which was replicated between 7 and 18 times due to observed ecological protection.

Imipenem degradation assay in LB media

To investigate if *S. maltophilia* breaks down imipenem when grown in LB, low starting densities of overnight SM-518951 was used to inoculate 15 ml of 128 µg/ml imipenem containing LB media. Bacteria were grown for 78 hours at 37°C with constant shaking and treatments were replicated three times for antibiotic free and 128 µg/ml imipenem treatments. Every 24 hours, three ml of culture was removed, sterilised with 0.22 nm filters, and 185 µl pipetted into 15 wells of a 96 well plate before being inoculated with 15 µl of diluted bacterial strains with previously established MICs. Four bacterial strains were used: PA-515698, PA-519857, PA01 and SM-521307, and each strain was replicated three times. Inoculated filtrates were grown at 37°C with shaking for 20 hours and the average OD595 score was taken across each set of three wells as a measure of bacterial growth.

Imipenem degradation assay in SCFM

To assess if *S. maltophilia* breaks down imipenem when grown in SCFM, the capacity of PA01:rfp to grow in periodically sampled and sterilised filtrate was assayed. Two treatment groups were used: Imipenem concentration (0 µg/ml, 1 µg/ml, 4 µg/ml and 8 µg/ml) and Bacterial inoculant (No bacteria, *P. aeruginosa*, *S. maltophilia* and a co-culture of the two), creating sixteen tubes of 20 ml SCFM. Imipenem concentrations were made by adding 2.5 ml filter sterilised imipenem containing SCFM(m-) to 16.0 ml and 14.5 ml SCFM(m+) for monocultures and co-cultures respectively. Cultures were then made by combining and diluting 0.6 ml of independently prepared overnight cultures with 11.4 ml SCFM(m+). 1.5 ml of diluted inoculum was added to 18.5 ml imipenem containing SCFM(m+) for monocultures, while 3.0 ml of diluted overnight PA01:rfp and SM-518951 were combined and

thoroughly mixed before 3.0 ml was added to 17.0 ml SCFM(m+) for co-cultures. In the no bacteria treatment, 2.5 ml of sterile filtered SCFM(m-) and 1.5 ml sterile SCFM(m+) was added to 16.0 ml SCFM(m+). Tubes were incubated at 37^{o}C with constant shaking for 48 hours, every eight hours two ml of media was sampled, syringe filtered through 0.22 um filters and 185 µl pipetted into six wells. 15 µl of diluted overnight PA01:rfp grown in SCFM(m-), was added to each well and grown for 24 hours. Every eight hours OD595 readings were taken to measure *P. aeruginosa* growth and averaged for each well to evaluate growth inhibition over the 24-hour period. Fresh batches of PA01:rfp were prepared for each eight hour interval to keep inoculating cell densities roughly equal.

Impact of mucin on imipenem sensitivity

To evaluate the relative impact of mucin on imipenem tolerance, MIC broth microdilution assays in pure LB media and LB(m+) media, supplemented with 5g/L porcine mucin were performed for monocultures of PA01:rfp and co-cultures of PA01:rfp and SM-518951. Pre-sterilised mucin was dissolved in 100 ml sterile water for 24 hours and added to 100 ml sterile LB media, this LB media contained double the concentration of its constituent components so the final nutrient composition of both LB and LB + 5g/L mucin was equal. The assay was replicated at least six times for monocultures and at least 12 times for co-cultures.

Long-term imipenem resistance evolution experiment on *P. aeruginosa* in the absence and presence of *S. maltophilia* in SCFM

PA01:rfp and SM-518951 were grown in mono- and co-cultures in 200 µl SCFM(m+) in the absence and presence of three imipenem concentrations (1 µg/ml, 4 µg/ml and 8 µg/ml), reflecting the typical clinical concentrations in lung sputum during conventional IV [159] and nebulised treatments [160] [161]. Imipenem solutions were prepared as previously described. To reduce the loss of mucin by filtration only 25 µl of imipenem containing SCFM(m-) was added to each well. 0 µg/ml treatments were made with 25 µl of imipenem free filter sterilised SCFM(m-) to keep mucin concentrations equal. On day zero, monocultures were made by diluting 10 µl overnight culture in 190 µl SCFM(m+) before 15 µl was transferred into 185 µl of antibiotic containing SCFM (approximately 3.75 x 10^5 CFU/ml). For co-cultures, 40 µl of each diluted strain was combined and thoroughly mixed before 30 µl was added into 170 µl of SCFM. Keeping the relative addition of each species equal meant any impact on growth or antibiotic resistance remained proportional. All treatments were replicated ten times and plates were incubated at 37^{o}C throughout the assay with constant shaking. Every two days, 15 µl of each evolved population was serially transferred into 185 µl of fresh SCFM containing imipenem relative to the starting treatment concentration. Mixing was not performed at any stage of the transfer to minimise biofilm disruption and model the colonisation of fresh sputum, as clinical treatment success is defined by growth inhibition rather than complete eradication [54, 55]. Although this introduced some random effects, this is the only stage where mixing was not performed, and relatively homogeneous population subsets were still transferred. Serial transfers were repeated 12 times over a 24-day period. To reduce the risk of contamination and potential human error individual populations were not randomised. Instead, plate positions and plate number for monoculture and co-culture replicates were randomised in sets of four imipenem concentrations (0 µg/ml, 1 µg/ml, 4 µg/ml and 8 µg/ml) at each transfer stage. All evolved bacterial populations were cryopreserved at -80^{o}C in 25% glycerol at days 4, 12, 18 and 24.

Isolation of *P. aeruginosa* colonies from evolved populations

Pseudomonas aeruginosa colonies were isolated from frozen glycerol stocks by spreading directly onto *Pseudomonas*-selective SCFM agar plates and grown for 16 hours at 37°C. A single colony from each individual population from all treatments was randomly selected, grown for 16 hours in 200µl SCFM(m+) at 37°C with shaking, and 25% glycerol stocks made, resulting in a single clonal evolved *P. aeruginosa* population isolated from each replicate treatment population.

Observing the presence of *S. maltophilia* in co-cultures

The presence of *S. maltophilia* in co-cultures from all four sampling time points was evaluated by plating roughly equal quantities of frozen whole population glycerol stocks straight onto tobramycin containing SCFM agar plates (64 µg/ml) preventing *P. aeruginosa* growth. Plates were grown for 24 hours at 37°C. *S. maltophilia* was considered present if there was any observable growth of dot-like white colonies characteristic of *S. maltophilia*. This was independently replicated twice for each sampling point.

Quantifying imipenem resistance of evolved populations

Imipenem broth microdilution assays were independently performed in SCFM(m+) twice for each isolated clone to measure change in imipenem tolerance over the course of the selection experiment. Each isolated clone from all imipenem culture concentrations at every sampling day was assayed as previously described. Briefly, twelve serially diluted imipenem containing wells (0-128 µg/ml) in a single row of a 96 well plate were inoculated with diluted overnight culture creating a starting OD595 below 0.6 (approximately 3.75×10^5 CFU/ml). The assay row for each clone was randomised within replicates as previously described. Plates were grown for 24 hours at 37°C with constant shaking before lids were removed and OD595 measurements taken. Growth inhibition curves were constructed from the average OD595 scores for each well concentration. An overall score of imipenem resistance was measured as Area Under the Curve (AUC), this integral was measured from the combined area in OD595 between each successive imipenem concentration, calculated using the trapezium rule. 0 µg/ml wells were not included because they did not contain any imipenem. To account for the opacity of mucin in SCFM(m+) raising the AUC of wells with no growth, a baseline for pure 200 µl SCFM(m+) incubated at 37°C for 24 hours with shaking was established (0.585) and subtracted from all OD595 values before integral calculation. Furthermore, broth microdilutions require antibiotics to be filter sterilised, removing mucin and lowering the base OD595 values in the three highest concentrations (32-128 µg/ml). To account for this the average difference in OD595 below the baseline (0.585) for each concentration was calculated from sterile imipenem containing wells grown for 24 hours at 37°C and added before AUC calculation. This ensured a consistently even background baseline for each dilution, as the OD595 of uninoculated SCFM(m+) was highly consistent across all days. Lastly outliers were identified using a simple conservative mathematical model: If the AUC of a clone lay outside triple the range of the remaining nine clones for a given treatment combination and sampling day it was excluded. In total, only 5 of 300 (1.7%) of datapoints were considered as outliers and removed.

Measuring pyoverdine and pyocyanin production of evolved *P. aeruginosa* clones

Pyoverdine and Pyocyanin were quantified as previously described in Sass et al. (2017) [162] and O'Loughlin et al. (2013) [163]. Briefly, inoculation cultures from evolved clonal populations at the final sample point (day-24) were diluted to between 0.05-0.06 OD595 and grown in 200 µl SCFM(m-) for 22 hours at 37°C with shaking. Afterwards OD595 measurements were taken before cultures were centrifuged at 200 x g at room temperature for 30 minutes. 100 µl of supernatant was then collected and measured at OD wavelength: 405, 595 and 695 nm. Supernatants exceeding an OD595 reading of 0.3 indicated pellet dispersal and were excluded from the analysis. The background OD595 of uninoculated centrifuged media was subtracted before measurements were normalised to starting culture OD595: Relative Pyoverdine production = OD405/OD595, Relative Pyocyanin production = OD695/595 nm. All clones were measured six times and the average taken from at least four replicates, depending on pellet dispersal.

Measuring the growth of evolved *P. aeruginosa* clones in the absence of imipenem

The relative growth of evolved *P. aeruginosa* clones sampled from day 24 was estimated from 22-hour growth curves in the absence of imipenem. Overnight inoculation cultures were diluted and mixed twice by 15 µl in 185 µl SCFM(m-) giving a starting OD595 of between 0.095 and 0.10 (approximately 5.5 x 10^5 CFU/ml). Plate and well number was assigned by randomly ordering all evolved clones in sets of two replicates and allocating sequentially to each plate position across two 96-well plates. The order was re-randomised for each pair of replicates. One randomly placed well in every plate contained the ancestral PA01:rfp strain to control for variation. Plates with notably different control wells were excluded from later analysis (three plates in total). Plates were Incubated at 37°C with lids on and constant shaking for 22 hours with OD595 readings taken every 20 minutes and red fluorescence every two hours. Two metrics of growth were calculated: Rate of exponential growth or 'Max-V' and Area under the curve or 'total growth' [164]. Max-V (mOD595/hour) was measured as the maximum rate of linear change in OD595 over a given five-hour period, based on simple linear regression. It describes the initial speed of fresh substrate colonisation. Optical density does not directly relate to population size instead being proportional to the log of population size [165]. Therefore, by measuring the rate of linear change during the exponential log-phase we estimate the overall growth rate, 'r'. Total growth was determined as the complete area under the 22-hour growth curve, calculated as previously mentioned by the sum of area change in OD595 between each 20-minute interval, using the trapezium rule. It represents the capacity of a bacterial population to maintain sustained high-density growth.

Evaluating relative fitness of evolved *P. aeruginosa* clones by competition with the ancestral PA01

The fitness of individual evolved clones sampled from day-24 were quantified by performing competition assays with the non-fluorescently tagged PA01 ancestor in SCFM(m+). Clones and PA01 were separately grown in SCFM(m+) inoculation cultures before 15 µl was diluted in 185 µl SCFM(m+). 15 µl of diluted evolved clone and 15 µl of PA01 ancestor were then combined with 170 µl SCFM(m+), mixed, and incubated at 37°C for 24 hours with constant shaking. The starting number of cells for each was kept roughly equal (approximately 5.35 x 10^5 CFU/ml each). OD595 and red fluorescence intensity readings were taken after 24 hours. The background OD595 (0.585) and RFI (450) values of uninoculated SCFM(m+) were subtracted from the final 24-hour OD595 and RFI

scores. RFI values were then normalised to final culture OD595 (RFI/OD595), showing the proportion of co-culture consisting of fluorescent cells, and averaged across at least ten replicates. Relative well and plate positions for each group of two replicates were randomised as previously described. At least one well per plate contained PA01 and PA01:rfp to measure the ancestral fitness and control for variation between plates and days. Five out of 22 plates were excluded in total due to contamination. The competitive growth scores of evolved clones were normalised against the competitive score of the ancestral PA01:rfp competing against PA01. The average competitive score for the ancestral PA01:rfp strain was subtracted from the average for each evolved clone before being divided by the ancestral value. This generated a competitive index relating to the relative increased in competitive ability, with the ancestral fitness represented as 0. A relative phenotypic increase of 1.0 represents a doubling compared to the ancestor. This same approach of normalising phenotypic changes relative to the ancestor was also performed for Max-V growth rate, Total growth rate, Pyocyanin production and Pyoverdine production. Since the relative proportion of difference between clones remains equal this transformation does not alter statistical testing. Lastly to assess if evolved clones had lost their ability to fluoresce over the course of the selection experiment, RFI scores from the 22nd hour of the previously performed monoculture growth curves were normalised against total growth AUC and compared. All clones had maintained roughly equal RFI relative to total growth except for two outliers that over-expressed red fluorescence and which were then removed from the final dataset.

$$\boldsymbol{Competitive\ ability} = \frac{(\boldsymbol{Culture\ RFI - Media\ RFI})}{(\boldsymbol{Culture\ OD - Media\ OD})}$$

$$\boldsymbol{Competitive\ index} = \frac{\boldsymbol{Evolved\ competive\ ability - Ancestral\ competitive\ ability}}{\boldsymbol{Ancestral\ competitive\ Ability}}$$

Sequencing of evolved *P. aeruginosa* clones

48 clonal isolates of *P. aeruginosa* were sequenced in total: Six from individual monocultured populations and six from individual populations co-cultured with *S. maltophilia*, sampled from day 24 for each imipenem culture concentration (0 µg/ml, 1 µg/ml, 4 µg/ml and 8 µg/ml). The starting ancestral PA01:rfp strain was also sequenced. Genome sequencing was provided by MicrobesNG using the following protocol: Three beads were washed with extraction buffer containing lysozyme (or lysostaphin for Staphylococcus sp.) and RNase A, incubated for 25 min at 37oC. Proteinase K and RNaseA were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on an Illumina instrument using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic (version: 0.30) with a sliding window

quality cut-off of Q15 [166]. De novo assembly was performed on samples using SPAdes (version: 3.7)[167], and contigs were annotated using Prokka (version 1.11) [168].

Genomic analysis of evolved clone sequences

SNP and indel variants were identified by assembling paired-fastq reads of each sequenced clone to the assembled ancestral PA01:rfp genome and the published PA01 reference genome 'AE004091.2' [169] using the program Snippy from Galaxy (version: 4.5.0) [170]. Nucleotide base differences with a minimum coverage of five or more and a proportional change of at least 0.9 were considered sufficient to indicate an SNP. Ancestral PA01:rfp paired-fastq reads were compared against its own assembled genome to identify errors in the assembly process, two SNP's which were also present in over 90% of clonal genomes were identified and excluded. Contigs were viewed using the integrative genomic viewer (version: 2.8.10). The chromosomal location and impact on protein translation of SNPs were identified using BLASTnt and BLASTx searches [171] on the surrounding 400bp region. The effect of codon changes on amino acid translation were calculated and compared against the annotated AE004091.2 genome [169]. Commonly evolved SNPs in non-coding regions were characterised by identifying upstream and downstream genes using BLASTnt. Association with resistance was defined as when the same SNP appeared at least twice in separate clones and when over 70% of mutants had evolved resistance. Lastly, the structural and functional properties of the proteins encoded by mutated genes were identified by comparing information across three databases: National Center for Biotechnology Information (NCBI) [171], UniProt [172], and *Pseudomonas* genome DB [173].

Statistical Analyses

Statistical significance of the effect of experimental conditions was primarily determined through direct mean comparisons between treatment groups using a standard least squares model with an emphasis on effect leverage. This gives reliably accurate significance scores and enables more in-depth comparisons compared with alternatives, such as mixed effect models which take population as a random effect. Significance to reject a null hypothesis of no effect was universally determined at $p<0.05$. Two tailed t-tests were used to determine statistical significance between paired treatment group means. Assumption of equality of variance was based on both the experimental design and the distribution of data points. When within group variation and samples sizes were equal pooled Students t-tests were used due to superior statistical performance [174]. When these assumptions were not met un-pooled Welch's t-tests were used. Assumptions of variance were confirmed using Levene's test. To evaluate if evolved populations statistically differed from the starting ancestral strain Dunnett's tests were used, independently comparing average scores of multiple treatment groups to the ancestral mean. The ancestor represents a single population and therefore replicate measurements were used to compare against multi-population treatment averages. High variation between ancestor replicates or evolved clones decreases the likelihood observed changes in treatment groups are significantly different from the ancestral phenotype.

To compare the effect of treatments with more than two levels, primarily imipenem culture concentration and presence of competitor species, two-way full-factorial ANOVA's or Analysis of Covariance (ANCOVA), were used for comparing variance between two multi-level treatment groups, and to determine the significance of potential interaction effects. These were notably used to determine if gradients between linear regressions were significantly different. When the direction

of effect was not linear across treatment groups post-hoc tests each pair-t or Dunnett's tests were also applied. Non-parametric tests were used when data violated the core assumption of normality, primarily during SNP analysis. Wilcoxon and Kruskal-Wallis tests of mean rank score were used for comparing two or more than two treatment levels respectively. Owing to the variety of tests used each will be clearly signposted. Data handling and outlier identification was performed in excel (version: 2009). Statistical analyses and initial graph drawing were performed in R (version: 4.0.1). Graphics were enhanced and theoretical diagrams drawn in Inkscape (version: 1.0.1). Genome circle plots were made in R using modified code originally made by Jack Law at the Friman lab group, University of York.

MIC Significance Testing

To avoid the statistical problems associated with semi-qualitative MIC scores [175], changes in antibiotic tolerance between LB media and SCFM(m-) grown strains were compared by taking the mean OD595 value across the imipenem concentrations of the MIC curves for each replicate, excluding 0 μg/ml. The significance of these averages was then evaluated using Student's t-tests. This took into account both the variance in MIC between and the degree of growth within individual antibiotic concentrations. To adjust for some strains growing at higher densities in SCFM(m-) compared to LB media, SCFM(m-) MIC curves were normalised against those for LB. This was done by averaging the difference in OD595 between the 0 μg/ml concentration wells in LB and SCFM(m-) for each strain-antibiotic pair. This difference was then subtracted from the OD595 scores for all other SCFM(m-) well concentrations. This difference was not subtracted from growth inhibited SCFM(m-) wells giving an equal baseline for both medias. These adjustments completely removed the bias between growth in SCFM(m-) and LB media. The reliability of this significance method was also assessed. Firstly, LB and SCFM(m-) replicates with equal MIC curve averages had equal MIC scores, demonstrating no false negatives. Secondly, Student's t-tests between the MIC curve averages of LB and SCFM(m-) replicates with equal MIC scores were all not statistically significant, indicating no false positives. For PA01:rfp polycultures this process was performed for RFI in place of OD595.

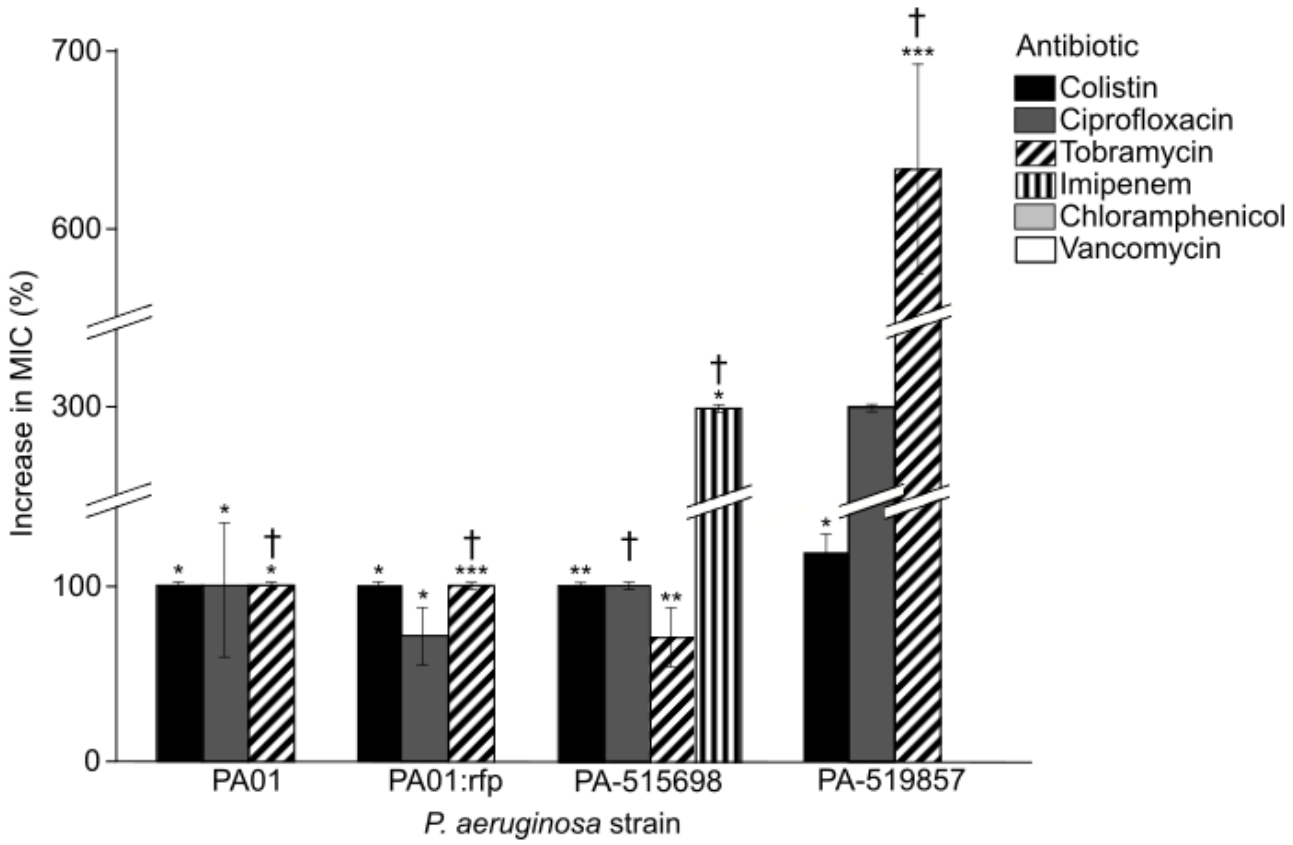
Results

Section 1: Screening CF bacterial strains

Initially, two primary research questions were asked at the start of this study. Does CF lung sputum significantly alter the antibiotic tolerance of bacterial pathogens and can clinical *S. maltophilia* or *S. aureus* protect sensitive *P. aeruginosa* against antibiotic treatment in co-cultures. The first question needed to be addressed so we could evaluate if clinically accurate growth media would be a necessity for later assays modelling *in vivo* resistance evolution. We proposed that the nutritional and structural complexity of SCFM and mucin could provide a generic protective effect against most antibiotics, increasing tolerance. The second question was aimed at identifying the presence and significance of potential interspecies interactions that may take place in CF lung communities. We hypothesised that clinical strains with high resistance could provide inter-specific protection to sensitive *P. aeruginosa,* manifesting as a capacity to grow beyond its normal MIC.

