COLLATERAL SELECTIVE LANDSCAPE OF DRUG RESISTANCE IN EXPERIMENTALLY EVOLVED BIOFILMS OF *PSEUDOMONAS AERUGINOSA*

By

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Abstract

Biofilms represent a discrete form of microbial life which possess physiological distinctions from free-living planktonic counterparts which may also elicit lifestyle-dependent adaptive responses to selective pressures. In this work, an experimental evolution model is validated and used to study the evolutionary trajectories to antimicrobial resistance in biofilms of *Pseudomonas aeruginosa*. The serial passage of biofilms selected for biofilm hyperproduction which was characterised by increased biomass deposition and phenotypic diversification but was not associated with intrinsic resistance to antibiotics. The mechanisms by which this was achieved appeared to be through constitutive increase of intracellular c-di-GMP concentrations via mutations in the phosphodiesterase *dipA,* the *yfiBNR* signalling complex and bifunctional diguanylate cyclase/ phosphodiesterase *morA*, Next, whether biofilm and planktonic lineages took disparate evolutionary trajectories to resistance to the antipseudomonal antibiotics, ciprofloxacin, ceftazidime and tobramycin was investigated. For each antibiotic, resistant mutants from both lifestyles possessed conserved 'driver' mutations, however mechanistic distinctions between lifestyles were identified based on the repertoire of secondary mutations. This included efflux pump regulators, porins and biofilm-dependent pathways including type IV pili and alginate biosynthesis genes. Finally, the effect of adaptation to a panel of 48 antimicrobial agents including antibiotics, biocides and metals on biofilm formation, relative fitness and antimicrobial susceptibility was investigated. Over half of the selective agents were able to collaterally select for reduced susceptibility to antibiotics indicating that the widespread use of these agents may act as a driver of clinically-relevant drug resistance. Cross-resistant mutants arose through a variety of mechanisms, including constitutive expression of multidrug efflux, hypermutation, cell wall remodelling and porin loss. This work provides novel insights into the role of lifestyle in adaptation to selective pressures and the importance of collateral selective effects in the evolution of drug resistance.

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List of Abbreviations

ABC	ATP-binding cassette
AHL	N-Acyl homoserine lactones
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
AUC	Area under curve
bp	Base pairs
CAUTI	Catheter-associated urinary tract infection
CDC	Centers for Disease Control and Prevention
c-di-GMP	Cyclic diguanylate
c.f.u.	Colony forming units
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HGT	Horizontal gene transfer
H-NS	Histone-like nucleoid structuring protein
ICU	Intensive care unit
IRIDA	Integrated Rapid Infectious Disease Analysis
LB	Lysogeny broth
LPS	Lipopolysaccharide
LTEE	Long-term evolution experiment
MATE	Multidrug and toxic compound extrusion family
MBEC	Minimum biofilm eradication concentration
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MSF	Major facilitator superfamily
MGE	Mobile genetic element
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical density
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDR	Pan-drug resistant

pmf	Proton-motive force
(p)ppGpp	Guanosine (pentaphosphate) tetraphosphate
PQS	*Pseudomonas* Quinolone Signal
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
RND	Resistance-nodulation-division superfamily
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCC*mec*	Staphylococcal cassette chromosome *mecA*
SCV	Small colony variant
SMR	Small molecule resistance family
SNP	Single nucleotide polymorphism
QAC	Quaternary ammonium compound
QRDR	Quinolone resistance determining region
UTI	Urinary tract infection
VBNC	Viable-but-nonculturable
WHO	World Health Organisation
XDR	Extensively drug resistant

CHAPTER 1. INTRODUCTION

1.1. Pseudomonas aeruginosa

Members of the genus *Pseudomonas* are non-fastidious Gram-negative, motile rods within the class Gammaproteobacteria (Baron, 1996). They are aerobes characterised by broad metabolic plasticity and widespread colonisation in the natural world. Most pseudomonads are non-pathogenic saprophytes ubiquitous to soil and waters, but important human and plant pathogens also exist, such as *Pseudomonas aeruginosa* and *P. syringae*, respectively. Pseudomonads are closely related to other genera of human pathogens including *Moraxella* spp. and *Acinetobacter* spp. residing within the order Pseudomonadales.

1.1.1. Clinical Manifestations

P. aeruginosa is an opportunistic pathogen of significant morbidity and mortality in the immunocompromised. It is responsible for approximately 10% of bacterial infections in nosocomial environments, which increases to over 20% in intensive care units (ICUs) (Gaynes and Edwards, 2005, Lister et al., 2009). Individuals with compromised pulmonary function or primary immunodeficiencies demonstrate an incidence of *P. aeruginosa* infection up to ten-times that of the immunocompetent (Bassetti et al., 2018). Lower respiratory tract infections are the most common manifestation of healthcare-associated *P. aeruginosa* infection, with the principal risk factor being structural lung disease, including chronic obstructive pulmonary disorder, bronchiectasis or cystic fibrosis (Restrepo et al., 2018). Unlike many opportunistic respiratory pathogens, *P. aeruginosa* is seldom commensally present in the respiratory microbiome. Carriage of *P. aeruginosa* in healthy individuals is normally below 5%, however colonisation rates can rise to over 50% after hospitalisation due to its propensity to contaminate the nosocomial environment (Cross, 1985, Morrison and Wenzel, 1984). *P. aeruginosa* is often the most frequently isolated Gram-negative aerobe from environmental surfaces and fomites within ICUs. Consequently, it is the most common aetiological agent of late-onset nosocomial and ventilator-associated pneumonia and second-most common cause of hospital-acquired pneumonia overall, after *Staphylococcus aureus* (Rello et al., 2002). Sequelae of pulmonary disease is associated with long-term respiratory colonisation in recently hospitalised individuals with structural lung disease or who have received broad-spectrum antibiotic therapy (Rello et al., 1998). *P. aeruginosa* pulmonary infection in the immunocompromised is associated with poorer clinical outcomes compared to other Gram-negative rods, with crude mortality often exceeding 50% (Bodey et al., 1983, Fujitani et al., 2011).

Consequently, *P. aeruginosa* infection has been observed to possess the highest burden of disease of any aetiological agent in intensive care units (Lambert et al., 2011).

P. aeruginosa is the second-most frequently isolated pathogen from the cystic fibrosis lung, after *S. aureus,* but the most significant contributor to infectious mortality (Parkins et al., 2018). Long-term colonisation with *P. aeruginosa* in cystic fibrosis sufferers is associated with aggressive decline in pulmonary function which effectuates an approximate ten-year reduction in life expectancy (Driscoll et al., 2007). In a healthy sinopulmonary tract, innate immunity and the ciliary clearance of mucous normally excludes pathogens from the lower tract. However, cystic fibrosis sufferers possess dysregulated epithelial chloride ion transport, causing net sodium resorption which dehydrates the airways of periciliary fluid (Faure et al., 2018). Consequently, mucous becomes thickened and unable to be expectorated, obstructing the respiratory tract with foreign material which predisposes individuals to chronic inflammation and colonisation with opportunistic pathogens. Approximately half of cystic fibrosis sufferers are asymptomatically colonised with *P. aeruginosa* within the first three years of life (Smith et al., 2017). By adolescence, in most individuals, this progresses into a chronic, unresolvable disease. This, over the course of decades, periodically exacerbates until death from loss of respiratory function (Bhagirath et al., 2016, Malhotra et al., 2019).

P. aeruginosa is also a significant cause of extra-pulmonary infections and is associated with a higher morbidity and mortality than most aetiological agents (Driscoll et al., 2007). It is the third most common cause of complicated urinary tract infections (UTIs), after *Enterobacteriaceae* and *Enterococcus* spp. (Lister et al., 2009). It is rarely seen in uncomplicated UTI as it is predominantly associated with long-term catheterisation due to its persistence as a wash solution contaminant and propensity to colonise the catheter lumen as a biofilm (Newman et al., 2017). Moreover, *P. aeruginosa* is the most common pathogen of late-onset burn wound infections, where it produces a characteristic blue-green purulence in wound dressings (Weinstein and Mayhall, 2003). It is also the fourth-most common pathogen of surgical wounds after *Staphylococcus* spp., *Enterococcus* spp. and *Escherichia coli*, and the most significant contributor to contact lens-related keratitis and infectious vision loss (Pruitt et al., 1998, Teweldemedhin et al., 2017, Yılmaz et al., 2006). Appropriate skin and wound antisepsis decolonises the commensally-present opportunistic pathogens which dominate the aetiology of early-onset infections. Consequently, infections which manifest after two weeks are predominantly caused by exogenous acquisitions from the hospital environment, of which *P. aeruginosa* is the most significant aetiology

(Church et al., 2006). Indeed, the transmission of *P. aeruginosa* in nosocomial environments is associated with its widespread endemicity in hospital plumbing systems which have been a reservoir of several fatal *Pseudomonas* outbreaks in the United Kingdom (Quick et al., 2014). Surveillance of potable water sources for *P. aeruginosa* in augmented care units is now extensively conducted and guidance outlining antipseudomonal decontamination measures is in place nationally. *P. aeruginosa* wound infections are a serious risk factor for progression to disseminated disease in individuals with wounds, primary immunodeficiencies, acute myeloid leukaemia or after haematopoietic stem cell transplantation (Funada and Matsuda, 1998, Lossos et al., 1995). *P.* aeruginosa bloodstream infections are up to six-times higher on burn wards than in the general hospital population and possesses the highest mortality in the neutropenic out of any aetiological agent (Chatzinikolaou et al., 2000, Song et al., 2001).

1.1.2. Pathogenesis

The versatility of *P. aeruginosa* as a pathogen can be attributed to an array of virulence factors which contribute to its ability to colonise diverse anatomical sites and persist in abiotic environments (figure 1.1.). Chronic pulmonary infection in cystic fibrosis, acute nosocomial pneumonia, UTIs and medical device-related infections possess distinct pathogeneses due to the remarkable metabolic flexibility facilitating pathoadaptation (Jurado-Martín et al., 2021). *P. aeruginosa* possesses an extensive repertoire of secreted products, regulatory networks and delivery apparatus to translocate virulence factors to their extracellular targets. The secretome of *P. aeruginosa* represents approximately 3% of genomic open reading frames and consists of cytotoxins, proteolytic and lipolytic enzymes, secondary metabolites and exopolysaccharides (Behzadi et al., 2021).

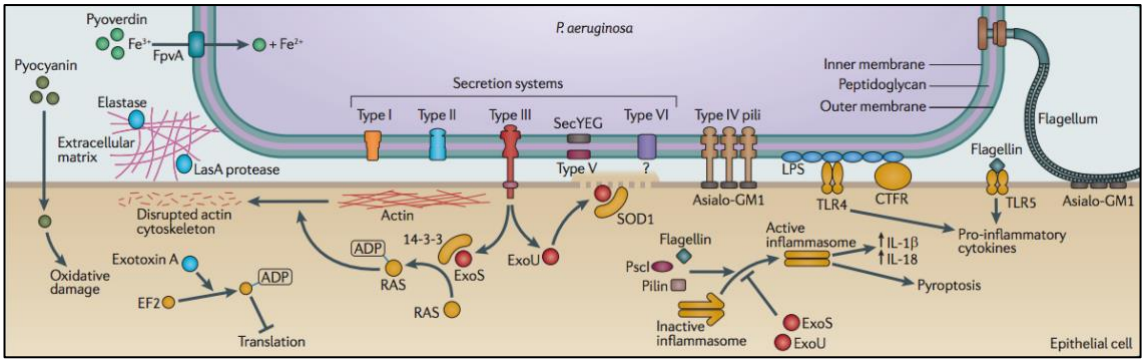

Figure 1.1. *P. aeruginosa* possesses a large repertoire of virulence factors which contribute to its diverse clinical presentation and broad host range. This includes several structurally distinct secretion systems which translocate toxin effectors into the extracellular milieu or directly into host cells in addition to secondary metabolites such as siderophores, oxidative agents and extracellular polymers. This acts to repress activation of toll-like receptors, inhibiting inflammasome formation disrupting cytoskeleton and inducing tissue necrosis. In this way, *P. aeruginosa* can remodel the host environment to support tissue infiltration and immunoevasion. Adapted from Hauser and Ozer (2011).

1.1.2.1. Secretion Systems and Toxin Effectors

Most of the protein components of virulence of *P. aeruginosa* are effectors for three major pathogenicity-associated secretion systems, type I, II and III (Behzadi et al., 2021). Proteolytic and lipolytic enzymes possess important roles in remodelling the lung microenvironment for colonisation however, the decompartmentalisation of infection relies on expression of potent secreted exotoxins (Kipnis et al., 2006). The most important secretion system in *P. aeruginosa* is the type III secretion system, which translocates effectors directly into neighbouring cells with a syringe-like appendage known as an injectisome (Klockgether and Tümmler, 2017). The substrates of the type III secretion system are exclusively cytotoxins: the guanosine triphosphatases ExoS/T, the adenylate cyclase ExoY and the phospholipase ExoU (Burstein et al., 2015). These toxins are differentially expressed in different strains of *P. aeruginosa,* but operate co-ordinately to lyse epithelial cells and resist phagosomal internalisation.

The type I secretion system is a tripartite complex with homology to an adenosine triphosphate-binding cassette (ABC) superfamily efflux pump (Bleves et al., 2010). *P. aeruginosa* possesses two type I secretion systems associated with virulence which possess monospecific substrate specificities: AprDEF, which secretes the alkaline protease AprA, and HasDEF, which secretes the heme chelator HasAp.

P. aeruginosa also possess two type II secretion systems, Hxp and Xcp (Filloux, 2011). Hxp has a single known virulence-associated effector, the alkaline phosphatase LapA. However, Xcp has a broader substrate repertoire including the elastases LasA/B, lipases LipA/C, phospholipases PlcB/H/N and protease IV PrpL (Jyot et al., 2011). These secreted products participate in the pathogenesis of *P. aeruginosa* through multifaceted proteolytic and lipolytic activity against essential structural components of the epithelium and innate immunity. Notable ligands include fibronectin, elastin, plasminogen and the pulmonary surfactant, impairing mechanical clearance and weakening cellular junctions to promote epithelial adhesion and colonisation (Jurado-Martín et al., 2021). Furthermore, these secreted effectors also have immunoevasive properties (Cigana et al., 2011). They are able to degrade complement components, interfere with toll-like receptor recognition, inhibit signalling molecules such as cytokines, chemokines and reactive oxygen species (ROS) and downregulate pathogen-associated molecular patterns such as acellular flagellin. The most cytotoxic virulence factor possessed by *P. aeruginosa* is the Xcp-dependent exotoxin ToxA (Filloux, 2011). ToxA is an adenosine diphosphate-ribosyltransferase which possesses specificity for the eukaryotic elongation factor 2 and indiscriminately inhibits eukaryotic protein synthesis leading to rapid tissue necrosis. Ultimately, compromised mucosal barriers, airway acidification and chronic inflammation leads to loss of respiratory function (Jurado-Martín et al., 2021, Soong et al., 2008).

1.1.2.2. Quorum Sensing

An important mechanism for the regulation of virulence factor production throughout the pathogenesis of *P. aeruginosa* infection is quorum sensing. Quorum sensing is a form of intercellular communication which regulates social behaviour through production of transcription-modulating secondary metabolites known as autoinducers (Jurado-Martín et al., 2021). In most Gram-negative organisms, canonical quorum sensing autoinducers are N-acyl homoserine lactones (AHLs) are passively released into the extracellular milieu and freely diffusible into neighbouring cells (Miller and Bassler, 2001). At a critical threshold concentration, autoinducers subsequently form complexes with transcriptional activators to access cognate recognition boxes within promoter sequences. Thus, gene expression is coordinated as a function of effective population size, which allows transcriptional optimisation dependent on stage of infection, adaptation to stress or changing environments (Moradali et al., 2017). Population-dependent phenotypes under the control of quorum sensing include biofilm

formation, carbon utilisation, transformation competence and conjugation, antimicrobial peptide and toxin production and motility.

The network of interactions underpinning quorum sensing is complex and it is estimated that 10% of the *P. aeruginosa* genome is under control from quorum sensing (Gellatly and Hancock, 2013). Quorum sensing is an instrumental regulator of pathogenesis and mutations in quorum sensing genes is characteristic of the transition from chronic carriage to acute infection in the cystic fibrosis lung (Winstanley and Fothergill, 2009). The *P. aeruginosa* quorum sensing circuitry is composed of four known systems with independent autoinducers which operate in a hierarchal manner (Jurado-Martín et al., 2021). There is a bias towards genes which encode secreted products in quorum sensing repertoires, selected to act as shared goods and offer their benefit to the community-at-large (Kostylev et al., 2019). Las is considered the master system in the quorum sensing hierarchy of *P. aeruginosa* and positively regulates the other systems. It consists of a divergently transcribed AHL autoinducer synthase LasI and a transcriptional activator LasR, which has cognate recognition sites upstream of Xcp- and Apr-dependent secretion system effectors (Miller and Bassler, 2001). Moreover, Las is primary regulator for expression of the high-affinity peptide siderophore, pyoverdine. Pyoverdine is a secondary metabolite which is responsible for the green pigmentation of *P. aeruginosa* and necessary for acute infection (Kang and Kirienko, 2017). It is the major siderophore of *P. aeruginosa* induced in iron-limited conditions to overcome the host response to limit infection by restricting labile iron concentrations (Cornelis and Dingemans, 2013). Pyoverdine expression is also intimately linked to biofilm formation though the Pqs quorum sensing system, with compromised expression of one possessing a deleterious effect on the other (Kang et al., 2017). Pqs utilises a variety of alkyl quinolone precursors known as the *Pseudomonas* Quinolone Signal (PQS) as autoinducers (Lee and Zhang, 2015). Pqs is involved in production of another important virulence factor, pyocyanin, a phenazine pigment which is responsible for the blue pigmentations of *P. aeruginosa*. It is secreted by the type II secretion system and generates ROS which controsl eDNA release into the biofilm matrix and induces inflammation in host tissue (Moradali et al., 2017). The PQS shares a similar substrate repertoire with a second AHL, encoded by the Rhl system, including pyocyanin, hydrogen cyanide and lectins (Turkina and Vikström, 2019, Venturi, 2006). Rhl also expresses the RhlAB rhamnosyltransferases through its regulation of RpoS. RhlAB controls rhamnolipid production, an amphipathic secreted product which degrades the pulmonary surfactant, increases surface hydrophobicity and facilitates swarming motility by lowering interfacial tension (Moradali et al., 2017).

A fourth quorum sensing system, Iqs has also been identified which uses the thiazole carbaldehyde, aeruginaldehyde, as an autoinducer (Cornelis, 2020). This system exists at the bottom of the hierarchy, however little else is known about its role.

1.1.3. Treatment and Drug Resistance

The clinical management of *P. aeruginosa* infections has always been made challenging by a broad pattern of intrinsic resistance to structurally diverse classes of antibiotics (Pang et al., 2019). Notable examples include aminopenicillins, first- and second- and orally-administered third-generation cephalosporins, macrolide-lincosamide-streptogramins, tetracyclines, co-trimoxazole and early-generation quinolones. This is mainly attributed to the characteristically impermeable membrane possessed by *P. aeruginosa*, even compared to other Gram-negative organisms, which impedes entry of antimicrobials (Chevalier et al., 2017). Membrane impermeability in *P. aeruginosa* is a multifaceted phenomenon, mediated by additive effect of several distinct mechanisms. Most notably, transmembrane import in *P. aeruginosa* is largely mediated by numerous substrate-specific porins instead of non-selective channels (Horcajada et al., 2019). Moreover, a high concentration of magnesium cations decorating the outer membrane contributes to repulsion of positively charged molecules. Inducible multidrug efflux systems and the chromosomal AmpC β-lactamase also participate in transient resistance to β-lactams. Consequently, a limited range of drugs with broad-spectrum Gram-negative cover also retain equivalent activity against *P. aeruginosa* (Driscoll et al., 2007).

The efficacy of antipseudomonal chemotherapy has become increasingly jeopardised due to acquisition of antibiotic resistance. The importance of drug resistance in the clinical management of *P. aeruginosa* is well recognised. *P. aeruginosa* is an ESKAPE pathogen, one of six bacterial pathogens of high clinical significance which have become a challenge to treat effectively due to the burden of drug resistance (Rice, 2008). It is also a World Health Organisation (WHO) critical priority for new therapeutics and a Centers for Disease Control and Prevention (CDC) serious threat to the efficacy of antimicrobial chemotherapy (Centers for Disease Control and Prevention, 2019, Tacconelli et al., 2018). Drug resistance is an important marker of poor clinical outcome of *P. aeruginosa* infection, including progression to bloodstream infection and sepsis, and complicates clinical management by reducing the efficacy of first-line therapy. Antipseudomonal chemotherapy for acute infection largely relies on parenteral combination therapy to achieve appropriate coverage. Failure to administer

an agent with appropriate activity during empiric therapy or prophylaxis is associated with significant increases in mortality (Lodise et al., 2007, Park et al., 2012). Consequently, antipseudomonal drugs-of-choice such as aztreonam, carbapenems, ceftazidime/avibactam, ceftolozane/tazobactam and piperacillin/tazobactam are combined with a high-dose aminoglycoside or fluoroquinolone until susceptibility information permits de-escalation to β-lactam monotherapy (Bassetti et al., 2018). Failure to achieve effective empiric therapy increases the risk of disease progression to bacteraemia and targeted therapy becomes reliant on post-first-line agents with inferior activity (Juan et al., 2017, Kang et al., 2003).

Multiple geographically diverse centres have observed an increased incidence of healthcare-associated *P. aeruginosa* infection resistant to clinically-relevant drugs, such as β-lactam/ β-lactamase inhibitors, carbapenems and fluoroquinolones since 2000 (Lautenbach et al., 2010, Xu et al., 2013, Yayan et al., 2015). The European Antimicrobial Resistance Surveillance Network reported that 30.2% of *P. aeruginosa* isolates are resistant to at least one first-line agent (European Centre for Disease Prevention and Control, 2020). Continent-wide multidrug resistance (MDR) in *P. aeruginosa*, as defined as insusceptibility to at least one agent in three or more classes of antibiotic, has remained largely stable since 2014. However, multidrug resistance remains unacceptably high in much of eastern and southern Europe, often exceeding 30%, and has seen dramatic increases in countries with weak antibiotic prescription regulations such as Cyprus and Greece. The genomic epidemiology of drug resistance in *P. aeruginosa* illustrates a multifactorial landscape of resistance mechanisms which contribute to the escalating challenge of treating *P. aeruginosa* infection. Chromosomally encoded resistance mechanisms mediated by accumulation of genomic point mutations are as clinically relevant as horizontally-acquired resistance mechanisms (Ruiz-Garbajosa & Cantón, 2017). Constitutive expression of the aforementioned intrinsic resistance mechanisms such as the AmpC β-lactamase, multidrug efflux pumps or inactivation of porins are well represented among clinical isolates of drug resistance *P. aeruginosa*. Resistance can then be compounded by the acquisition of globally distributed mobile genetic elements, most notably β-lactamase-encoding plasmids, many of which are capable of hydrolysing carbapenems, such as VIM- and IMP-types. Consequently, the combinatorial nature of resistance has necessitated use of escalated terminologies such as extensive drug resistance (XDR) and pan-drug resistance (PDR) due to increasing incidences of isolates resistant to second- and third-line therapy (Horcajada et al., 2019, Pérez et al., 2019). Prescription habits have also changed with the landscape of drug resistance. An increasing reliance

on carbapenems, novel cephalosporin/ β-lactamase combinations and the revival of colistin are the consequence of mounting resistance which now extends across all classes of antipseudomonal agents (Horcajada et al., 2019, Mladenovic-Antic et al., 2016, Rahal et al., 2002, Zou et al., 2015).

1.2. Biofilms

1.2.1. Structure and Lifecycle

Bacterial biofilms are higher-order microbial structures composed of an aggregated microconsortium of cells immobilised within a secreted matrix of extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). Biofilms are the typical state for most microbial life in the environment, with up to 90% of bacteria in aquatic and terrestrial ecosystems forming polymicrobial communities within biofilms. Biofilms represent a distinct form of bacterial life which possess numerous physiological distinctions from their free-living planktonic counterparts. The composite architecture, heterogeneity and social interactions associated with the biofilm lifestyle give rise to altered stress responses, growth kinetics and selective outcomes referred to as ‘emergent properties’ (Karygianni et al., 2020). Biofilms have been described as an intermediate lifeform between unicellular and multicellular organisms based on their capacity for self-organisation, transcriptional differentiation and cooperative action (Flemming et al., 2016). Consequently, biofilms are a form of transient multicellularity emerging from often non-clonal, unicellular organisms, which facilitates survival and proliferation in spite of unfavourable conditions.