The growth substrate of the CF lung substantially impacts the standing minimum inhibitory concentration of bacterial species

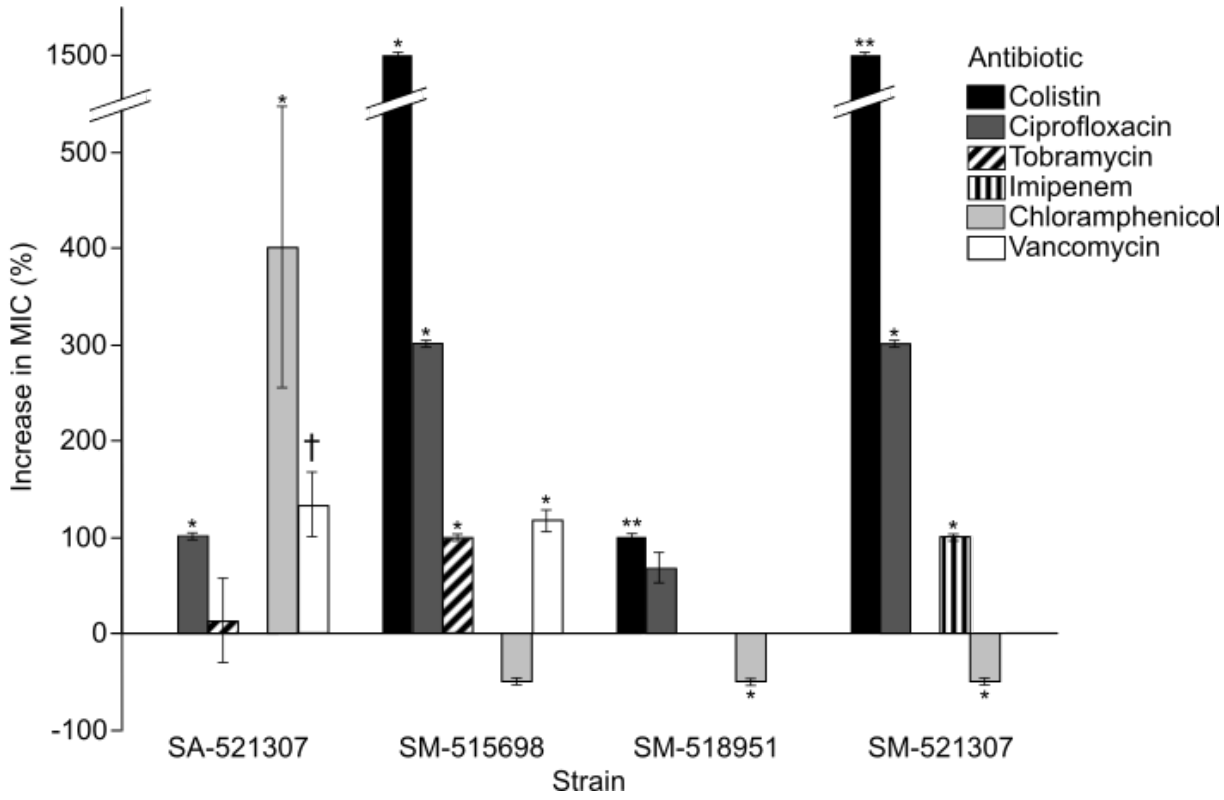
To study the effect CF lung sputum substrate has on antibiotic tolerance, selected bacterial strains were grown in SCFM(m-) and LB media across a range of antibiotics and concentrations. Overall, bacteria reached higher MIC scores in SCFM(m-) compared to LB media (ANOVA: $F(1,77)=11.6302$, $p=0.0010$), and this was notably associated with a clear interaction effect between antibiotic and species (ANOVA: $F(10,77)=2.1899$, $p=0.0271$). Interestingly, within species the degree of this effect often followed consistent trends, while also depending on the individual strain in question. In *P. aeruginosa*, growth in SCFM(m-) universally increased antibiotic tolerance to tobramycin, colistin and ciprofloxacin, with clinical strains showing markedly higher increases compared to laboratory PA01 for tobramycin, ciprofloxacin and imipenem (Fig. 1). The MIC of *P.* aeruginosa against vancomycin and chloramphenicol was not affected by media type, likely due to all strains possessing high innate resistance to these antibiotics. Generally, higher MIC scores in LB were not associated with greater percentage MIC increases in SCFM(m-) (ANOVA: $F(44,1)=2.7424$, $p=0.1048$), demonstrating that strains with higher tolerance in LB media did not undergo greater increases in SCFM(m-).



<u>Figure 1</u>: Increase in the antibiotic tolerance of *P. aeruginosa* in SCFM relative to LB media (0 denotes baseline of no difference).

The MIC for clinical and laboratory *P. aeruginosa* strains grown in LB media and SCFM(m-) were taken across six antibiotics: colistin (black), ciprofloxacin (dark grey), tobramycin (dashed), imipenem (vertical striped), chloramphenicol (light grey) and vancomycin (white). Represented as the relative percentage increase in MIC (μg/ml) from LB media to SCFM(m-). Culturing in SCFM causes both general and isolate-specific increases in antibiotic MIC score for *P. aeruginosa*. MIC scores in each media were replicated at least three times. Error bars are constructed from ± one standard error of the mean from both LB media and SCFM(m-) replicates. Statistical significance at $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***) was determined using Students t-tests between the mean MIC curve OD595 values of media replicates normalised to the difference in growth in 0 μg/ml. The cross demarcation represents shifts from antibiotic sensitivity to clinical resistance as defined by the ECUAST 2019 clinical breakpoints [158].

Like *P. aeruginosa*, *S. maltophilia* strains demonstrated both consistent and strain specific changes in MIC when grown in SCFM(m-) across the assayed antibiotics. Synthetic CF sputum consistently increased antibiotic tolerance against colistin and ciprofloxacin. Although the percentage increase in MIC for strain SM-515698 and SM-521307 was identical, SM-518951 demonstrated substantially lower increases, potentially linked to its lower overall resistance in LB media (Fig. 2). Strain specific increases in tolerance were notably observed in individual isolates against tobramycin, imipenem and vancomycin. *S. maltophilia* also demonstrated the only observed case of SCFM substrate decreasing antibiotic tolerance, reducing the MIC of all three strains equally against chloramphenicol. Since only one clinical *S. aureus* strain was tested we cannot infer potential inter-strain differences, however its profile of MIC change across antibiotics was notably different from both *P. aeruginosa* and *S. maltophilia*.



<u>Figure 2</u>: Impact of SCFM on the antibiotic tolerance of clinical CF isolates relative to LB media (0 denotes baseline of no difference).

The MIC for four clinical isolates of *S. aureus* and *S. maltophilia* in colistin (black), ciprofloxacin (dark grey), tobramycin (dashed), imipenem (vertical striped), chloramphenicol (light grey) and vancomycin (white), were tested in LB media and SCFM(m-). Displayed in relative percentage increase in MIC (μg/ml) from LB to SCFM(m-). SCFM induces increases and decreases in antibiotic tolerance, varying across species, strain and antibiotic. Each treatment was replicated at least three times. Error bars are constructed from ± one standard error of the mean from replicates for both medias. The 100% increase in colistin for SM-518951 exceeded the upper concentration of the MIC assay meaning it could be an underestimate. Statistical significance at $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***) was measured using Students t-tests between mean MIC OD595 values of LB media and SCFM(m-) replicates normalised to differences in total growth at 0 μg/ml. The cross represents shifts from antibiotic sensitivity to clinical resistance as defined by the ECUAST 2019 clinical breakpoints [158].

Most observed increases in MIC from LB to SCFM for individual strain-antibiotic pairings were significant (Table 1). Insignificant increases represent strains with only marginally increased growth in higher antibiotic concentrations. The clinical isolate PA-519857 is particularly interesting because although it possessed lower levels of tolerance to tobramycin in LB media compared with strain PA-515698, it demonstrated substantially higher MIC values when grown in SCFM. PA-515698 displayed a similar response against imipenem, increasing its MIC from LB to SCFM while all other strains demonstrated no altered tolerance between the two medias. This confirms that while using substrate that mimics CF lung sputum results in increased tolerance to a range of antibiotics, the degree of this effect varies considerably between clinical strains and individual species.

	Effect of Growth Substrate on Average MIC											
Strain	Colistin [2]		Ciprofloxacin [0.5]		Tobramycin [4]		Imipenem [4]		Chloramphenicol [8]		Vancomycin [2]	
P. aeruginosa	LB	SCFM	LB	SCFM	LB	SCFM	LB	SCFM	LB	SCFM	LB	SCFM
PA01	2.0	4.0 (*)	0.083	0.167 (*)	2.0	4 (*)	2.0	2.0	64	64	>128	>128
PA01:rfp	2.0	4.0 (*)	0.073	0.125 (*)	2.0	4 (***)	2.0	2.0	64	64	>128	>128
PA-515698	2.0	4.0 (**)	0.5	1.0	4.7	8 (**)	1.0	4 (*)	512	512	>128	>128
PA-519857	1.8	4.0 (*)	0.25	1.0	2.0	14.7 (***)	2.0	2.0	512	512	>128	>128
S. aureus												
SA-521307	>128	>128	0.125	0.25 (*)	2.7	3.0	<0.125	<0.125	5.3	26.7 (*)	1.0	2.3
S. maltophilia												
SM-521307	2.0	32 (**)	4.0	16 (*)	>256	>256	4.0	8 (*)	16.0	8 (*)	>128	>128
SM-515698	0.5	8 (*)	4.0	16 (*)	64	128 (*)	>256	>256	16.0	8.0	58.7	128 (*)
SM-518951	64.0	128 (**)	4.0	6.7	>256	>256	>256	>256	16.0	8 (*)	>128	>128

Table 1: Average antibiotic MIC of bacteria cultured in LB media and SCFM.

MIC scores of *P. aeruginosa*, *S. aureus* and *S. maltophilia* against colistin, ciprofloxacin, tobramycin, imipenem, chloramphenicol and vancomycin when grown in either standard LB media or SCFM(m-). Growth substrate alters antibiotic sensitivity depending on species, isolate and antibiotic. The clinical breakpoint for each antibiotic is included in '[]' µg/ml. Each MIC treatment combination was replicated at least three times. Statistical significance of change from LB to SCFM is measured at $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***) and was determined by paired Student's t-tests between average MIC curve OD595, normalised to total growth in 0 µg/ml.

While resistant *S. aureus* and *S. maltophilia* do not confer protection to sensitive *P. aeruginosa* against most antibiotics, *S. maltophilia* does provide protection against imipenem in SCFM

To investigate if antibiotic resistant clinical strains could confer protection to *P. aeruginosa*, sensitive PA01:rfp was grown in co-cultures with *S. maltophilia* (SM-518951) and *S. aureus* (SA-521307) in both SCFM(m-) and LB media. *P. aeruginosa* specific growth was measured through intensity of red fluorescence. There was not a measurable difference in *P. aeruginosa*'s MIC against tobramycin, colistin, ciprofloxacin, chloramphenicol and vancomycin when grown in LB or SCFM(m-) in *S. aureus* and *S. maltophilia* polycultures. In imipenem, clinical *S. maltophilia* consistently raised the MIC of clinically sensitive PA01:rfp from 2 μg/ml to 8 μg/ml in SCFM(m-) (Fig. 3A). This extended beyond the 4 μg/ml breakpoint used for determining clinical imipenem resistance [158], conferring passive resistance to PA01:rfp. Moreover, a full-factorial ANOVA of PA01:rfp's imipenem MIC AUC, measured in red fluorescence intensity, was performed across monoculture, *S. maltophilia* co-culture and *S. aureus* trio-culture treatments. The presence of *S. maltophilia* alone did not significantly impact *P. aeruginosa* tolerance (AUC: $F(1,70)=0.0036$, $p=0.9522$; Fig. 3B). However it did increase tolerance when interacting with SCFM(m-) (AUC: $F(1,70)=8.5217$, $p=0.0047$). *S. maltophilia* protection was observed in SCFM(m-) for both duo-cultures with *P. aeruginosa* and trio-cultures with *P. aeruginosa* and *S. aureus*. Although PA01:rfp exhibited lower red fluorescence intensity at 1 μg/ml and 2 μg/ml imipenem concentrations when grown in the trio-culture (Welch's t-test: $t(46.7)=2.3721$, $p=0.0219$), indicating reduced protection when *S. aureus* is present. It was also observed that SCFM(m-) significantly increased monocultured *P. aeruginosa*'s growth at lower imipenem concentrations (AUC: $F(1,70)=5.7787$, $p=0.0186$), showing SCFM can still improve growth at sub-inhibitory concentrations even when it does not alter the overall MIC score.

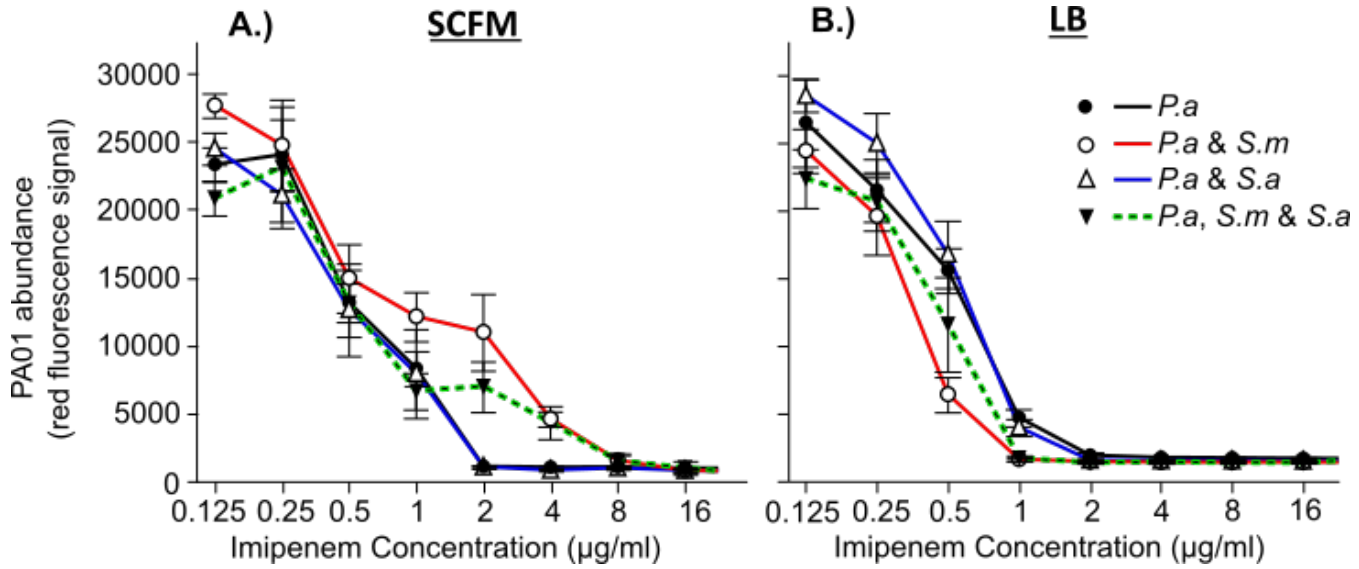


Figure 3 – ***S. maltophilia*** **increases** ***P. aeruginosa*****'s tolerance to imipenem in SCFM.**

Plot of average imipenem MIC curves for monocultures (black circle), co-cultures (white circle/triangle) and trio-cultures (black inverted triangle) of laboratory PA01:rfp in the absence and presence of clinical *S. maltophilia* (red) and *S. aureus* (blue) cultured in SCFM(m-) (A) and LB media (B). Curves are constructed from average red fluorescence intensity measurements (ex:em 532:588nm) and indicate PA01:rfp-specific growth. The presence of *S. maltophilia* confers passive protection to sensitive *P. aeruginosa* against imipenem in SCFM, enabling it to grow beyond the threshold for clinical resistance. MIC curves were replicated at least seven times and error bars show ± 1 standard error of the mean. Dotted green lines represents trio-cultures.

In summary, comparing MIC scores between LB media and SCFM(m-) demonstrated lung substrate does markedly alter the resistance profile of bacterial isolates, in several instances passing beyond the Eucast clinical breakpoint for resistance determination [158]. This means that not only is standard laboratory media unsuitable for *in vitro* assays attempting to model *in vivo* antibiotic treatment, but that lung sputum is likely responsible for clinical sensitivity screening poorly correlating with successful treatments [113]. Furthermore, although interspecific protection was not found to emerge in the vast majority of cases, *S. maltophilia* was able to provide significant protection to *P. aeruginosa* against imipenem when grown in SCFM(m-). Considering that this effect occurred at clinically significant concentrations and emerged from low starting cell densities, it warranted further investigation, since it is highly likely to take place in *in vivo* CF lung communities and therefore potentially impact treatment outcomes.

Section 2: Investigating *S. maltophilia*-mediated protection of *P. aeruginosa* from imipenem

Having established that *S. maltophilia* can protect *P. aeruginosa* against imipenem by enabling growth beyond the clinical resistance breakpoint, we proposed two potential mechanisms to help explain this finding and design subsequent assays. Particularly focusing on why this protective interaction only emerged for this specific antibiotic and when cells were grown in media that mimicked CF growth conditions.

Hypothesis 1.) *S. maltophilia* drives imipenem detoxification

S. maltophilia possesses two genomic beta-lactamase genes bla_{L1} and bla_{L2} [176] and the extracellularly secreted beta-lactamase L1 confers broad spectrum carbapenem resistance [177] by hydrolysis [178]. This protein is constitutively expressed in many *S. maltophilia* strains [179] and explains the high resistance observed in 2/3 tested clinical isolates. We would expect imipenem to naturally break down in solution incubated at 37^0C, meaning the presence of *S. maltophilia* alters the dynamics of imipenem degradation from negative linear to negative exponential, as increasing cell density generates more beta-lactamases at a higher rate over time. This would substantially reduce the total time *P. aeruginosa* has to survive in inhospitable media. Since imipenem has bactericidal activity by disrupting cell wall synthesis [180], *P. aeruginosa* is actively killed in concentrations above its MIC in contrast to simply having its growth inhibited. As a result, the longer populations spend in environments containing lethal levels of imipenem, the less likely individual cells will survive to colonise the media after antibiotic concentrations fall. However, the observation that protection was only conferred when co-cultures were grown in SCFM demonstrates that the presence of *S. maltophilia* alone does not explain the interaction. We would not expect the production of constitutively expressed factors to substantially vary between medias. Therefore, the growth substrate itself plays an equally substantial role in enabling *S. maltophilia* to provide a protective effect.

Hypothesis 2.) Mucin derived protection

The mesh-like structure of aqueous mucin in SCFM has the potential to both increase the survival of *P. aeruginosa* in lethal imipenem concentrations and enhance the relative effect of *S. maltophilia*-mediated antibiotic degradation. Mucin causes a two-fold delay in beta-lactam diffusion rates [181] and binds several antibiotic classes reducing their efficacy [182, 183]. This may lower the average exposure of *P. aeruginosa* to imipenem, reducing rate of cell death at otherwise lethal concentrations. Furthermore, the composition of mucin imposes a physical barrier that likely enables the formation of structural pockets, generating reservoirs of *P. aeruginosa* cells partially isolated from the media. This effect would be amplified by biofilm production, as mucin serves as an attachment surface promoting biofilm formation in *P. aeruginosa* [184]. Together, these factors could increase survivability until *S. maltophilia* detoxifies the global environment. However, they may also reduce the diffusion of nearby beta-lactamases away, enabling *S. maltophilia* to detoxify the local environment. The structural heterogeneity imposed by mucin could lead to structural reservoirs detoxifying at a faster rate, enabling *P. aeruginosa* to survive until the whole environment becomes habitable for colonisation.

Mucin alone is not the component of SCFM that enables *S. maltophilia* to protect *P. aeruginosa* against imipenem

We hypothesised that if mucin was the active component of SCFM, the addition of mucin to standard LB media would enable *S. maltophilia* to protect *P. aeruginosa* against imipenem. Imipenem broth microdilution assays were performed for *P. aeruginosa* monocultures and *S. maltophilia* co-cultures in LB media and LB supplemented with 5g/L mucin. *P. aeruginosa* specific growth was measured in red fluorescence intensity (ex:em 532:588nm). It was observed that LB + mucin did not induce any change in PA01:rfp's MIC when co-cultured with *S. maltophilia* (SM-518951) compared to standard LB media, remaining at 4 µg/ml (Fig. 4). This was reflected in there being no significant difference between MIC AUC scores for co-cultures in both media types (Student's t-test: MD=5928, SD=5499; t(12)=1.08, P=0.3031). Following previous results, *S. maltophilia* presence did not significantly impact *P. aeruginosa*'s tolerance in standard LB media compared to monoculture (Students t-test: MD=7681, SD=7151; t(22)=1.07, P=0.2945). However, we did observe that including clinical levels of mucin did increase *P. aeruginosa*'s tolerance to imipenem, raising the functional MIC of PA01:rfp from 2 µg/ml to 4 µg/ml (MIC AUC Students t-test: MD=52601, SD=6159; t(11)=8.54, p=<0.0001). Therefore, despite providing its own protective effect, mucin alone was not responsible for *S. maltophilia* protecting *P. aeruginosa* against imipenem in SCFM.

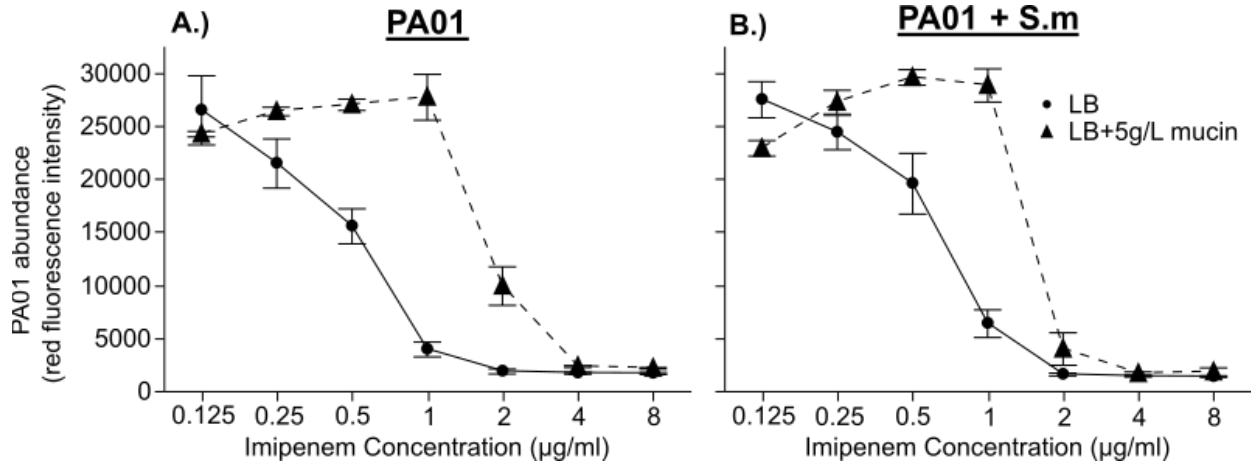


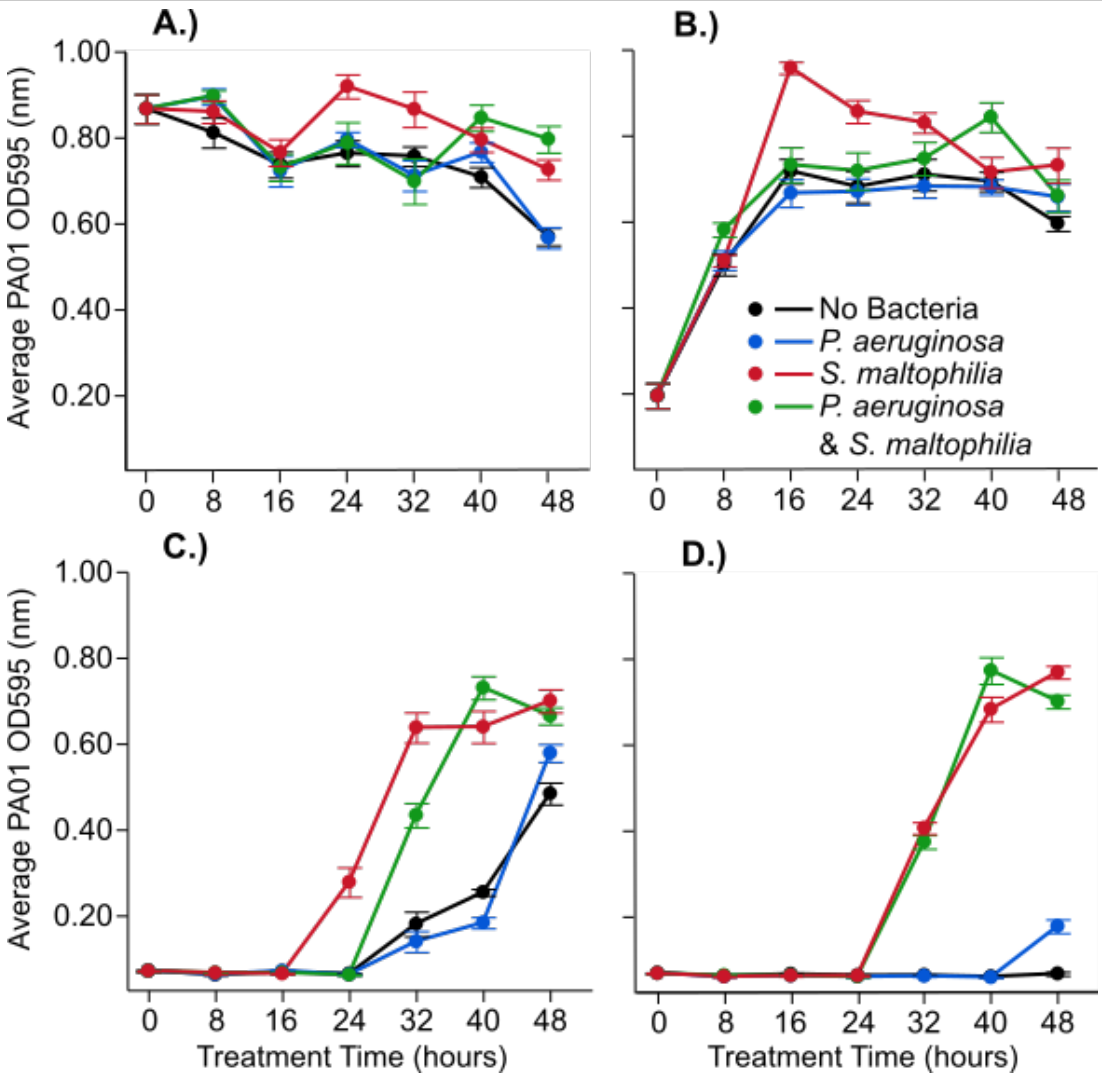
Figure 4 – Impact of mucin on *P. aeruginosa* imipenem tolerance.