1.2.1.1. Initial Attachment

Biofilms which form on inert surfaces share a life cycle from free-living individual cells to aggregate microcolonies and finally mature biofilms which can, in turn disperse to colonise new environments (figure 1.2.) (Flemming et al., 2016). Solid surfaces offer some distinct environmental advantages when colonised, most notably, biological material sediments and adsorbs increasing localised nutrient concentrations (Petrova and Sauer, 2012). Moreover, surface environments are often more stable as the interface buffers changes in physicochemical conditions such as heat, pH and flow rate and protects from protozoal predation (Tuson and Weibel, 2013). Consequently, biofilms typically form at interfaces; the solid-liquid interface being the most common, but the solid-air and liquid-air interfaces are also well represented. There are few

prerequisite requirements for a surface to be amenable to biofilm formation and abiotic surfaces are often made more amenable by anthropogenic processes such as painting, plasticising, and alloying (Garrett et al., 2008, Whitehead and Verran, 2009). Consequently, biofilms can form on a wide variety of surfaces including plastics, metal and glass. Exhaustive work has been undertaken defining the surface properties which predispose to biofilm contamination (Zheng et al., 2021). Positively charged, hydrophobic and non-shedding surfaces particularly support biofilm formation, with surface roughness and wettability also affecting the strength of adhesive forces. Moreover, in biofilms grown at an interface with a liquid phase, surface properties are interdependent with fluid dynamics. Low shear rate environments, a low interfacial tension and low-density liquids confer little mechanical resistance for cells to interact with surfaces.

The initial transition from motility to sessility is generally preceded by the conditioning of surfaces into a substrate more amenable for colonisation (Donlan, 2002). Surface conditioning is generally a passive process through which the physicochemical characteristics of non-shedding surfaces are altered by the deposition of heterogenous biological material (Kimkes and Heinemann, 2019). Unlike adhesion to biotic surfaces such as tissue, which generally relies on specific ligand binding, adhesion to abiotic surfaces is mediately by non-specific intramolecular forces (Dunne, 2002). Consequently, conditioning increases the affinity of ionic interactions and hydrogen bonding can occur between cells and the substrate via charged, hydrophobic organic compounds. In the earliest stages of biofilm formation, excreted products and lysis debris from planktonic cells composes most of the bacterially-derived conditioning film (Moreira et al., 2017). Subsequently, a monolayer of cells adsorb onto the surface through Brownian motion and flagellar motility (Busscher et al., 2010). Mechanical positioning of the long axis of the cell to maximise contact area, elimination of interfacial water and alteration of cell surface charge supports attachment to the substrate for further colonisation.

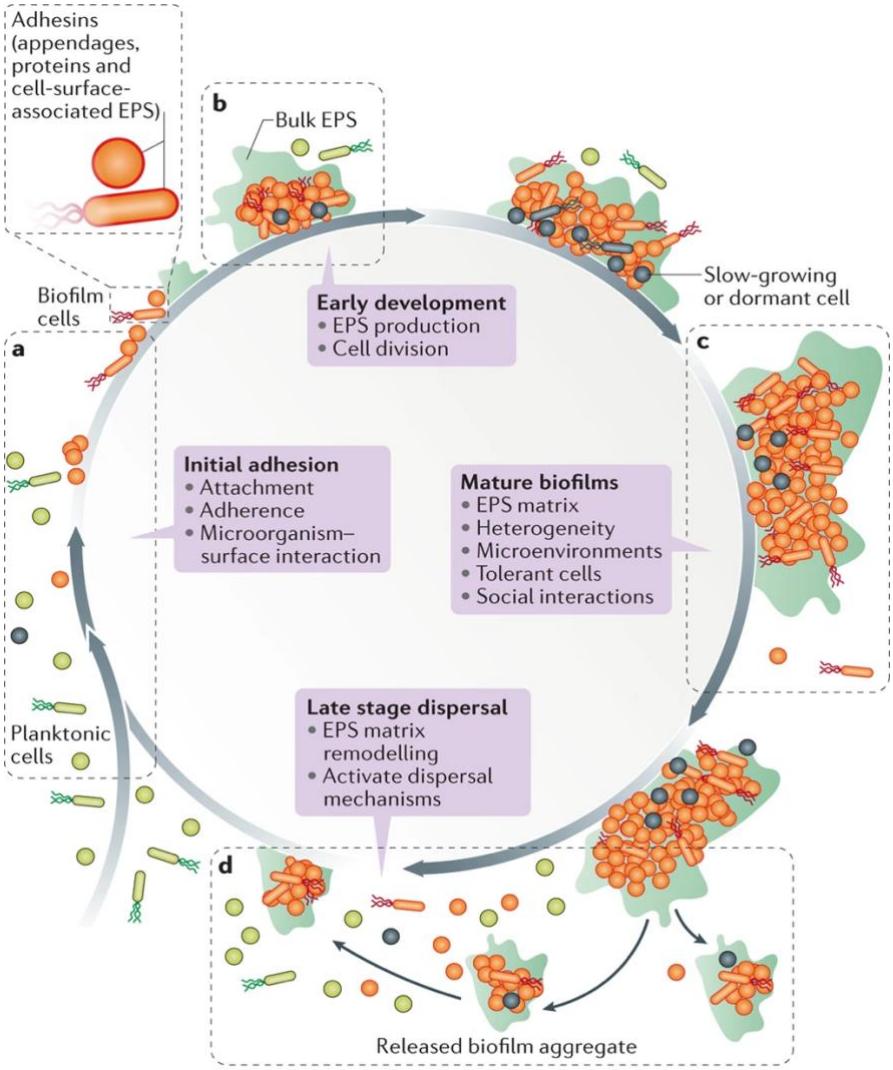

Figure 1.2. Liquid interface-associated biofilms are considered to have a prototypical life cycle beginning with the reversible adherence of planktonic cells to a surface through intramolecular forces. Cells then undergo a transcriptional reorganisation known as the motile-sessile switch which supports the secretion of an extracellular matrix of biopolymers through which they irreversibly adhere via ligand binding interactions. As cells continue to divide and secrete matrix, the biofilm matures to yield steep gradients in solute chemistry and spatially-stratified microenvironments. This gives rise to emergent properties characteristic of biofilms, including population heterogeneity, sociality and antimicrobial tolerance. Environmental factors such as flow rate and nutrient availability dictate the extent to which the biofilm can grow before dispersal mechanisms take hold. Once this critical threshold is surpassed, passive sloughing of aggregates into bulk flow or transcriptional upregulation of flagellar motility and matrix hydrolysis, allow for the biofilm to disseminate to new environments, thus restarting the life cycle (Koo et al., 2017)

1.2.1.2. Motile-Sessile Switch

Bacteria attached to the conditioned surface depend on weak intramolecular forces with nanometre ranges to maintain attachment (Akhidime et al., 2020). Surface-bound bacteria can commit to long-term surface attachment in a process known as the motile-sessile switch which results in upregulation of genes involved in adhesion, EPS production and cell-to-cell signalling and downregulation of virulence and motility (Chang, 2018). One of the most important regulatory components controlling this lifestyle switch in many clinically-important Gram-negative organisms, particularly *P. aeruginosa*, is the bacterial second messenger cyclic diguanylate (c-di-GMP) (figure 1.3.). c-di-GMP is one of the foremost global regulators of the biofilm lifestyle and operates at a transcriptional, translational and post-translational level (Römling et al., 2013). Its transduction networks are staggeringly complex and the intracellular level of c-di-GMP dictates important cellular behaviours including pathogenicity, cell division, central metabolism and numerous stress responses, in addition to biofilm formation. Concomitant synthesis and degradation of c-di-GMP by diguanylate cyclases and phosphodiesterases, respectively, tightly controls intracellular concentrations of c-di-GMP (Galperin, 2005). Cellular perturbations affecting the expression and function of these enzymes act to enrich or deplete reserves of c-di-GMP. The effector systems under allosteric control from c-di-GMP are widespread and varied in regulatory mechanism and activation stoichiometry (Hengge, 2009, Whitney et al., 2015). Examples includes the PilZ-like protein family post-translational modifiers, the c-di-GMP-dependent transcriptional activator PelD and transcriptional repressor FleQ. Many of these c-di-GMP-binding regulatory elements are themselves induced by specific conditions, providing an additional layer of regulation to control the specificity of the phenotypic output (Boyd and O'Toole, 2012).

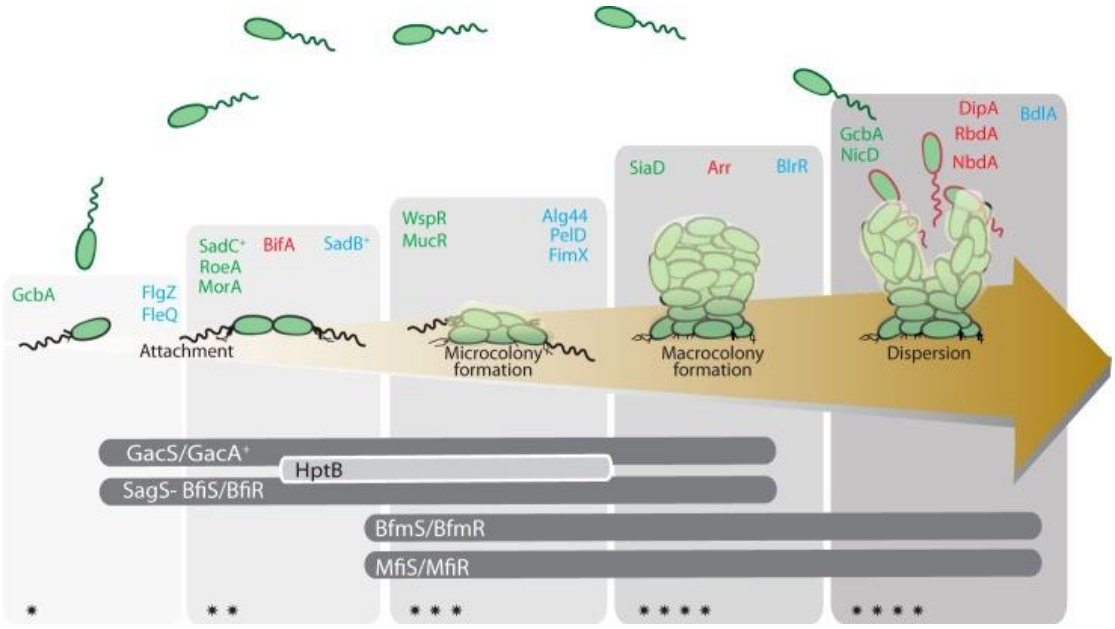

Figure 1.3. The bacterial second messenger c-di-GMP is a central signalling molecule in *P. aeruginosa* and controls numerous important physiological processes, including virulence and biofilm formation. The intracellular c-di-GMP reservoir is controlled by a sophisticated network of diguanylate cyclases and phosphodiesterases which regulate the transition to sessility and progression through the biofilm life cycle. Upon adherence, surface-sensing two-component systems upregulate the production of c-di-GMP which in turn leads to activation of signalling cascades such as Gac/Rsm. Such cascades possess large regulons involved in expression of matrix constituents and repression of flagellar motility and pathogenicity. In this way, c-di-GMP signalling enables a dynamic reorganisation of gene expression to promote and maintain a sessile lifestyle, with each stage of the biofilm life cycle being a transcriptionally distinct period (Valentini and Filloux, 2016).

Bacteria can directly sense surface attachment to instigate the motile-sessile switch, for example via the chemoreceptors CheA in *E. coli* and WspA in *P. aeruginosa* which activate signal transduction cascades to express the CheY and WspR diguanylate cyclases (O'Connor et al., 2012). Moreover, indirect sensing pathways also have been shown to play a role in increasing c-di-GMP concentrations during adhesion. In *E. coli*, adhesion can activate the CpxAR membrane stress response pathway, which upregulates expression of the diguanylate cyclase DgcZ (Otto and Silhavy, 2002). Indeed, during the motile-sessile switch, intracellular c-di-GMP concentrations increase up to three-fold which regulates a number of important physiological changes to support adhesion (Chang, 2018).

The secretion of exopolysaccharides and upregulation of adhesins facilitate ligand-binding interactions up to ten times stronger than non-covalent intermolecular bonds (Dufrêne and Viljoen, 2020). Consequently, motility and sessility are inversely regulated by c-di-GMP, and negative regulation of motility is accompanied by positive

regulation of exopolysaccharide, fimbrial and pilin biosynthesis. Exopolysaccharides are high-molecular weight polymers secreted into the extracellular milieu which act as the primary mediators for adhesion to substrates (Flemming et al., 2016). The *P. aeruginosa* extracellular matrix is characterised by high abundances of three exopolysaccharides: alginate, Psl and Pel (Ghafoor et al., 2011). Psl and Pel are aggregative polysaccharides important in microcolony formation, adhesion to host cells and maintaining the structural integrity of mature biofilms (Jurado-Martín et al., 2021). The specific roles of Psl and Pel are highly redundant, with one able to compensate for compromised production of the other in a strain-specific manner. *P. aeruginosa* PA14 possesses a truncated Psl biosynthesis operon and relies exclusively on Pel for biofilm formation (Colvin et al., 2012). In contrast, Psl has little contribution to the extracellular matrix of *P. aeruginosa* PAO1, with Pel being the primary structural component. In most strains of *P. aeruginosa* however, Pel and Psl are co-ordinately expressed and contribute in varying degrees to biofilm formation. Psl is a mannose- and galactose-rich polysaccharide expressed during planktonic growth to promote early irreversible adhesion to surfaces. As the biofilm matures, Psl becomes a shared good, localised around the periphery of the colony. Consequently, cells dispersed from the biofilm express Psl to assists with formation of subsequent aggregate microcolonies. Pel is a cellulose-like polysaccharide which generates a scaffold of crosslinked polymers which interact with eDNA and carbohydrate-binding proteins, such as lectins, to facilitate cell-to-cell interactions (Jennings et al., 2015). It is essential for the characteristic rugosity of biofilm colonies and for forming pellicles at the air-liquid interface. The most abundant exopolysaccharide in mature biofilms is the capsular polysaccharide alginate. Alginate is a copolymer of partially acetylated D-mannuronic and L-glucuronic acid and the main determinant of the mucoid phenotype (Heredia-Ponce et al., 2021). *P. aeruginosa* mutants which constitutively express alginate are frequently isolated from the cystic fibrosis lung and are indicative of conversion from an acute to chronic infection (Moradali et al., 2017, Thi et al., 2020). It possesses a limited role as a structural element of the biofilm, however, is important in conferring mechanical protection from phagocytosis, antimicrobial stress and desiccation.

Most exopolysaccharides are induced upon attachment of surfaces, with the exception of Psl which is constitutively expressed and mediates cellular aggregation at the air-liquid interface. The Pel polysaccharide, on the other hand, is under transcriptional and post-translational control from c-di-GMP (Römling et al., 2013). The Gac/Rsm cascade in *P. aeruginosa* regulates expression of a number of matrix constituents among a regulon of approximately 500 genes through the transcriptional repressor RsmA

(Moscoso et al., 2014). This includes upregulation of exopolysaccharides and carbohydrate-binding proteins and downregulation of type IV pili and type III secretion systems, qualities associated with a sessile lifestyle (Brencic and Lory, 2009). The Gac/Rsm cascade is cascade is repressed by RsmA at low c-di-GMP conditions, however upon activation of the GacSA two-component system, two antisense RNAs RsmY/Z, are expressed (Valentini and Filloux, 2016). RsmY/Z sequester RsmA permitting expression of the regulon and activation of the post-translationally repressed cyclic diguanylase SadC, leading to increased c-di-GMP production. The activating signal of GacA is not known, however it appears to be intimately linked to the motile-sessile switch (Latour, 2020).

Alginate and cellulose biosynthesis are also under regulatory control from c-di-GMP through the synthases Alg44 and BscE, respectively (Liang, 2015). Alg44 and BscE are constituents of complexes which catalyse the polymerisation of carbohydrate monomers into polysaccharides and translocate polymers into the periplasm for extracellular export (Römling and Galperin, 2015). However, they are only able to assume an appropriate conformation for polymerisation when bound to c-di-GMP and possess local diguanylate cyclases to ensure expression in a feedback system. In the alginate biosynthesis pathway this is MucR, and in the cellulose pathway it is AdrA, which are also under transcriptional repression from global regulators, AlgU and CsgD, respectively (Brombacher et al., 2006, Hay et al., 2014). In *P. aeruginosa*, AlgU is a homologue to RpoE and involved in the membrane stress response. CsgD is the master biofilm regulator in *Enterobacteriaceae* and an effector of c-di-GMP (Ahmad et al., 2017). These regulators respond to a variety of physiological signals including nutrient starvation, membrane stress, osmolarity, oxygen tension and heat (Gerstel and Römling, 2003, Wang et al., 2021). Consequently, initiation of biofilm formation is intrinsically linked to evasion of stress and is often thought of as a stress response itself.

Secretion of exopolysaccharides provides a nascent extracellular matrix for cells to occupy and provide ligand binding sites for irreversible adhesion. Consequently, the motile-sessile switch induces the upregulation of surface appendages such as pilin and fimbrial adhesins to promote attachment (Blumer et al., 2005). Pili and fimbriae are structurally similar, except pili tend to possess ligand-dependent specificities whereas fimbriae mediate non-specific binding. In this regard, the importance of fimbriae in facilitating irreversible adhesion is well characterised (Johnson et al., 2011, Lasaro et al., 2009, Schroll et al., 2010). The adhesin which mediates ligand binding is located at the tip of the fimbrial complex and is typically a carbohydrate-binding protein known as

a lectin which is able to interface with matrix exopolysaccharides. Fimbriae are particularly important in *P. aeruginosa* where the CupA/B/C fimbria and CdrA adhesin mediate adhesion to Psl and Pel (Meissner et al., 2007). Though fimbriae such as the mannose-specific adhesin FimH also play a role in adhesion in *Enterobacteriaceae,* the most important proteinaceous component of biofilms in is the Curli pilus (Barnhart and Chapman, 2006). Curli pili are fibrillar amyloid proteins expressed on the outer leaflet of the membrane which interact with cellulose to immobilise cells. Biosynthesis of Curli relies on two divergently transcribed operons, *csgBAC* and *csgDEFG* (Ahmad et al., 2017). CsgD, as discussed earlier, is the c-di-GMP-dependent master biofilm regulator in *Enterobacteriaceae* and has a pleiotropic role in regulating the motile-sessile switch. Transcriptional de-repression of *csgDEFG* is mediated by stress responses such as RpoS, OmpR-EnvZ or the histone-like nucleoid structuring protein (H-NS) (Gerstel et al., 2003). Upon expression, c-di-GMP must bind to CsgD to allow it to activate transcription of *csgBAC.* CsgA/B are the major and minor Curli subunits, which are chaperoned by CsgC and polymerised by CsgEFG at the envelope. The pilus extends into the extracellular milieu and interfaces with the cellulose component of the biofilm matrix (Saldaña et al., 2009).

The hydrophobicity of the bacterial flagellum often contributes to the mechanical adhesion of cells to the substrate and flagella-deficient mutants often show compromised initial attachment (Kimkes and Heinemann, 2019). However, continued flagellar expression becomes a detriment when attempting to irreversibly attach (Römling et al., 2013, Vatanyoopaisarn et al., 2000). Consequently, c-di-GMP downregulates flagellar motility in *P. aeruginosa* after adhesion through binding and repression of FleQ, a transcriptional activator of flagellar biosynthesis (Hickman and Harwood, 2008). Furthermore, c-di-GMP can also post-translationally modify flagellar activity by interfering with rotation machinery (Sadiq et al., 2017). The regulator of flagellar rotation in *Enterobacteriaceae* YcgR, is also an effector of c-di-GMP. It plays an essential role in chemotaxis and is under control from the master flagellar regulator FlhD along with the c-di-GMP phosphodiesterase YhjH. High concentrations of c-di-GMP cause conformational changes to allow it to bind with greater affinity to its receptor, either the flagellum switch complex FliGMN, or the stator subunit MotA. With the substrate binding site permanently occupied, the ability for chemotactic signals to be translated into flagellar rotation is greatly diminished. Downregulating flagellar motility eliminates the main mechanism for cells from overcoming the steric forces adhering the cell to the surface (Jefferson, 2004).

1.2.1.3. Self-Organisation and Maturation

Immobilisation of cells within an extracellular matrix begins the process of self-organisation into microcolonies, in which daughter cells aggregate due to electrostatic interactions with the EPS, forming small globular clusters (Flemming and Wingender, 2010). Each microcolony is a clonal, independent microconsortia formed from a single cell. Polymicrobial variation in biofilms is largely generated through secondary colonisation events, in which planktonic cells become immobilised within the matrix *post hoc* (Chenicheri et al., 2017). As microcolonies mature, they begin to adopt complex structures, with neighbouring microcolonies spatially segregated by interstitial voids for nutrient and gas exchange (Donlan, 2002). The topography of microcolony formation varies with colonising species, environmental stress and nutritional conditions. The “mushroom-shaped” structures of *P. aeruginosa* were long considered the archetypal microcolony morphology, with much work postulating how such a topography supports vertical colonisation and dispersal (Ghanbari et al., 2016). However, this phenomenon is dependent on glucose-rich conditions and visualisation of biofilms in infection models *in vivo* do not recognise these structures (Tolker-Nielsen et al., 2015). It hard to generalise how these spatial interactions between cells manipulate biofilm maturation and the mechanisms which control microcolony topography are poorly understood. It has been suggested that self-organisation into microcolonies is a dynamic feedback system whereby migrating bacteria become preferentially immobilised in exopolysaccharide-rich regions, further increasing exopolysaccharide deposition and cellular entrapment (Zhao et al., 2013). Such a model provides rationale why cells self-organise into microcolonies instead of swarming across the substrate as a monolayer. However, the mechanisms by which microcolonies organically form into complex structures such as mushrooms, stalks and caps remain broadly unknown. Social organisation likely involves other selectively-driven feedback systems, of which eDNA appears to play an important role, in addition to broader transcriptional changes specific to changing microenvironments (Gloag et al., 2013).

As the biofilm matures, the composition of the EPS becomes more diverse as ancillary matrix constituents are secreted for long-term stabilisation of the matrix and cellular immobilisation (Flemming and Wingender, 2010). Such constituents, including extracellular proteins, nucleic acids, secondary metabolites, and to a lesser extent, lipids and lipopolysaccharide (LPS) or teichoic acids, generally play a limited direct role in adhesion to substrates (Karygianni et al., 2020). Instead, they interface with the matrix to provide structural reinforcement through polymer-polymer, cell-polymer and

cell-cell interactions. The EPS is likely abundant in proteins derived from lysed cells, however relatively few possess characterised structural roles. In *P. aeruginosa*, the most structurally significant extracellular proteins are the lectins LecA/B (Fong and Yildiz, 2015). LecA is essential for biofilm formation on polystyrene and stainless steel, whereas LecB is involved in colonisation of glass surfaces. Lectins are carbohydrate-binding proteins which possess sugar-dependent specificity. LecA binds to galactose, N-acetyl-D-galactosamine and glucose, although its ligand in the biofilm matrix remains unknown, and LecB binds to fructose, galactose and mannose. Expression of LecA/B is under the control of the Rhl quorum sensing system in addition to the Gac/Rsm cascade (Landi et al., 2019, Pessi et al., 2001). LecB is a mediator of cell-exopolysaccharide interactions by binding with Psl and OprF on the cell membrane and involved in localising Psl to the colony peripheries (Borlee et al., 2010, Passos da Silva et al., 2019).

In addition to lectins and exopolysaccharides, extracellular double-stranded genomic DNA (gDNA) fragments are essential components of the matrix of *P. aeruginosa* biofilms. eDNA is essential during early stages of biofilm maturation, without which, biofilms fail to establish microcolonies (Steinberger and Holden, 2005, Whitchurch et al., 2002). eDNA can form ionic complexes with both major structural exopolysaccharides in *P. aeruginosa,* Pel and Psl, which crosslink the matrix to support structural integrity (Jennings et al., 2015, Wang et al., 2015). Moreover, eDNA can also bind to type IV pili, forming exopolysaccharide-eDNA-cell complexes which inhibit the retarding effect of DNase on biofilm formation (Nolan et al., 2020). The biofilm can acquire eDNA through cell lysis or vesicular blebbing which both occur passively during exponential growth (Sarkar, 2020). Subsequently, in mature biofilms DNA release becomes altruistically regulated through endolytic pathways comparable to eukaryotic apoptosis. Activation of the Pqs quorum sensing system can induce oxidative stress in a population-dependent manner by virtue of its transcriptional control of the Phz phenazine biosynthesis operons (Allesen-Holm et al., 2006). The most abundant product of the phenazine biosynthetic pathway, pyocyanin, is an inhibitor of catalase, which processes hydrogen peroxide to non-toxic products (O'Malley et al., 2003). Consequently, elevated expression of pyocyanin is associated with an increase in hydrogen peroxide leading to increased rates of cell lysis (Das and Manefield, 2012, Das et al., 2015). Increased oxidative stress can also occur organically as the biofilm matures, as physicochemical gradients become steeper and senescent subpopulations experience oxygen tension, accumulation of excreted material and carbon limitation. Such endogenous oxidative stress has been shown to activate the RecA-dependent

SOS stress response in biofilms (Boles and Singh, 2008). In *P. aeruginosa* some of the most significantly upregulated genes in the SOS regulon are lytic prophages and associated constituents such as pyocins and holins (Cirz et al., 2006). The Lys pyocin and Hol holin have been shown to play an important role in the vesicularisation and release of eDNA into the biofilm matrix in *P. aeruginosa* through explosive cell lysis (Turnbull et al., 2016). Therefore, pyocin-mediated cell lysis is likely a mechanism employed to liberate eDNA from stressed subpopulations which have limited contribution to continued biofilm growth.

1.2.1.4. Dispersal

The terminal stage in the biofilm life cycle is dispersal, which allows the biofilm to disseminate for further surface colonisation or phenotypically switch to a planktonic lifestyle (McDougald et al., 2012). Dispersal is a heterogenous process which involves the erosion of cells from the colony surface, mechanical sloughing of microcolonies into flocs or enzymatic dissociation of the EPS (Kaplan, 2010). It can occur passively due to the effect of rheological forces or be induced by environment-sensing signal transduction systems.

Passive dispersal, also known as detachment, is most relevant to the life cycle of biofilms at a liquid interface (Rumbaugh and Sauer, 2020). Bulk flow is the primary mechanism by which biomass accumulation is limited and how detached cells migrate away from the parental colony. Passive dispersal occurs when the biofilm attains a critical volume at which viscoelasticity is overcome by shear stress, collisions with solid material or substratum disruption (Wille and Coenye, 2020). It is mainly characterised by the capture of small aggregates of loosely adherent, non-motile cells from the biofilm surface which travel passively until they sediment out of bulk flow. However, hydrodynamic forces can also remodel biofilm architecture to increase colonised surface area and rates of dispersal through rippling effects and generation of filamentous microcolonies of streamers (Prades et al., 2020).

Whereas passive dispersal is an abiotic process mediated by mechanical erosion of sessile cells from the biofilm surface, active dispersal is associated with transcriptional changes which reverse the motile-sessile swich (Kostakioti et al., 2013). Though dispersal facilitates dissemination, excessive dispersal can compromise the biofilm's structural integrity and strip it of its emergent properties. Consequently, biofilm dispersal is as tightly controlled as biofilm formation, and both are regulated through many shared signal transduction systems. Active dispersal is primarily induced to

escape from unfavourable conditions, through which physicochemical gradients provides a mechanism for the biofilm to sense stress (Kaplan, 2010). Positive or negative gradients of redox, pyruvate, fatty acids, glutamate and nitric oxide are native signals attributed to induction of active dispersal in *P. aeruginosa* (Rumbaugh and Sauer, 2020). The topography and magnitude of dispersal is highly dependent on the nature and strength of the inducing signal but rarely involves the entire colony. Native signals induce cavitating dispersal from the biofilm interior due to the accumulation of toxic products and nutrient deficiency associated with the deep biofilm strata.

Most signal sensing pathways interface with biofilm dispersal by downregulating c-di-GMP (Wille and Coenye, 2020). In *P. aeruginosa*, nitric oxide stress and nutrient abundance are sensed by their respective membrane sensors NbdA and NicD, which recruits the chemotaxis protein BdlA activating the c-di-GMP phosphodiesterases DipA and RbdA (McDougald et al., 2012). The resulting decrease in concentrations of c-di-GMP results in reversion of the motile-sessile switch, however cells are still largely immobilised by the biofilm matrix. Consequently, liberation of motile cells from the matrix in *P. aeruginosa* also relies on expression carbohydrate-degrading proteins, nucleases and the lysogenic prophage Pf4 (Kaplan, 2010). The most important determinants of matrix degradation are the AlgG alginate lyase, PelA and PslG glycoside hydrolases and the EndA and EddA/B DNases (Rumbaugh and Sauer, 2020). Concomitant degradation of the matrix and re-acquisition of motility allows cells to disperse from the biofilm and resume a planktonic existence.

1.2.2. Emergent Properties Involved in Antimicrobial Tolerance

The biofilm lifestyle instigates a fundamental shift in bacterial physiology which has consequences for both the individual cells and the colony community. The altered transcriptional landscape has pervasive phenotypic outcomes, and the biofilm ultrastructure gives rise to population-dependent phenomena facilitating social dynamics such as cheating, bet-hedging, and intraspecific competition (Flemming et al., 2016). The phenotypic presentation of the social lifestyle specific to biofilms are known as ‘emergent properties’ and include many characteristics which contribute to environmental persistence, stress tolerance and niche adaptation. Though most biofilms are polymicrobial and multispecies diversity contributes to emergent properties, multicellular action arises even in isogenic cultures grown as a biofilm. The EPS is the most salient difference between planktonic and biofilm lifestyles and facilitates emergent properties through the physiological gradients, cellular

immobilisation, resource capture and barrier functions it confers (figure 1.4.) (Flemming et al., 2016). Consequently, emergent properties can be seen as an intrinsic quality of biofilms facilitated by the structural complexity, social interactions and heterogeneity inherent to an EPS-bound lifestyle.

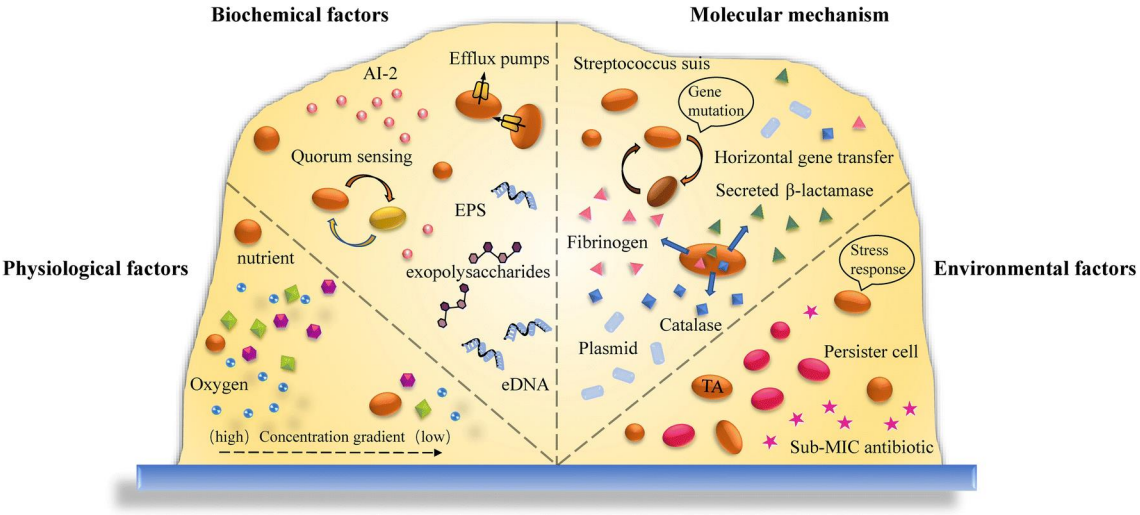

Figure 1.4. The biofilm lifestyle elicits emergent properties which can confer reduced susceptibility to antimicrobials through a variety of mechanisms. An intrinsic property of the biofilm is the steep physicochemical gradients given rise by deficits in penetration of gases and nutrients. Whilst this may have a direct impact on the capacity for antimicrobials to diffuse into the biofilm, it also induces the formation of metabolically quiescent subpopulations in resource-deficient strata. With diminished metabolic activity, the activity of antimicrobials which rely macromolecular turnover to exert a bactericidal effect are diminished. Moreover, the accumulation of secreted products within the matrix can also potentiate the action of acquired resistance mechanisms such as antibiotic-modifying enzymes or reactive oxygen scavengers which sequester and inactivate toxic compounds. Similarly, the close localisation of cells can also increase the efficiency of horizontal gene transfer allowing for resistance-conferring mobile genetic elements to disseminate through the biofilm (Yi et al., 2020).

1.2.2.1. Matrix Diffusion Barrier

Survival under gross environmental insults such as nutrient poverty or desiccation is a characteristic feature of the biofilm, to which the EPS is a direct contributor. The EPS is a highly viscoelastic structure which is able to swell or shrink to maintain saturation in response to large changes in water content (Ido et al., 2020). The main mediator of this is the exopolysaccharide matrix constituents, most notably alginate in *P. aeruginosa*, which are highly hygroscopic and store approximately 90% of the biofilm mass as EPS-bound water (Schmitt and Flemming, 1999). The storage of water through the hydrolysis of polysaccharides prevents loss from evaporation, osmotic stress or capillary action (Guo et al., 2018). Biofilms of *P. putida* have been shown to possess

higher viability under desiccated conditions than planktonic counterparts (van de Mortel et al., 2004). Alginate deficiency severely compromises desiccation survival however, overexpression of alginate biosynthesis has little impact on surviving dry conditions in *P. fluorescens* (Chang et al., 2007, Schnider-Keel et al., 2001). Consequently, the benefit alginate production possesses under desiccated conditions appears to be directly dependent on water retainment instead of some intrinsic structural role. Possessing a water-saturated matrix also yields a rich reservoir of dissolved trace elements and nutrients which can be sequestered from the liquid phase (Flemming et al., 2016). The chemical heterogeneity of the EPS confers a broad capacity for ion exchange, particularly due to the abundance of carboxyl and amino groups mediating electrostatic interactions within the matrix (Flemming, 1995, Kurniawan et al., 2012). Thus, the matrix facilitates the capture of nutrients for an immobile community which lacks the agency to act upon chemotactic signals which facilitates growth in highly oligotrophic conditions.

The same matrix properties which contribute to nutrient sorption have been suggested to be involved in sequestering antimicrobial compounds and contributing to detoxification of the biofilm (Davies, 2003). Tolerance to antimicrobials is a central feature of the biofilm lifestyle and characterised by up to a three-log reduction in the efficacy of antimicrobial killing compared to planktonic cultures. Tolerance is mechanistically distinct to canonical antimicrobial resistance as it is not mediated by *de novo* resistance determinants. Instead, it is an emergent property in which the action of antimicrobials is subverted by contextually-dependent mechanisms of insusceptibility which allows the cell to survive for extended periods of time under stress (Balaban et al., 2019, Ciofu and Tolker-Nielsen, 2019). It is a transient, non-heritable, non-specific phenomenon in which sensitivity is restored if grown planktonically. Early studies focused on the perception that the EPS acts as a physical diffusion barrier to antimicrobial penetration, particularly to tobramycin and β-lactams (Hoyle and Costerton, 1989, Hoyle et al., 1992, Nichols et al., 1988). Indeed, the matrix has shown to possess a broad capacity to sequester toxic compounds, including the organic pollutants *m*-xylene, benzene and toluene, and chelate heavy metals such as cadmium, copper and zinc (Flemming, 1995, Späth et al., 1998). However, the extent to which sequestering toxic compounds participates in tolerance is contentious, and accounts of matrix constituents contributing to drug resistance largely vary with experimental format, agent and organism (Hall and Mah, 2017). It is now generally accepted that the EPS as a non-specific diffusion barrier is not, for the most part, a major contributor to biofilm-associated antimicrobial tolerance. Nevertheless, some

consistent examples of the matrix contributing to antimicrobial tolerance have been demonstrated, whereby the compartmentalisation of secreted products potentiates their action (Sharma et al., 2019). Ampicillin can only penetrate biofilms of *Klebsiella pneumoniae* in β-lactamase-deficient strains, which was attributed to inactivation of ampicillin at a rate greater than the drug was able to diffuse (Anderl et al., 2000, Zahller and Stewart, 2002). Similarly, in *P. aeruginosa*, alginate has been shown to bind tobramycin and reduce susceptibility in a concentration-dependent fashion (Nichols et al., 1988, Cao et al., 2015). Moreover, modelling suggests that as a polyanion, alginate possesses sufficient ligand-binding capacity to attenuate inhibitory concentrations of negatively-charged aminoglycosides (Cao et al., 2016). Consequently, matrix components appear to contribute to insusceptibility in specific circumstances through a mechanism of ligand-specific diffusion-reaction inhibition. Therefore, though biofilms acquire nutrients through generalised resource capture, this appears not to be recapitulated with antimicrobial detoxification. A broader underpinning role of the matrix in antimicrobial susceptibility may exist in spite of this. Even the modest action of slowed accumulation of antimicrobials to bactericidal concentrations may still potentiate resistance mechanisms such as multidrug efflux.

1.2.2.2. Horizontal Gene Transfer

The high cell density, spatial stability and abundance of eDNA in the EPS provides a highly efficient environment for horizontal gene transfer (HGT) (Madsen et al., 2012). Conjugation is facilitated by cell-to-cell contact which seldom occurs during planktonic growth, but has ample opportunity in biofilms due to the dense, immobilised arrangement of cells which stabilises mating-pair formation (Roberts and Kreth, 2014). Conjugative interactions have been shown to induce the motile-sessile switch, supporting the importance of biofilms in facilitating HGT (Ghigo, 2001). Moreover, many of the global regulatory components controlling the biofilm lifestyle, such as quorum sensing and stress responses, are also involved in expression of genetic competence and conjugative machinery (Jefferson, 2004). In addition to cell density, the energy conservation from immobility and auxotrophy inherent to the biofilm lifestyle enables the maintenance of plasmids at higher copy numbers, increasing their selective advantage (O'Connell et al., 2007). There may be a selective advantage for biofilms to disseminate MGEs as indiscriminately as possible, as sharing MGEs increases species relatedness which supports social behaviour (Madsen et al., 2012). However, there are also spatiotemporal constraints to the dissemination of MGEs which inhibit a wavefront of HGT travelling throughout the entire colony. Invasion of

MGEs is most active during the initial establishment of the biofilm and typically occurs at the biofilm peripheries where cells are most metabolically active (Cook and Dunny, 2014, Stalder and Top, 2016). Consequently, this selectively favours resident MGEs as it deters competition with other molecular parasites which may displace them and generally restricts dissemination of colonising MGEs to local subpopulations.

In addition to being an important structural component for biofilms of many species, eDNA also provides a highly accessible substrate for genetic transformation in naturally-competent species (Hannan et al., 2010). Transformation success is under control from kin-recognition systems which will degrade internalised eDNA if it is deemed foreign. Therefore, the localisation of individuals from the same species in biofilms is a significant contributor to increased transformation efficiency. Generally, eDNA is released in biofilms through cell lysis where it associates with matrix proteins to become part of the EPS ultrastructure. Conversely, eDNA released from planktonic cells is rapidly degraded by nucleases in the extracellular milieu. Consequently, in addition to the increased the opportunity for transformation events to occur from cellular immobilisation, the chance of encountering undegraded DNA is similarly increased (Abe et al., 2020). Cells must be genetically competent to allow the translocation and recombination of extrachromosomal DNA into the recipient genome. Natural transformation competence has been shown to be induced by biofilm growth in a variety of species, including *P. aeruginosa,* where it is dependent on the type IV pilus or pseudopilin homologues (Nolan et al., 2020). The type IV pilus is an important determinant of biofilm formation involved in initial surface attachment and is also the main mediator of eDNA binding in mature *P. aeruginosa* biofilms (Flemming and Wingender, 2010).

1.2.2.3. Persister Cells

The EPS also gives rise to steep physiological gradients of oxygen, water, pH and redox (Flemming and Wingender, 2010). Cells respond to this by becoming stratified into subpopulations with varying degrees of metabolic latency, phenotypically manifesting as a protracted lag phase with increased doubling times (Flemming et al., 2016, van Gestel et al., 2015). Consequently, as many antimicrobials are specific inhibitors of essential macromolecular synthesis, they demonstrate reduced activity in cells which are not undergoing rapid growth (Kaldalu et al., 2016). Though antimicrobial tolerance can be an intrinsic property of starved cells unable to commit sufficient resources to growth and replication, mechanisms of biochemically-regulated dormancy, known as persistence, also exist. Persistence is a hedge-betting mechanism which

induces an almost entirely quiescent subpopulation characterised by protracted antimicrobial tolerance of which magnitude of stress has little impact (Maisonneuve and Gerdes, 2014). Persister cells are able to survive antimicrobial challenge in a non-replicating state until dissipation of the stress permits metabolic resuscitation and proliferation. Persister cells are ubiquitously present in biofilms, even in communities without exogenous stress, often representing the populations inhibiting the deepest, most nutrient impoverished, anoxic strata of the biofilm (Yan and Bassler, 2019). They are the main determinant of recalcitrance in biofilms after eradication which contributes to the characteristic morbidity and unresponsiveness to antimicrobial chemotherapy associated with biofilm-related infections (Fisher et al., 2017). However, persisters are not unique to biofilms and were first identified in planktonic cultures exposed to β-lactams (Bigger, 1944, Hobby et al., 1942). Persisters form from clonal populations and constitute approximately between 1 in 10^6 cells, however persister fractions can increase to up 1 in 10^3 cells under stress (Balaban et al., 2019). Moreover, persistence is not a homogenous phenomenon; persisters induced by nutrient starvation and β-lactam stress demonstrated divergent morphological and stress tolerance phenotypes (Paranjape and Shashidhar, 2019).

Regulatory control of persistence is not well understood. Maintaining a population in early exponential phase inhibits the formation of persisters which would not be expected if persistence was an entirely stochastic phenomenon (Keren et al., 2004). However, transposon screens for persister-deficient mutants have had limited success. It is now believed that persistence is mediated by multiple redundant pathways which respond to stress signals such as oxidative stress, membrane damage and cellular senescence to quench macromolecular synthesis (Luidalepp et al., 2011). In this regard, chromosomal toxin-antitoxin systems, the SOS stress response and the stringent response have accumulated evidence bases for their roles in persister generation (Lewis, 2010). However, the molecular mechanisms of persister induction and resuscitation are largely unknown and several high-profile publication retractions have further confused understanding of persistence (Chen et al., 2019, Germain et al., 2019, Maisonneuve et al., 2018). Moreover, most investigations into persister determinants have been conducted in planktonic cultures after acute stress and one must be careful not to overgeneralise these mechanisms in biofilms (Hall and Mah, 2017).

1.2.3. Clinical and Industrial Relevance

Biofilms are ubiquitous and encountered in a variety of anthropologically-relevant settings. They cause significant challenges in both clinical and industrial environments due to their characteristic recalcitrance and mucoid nature which predisposes to persistence on industrial surfaces and medical devices. Biofilms contribute to the complication of clinical management of infection and substantial economic losses encountered by a variety of important industries (Mattila-Sandholm and Wirtanen, 1992). It is estimated that up to 70% of bacterial infections possess a biofilm component somewhere in the chain of infection from infection control to antimicrobial chemotherapy (Bryers, 2008).

Biofilms are an important environmental reservoir for pathogens which contribute to infection through contamination of fomites in the healthcare environment or consumer goods at the stage of manufacturing (Van Houdt and Michiels, 2010). Biofilms at a solid-air interface, referred to as ‘dry’ biofilms, which colonise medical devices, flooring and fixed furnishings are typically highly desiccated and nutrient-starved (Almatroudi et al., 2015, Hu et al., 2015). Dissemination of the biofilm typically takes place through direct transmission on the hands of healthcare workers. Appropriate disinfection regimes of high-touch surfaces and adherence to infection prevention protocols can effectively control transmission of pathogens from biofilms at a solid-air interface. Nevertheless, their ubiquity presents a constant challenge to infection control measures. Biofilms at solid-liquid interfaces often represent an even greater challenge to infection control as they form at sites not easily accessible for environmental monitoring or decontamination. This particularly includes pipework, drains, boiler tanks, and sink and shower faucets (Chaves Simões and Simões, 2013). These ‘wet’ biofilms are able to achieve higher cell densities, form more EPS and disseminate through sloughing of microcolonies and planktonic cells due to shear stress (Parsek and Singh, 2003). Consequently, they can transmit pathogens from cryptic sources. The archetypal example of this is the contamination of hospital water distribution systems by biofilms of *P. aeruginosa* and *Legionella pneumophila* (Wingender and Flemming, 2011). Up to half of *Pseudomonas* infections in ICUs can be attributed to contaminated drinking water (Abdel-Nour et al., 2013, Bédard et al., 2016). Resolution of outbreaks associated with contaminated water systems is difficult as the source of the outbreak is not always obvious and the efficacy of chemical disinfection is attenuated by dilution effects (Kanamori et al., 2016). Moreover, exclusion of the biofilm often requires mechanical removal of the contaminated device, a process made non-trivial when entire plumbing systems are fouled by EPS.

Biofilms can also cause infections directly, primarily due to colonisation of indwelling medical devices. Over half of healthcare-associated infections can be attributed to contamination of medical devices (Darouiche, 2004). In the US, this amounts to approximately 17 million infections per year, which leads to at least 550,000 deaths (Kovach et al., 2017). Moreover, utilisation of medical devices has been consistently increasing due to an aging population with increasing rates of comorbidities and immunocompromised individuals. Consequently, the clinical relevance of medical device-related infections is huge and is only set to increase in coming years (VanEpps and Younger, 2016). Indwelling medical devices are highly amenable for biofilm formation as they are non-shedding surfaces in a fluid-rich environment which abrogate the body's intrinsic colonisation resistance through innate immunity and cellular turnover (Weinstein and Darouiche, 2001). Implanted medical devices such as prosthetic joints, ventricular assist devices and cosmetic implants provide substrates for biofilm formation and increase the risk of disseminated disease. Moreover, many medical devices provide a route for exogenous microbes to enter a sterile organ, such as urinary catheters, central venous catheters and endotracheal tubes (Percival et al., 2015). Contamination of such devices is difficult to resolve with antimicrobial chemotherapy, leaving surgical removal the only recourse, increasing the morbidity of many conditions. The most common biofilm-associated infection is catheter-associated urinary tract infections (CAUTI) (Cortese et al., 2018). Long-term catheterisation is associated with a very high risk of UTI due to colonisation along the catheter lumen into the bladder, primarily from peri-urethral commensals or contaminated drainage bags. Ascending CAUTI cannot be avoided through aseptic measures and current management guidelines involve removal and replacement of the contaminated catheter. Biofilms can also grow on organic substrates such as non-shedding tissues causing septic arthritis, endocarditis and osteomyelitis, and with impaired clearance mechanisms such as the cystic fibrosis lung. Cystic fibrosis is associated with chronic carriage of respiratory pathogens such as *P. aeruginosa* and *Burkholderia cepacia* complex which can last for decades. Consequently, pathogens of cystic fibrosis lung have a unique opportunity to undergo within-host evolution to adapt to the lung environment (Luján et al., 2011). Evolutionary trajectories in these strains demonstrate selection for increased biofilm formation to facilitate subclinical persistence through immunoevasion and reduced acute virulence. In general, understanding of the physiology of the *in vivo* biofilm is poor and applying the archetypal biofilm life cycle on abiotic surfaces to them is probably misleading. Biofilms in the cystic fibrosis lung are not associated with an interface, instead they persist as clustered microcolonies

suspended in mucous which may deter phagocytosis. One of the characteristic features of lung-adapted *P. aeruginosa* is the conversion to mucoidy due to constitutive de-repression of alginate biosynthesis through mutation in the alginate repressor MucA (Pedersen et al., 1992). End-stage pulmonary infection in cystic fibrosis is associated with reversion of the biofilm phenotype whereby the quorum sensing network is compromised through mutations in the master transcriptional activator LasR (Ciofu et al., 2010). Consequently, biofilm formation is used as a strategy to persist in an organic environment with compromised biomechanical removal.

Biofilms also pose a particularly significant challenge to the efficiency of process manufacturing, wastewater treatment and the food industry (Stepanovic et al., 2004). The mechanical impacts of biofilms growing in industrial surfaces contribute to reduced efficacy of heat transfer, increased frictional resistance requiring higher energy consumption and impediment of membrane or ion exchange processes. Areas vulnerable to biofilm formation include surface defects where penetration of chemical disinfectants is limited, points of reduced flow, notably bends in pipework, and at the air-water interface (Galié et al., 2018). Biofilms accelerate the corrosion of surfaces, blockage of pipework and contaminate manufactured goods. Aggressive mechanical decontamination or replacement of the contaminated surface is often required for removal of mature biofilms (Van Houdt and Michiels, 2010). Consequently, the economic burden of product contamination, manufacturing stoppage and energy loss accountable to biofilm formation is estimated to exceed $100 billion per annum (Worthington et al., 2012)

1.3. Antimicrobial Resistance

1.3.1. Antibiotics and Mechanisms of Resistance

Antimicrobial resistance is an archetypal example of Darwinian natural selection observable within the course of a human lifetime. Simple selective principles applicable to any evolutionary process have governed the emergence and proliferation of antibiotic-resistant bacteria to create the single largest public health crisis in modern history (Martens and Demain, 2017). Scientists and physicians have been aware of antimicrobial resistance since at least the introduction of penicillin (Fleming, 1944). Fleming cautioned in his 1945 Nobel prize lecture that laboratory culture of staphylococci could be “educated” to resist the action of penicillin. However, even earlier observations of resistance have been made. James McDonagh, a physician and the foremost authority on Salvarsan in England in the early 20th century, described

"immunity" of *Plasmodium* parasites to Salvarsan when pre-treated with other arsenicals (Williams, 2009). Similarly, sulfonamide-resistant infections were observed extensively in American G.I.'s during the Second World War, many of whom were receiving sulfonamides prophylactically to avoid sexually transmitted infections (Levy, 1982). Furthermore, within only a few years after the mass-production of penicillin, penicillin-resistant *S. aureus* was being isolated in London hospitals (Barber and Rozwadowska-Dowzenko, 1948). Moreover, post-war Japan experienced an epidemic of bacillary dysentery caused by multidrug-resistant *Enterobacteriaceae* resistant to streptomycin, tetracycline and chloramphenicol (Watanabe, 1963). Though provident physicians began expressing anecdotal concerns at the pace at which resistance was developing, mainstream clinical opinion, bolstered by the success of the period's profligate antibiotic use, remained unchanged (Barber, 1948).