Plots of the average imipenem MIC curves for monocultured PA01:rfp (A) and PA01:rfp co-cultured with clinical *S. maltophilia* (B) in LB media (circles) and LB media + 5g/L porcine mucin (triangles) measured in red fluorescence intensity (ex:em 532:588nm). The red fluorescence of LB + 5g/L mucin was normalised to match the baseline of LB media. Mucin does provide a protective by increasing imipenem tolerance in monocultures, however it does not enable the emergence of *S. maltophilia* mediated protection in co-cultures. Error bars represent ± one standard error from the mean.

Inoculating with *S. maltophilia* increases the rate imipenem treated SCFM becomes habitable for *P. aeruginosa* at higher clinical concentrations

To establish if *S. maltophilia* actively degraded imipenem its rate of loss from imipenem treated SCFM(m+) was indirectly measured by observing how well sensitive *P. aeruginosa* could grow in its sterile filtrate. We anticipated that *S. maltophilia* could confer antibiotic protection even after it was removed from culture, leading to higher overall *P. aeruginosa* growth. SCFM(m+) was treated with four imipenem concentrations: 0 μg/ml, 1 μg/ml, 4 μg/ml and 8 μg/ml, and inoculated with either no bacteria, *P. aeruginosa* (PA01:rfp), *S. maltophilia* (SM-518951) or them both. This media was then periodically sampled and filtered before being re-inoculated with PA01:rfp, measuring imipenem degradation indirectly. As expected, higher imipenem concentrations resulted in significantly lower *P. aeruginosa* growth, measured in average OD595 over 24 hours of filtrate growth (ANOVA: $F(3,96)=57.4627$, $p<0.0001$). Bacterial treatment had a significant effect on PA01:rfp's growth in filtrate as imipenem was degraded at a faster rate when SCFM(m+) was inoculated with clinical *S. maltophilia* (ANOVA: $F(3,96)=4.0936$, $p=0.0088$; Fig. 5). However, this effect only emerged at higher imipenem concentrations (4 μg/ml: $F(3,20)=3.7411$, $p=0.0278$ and 8 μg/ml: $F(3,20)=9.2671$, $p=0.0005$) with no significant effect of bacterial treatment at lower concentrations (0 μg/ml: $F(3,20)=2.5418$, $p=0.0853$ and 1 μg/ml: $F(3,20)=0.5481$, $p=0.6551$). This effect was likely associated with the observation that the innate degradation of imipenem over time in SFCM(m+), reduced its antimicrobial activity at 1 μg/ml and 4 μg/ml treatment concentrations in the absence of bacteria.

In 4 μg/ml imipenem treated SCFM(m+), *S. maltophilia* monoculturing enabled PA01:rfp to grow in filtrate within 24 hours, compared to 32 hours in the no bacteria control treatment, while also enabling significantly higher growth overall, measured in 48-hour AUC growth (Welch's t-test: $MD=9.3162$, $SD=0.7245$; $t(6.90)=12.85887$, $P<0.0001$; Fig. 5C). *P. aeruginosa* alone had no effect on the rate of imipenem degradation, causing an insignificant change in 48-hour AUC compared to the no bacteria treatment (Welch's t-test: $MD=0.5107$, $SD=0.4517$; $t(9.80)=1.13062$, $p=0.2851$). Although interestingly co-culturing with *S. maltophilia* led to significantly reduced speed of detoxification and lower total PA01:rfp growth compared to the *S. maltophilia* monoculture (Welch's t-test: $MD=2.7551$, $SD=0.80375$; $t(8.89)=3.42779$, $p=0.007$). By the 40th hour of bacterial growth there was no substantial difference in PA01:rfp filtrate growth between either treatment. In contrast, there was no significant difference between *S. maltophilia* monoculture and *P. aeruginosa* co-culture treatments on PA01:rfp's growth in filtrate in SCFM(m+) treated with 8 μg/ml (Welch's t-test: $MD=0.2005$, $SD=0.4869$; $t(9.58)=0.41172$, $p=0.6896$; Fig. 5D). Although *P. aeruginosa* monoculturing did lead to marginal growth after 48 hours of treatment, compared to the no bacteria group where PA01:rfp filtrate growth was completely inhibited (Welch's t-test: $MD=0.341453$, $SD=0.119$; $t(9.53)=2.86261$, $p=0.0177$). Lastly we observed that SCFM gradually reduced its capacity to support PA01 growth over time in the absence of imipenem likely due to consumption of resources in SCFM(m+) (ANOVA: $F(1,20)=20.4508$, $p=0.0002$).



<u>Figure 5</u> – *S. maltophilia* increases the media habitability of imipenem treated SCFM.

SCFM(m+) containing 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) and 8 µg/ml (D) of imipenem was treated with no bacteria (black), PA01 (blue), clinical *S. maltophilia* (red) or a co-culture of the two (green) over a 48-hour period. The media was sampled in eight hour intervals and filter sterilised before PA01:rfp was grown in the filtrate to indirectly measure imipenem degradation. Growth was measured in OD595 (nm) every eight hours and averaged across 24 hours. Culturing *S. maltophilia* enabled *P. aeruginosa* to start growth earlier in 4 µg/ml and 8 µg/ml imipenem treated SCFM, while co-culturing with *P. aeruginosa* reduced this effect in 4 µg/ml. Each sampling point was replicated six times and error bars correspond to ± one standard error from the mean.

Lastly, to explore the possibility that *S. maltophilia* only detoxified imipenem when grown in SCFM, its capacity to degrade imipenem in LB media was also assessed. We anticipated that the constitutive expression of beta-lactamases should enable *S. maltophilia* to actively degrade environmental imipenem independent of substrate. Clinical *S. maltophilia* (SM-518951) was cultured in LB media containing 128 µg/ml imipenem, before the culture was filter sterilised and used to grow four sensitive bacterial strains for 24 hours. The filtrate of media not originally inoculated with *S. maltophilia* completely inhibited all bacterial growth over all sampling time-points (Fig. 6A), while growing clinical *S. maltophilia* in imipenem treated LB media had a significant effect of promoting the growth of otherwise susceptible *P. aeruginosa* isolates after 72 hours (Average OD595: $F(1,23)=6.0716$, $p=0.0216$); Fig. 6B). Notably, at the 48-hour mark there was minor growth of SM-521307 in both *S. maltophilia* absent and present treatment, which may represent its own beta-lactamase production. However, strains in general did not have a significant effect on average growth ($F(3,23)=0.0391$, $p=0.9894$). Although this data demonstrates that *S. maltophilia* does degrade imipenem in when cultured in LB, this was a preliminary assay performed before the experimental design was improved for SCFM(m+). Therefore, since the starting imipenem concentrations were different these results do not show that the rate of *S. maltophilia* mediated detoxification is slower when grown in LB media.

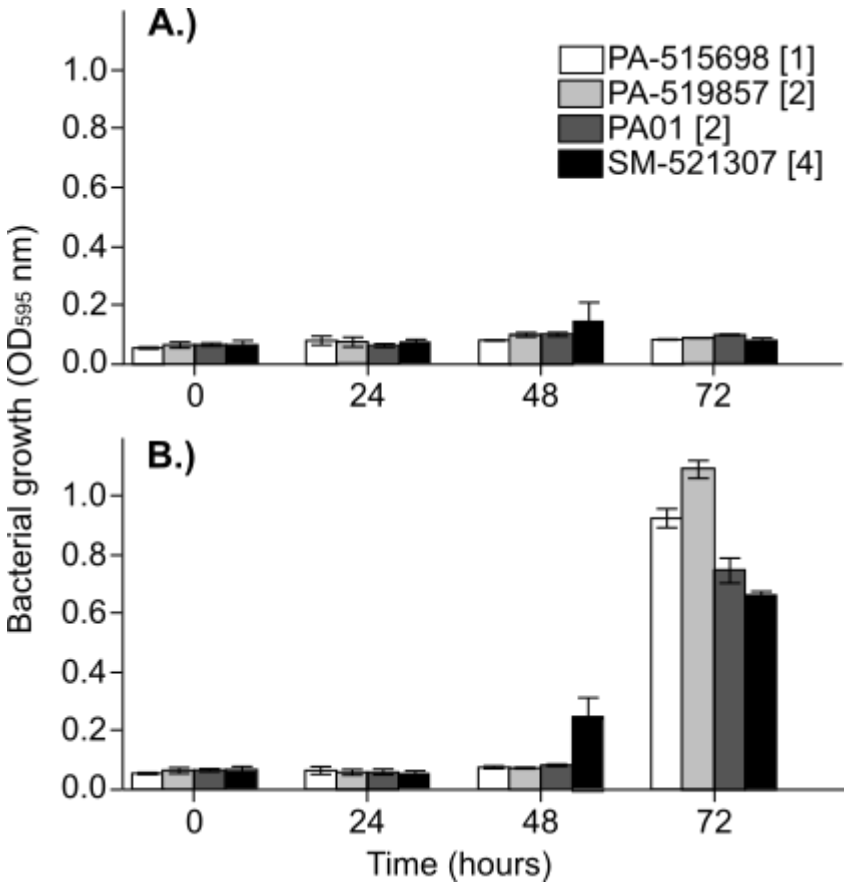


Figure 6 - *S. maltophilia* increases the habitability of imipenem treated LB media.

LB media containing 128 µg/ml imipenem was left sterile (A) or inoculated with clinical *S. maltophilia* (B) and periodically sampled and filtered every 24 hours for three days. Laboratory (white) and clinical (light and dark grey) isolates of *P. aeruginosa* alongside a low resistance *S. maltophilia* isolate (black) were grown in the filtrate for 24 hours before OD595 readings were taken. Previous growth of a highly imipenem resistant *S. maltophilia* isolate increased the capacity for sterile media to support sensitive bacterial growth. Averages for each strain were taken across three replicates for each sampling point. Error bars represent ± one standard error from the mean. Previously established imipenem MIC µg/ml scores when grown in LB are noted in '[]' µg/ml.

In conclusion, we found clear support for imipenem degradation by *S. maltophilia* being responsible for enabling *P. aeruginosa* to grow beyond its clinical breakpoint. Clinical *S. maltophilia* substantially protected PA01:rfp at 4 µg/ml and 8 µg/ml clinical imipenem concentrations, with some evidence that the presence of *P. aeruginosa* slightly reduced this effect. The inclusion of the structural component mucin alone was not responsible for explaining why this interaction only emerged in SCFM. However, considering that *S. maltophilia*'s capacity to degrade imipenem was not inhibited when grown in LB, it suggests another component of the SCFM is likely responsible. Having firmly established the dynamics and likely clinical importance of this interaction from an immediate ecological perspective we shifted our focus to investigate how the presence of *S. maltophilia* could affect the long-term emergence of imipenem resistance on an evolutionary scale.

Section 3: Experimental evolution in imipenem and SCFM

While it was found that the presence of *S. maltophilia* could increase *P. aeruginosa*'s tolerance to imipenem in short-term cultures, it was unclear if this relationship was evolutionarily stable and how it would affect resistance development in the long-term. We predicted that if there is inter-specific protection, there would be weaker selection for resistance evolution. This relationship could in turn change as a function of resistance, where the evolution of increased tolerance in *P. aeruginosa* reduces the relative benefit of the protective effect leading to increased selection. Initially, such reduced selection could promote smaller stepwise increases in resistance compared to the single large mutations often associated with stronger selection pressures. Furthermore, adaptations linked to antibiotic resistance could lead to reduced growth and fitness in the absence of antibiotics due to associated mechanistic costs. To evaluate if the presence of clinical *S. maltophilia* strain (SM-519851) had any impact on the evolution of imipenem resistance in *P. aeruginosa*, a 24-day selection experiment was performed in SCFM(m+) across four imipenem concentrations: 0 μg/ml, 1 μg/ml, 4 μg/ml, and 8 μg/ml. A subset of either monoculture PA01:rfp or co-cultures with *S. maltophilia* were passaged into fresh imipenem treated SCFM(m+) every two days simulating the repeated colonisation of CF lung sputum during antibiotic treatment. Ten replicate populations were evolved per treatment combination, and individual clones were isolated from each at day 4, 12, 18 and 24, before a series of phenotypic assays were performed including antibiotic tolerance, competitive fitness and pyoverdine and pyocyanin virulence factor production. The survival of *S. maltophilia* was confirmed using selective plating, and 48 evolved clones alongside the ancestral strain were genetically sequenced.

Imipenem treatment induces the competitive exclusion of resistant *S. maltophilia* by *P. aeruginosa*

To observe if *S. maltophilia* persisted with *P. aeruginosa* in co-cultures, its presence from each sampling timepoint was tested by plating glycerol stocks onto *S. maltophilia* selective agar. *S. maltophilia* was stably maintained in the majority of *P. aeruginosa* co-cultures not exposed to imipenem (Fig. 7). However, was rapidly lost when media was treated with the antibiotic. In the absence of imipenem 80% of co-cultures maintained a measurable level of *S. maltophilia* throughout the 24 days, while only 40% of co-cultures treated with 1 μg/ml imipenem stably retained *S. maltophilia*. In both 0 μg/ml and 1 μg/ml treatments all cultures that lost *S. maltophilia* had done so by the fourth day. Comparatively, in 4 μg/ml and 8 μg/ml 60% and 80% of cultures from day-4 respectively had retained *S. maltophilia*. However, by day-24 all had lost the bacterium, with the exception of one culture in the 4 μg/ml treatment. The majority of these co-cultures lost *S. maltophilia* by day twelve although a small number lost it at day-18 and day-24. Although the CFU/ml of *S. maltophilia* in co-cultures was not directly measured, in stable co-cultures the number of plated colonies ranged between 20 and 100 for 0 μg/ml imipenem and between 1 and 20 for 1 and 4 μg/ml imipenem treatments. Finally, all *S. maltophilia* monocultured populations grown in the absence of *P. aeruginosa* retained *S. maltophilia* after 24 days regardless of imipenem treatment concentration. This shows that although *S. maltophilia* can be stably be maintained in SCFM with either *P. aeruginosa* or imipenem, it cannot do so with both combined.

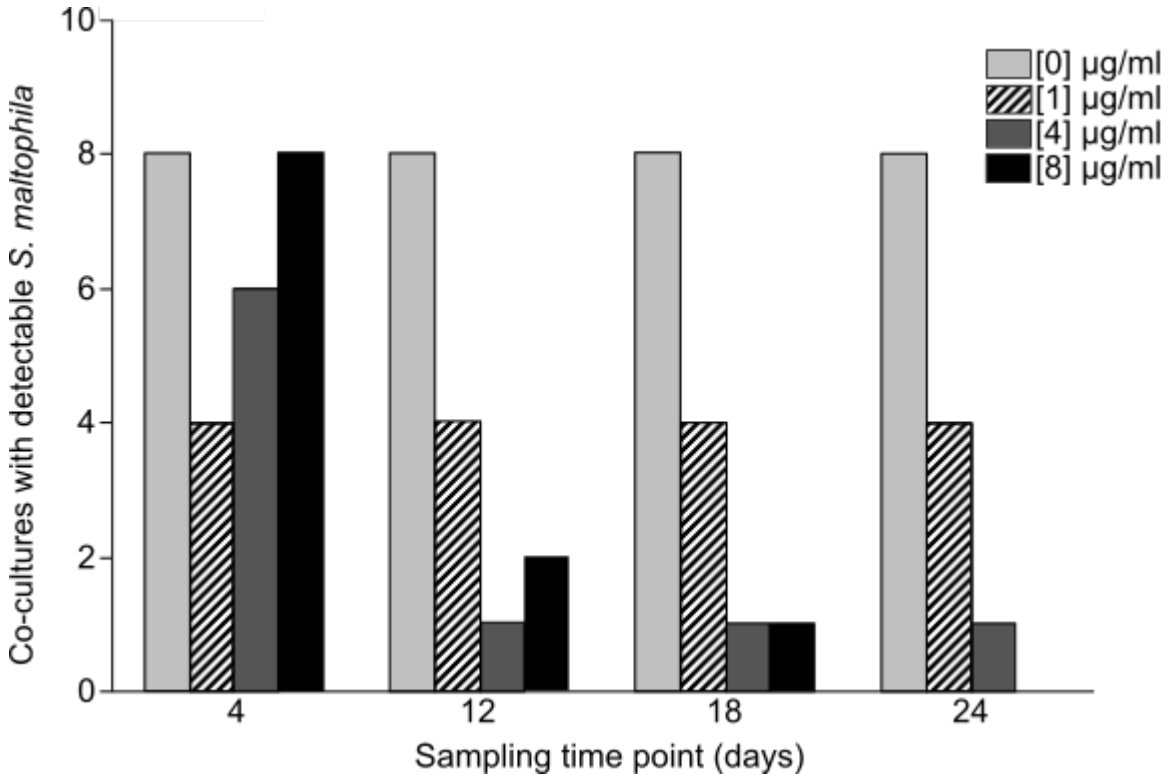
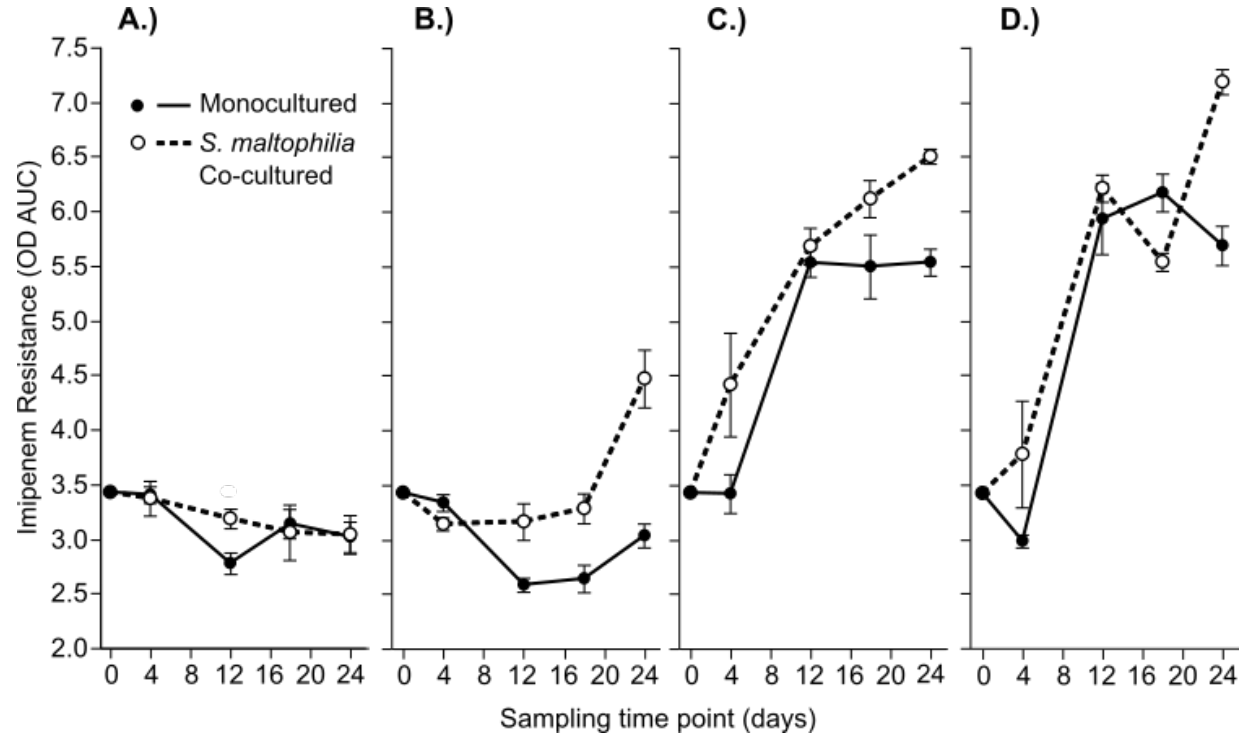


Figure 7 – Presence of *S. maltophilia* in *P. aeruginosa* co-cultures.

Presence of *S. maltophilia* was detected by streaking co-cultures with PA01:rfp onto *S. maltophilia* selective SCFM agar and identifying colony formation after 24 hours. This was independently performed twice for every set of ten population from each imipenem treatment 0 µg/ml (light grey), 1 µg/ml (striped), 4 µg/ml (dark grey) and 8 µg/ml (black), from each sampling time point over the 24-day selection experiment in SCFM(m+). *S. maltophilia* is stably maintained in co-cultures with *P. aeruginosa* in the absence of antibiotics, while it is steadily lost in a dose-dependent manner when co-cultures are treated with imipenem . Error bars are not included as all replicates were consistent. Only eight populations were sampled for each treatment on day four, the presence of *S. maltophilia* in one culture from 0 µg/ml was inferred based on presence in later sampling points.

Co-culturing with clinical *S. maltophilia* leads to faster emergence and higher overall levels of evolved imipenem resistance in *P. aeruginosa*

The evolution of imipenem resistance was determined by comparing the resistance of evolved *P. aeruginosa* clones from each sampling time point. Broth microdilution assays were performed in SCFM(m+) and the AUC of the resulting MIC curves were taken as an overall measure of resistance. Treating *P. aeruginosa* cultures with higher imipenem concentrations significantly increased the resistance of evolved clones (ANOVA: $F(3,292)=150.2280$, $p<0.0001$), and this effect increased over time, significantly interacting with sampling time point (ANOVA: $F(3,292)=53.9117$, $p<0.0001$; Fig. 8). Interestingly, co-culturing *P. aeruginosa* with *S. maltophilia* led to higher overall resistance on average compared to monocultures (ANOVA: $F(1,71)=13.7649$, $p<0.0004$). However, the presence of *S. maltophilia* did not significantly interact with sampling time to alter resistance ($F(1,71)=0.1617$, $p=0.6888$) indicating it only increased the magnitude of evolved resistance, not its overall rate. However, *S. maltophilia* co-culturing did lead to the faster emergence of resistance, as clones with increased resistance appeared by day-4 compared to day-12 for monocultures. Furthermore, by the end of the 24-day selection experiment higher levels of resistance for *S. maltophilia* co-cultured clones were still maintained (Welch's t-test - 1 µg/ml: $t(12.01)=5.030272$, $p=0.0003$, 4 µg/ml: $t(14.06)=6.885012$, $p<0.0001$ and 8 µg/ml: $t(13.76)=7.030621$, $p<0.0001$). This effect was particularly pronounced in the 1 µg/ml treatment where initial co-culturing with *S. maltophilia* entirely determined if *P. aeruginosa* evolved increased resistance. The increased resistance of *S. maltophilia* co-cultured clones, measured in MIC AUC, was also reflected in their functional MICs. The average MIC of monocultured *P. aeruginosa* clones from day-24 was: 1.7 (SD=0.95) in 0 µg/ml, 1.6 (SD=0.52) in 1 µg/ml, 16.0 (SD=0) in 4 µg/ml and 16.0 (SD=0) in 8 µg/ml imipenem treatments. While clones co-cultured with *S. maltophilia* had mean imipenem MIC scores of: 1.9 (SD=0.88) in 0 µg/ml, 5.2 (SD=2.53) in 1 µg/ml, 25 (SD=7.60) in 4 µg/ml and 32.0 (SD=0) in 8 µg/ml treatments. Over the course of the selection experiment there was only one instance of *P. aeruginosa* being completely eradicated from SCFM(m+) culture, this happened by the fourth day in a monoculture population treated with 8 µg/ml imipenem. Lastly, selection in SCFM in the absence of imipenem lead to significantly reduced imipenem resistance in evolved clones compared to the ancestral strain (Welch's t-test: $t(25.7)=3.89935$, $p= 0.0006$), with no significant effect of *S. maltophilia* co-culturing (Welch's t-test: $t(17.11)=0.099376$, $p=0.9220$). This means the evolution of resistance in *P. aeruginosa* is not a generic adaptation to *in vitro* SCFM(m+) condition and that the presence of clinical *S. maltophilia* is most substantial during imipenem treatment.



<u>Figure 8</u> – *S. maltophilia* enhances imipenem resistance evolution in *P. aeruginosa*.