From the 1950s, an increasing volume of evidence from academic workers forewarned that drug resistance was a greater problem than was widely appreciated at the time (Dowling et al., 1955). The discovery of the horizontal gene transfer of resistance determinants and the evolution of multidrug resistance to methicillin combination therapy in *S. aureus* was a portent of things to come (Akiba et al., 1960). However, it was not until the 1980s that antimicrobial resistance was recognised as a significant clinical problem and prescription practice began to change. Up to this point, the corollary of resistance on clinical outcome was ameliorated by the continuous development of new classes of antibiotic (Aminov, 2010). The period from the introduction of penicillin to the 1980s was known as the 'Golden Age of Antibiotics' due to the vast numbers of antibiotics developed at this time. Antibiotic discovery programmes of the age largely relied on soil and fungal screens for organisms with inhibitory activity on human pathogens, followed by chemical purification of the antimicrobial compound (Aminov, 2017). These methodologies were based on some of the earliest work identifying new penicillin analogues and changed comparatively little over the course of 40 years. Such programmes were highly productive and yielded some of the most widely used antibiotics in use today including aminoglycosides, tetracyclines, β-lactams, macrolides, phenicols and glycopeptides. However, by 1980, almost all of the 'low-hanging fruit' had been picked, and efforts to recapture earlier success with genetic and biochemical approaches were largely underwhelming (Davies and Davies, 2010). Thus, the 1980s marked the beginning of a discovery void in antibiotic development which frustrated the prevailing strategy to circumvent resistance by prescribing a newer agent.

Drug resistance consistently follows the clinical introduction of antibiotics very quickly (Centers for Disease Control and Prevention, 2013). This aptitude for selective change is the product of billions of years of adaptation in the face of antimicrobials in nature. Consequently, homeostasis mechanisms which maintain benign intracellular concentrations of these compounds evolved to become universally disseminated amongst bacteria (D'Costa et al., 2011). Moreover, most clinical antibiotics used for chemotherapy in humans are derivatives of compounds produced by microorganisms in nature for interspecific competition (Larsson and Flach, 2021). Consequently, resistance is an intrinsic necessity of antibiotic production as a mechanism to prevent autotoxicity. Thus, long before humans use of antibiotics, a large reservoir of resistance genes have existed which can be repurposed, optimised, mobilised and selected to adapt to new antimicrobial stresses. Acquired resistance mechanisms fall into four broad categories based on their method of drug detoxification: enzymatic alteration of the drug, protection of the target, prevention of access of the drug to its target and metabolic bypass where the essentiality of the target is lost due to gain of an alternative gene (figure 1.5.) (Blair et al., 2015).

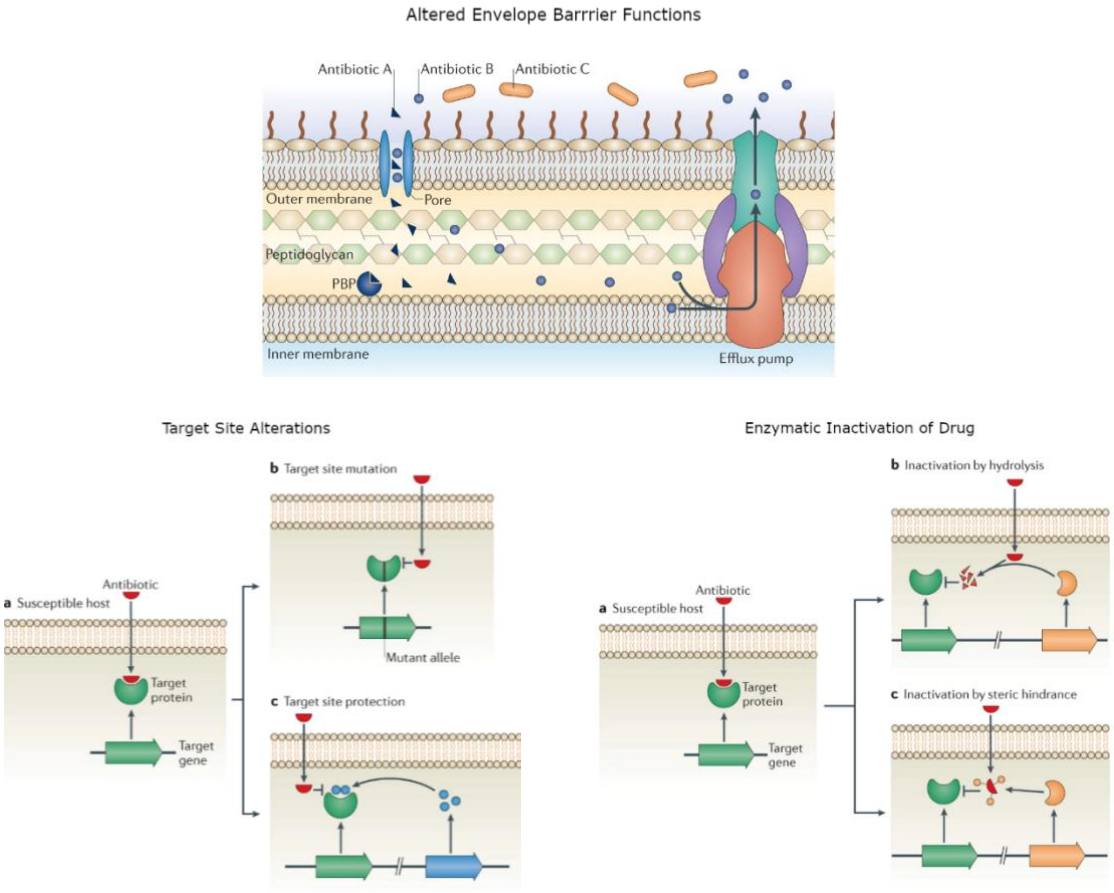

Figure 1.5. There exist many mechanisms by which bacteria can resist the action of antimicrobials however, most fall into one of three main categories. Firstly, chromosomal mutations which alter envelope permeability can reduce susceptibility to antimicrobials by limiting their intracellular accumulation. This can be achieved primarily via downregulation of porin channels, thus reducing antimicrobial import, or upregulation of multidrug efflux pumps, thus increasing export. Antimicrobial agents can also be inactivated by antibiotic-modifying enzymes, generally via hydrolases or transferases which inhibit target site binding. Such mechanisms are often horizontally acquired and represent some of the most clinically significant mechanisms of resistance, the most notable example of which being plasmid-encoded β-lactamases. The antibiotic target can also be directly modified to prevent drug binding. This can be achieved through chromosomal mutations in the antimicrobial target, most notably ribosomal subunits, penicillin-binding proteins and topoisomerases, or via post-translational modifications to the target site to protect the targe from drug binding. Adapted from Blair et al. (2015).

Enzyme-catalysed inactivation of antibiotics is an important mechanism of resistance due to the high-level, often horizontally transferable resistance it confers (Egorov et al., 2018). Enzymatic degradation or modification of antibiotics exists for a wide variety of different classes, including β-lactams, tetracyclines, phenicols, macrolides and aminoglycosides (Blair et al., 2015). The most pertinent example of antibiotic-degrading enzymes are β-lactamases, enzymes which sequester β-lactams through acetylation and hydrolyse the β-lactam ring to prevent binding to penicillin-binding proteins (PBPs) (Bonomo, 2017). They are widespread throughout both Gram-positive and -negative

pathogens, most notably *S. aureus*, *Streptococcus pneumoniae, Enterobacteriaceae* and *P. aeruginosa*. Clinically-relevant chromosomal β-lactamases exist, such as AmpC in *P. aeruginosa*, however most are mobilised on conjugative plasmids from phylogenetically ancient, naturally occurring β-lactamases in soil bacteria (Bush, 2018). The first detection of a penicillinase was made by Abraham and Chain (1940) in *E. coli* before penicillin even entered clinical practice. However, β-lactamases now exist which are able to inactivate all classes of β-lactam. Extended-spectrum β-lactamases such as CTX-M are able to hydrolyse third-generation cephalosporins and monobactams, and the KPC and VIM carbapenemases can hydrolyse β-lactamase inhibitors in addition to carbapenems (Sawa et al., 2020). Similarly, aminoglycoside-modifying enzymes are also encoded on mobile genetic elements. However instead of degrading drugs to non-toxic analogues, they exploit steric hindrance to prevent ligand binding (Ramirez and Tolmasky, 2010). Aminoglycosides are large polycations with multiple hydroxyl and amide groups susceptible to chemical modification at charged sites used for recognition of the 30S ribosomal subunit binding cleft. Consequently, a variety of phylogenetically-distinct enzymes including acetyl-, phospho- and nucleotidyltransferases have evolved to modify the aminoglycoside molecule to an inactive form.

The highly specific mode of action which affords the selective toxicity that an antibiotic's clinical value relies on, is also a source of vulnerability to resistance. Small conformational changes in drug targets, such as bacterial topoisomerases, transpeptidases and ribosomal subunits which reduce drug binding affinity can confer large reductions in susceptibility (Lambert, 2005). Expression of β-lactam insensitive PBPs is another major mechanism of β-lactam resistance. In *S. aureus,* this is mainly mediated by PBP2a, a non-native PBP encoded by *mecA* on a *Staphylococcus*-specific mobile genetic element known as a staphylococcal cassette chromosome (SCC*mec*) (Hanssen and Ericson Sollid, 2006). It is expressed in addition to genomic β-lactam sensitive PBPs, can be horizontally transferred and is the main determinant for the global spread of methicillin-resistant *S. aureus* (MRSA). Similar mechanisms of manipulating insensitive PBPs for reduced susceptibility to β-lactams have been observed in a number of other important pathogens (Zapun et al., 2008). Point mutations in the *pon* PBPs in *Neisseria gonorrhoeae* can reduce susceptibility to penicillins by decreasing the acetylation rate of the β-lactam molecule. Moreover, the low-affinity PBPs 4 and 5 in *Enterococcus faecium* can be overexpressed through mutations in the promoter region to compensate for inactivation of the β-lactam sensitive PBPs (Montealegre et al., 2016).

Macromolecular synthesis is a highly selectively optimised process, therefore almost any functional change can introduce collateral trade-offs from perturbations in protein synthesis, DNA supercoiling or cell wall polymerisation (Melnyk et al., 2015). This is particularly the case for mutations in ribosomal RNA (rRNA); even small reductions in the efficiency of protein synthesis can give rise to punitive fitness costs. Consequently, in addition to *de novo* mutation of cellular targets, target-modifying enzymes also exist to protect the target at the post-translational level without requiring target mutations which may not be tolerated (Connell et al., 2003). Target protection proteins are generally plasmid-encoded and include the *erm* 16S rRNA methyltransferase which confers resistance to macrolides, lincosamides and streptogramins and the *cfr* 23S rRNA methyltransferase which confers phenicol resistance (Blair et al., 2015). These proteins catalyse the methylation of a single adenine base in the rRNA macromolecule altering the electrostatic charge and produce a hostile environment at the specific drug binding site (Dzyubak and Yap, 2016). Protection proteins also exist which do not alter the target ultrastructure, and instead operate through a mechanism of competitive inhibition. For example, the *tet* 16S rRNA GTPases bind to post-translocation ribosomes and physically displace tetracycline molecules allowing for translation to continue (Wilson et al., 2020).

Cell wall barrier functions and transmembrane export impede the intracellular accumulation of drugs to toxic concentrations (Blair et al., 2015). Such mechanisms are generally chromosomally-encoded and can confer modest reductions in susceptibility to a broad repertoire of agents simultaneously (Delcour, 2009). Moreover, they possess a distinct benefit by being able to act upon agents which do not possess a specific mode of action. They are often integral parts of the cellular stress response and constitutive expression relies on release from regulatory control, usually through mutation in repressor genes (Blair et al., 2014). Consequently, they are often considered an intermediate step between transient adaptation and *de novo* resistance. Multidrug efflux, downregulation of size-selective outer membrane proteins and changes in membrane fatty acid composition are all examples of concentration-limiting mechanisms and will be discussed in detail in the next subchapter.

As immunocompromised populations grow due to an aging population and better clinical outcomes for the immunocompromised, efficacious antimicrobial chemotherapy becomes ever more necessary (Beckett et al., 2015). The loss of efficacy of antimicrobial chemotherapy would have catastrophic consequences, not only on treatment of infectious disease, but also clinical interventions which reliant on antibiotics, such as cancer chemotherapy, surgical prophylaxis, and

immunosuppressive treatment (Teillant et al., 2015). To overcome the current pace of resistance, it has been recommended that nineteen new antibiotics are required per decade, with two novel classes of both empiric and targeted agents (The Review on Antimicrobial Resistance, 2015). However, in the last 40 years only two new classes of antibiotics, the oxazolidinones and lipopeptides, have been introduced into clinical practice. Multinational pharmaceutical companies have largely abandoned antibiotic discovery programmes due to the high attrition rate of drug candidates in clinical trials and modest economic return (Ventola, 2015). As the use of new antibiotics is stringently minimised to avoid resistance, and when prescribed, courses last in the magnitude of weeks, relying on meagre sale revenue hinders commercial viability (Rahman et al., 2021). This contrasts with therapy for autoimmune disease, diabetes and cardiovascular disease, which are prescribed generously throughout the patient's lifetime. Consequently, there has been an overwhelming financial incentive to divest from antibiotic development by the multinational pharmaceutical industry. Consequently, clinicians have been forced to reimplement old and inferior antibiotics which have been safeguarded from resistance due to lack of use (Theuretzbacher et al., 2015). Antibiotics such as colistin now have central roles in treating antibiotic-resistant infection. However, resistance to even this last line of antibiotic therapy is now emerging which presents a serious challenge to the long-term efficacy of antibiotic therapy. The progression of drug resistance has already fundamentally altered management of infectious disease and antibiotic prescription habits. Antibiotic resistant infections, as defined by resistance contributing to failure in clinical management, cause approximately 50,000 deaths per year in the United States and Europe (Martens and Demain, 2017). Extrapolating from current trends, it has been estimated that antibiotic-resistant infections will contribute to ten million deaths per annum by 2050 (The Review on Antimicrobial Resistance, 2016).

1.3.2. Collateral Selection of Antibiotic Resistance by Non-Therapeutic Antimicrobials

Antibiotics are usually naturally derived, or semi-synthetic modifications of chemistries isolated from soil microbes. They evolved in nature to act as interspecies niche competition molecules and as such, they possess well defined cellular targets to prevent autotoxicity (Blair et al., 2015). Non-therapeutic antimicrobials on the other hand, are often synthetic or inorganic, produced independently of microbes, ergo their activity is not constrained by the necessity for intrinsic resistance of the producer. Accordingly, non-therapeutic antimicrobials often possess non-specific mechanisms of action which exert antimicrobial effects through gross cellular damage (Walsh et al.,

2003). This is achieved primarily through membrane disruption or coagulation of cytosolic constituents (Ashraf et al., 2014, Russell, 2003). These mechanisms confer a broader spectrum of activity than most antibiotics, but often include a degree of cytotoxicity. Consequently, the lack of selective toxicity impedes the development of high-level resistance but renders most man-made antimicrobials unable to be used therapeutically.

The definition of resistance describes a degree of insusceptibility which surpasses an arbitrary breakpoint condition (Turnidge and Paterson, 2007). Breakpoints represent a binary metric which deconvolute a scalar phenotype of susceptibility by estimating the threshold which an agent can no longer exert a successful antimicrobial effect in clinical practice. This has proven useful at predicting the outcome of antimicrobial chemotherapy, as antibiotic resistance often presents as magnitude reductions in susceptibility. This paradigm seldom applies with non-therapeutic antimicrobials. Unlike antibiotics, non-therapeutic antimicrobials possess non-specific modes of action which rarely elicit resistance due to a multiplicity of targets (Maillard, 2018). Additionally, working concentrations are not limited by *in vivo* tolerability and can are often applied at concentrations rarely amenable to resistance. Moreover, antibiotics generally possess linear dose-dependent activity, whereas non-therapeutic antimicrobials often have an inhibitory threshold at which a sub-inhibitory dose rapidly becomes potently bactericidal with a small increase in concentration (Russell, 2003). This phenomenon is likely a product of their non-specific mode of action causing membrane leakage, cytosolic coagulation and collapse of the electron transport chain. Consequently, when the enzymatically competitive mechanisms to maintain homeostasis under such gross stress become saturated, an irreversible cascade of cellular perturbations soon ensues. The repertoire of mechanisms which can reduce such a degree of insult in any significant way is very limited (McDonnell and Russell, 1999). Thus, the mutational space in which bacteria can become definitively resistant to agents with non-specific mechanisms of action is small.

Despite non-therapeutic antimicrobials rarely yielding mutants with high-level resistance, this does not mean they are selectively neutral. In practice, sub-inhibitory concentrations of non-therapeutic antimicrobials are more likely to be encountered than equivalent concentrations of antibiotics by virtue of the larger volumes consumed and routes of application (Maillard et al., 2013). Dosing regimens for antibiotics have been pharmacodynamically optimised to maintain super-inhibitory concentrations of drug throughout the course of therapy. This is in contrast to non-therapeutic antimicrobials which generate biologically-active residual concentrations many orders of magnitude

below the working concentration for extended periods of time (Thomas et al., 2000). For example, non-shedding surfaces in between cleaning regimes yield sub-inhibitory concentrations of antimicrobials, as does the livestock gut between feeds. Furthermore, the environment now contains appreciable amounts of many common biocidal agents at a range of concentrations, most notably triclosan (Yueh and Tukey, 2016). Given the non-specific targets of most non-therapeutic antimicrobials, often only mechanisms which prevent molecules from entering the cell, or evacuate them upon entry, are able to confer reductions in susceptibility (McBain and Gilbert, 2001). Workers have been reluctant to describe this as resistance, given that adaptation manifests with such marginal reductions in susceptibility which seldom surpasses to the working concentration of the agent. The modest sub-fold changes in inhibitory concentration or has been described by others as ‘tolerance’. However, this too has become a misnomer, given that tolerance has acquired a new definition in recent years referring to non-hereditary mechanisms of resisting antimicrobial killing (Levin-Reisman et al., 2017). For the purposes of this report, significant reductions in phenotypic susceptibility to non-therapeutic antimicrobials will nonetheless be referred to as resistant.

An interesting property of the generic resistance mechanisms which underpin reductions in susceptibility to non-therapeutic antimicrobials is the breadth and lack of functional discrimination within their substrate repertoires (White and McDermott, 2001). Consequently, despite the change in susceptibility associated with these mechanisms being small, they possess collateral selective effects on a broad range of chemically-diverse agents which also lie within their repertoire. Hence, decreased susceptibility can be selected to functionally diverse antimicrobial agents, even if they have never before been encountered (Oggioni et al., 2013). This phenomenon is known as cross-resistance and has elicited concerns particularly regarding the possibility that non-therapeutic antimicrobials could act as non-canonical drivers of antibiotic resistance. Clinical and environmental isolates resistant to non-therapeutic antimicrobials often correlate with reduced susceptibility to antibiotics and higher incidences of multidrug resistance (Akimitsu et al., 1999, Alotaibi et al., 2017, Cottell et al., 2009, Guo et al., 2015b, Koljalg et al., 2002, Oethinger et al., 1998, Timoney et al., 1978). However, it is difficult to conclude a causative relationship between the selective properties of non-therapeutic antimicrobials and antibiotics from susceptibility profiles alone. In this regard, epidemiological and experimental studies designed to impose a selective pressure in a controlled environment are far more valuable for understanding cross-resistance. The earliest observations of non-antibiotic stresses directly modulating antibiotic susceptibility were made in the 1980s. Bacteria isolated from

chlorinated tanks in wastewater treatment facilities have been shown to be more likely to be resistant to drugs than the non-chlorinated inflow water (Armstrong et al., 1982, Calomiris et al., 1984, Murray et al., 1984).

The first correlation between heavy metals as livestock supplements and antibiotic resistance was made by Huysman et al. (1994). Microbial populations from swine manure were more likely to surpass clinical breakpoints for a wide variety of drugs from pigs consuming copper as an in-feed additive. Since then, several workers have demonstrated that experimental supplementation of copper or zinc in swine diets can increase the proportion of drug resistant organisms in the porcine microbiome and faeces (Bednorz et al., 2013, Holzel et al., 2012, Li et al., 2015). The dependent variable in these studies was the presence of heavy metal additives in different groups of animals. However, the results have also been substantiated temporally using piglets before and after transitioning onto a solid diet with in-feed copper. Pairwise comparisons of *Enterococcus* spp. and *Lactobacillus* spp. isolated from weaning piglets demonstrated a significant increase in isolates with drug resistance after receiving the copper-supplemented diet (Zou et al., 2017). Moreover, these observations are not limited to bacteria encountered *in vivo*. Communities in agricultural soil dominated by Gram-positive organisms have also shown increased incidences of multidrug resistance in microcosms with historical copper pollution or experimental supplementation with heavy metals (Berg et al., 2005, Berg et al., 2010, Fernández-Calviño and Bååth, 2013).

The extent to which heavy metals can select for decreased susceptibility to drugs appears to be broadly dose-dependent. After five years of exposure to either copper or nickel at stress concentrations doubling from 50 to 800 mg metal kg^{-1} soil, significant increases in the abundance of antibiotic resistance genes peaking at either 400 or 800 mg kg^{-1} (Hu et al., 2016, Hu et al., 2017). Moreover, there was significant enrichment of mobile genetic elements, particularly the integrase *intI1*. Whether this represents *de novo* adaptation or an artifact of changing community structure favouring intrinsically resistant taxa remains unknown. However, horizontal gene transfer has been established as one of the most important factors governing resistance to antibiotic growth promoters (Zhu et al., 2013). Supporting the extension of this to non-therapeutic antimicrobials; zinc, copper, cadmium, mercury and arsenic resistance determinants and antibiotic resistance genes have been found co-localised on mobile genetic elements from agricultural sources (Amachawadi et al., 2015, Andrade et al., 2018, Fang et al., 2016, Levings et al., 2007). Thus, as horizontal gene transfer transmits extrachromosomal elements as a single heritable unit, heavy metal exposure can co-

select for antibiotic resistance. Accordingly, plasmid-encoded tetracycline, macrolide and sulfonamide resistance are particularly associated with heavy metal exposure in livestock, presumably due to their widespread historical use as growth promoters (Song et al., 2017, Vahjen et al., 2015). Moreover, Hasman and Aarestrup (2002) identified a correlation between reduced vancomycin susceptibility and copper in-feed additives in *Enterococcus* spp. isolated from swine. Experimental selection under copper stress was able to co-select for vancomycin resistance in a naïve host (Hasman et al., 2006). The mechanism of this was determined to be the presence of a conjugative plasmid bearing the CPx-type copper exporting ATPase *tcrB*, alongside the glycopeptide resistance determinant Tn*1546*-*vanA* (Hasman, 2005, Hasman and Aarestrup, 2005).

Similar results have been found in MRSA, in which reduced susceptibility to cadmium and mercury was abolished alongside loss of methicillin resistance through serial passage (Poston and Li Saw Hee, 1991). Zinc, copper, cadmium and arsenic insusceptibility determinants have been found co-localised on *mecA*-positive SCC*mec*'s (Aarestrup et al., 2010, Ito et al., 2001, Li et al., 2011). Moreover, exposure to in-feed zinc has been associated with a dose-dependent increase in the prevalence of MRSA among swine due to the carriage of *czcA* within the SCC*mec* element (Amachawadi et al., 2015, Slifierz et al., 2015a, Slifierz et al., 2015b).

Substantiating such causative relationship between use of non-therapeutic antimicrobials and antibiotic resistance is more challenging in clinical settings (Maillard, 2005). Limitations in experimental design caveat the widespread and obligatory use of both compounds confounds the drawing of strong epidemiological associations. However, an association between resistance to antibiotics and reduced susceptibility to quaternary ammonium compounds (QACs), cationic surfactants in widely used as disinfectants in clinical environments, has been identified (Furi et al., 2013). The main mechanism for reduced susceptibility to these agents is a group of plasmid-encoded efflux pumps, QacA/B, Smr and QacE, which are present in both Gram-positive and -negative organisms (Slipski et al., 2021). In addition to cationic surfactants, their substrate repertoire also includes DNA-intercalating dyes, diamidines and guanylhydrazones, but not antibiotics. Despite this, *qacE* bears a close association with type I integrons, widely disseminated mobile genetic elements and important determinants of antibiotic resistance (Gaze et al., 2013). Integrons possess a promoter-activated integrase and a recombination site with which they accumulate new gene cassettes encoding reduced susceptibility to drugs, metals and biocides. They leverage transposition machinery to horizontally transfer and can be chromosomally- or plasmid-

encoded. Environmentally-derived type I integrons typically possess a Tn*402* transposon flanking a recombination site containing *qacE* and a metal insusceptibility and antibiotic resistance cassette (Gillings et al., 2008). These integrons become enriched in QAC contaminated environments leading to concerns that they can co-select for drug resistance (Gaze et al., 2005, Gaze et al., 2013). Despite this, clinically-relevant type I integrons, known as class I integrons typically possess a defective *qacE* allele known as *qacEΔ1* truncated by the 3' capture of the sulfonamide resistance gene *sul1* within the recombination site (Paulsen et al., 1993). Class I integrons typically possess a more diverse array of antibiotic resistance genes and transposon backbones. Moreover, *qacEΔ1* itself does not confer reduced susceptibility to QACs (Jennings et al., 2017, Kücken et al., 2000). Consequently, it was hypothesised that acquisition of *qacE* was an intermediate step in the evolution of class I integrons which allowed for adaptation to anthropogenic environments and transmission to humans (Ghaly et al., 2017). Subsequent evolution selected against the *qacE* gene in favour of molecular diversification of drug resistance in human pathogens.

Finally, triclosan is a biocide which has been used extensively as a preservative for consumer goods since the 1970s. Since its introduction, staphylococci isolated from blood cultures have become approximately 6-fold less susceptible to triclosan over the course of 50 years (Skovgaard et al., 2013). Trends of reduced susceptibility to antibiotics have also been observed in experimental environments exposed to triclosan. Dietary exposure of mice to triclosan resulted in an increase in abundance of antibiotic resistance and heavy metal insusceptibility determinants (Gao et al., 2017). Furthermore, incidences of drug resistance were higher in dust particles from triclosan-utilising facilities and after a year of exclusive triclosan usage in domestic households (Aiello et al., 2004, Fahimipour et al., 2018). Unusually for a biocide, triclosan possesses a defined primary target in the FabI enzyme involved in type II fatty acid biosynthesis (Heath et al., 1999). Unlike prior examples with heavy metals and cationic surfactants, mobile genetic elements do not mediate widespread resistance to triclosan. Triclosan resistance mechanisms are primarily chromosomal and are selected through *de novo* mutation instead of horizontal gene transfer (Webber et al., 2008a). Such changes in the core genome are largely opaque to associations with cross-resistance as a single mechanism mediates reduced susceptibility to both agents. As a consequence of this, definitive mechanistic associations of triclosan with resistance to antibiotics derived from epidemiological investigations have been nebulous. Nevertheless, perceived concerns regarding triclosan resistance and antibiotic cross-resistance has led to a Food and Drugs Administration (FDA) ban on

triclosan in antibacterial soaps in the US (Weatherly and Gosse, 2017). The mechanisms underpinning the molecular basis of cross-resistance will be discussed exhaustively in the next subchapter.

1.4. Molecular Mechanisms of Cross-Resistance

1.4.1. Multidrug Efflux

Efflux systems are membrane-bound protein complexes which act to extrude intracellular molecules into the extracellular environment (Blair et al., 2015). Their main physiological role is cellular detoxification through expulsion of toxic compounds including metabolic byproducts and xenobiotics. Efflux likely evolved in nature to provide autoimmunity or transient resistance during niche competition to toxic natural products such as antibiotics, bacteriocins or bile salts (Du et al., 2018). In bacteria they also have been shown to possess roles in modulating pathogenicity, biofilm formation and cell-to-cell signalling (Henderson et al., 2021). The action of some bacterial efflux pumps, such as metal ion antiporters or tetracycline exporters, can be very narrow with monospecific substrate specificities. Conversely, multidrug efflux pumps possess very broad substrate specificities which confer low- to moderate-level resistance to multiple classes of antimicrobials simultaneously (Edgar and Bibi, 1997). Multidrug efflux pumps often act as an adaptively-acquired intermediate step between sensitivity and acquisition of *de novo* resistance by mutation (Ebbensgaard et al., 2020). When a multidrug efflux pump is expressed, it not only expels any inducing compound, but is also able to extrude other substrates which share specificity for the pump. This can include antibiotics, organic solvents, non-antimicrobial pharmaceuticals, biocides and metals (Toba et al., 2019). Consequently, expression of multidrug efflux by non-antibiotic substrates can result in reduced susceptibility to clinically-relevant antibiotics, and vice versa.

Several families of multidrug efflux pumps exist. Most are chromosomally-encoded but however, others, such as the Qac pumps, are commonly found on plasmids (Piddock, 2006). Some families show preferential distribution among Gram-positive or -negative organisms. Major facilitator superfamily (MFS) pumps are the most important in Gram-positive bacteria, and notably include NorA in Staphylococci and Cme in *Clostridium difficile* (Handzlik et al., 2013). In Gram-negative bacteria, the resistance-nodulation-division superfamily (RND) is the most clinically relevant (Blair et al., 2015). RND pumps are composed of a transporter, accessory protein and outer membrane channel assembled into a tripartite complex which spans the periplasm and both membrane

leaflets. Archetypal RND efflux systems include AcrAB-TolC in *Escherichia coli* and *Salmonella* spp. and MexAB-OprM in *Pseudomonas aeruginosa*. Efflux is a form of active transport, and most families, including the RND and MFS families, utilise the process of translocating hydrogen ions down an electrochemical gradient known as the proton-motive force (pmf) (Blanco et al., 2016). ABC pumps are distinct in that they are directly ATP-dependent. Despite structural differences between families, ligand extrusion by efflux pumps follows similar biochemical principles (Du et al., 2018). Within the pump tertiary structure, occupied ligand docking sites reveal proton loading domains. This induces conformational changes within the pump allowing the docked substrate to migrate out of the pump. The captured protons migrate down the electrochemical gradient until they are released which returns the pump to its native conformation.

Most pumps are quiescent in unstressed conditions, with expression tightly regulated as a result of the fitness costs associated with collateral expulsion of essential substrates (Piddock, 2006).. Expression of RND multidrug efflux pumps is often regulated by transcriptional repressors encoded locally of pump operon, or through global regulators of the general stress response (Holden and Webber, 2020). It is not unconditional that exposure to an efflux inducer will always confer reduced drug susceptibility. The broader regulatory architecture which controls expression of multidrug efflux also includes stress-sending two-component systems, trans-acting antisense RNAs and selective proteases (Sun et al., 2014). These co-dependencies have their own induction mechanisms which adds additional orders of positive and negative regulation acting transcriptionally and translationally, complicating the global regulatory landscape of efflux de-repression.

Transcriptional regulators repress expression of the pump operon by binding to a cognate operator, thus preventing transcriptional access of RNA polymerase. However, when a pump inducer is present, the repressor protein undergoes conformational change, permitting expression of the operon. For this reason, expression of multidrug efflux is induced by stress and associated with reversion to a susceptible phenotype when stress is ablated (Alcalde-Rico et al., 2016). However, under sustained stress, this can be a detriment to biological fitness as cells struggle to achieve appropriately high levels of expression when under strict transcriptional control (Blair et al., 2014). Consequently, mutations allowing multidrug efflux to escape from regulatory constraints can undergo purifying selection which constitutively overexpress pumps. In this regard, loss-of-function mutations in pump repressors are one of several

mechanisms by which decreased susceptibility to antimicrobial insults can be selected via multidrug efflux (Webber and Piddock, 2001, Wang et al., 2001).

1.4.1.1. Local De-Repression

1.4.1.1.1. *P. aeruginosa*

Polychlorinated phenol biocides, including pentachlorophenol, 2,4-dichlorophenoxyacetic acid, and paraquat, have been shown to alter susceptibility to carbenicillin, quinolones and tetracycline in *P. aeruginosa* through de-repression of MexAB-OprM (Muller et al., 2007, Muller et al., 2015, Starr et al., 2012). MexAB-OprM is one of five homologous Mex-type pumps of *P. aeruginosa* and possesses the most complex regulatory architecture as it is constitutively expressed at low levels (figure 1.6.). MexAB-OprM is the most clinically-relevant of the five RND efflux pumps in *P. aeruginosa*. It plays an instrumental role in the broad pattern of intrinsic resistance possessed by *P. aeruginosa* to tetracyclines, macrolides, penicillins and oral cephalosporins (Li et al., 1994). Constitutive overexpression of MexAB-OprM in clinical isolates is caused by mutations in its three repressors and associated with acquired resistance to fluoroquinolones, chloramphenicol and antipseudomonal β-lactams, excluding imipenem (Blair et al., 2014). Transcription of MexAB-OprM is regulated through interplay between local repressors MexR and NalD. *mexR* is encoded upstream of *mexAB-oprM* and its transcription is controlled by an antirepressor, ArmR. Transcription of the *armR* antirepressor is in turn repressed by the divergently transcribed product of *nalC*. Repression of NalC permits transcription of *armR,* leading to formation of the MexR-ArmR complex, thus allowing for overexpression of MexAB-OprM. Ligand binding studies have established a broad affinity of chlorophenols for the NalC repressor of MexAB-OprM which contributes to decreased antibiotic susceptibility in their presence (Ghosh et al., 2011).

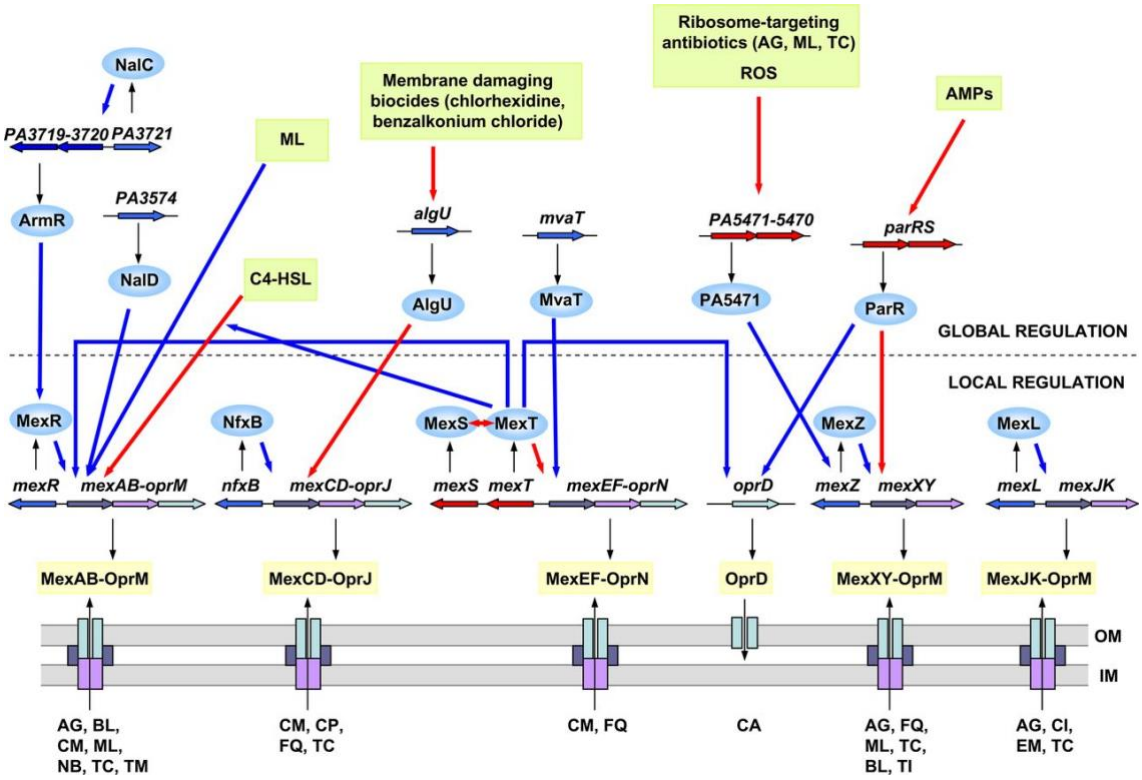

Figure 1.6. *P. aeruginosa* possesses several clinically-relevant resistance-nodulation-division multidrug efflux pumps which are involved in extrusion of an array of chemically diverse molecules including a variety of classes of antibiotics and biocides. Each RND efflux pump is a tripartite complex composed of a pump protein, an accessory protein and a porin channel each with a unique repertoire of substrates and inducers. Multidrug efflux pumps are generally quiescent in unstressed conditions, with tight regulation operating at both the local and global level. However, upon exposure to an appropriate inducer, repressors are sequestered permitting expression of the pump operon and expulsion of the offending compound in addition to any other compounds present in the substrate repertoire. Mutations in the regulatory network controlling efflux either at the local or global level can induce constitutive overexpression of multidrug efflux pumps and reduce susceptibility to agents within its substrate repertoire. In this way, resistance mediated by multidrug efflux acts polygamously, conferring resistance to the selective agent in addition to cross-resistance to any other agent within the substrate repertoire (Fernández and Hancock, 2012)

The phenolic biocide triclosan, which specifically inhibits FabI, the enoyl-acyl carrier protein (ACP) reductase involved in type II fatty acid biosynthesis, has also been implicated in cross-resistance in *P. aeruginosa*. However, de-repression of MexAB-OprM has been shown to induce transient resistance to ciprofloxacin in the presence of triclosan, chloroxylenol or ortho-benzyl-para-chlorophenol. (Chuanchuen et al., 2003, Muller et al., 2007). *P. aeruginosa* possesses a triclosan insensitive enoyl-ACP reductase allele known as FabV, conferring intrinsic resistance (Zhu et al., 2010). Despite this, deletion of MexAB-OprM or collapse of the proton-motive force results in abolition of triclosan resistance in *P. aeruginosa* (D'Arezzo et al., 2012, Mc Cay et al., 2010, Schweizer, 1998) This is an example of how multidrug efflux can be considered

a force multiplier; an essential component of cellular detoxification without which cells become hypersusceptible to antimicrobials (Webber et al., 2008a). Similarly, *gyrA* DNA gyrase mutants in *E. coli* do not possess quinolone resistance in the absence of efflux (Oethinger et al., 2000). Though MexAB-OprM is the main effector through which cross-resistance between triclosan and antibiotics is mediated, a number of other multidrug pumps in *P. aeruginosa* have also been implicated. MexCD-OprJ is kept quiescent in unstressed conditions by the local repressor NfxB, however it can be reversibly de-repressed by a number of antimicrobials including triclosan, cationic surfactants and the pharmaceuticals procaine and atropine (Issa et al., 2018, Laborda et al., 2019). MexCD-OprJ possesses a similar substrate repertoire as MexAB-OprJ and is kept quiescent in unstressed conditions by NfxB. Mutations in *nfxB* possess clinical relevance and have been selected by triclosan in a Δ*mexAB* strain of *P. aeruginosa* to confer multidrug resistance though constitutive overexpression of MexCD-OpJ (Chuanchuen et al., 2001). Continuing this trend, in triclosan-resistant mutants selected from a Δ*mexAB* Δ*mexCD* background, multidrug resistance was acquired due to the de-repression of MexXY-OprM (Chuanchuen et al., 2002). Expression of OprM was required for resistance to both triclosan and antibiotics, however, triclosan resistance appeared independent of MexXY. OprM is promiscuous can form complexes with multiple efflux systems in *P. aeruginosa* (Issa et al., 2018). Consequently, triclosan resistance was attributed to two novel RND efflux pump genes designated as *mexJK*, which was overexpressed due to a single mutation in the transcriptional repressor *mexL*. Therefore, in the absence of MexAB-OprM and MexCD-OprJ, multidrug resistance was transiently acquired via de-repression of MexXY-OprM, and triclosan resistance was attained via constitutive expression of MexJK-OprM. This exemplifies the broad regulatory amplitude that *P. aeruginosa* possesses to adapt to stress via controlled expression of a hierarchy of multidrug efflux systems.

The oxidising agents hypochlorous acid and hypothiocyanous acid have been shown to de-repress MexEF-OprN via its transcriptional activator MexT (Farrant et al., 2020). Expression of *mexT* is negatively regulated by the repressor MexS in a redox system induced by electrophilic and nitrosative stress. MexEF-OprN is highly pleiotropic and also has possesses roles in cell-to-cell signalling by exporting phenazine pigments and quorum sensing autoinducers. Consequently, the phenazine pigment pyocyanin can also induce de-repression of MexEF-OprN, in addition to MexGHI-OpmD, to confer reduced susceptibility to quinolones (Meirelles et al., 2020). These pumps have also been implicated in adaptation to isothiazolinone biocides. Exposure to an

isothiazolinone-containing laundry detergent induced overexpression of MexGHI-OpmD and mutants selected by a methylisothiazolinone and chloromethylisothiazolinone biocide formulation demonstrated MexEF-OprN-dependent cross-resistance to ciprofloxacin (Green et al., 2018, Zhou et al., 2014). A secondary bactericidal effect of isothiazolinone stress appeared to be accumulation of intracellular nitric oxide due to upregulation of nitrogen metabolism genes (Zhou et al., 2016). The role of nitrogenous stress in de-repression of MexEF-OprN is well established, therefore isothiazolinones may induce multidrug efflux through downstream generation of reactive nitrogen species.

1.4.1.1.2. *Enterobacteriaceae*

Salmonella enterica serovar Typhimurium adapted to triclosan through serial passage demonstrated inducible resistance to antibiotics through transient de-repression of AcrAB-TolC. (Copitch et al., 2010, Gantzhorn et al., 2015). AcrAB-TolC is the archetypal RND efflux pump in Enterobacteriaceae including *E. coli, Salmonella* spp. and *K. pneumoniae* and possesses close homologues in most other genus members (Piddock, 2006). It is under local regulation from the transcriptional repressor AcrR which can be de-repressed by bile salts, ethanol, surfactants and a wide variety of antibiotics including quinolones, β-lactams, chloramphenicol, tetracycline and macrolides (Jun et al., 2019). Similar observations of AcrAB-TolC-inducible cross-resistance have also been made in *Salmonella enterica* sv. Heidelberg exposed to cetylpyridinium chloride and acidified calcium hypochlorite, and *E. coli* exposed to benzalkonium chloride (Cadena et al., 2019, Moen et al., 2012). Cross-resistant mutants constitutively overexpressing multidrug efflux pumps have also been observed. *Salmonella enterica* sv. Typhimurium passaged daily in sublethal concentrations of several biocide formulations exhibited agent-specific modulation of antibiotic susceptibility (Karatzas et al., 2007). An aldehyde-containing quaternary ammonium formulation was able to select for decreased susceptibility to tetracycline and ampicillin when serially passaged at subinhibitory stress. Multidrug resistant mutants were also isolated from lineages passaged under stress from triclosan increasing in a stepwise fashion. Cross-resistant mutants were harder to select with an oxidising biocide or a phenolic-organic acid formulation. When assayed as a population, these lineages exhibited no decreased susceptibilities. However, a subpopulation of multidrug resistant mutants could be isolated with significantly higher fitness costs than mutants selected by other agents in both competitiveness in broth and virulence (Karatzas et al., 2008). It was revealed that expression of AcrAB-TolC

was significantly increased in populations exposed to the QAC-aldehyde formulation, the oxidising biocide and triclosan, but not the phenolic-organic acid biocide. Further work revealed that it was not possible to select for cross-resistant mutants in Δ*acrB* or Δ*tolC* backgrounds, and high level triclosan resistance was abolished upon deletion of either of these genes (Webber et al., 2008b). In an AcrAB-TolC inactivated background, such hypersusceptibility may be the result of continuous intracellular accumulation of triclosan. At such concentrations, *fabI* mutants can no longer outcompete the ancestor as the mechanism of killing is gross cellular damage.

Adaptation of *Salmonella enterica* sv. Typhimurium to benzalkonium chloride in a stepwise manner chloride resulted in selected for decreased susceptibility to quinolones, chloramphenicol and tetracycline (Guo et al., 2014). Abolition of resistance, and in some cases hypersusceptibility, was observed when either *acrAB* or *tolC* was deleted, indicating that the AcrAB-TolC pump was responsible for this phenotype. Furthermore, expression of *acrB* was upregulated in these mutants between 2- and 4-fold relative to the ancestor. In an Δ*acrAB* mutant passaged under the same regime, cross-resistance developed to the same magnitude as the wild-type, despite the AcrAB-TolC system being inactive (Guo et al., 2014). Investigation of other efflux systems in *Salmonella* revealed that *acrEF* was overdressed by approximately 100× to 250× in the Δ*acrAB* mutant, and knockout of *acrEF* or *tolC* fully abolished the cross-resistant phenotype. This indicates that overexpression of AcrAB-TolC is the preferential mechanism for cross-resistance between benzalkonium chloride and antibiotics in *Salmonella enterica* sv. Typhimurium. However, when AcrAB-TolC is inactive, homologues such as AcrEF-TolC can ameliorate this and become the dominant mechanism of resistance.

Serratia marcescens ATCC 13880 adapted to cetylpyridinium chloride demonstrated a 3- to 4-fold decrease in susceptibility to multiple fluoroquinolones and a 3-fold decrease to chloramphenicol (Maseda et al., 2009). *S. marcescens* was sequentially passaged in a range of cetylpyridinium chloride concentrations from 1.56- to 3.05-fold above MIC. The highest concentration which supported growth was then subcultured into another range of concentrations which progressively increased over the course of the experiment. After 13 passages, a stable 3-fold decrease in cetylpyridinium chloride susceptibility was observed, in addition to reduced susceptibility to other cationic surfactants such as chlorhexidine and benzalkonium chloride. Norfloxacin uptake experiments revealed that the cetylpyridinium chloride-adapted strain accumulated only a sixth of the norfloxacin than the ancestor. Furthermore, when the efflux inhibitor carbonyl cyanide *m*-chlorophenyl hydrazine was added, the reduction in antibiotic

accumulation in the adapted strain was abolished, implicating multidrug efflux in the phenotype.

Transposon mutants of the cetylpyridinium chloride-adapted strain were screened for abolition of the quinolone-resistant phenotype. Two quinolone-susceptible mutants were identified which both possessed transposon insertions within *hasF*, a *tolC* homologue in *S. marcecsens* which forms the porin channel to the tripartite efflux pump SdeAB-HasF (Maseda et al., 2009). Complementation of *hasF* into the transposon mutants resulted in re-acquisition of cetylpyridinium chloride and quinolone resistance. Furthermore, construction of *sdeB::xylE* gene fusions revealed that *sdeB* was transcribed in the cetylpyridinium chloride-adapted strain whilst being quiescent in the ancestor. Sequencing of the local transcriptional regulator of SdeAB, *sdeS* revealed a nonsense mutation in the adapted strain which led to constitutive overexpression of SdeAB-HasF (Maseda et al., 2011).

1.4.1.1.3. *Staphylococcus* spp.

There is a well-documented correlation between antiseptics and multidrug resistance in staphylococci (Costa et al., 2013a). Hardy et al. (2018) observed a correlation between increasing use of chlorhexidine and octenidine for skin decolonisation and multidrug resistance. Moreover, an MIC greater than 2 µg/mL to either benzalkonium chloride or triclosan was associated with a significant increase in the incidence of multidrug resistant in clinical isolates of *S. aureus* (Coelho et al., 2013). DeMarco et al. (2007) identified *S. aureus* bloodstream isolates with reduced susceptibility to quinolones, DNA-intercalating dyes and cationic surfactants which could be abolished by efflux inhibitors. Similarly, *Staphylococcus epidermidis* and *S. aureus were* adapted to ethidium bromide, a DNA-intercalating dye, also demonstrated reductions in susceptibility to quinolones which could be abolished by efflux inhibitors. (Costa et al., 2018, Couto et al., 2008).

S. aureus possesses eight clinically-relevant multidrug efflux pumps, most of which are MFS or multidrug and toxic compound extrusion family (MATE) pumps with broad substrate specificity for fluoroquinolones, cationic surfactants and DNA-intercalating dyes (Costa et al., 2013b). Huet et al. (2008) showed that the biocides ethidium bromide, rhodamine 6G or dequalinium chloride induced upregulation of multiple efflux pumps, including NorA, NorC, MepA and MdeC. Only NorA however was associated with quinolone resistance. NorA, the archetypal multidrug efflux pump of *S. aureus* is broadly distributed among staphylococci and regulated by the transcriptional

repressors MgrA and NorG, which also regulate NorB/C (Costa et al., 2013b). Unlike Gram-negative bacteria, mutations in transcriptional repressors are not a widely disseminated mechanism of constitutive efflux overexpression. Instead, most efflux overexpressing-clinical isolates of staphylococci possess mutations in the gene promoter region (Guirao et al., 2001). Furi et al. (2013) observed 14 unique single nucleotide polymorphisms (SNPs) in *S. aureus* within the *norA* promoter region, selected by ethidium bromide, acriflavine, benzalkonium chloride or chlorhexidine after serial passage of in broth. Likewise, strains of *S. aureus* adapted to triclosan, acriflavine, acrinol, benzethonium chloride or benzalkonium chloride also demonstrated overexpression of NorA through mutation in promoter sequences (Noguchi et al., 2002, Tkachenko et al., 2007).