The evolution of imipenem resistance in PA01:rfp clones was measured by comparing average MIC AUC OD595 (nm) scores from broth microdilution assays in SCFM(m+). Each point represents the average imipenem resistance score across ten clones isolated from independent populations of PA01:rfp monoculture (black circles) and clinical *S. maltophilia* co-culture (white circles). Populations were regularly treated with either 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) or 8 µg/ml (D) imipenem. The presence of *S. maltophilia* leads to the faster emergence and higher overall levels of imipenem to resistance. MIC scores for each clone was measured twice. Error bars are constructed from ± one standard error of the mean. In total 5/300 data-points were identified as outliers and removed; they did not observed relationships.

P. aeruginosa clones evolved reduced maximum growth rates in SCFM while *S. maltophilia* co-culturing leads to lower reductions

Growth curve profiles of evolved *P. aeruginosa* clones sampled from day-24 of the selection experiment were assayed over 22 hours in SCFM(m-) to evaluate if changes in growth had been evolved in response to experimental selection. The maximum growth rate of evolved clones decreased over the course of the selection experiment compared to the starting ancestral strain (Fig. 9). Interestingly this decrease was more pronounced in the absence of *S. maltophilia*, as co-cultured clones possessed significantly lower reductions in Max-V on average (ANOVA: $F(1,69)=5.6829$, $p=0.0199$). This effect was most pronounced at 0 µg/ml and 1 µg/ml imipenem concentrations and reflects how monocultured clones had significantly decreased maximum growth rates compared the ancestral control group (N=12) (Dunnett's test, 0 µg/ml ($p=0.0299$), 1 µg/ml ($p=0.0003$), 4 µg/ml ($p=0.0045$) and 8 µg/ml ($p=0.0372$) imipenem). While the difference for *S. maltophilia* co-cultured clones was not significant (Dunnett's test, 0 µg/ml ($p=0.1541$), 1 µg/ml ($p=0.0628$), 4 µg/ml ($p=0.1802$) and 8 µg/ml ($p=0.0538$) imipenem). Change in Max-V growth rates was independent of imipenem culture concentration (ANOVA: $F(3,69)=1.5023$, $p=0.2216$), or an interaction with *S. maltophilia* (ANOVA: $F(3, 69)=1.4781$, $p=0.2281$). In comparison to maximum rate of growth, total growth remained roughly equal compared to the ancestral strain, with no significant effect of imipenem culture concentration (ANOVA: $F(3,69)=1.4475$, $p=0.2374$), *S. maltophilia* co-culturing (ANVOA: $F(1,69)=0.9671$, $p=0.3292$), or an interaction between them (ANOVA: $F(3,69)=1.6758$, $p=0.1812$) on growth curve AUC (Fig. 10). Furthermore, total growth values within treatment combinations did not significantly differ from the starting ancestral control group (N=12) for both monoculture and *S. maltophilia* co-culture treatments (Dunnett's test, 0 µg/ml ($p=0.7406$ & $p=0.9183$), 1 µg/ml ($p=0.3900$ & $p=0.9046$), 4 µg/ml ($p=0.7617$ & $p=0.7497$) and 8 µg/ml ($p=0.4497$ & $p=0.5511$) imipenem, respectively). *S. maltophilia* co-culturing did result in higher total growth in the 8 µg/ml treatment compared to monocultures, (Welch's t-test: $t(14.98)=2.34539$, $p=0.0332$) however, considering this was only marginally significant and the effect did not appear in 4 µg/ml imipenem it was likely the result of experimental error.

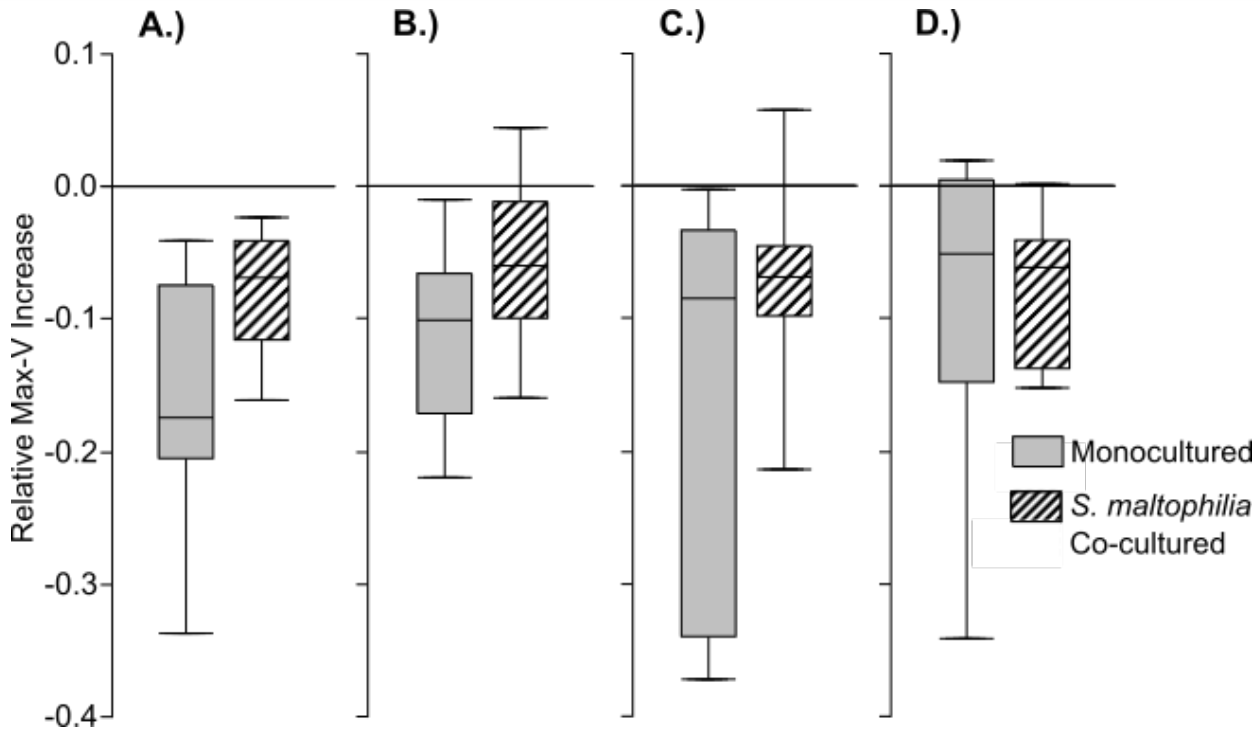


Figure 9: SCFM adaptation leads to reduced Max-V growth rates in *P. aeruginosa.*

The maximum rate of exponential growth (Max-V) was measured in SCFM(m-) for isolated monocultured (solid grey) and *S. maltophilia* co-cultured (dashed grey) *P. aeruginosa* clone from day-24 of the selection experiment in SCFM(m+). Clones came from independent populations regularly treated with either 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) or 8 µg/ml (D) imipenem. Rates were normalised to the ancestor to give relative increase. The average ancestral Max-V rate is represented as y=0. After long-term selection in SCFM(m+) Max-V growth rates decreased independently of imipenem culture concentration, however this effect was less pronounced in clones that had been originally cultured with clinical *S. maltophilia*. Max-V growth rates were replicated at least six times for each clone.

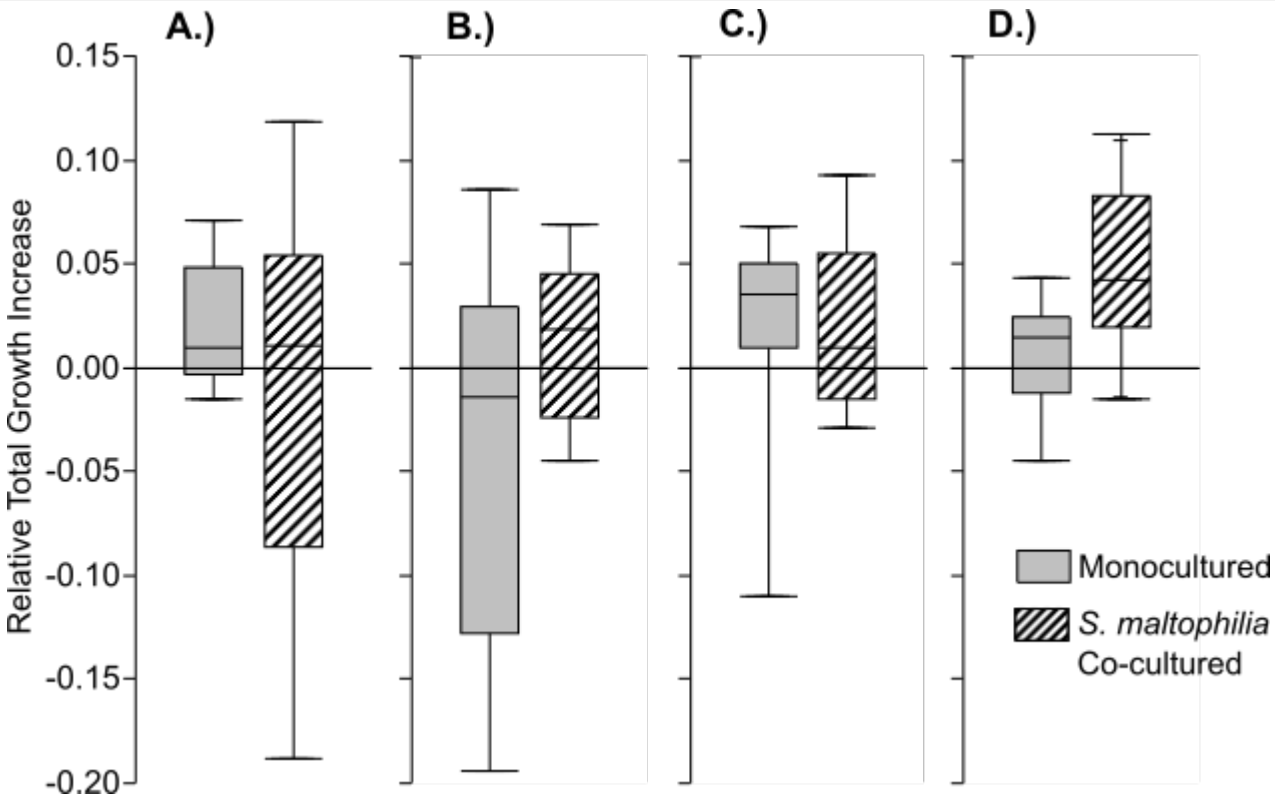


Figure 10: Total growth rate of SCFM evolved *P. aeruginosa.*

Area under the 22 hour growth curve (total growth) was measured in SCFM(m-) for monocultured (solid grey) and *S. maltophilia* co-cultured (dashed grey) PA01:rfp clones from 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) and 8 µg/ml (D) imipenem, sampled at day-24 from the selection experiment. Total growth was converted to relative total growth increase and the ancestral average is represented as y=0. Culturing in SCFM(m+) and the presence of imipenem or *S. maltophilia* did not have a significant effect. Total growth rate was calculated from at least four replicates for each clone

Evolving in SCFM increases the competitive ability of *P. aeruginosa* clones while imipenem treatment is associated with reduced adaptation

To investigate if SCFM adaption impacted competitive fitness, evolved *P. aeruginosa* clones were grown in SCFM(m+) alongside the non-red fluorescing variant of the ancestral PA01 in the absence of antibiotics. Culturing in higher imipenem culture concentrations was significantly associated with a clear reduction in competitive ability increase in *P. aeruginosa* clones (ANOVA: $F(1,70)=5.8578$, $p=0.0013$; Fig. 11). By the end of the selection experiment almost all *P. aeruginosa* clones across all treatments possessed at least a marginal increase in competitive ability compared to the ancestral PA01:rfp strain. On average, monocultured clones had significantly increased competition indexes compared to the ancestral control group (N=18) (Dunnett's test - 0 µg/ml: ($p=0.0003$), 1 µg/ml: ($p=0.0004$), 4 µg/ml: ($p=0.0033$), 8 µg/ml: ($p=0.0191$). This was shared by *S. maltophilia* co-cultured *P. aeruginosa* clones (Dunnett's test - 0 µg/ml: ($p=0.0015$), 1 µg/ml: ($p=0.01872$) and 4 µg/ml: ($p=0.0292$)) but was not significant in 8 µg/ml imipenem ($p=0.2018$). Lastly, evolving in the presence of *S. maltophilia* competitor did not appear to significantly affect competitive ability (ANOVA: $F(1,70)=1.1604$, $p=0.2851$), or significantly interact with imipenem concentration (ANOVA: $F(3,70)=2.6158$, $p=0.0578$). This was reflected when competitive indexes were compared between monocultures and *S. maltophilia* cocultured clones within imipenem concentrations (Welch's t-test - 0 µg/ml: ($t(11.9)=1.17924$, $p=0.2614$), 1 µg/ml: ($t(14.9)=2.0716$, $p=0.0561$), 4 µg/ml: ($t(17.9)=1.1112$, $p=0.2812$) and 8 µg/ml: ($t(16.1)=1.2335$, $p=0.2351$). Overall, these results suggest selection in SCFM does lead to media adaptation in *P. aeruginosa*, which is limited during clinical imipenem treatment.

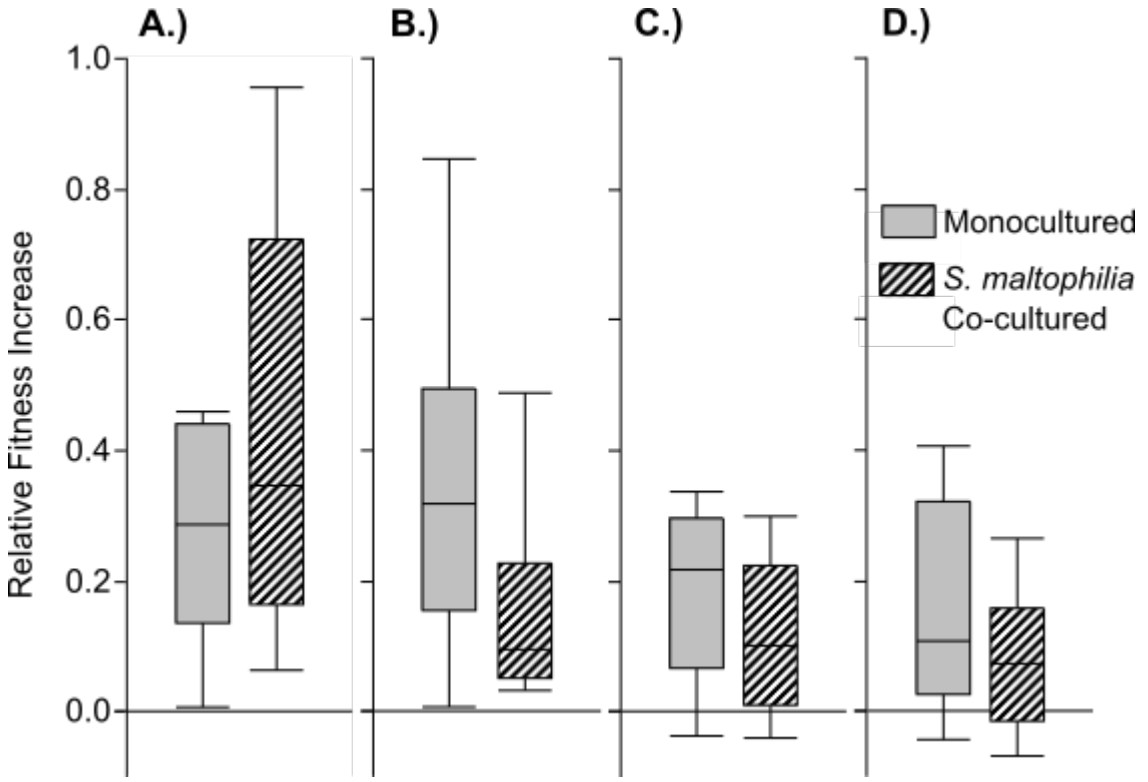


Figure 11: Competitive index of evolved *P. aeruginosa* clones.

Relative increase in competitive fitness was measured from the red fluorescence intensity of evolved *P. aeruginosa* clones from day-24 of the selection experiment competed against their non-red fluorescing ancestral strain. Cells were grown in SCFM(m+) for 24 hours in the absence of antibiotics. Each box plot consists of ten clones from monocultured (solid grey) and *S. maltophilia* co-cultured (striped grey) treatment regularly treated with either 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) and 8 µg/ml (D) imipenem. Selection in SCFM increases competitive ability and the relative increase is lower at higher imipenem culture concentrations. The average for each clone was calculated from at least ten replicates. 2/79 clones from different treatments were identified as outliers and removed due to the over expression of red fluorescence.

The evolution of imipenem resistance by *P. aeruginosa* in SCFM imposes a distinct fitness cost in terms of reduced competitive ability without an observable trade-off in growth rate

Competitive index was used as a measure of relative fitness for performing linear regression analysis to evaluate if evolved imipenem resistance in *P. aeruginosa* clones imposed an overall growth cost. Clones with increased imipenem resistance performed worse competitively compared to those with low resistance, with a whole model ANOVA demonstrating a significant association ($F(1,75)=24.1023$, $p=<0.0001$, $R^2=0.2432$; Fig. 12) that was reflected within both monocultured ($F(1,37)=9.6299$, $p=0.0037$, $R^2=0.2065$) and *S. maltophilia* co-cultured treatment groups ($F(1,36)=12.1166$, $p=0.0013$, $R^2=0.2518$). In contrast to our original hypothesis suggesting that *S. maltophilia* presence would select for more costly resistance phenotypes, we observed almost identical linear trade-offs between both treatment groups. ANCOVA on competitive fitness demonstrates an insignificant effect of *S. maltophilia* co-culturing ($F(1,73)=0.1382$, $p=0.7112$) and notably an interaction effect with imipenem tolerance ($F(1,73)=0.0012$, $p=0.9730$), showing both linear gradients to be equal. Level of evolved imipenem resistance explained a significantly substantial proportion of the variation in competitive ability demonstrating a clearly distinct fitness trade off associated with increased imipenem resistance.

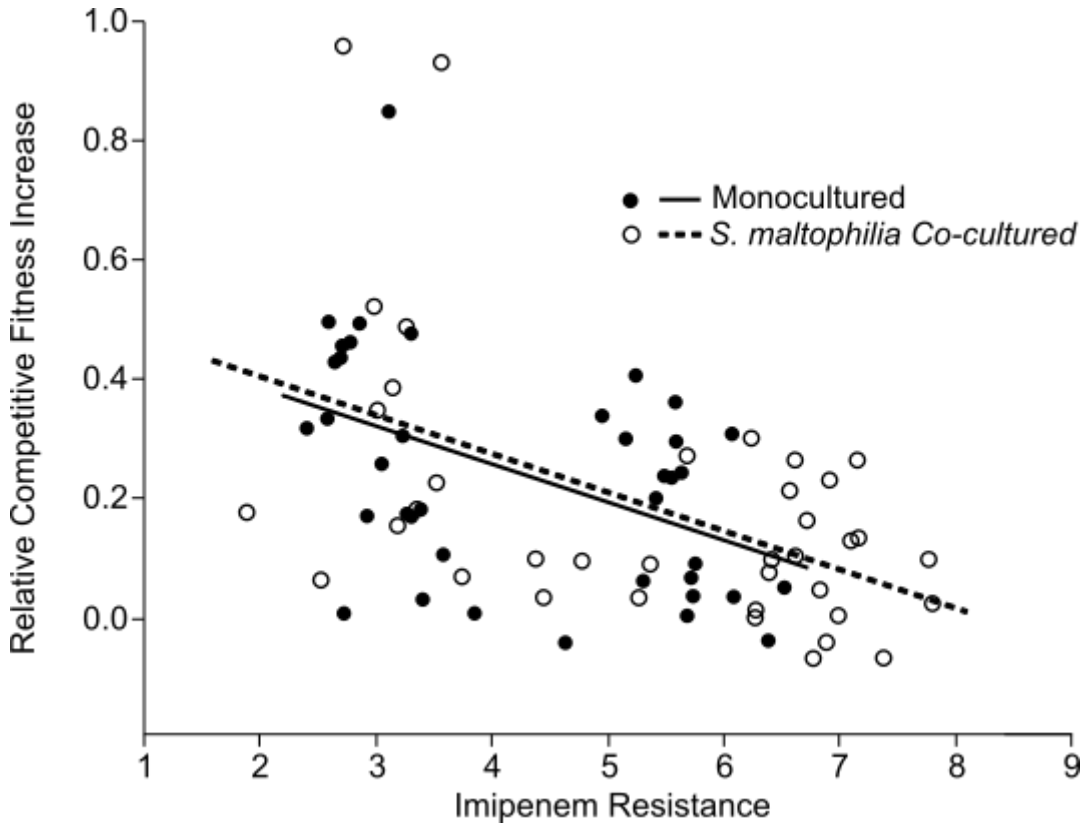
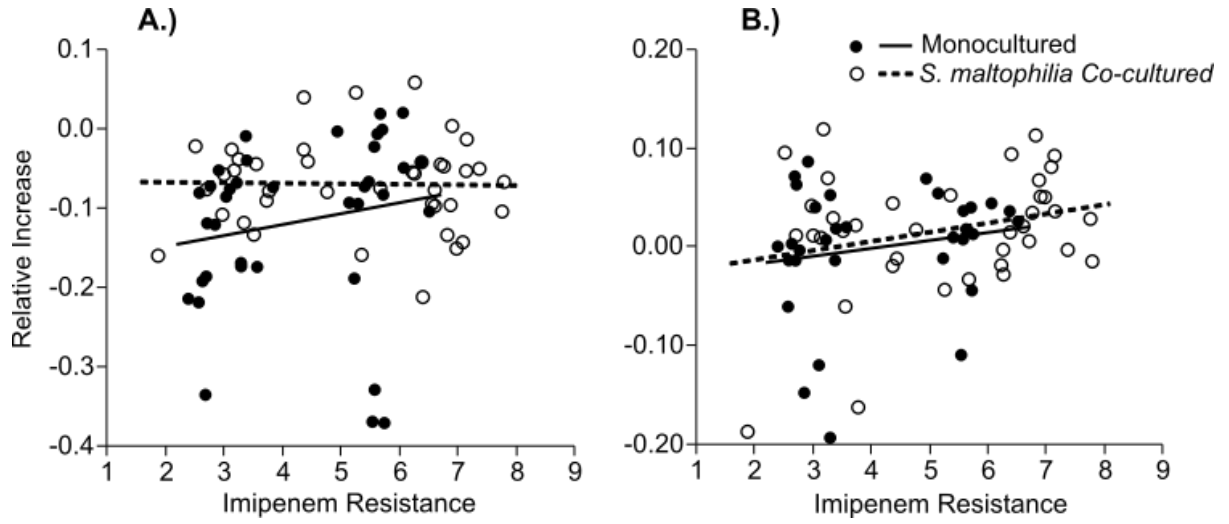


Figure 12: Evolving imipenem resistance results in a trade-off with competitive fitness.

Linear regressions between competitive fitness index in SCFM(m-) and imipenem MIC AUC resistance in SCFM(m+), were performed for monocultured (black circle) and *S. maltophilia* co-cultured (white circle) *P. aeruginosa* clones from day-24 of the selection experiment. We found the evolution of higher levels of imipenem resistance was significantly associated with reduced competitive ability, indicating a distinct fitness-resistance trade-off. 2/79 clones were identified as outliers and excluded due to the over expression of red fluorescence, both possessed low imipenem resistance and did not augment the overall relationship observed.

To evaluate if this imipenem resistance-fitness trade-off was also reflected in reduced growth rates further linear regression analyses were applied. We found that evolving increased levels of imipenem tolerance did not impose a significant growth cost in either maximum growth rate (ANOVA: $F(1,67)=1.3012$, $p=0.2582$, $R^2=0.0274$, Fig. 13A), total growth rate (ANOVA: $F(1,67)=3.4693$, $p=0.0669$, $R^2=.0637$, Fig. 13B), or an interaction between them (ANOVA: $F(1,67)=0.0057$, $p=0.9399$). The lack of a clear association between growth rate and imipenem AUC resistance was reflected within treatment groups as in monocultured *P. aeruginosa* clones the maximum growth rate (ANOVA: $F(1,29)=0.2335$, $p=0.6326$), total growth rate (ANOVA: $F(1,29)=1.7962$, $p=0.1906$), and their interaction (ANOVA: $F(1,29)=2.6069$, $p=0.1172$) were all not significantly associated with imipenem resistance. Although imipenem resistance was not linked to variation in maximum growth rate (ANOVA: $F(1,34)=0.0122$, $p=0.9127$) or total growth rate (ANOVA: $F(1,34)=0.3130$, $p=0.5795$) in *S. maltophilia* co-cultured clones there was a marginally significant positive interaction term between them (ANOVA: $F(1,34)=4.4624$, $p=0.0421$). This means that *S. maltophilia* co-cultured clones with higher maximum and total growth rates possessed greater imipenem resistance, suggesting slightly improved growth rates in SCFM may be responsible for higher imipenem AUC resistance scores.