1.4.1.2. Global Stress Responses

1.4.1.2.1. Mar and Homologues

Buffet-Bataillon et al. (2011) observed a strong association between ciprofloxacin resistance and reduced susceptibility to quaternary ammonium compounds in bloodstream isolates of *E. coli*. In these isolates, AcrAB-TolC was de-repressed via constitutive expression of the Mar global stress response through a mutation in its transcriptional repressor *marR* (Buffet-Bataillon et al., 2012). MarR is the regulator of the master transcriptional activator MarA which induces the large pleiotropic Mar regulon. The Mar regulon includes genes involved in drug resistance, stress responses, virulence factors and biofilm formation (Hao et al., 2014). RND efflux pumps are broadly under the control of Mar and efflux mutants have been widely selected by a broad range of biocides through constitutive de-repression of the Mar regulon.

Mar mutants of *Salmonella enterica* sv. Typhimurium demonstrated constitutive expression of AcrEF-TolC selected by a single inhibitory challenge with working concentrations of the quaternary ammonium biocide Superkill and tertiary amine biocide Trigene (Whitehead et al., 2011). Moreover, exposure of *Salmonella enterica* sv. Typhimurium to a single challenge from acetic acid sodium benzoate or nitrite was also able to select for Mar-mediated reductions in antibiotic susceptibility (Potenski et al., 2003). Supporting this, *marR* mutants of *E. coli* and *K. pneumoniae* have been selected by exposure to benzalkonium chloride and triclosan which resulted in significant increases in expression of AcrAB-TolC and MdtEF-TolC (Bore et al., 2007, Curiao et al., 2015).

marR mutants of *E. coli* have also been selected *in vitro* by exposure to organic solvents and oxidative biocides (Asako et al., 1997, Moken et al., 1997). Gradient plating on solid media containing pine oil, a terpene alcohol formulation, resulted in decreased susceptibility to ampicillin, chloramphenicol and tetracycline. Moreover, strains adapted to cyclohexane through serial passage demonstrated the ability to grow up to 20% v/v cyclohexane in addition to reduced susceptibility to ampicillin, chloramphenicol and nalidixic acid (Aono et al., 1991, Komatsu et al., 1994). MarA was overexpressed in all solvent-adapted mutants due to a mutation in *marR*. Deletion of the entire *marRAB* locus in the pine oil-adapted strain resulted in abolition of cross-resistance. Furthermore, introduction of the mutant cyclohexane-selected *marR* allele to its ancestral strain recapitulated the multidrug resistant phenotype. Similar *marR*-dependent multidrug resistant phenotypes were selected when *E. coli* was exposed to subinhibitory stress from the wastewater disinfection byproducts iodoacetic acid and sodium chlorite through serial passage (Li et al., 2016).

Inducers of Mar are varied and include chlorine, antibiotics and phenolic compounds, (Ariza et al., 1994, Potenski et al., 2003). Expression of MarA relies on a copper-dependent mechanism which liberates MarR from its cognate operator sequence by catalysing its covalent dimerization through the oxidation of cysteine residues (Hao et al., 2014). The bacterial cytosol is a highly reduced environment; labile metal ions occupy their lowest stable oxidation state by the action of enzyme proton scavenging. Gross membrane perturbations and ROS often impede the reduction and chelation of metal oxidants by membrane-spanning enzymes. These processes increase the ionic load of labile copper within the cytosol, which a conserved feature of exposure to a number of Mar inducers, including salicylate, norfloxacin and ampicillin. Ergo, MarA de-repression is dependent upon exposure to agents which liberate, and inhibit reduction of, oxidised copper species. However, direct exposure to copper compounds also induces expression of MarA and reduces susceptibility to antibiotics.

Not all Mar inducers are able to select for constitutive overexpression of multidrug efflux. Benzoate is a phenolic aromatic acid widely used as an antimicrobial preservative. Despite this, adaptation of *E. coli* to potassium benzoate resulted in the progressive loss of salicylate- or benzoate-inducible chloramphenicol resistance with increasing benzoate stress (Creamer et al., 2017). This is an unexpected trade-off given the role of benzoate as an archetypal inducer of Mar, and the role of the Mar regulon in the adaptive response to organic acid stress. All but one mutant possessed mutations in *marAB*, including a 6 Kbp deletion which removed the entire *marRAB* operon. Weak acids are partial uncouplers the proton-motive force, therefore under

continuous stress, the efficacy of multidrug efflux would be diminished. Furthermore, depletion of the reservoir of protons puts pressure other essential cellular processes, increasing the fitness cost of constitutive expression (Moore et al., 2019). In this way, it is possible that it became selectively advantageous to suppress MarA-dependent expression of efflux by removing the mechanism of induction.

In addition to Mar, *Enterobacteriaceae* also possesses two other global stress response systems: Sox and Rob, or in *Salmonella* spp. Ram, which regulate stress-induced regulons which include multidrug efflux. *Salmonella enterica* sv. Typhimurium adapted to quaternary ammonium-based biocide formulations possessed mutations in *ramR*, conferring quinolone, chloramphenicol and tetracycline resistance (Webber et al., 2015). Multidrug resistance was abolished in the presence of efflux inhibitors and Δ*acrB* and Δ*tolC* mutants demonstrated significantly increased susceptibility to biocides (Whitehead et al., 2011). SoxS is involved in resolution of superoxide and nitric oxide stress and inducers include paraquat and organic solvents and organomercury which results in de-repression of multidrug efflux (Fuentes and Amabile-Cuevas, 1997, LaVoie and Summers, 2018, Nakajima et al., 1995b). Overexpression of *soxS* has previously been shown to induce triclosan resistance by a mechanism of AcrAB-TolC de-repression, constitutive efflux mutants have been selected through *soxR* mutation (Lu et al., 2018a, McMurry et al., 1998). Paraquat can induce the SoxS regulon in *P. aeruginosa*, which leads to overexpression of MexGHI-OpmD, involved in export of β-lactams, aminoglycosides, quinolones, chloramphenicol and rifampicin (Aendekerk et al., 2005, Palma et al., 2005). Similarly, the herbicide dicamba has also been shown to de-repress multidrug efflux in *E. coli* and *Salmonella enterica* sv. Typhimurium in a SoxS-dependent manner (Kurenbach et al., 2015). Rob is a paralogue within the AraC/XylS family but possesses structural and functional dissimilarities from Mar, Ram or Sox (Holden and Webber, 2020). It is constitutively expressed but lacks a repressor, instead regulated by a mechanism by which Rob proteins form an oligomer unable to bind to cognate operators in the absence of an appropriate inducer. It is important in reduced susceptibility to antibiotics, organic solvents and heavy metals (Nakajima et al., 1995a). The pharmaceutical antidepressant fluoxetine is a non-antimicrobial compound which has been shown to de-repress AcrAB-TolC in *E. coli* selected via a mutation in Rob which quinolones, cephalosporins, tetracycline and chloramphenicol. (Jin et al., 2018). Consequently, by virtue of their structural homology to natural efflux substrates, many anthropogenically-derived compounds can possess off-target antimicrobial activity and consequently may

select for cross-resistance to antibiotics via constitutive de-repression of multidrug efflux.

1.4.1.2.2. BaeSR and CpxAR Membrane Stress Response Pathways

The presence of metal compounds such as zinc and copper has been shown to be correlated with an increase in the incidence of multidrug resistant *E. coli* in pig manure (Holzel et al., 2012). Metals are essential trace elements which act as and co-factors in many metabolic processes but are toxic in excess, so mechanisms to regulate them are extensive. Metal chelation proteins and metal-dependent riboswitches play a role in detoxification in addition to a diverse array of transporters (Lee et al., 2005). Membrane transport systems including ZnuABC, which regulates zinc uptake in *E. coli*, or metal-specific efflux pumps such as CusCFBA, a copper ion exporter, are some of the most well characterised (Chandrangsu et al., 2017). Less well studied however, are multidrug transporters with broad substrate specificities and include other types of antimicrobials, including antibiotics.

It is known that some multidrug efflux pumps can be induced by exposure to metals. In *P. aeruginosa*, MexGHI-OpmD confers adaptive resistance to vanadium (Aendekerk et al., 2002). *Salmonella enterica* sv. Typhimurium exposed to 2 mM copper or 1 mM zinc in a laboratory setting demonstrated an increase in expression of *mdtA* and *acrD* (Nishino et al., 2007). MdtABC forms a tetrapartite complex with TolC capable of extruding β-lactams and bile salts in which both MdtBC act as pump transporters (Nishino et al., 2010). AcrD is an aminoglycoside efflux pump which complexes with AcrA and TolC as it does not possess a dedicated membrane fusion protein (Yamasaki et al., 2011). When overexpression is induced in stress conditions, it destabilises unsaturated AcrAB-TolC complexes and exchanges itself with AcrB to form AcrAD-TolC (Yamamoto et al., 2016). Both AcrD and MdtABC do not possess native repressors, they are constitutively expressed at low levels and overexpressed when an activator binds to a site in the promoter region stimulating transcription (Baranova and Nikaido, 2002, Nagakubo et al., 2002). These pumps are members of the RND family of efflux pumps, and both are well described antibiotic transporters. It is unlikely that copper or zinc acts directly on pump repressors given the ubiquitous presence of metals in cells for maintaining metabolic processes. Screening for determinants of metal-induced *mdtABC* overexpression revealed that de-repression could be induced by either BaeR or NlpE, two regulators involved in resolving membrane stress (Nishino et al., 2010) (Figure 1.7.).

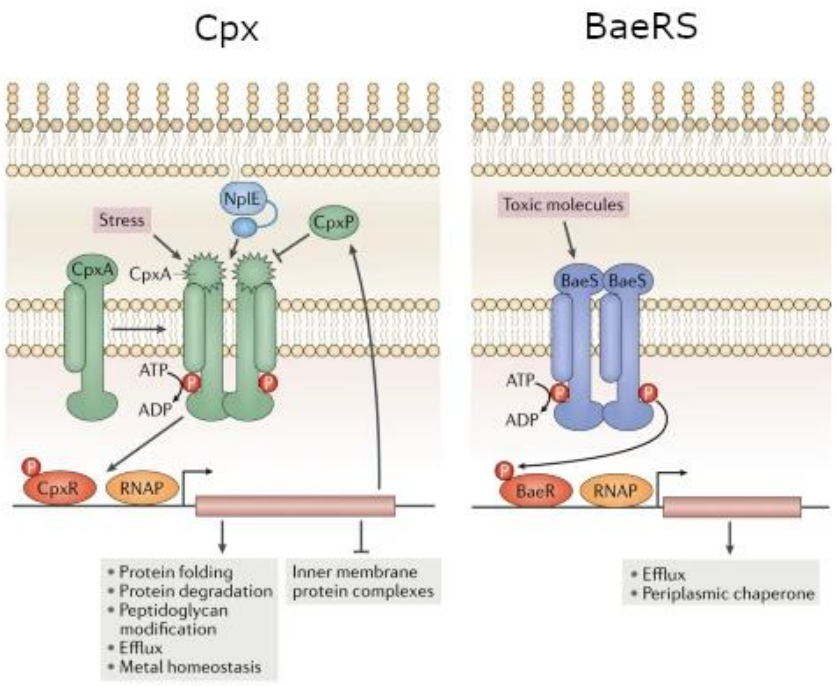

Figure 1.7. The Cpx and BaeRS two-component systems are important regulators involved in the membrane stress response of bacteria. The histidine kinases CpxA and BaeS detect cues associated with membrane perturbations such as altered phospholipid fluidity, misfolded envelope proteins, disrupted periplasmic redox and membrane-damaging antimicrobials. This leads to phosphorylation of the response regulators CpxR and BaeR permitting expression of their respective regulons which are largely involved in restoring membrane integrity by re-establishing envelope homeostasis. This includes expression of the multidrug efflux pumps AcrCD-TolC and MdtABC-TolC and repression of porins, in addition to upregulation of periplasmic lipoprotein homeostasis machinery including molecular chaperones and proteolytic enzymes. Therefore, by limiting the intracellular accumulation of envelope-damaging agents and diminishing the associated downstream impairment of envelope maintenance, the Cpx and BaeRS two-component systems ameliorate cellular damage during acute membrane stress. When envelope damage subsides, CpxA and BaeS stop phosphorylating their respective response regulators, once again repressing their regulons to return expression of envelope homeostasis machinery to its baseline level. Adapted from Mitchell and Silhavy (2019).

BaeR is a response regulator in a two-component system with BaeS which senses a variety of membrane-modulating chemicals including metals, acids and indole (Appia-Ayme et al., 2011). The regulon controlled by BaeRS is small, comprising just the spheroplast formation protein, *spy,* and the aforementioned efflux pumps, one of which it shares the *mdtABCD-baeRS* operon with. Not all membrane-damaging agents induce signal transduction of BaeRS, indicating that it is likely not responding to gross signals of membrane disruption (Leblanc et al., 2011). Although the specific cue of BaeSR activation is not known, it has been hypothesised that an oxidation product generated directly or liberated from the membrane in the presence of inducing compounds may be responsible. This would account for a narrow substrate specificity

that BaeSR possesses, relative to other membrane stress responses. When BaeR is phosphylated by an activated BaeS in the presence of an inducer, it is able to act on binding sites within the *acrD* and *mdtABCD* promoter regions to induce overexpression of multidrug efflux (Nishino et al., 2007).

The other detected regulator was NlpE, an outer membrane lipoprotein which senses adhesion to hydrophobic surfaces and disruption of periplasmic redox (Nishino et al., 2010). It is involved in activation of the CpxAR membrane stress response which detects misfolded proteins in the cell envelope. Conformational changes in NlpE induced by substrate binding appears to directly interact with CpxA in the inner membrane when the cell adheres to a surface (Vogt and Raivio, 2012). However, metals have not been shown to bind NlpE and its role in responding to metal stress is not fully understood. Despite this, mislocalisation of NlpE has also been shown to activate CpxAR, even in the absence of a known substrate (Hews et al., 2019). This indicates that NlpE may have a secondary, indirect interaction with CpxAR in regulating the stress response. Through an unknown mechanism, NlpE is necessary for operation of DsbAB, a two component system regulating disulphide bond formation in periplasmic proteins (Delhaye et al., 2019). Lipoprotein trafficking to the outer membrane is disrupted during membrane stress, therefore mistrafficked NlpE may cause oversaturation of the DsbAB folding machinery. An abundance of misfolded proteins in the periplasm is a cue for CpxA activation, thus activating the stress response (Hirano et al., 2007).

The Cpx stress response regulates pili, fimbrial and flagellar biogenesis, peptidoglycan synthesis and proteolysis at the inner membrane (Raivio, 2014). CpxAR also plays an important role in resistance to chemical stresses by upregulating expression of multidrug efflux systems and negatively regulating expression of outer membrane porins (Kenyon et al., 2002). Mutations at the *cpx* locus conferring aminoglycoside resistance have been observed for decades (Bryan and Van Den Elzen, 1977). Originally believed to interfere with the self-promoted mechanism of aminoglycoside uptake, it is now known that de-repression of AcrD is the principal mechanism of resistance. Moreover, constitutively activated CpxR confers broad spectrum β-lactam resistance in *E. coli* by de-repressing expression of MdtABC (Hirakawa et al., 2003a, Hirakawa et al., 2003b). Like BaeR, when CpxR is phosphorylated, it is able to act directly on binding sites within the *acrD* and *mdtABCD* promoter regions to induce overexpression of multidrug efflux to detoxify the cell (Hirakawa et al., 2005).

Inactivation of BaeSR or NlpE abolished induction of AcrD or MdtABC by either copper or zinc (Nishino et al., 2007, Nishino et al., 2010). Moreover, when either pump was

inactivated, susceptibility to either metal increased indicating that the pumps played an active role in metal detoxification. The mechanism of metal-mediated induction of multidrug efflux may relate to membrane perturbations induced by metals at subinhibitory concentrations. Approaching inhibitory concentrations, metals demonstrate a nonspecific mechanism of action disrupting most macromolecular processes (Espírito Santo et al., 2011). Of these, interrupting envelope integrity is likely to be one the more bactericidal activities. De-repression multidrug efflux cascading from the activation of BaeS or NlpE would act to expel the metal and detoxify the cell, which would also confer transient resistance to other pump substrates, such as the antibiotics observed by Nishino et al. (2007).

1.4.1.2.3. RpoE and AlgU Alternative Sigma Factors

Sigma factors (σ-factors) are bacterial transcription factors which mediate the binding of RNA polymerase to promoter regions and play a broad role in global gene regulation. Housekeeping σ-factors, such as RpoD, regulate most essential transcription during exponential growth (Potvin et al., 2008). Others, including most σ-factors with extracytoplasmic function, are induced upon exposure to specific challenges to express a regulon which often contains genes involved in stress amelioration. The archetypal regulator of the membrane stress response is the broadly conserved alternative σ-factor, RpoE (figure 1.8.) (Rhodius et al., 2005). It is involved in homeostasis of lipopolysaccharides, trafficking of outer membrane proteins and regulation of transcriptional circuitry, including other σ-factors with their own downstream regulons and antisense RNAs (Shetty et al., 2019).

It is believed that RpoE is induced by the interrupted assembly or misfolding of outer membrane proteins associated with loss of membrane integrity (Rhodius et al., 2005). Noteworthy chemical inducers of the RpoE-dependent stress response include metal ions, cationic surfactants, antimicrobial peptides and ethanol (Crouch et al., 2005, Egler et al., 2005, Haines-Menges et al., 2014). In unstressed conditions, RpoE is expressed but quiescent through sequestration by its inner membrane-bound antisigma factors RseAB. Mistranslocated outer membrane proteins accumulating in the periplasm induce conformational changes in the proteolytic enzymes DegS and RseP at the inner membrane. Sequential cleavage by DegS and RseP, in coordination with the cytosolic proteases ClpXP and Lon, fully liberates RpoE from RseAB. RpoE is then able to form a holoenzyme with RNA polymerase permitting access to cognate promoter regions, thus inducing the regulon.

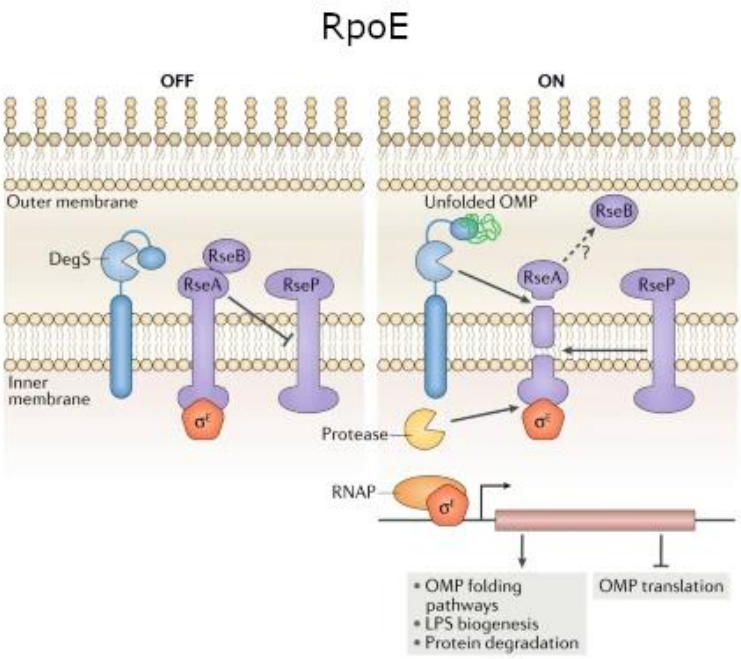

Figure 1.8. The RpoE alternative σ-factor is a widely conserved component of the membrane stress response involved in trafficking proteins to the outer membrane, lipopolysaccharide homeostasis and regulating the expression of other σ-factors. It is activated by the misfolding of outer membrane proteins in the periplasm associated with membrane disruption. This activates proteolytic enzymes to liberate RpoE rendered quiescent via binding to its antisigma factor RseA. RpoE forms a holoenzyme with RNA polymerase which permits access to cognate promoter regions and induces expression of the RpoE regulon. The RpoE-dependent stress response is characterised by increases expression of membrane-stabilising lipoproteins in addition to chaperones and proteases which facilitate translocation of outer membrane proteins through the periplasmic space. Moreover, through expression of the antisense RNAs MicA and RybB, RpoE is also able to negatively regulate expression of the major porins OmpA and OmpC at a post-translational level. In addition to limiting uptake of antimicrobials, this also helps resolves the accumulation of misfolded outer membrane proteins in the periplasm which contribute to the gross failure of envelope homeostasis associated with exposure to membrane-damaging agents. Adapted from Mitchell and Silhavy (2019).

Much of the downstream effects of RpoE activation are involved in resolution of membrane damage. Expression of a complement of periplasmic chaperones, folding apparatus, and proteases promote the correct translocation of outer membrane proteins in a milieu of acute membrane stress (Rowley et al., 2006). Similarly, the RpoE-dependent stress response also upregulates expression of a repertoire of genes involved in phospholipid and lipopolysaccharide biogenesis and stabilisation. Moreover, the small regulatory RNAs (sRNAs) MicA, MicL and RybB are expressed as a mechanism to self-regulate the RpoE-dependent stress response. By selectively downregulating cognate transcripts encoding outer membrane proteins and

lipoproteins, the accumulation of cascade-activating inducers is limited, preventing runaway expression of the stress response.

Many of the regulatory components of σ-factor-dependent stress responses have downstream collateral effects on other physiological processes. The mechanisms by which σ-factors modulate expression of pathogenicity islands and virulence factors have been well described (Hews et al., 2019, Rowley et al., 2006). Activation of σ-factors can also influence susceptibility to antimicrobials (Felden and Cattoir, 2018). Given the role of outer membrane proteins in activating the RpoE-dependent stress response, the sRNAs MicA and RybB have broad post-transcriptional repression of porins (Vogel and Papenfort, 2006). RpoE has been implicated in metal tolerance through downregulation of OmpA and OmpC in *E. coli*, and by the same mechanism, RpoE can induce transient resistance to β-lactams in *Salmonella enterica* sv. Typhi (Egler et al., 2005, Xie et al., 2016). Moreover, through the overexpression of envelope structural components and maintenance apparatus, the RpoE-dependent stress response can confer reduced susceptibility to membrane-active agents, including chlorhexidine and polymyxins (Kenyon et al., 2002, Loutet et al., 2011, Vidovic et al., 2018). Furthermore, expression RpoE is co-regulated interdependently with other σ-factors including RpoS which can be induced by biocides including triclosan, metals and cationic surfactants (Gantzhorn et al., 2015).

The RpoE homologue in *P. aeruginosa* is AlgU, a σ-factor characterised by the distinct mucoid phenotype it induces through overexpression of the alginate exopolysaccharide biosynthesis gene *algD* (Fraud et al., 2008). The activation cascade of AlgU shares strong homology with RpoE (Damron and Goldberg, 2012). MucAB act as antisigma factors, cleaved from the membrane by AlgW and MucP, homologous to DegS and RseP, and then again by the conserved cytosolic protease ClpXP (Qiu et al., 2007, Qiu et al., 2008). Like RpoE, AlgU plays an important role in resolving membrane damage through regulation of structural lipoproteins, an array of outer membrane proteins including *oprF* and the membrane fluidity-modulating osmotic shock regulators *osmC* and *opuC* (Firoved and Deretic, 2003). Notably, activation of AlgU results in extensive downregulation of protein synthesis regulators. Furthermore, AlgU has also been shown to have a direct interaction with the multidrug efflux pump MexCD-OprJ (Fraud et al., 2008). Bisbiguanides such as chlorhexidine can induce expression of MexCD-OprJ in wild-type *P. aeruginosa* PAO1 (Morita et al., 2003). Moreover, in the presence of chlorhexidine, Δ*algU* mutants demonstrate repressed MexCD-OprJ expression, a phenotype which is preserved even when NfxB, the local repressor of *mexCD-oprJ,* was inactivated. Furthermore, transcriptional de-repression of MexCD-OprJ was

restored in the Δ*nfxB* mutants upon complementation of AlgU in the Δ*nfxB* mutants, indicating a direct role for AlgU in inducing MexCD-OprJ expression. These observations of AlgU-dependent de-repression of MexCD-OprJ of have been extended to other membrane-active agents including: polymyxin B, sodium dodecyl sulfate, ethanol and antimicrobial peptides. Chlorhexidine-mediated de-repression of MexCD-OprJ was also associated with modest reductions in susceptibility to quinolones and macrolides.

The transcriptional regulator of the AlgD-dependent mucoidal phenotype is AlgR, a member of the AlgU regulon (Martin et al., 1994). AlgR is in a two-component system with FimS, a sensor kinase controlling fimbrial biosynthesis, through which the AlgU-dependent stress response coordinately regulates both mucoidy and twitching motility (Whitchurch et al., 1996). AlgU consequently possesses an extended regulon via the downstream activity of the AlgR-FimS operon. In addition to alginate production and fimbrial biosynthesis, AlgR regulates the multidrug efflux pump MexEF-OprN, a pump with known roles in resistance to fluoroquinolones, chloramphenicol and trimethoprim (Kohler et al., 1997). Ergo, through AlgU-mediated expression of AlgR, multidrug efflux can be induced to confer transient resistance to antibiotics. Supporting this, the induction of AlgR-dependent mucoidy by antibiotics, metals and biological surfactants has been well described (Bagge et al., 2004, LaBauve and Wargo, 2014, Teitzel et al., 2006, Wood et al., 2006).

1.4.2. Envelope Barrier Functions

In addition to upregulating export mechanisms to prevent antimicrobial accumulation, inhibition of transmembrane import also confers reduced susceptibility to antimicrobial agents (May and Grabowicz, 2018). This can be achieved via several mechanisms involving the downregulation of low-selectivity membrane transporters or altering envelope architecture to reduce the electrostatic attraction between the cell and antimicrobial. As many antimicrobials share conserved import pathways, such mechanisms can alter susceptibility to a broad range of antimicrobials, most notably carbapenems and cationic surfactants (Gogry et al., 2021, Meletis, 2016). Moreover, altered transmembrane import is often selected alongside de-repression of multidrug efflux as functionally independent mechanisms which act synergistically to limit intracellular concentrations of antimicrobials (Nicolas-Chanoine et al., 2018).

1.4.2.1. Downregulation of Transmembrane Importers

The hydrophobic bacterial envelope acts as a permeability barrier which regulates transport of compounds into cells through membrane-spanning protein channels. β-barrelled outer membrane proteins known as porins form water-filled pores in the outer membrane which control the uptake of hydrophilic solutes in a charge- and size-dependent manner (Ghai and Ghai, 2017). Some porins have narrow ligand specificities such as the maltoporin LamB or the nucleoside channel Tsx in *E. coli*. Others, such as OmpC/F, are constitutively expressed generic importers which possess broad substrate repertoires (Prajapati et al., 2021). Such porins are the primary determinants for entry of hydrophilic antimicrobials into the cell, including β-lactams, tetracyclines, chloramphenicol, fluoroquinolones and metal cations (Ghai and Ghai, 2018). Consequently, porin expression is under complex regulatory control by stress-sensing two-component systems, antisense RNAs and global transcriptional repressors. The primary regulator of OmpC/F is EnvZ-OmpR which is activated at high osmolarity to increase transcription of OmpC and repress OmpF (Pratt et al., 1996). OmpF possesses a larger porin eyelet aperture and thus possesses a higher import capacity which is beneficial in low osmolarity environments, whereas solute-rich environments favour OmpC which mitigates entry of toxic compounds. Differential expression of OmpC and OmpF is characteristic of most porin regulators, including the antisense RNA *micF* and the regulatory proteins H-NS, RpoE and CpxAR (Vergalli et al., 2020). Via this complex regulatory network, numerous extracellular signals can manipulate porin expression including temperature, nutrient poverty, pH, ROS and growth rate. Moreover, porin genes exist within the regulons of the global stress responses Mar, Rob and Sox, providing a direct mechanism through which antimicrobials can also modulate porin expression (Chubiz and Rao, 2011).

Altered porin expression possesses pleiotropic effects on antimicrobial susceptibility by virtue of the broad reliance on porins for translocation of molecules into cells (Fernández and Hancock, 2012). Consequently, porin loss is associated with modest reductions in susceptibility to a variety of antimicrobials including antibiotics, biocides and heavy metals. Exposure of *Salmonella enterica* sv. Typhi to cadmium has been observed to reduce susceptibility to β-lactams, ciprofloxacin and chloramphenicol and was associated with broad downregulation of outer membrane proteins, although specific determinants were not identified (Kaur et al., 2018). Similarly, vanadium has been observed to induce reductions in susceptibility to erythromycin, tetracycline and fluoroquinolones in *Enterobacter hermannii* via downregulation of two outer membrane proteins termed Omp43 and Omp48 (Hernandez et al., 1998). Moreover, arsenic ions

induced reductions in susceptibility to aminoglycosides, tetracycline and ciprofloxacin by downregulating OmpA expression in *Yersinia enterocolitica* (Mallik et al., 2012).

Unlike *Enterobacteriaceae*, *P. aeruginosa* does not possess general diffusion porins; instead, transmembrane import is controlled by 40 known porin channels with distinct substrate repertoires and low molecular size limits (Ude et al., 2021). Each porin often shows specificity for a primary ligand, and other substrates are transported by virtue of possessing a similar chemical moiety. This strategy contributes to the relative impermeability of *Pseudomonas* spp. compared to other Gram-negative species. The most significant porin involved in antibiotic transport is OprD, the importer of basic amino acids arginine, histidine, lysine and ornithine, however it is also able to translocate imipenem into the cell (Chevalier et al., 2017). The transcriptional activator ArgR increases expression of OprD in the presence of arginine to capitalise on nutrient availability. OprD is also under positive regulatory control from the SigX membrane stress response, FliA flagellar biosynthesis and RpoN nitrogen limitation σ-factors. Moreover, OprD is coregulated with the cobalt-zinc-cadmium efflux pump CzcCBA via heavy metal-sensing two-component system CzcRS (Perron et al., 2004). Activation of the transcriptional regulator CzcR results in repression of *oprD* and zinc eluate was observed to transiently induce imipenem resistance in *P. aeruginosa* isolates from silicon-latex urinary catheters (Conejo et al., 2003, Dieppois et al., 2012, Martínez-Martínez et al., 1999). Exposure to inhibitory concentrations of zinc selected for mutants with constitutive imipenem resistance via missense mutations in CzcS which constitutively activated CzcR. Similarly, adaptation to copper was also associated with imipenem resistance via downregulation of OprD and upregulation of CzcCBA via the action of the copper-sensing CopRS two-component system (Teitzel et al., 2006). CopRS controls the CopABCD copper transporter however, Caille et al. (2007) demonstrated that overexpression of CopR also resulted in de-repression of CzcCBA due to promoter homology between the two metal transporter operons. Copper was not as strong of an effector as zinc on OprD, however it was still able to induce an imipenem-resistant phenotype. The role of OprD repression during metal stress is not understood, however a second mechanism co-regulating CzcCBA and OprD expression has been identified, indicating that a selective advantage likely exists (Ducret et al., 2016). The RNA-binding protein Hfq is a pleiotropic regulator involved in resolving membrane stress, carbon catabolite repression and oxygen tension through activation of small non-coding RNAs. Hfq possesses a documented role in regulation of OprD inducible by carbapenems whereby the antisense RNAs EsrA and Sr0161 associate with Hfq allowing them to sequester OprD mRNA transcripts. Abatement of

stress results in expression of the small RNA CrcZ which titrates Hfq, thus relieving translational control of OprD (Sonnleitner et al., 2020). Moreover, this mechanism was also inducible by exposure to zinc or copper exerting post-transcriptional repression of OprD independently from CzcRS or CopRS signal transduction systems.

1.4.2.2. Cell Surface Charge Modification

The bacterial envelope possesses a net negative charge due to a carboxyl and amino group-rich peptidoglycan layer, anionic membrane phosphatidylglycerols and LPS in Gram-negative or wall teichoic acids in Gram-positive organisms (Silhavy et al., 2010). Cationic membrane-active antimicrobials such as polymyxins, lipopeptides or QACs exploit envelope potential to facilitate binding and ionic dissociation of membrane integrity. As this interaction is facilitated by electrostatic forces, the strength of association is dipole-dependent which can be manipulated by cells to mitigate antimicrobial damage. Mechanisms to reduce the net negative charge at the envelope, and thus the attractive force between the cell and antimicrobial cations, act primarily on the lipid A component of LPS. *P. aeruginosa* and most *Enterobacteriaceae* possess two major regulatory systems involved in lipid A fatty acid modification, PhoPQ and PmrAB, through which the cell responds to perturbations in envelope potential (figure 1.9.) (Nuri et al., 2015). PhoPQ is a prototypical two-component system which senses low pH, scarcity of divalent magnesium cations and cationic antimicrobials. Similarly, PmrAB is also a two-component system, activated by low pH, excess of iron and aluminium cations and cationic antimicrobials. The activation of these systems results in transcription of the *pmrFHIJKLM* operon which synthesises the cationic sugar 4-amino-4-deoxy-L-arabinose and catalyses its transfer to phosphate groups on nascent lipid A molecules (Aghapour et al., 2019). In *Enterobacteriaceae,* the response regulator PmrA can also be activated independently of the sensor kinase PmrB by the alternative signal transducer PmrD which is activated by PhoPQ (Charles et al., 2009). Consequently, PhoPQ can activate expression of the *pmrFHIJKLM* operon indirectly. *P. aeruginosa* lacks a known PmrD homologue, however *pmrFHIJKLM* still appears to be under regulatory control from PhoPQ, and thus able to elicit lipid A modifications through an unknown mechanism (Miller et al., 2011, Moskowitz et al., 2004). With an increase in the number of cationic moieties decorating the LPS, the negative charge on the cell envelope is diminished, limiting the electrostatic attraction between the cell and positively charged molecules.

Lipid A modifications are the most clinically-relevant mechanism of chromosomally-encoded resistance to colistin in Gram-negative bacteria (Gogry et al., 2021). Moreover, alterations in envelope fatty acids and increased membrane isoelectric potential have been broadly observed in Gram-negative bacteria selected by cationic surfactants (Bruinsma et al., 2006, Guerin-Mechin et al., 1999, Guerin-Mechin et al., 2000). Adaptation of *P. aeruginosa* to the QACs didecyldimethylammonium bromide and benzyldimethyltetradecylammonium chloride was associated with increased β-hydroxylated fatty acids and a decrease in palmitic acid (Mechin et al., 1999). Moreover, changes in phospholipid composition selected by tetradecyltrimethylammonium chloride exposure in *P. putida* which resulted in an increase in phosphatidic acid and phosphatidylglycerol content and a decrease in cardiolipin (Boeris et al., 2007). Such changes in fatty acid composition have also been selected by benzalkonium chloride which was associated with a reduction in cell envelope electrostatic negativity and cross-resistance to polymyxin B (Loughlin et al., 2002).

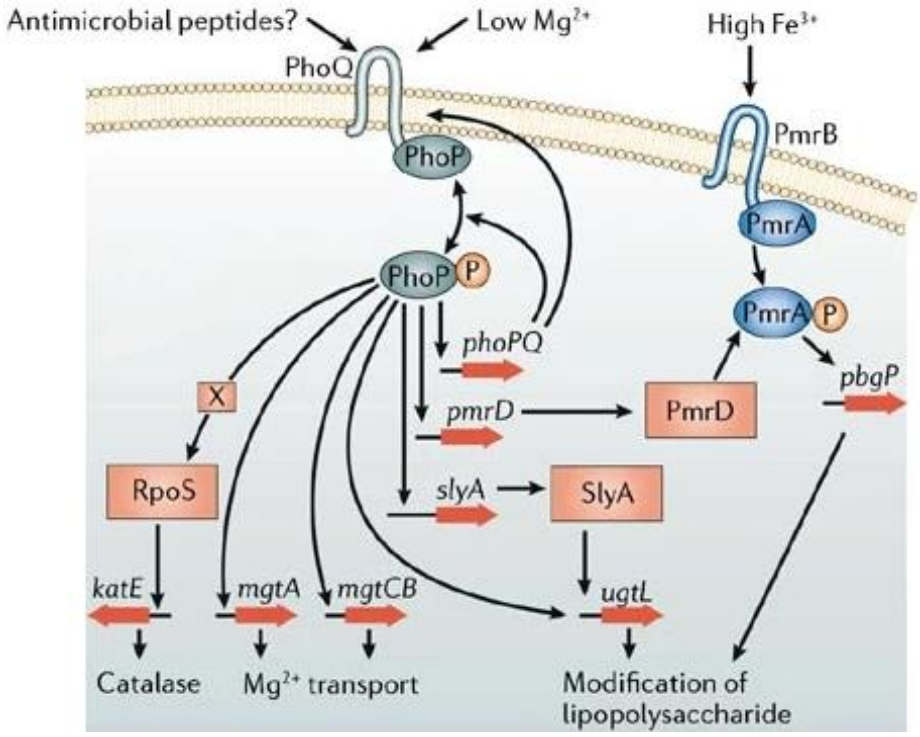

Figure 1.9. Regulation of the PhoPQ and PmrAB lipid A-modifying two-component systems. Adapted from Groisman and Mouslim (2006)

Mutants of *P. aeruginosa* resistant to polymyxins have been isolated from experimentally-controlled aerobic bioreactors supplemented with benzalkonium chloride which demonstrated mutations in *pmrB* (Kim et al., 2018b). Notably, all unique

pmrB substitutions were able to confer decreased susceptibility to benzalkonium chloride, but not all yielded cross-resistance to polymyxin B (Kim et al., 2018a). This indicates that the targets of selection involved in decreased benzalkonium chloride susceptibility and cross-resistance to polymyxins may not be the same. The main effector through which PmrAB elicits decreased susceptibility to cationic peptides and surfactants is the lipid A-modifying PmrFHIJKLM (Charles et al., 2009). However, other members of the PmrAB regulon have been observed to play roles in envelope charge homeostasis in *Enterobacteriaceae*, most notably PmrC which catalyses the incorporation of phosphoethanolamine into lipid A (Steinbuch and Fridman, 2016). The PmrC homologue in *P. aeruginosa* EptAs, has only recently been described (Freire et al., 2021). Therefore, it is possible that the decreased susceptibility to polymyxin B and benzalkonium chloride may be regulated by different lipid-A modifying members of the PmrAB regulon. Various PmrB alleles may exert different strengths of PmrA activation which elicits differential expression of the PmrAB regulon. Similarly, adaptation of *K. pneumoniae* to chlorhexidine digluconate was able to select for cross-resistance to colistin (Zhang et al., 2019b). Wand et al. (2017) identified two mutational targets exhibiting evolutionary parallelism, *smvR*, the transcriptional repressor of the MFS efflux pump SmvA, and *phoPQ*. SmvA was de-repressed in *smvR* mutants and contributed to reduced susceptibility to chlorhexidine but did not appear to play a role in colistin cross-resistance. Mutations in *phoPQ*, however, were able to confer reduced susceptibility to both chlorhexidine and colistin and were associated with upregulation of the PmrB-independent PrmA activator *pmrD*, and the lipid A modifier *pmrFHIJKLM*. The axis of cross-resistance underpinning the selective relationship between chlorhexidine and colistin was unidirectional. Adaptation of *K. pneumoniae* to colistin was no able to select for cross-resistance to chlorhexidine despite *phoPQ* mutants being selected in abundance. This further indicates that cationic surfactants and polymyxins have separate effectors in the envelope charge homeostasis network.

1.4.3. Efflux-Independent Stress Reponses

Stress responses which do not involve membrane transport have also been implicated in cross-resistance between non-therapeutic antimicrobials and antibiotics. The collateral implications of such mechanisms are generally a result of downstream effects of stress response activation on other physiological properties which are under co-regulation (Webber et al., 2017). This is in contrast to envelope-acting mechanisms which possess polygamous action on multiple antimicrobials directly. Consequently, such mechanisms are generally selectively unidirectional in that one agent may confer

cross-resistance via activation of a stress response, but the other agent cannot (Gantzhorn et al., 2015).

1.4.3.1. Interruption of Macromolecular Syntheses

A significant mechanism of chromosomally-encoded resistance to antibiotics is target alteration, characterised by point mutations at sites of drug binding. This mechanism is however dependent on the antimicrobial agent possessing a specific mode of action through a single molecular target which facilitates selective toxicity. The fact that most non-therapeutic antimicrobials inhibit multiple targets makes single target mutations that impair molecular binding ineffective and simultaneous mutation of multiple targets is statistically infeasible (Russell, 2004). Despite this, sufficient perturbations of macromolecular syntheses, such as impaired protein synthesis or DNA replication can have far-reaching physiological consequences including metabolic slowdown and activation of stress responses (Webber et al., 2017). Consequently, mutations affecting essential cellular processes can confer increased fitness to mechanistically-independent antimicrobials by virtue of their growth-dependent modes of action.

Quinolone-resistant *gyrA* DNA gyrase mutants of *E. coli* and *Salmonella* sv. Typhimurium have been shown to possess decreased triclosan susceptibility (Webber et al., 2013). This is despite triclosan operating as a fatty acid inhibitor, functionally independent from the action of DNA gyrase as a mediator of negative DNA supercoiling (Webber et al., 2017). Moreover, whilst ciprofloxacin susceptibility decreased proportionally with supercoiling capacity in mutants with different amino acid substitutions in GyrA, reduced triclosan susceptibility was independent of DNA supercoiling. It was further revealed that the severe supercoiling defect *gyrA* mutants possessed significantly upregulated expression of a number of stress responses. In *E. coli*, the SOS stress response, RpoD, RpoH and RpoN σ-factors were upregulated and in *Salmonella*, RpoS and RpoN was expressed. Such stress responses have documented roles in resistance to triclosan (Bailey et al., 2009, Gantzhorn et al., 2015). Whilst mutants conferring reduced triclosan susceptibility appear rapidly under selection from fluoroquinolones, selection under triclosan stress has thus far, been able to recapitulate the *gyrA*-mediated cross resistance. This may be due to other mechanisms which select for greater degrees of altered susceptibility with milder fitness defects, such as *fabI* mutations or constitutive de-repression of efflux.

Similarly, benzalkonium chloride-resistant mutants of *A. baumannii* demonstrated reduced susceptibility to ciprofloxacin through mutations in ribosomal subunit proteins

(Knauf et al., 2018). Neither QACs nor ciprofloxacin are ribosome-acting translation inhibitors. However, low-level ciprofloxacin resistance has been observed to be selected through a mutation in the 30S ribosomal subunit gene *rpsK* which slowed growth rates in *E. coli* (Ching and Zaman, 2020). Moreover, benzalkonium chloride can possess a narrowed target specificity at sub-bactericidal concentrations, preferentially causing cytosolic poisoning through the coagulation of proteins. Consequently, proteostasis mechanisms are broadly upregulated and experience positive selection under subinhibitory QAC stress (Casado Muñoz et al., 2016, Mangalappalli-Illathu and Korber, 2006, Moen et al., 2012). Mutations in the ribosomal proteins L23 and L24 of *A. baumannii* were hypothesised to protect against the aggregative action of benzalkonium chloride by increasing polypeptide-chaperone interactions. Moreover, the impact these mutations had on ciprofloxacin susceptibility was independent of growth rate. How ribosomal subunit mutations were able to select for altered ciprofloxacin susceptibility without inducing a constitutively tolerant phenotype remains unknown.

One of the most common phenotypic manifestations of constitutively interrupted macromolecular synthesis is the formation of small colony variants (SCVs) on solid culture media. SCVs are characterised by slowed growth rates mediated by specific deficiencies in the electron transport chain, including impaired thymidine, haemin and menadione biosynthesis (Proctor et al., 2006). SCVs can be constitutively produced through mutations in Kreb's cycle enzyme biosynthetic pathways such as *menD, hemB* and *ctaA* and often yield auxotrophic mutants. In Gram-positive species, SCVs selected by triclosan which possess cross-resistance to antibiotics have been commonly observed (Bayston et al., 2007, Latimer et al., 2012). SCVs of *S. aureus* and *L. monocytogenes* selected by serial passage under subinhibitory triclosan stress demonstrated decreased susceptibility to aminoglycosides (Christensen et al., 2011, Seaman et al., 2007). *L. monocytogenes* SCVs possessed broadly altered carbon utilisation, loss of haemolysin and catalase activity in addition to mutations in ATP synthases *atpA/D* and *fusA* elongation factor (Kastbjerg et al., 2014). The mechanism responsible for the transition to the SCV morphotype appeared to be specifically regulated by the heme biosynthesis genes *hemA/H*, as addition of hemin restored the ancestral colony morphology. This did not impact aminoglycoside susceptibility however, and tolerance associated with interrupted macromolecular synthesis would not be expected to confer reduced susceptibility to a single class of antibiotics. Consequently, it was hypothesised that respiratory arrest in SCVs may specifically

interfere with the ATP-dependent self-promoted uptake mechanism of aminoglycosides into cells.

SCVs are characterised by non-lethal respiratory depression which elicits a reduction in gross macromolecular activity, conferring reduced susceptibility to antimicrobials which exhibit growth rate-dependent activity (Proctor et al., 2006). The SCV phenotype is generally constitutively expressed, due to rate-limiting mutations in respiratory enzymes. However, other mechanisms to circumvent antimicrobial stress exist, including entry into a viable-but-nonculturable (VBNC) state. VBNC cells exist on the spectrum of tolerant phenotypes which also includes SCVs and persister cells. The exact nature of VBNC and their distinction from persisters, if any, remains a contentious issue (Song and Wood, 2021). Like most tolerant phenotypes, the VBNC state is characterised by the loss of culturability, arrest of aerobic respiration and increased tolerance to environmental stress (Zhao et al., 2017). Unlike SCVs, VBNC cells are induced in a subset of the population which quiescently persist through unfavourable environmental conditions and resume normal metabolic activity when the stress subsides. As the tolerant phenotype elicited is mediated by metabolic quiescence, induction confers decreased susceptibility to a broad range of antimicrobial insults including nutrient poverty, oxygen tension, pH, temperature, desiccation, biocides and antibiotics (Li et al., 2014). Sodium hypochlorite and benzalkonium chloride have been shown to induce a VBNC states in *Listeria monocytogenes* which increased cell viability after exposure to inhibitory concentrations of antibiotics (Highmore et al., 2018, Lin et al., 2017, Noll et al., 2020). Similarly, a VBNC state in *Vibrio vulnificus* was able to reduce susceptibility to antibiotics, ethanol and zinc, in addition to gross environmental insults including heat, pH, oxidative and osmotic stress (Nowakowska and Oliver, 2013).

The mechanisms which regulate persistent phenotypes including the VBNC state are not well understood, however the stringent response appears to play an important role. The stringent response is regulated by the alarmones guanosine tetraphosphate and guanosine pentaphosphate ((p)ppGpp). In *E. coli*, (p)ppGpp is synthesised by the pyrophosphatase RelA and bifunctional alarmone synthase/ hydrolase SpoT, activated by ribosome-bound deacetylated tRNA indicative of cellular stress (Agirrezabala et al., 2013). (p)ppGpp is a global transcriptional regulator which binds RNA polymerase to exert broad activity downregulating energy-intensive cellular processes including DNA replication, nucleotide biosynthesis, protein synthesis and lipid metabolism (Irving et al., 2021). Notably, one of the few pathways positively regulated by the stringent response is amino acid synthesis which results in the re-acetylation of tRNA molecules

and deactivation of RelA (Chatterji and Kumar, 2001). Intracellular concentrations of (p)ppGpp then return to cellular baseline as SpoT hydrolyses (p)ppGpp to GTP, and normal macromolecular functions resume. How the stringent response specifically induces VBNC cells remains unknown. However (p)ppGpp-deficient mutants demonstrated a reduced capacity to form VBNC cells and RelA-dependent VBNC states which possess reduced susceptibility to non-therapeutic antimicrobials and antibiotics have been observed (Ayrapetyan et al., 2018, Westfall et al., 2019).

1.4.3.2. SOS-Induced Horizontal Gene Transfer and Mutagenesis

The mobilisation of MGEs relies on cellular factors which mediate the uptake and transfer of exogenous DNA. Most notably, expression of conjugative machinery and transformation competence are under regulatory control from stress responses which facilitate increases in HGT in unfavourable conditions, presumably as a bet-hedging mechanism. An array of antimicrobials and adverse environmental conditions which interact with such stress responses have been implicated in mobilising MGEs (Pearce et al., 1999, McMahon et al., 2007). Exposure to antibiotics has been extensively observed to increase conjugative transfer and transformation with eDNA (Liu et al., 2019). Many MGEs possess antibiotic resistance determinants and therefore their transmission throughout populations increases fitness of the community under stress. However, in addition to antibiotics, livestock feed adjuvants, ionic solvents, chlorination byproducts, metals and biocides have also been implicated in stress-induced HGT (Guo et al., 2015a, Luo et al., 2014, Rodríguez-Beltrán et al., 2013, Zhang et al., 2017). Though the selective advantage of transformation competence, as a phenotype which exclusively benefits the recipient, is clear, why increased conjugation also came under stress-induced regulatory control is less obvious. Such altruism mainly benefits the community at a detriment to donor competitiveness, however kin-discrimination mechanisms may act as a natural bottleneck, impairing plasmid uptake and limiting fitness gains in individuals with low relatedness. Exposure to stress does not always have a positive regulatory effect on HGT, however. A number of antimicrobials have been observed to suppress conjugative transfer, including sodium dodecyl sulfate, mupirocin, organomercury and ionic metals (al-Masaudi et al., 1991, Buberg et al., 2020). Moreover, in a bacterial community experiencing copper stress, plasmid uptake was impaired, but adaptation of the community to copper restored unstressed conjugation frequencies (Klümper et al., 2017, Klümper et al., 2019).