<u>Figure 13</u>: Evolving imipenem resistance does not reduce growth rate in *P. aeruginosa*.

Linear regression graphs comparing imipenem tolerance measured in MIC AUC in SCFM(m+) against relative increase in maximum growth rate (A) and total growth rate (B) in SCFM(m-) for PA01:rfp clones from day-24 of the selection experiment across all imipenem treatments. Regression lines are grouped by monoculture (black circle) and *S. maltophilia* co-culture (white circle) treatments. There was no significant relationship found between maximum growth rate or total growth on imipenem tolerance, suggesting no growth trade-off.

Lastly, to investigate if the ability of *P. aeruginosa* clones to grow in SCFM affected their ability to compete against the ancestral strain, maximum growth rate and total growth rate was compared against competitive index scores. Overall, maximum rate of growth was not associated with improved competitive fitness (ANOVA: [$F(1,67)=0.1707$, $p=0.6808$, $R^2 = 0.0085$) as clones capable of faster exponential growth were not better at outgrowing the ancestral strain. Interestingly, clones with higher total growth rates were significantly linked with lower competitive indexes on average (ANOVA: $F(1,67)=7.7802$, $p=0.0069$, $R^2 = 0.1238$). Furthermore, there was a strong positive interaction term between both growth metrics (ANOVA: $F(1,67)=8.5507$, $p=0.004$)], meaning that clones possessing high values in both traits were relatively more competitive against the ancestral strain than clones with high scores in only one. This effect was most pronounced in *P. aeruginosa* monocultures where high total growth rate significantly reduced competitive ability (ANOVA: $F(1,31)=12.4832$, $p=0.0013$), while also interacting with maximum growth rate to increase it (ANOVA: $F(1,31)=17.4820$, $p=0.0002$). In contrast, *S. maltophilia* co-cultured clones demonstrated no significant effect of total growth rate (ANOVA: $F(1,32)=0.4883$, $p=0.4897$) or its interaction with maximum growth rate (ANOVA: $F(1,32)=0.0214$, $p=0.8839$). These results indicate that faster growth rates do not increase fitness when grown in SCFM, and likely explain why growth does not correlate with evolved imipenem resistance. Overall, the costs of evolved imipenem resistance and the advantages of SCFM adaptation are reflected in the capacity to outcompete competitors and not simply in growth rate, suggesting more complex adaptations are responsible for improved competitive media colonisation.

Evolutionary changes in pyoverdine and pyocyanin production depend on the presence of *S. maltophilia* and imipenem concentration, while increased pyoverdine is linked to improved competitive ability

Change in the virulence of evolved clones was measured through the production of pyoverdine and pyocyanin in SCFM(m-), which are linked to iron sequestration and cytotoxic ability, respectively. Pyoverdine production universally increased across all evolved clones and when compared to the ancestral replicates (N=12) showed a significant increase on average for monocultured (Dunnett's test: $p<0.0001$) and *S. maltophilia* co-cultured groups (Dunnett's test: $p<0.0001$; Fig.14). Overall, there was not a significant effect of imipenem culture concentration (ANOVA: $F(3,72)=0.1891$, $p=0.6649$), *S. maltophilia* presence (ANOVA: $F(1,72)=1.4824$, $p=0.2265$), or an interaction between the two (ANOVA: $F(3,70)=0.6628$, $p=0.5776$). Indicating increased pyoverdine production is a universal adaptation to the SCFM. In contrast, pyocyanin production typically decreased in *P. aeruginosa* clones evolved in SCFM and that this effect was stronger in the presence of *S. maltophilia* (Fig. 15). Comparison against the ancestral replicate control group (N=12) found significant decreases in pyocyanin production for both monocultured (Dunnett's test: $p=0.0001$) and *S. maltophilia* co-cultured (Dunnett's test: $p<0.0001$) evolved clones. The significant decrease was primarily observed in the absence of imipenem and although *S. maltophilia* presence alone had little effect (ANOVA: $F(1,72)=0.1333$, $p=0.7161$) its interaction with imipenem concentration was significant (ANOVA: $F(3,72)=8.0149$, $p=0.0001$). This reflects how relative pyocyanin production was higher in *S. maltophilia* co-cultured evolved clones in 0 μg/ml (Students t-test: $t(15)=2.16979$, $p=0.0463$) and 1 μg/ml (Student's t-test: $t(17)=2.10659$, $p=0.0499$) imipenem treatments. While monocultures had significantly higher production in 4 μg/ml (Student's t-test: $t(16)=2.57946$, $p=0.0199$) and 8 μg/ml imipenem (Student's t-test: $t(18)=3.13566$, $p=0.0057$).

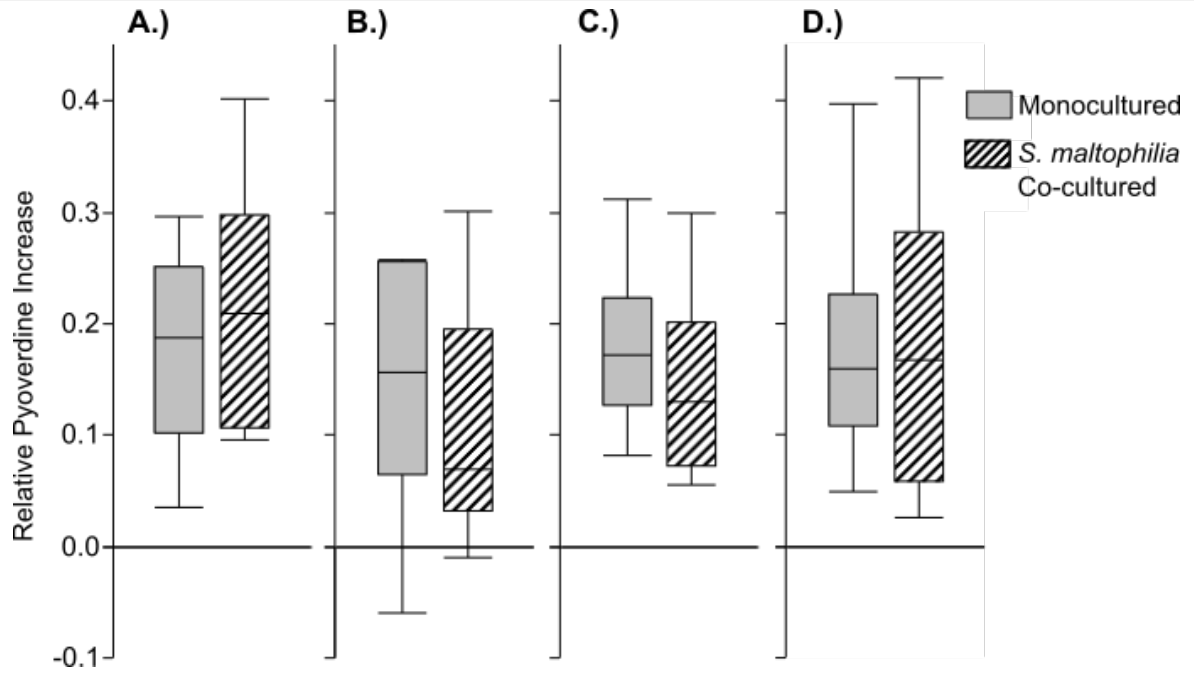


Figure 14: - *P. aeruginosa* increases pyoverdine production when evolved in SCFM.

The pyoverdine production of isolated PA01:rfp clones from day-24 of the selection experiment in SCFM(m+) was assayed in SCFM(m-) by measuring the OD405 (nm) of supernatants divided by the OD595 (nm) of cultures pre-centrifugation. Production was then normalised to the ancestral level giving the relative increase. Box plots represent ten monocultured (solid grey) or *S. maltophilia* co-cultured (dashed grey) independent *P. aeruginosa* clones from the 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) and 8 µg/ml (D) imipenem treatments. Culturing in SCFM(m+) lead to the universal increase in pyoverdine production across all treatment groups. The assay was replicated at least four times for each clone.

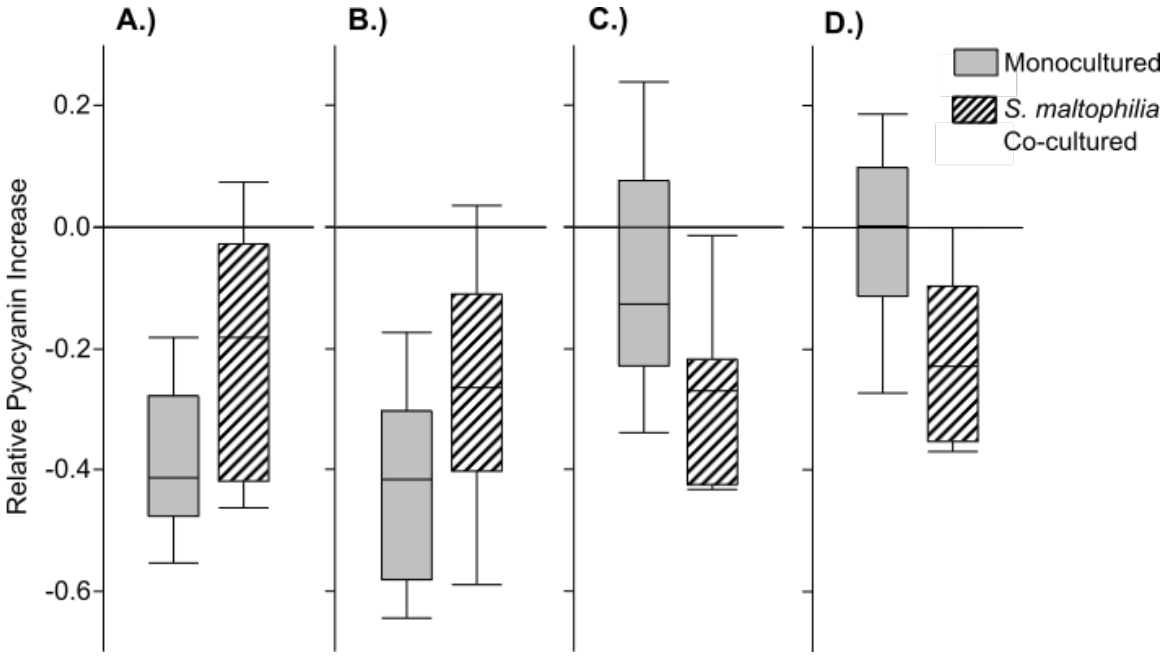


Figure 15 – Pyocyanin production of SCFM evolved *P. aeruginosa.*

Box-plots represent the average pyocyanin production across 10 independent monocultured (solid grey) and *S. maltophilia* co-cultured (dashed grey) PA01:rfp clones grown in SCFM(m+) for 24 days under regular 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) and 8 µg/ml (D) imipenem treatments. Relative increase in production was calculated by normalising the OD695/OD595 of supernatants and pre-centrifuged cultures to the ancestral pyocyanin level. Selection in SCFM(m+) led to decreased pyocyanin production while imipenem treatment limited this reduction in monocultures. Co-culturing with *S. maltophilia* led to relatively equal pyocyanin production levels across imipenem treatments. Each clone was independently assayed at least four times.

Further linear regressions were performed to find out if competitive ability was associated with pyocyanin or pyoverdine production in SCFM. Increased production of pyoverdine in evolved *P. aeruginosa* clones was significantly associated with improved competitive fitness, (ANOVA: $F(1,75)=8.6765$, $p=0.0043$, $R^2=0.1037$). This trend was present within both monoculture (ANOVA: $F(1,37)=2.1984$, $p=0.1466$, $R^2 = 0.0561$) and *S. maltophilia* co-culture (ANOVA: $F(1,36)=5.8305$, $p=0.0210$, $R^2=0.1394$) treatment groups, however it was only significant in the latter. Overall, higher pyocyanin production was not significantly correlated with altered competitive ability (ANOVA: $F(1,75) = 1.6006$, $p=0.2097$, $R^2 = 0.0209$). However, within the monoculture treatment group its production was significantly linked to reduced competitive fitness (ANOVA: $F(1,37) = 5.3805$, $p=0.0260$, $R^2= 0.1270$). In contrast, pyocyanin production was completely independent of competitive ability among *S. maltophilia* co-cultured clones (ANOVA: $F(1,36) = 0.1182$, $p=0.7330$, $R^2=0.0033$). It is possible that since imipenem resistance is a primary predictor of competitive fitness that pyocyanin and pyoverdine are simply covarying with evolved tolerance and are not actually associated with competitive ability. Comparing virulence factor production against imipenem AUC resistance demonstrated that pyocyanin production was positively correlated with evolved resistance, (ANOVA: $F(1,74) = 13.2299$, $p=0.0005$, $R^2=0.1517$), while any association with pyoverdine was insignificant, (ANOVA: $F(1,74)=0.0283$, $p=0.8668$, $R^2=0.0004$). This indicates the relationship between increased pyocyanin production and decreased competitive fitness is likely the result of co-variance with imipenem tolerance and that pyoverdine production is likely directly connected to improved competitive ability.

Mutations in five common gene regions were significantly associated with imipenem resistance in evolved *P. aeruginosa* clones

To investigate how genomic changes underpinned the evolution of phenotypic traits, one clone from six replicates within each treatment was randomly chosen and sequenced. The presence of SNP mutations were identified in protein coding and non-coding regions and compared across *S. maltophilia* and imipenem culture treatments (Fig. 16C, Fig. 16D). The functional role of these genes was identified using protein databases [171, 172, 173]. Imipenem resistance was notably associated with mutations in a small suite of five chromosomal regions, two genic and three intergenic. Mutations in the *oprD* gene, encoding the OprD porin, emerged only in imipenem resistant clones and 63% (15/24) of all clones from 4 μg/ml and 8 μg/ml imipenem treatments had mutations in the *oprD* gene. These mutations consisted of a wide variety of insertions, deletions and single base substitutions across a 1119 bp region near the centre of the gene, all resulting in either novel stop codon expression or frameshifts. Mutations in the *PA1874* gene, encoding an outer-membrane glycoprotein, were only present in two resistant clones. In both instances these were the same synonymous C to T substitution at AA position 775 (Threonine). One of these *PA1874* mutants from the *S. maltophilia* co-culture treatment also had an *oprD* mutation and a higher AUC resistance score compared to the other *PA1874* mutant, from the monoculture treatment, without an *oprD* mutation. This suggests that these mutations may have had synergistic effects on imipenem resistance.

Mutations in three intergenic regions were also notably associated with imipenem resistance. An individual C to T mutation between the *ppiD* and *fabL* genes was present in 44% (21/48) of clones, 71% (15/21) of which had evolved increased resistance. These two genes encode a periplasmic folding chaperone and an NADPH dependent enoyl-ACP reductase, respectively. Mutations in this

region were particularly common in the 8 μg/ml imipenem treatment as 75% (9/12) of clones were *ppiD-fabL* mutants. It also appeared in 50% (3/6) of the moderately imipenem resistant clones from the co-cultured 1 μg/ml treatment, suggesting its effects are likely quite minimal. Mutations in the second intergenic region appeared in seven clones, 86% (6/7) of which possessed increased imipenem resistance. This region consisted of a relatively long inverted repeat sequence between the *PA3463* and *PA3464* genes, encoding a hypothetical protein of unknown function and a zinc dependent phospholipase nuclease. All mutations were within 22bp of one another and consisted primarily of single C to G transitions, however one clone possessed a complex CGAAAC to GAAAAA. Lastly, a single A to C SNP between *PA4838*, encoding an uncharacterised membrane protein, and *speA*, coding an arginine decarboxylase, appeared in 54% (26/48) of all evolved clones, and 81% (21/26) of these possessed increased imipenem resistance.

On average, clones exposed to higher imipenem concentrations possessed mutations in a significantly greater number of these regions (Kruskal-Wallis test: $X^2(3)=12.0139$, p=0.0073, with mean rank scores of 17.0, 20.0, 26.1 and 34.8). The mean number of mutated regions per clone was: 0.833 (SD=0.83485) for 0 μg/ml, 1.08 (SD=1.0836) for 1 μg/ml, 1.67 (SD=1.3027) for 4 μg/ml and 2.41 (SD=0.9962) for 8 μg/ml. Although evolved clones cultured with *S. maltophilia* possessed mutations in a greater number of resistance-associated regions in 1, 4 and 8 μg/ml imipenem concentrations, this increase was not statistically significant (Wilcoxon test Z=1.29952 p=0.1938, with mean rank scores of 16.25 and 20.75). The difference in SNP number between *S. maltophilia* co-culture and monoculture groups remained not significant when comparing just 4 μg/ml and 8 μg/ml treatments (Wilcoxn: Z=0.35672, p=0.7213, with mean rank scores of 11.96 and 13.04). This means that *S. maltophilia* co-cultured clones did not possess significantly more mutations in the five genomic regions we have identified as associated with imipenem resistance. Considering we only observed slight differences between monocultured and co-cultured *P. aeruginosa* clones, this suggests the difference observed in evolved resistance may be the indirect result of an improved ability to grow in SCFM at sub-inhibitory concentrations. Alongside possible epistatic interactions with other non-resistance associated mutations.

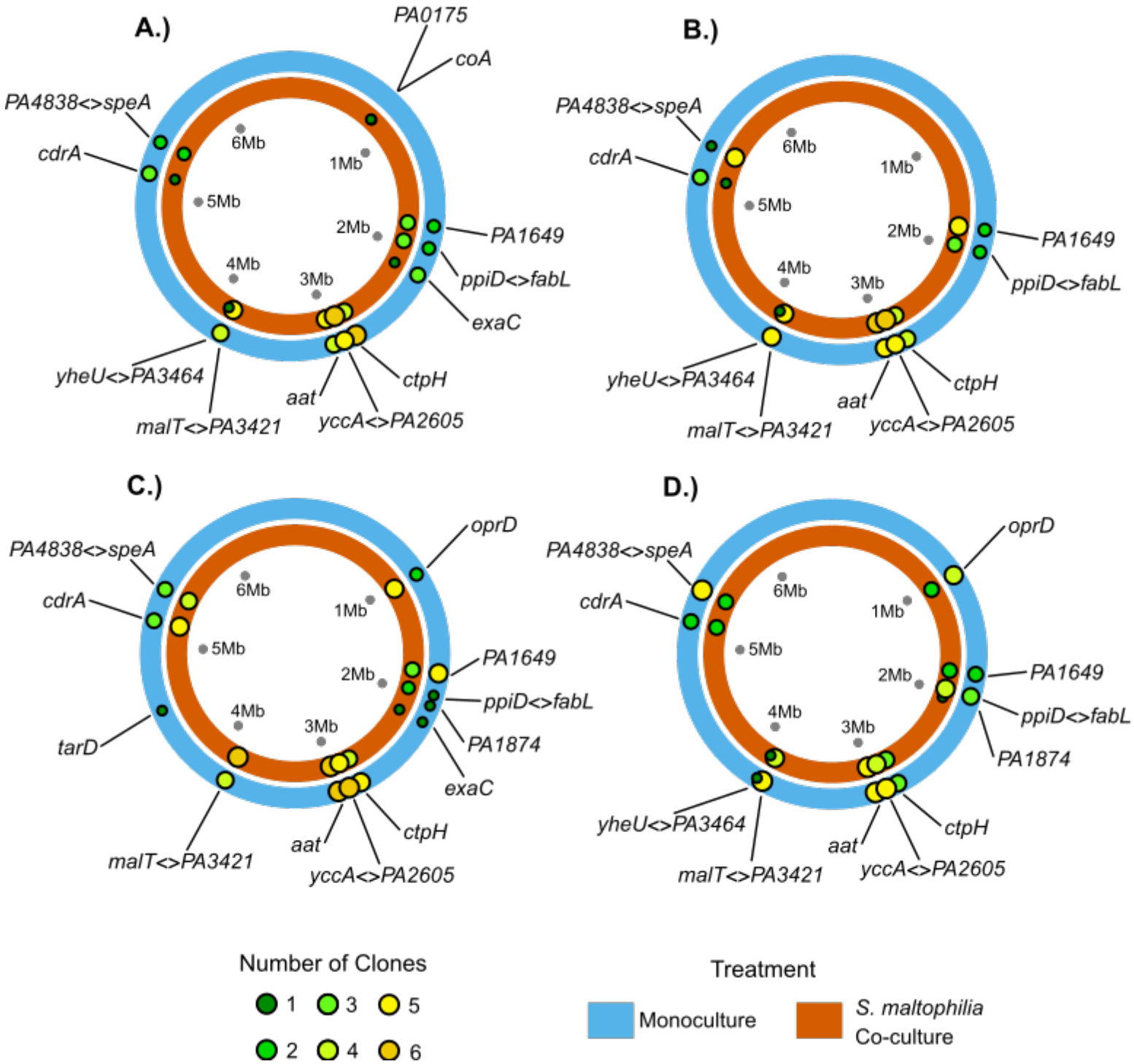


Figure 16: Coding region SNPs in evolved *P. aeruginosa* clones.

Frequency and position of SNP variants for six out of ten randomly selected *P. aeruginosa* clones from day-24 of the selection experiment for each treatment combination. Mutations in the *oprD* and *PA1874* gene are associated with increasing imipenem treatment concentrations: 0 µg/ml (A), 1 µg/ml (B), 4 µg/ml (C) and 8 µg/ml (D). Mutation in several genes commonly emerge regardless of monoculture (blue) or *S. maltophilia* co-culture (red) treatment conditions. 48 evolved clones were sequenced in total. The number of clones possessing mutations in individual genes are represented by circles of increasing size (dark green-dark yellow). Clones with multiple SNPs in the same gene were only counted once. Intergenic regions are noted by '<>' between the flanking genes.

Long term evolution in SCFM resulted in several commonly mutated genomic regions independent of imipenem or *S. maltophilia* treatment

Beyond imipenem resistance, mutations in several other genomic regions regularly emerged in clones evolved independently in SCFM, regardless of antibiotic treatment. Over the 24 days of selection PA01 clones accrued 10.21 (SD=3.10) SNPs on average (0.04165 mutations per generation) A total of 92% (44/48) of all clones possessed an identical missense mutation in the *aat* gene, responsible for coding a leucyl/phenylalanyl-tRNA protein transferase. A single A to T substitution led to the conversion of lysine to asparagine at AA position 33. Mutations in the *ctpH* gene, encoding a putative chemotaxis transducer, appeared in 73% (35/48) of clones. All SNPs were within the same 6 bp region, causing silent alanine to alanine or glycine to glycine substitutions at AA position 162 and 163, respectively. Around 54% (26/48) of all clones evolved a missense mutation in the *PA1649* gene, a homologue of *linC*, which codes a short chain dehydrogenase protein. All mutations were a single G to C SNP causing a Glycine for Arginine substitution at AA position 217. Mutants were also slightly more common in clones with evolved imipenem resistance: 63% (19/30) of resistant and only 39% (7/18) of non-resistant clones had a mutation in *PA1649*. Interestingly nine of these resistant mutants did not possess a mutation in the *oprD* porin gene, suggesting *linC* may aid the evolution of increased resistance in the absence of other changes. Roughly half (7/14) of all *PA1649* mutants from 4 μg/ml and 8 μg/ml imipenem concentrations were also double mutants with *oprD*. Furthermore, double mutants were not more common in the *S. maltophilia* co-cultured groups. Lastly, we observed a recurrent missense mutation leading to the substitution of Alanine to Serine at AA position 53 in the *exaC* gene, which is linked to the expression of an Acyl-CoA reductase-like dependent aldehyde dehydrogenase - present in 13% (6/48) of evolved *P. aeruginosa* clones.

Mutations in the *cdrA* gene, which encodes a large exoprotein involved in biofilm development, appeared in 46% (22/48) of clones. These mutations were all synonymous and varied in position within a single 51 bp region. The most common SNPs were: C to T at AA position 1572 (Asparagine), A to G at AA position 1589 (Alanine) and a complex TAGC to CAGT at AA position 1582 (Asparagine). 72% (13/22) of these clones possessed more than one mutation in this region. The complex mutation at AA position 1582 is particularly interesting because it appeared at notably higher levels in *S. maltophilia* co-cultured clones in 4 μg/ml and 8 μg/ml imipenem. Roughly 78% (7/9) of clones with evolved resistance and this specific SNP had been co-cultured with *S. maltophilia*. It is also worth mentioning a few notable genic mutations that only appeared once in a single clone. One mutant from the 0 μg/ml *S. maltophilia* co-culture treatment possessed a pair of missense mutations in the *PA0715* gene, which encodes a reverse transcriptase family protein. The mutations caused Cystine to Tyrosine and Lysine to Glutamine substitutions at AA positions 166 and 192, respectively. The same clone also possessed a G to A mutation in *coaA,* a phage coat gene, substituting Glycine for Arginine. Lastly one clone from the 4 μg/ml monoculture treatment possessed a T to G missense mutation in *tarD*, coding a hypothetical tartrate dehydrogenase, changing Tyrosine to Asparagine at aa position 324.

There were also mutations in a three notable intergenic region that appeared at very high frequencies. One individual C to G SNP appeared in 92% (44/48) of all clones, in the non-coding region between the *PA2604* gene encoding a YcaA-like hypothetical protein, and *PA2605* encoding the sulfurtransferase TusD. Secondly, a variety of SNPs were observed in 83% (40/48) of clones in the region between *malT* and *PA3421*, encoding a transcriptional regulator and a hypothetical

protein of unknown function, respectively. Two were notably common, emerging in a Rho-independent transcription terminator region. At least one appeared in 67% (32/48) of all clones. Several clones had multiple mutations in this intergenic region. Mutations in the last intergenic region between *yheU* and *PA3464* genes, emerged in just 8% of clones (4/48). Considering that both genes encode hypothetical proteins of unknown function and the relatively low frequency mutations in this region were likely of limited importance

To evaluate if imipenem or *S. maltophilia* treatment resulted in clones accruing a larger number of SNPs, several Kruskal-Wallis tests were applied. Overall, there was not a significant difference in number of SNPs for clones evolved in different imipenem concentrations ($X^2(3)=1.6386$, $p=0.6507$), with mean rank scores of 23.25 for 0 μg/ml, 21.13 for 1 μg/ml, 27.958 for 4 μg/ml and 25.67 for 8 μg/ml treatments. Furthermore, the average number of SNPs per clone did not significantly vary between monocultured and co-cultured clones in 0 μg/ml, 1 μg/ml and 8 μg/ml. However, in 4 μg/ml imipenem *S. maltophilia* co-cultured clones did have significantly more SNPs on average ($X^2 = 4.5231$. $p=0.0344$, mean rank scores of 4.33 for monocultures and 8.67 for co-cultures). This indicates the increased imipenem resistance of co-cultured clones did not come from more mutations across most genes associated with SCFM adaptation, but is instead likely linked to subtle differences in a few individual genes or their interactions with one another.

Discussion

The aim of this study was to understand how *P. aeruginosa*'s susceptibility to antibiotics is affected by the presence of other species in conditions that mimic the environment of the CF lung. Accurate *in vitro* models provide a valuable insight into how antibiotic resistance and pathogenicity develop over the course of patient treatment. Imipenem is one of the most effective drugs available for treating *Pseudomonas* [185, 186], making the emergence of resistance to this antibiotic particularly concerning [68]. Despite this there have been no *in vitro* studies investigating how imipenem resistance evolves in an environment attempting to replicate *in vivo* conditions, especially in the presence of clinical *S. maltophilia*. *S. maltophilia* persistently infects up 30% of CF patients and transiently infects many more [187, 188]. Due to its high innate resistance is it capable of persisting in the lung when most other species are eradicated [127] making it an important commensal to focus on. Increased experimental complexity does however come with clear trade-offs in time, efficiency and analytical certainty. This is in contrast to many studies in the field which appear to focus on outcome driven assay designs that are often unrepresentative of clinical evolution, yet still apply their conclusions to CF infection. Here we take a clear step towards demonstrating the impact sputum substrate and community members can have on imipenem resistance evolution in *P. aeruginosa*, while simultaneously proving why more considered assay design is required when attempting to apply *in vitro* findings to clinical infections.

Chapter 1: Designing a clinically representative selection model

A common theme observed in literature looking to explore how *P. aeruginosa* behaves and evolves in the CF lung through *in vitro* assays is the use of experimental designs that are unrepresentative of *in vivo* growth, and which are often biased towards desired outcomes. To avoid this problem in our selection experiment, we developed a model of pathogenic *P. aeruginosa* growth in synthetic CF lung media (SCFM), based on clinical evidence and antibiotic pharmacokinetics. Imipenem treatment concentrations were based on the average concentration of imipenem recovered from patient lung sputum shortly after therapy. Intravenously administered imipenem rarely exceeds 1 to 2 µg/ml in lung sputum [159] while nebulised treatment achieves higher concentrations [161] and more uniform distribution [189]. Generally, the mean concentration of imipenem is roughly 4-fold higher for inhaled compared with I.V. imipenem [160], and this was the primary basis for choosing 1, 4 and 8 µg/ml as our culture concentrations. Using excessively high antibiotic concentration not only increases the likelihood of eliminating *P. aeruginosa* from culture but also exposes it to unrealistic selection pressures for resistance evolution. Deciding what level of shaking should applied to cultures was another important decision as the thick dehydrated mucous of the CF lung impairs proper mucocilliary clearance [190]. Although cilia do not perform their function they do still move [191], and minor mixing in sputum would still be expected. To model this we used low shaking speeds of 220 rpm accompanied with a small orbital diameter, which ensured there was enough shaking for low levels of solute flow but not enough to break up forming biofilms.

Low inoculation cell densities and a 48-hour transfer window were chosen to model the two key phases of infective growth: colonisation of fresh sputum and persistence in developed biofilms. A common misconception is that since *P. aeruginosa* develops biofilms during infection, investigations into antibiotic resistance evolution should focus on treating colonies grown in high density biofilms [81, 82, 192, 193]. This approach fails to take into account the fact that biofilms are so antibiotic resistant that they are nearly impossible eradicate without using lethal levels of antibiotics [53, 54]. For example, only 12-19% of fluorescently tagged colistin is able penetrate into established SCFM biofilms [52]. Typically, biofilms are treated clinically by the mechanical removal of lung sputum [194, 195, 196], which makes continual colonisation essential for long term pathogenic persistence in the CF lung. This is why the primary aim of antibiotic treatment in CF patients is not clearing infection but preventing new growth to control infection and pathology in freshly produced sputum [54 & 55] (Fig.17). Therefore, the key stage of infective growth that should be studied for antibiotic resistance evolution is the colonisation of freshly treated lung sputum. Antibiotic treatments are usually prescribed twice a day for a month before antibiotics are changed [197, 198, 199] meaning a potential criticism of our model is that we simulate treatment every 48 hours. However, when considering that *P. aeruginosa* forms biofilms within 8 hours of inoculation [200], keeping one antibiotic treatment per pre-biofilm formation event is reasonable. Furthermore, constantly replenishing the media to perform daily treatments would lead to another common error in selection experiments [149], keeping *P. aeruginosa* in a state of perpetual exponential growth. Although CF lung sputum is very nutritionally rich, since *P. aeruginosa* does grow to high cell densities, nutrient limitation is a key feature of the host environment to include [201, 202]. Capacity to survive in established biofilms at low growth, low nutrient concentrations until fresh sputum is secreted is a major selective pressure that needs to be taken into account because it moderates unrealistic selection for excessively fast growth dynamics.

Although using low starting densities of *P. aeruginosa* was clinically sound, the amount of *S. maltophilia* that should be used was not as easy to determine. Since it is completely resistant to imipenem and can grow in sputum outside of biofilms it is feasible to propose that it should exist at high cell densities at the beginning of all transfers. This would also have the added benefit of likely increasing the effect of ecologically derived resistance, as more *S. maltophilia* would mean more beta-lactamase production and enhanced imipenem degradation. We chose low inoculation densities of *S. maltophilia* because we are only investigating a small snapshot of the total CF microbiome and the constitutive expression of several resistance mechanisms leads to *S. maltophilia* having a moderately slow growth rate. Together these factors mean we would not expect *S. maltophilia* to grow to high cell densities *in vivo* making it unrealistic for us to culture *P. aeruginosa* in an environment already completely colonised by an *S. maltophilia* competitor. Therefore, it must be able to provide its protective effect at relatively low densities, as would be expected when existing in a complex microbial community. Being able to demonstrate ecological and evolutionary effects on imipenem resistance in *P. aeruginosa* at low starting cell densities means this interaction is considerably more likely to take place *in vivo*. It also meant we did not have to add *S. maltophilia* into the selection experiment at regular intervals, which enabled us to gauge its capacity to coexist with *P. aeruginosa* over time.

This brief conceptual framework enables the dynamics of *P. aeruginosa* infection and antibiotic treatment to be incorporated into accurate *in vitro* assay designs, providing much greater certainty in applying observations of ecological and evolutionary interactions to clinical infections. Ultimately, it demonstrates the need to fully understand the system being investigated and to not develop assays which bias the results in the direction of the desired outcome, because as we found throughout this study our initial hypotheses were often completely wrong.

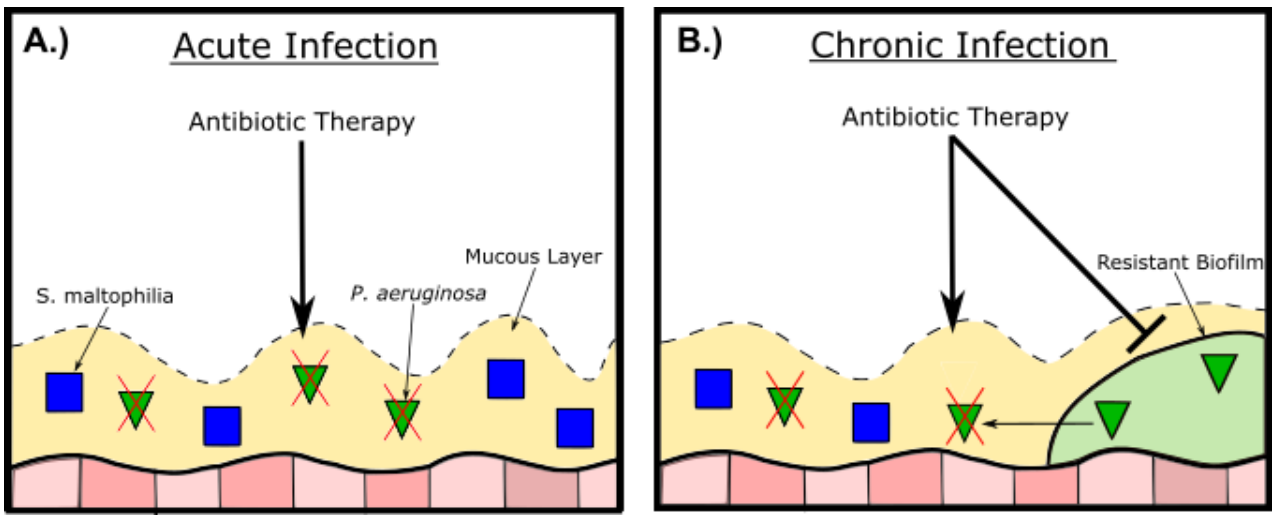


Figure 17 – Theoretical diagram of antibiotic treatment in the CF lung.

Figure illustrating the mode of operation for antibiotic therapy against pathogenic *P. aeruginosa* (green triangle) in the CF lung during acute (A) and chronic (B) infection. Resistant *S. maltophilia* community members (blue square) are included in the sputum. During acute infection, antibiotic therapy successfully eliminates *P. aeruginosa* (red cross) preventing long-term infection from establishing. In chronic infection, *P. aeruginosa* has formed antibiotic resistant biofilms that enable its survival during antibiotic therapy. Here therapy is targeted at preventing growth in the surrounding sputum, controlling infection and limiting pathology, rather than completely clearing infection.

Chapter 2. The gap between *in vitro* susceptibility testing and treatment success

One of the primary goals of this study was to investigate the impact CF lung sputum has on antibiotic susceptibility testing. We observed that culturing clinical CF isolates in SCFM led to significantly increased antibiotic tolerance compared to standard laboratory LB media, in several instances elevating sensitive isolates beyond the clinical breakpoint for resistance determination. Protection was not universal across antibiotics with species and strain having a significant impact, indicating both generic media protection and active bacteria-environment interactions. In the case of imipenem, clinical mucin levels alone conferred a substantial protective effect which likely applies to other antibiotic groups. This evidence suggests clinically unrepresentative media is responsible for the observation that antibiotic treatments in CF patients often don't control pathogenic strains that test as sensitive *in vitro*. To address this issue we provide preliminary data for generating a quantitative framework that could be used to translate *in vitro* testing into accurate predictions of *in vivo* efficacy.

Bacterial strains within species demonstrated relatively high consistency in the degree of tolerance increase observed when grown in SCFM compared to LB, with substrate-mediated protection against each tested antibiotic emerging at least once. This data suggests CF lung sputum has a relatively generic protective effect independent of antibiotic class. The main candidate likely responsible is mucin, which reduces bacterial exposure to antibiotics by limiting diffusion and acting a physical barrier. Adding mucin at clinical concentrations to LB media quadrupled the MIC of PA01 from 1 to 4 µg/ml against imipenem, however this was only tested for this one antibiotic. It is important to note that since antibiotic susceptibility testing was performed in SCFM(m-) with reduced mucin the relative increases observed for each isolate are likely underestimates compared to *in vivo* sputum. Although SCFM does appear to provide a universal protective effect, the relative increase in tolerance observed between species and strains within species suggests active interactions between bacteria and the environment. Since genetic background does impact how the tolerance of individual clinical isolates change when grown in sputum-like substrate, it implies the nutritional or structural composition of SCFM is either altering gene expression or interacting with resistance-associated phenotypes. Media induced changes in gene expression are relatively common throughout bacteria [52] and the availability of a solid mucin scaffold could enable protective biofilms to form faster. Therefore, isolates adapted to CF lung sputum or strains who overexpress biofilm components would have considerably greater protection in SCFM. Alternatively, mucin may provide passive protection by simply binding antibiotic, which has been observed in several antibiotics, significantly reducing their efficacy [182, 183].

P. aeruginosa was the only species for which we tested both laboratory and clinical strains, and the highest increases in antibiotic tolerance from LB media to SCFM appeared in clinical isolates. This suggests pre-existing antibiotic resistance or adaptations to *in vivo* growth conditions may predispose clinical isolates to become more tolerant in SCFM, or conversely more sensitive in laboratory media. Interestingly we found no clear link between antibiotic sensitivity in LB and the degree of percentage increase in SCFM, implying that pre-existing resistance did not tend to pre-dispose isolates to higher increases when grown in SCFM. We also observed that despite there being variation between isolates, each species displayed consistent patterns of resistance increase

between LB and SCFM. Inter-species differences could be explained by innate antibiotic resistance, as membrane permeability substantially differs between all three species [203] and inter-species variations in pore dimensions could impact the effectiveness of mucin as a structural barrier. Alternatively, in several instances isolates grew outside of the microdilution range, meaning that greater changes between LB and SCFM might have been observed at higher concentrations, although this would be of limited clinical relevance. It is important to note that these SCFM induced changes in antibiotic tolerance are clinically relevant because on several occasions it elevated clinical isolates that tested as sensitive in LB beyond the breakpoint for resistance determination. This provides clear evidence that CF lung sputum does alter the antibiotic sensitivity of a range of clinical isolates through a mixture of passive and active effects with bacterial physiology.

Our finding that SCFM can significantly increase the MIC in planktonic cells is surprisingly novel as it has not been investigated or reported in previous studies [134]. One study by Kirckner et al. (2012) performed similar MIC susceptibility testing in ASM(m-) finding only minor evidence for increased planktonic cell tolerance. However, they focused more on pre-biofilm formation and microaerophilic growth which universally reduced sensitivity [153]. Despite the ASM also containing low mucin levels little to no protective effect was observed, further supporting our hypothesis that the nutritional content of SCFM substantially contributes to the decreased susceptibility to antibiotics. Several papers have shown SCFM to change the gene expression and physiological properties of *P. aeruginosa* [106, 149, 204, 205], particularly the upregulation of biofilm formation. The physiochemical environment of the host has been well documented to alter bacterial responses [52] and several key properties of *P. aeruginosa* biofilms are altered by environmental conditions [206]. Therefore, it is highly likely our results captured SCFM-induced change in plastic *P. aeruginosa* resistance phenotypes, probably early biofilm formation.

Mucin is likely the main component providing this protection as it induces enhanced biofilm formation which can increase PA01's resistance to tobramycin when grown on solid substrate [184]. Exposure to 2.0 μg/ml tobramycin leads to only 24% (±10%) killing when biofilms are grown for 24 hours on mucin compared with 83% (±8%) on glass. Sterile human mucous and 0.5% mucous buffer have also been shown to increase the survival of PA01 cells during two-hour exposure to 64 μg/ml of colistin and 0.25 μg/ml of ciprofloxacin [182]. A few papers have investigated the impact of mucin on *P. aeruginosa* tolerance in the context of SCFM, but not in simple laboratory media, finding mucin-dependent changes in PA14 motility provided broad-spectrum antibiotic resistance in SCFM. In the presence of 0.4% mucin 'free-swimming' cells switch to a 'surfing' motility giving increased movement across SCFM-agar [152]. This led to a significantly smaller zone of inhibition for tobramycin, imipenem, ciprofloxacin, colistin, chloramphenicol and seven others antibiotics not examined in our study [175], indicating increased tolerance. However, this effect was not only due to the presence of mucin as 0.4% mucin-agar without SCFM only significantly increased disk diffusion resistance against just tobramycin, ciprofloxacin, colistin and two others. Interestingly, in SCFM broth microdilutions 0.4% mucin significantly increased PA14's MIC against colistin by 700%, while insignificantly increasing chloramphenicol and ciprofloxacin by 100% each and decreasing imipenem by 100% [175]. This represents a noticeable difference from our LB(m+) experiment, however our study benefited from replicating our data which enabled more comprehensive tests for significance in place of the simpler 'three-fold increase'. Furthermore, we used considerably higher mucin levels (5%) which suggests that the protective effect of mucin is most pronounced at more clinically relevant concentrations. However, for our initial susceptibility testing mucin was at analogous

quantities (0.5%), indicating the protective effect of mucin varies depending on if bacterial growth is in liquid or on solid culture.

One potential limitation of our approach is that MIC scoring happens on a $\log_2$ scale meaning a 100% increase from 2 to 4 µg/ml only changes the MIC by 1/4th of a 100% percentage increase from 8 to 16 µg/ml, meaning smaller increases appear more significant. This is an inherent problem with the broth microdilution assay design, which sacrifices a linear scale for wider ranges. However, since we found lower scores did not lead to higher increases, and vice versa, this effect likely had limited impact. Secondly, when comparing tolerance in LB and SCFM we avoided trying to statistically analyse qualitative MIC scores, instead comparing average growth across concentration wells. This took into account both ability to grow in higher concentrations and the level of that growth, while normalising differences in maximum growth capacity between medias. In several instances, MIC increases from SCFM to LB were not significant because they only reflected marginal changes between wells. This method offers a sound alternative approach at the cost of requiring multiple replicates. Another limitation was our relatively small sample size of clinical isolates, especially considering we only tested one isolate of *S. aureus*.

One of the most substantial challenges facing medical professionals when treating CF patients is finding and prescribing antibiotic treatments that control infection successfully [194]. Despite resistance being high in clinical isolates, it often varies considerably between patients making antibiotic susceptibility tests of vital importance. However, they regularly fail to predict *in vivo* success [207, 208, 209]. Here we demonstrate clearly that CF sputum plays a major role in conferring protection to clinical isolates that test as susceptible during standard *in vitro* testing. SCFM does however require high preparation times, measuring dozens of individual components and using stock solutions with different shelf lives makes it an expensive and inviable alternative media for clinical laboratory susceptibility testing. However, we did find that for individual species the relationship between MIC in LB and SCFM are often highly conserved across different strains, meaning with enough data it could be possible generate a quantitative framework that clinicians could use for predicting the actual *in vivo* tolerance of isolates from basic *in vitro* tests. Alternatively, the addition of pre-sterilised porcine mucin to simple laboratory media is a relatively simple change that could be implemented when evaluating CF patient isolates specifically. Lastly, the fact that *S. maltophilia*-conferred imipenem resistance only occurred in SCFM demonstrates that ecological interactions can be heavily moderated by the growth substrate. In this study we only focused on antibiotic tolerance. However, there is a whole range of phenotypic traits that may be augmented by interspecies interactions depending on the media used. It is feasible to assume that reduced sensitivity could lead to higher minimum selective concentrations (MSCs), while increased structural complexity may augment intra-specific interactions, which together could change evolutionary outcomes. Future *in vitro* studies of antibiotic resistance evolution should thus utilise more clinically representative media such as SCFM or ASM

Chapter 3: The dynamics of *S. maltophilia* mediated imipenem degradation in CF lung sputum and its impact of *P. aeruginosa* growth

Despite existing in abundance in the CF lung, the impact of resistant competitors on *P. aeruginosa*'s capacity to tolerate antibiotic treatment under clinically relevant growth conditions has not yet been explored. Clinical isolates of *S. aureus* and *S. maltophilia* did not confer protection against most antibiotics. However, co-culturing with *S. maltophilia* did significantly increase the MIC of sensitive *P. aeruginosa* against imipenem when grown in SCFM. This provides clear evidence that *S. maltophilia* can substantially limit the effectiveness of imipenem therapy in CF patients even at low cell densities, highlighting the potential need to test for the presence of *S. maltophilia* in CF patient microbiomes before imipenem is considered as a treatment.

The increase in functional imipenem MIC for sensitive PA01 grown in co-cultures with *S. maltophilia* enabled *P. aeruginosa* to grow in concentrations beyond its clinical resistance breakpoint, demonstrating the interaction can impact clinical outcomes. We observed pre-culturing *S. maltophilia* in imipenem treated SCFM did substantially increase the rate at which filtrate supported sensitive *P. aeruginosa* growth. Filter sterilising the media physically separated the two bacteria, meaning *S. maltophilia* could only provide a protective effect indirectly through the detoxification of environmental imipenem and not through direct cell-cell interactions. This distinction is important because clear metabolic variation is observed when *P. aeruginosa* competes against other respiratory pathogens [210], and direct competition may have induced more antibiotic tolerant phenotypes. However, this protective effect only significantly reduced the antibiotic control of *P. aeruginosa* at 4 μg/ml and 8 μg/ml clinical imipenem concentrations, which are only achieved in the lung lumen through aerosolization meaning it is not likely relevant to IV treatment. Interestingly the emergence of protection was entirely dependent on culturing in SCFM, despite *S. maltophilia* degrading imipenem in both SCFM and LB media.

In contrast to our initial hypothesis mucin was not the sole compound responsible for enabling this interaction, as *S. maltophilia* did not protect PA01 when grown together in 5 g/L mucin supplemented LB. We expected the complex mesh formed by long chain mucin proteins to create added structure in the media, which would enable the formation of multi-species pockets. These would reduce solute diffusion while allowing the local degradation of imipenem. The fact we observed a protective effect of *S. maltophilia* when culturing in SCFM(m-) containing reduced mucin levels indicates that the nutritional complexity of sputum is responsible for the emergence of this interaction. The difference in gene expression between *P. aeruginosa* grown in basic laboratory media and SCFM is considerable [112] and SCFM likely induced a more tolerant phenotype to emerge. Although we observed that SCFM increased the imipenem MIC of one clinical *P. aeruginosa* isolate, it had no effect on monocultured PA01, demonstrating that the media operated to increase cell survival in place of enabling growth. Higher survival rates means some cells would still be alive when *S. maltophilia* reaches a suitable density to start significantly decreasing the concentration of imipenem in solution. This local degradation would ultimately enable the global colonisation of the media by the remaining sensitive *P. aeruginosa*. Future research could focus on evaluating if the effect of SCFM is more pronounced during kill-curve resistance assays, which would help distinguish its effect on bacterial survival specifically. Furthermore, the mechanism behind this effect is

unknown: the media could reduce growth rates, enhance biofilm formation, or alter metabolic requirements reducing the activity of imipenem related porins, and mucin may still play a synergistic role. Ultimately, we show that substrate dependent emergence of community-level resistance has the potential to substantially alter the success of clinical imipenem treatment in CF patients. This demonstrated that lung sputum and relatively non-pathogenic community members can interact to enhance the antibiotic tolerance of *P. aeruginosa*.

How novel this finding is in the context of the wider literature is somewhat debateable. One review paper has stated *S. maltophilia* provides protection to *P. aeruginosa* [134]. However, the referenced paper only demonstrated imipenem degradation in *S. maraceans* and did not show any impact on the functional MIC of *P. aeruginosa* [211]. A previous paper by the same author did find experimental evidence [212] but in basic laboratory media. Moreover, both bacterial species were grown at very high starting densities before imipenem treatment, artificially increasing the detoxification rate and survival time. Additionally, resistance was measured using the disk diffusion method which has been repeatedly reported as highly inaccurate and unrepresentative of clinical and broth-based resistance scores [213, 214, 215]. Together this shows that although the concept of *S. maltophilia* mediated protection against imipenem towards *P. aeruginosa* and other microbial species has been considered previously, this study is the first to extensively investigate the interaction in the context of clinically representative media.

Discussing the changes observed in imipenem treated SCFM's capacity to inhibit sensitive *P. aeruginosa* growth after previous bacterial treatment provides several useful insights into the dynamics of imipenem therapy. Firstly, the capacity for *P. aeruginosa* to grow in sterile 1 μg/ml and 4 μg/ml imipenem treatments supports previous studies demonstrating imipenem is innately degraded in solution at 37^{o}C [216]. In 1 μg/ml this effect, combined with the decreased sensitivity afforded by the SCFM, resulted in only minimal control of bacterial growth with filtrate colonisation starting within eight hours of treatment. This shows that the lung lumen concentrations typically achieved through IV administered imipenem treatment is not suitable for treating sensitive *P. aeruginosa* isolates even if treatment is administered twice daily [197, 198, 199]. This likely explains why in the past the drug was considered clinically ineffective and has only recently undergone a recent resurgence as an aerosolised treatment for respiratory pathogens [160, 217], particularly when treating acute infection in juvenile CF patients [218]. Our results support the effectiveness of nebulised concentrations as *P. aeruginosa* growth was completely controlled beyond the 12-hour sampling point in both 4 μg/ml and 8 μg/ml regardless of previous bacterial treatment. This does not however, mean that *S. maltophilia* has little effect on clinical treatment, as thorough mixing during the filtration stage likely disrupted local imipenem gradients, averaging the concentration throughout the media. Furthermore, *S. maltophilia* likely exists at higher densities in the lung and may partly colonise sputum in between treatments, leading to faster imipenem degradation rates and likely earlier growth of *P. aeruginosa*. Conversely, the PA01 strain used was relatively sensitive to imipenem and many sensitive clinical *P. aeruginosa* isolates may still be more tolerant and capable of growth at marginally higher concentrations. Even in the absence of *S. maltophilia*'s ability to detoxify imipenem, concentrations achieved from IV administration only provide very short-term inhibition of sensitive *P. aeruginosa* in lung sputum, suggesting that nebulisation is required for effective control.

Some evidence was also found that shows *P. aeruginosa* has an antagonistic effect on *S. maltophilia's* capacity to degrade imipenem. Despite using equal starting cell densities of *S. maltophilia* for both monocultured and co-cultured treatments, co-culturing with *P. aeruginosa* significantly reduced the rate of imipenem detoxification in 4 μg/ml imipenem. A similar observation was observed in the 1 μg/ml treatment, where monocultured *S. maltophilia* caused greater *P. aeruginosa* filtrate growth compared to co-cultured and no bacteria treatments. This means that despite benefiting from *S. maltophilia*'s degradation of imipenem, *P. aeruginosa* actively disrupts this process, likely through indirect competition or active competitor killing. Interestingly, co-culturing *P. aeruginosa* with *S. maltophilia* does not reduce the rate of imipenem degradation in 8 μg/ml SCFM. This demonstrates that imipenem treatment can modulate interspecies antagonism, probably through the more effective killing and inhibition of *P. aeruginosa* at higher concentrations. We also observed that SCFM is not stable over time at 37^{o}C, as *P. aeruginosa*'s growth in filtrate from the 0 μg/ml no bacteria treatment reduced over time. This suggests that SCFM gradually loses its capacity to support growth and demonstrates a potential limitation when using SCFM as a model for long-term interactions. Although this is a common feature of most medias, standard laboratory medias tend to be more stable over time.

Although the focus has been on *S. maltophilia* and imipenem, it is important to reiterate that against the majority of antibiotics tested resistant CF community members did not provide ecological protection to *P. aeruginosa*. This could be for a variety of reasons: many resistance mechanisms such as efflux pumps do not actively reduce antibiotic concentration, *S. aureus* and *S. maltophilia* may not achieve high enough cell-densities, or alternatively, *P. aeruginosa*-mediated killing might prevent them from providing any protection. Furthermore, *P. aeruginosa*'s capacity to survive at growth-inhibiting antibiotic concentrations is likely what enables it to colonise the media after detoxification. SCFM might not cause the same increase in survival against other antibiotics, leading to more consistent eradication at lower concentrations.

One arguable limitation of this study is that we might have recorded a larger number of inter-species protective interactions if we had grown *P. aeruginosa*, *S. aureus* and *S. maltophilia* to high cell densities and then applied antibiotic treatment, measuring resistance through elimination-kill curves. However, by keeping starting cell densities low in our assays, we created a more realistic representation of *in vivo* lung conditions. It is unlikely two individual species would both exist at high cell densities when living in large multi-species communities [128, 129, 130], even if the sputum itself can support high bacterial growth overall. Furthermore, antibiotic therapy in the CF lung is primarily targeted at preventing new growth, rather than attacking already established colonies [54, 55], making early sputum colonisation the key stage to investigate the efficacy of antibiotic treatments. Naturally we would expect the protective effect from *S. maltophilia* to be magnified in pre-established multi-species biofilms. Ultimately, pre-culturing bacteria before antibiotic treatment biases the outcome towards our preferred result and greatly increases the likelihood any observed *in vitro* interactions do not actually happen *in vivo*.

A more serious limitation of our approach is that we did not explicitly prove our clinical *S. maltophilia* strain actively degraded imipenem. By using media habitability as a proxy, we may have been quantifying the effect of multiple different factors. However, media habitability does work

very effectively as a proxy when simultaneously taking into account the relative half-life of imipenem in solution, and physically removing *S. maltophilia* means only the environment itself is being measured. Furthermore, there is extensive evidence demonstrating that many *S. maltophilia* strains constitutively express beta-lactamases [176], one of which degrades imipenem [177, 178]. One last limitation is that the regular sampling required for the imipenem degradation assay meant container size had to be scaled up from 200ul to 20ml. This caused more mixing, improved nutrient flow and likely increased antibiotic exposure. The lower surface area to volume ratio could also reduce attachment surface availability and reduce the rate of biofilm formation [219]. This means we probably observed higher levels of bactericidal activity than would be seen in a 96-well plate or the cilia of the lungs. Lastly, measuring in eight-hour increments creates a relatively wide time window where the media could have become habitable for *P. aeruginosa* considerably earlier than measured.

Bacterial microflora in the CF lung are usually thought to be inconsequential when evaluating therapeutic options due to low virulence and regular loss over the course of chronic *P. aeruginosa* infection. Here we demonstrate *S. maltophilia* substantially reduces the efficacy of imipenem therapy. Considering over a quarter of CF patients have simultaneous *S. maltophilia* infections [220], this raises an important consideration when evaluating treatment options. *S. maltophilia* can be pathogenic in its own right and infection is often associated with an increased risk of mortality [221, 222], especially in patients where the focal pathogens have been cleared [223]. Quantitative real-time PCR assays have already been demonstrated to rapidly identify *S. maltophilia* with high accuracy [224] and could be relatively easily integrated into normal microbiome diagnostics for CF patients. Our study demonstrates that there is a potential medical benefit to be gained from identifying the presence of *S. maltophilia* even when it is not the primary infective pathogen. Although it is important to note that just because *S. maltophilia* is present in the lung, it does not mean it will definitely provide a protective effect, indicating the need to perform susceptibility testing first. Ultimately, we established that *S. maltophilia* CF isolates can confer ecologically mediated antibiotic protection to *P. aeruginosa* under clinically relevant conditions in SCFM.

Chapter 4. The evolution of imipenem resistance and impact of *S. maltophilia* on *P. aeruginosa* in SCFM

The primary research objective of this part of the thesis was to investigate if ecologically derived antibiotic protection by *S. maltophilia* could alter the evolution of resistance, specifically in the context of CF sputum. We proposed that the local degradation of imipenem by *S. maltophilia* would reduce selection for resistance in *P. aeruginosa* leading to lower levels of evolved tolerance. Instead we found the opposite: *S. maltophilia* co-culturing led to faster rates and higher overall levels of imipenem resistance. This effect was most likely driven by resistance-enhancing inter-specific competition during early sputum colonisation. Several regularly evolved resistance associated mutations, most notably loss of function in the *oprD* porin gene, were consistently evolved alongside a clear fitness trade-off in competitive ability. These results show community derived antibiotic tolerance can affect the long-term evolution of resistance and may enhance the emergence of clinical imipenem resistance, further highlighting the importance of testing for *S. maltophilia* presence in patients' lung microbiomes before using imipenem as a treatment antibiotic.

Comparing clonal imipenem resistance across sampling points demonstrates *S. maltophilia* co-culturing led to the earlier emergence of imipenem resistance by several days, which could be attributed to *S. maltophilia*'s degradation of imipenem. Our habitability assay showed co-culturing enabled *P. aeruginosa* to begin growth over eight hours earlier in imipenem treated SCFM. High growth would increase the number of *de novo* mutations in the population and enable the more rapid fixation of low-frequency mutants, increasing the likelihood of resistance emergence. However, this does not explain why *P. aeruginosa* originally co-cultured with *S. maltophilia* was more resistant by the end of the selection experiment compared to monocultures. Higher resistance was reflected in both MIC and AUC scores showing this difference was not simply the result of an improved ability to grow in sub-inhibitory concentrations. Since *S. maltophilia* imipenem degradation could have enabled the earlier adaptation of *P. aeruginosa* in co-cultures, monocultures may have evolved equal imipenem resistance if selection had continued for longer. This however seems unlikely, as the AUC scores of monocultured clones plateaus from day-12, indicating a stable resistance phenotype.

Originally, we proposed *S. maltophilia* would reduce resistance emergence. The regular detoxification of imipenem should lower selection for resistance while direct competition could constrain adaptation by reducing the effective population and therefore the supply of novel resistance mutations. This follows a common trend in bacterial ecology that competition between species constrains abiotic adaptation [125, 225], in this case imipenem. Instead, we found that competition actually promoted resistance evolution, which could happen if competitive exclusion requires higher level resistance mutations to get fixed. In the presence of the highly resistant *S. maltophilia*, low resistance *P. aeruginosa* mutants are not favoured because these they still have lower fitness compared to the competitor, despite having higher fitness relative to the ancestral PA01 when imipenem is present in the environment. This theory is supported by previous evidence which shows the primary selective pressure in bacterial polycultures is the out competition of rival competitor species [226, 227]. Furthermore, the observation that in 1 µg/ml imipenem increased resistance only evolved in *P. aeruginosa* grown with *S. maltophilia*, indicates imipenem resistance is not being evolved as a direct adaptation to the antibiotic at low culture concentrations. By day-24,

9/10 populations still contained *S. maltophilia*, and mild imipenem resistance likely provided a competitive advantage during initial SCFM colonisation. This means that the presence of *S. maltophilia* lowers the MSC for imipenem resistance. It was not however particularly clear why AUC resistance scores continued to increase in 4 μg/ml and 8 μg/ml treatments several days after *S. maltophilia* had been excluded. Although this can perhaps be attributed to the evolution of secondary adaptations that improved *P. aeruginosa*'s capacity to grow in SCFM, which probably emerged after the first twelve days, when *S. maltophilia* was present in the environment. In summary, this data is the first evidence that ecological interactions between species can increase both the short- and long-term evolution of antibiotic resistance, as co-culturing with *S. maltophilia* lowered the MSC for imipenem resistance in *P. aeruginosa* and increased the maximum threshold for evolved resistance in clinical concentrations.

Having explained the evolution of imipenem resistance in terms of ecological interactions, we then analysed the genetic sequence of a sub-set of evolved *P. aeruginosa* clones. We found that high resistance clones possessed mutations in a larger number of resistance associated regions. However, this difference did not significantly explain variation observed between monocultured and co-cultured treatment groups and reflects a key-limitation when trying to use single-gene explanations to explain relatively complex phenotypes. The observed differences in resistance are therefore likely the effect of positive epistatic interactions between multiple mutations with small individual effects. Increasing evidence is demonstrating the important role epistasis plays in resistance evolution, particularly when populations are evolving in sub-lethal levels of antibiotics [228]. Furthermore, the functional overlap between mutated genes associated with imipenem resistance and adaptation to the SCFM indicates improved media growth and colonisation may also be linked to differences in resistance phenotype.

So far, there has yet to be a study investigating the evolution of imipenem resistance in *P. aeruginosa in vitro.* Our results demonstrate a small suite of mutations are likely involved, with the primary genetic determinant being frameshift and stop codon insertion mutations in the *oprD* gene. *oprD* encodes the outer membrane porin OprD, which is involved in the uptake of amino acids, peptides and carbapenem antibiotics [229]. These mutations were likely loss of function as they appeared in the centre of the gene, probably leading to a non-functional or misfolded protein [89, 90], reducing porin expression and limiting imipenem uptake. A relatively uncommon synonymous mutation in the *PA1874* gene was also associated with increased resistance. This gene encodes a hypothetical outer-membrane glycoprotein which has been found to confer biofilm specific resistance against several antibiotics in *P. aeruginosa* [230]. Although the effects of synonymous mutations can be hard to predict, they can alter transcription factor and ribosomal binding, substantially altering the expression of individual genes, or alter the folding of the resulting protein. Considering that deletion of this gene is associated with increased antibiotic sensitivity [230], it is feasible to propose that this mutation likely increased expression. It has previously been hypothesised the PA1874 may form one component of a mostly uncharacterised efflux pump [230], however it may just as easily reduce the impermeability of the envelope to imipenem. However, many resistant clones did not possess mutations in either gene, indicating they are not the sole determinants of imipenem resistance. Although it is worth noting that *oprD* is highly regulated at both pre- and post-transcriptional levels [229], meaning downregulation may be achieved through transcriptional regulation instead of direct and more permanent mutation-driven change. Performing transcriptomics analysis on evolved clones would have been the next clear step in this

project to investigate how differences in gene expression were associated with resistance phenotypes.

Mutations in three intergenic regions were also notably associated with imipenem resistant clones. However, they also appeared at very low levels in sensitive lineages, indicating there is possibly some cross-over between adaptations for resistance and improved SCFM colonisation. Attempting to determine their effects is more speculative because the non-coding regions between genes often have unknown functions. Firstly, mutations in the intergenic repeat between the *PA3463/4* genes were very significantly associated with increased imipenem resistance. While the upstream gene PA3463 is entirely uncharacterised, the downstream *PA3464* codes for a zinc dependent Phospholipase Nuclease (PLC). PLC's are key for developing twitching motility phenotypes in *P. aeruginosa*, which are a common evolved train in resistant clinical isolates [231]. Performing twitching motility assays on evolved clones may have helped to clarify the importance of this mutation. The second intergenic region, between the *PA4838* and *speA* gene, was also associated with imipenem resistance. The upstream *PA4838* codes an uncharacterised membrane protein, while *speA is* a member of a superantigen toxin family heavily associated with virulence and reduced antibiotic resistance in *Streptrococcus* [232]. Implicating minor changes in expression may subtly increase imipenem resistance. Thirdly, mutations were identified in the *ppiD-fabL* region, which could have improved outer-membrane stability. The upstream *ppiD* gene encodes a periplasmic folding chaperone involved in cell envelope maintenance and has been associated with ciprofloxacin resistance at low concentrations in *E. coli* [233]. The downstream *fabl* gene encodes an NADPH dependent enoyl-ACP reductase involved in fatty acid synthesis and is a common antibiotic target across bacterial species [234]. Considering the bactericidal activity of imipenem stems from cell-wall synthesis disruption [180], this mutation could have enhanced the repair of the outer envelope. In addition, improved maintenance of external barrier may provide a simultaneous advantage in the presence of *S. maltophilia* because clinical strains consistently secrete extracellular lipases and proteases [235]. Overall, all three intergenic regions were considerably more common in clones evolved at higher imipenem concentrations, supporting the hypothesis that imipenem resistance was likely caused by synergistic effects between a variety of different mutations.

In the wider literature, several beta-lactam selection experiments in *P. aeruginosa* have also tested imipenem sensitivity. Beta-lactam antibiotics operate by binding to inner-membrane Penicillin Binding Proteins (PBPs) and preventing the formation of peptidoglycan. Resistance mechanisms primarily involve increasing outer membrane impermeability and the upregulation of efflux pumps [236]. This corroborates our findings that imipenem resistance is likely gained through reducing outer membrane permeability, increasing biofilm formation and maintaining cell envelope integrity. Mutations in the Mex-AB-OprM efflux pump are commonly associated with meropenem resistance but not imipenem resistance [237], further in line with our results. During a seven-day selection experiment in meropenem, three mutations were commonly evolved: *oprD* inactivation, efflux pump overexpression, and alteration of the binding site of PBP-3 [238]. Only *oprD* inactivation was associated with decreased imipenem sensitivity, and this is supported by cross-resistance studies that show *oprM* increases resistance against meropenem but not imipenem [239]. In line with previous findings, our study explicitly proves *oprD* inactivation is one of the major mechanisms for imipenem resistance, while the *oprM* efflux pump is left unaffected. This may be because OprM is a broad activity efflux pump [236] and could incur higher fitness costs relative to mutations in *oprD*, which performs fewer functional roles. Our findings are also supported by research in clinical CF

isolates, which found reduced *oprD* expression was only correlated with increased carbapenem resistance, and that in some isolates loss of *oprD* correlated with increased imipenem resistance [240]. This may directly demonstrate that the presence of mutations in other regions are required for *oprD* to confer clinical imipenem protection and helps explain the common emergence of mutations not mentioned in other selection experiments. However, it is very important to stress that direct comparisons with *in vitro* selection studies from the literature are inherently limited because in their assays concentrations often reach up to 10-100x the clinical breakpoint [237, 238]. This would lead to selective pressures entirely unrepresentative of clinical evolution and likely explains why such high numbers of inherently costly mutations are selected for. Furthermore, high degrees of evolved resistance gained through epistatic interactions are considerably more common when bacteria are exposed to clinical levels of antibiotics [228]. Interestingly, clinical *P. aeruginosa* isolates also tend to have a small number of core resistance determining genes [240], which further supports our suggestion that we have captured an accurate representation of clinical resistance evolution.

Contrary to our original hypothesis, the removal of imipenem by *S. maltophilia* in co-cultures did not reduce selection for imipenem resistance in *P. aeruginosa*. However, we also anticipated that this effect would manifest in a reduced fitness trade-off in growth. Although not finding a clear cost in terms of growth, we did identify distinct trade-offs in competitive ability associated with increasing imipenem resistance. The gradient of fitness loss was practically identical between monoculture and *S. maltophilia* co-culture groups, despite significant differences in imipenem resistance between independently evolved clones. This is unsurprising considering competitors were driven to extinction and antibiotic concentration was the primary factor affecting competitive cost. This indicates imipenem resistance consistently evolved through the same genetic pathways and caused proportional costs to competitive ability. This is similar to previous meropenem selection experiments where a low supply of available mutations created a bottleneck which drove the selection and fixation of high-cost resistance [238]. In contrast, ceftazidime resistance can evolve through a large pool of potential resistance mutations which does enable the stepwise evolution of low-cost resistance through multiple small resistance determinants [238]. Interestingly large genome deletions were found to be a common cause of meropenem resistance [238] but were completely absent in our clones, likely due to their considerably high fitness cost and the clear importance of competitive ability when growing in SCFM.

Unexpectedly, no compensatory mutations that restored the fitness of antibiotic resistant clones were observed, even though they are often regularly reported for other antibiotics [101, 102, 103]. However, some evidence is suggesting that the actual impact of compensatory mutations are often overestimated *in vitro* studies [241] and that they more typically resolve costs associated with single gene mutations [242], in-place of complex multi-gene phenotypes. Furthermore, at higher culture concentrations in our assays, most growth within the transfer window occurs in the presence of antibiotics and therefore selection for growth in their absence is limited. Most compensatory mutations associated with carbapenem resistance in *P. aeruginosa* either alter periplasmic proteases such as *ctpA* or the structural elements of mutated efflux pumps [237]. As observed, none of our clones possessed efflux pump mutations which may have limited their capacity for compensatory mutations. Although, since competitive ability was only assayed at day-24, resistant clones from earlier time points may have had greater reductions in competitive fitness, meaning that compensatory mutations may have emerged that only partly restored fitness. Our results clearly indicate the evolution of resistance during clinical imipenem treatment does cause a fitness cost

reflected in reduced competitive ability. This is not lost over successive generations, demonstrating there is a persistent genetic load that likely stacks when combined with resistance to other antibiotics.

The approach used in this study was designed to maximise accuracy and clinical relevance, however several limitations need to be discussed. A key issue is that we only isolated, assayed and sequenced individual clones in place of comparing whole populations, meaning some variation was likely missed as levels of resistance can vary among individuals in the same culture. However, by choosing one clone from entirely independently evolved population we gained a more representative picture of consistently evolved evolutionary trends. One technical limitation is that some clones were sequenced to considerably higher read depths than others, meaning some mutations may not have been identified, particularly at the start and end of assembled contigs. We used moderately generous SNP definition parameters to adjust for this, which gave a wider range of potential SNPs. Comparing against both the reference genome assembly and the PA01 reference genome (AE004091.2) greatly improved our accuracy at identifying sequencing errors. The semi-independence of clones between sampling points within cultures was also an issue as there was no way to standardise how closely they were related to previous isolates. This caused a problem in day-18 8 µg/ml imipenem where a pair of *S. maltophilia* co-cultured *P. aeruginosa* clones had noticeably lower imipenem resistance scores and dragged down the average. However, by using a relatively large sample size of ten populations for each treatment combination we mostly avoid this problem, demonstrated by reasonably smooth plots of resistance between sampling points. Lastly, growth in SCFM exposed *P. aeruginosa* to another adaptive force making it hard to distinguish between substrate and resistance adaptations, or mutations that were linked to both. Although this was a key part of our study, because the sputum likely augments the evolutionary trajectory of resistance evolution, an alternative approach would be to evolve the bacteria in SCFM first, perform some sequencing, then treat with imipenem. This way resistance develops in a genetic background already adapted to SCFM, and imipenem mutations are easier to distinguish because they are not evolving at the same time with media adaptations.

In summary, this research provides the first evidence that ecological interspecies interactions between pathogen and microbial community members can significantly increase the evolution of antibiotic resistance. This demonstrates that other species may have a more nuanced role than simply providing novel genetic material for HGT. Since this was demonstrated in clinically relative conditions, it has implications beyond the field of evolutionary ecology. It reiterates the need to take the wider diversity of patient microbiomes into consideration, if we are to reign in the local emergence of MDR *P. aeruginosa* in hospitals, which is already a major problem for both CF and non-CF patients. This is especially important as imipenem is currently one of the most efficacious drugs for treating severe pulmonary *P. aeruginosa* and there is strong evidence that extensive clinical imipenem use is also selecting for more generalist carbapenem resistant strains [243]. Carbapenem cross-resistance is particularly concerning because the newest carbapenem, doripenem, is one of the most promising emerging antibiotic therapies for treating *P. aeruginosa* in CF patients [69]. Future directions would include long-term selection experiments with two or three species, taking particular care to keep conditions as clinically accurate as possible, despite the risk of not achieving predicted outcomes. Over time, this would enable the construction of more complex *in vitro* CF communities where individual interactions have already been previously identified.

Chapter 5. The interaction between *P. aeruginosa* and imipenem treatment on *S. maltophilia* persistence in evolved co-cultures

Interspecific interactions extend beyond community-level resistance and the loss of *S. maltophilia* from *P. aeruginosa* co-cultures treated with imipenem demonstrates that antibiotic treatment may indirectly reduce lung microbial diversity which could further impact patient health. During the selection experiment, dual-cultures of both bacteria were able to stably co-exist when grown in the absence of antibiotics. However, exposure to imipenem led to the consistent loss of *S. maltophilia* in 4 μg/ml and 8 μg/ml. The formation of stable communities contrasted with our original hypothesis that anticipated the competitive exclusion of *S. maltophilia*, since *P. aeruginosa* has a faster intrinsic growth rate and is regularly observed to eliminate competing species *in vitro* [120, 244, 245, 246, 247]. Previous *in vitro* studies on interspecies persistence with *P. aeruginosa* utilised simple laboratory medias, indicating that the SCFM might be responsible for enabling stable co-existence. The nutritional and structural complexity of CF sputum could provide a wider range of ecological niches, alongside the physical separation of cells. Generally, competition is reduced in static cultures or biofilms compared with well mixed liquid cultures [227, 248], and the selection experiment used growth conditions between these extremes: liquid cultures with low shaking, regular nutrient replenishment and the presence of structural mucin. This likely generated some additional spatial heterogeneity in the cultures, partially limiting inter-species interactions and therefore reducing *P. aeruginosa*'s ability to eliminate competitors, as is observed *in vivo*.

Furthermore, the SCFM substrate itself may have modulated antagonistic behaviour in *P. aeruginosa*, leading to more benign inter-species interactions, particularly if gene expression shifts towards more varied resource acquisition. This was partially observed with most clones from 0 μg/ml treatments evolving reduced pyocyanin production. Pyocyanin is a bactericidal virulence factor [249] used for controlling the growth of competitors [250]. A common observation in CF patients is that as *P. aeruginosa* infection progresses, clinical isolates become less aggressive over time to both the host and microbiome [251]. This is a trend we might have captured however, we also observed lower pyocyanin reduction when *P. aeruginosa* was evolved with *S. maltophilia* in the absence of imipenem. Therefore, although SCFM modulated antagonistic interactions, the presence of *S. maltophilia* reduced this effect. This may be reflective of early *in vivo* adaptation, during which increased pyocyanin production is a more common phenotype in clinical isolates [252, 253]. Together this implies competition against other microbes may be a notable driving force for increased virulence *in vivo* and may explain why clinical production decreases over time [254] as microbial diversity reduces.

An unexpected result was that the competitive exclusion of *S. maltophilia* by *P. aeruginosa* is induced by antibiotic treatment, providing evidence for a novel factor that might contribute towards the clinical trend of reduced lung diversity over time. Initially, we proposed antibiotic treatment would curtail *P. aeruginosa* growth, increasing reliance on *S. maltophilia* for survival and therefore limiting competition. Instead the opposite was observed: *S. maltophilia* was lost from co-cultures treated with imipenem. Considering that all monocultured *S. maltophilia* populations survived imipenem treatment and their loss from co-cultures increased with imipenem concentration, it demonstrates a clear interaction between imipenem treatment and *P. aeruginosa*. At higher imipenem concentrations, resistant *S. maltophilia* is capable of outgrowing initially sensitive *P.*

aeruginosa, increasing competition and potentially selecting for more antagonistic interactions. Species driven competition aligns with the observation that *S. maltophilia* co-culturing led to *P. aeruginosa* clones having higher max-V growth rates on average, particularly at low-moderate imipenem culture concentrations. Together this supports our previous explanation that enhanced interspecies competition led to increased imipenem resistance evolution in *P. aeruginosa*.

However, the mechanism of competitive exclusion is not entirely clear as pyocyanin production was roughly equal in *S. maltophilia* co-cultured clones across imipenem treatments, suggesting it might instead have played a role in controlling competitor growth. *Pseudomonas* controls several community-level behaviours through the Pseudomonas Quinolone Signal (PQS) QS system [255], most notably phenazine production. Phenazine is used by *P. aeruginosa* to kill *E. coli* in co-cultures [247] and provides a likely candidate mechanism for the removal of *S. maltophilia* from cultures. Worth noting is that PQS-controlled systems are often induced during stress responses [255], making it possible *S. maltophilia* was excluded due to a generic stress response to imipenem by *P. aeruginosa*. This is supported by enhanced phenazine production specifically being found to increase antibiotic tolerance [192], however we did not observe other markers associated with increased stress response such as elevated pyocyanin [256]. The use of the nitrate reductase activity test [257] to test for relative phenazine production could help explain the mechanism of imipenem-induced *S. maltophilia* loss in future research.

One limitation with our approach is that we only tested for the observable presence of *S. maltophilia*, not relative percentage of biovolume, meaning although present the bacteria may have simply existed at inconsequential densities. However, it is worth noting is that since *S. maltophilia* is slow growing we would not expect high densities *in vitro* or *in vivo*, and although we did not quantify CFU/ml, we did qualitatively observe that selectively plating small amounts of 0 µg/ml co-cultures led to substantial colony formation, indicating at least moderate levels of persistence. Furthermore, since each population was independently evolved, and each treatment replicated ten times, the consistent parallel development of community composition is indicative of a significant presence of *S. maltophilia*. One more constraint is that we only tested long term persistence in SCFM and not LB media. However, this would not have helped explain *in vivo* interactions, and there is already extensive evidence that *P. aeruginosa* simply outcompetes other species in simple laboratory medias [120, 244].

Ultimately, our results demonstrate antibiotic treatment induces more antagonistic and competitive behaviours in *P. aeruginosa,* leading to the out-competition of other microbial species. Across CF patients there is a consistent trend of decreasing microbial lung diversity with time [258, 259], associated with antibiotic treatment removing sensitive species [128] and focal pathogens directly altering community composition [260]. Here we indicate there is likely an interaction effect between *P. aeruginosa* and antibiotic regime which also drives the loss of community members *in vivo*, particularly those already possessing antibiotic resistance. This highlights another danger of prescribing ineffective antibiotics, as changes in microflora are connected to severe decline in lung function [261] and worsened pulmonary exacerbations [48]. Antibiotic treatment could inadvertently remove resistant low virulence community members that would otherwise help control *P. aeruginosa* growth through direct competition. These results simultaneously have useful

applications for the field of evolutionary ecology, as our assay demonstrates growth substrate and media complexity may be key to creating stable polymicrobial communities *in vitro.* The inclusion of the wider microbiome is one of the major next frontiers for CF assay design, however these assays are rarely performed with even two or three species communities because *P. aeruginosa* regularly outcompetes other microbes *in vitro* [193, 262]. Our results suggest the use of more clinically relevant growth media, alongside the inclusion of components that add structural complexity such as mucin, may increase the capacity for bacterial species to persist together. Future research could investigate if imipenem induced competitive exclusion still happens when both species are left to adapt together in SCFM several days before antibiotic treatment is applied.

Chapter 6. Adaptation to CF lung sputum

Inclusion of growth substrate representative of *in vivo* lung sputum was a key element of our assay design and we observed that SCFM imposed significant evolutionary pressures on *P. aeruginosa*. During the selection experiment in SCFM, clones underwent parallel evolutionary changes in several phenotypic traits and genomic regions, emerging independent of imipenem or *S. maltophilia* treatment conditions. These provide several insights into the specific influence of CF sputum during the early stages of lung infection and adaptation, helping to identify new candidate genes likely involved in the process.

Mutations commonly evolved in several resource metabolism genes that are likely associated with *P. aeruginosa* using a wider variety of nutrients as primary metabolites. Dehydrogenase based metabolism was notably affected, as over half of all clones underwent a missense substitution in the *PA1649* (*linC)* gene which encodes an uncharacterised short chain dehydrogenase. Furthermore, 10% of clones possessed mutations in the *exaC* gene, encoding a reductase-like aldehyde dehydrogenase. Dehydrogenases operate on a wide range of substrates [263] but are primarily involved in short chain fatty acid (SCFA) and sugar metabolism [264, 265, 266]. Altered amino acid metabolism was also a likely evolved adaptation as a single SNP appeared in 92% of all clones in the non-coding region between the *PA2604/5* genes. The upstream *PA2604* is entirely uncharacterised, however the downstream *PA2505* encodes the TusD sulphur-transferase subunit, part of the sulphur relay system in *Pseudomonas* [267]. This system is most notably responsible for the metabolism and catabolism of sulphur containing amino acids, such as methionine and cysteine [268], both represented in SCFM. Mutations in this region likely help regulate downstream TusD expression to take advantage of the wider range of amino acids available. Mutations in these metabolic pathways are consistent with the observation that *P. aeruginosa* predominantly uses SCFAs and amino acids as carbon sources when grown in SCFM, even when glucose is readily available [52, 106]. These metabolic preferences are also reflected during *in vivo* growth [269]. Lastly, the broad functionality of many dehydrogenases may explain why *PA1649* (*linC*) was notably more present in imipenem resistant clones and suggests some of these metabolic adaptations may extend towards increasing antibiotic tolerance, particularly as most imipenem resistant *PA1649* mutants did not possess *oprD* mutations. Although they could also alternatively be compensatory mutations to overcome the costs of evolved imipenem resistance. Our results provide strong evidence *P. aeruginosa* evolves a selection of metabolic adaptations when cultured in SCFM, demonstrating that the nutritional complexity of CF lung sputum does likely directly influence genomic adaptation during chronic infection.

Changes in metabolism are often controlled by plastic gene expression and a substantial proportion of SCFM evolved clones possessed mutations in two regions associated with genomic and proteomic regulation, indicating sputum adaptation is linked to non-plastic changes in wider gene expression. Over 90% of clones had the same missense amino acid substitution in the *aat* gene, which codes a leucyl/phenylalanyl-tRNA protein transferase. This transferase plays a key role in protein degradation by N-terminal conjugation [270], suggesting that altering the post-translational regulation of proteins is a key adaptation to SCFM. A variety of SNPs were also evolved in over 85% of clones in the intergenic region between *PA3421 and MalT*. *PA3421* is entirely uncharacterised, however *MalT* encodes a transcription factor involved in the upregulation of genes involved in the uptake and metabolism of malto-oligosaccharides [271]. This could reflect that the use of substrate

which mimics CF lung sputum leads to commonly evolved changes in pre-transcriptional and post-translational regulatory networks. Although rare, one clone underwent several mutations in the *PA0715* gene which codes for a reverse transcriptase family protein. However, due to its singular appearance it was most likely the result of random genetic drift. Considering that growing PA14 in isolated CF sputum upregulates many genes involved in amino acid, SCFA and sugar uptake and metabolism [272], it is likely that mutations in these regulatory gene regions are also likely impacting the core metabolism of *P. aeruginosa*, further implicating the nutritional complexity of SCFM as a major selective pressure.

Selection for the acquisition of inorganic compounds also appeared to be a considerable adaptive force in SCFM. Increased pyoverdine production was universally evolved across all clones, alongside mutations in the phosphate chemotaxis gene *ctpH* which emerged in over 70% of evolved clones. The consistent appearance of these changes demonstrates that iron and phosphate are particularly limited in this environment and that *P. aeruginosa* alters its capacity to sense and acquire these resources when adapting in SCFM. Pyoverdine is a siderophore commonly secreted by *P. aeruginosa* to scavenge essential iron from both the environment and host cells [273] when local iron is limited. Transcriptional changes are also observed in the upregulation of pyoverdine biosynthesis and synthase genes when *P. aeruginosa* is cultured in isolated CF sputum [272], reflecting strong similarities in the effects of SCFM and CF lung sputum on *P. aeruginosa*'s iron acquisition. The *ctpH* gene on the other hand encodes a chemotaxis receptor involved in sensing low to high inorganic phosphate gradients [274]. Considering all mutations in this gene were silent, and appeared within the same six bp region, they could have operated to alter expression while keeping the protein structure in-tact. Most likely it led to the upregulation of the ctpH receptor, as CF sputum also upregulates several chemotaxis genes in *P. aeruginosa* [272], enabling lower levels of phosphate to be detected and acquired. Together, this suggests iron and phosphate limitation is a major adaptive barrier to growth in SCFM, which *P. aeruginosa* overcomes through non-plastic changes in pyoverdine and chemotaxis receptor expression.

Iron sequestration is a common host strategy to limit bacterial growth, and although CF sputum iron concentrations are roughly four times higher than healthy sputum [275], it is still environmentally limited [276]. Since pyoverdine is also involved in the upregulation of biofilm formation [277] and several virulence genes [278], adaptation to lung sputum may intrinsically drive enhanced virulence through selection for improved iron acquisition. However, high iron availability intrinsically increases growth rates and pathogenesis, as there is a very close relationship between sputum iron availability and *P. aeruginosa* bacterial load in CF patients [279]. Clinically, CF isolates tend to have reduced [280], and sometimes completely absent [281], levels of pyoverdine production, while longitudinal studies demonstrate production decreases over the course of infection [273]. Initially this appears to contradict our findings. However, pyoverdine production is upregulated in clinical CF *Pseudomonas* isolated from acute infections [282, 283]. A proposed mechanism is that pyoverdine production decreases as more efficient iron acquisition develops [282], primarily membrane-bound heme transporters [284]. Interestingly, the low iron environment of the SCFM has recreated the adaptation of increasing energetically costly [285] pyoverdine production, typically observed in clinical strains during the initial stages of lung infection.

Curiously this result was is in complete contrast to a previous study, Schick et al. (2018) that performed a similar SCFM selection experiment for 220 generations, or approximately five days [286], with PA14 and without the inclusion of antibiotics or community members [149]. They found pyoverdine production levels decreased and concluded they had successfully recreated *in vivo* adaptation. However, reduced production levels were identical in both SCFM and Minimal media treatments. The growth model used in their study likely resulted in cultures being in continual exponential growth due to performing small transfers into high volume media every 24 hours. This would result in iron being in abundance and limit cell-density controlled QS induction of pyoverdine. Furthermore, they did suggest some of their results may have been from adaptation to *in vitro* growth conditions instead of the SCFM specifically. Interestingly, Schick et al. (2018) did find high mucin concentrations led to lower reductions in pyoverdine, suggesting the presence of mucin in our assay likely played a key role in stimulating increased production. High viscosity would limit nutrient flow and reduce local iron levels, increasing the need for iron scavenging while simultaneously reducing diffusion efficiency. In support of this, no change in pyoverdine production was observed during an SCFM selection experiment using *P. aeruginosa* strain AA2 which did not include mucin [193]. Including mucin and non-mucin treatments in our own selection experiment would have enabled more pronounced insight into its effects. Ultimately, *P. aeruginosa* universally upregulates pyoverdine production to improve the acquisition of limited iron in the sputum, capturing trends observed during acute infection stages. Increased production is directly associated with stimulating the production of other virulence factors and supporting overall biofilm development until alternative iron acquisition mechanisms evolve.

Despite biofilm formation not being directly assayed, we observed that mutations in the biofilm-associated *cdrA* gene emerged in over 45% of all clones. CdrA is a versatile biofilm matrix protein, regulated by the cyclic-di-GMP signalling system. It binds indiscriminately to extracellular polymeric substances and supports the agglutination of cells into aggregate biofilms [287, 288, 289]. Similar to the *ctpH* gene, all mutations were silent but appeared within a very narrow region, indicative of altered transcription through changes in ribosomal or transcription factor affinity. The deletion of the *cdrA* gene leads to significant biofilm defects [287] and since biofilm production factors are upregulated in sputum [272], these mutations likely stimulate the expression of CdrA. Interestingly there was one variant form of this mutated gene that was notably more present in imipenem resistant *S. maltophilia* co-cultured clones compared with other treatment groups. Altered biofilm production may operate to decrease antibiotic sensitivity while simultaneously reducing harmful interactions with competitors, providing a potential mechanism to explain disparities observed among evolved imipenem phenotypes. Mutations in this gene support the concept that biofilm formation is a key adaptive phenotype in CF sputum while simultaneously indicating that *P. aeruginosa* might adapt to both competitors and antibiotic treatment through permanent changes to biofilm development.

Reductions in pyocyanin production also appeared to be an adaptive response to long term growth in SCFM, suggesting adaptation to CF lung sputum may be a primary driver of reduced virulence. The redox activity of pyocyanin is used by *P. aeruginosa* to attack host cells and competing microbes [290] by interfering with an array of core cellular functions from energy metabolism to innate immunity [254]. Since the main targets of pyocyanin, lung epithelial cells and innate immune factors, were not included in this study, the lack of increase in production makes logical sense and indicates adaption to growing in lung sputum does not in itself increase virulence. In fact the reverse was

observed as pyocyanin levels decreased which suggests adaptation to the lung sputum substrate actively drives decreased pathogenicity. Pyocyanin is one of the major virulence factors produced by *P. aeruginosa* and is directly associated with pulmonary exacerbation and increased severity of disease [291]. In murine models, *P. aeruginosa*'s capacity to produce pyocyanin is strongly linked with mortality [292], disease progression, and capacity to clear infection [293]. A well-established trend is that virulence reduces over the course of chronic *P. aeruginosa* infection in the CF lung [252, 294]. Reduced virulence is associated with increased persistence and there is increasing evidence suggesting the trend also applies to pyocyanin [254]. Although pyocyanin is regularly recovered from CF patient sputum [295, 296], and some clinical *P. aeruginosa* isolates overproduce pyocyanin [297], phenotype diversity is relatively high [291] and *in vivo* concentrations can range between 0.00 to 27.3 μg/ml across patients [296]. Our results indicate that moderate to long term growth in CF sputum-like conditions reduces pyocyanin production and is therefore likely a key driver of the relationships of reducing virulence over time.

A common by-product of SCFM adaptation in evolved clones was a significantly reduced Max-V growth rate. We observed clear increases in competitive fitness, especially in 0 μg/ml imipenem, indicating that this change is linked to SCFM adaptation and is not the result of random drift. Considering the range of metabolism-linked mutations, it is likely that improved sputum colonisation comes at a distinct cost in decreased exponential growth. SCFM is a more complex nutritional environment and although consuming a wider range of nutrients is advantageous for long-term colonisation, upregulating more varied nutrient uptake systems is energetically costly, providing long term success at the cost of short-term growth. This further highlights the problem with using growth rates to measure fitness, as they are not necessarily representative of long-term survival. Alternatively, the reduction in Max-V growth rates may simply have been a result of adaptation to *in vitro* growth conditions. Populations typically entered stationary growth within 16-22 hours and then had to persist in high densities for another day. Reduced nutrient availability towards the end of each transfer window may have selected for lower metabolic rates, which improved long-term survival between transfers. This seems unlikely because not only could filtered SCFM support multiple rounds of moderate density growth, but evolved strains with reduced Max-V rates also competitively outperformed the ancestor over 24 hours. Furthermore, the total growth rate remained unchanged indicating growth capacity had not been fundamentally reduced.

Overall, our finding that metabolism-associated genes are consistently evolved in parallel populations grown in SCFM is in agreement with previous evidence that found the nutritional diversity of SCFM is the main driver of within-population diversity [149]. Interestingly the genomic regions we observed to be regularly mutated were not in the group of 20-54 'pathoadaptive' genes identified as most often evolving in clinical CF isolates [298, 299, 300], with the exception of *oprD*. However, there is considerable overlap between the functionality of mutated genes, such as: aldehyde dehydrogenase [299], amino acid metabolism [298, 299], chemotaxis [298], degradative protein regulation [299, 300] and cyclic-di-GMP regulated biofilm formation [301, 298, 299, 300]. The primary differences were that the vast majority of clinical mutations were caused by nonsense loss of function mutations which were non-existent in our genomes, excluding *oprD*. Clinical isolates have a high prevalence of large genomic deletions [300], and in one study, mutations in 45/52 pathoadaptive genes were nonsynonymous [298]. This suggests that although laboratory SCFM captures some of the selection pressures operating *in vivo,* the actual lung environment is considerably more complex probably due to selection by the host. The presence of an active

immune system, a wide variety of antibiotic treatments and more aggressive competitors likely drives more rapid adaptation in the form of severe genomic deletions and loss of function mutations. Adapting to SCFM alone results in relatively modest adaptations that are not particularly deleterious outside of the SCFM environment, however this could be further tested by performing competition assays against the ancestor in standard laboratory media.

One major draw-back with analysing our sequence data is that comparing SNP presence does not in itself explain their effect on gene function. Predicting the effect of mutations in genes involved in broad metabolic processes is particularly difficult, especially when observed genetic changes were synonymous, having no clear effect on protein structure. However, we can still make reasonable hypotheses for potential effects on expression levels based on our understanding of transcriptional control and the general trends of gene regulation observed in *P. aeruginosa*. Furthermore, despite being descriptive, simply observing mutations at high frequencies suggests consistent parallel evolution, especially as clones were isolated from populations that were evolved independently to one another. A more practical consideration is that we measured relative pyoverdine production using the same media as the selection experiment, contrasting other studies which tested evolved traits in a neutral laboratory media [149]. Being unable to quantify media-based expression level reduces our capacity to interpret the relative effect of SCFM. However, this insight would be inherently limited as testing phenotypes in basic laboratory media is mostly irrelevant to answering the clinical aspect of our research questions. Fundamentally, our selection experiment was limited by not simultaneously selecting populations grown in LB media, making it impossible to disentangle SCFM-specific adaptations. Doing so would have resulted in halving the number of replicates for each treatment, considerably decreasing our reliability and statistical power. Furthermore, commonly observed *in vitro* adaptations did not emerge in our clones or were already evolved in the ancestor. Lastly, this problem was why such care was taken for creating clinically representative assay designs, as we are measuring adaptation to our clinical growth model specifically, which is ultimately only a proxy for *in vivo* infection.

To summarise, SCFM does impose clear selective pressures on *P. aeruginosa*, most likely to utilise the wider array of nutrients available, enhance biofilm formation and improve acquisition of limited iron in the environment. This manifests through a mixture of mutations affecting both specific mechanisms, primarily amino acid and sugar metabolism, alongside broader changes to pre- and post-transcriptional regulatory systems. There is clear evidence for synergistic interactions between media adaptation, competitor presence and resistance evolution mutations, demonstrating the importance of accurate growth substrate. However, in comparison to clinical strains, observed mutations emerged in genes with more narrow functions compared to broader regulators. It seems likely that selection by the host immune system and the complexity of the lung environment pose a wider range of stronger selection pressures than our *in vitro* assay. Here we provide some new candidate genes likely involved in *in vivo* lung adaptation and begin to explain the evolutionary effect of CF sputum substrate individually, separate from the context of wider infection.

Conclusion

The emergence of antibiotic resistance in *Pseudomonas aeruginosa* is the major driver of death in cystic fibrosis patients, the most significant hereditary genetic disease in the European population. Despite the complexity of *in vivo* infection, key clinical elements such as growth substrate and microbial community members are excluded from most *in vitro* assays of resistance evolution, severely limiting the relevance of their findings to the clinical context. We have demonstrated that sputum substrate significantly decreases antibiotic susceptibility in clinical isolates and enables *S. maltophilia* to confer ecological protection to sensitive *P. aeruginosa*, reducing the clinical efficacy of imipenem. Long-term imipenem treatment in SCFM resulted in the competitive exclusion of resistant *S. maltophilia*, which would have otherwise stably coexisted in co-cultures with *P. aeruginosa*. Furthermore, the initial presence of *S. maltophilia* resulted in faster evolution and higher overall levels of imipenem resistance in *P. aeruginosa*. Resistance was conferred by a small suite of mutations, primarily loss of the OprD porin, and was heavily associated with a fitness trade-off in competitive ability. Synergistic interactions with adaptations to the SCFM itself were also associated to differing levels of resistance. Evolution in CF sputum is geared towards improved nutrient metabolism, biofilm formation and iron acquisition, however mutational changes were considerably less severe than those observed in clinical isolates. Our results demonstrate that considering the impact of CF lung sputum should not be overlooked when investigating the evolution of antibiotic resistance in *P. aeruginosa*, as the use of sputum replicating media can significantly alter the outcome of evolutionary studies. This indicates a need for the sensitivity testing of clinical isolates to incorporate basic sputum components, or for the creation of an advisory framework detailing likely species-antibiotic-media interactions. Here, we provide the first evidence in the field that ecologically conferred antibiotic tolerance can increase the long-term evolution of resistance at both the phenotypic and genotypic level. This interaction very probably emerges during *in vivo* infections, stressing the importance of performing diagnostic testing on patient lung microflora before administering specific last-line antibiotics, such as imipenem. Ultimately, the CF lung contains dozens of microbial species many of which possess intrinsic antibiotic resistance, meaning this study has likely only scratched the surface of the scope of these interactions and their potential implication for resistance evolution.

Abbreviations

CF	Cystic Fibrosis
SCFM	Synthetic Cystic Fibrosis Media
SCFM(m+)	Synthetic Cystic Fibrosis Media + 5 g/L porcine mucin
SCFM(m-)	Synthetic Cystic Fibrosis Media + 0.5 g/L porcine mucin
LB media	Luria-Bertani media
LB(m+) media	Luria-Bertani + 5 g/L porcine mucin media
WHO	World Health Organisation
MDR	Multi Drug Resistant
SNP	Single Nucleotide Polymorphism
HGT	Horizontal Gene Transfer
QS	Quorum Sensing
MSC	Minimum Selective Concentration
HQNO	2-heptyl-4-hydroxyquinoline N-oxide
DSF	Diffusible Signal Factor
ASM	Artificial Sputum Media
MIC	Minimum Inhibitory Concentration
RFI	Red Fluorescence Intensity
OD	Optical Density
CFU	Colony Forming Units
PA01	*Pseudomonas aeruginosa 01*
PA01:rfp	*Pseudomonas aeruginosa 01 : red fluorescence protein*
Max-V	Maximum rate of growth
AUC	Area Under the Curve
ANOVA	Analysis of Variance
AA	Amino Acid
PLC	Phospholipase Nuclease
PBP	Penicillin Binding Protein
PQS	*Pseudomonas* Quinolone Signal
SCFA	Short Chain Fatty Acid

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