Colloidal nanoparticles of aluminium, cadmium, copper, silver and titanium oxide elicited a dose-dependent increase in transformation competence and conjugative transfer of a MDR IncP plasmid in *E. coli* (Ding et al., 2016, Qiu et al., 2012, Qiu et al., 2015, Zhang et al., 2019a). Similar observations have also been made after exposure to triclosan, sodium hypochlorite and the anticonvulsant drug carbamazepine (Jin et al., 2020, Lu et al., 2018b, Wang et al., 2019, Zhang et al., 2018a). Exposure to these agents shared a conserved cellular response, the induction of ROS leading to activation of the SOS stress response to resolve acute oxidative stress (Lu et al., 2020). The SOS stress response is kept quiescent in unstressed conditions by the transcriptional repressor LexA. However, the accumulation of single-stranded DNA at replication forks due to double-stranded breaks or genomic lesions of non-canonical bases blocking DNA polymerase progression, activates the DNA recombination protein RecA (Baharoglu and Mazel, 2014). RecA proteolytically cleaves the LexA repressor into an inactive form, permitting the transcriptional cascade of the SOS regulon. Single-stranded DNA is the sole inducer of RecA, and by extension, the SOS response; however, they can be generated by a variety of stress conditions, including the action of ROS. Subsequently, a regulon of over 50 genes with diverse functions including cell division arrest, DNA repair and oxidative stress resolution (Maslowska et al., 2019).

Indeed, nanometal-exposed cells exhibited an increase in antioxidant enzymes including superoxide dismutase SodA, catalase KatB and glutathione reductase Gor. However, SOS-dependent promotion of conjugative mating was dependent on de-repression of the mating-pair formation gene *trbB* and the plasmid replication initiation gene *trfA* (Pu et al., 2020). The transcriptional repressor of the *trb* and *trf* operons is KorB, which was downregulated in SOS-expressing cells. How the SOS response regulates KorB is not understood. However, like *trb* and *trf,* the plasmid transfer *tra* operon also lacks a predicted LexA operator but was observed to be induced by antibiotics in SOS-dependent manner (Beaber et al., 2004). It was revealed that RecA appeared to directly interact with the repressor SetR, allowing expression of the transcriptional activators of *tra, setC/D*. Consequently, the proteolytic activity of RecA may also result in the inactivation of KorB, however this remains hypothetical.

The primary function of the SOS response is the resolution of DNA damage and within the regulon are genes encoding homologous recombination, nucleotide excision repair and translesion synthesis (Baharoglu and Mazel, 2014). The order in which the SOS transcription cascade takes place is controlled by the binding affinity of operator sequences for LexA, with recognition sequences possessing closer homology being expressed later. In this way, the strength of the stress response is be modulated

commensurately to the magnitude of stress. Translesion synthesis is often the last DNA repair mechanism to be mobilised by the SOS response by which DNA polymerases replicate past template errors, resolving the stalled replication fork. The primary mediators of translesion synthesis are the error-prone DNA polymerases DinB (Pol IV) and UmuDC (Pol V) which lack proofreading activity (Goodman, 2002). Consequently, translesion synthesis is associated with a two-orders of magnitude increase in base misincorporation (Patel et al., 2010). Constitutive SOS mutants possess increased mutation frequencies and this process has been suggested to accelerate the adaptive process through growth rate-independent mutagenesis (Torres-Barceló et al., 2015). Supporting this, many of the antimicrobial agents able to induce the SOS response to confer elevated HGT, such as metal nanoparticles and disinfection byproducts also possessed markedly increased mutation frequency (Lv et al., 2015, Zhang et al., 2018b). Notably, mutation frequencies increased in a dose-dependent manner, and the rate of emergence of antibiotic-resistant mutants increased generally linearly as well (Li et al., 2019).

1.5. Aims and Objectives

The contribution of the phenotypic properties of the biofilm to antimicrobial tolerance and persistence in environmental niches is well acknowledged. However, an integrated understanding of the role of the biofilm lifestyle in modulating the evolutionary outcomes of adaptation to antimicrobial selective pressures is less obvious. This work aims to characterise the molecular mechanisms and evolutionary trajectories under selection in biofilms which confer biofilm hyperproduction and reduced susceptibility to clinically-relevant antibiotics in *P. aeruginosa*. A novel experimental evolution platform was optimised for this purpose to select for biofilm hyperproduction and antimicrobial resistance in biofilms. From the evolved lineages, mutant phenotypes were identified and genome sequenced. Firstly, the mechanisms underpinning biofilm hyperproduction and its intrinsic role in altered antimicrobial susceptibility was investigated. It was hypothesised that biofilm hyperproduction would be selected through mutation in the regulatory genes controlling expression of biofilm matrix constituents such as local repressors, type IV pili or the c-di-GMP signalling network. Moreover, it was expected that biofilms which produce more matrix biomass would be less fit in broth and less susceptible to antimicrobial stress. Secondly, I investigated how selection for increased biofilm formation modulates evolutionary trajectories towards acquired antibiotic resistance and the collateral trade-offs which precluded specific mechanisms from being selected in different lifestyles. It was hypothesised that planktonic and biofilm

lifestyles would take different evolutionary trajectories to drug resistance due to disparate selective pressures and repertoires of essential genes. Finally, I explored if the adaptation of biofilms to non-therapeutic antimicrobial agents could collaterally select for reduced susceptibility to antibiotics. It was hypothesised that selection of cross-resistance by non-therapeutic antimicrobials would occur through polygamous mechanisms of resistance such as multidrug efflux, porin loss, envelope fatty acid modification or respiratory depression. Overall, it was expected that the experimental evolution of biofilms would yield novel mechanisms of antimicrobial resistance.

CHAPTER 2. MATERIALS AND METHODS

2.1. Bacterial Strains

Pseudomonas aeruginosa UCBPP-PA14 was employed as a model organism for experimental evolution. *P. aeruginosa* PA14 was originally isolated from a burn wound from a patient at Mercy Hospital, Pennsylvania, USA in the 1970s (Mathee, 2018). It is more virulent than *P. aeruginosa* PAO1 and causes disease in more diverse hosts including humans, *Drosophila melanogaster*, and *Arabidopsis thaliana*. It possesses a well annotated, circularised genome sequence freely available, can be readily genetically modified and has been widely used to investigate biofilm formation and drug resistance (Dötsch et al., 2009, Ha et al., 2015, Lee et al., 2006).

2.2. General Microbiological Techniques

Overnight cultures were grown in 5 mL of lysogeny broth (LB) and incubated at 37 °C, shaking at 250 rpm for 18 to 24 hours. Strains were archived for long-term storage on cryopreservation beads (Technical Service Consultants Ltd, Heywood, UK) at -70 °C. For working stocks and short-term storage, strains were stored in 20% v/v glycerol at -20 °C. Strains were resuscitated from cryogenic storage via growth overnight in liquid media and subculture onto LB agar by streaking to single colonies. A discrete colony was taken from solid media and grown overnight.

2.3. Evolution Experiments

2.3.1. Biofilm Propagation Medium Optimisation

Biofilms of *P. aeruginosa* PA14 were cultured on 5 mm polyvinyl chloride (PVC) beads (Xinlin Industrial Company Ltd, Ningbo, China) in an untreated 24-well cell culture plate (Eppendorf UK Ltd, Stevenage, UK) shaking at 60 revolutions per minute (rpm) for 24 hours at 25 °C. Four lineages of biofilms were propagated for 30 passages in 1 mL of one of five common microbiological culture media. These media were: LB, LB without salt, M9 minimal, Mueller-Hinton broth and brain-heart infusion. Planktonic controls were also propagated via 1:100 dilutions in each medium in 1 mL wells without any beads at 25 °C for 24 hours. At each timepoint, the beads were moved to a well containing 1 mL PBS using a 10 μL inoculation loop where they were washed via gentle agitation and moved to fresh broth containing three more sterile beads. Biofilms were then serially passaged by transferring seeded beads to fresh wells containing three sterile beads of a different colour, allowing biofilms to passively disseminate to a fresh substrate (Fisher Scientific, Loughborough, UK). At each timepoint, one bead was

stored in 1 mL 20% v/v glycerol in a 2 mL 96-well deep-well plate (Starlab (UK) Ltd., Milton Keynes, UK), one bead was used to propagate to the next transfer and the third bead was kept as a spare.

Biofilms were recovered from the archive by sealing the plate with a 96-well sealing mat (ThermoFisher Scientific, Basingstoke, UK) and vortexing at high speed for approximately one minute on a Vortex-Genie (Scientific Industries, New York, USA). Biofilm recovery was performed this way throughout all subsequent experiment. All populations from passages 10, 20 and 30 were resuscitated and the biofilm production was quantified at each timepoint. The medium which supported the greatest increase in biofilm formation after 30 successive subcultures was chosen as the optimum biofilm propagation medium for downstream evolution experiments.

2.3.2. Optimisation of the Selective Conditions for Adaptation to Antimicrobial Stress

Biofilms of *P. aeruginosa* PA14 were cultured on 5 mm PVC beads at 25 °C for 24 hours in 1 mL LB shaking at 60 rpm. Four lineages of biofilms were exposed to antimicrobial stress from ciprofloxacin, colistin, copper sulphate, zinc sulphate or benzalkonium chloride in two formats. One set of biofilms were exposed to each agent at a static concentration of 0.25× MIC of each agent for thirty passages. The second set were exposed to stress increasing in a stepwise manner starting from 0.25× MIC and increasing every three passages until growth failure. At each timepoint, the beads were moved to a well containing 1 mL PBS using a 10 μL inoculation loop where they were washed via gentle agitation and moved to fresh broth containing three more sterile beads. A bead was then stored in 1 mL 20% v/v glycerol in a 2 mL 96-well deep-well plate, another bead was used to propagate to the next transfer allowing biofilms to passively disseminate to a fresh substrate and the third was kept as a spare. The endpoint biofilms were recovered from the archive as described in section 2.3.1. and phenotyped for changes in antimicrobial susceptibility to the selective agent, according to the MIC.

2.3.3. Selection for Biofilm Hyperproduction

Biofilms of *P. aeruginosa* PA14 were cultured on 5 mm PVC, glass (Sigma-Aldrich Corporation, Gillingham, UK) or type-316 stainless steel (Simply Bearings Ltd, Leigh, UK) beads at 25 °C, shaking at 60 rpm for 24 hours in 1 mL LB. Planktonic cultures were grown in 1 mL LB at 25 °C, shaking at 60 rpm for 24 hours. Four lineages of

biofilms were propagated on each substrate, in addition to four lineages of planktonic controls, for thirty passages. At each timepoint, a bead from each lineage was stored in 1 mL 20% glycerol, in addition to 100 µL of planktonic culture diluted 1:10 in 20% v/v glycerol. A second bead was used to propagate biofilms to the next transfer allowing biofilms to passively disseminate to a fresh substrate, with the planktonic lineages propagated via a 1:100 dilution of culture into fresh broth. A third bead was kept as a spare. All populations from passage 10, 20 and 30 were recovered from the glycerol archive as described in section 2.3.1. for phenotyping and genome sequencing. The populations were assayed for changes in biofilm formation, colony morphology, antimicrobial susceptibility, stress tolerance and fitness in broth.

2.3.4. Evolutionary Trajectories to Drug Resistance in Biofilm and Planktonic Lifestyles

Biofilms of *P. aeruginosa* PA14 were cultured on 5 mm PVC beads at 25 °C for 24 hours, shaking at 60 rpm in 1 mL LB. Twelve replicate lineages of biofilms were initially seeded onto beads in unstressed conditions by diluting overnight cultures 1:100 into 1 mL of LB, with three beads in each well. In addition, twelve replicate lineages of planktonic cultures were grown in 1 mL of LB at 25 °C for 24 hours, shaking at 60 rpm. At each timepoint, the beads were moved to a well containing 1 mL PBS using a 10 µL inoculation loop where they were washed via gentle agitation and moved to fresh broth containing three more sterile beads and a defined concentration of ciprofloxacin, ceftazidime or tobramycin. A bead from each lineage was then stored in 1 mL 20% v/v glycerol, in addition to 100 µL of planktonic culture diluted 1:10 in 20% v/v glycerol. A second bead was used to propagate biofilms to the next transfer allowing biofilms to passively disseminate to a fresh substrate, with the planktonic lineages propagated via a 1:100 dilution of culture into fresh broth. A third bead was kept as a spare. The concentration of antibiotic doubled every three passages. In this way, lineages were passaged under stress from 0.25× MIC to 8× MIC over the course of 18 transfers. Four random biofilm and planktonic populations were selected from transfer 6, 12 and 18 and re-adapted for a further ten transfers under stress from the maximum concentration that their descendent population went on to be exposed to. The stepwise-adapted lineages and the endpoint re-adapted lineages were then recovered from the archive as described in section 2.3.1. and phenotyped for changes in biofilm formation, fitness and antimicrobial susceptibility.

2.3.5. Collateral Modulation of Drug Susceptibility by Non-Therapeutic Antimicrobials

The capacity of 48 distinct antimicrobials to select for antimicrobial resistance in biofilms was determined (table 2.1.). Biofilms of *P. aeruginosa* PA14 were cultured on 5 mm PVC beads at 25 °C for 24 hours, shaking at 60 rpm in 1 mL LB. Four replicate lineages of biofilms were initially seeded onto beads in unstressed conditions by diluting overnight cultures 1:100 into 1 mL of LB. Biofilms were then washed in PBS and transferred to fresh wells containing 0.25× MIC of one of the 48 stress conditions, including a naïve unstressed control to give 196 lineages total, passaged in parallel (table 2.3.). At each timepoint, the beads were moved to a well containing 1 mL PBS using a 10 μL inoculation loop where they were washed via gentle agitation and moved to fresh broth containing three more sterile beads. One bead was then used to propagate biofilms passively disseminate to a fresh substrate for the next transfer for up to 63 transfers or until growth failure, a second bead from each lineage was stored in 1 mL 20% glycerol every three transfers and a third bead was kept as a spare. From passage one to 15, the concentration doubled every three passages. From passage 15 onward, the concentration doubled every six passages, with an intermediate increase in concentration equidistant between doublings at every third passage. The adapted lineages were tested for viability after every increase in concentration. If lineages failed to grow, they were removed from the experiment. The last successful passage was recovered from the archive as described in section 2.3.1. and phenotyped for changes in biofilm formation, fitness and antimicrobial susceptibility. Lineages demonstrating significant changes in antibiotic susceptibility were genome sequenced.

Table 2.1. Antimicrobial used to investigate the collateral section of drug resistance

Class	Agent	Application
Antibiotics	Chloramphenicol	Protein synthesis inhibitor drug
	Rifampicin	RNA polymerase inhibitor drug
	Piperacillin	Cell wall synthesis inhibitor drug
	Meropenem	Cell wall synthesis inhibitor drug
	Aztreonam	Cell wall synthesis inhibitor drug
	Fosfomycin	Cell wall synthesis inhibitor drug
	Colistin	Cell membrane disruptor drug
Metals	Copper sulfate	Livestock feed additive
	Zinc sulfate	Livestock feed additive
	Silver nitrate	Antiseptic
	Thiomersal	Pharmaceutical preservative
	Mercurochrome	Livestock feed additive
	Arsanilic acid	Livestock feed additive
	Cobalt chloride	Environmental stressor
	Nickel chloride	Environmental stressor
	Aluminium acetate	Antiseptic
	Bismuth subgallate	Internal deodorant
Phenolics	Benzylalcohol	Antimicrobial preservative
	Phenoxyethanol	Antimicrobial preservative
	Chloroxylenol	Disinfectant
	Methylparaben	Antimicrobial preservative
	Chlorocresol	Antimicrobial preservative
	Pentachlorophenol	Antimicrobial preservative
Cationic surfactants	Benzalkonium chloride	Antimicrobial preservative
	Cetylpyridinium chloride	Antimicrobial preservative
	Benzethonium chloride	Antimicrobial preservative
	Cetrimonium bromide	Antimicrobial preservative
	Chlorhexidine gluconate	Antiseptic
	Octenidine dihydrochloride	Antiseptic
Salts	Sodium chloride	Environmental stressor
	Magnesium chloride	Environmental stressor
	Sodium benzoate	Antimicrobial preservative
	Potassium sorbate	Antimicrobial preservative
	Sodium nitrite	Antimicrobial preservative
	Potassium nitrate	Antimicrobial preservative
Acids	Benzoic acid	Antimicrobial preservative
	Sorbic acid	Antimicrobial preservative
	Citric acid	Flavouring and antimicrobial preservative
	Pelargonic acid	Pesticide
	2,4-Dichlorophenoxyacetic acid	Pesticide
Oxidisers	Sodium hypochlorite	Disinfectant
	Iodopovidone	Antiseptic
	Paraquat	Pesticide
Isothiazolinones	Methylisothiazolinone	Antimicrobial preservative
	Benzisothiazolinone	Antimicrobial preservative
Aldehyde releasers	Glutaraldehyde	Disinfectant
	Imidazolidinyl urea	Antimicrobial preservative

2.4. Phenotyping

2.4.1. Biofilm Assays

2.4.1.1. Biofilm Productivity Assay

Overnight cultures were diluted 1:100 into 1 mL of selected microbiological culture medium in an untreated 24-well cell culture plate containing beads of a chosen substrate and incubated at 25 °C, shaking at 60 rpm for 48 hours. Beads were washed twice in 1 mL phosphate buffered saline (PBS) and transferred to a 1.5 mL microcentrifuge tube (Fisher Scientific, Loughborough, UK) containing 1 mL PBS. Cells were harvested via agitation at 2000 rpm for two minutes in an Eppendorf ThermoMixer (Eppendorf UK Ltd, Stevenage, UK). The harvested cells were then diluted 1:10 into the first row of a microtiter tray (Greiner Bio-One, Kremsmünster, Austria) containing 180 µL PBS and serially diluted down the columns of the tray. After dilution, 5 µL of each well was spotted onto a square LB agar plate (R & L Slaughter Ltd, Basildon, UK) which were incubated for 24 hours at 37 °C. The number of colony forming units (c.f.u.) was then calculated per unit area.

2.4.1.2. Crystal Violet Biomass Assay

Overnight cultures were diluted 1:10,000 by diluting 1:100 twice in microtiter trays containing 180 µL of LB. The inoculated plates sealed with a gas-permeable membrane (4titude Ltd, Wotton, UK) and incubated for 48 hours at 30 °C. After incubation, the culture was discarded into a high-level biocide such as Bioguard (Bioguard Hygiene Solutions Ltd, Northampton, UK). and the wells were washed several times with tap water. Into each well, 200 µL of 0.1% crystal violet solution (Sigma-Aldrich Corporation, Gillingham, UK) was added and incubated at room temperature for 15 minutes. The crystal violet was then discarded, and the wells were again washed several times with tap water. Finally, 70% ethanol Sigma-Aldrich Corporation, Gillingham, UK) was added to each well and the optical density as measured by absorbance at 595 nm (OD_{595}) was read in a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK).

2.4.1.3. Congo Red-Coomassie Blue Colony Morphology Assay

Biofilm morphology agar plates containing 1% w/v bacteriological agar, 1% w/v tryptone, 40 µg/ mL Congo red (Sigma-Aldrich Corporation, Gillingham, UK) and 20 µg/ mL Coomassie Brilliant Blue-R (Sigma-Aldrich Corporation, Gillingham, UK) were

poured into 50 mm petri dishes (R & L Slaughter Ltd, Basildon, UK). Overnight cultures were diluted to an OD_{595} of 0.5 (± 0.05) in PBS using a Jenway 7200 spectrophotometer (Cole-Parmer, Stone, UK) and 10 µL was spotted onto biofilm morphology agar plates. The plates were incubated at 20 °C for ten days and then imaged using a Canon PowerShot SX210 digital camera (Canon (UK), Reigate, UK). Morphometric properties including colony area, agar invasion area, colony rugosity were determined using Fiji v2.1.0 (Schindelin et al., 2012).

2.4.2. Antimicrobial Susceptibility Testing and Stress Tolerance Assays

2.4.2.1. Minimum Inhibitory Concentration by Microbroth Dilution

MIC determination by microbroth dilution was performed following European Committee for Antimicrobial Susceptibility Testing (EUCAST) recommendations (EUCAST, 2021). The second to last columns of a microtiter tray were filled with 100 µL Mueller-Hinton broth. In the first and second columns of the microtiter tray, 100 µL of the selected antimicrobial diluted to an appropriate concentration in Mueller-Hinton broth was added. The antibiotic was then double diluted across column two to column eleven. Overnight cultures were diluted 1:100 twice in Mueller-Hinton broth and 100 µL was added to each well. The plate was sealed with a gas-permeable membrane and incubated at 37 °C for 24 hours. The lowest concentration in which no growth was observed was designated as the MIC.

2.4.2.2. Minimum Inhibitory Concentration by Agar Dilution

Mueller-Hinton agar was melted and allowed to cool to approximately 50 °C before being poured into a 50 mL falcon tube. The first tube contained 50 mL of Mueller-Hinton agar and subsequently tubes contained 25 mL. An appropriate concentration of the chosen antimicrobial was added to the first tube and was homogenised by inversion. Once homogenised, 25 mL of the agar was poured into a square petri dish and the remaining 25 mL was poured into the next tube. This was repeated for the required number of dilutions. In this way, the antibiotic was sequentially double diluted in each tube and contained half of the antimicrobial concentration of the previous plate. The plates were allowed to set in a laminar flow cabinet for one hour and used within 48 hours of pouring. Overnight cultures were diluted 1:1,000 in a microtiter tray in 200 µL of PBS and approximately 2 µL was spotted onto the antibiotic plates using an automated 96-pin multipoint inoculator (A.Q.S. (Manufacturing) Ltd, London, UK). The

spots were allowed to dry, and the plates were incubated at 37 °C for 24 hours. The lowest concentration in which no growth was observed was designated as the MIC.

2.4.2.3. Minimum Biofilm Eradication Concentration

A modified broth microdilution assay was used to determine the minimum biofilm eradication concentration (MBEC). Overnight cultures were diluted 1:100 twice in microtiter trays containing 180 µL of LB. A -skirted 96-well polymerase chain reaction (PCR) plate (Starlab, Milton Keynes, UK) was then allowed to sit within the wells of the microtiter tray. The plate was incubated for 48 hours at 30 °C allowing a biofilm to form on the outside of the wells of the PCR plate. After incubation the microtiter tray was discarded and the outside surface of the wells of PCR plate was washed in 200 µL of PBS in a microtiter tray. The PCR plate was then allowed to rest within another microtiter tray in the same format for an MIC assay described previously, which was incubated at 37 °C for 24 hours. The MIC plate was then discarded, and the PCR plate was placed in a fresh microtiter tray containing 200 µL of LB which was incubated for 24 hours at 37 °C. The corresponding concentration from the first well which the biofilm could not reseed was determined to be the MBEC.

2.4.2.4. Stress Tolerance Assays

Strains were grown overnight, diluted 1:1,000 into LB containing 8% w/v sodium chloride or in LB adjusted to either pH 4 or pH 11. In a microtiter tray, 200 µL of the diluted stock was added and incubated at 30 °C for 48 hours. The cultures were then serially diluted in PBS and 5 µL of diluent was spotted onto a square LB agar plate and incubated at 37 °C for 24 hours. The viability of each strain after stress challenge was determined by counting c.f.u.

2.4.3. Growth Kinetics

Overnight cultures were diluted 1:1,000 into a microtiter tray in 200 µL of LB. The plate was incubated for 20 hours at 37 °C and the OD_{600} was measured every 15 minutes using a FLUOstar Omega plate reader. The plate was homogenised by orbital shaking at 300 rpm for three seconds before each read. The integral area under the curve (OD_{600} hours) was calculated exponential phase growth velocity was calculated.

2.5. Genome Sequencing

2.5.1. DNA Extraction via Zymo *Quick*-DNA 96-Well Kit

Extraction of gDNA for whole genome sequencing of experimentally evolved mutants was performed with the Zymo Quick-DNA 96-Well Kit (Zymo Research, Irvine, California, USA). Strains were grown overnight, and 2 mL of culture was transferred to a well of a deep-well plate. The deep-well plate was centrifuged for 15 minutes at 3,000 × g to pellet cells and the supernatant was discarded. The cells were resuspended in 400 µL BashingBead buffer and transferred to the tubes of a ZR BashingBead Lysis Rack. The tubes were vortexed at high speed for 10 minutes and 250 µL of the BashingBead buffer was transferred to the wells of a 96-well block along with 750 µL of genomic lysis buffer. The 96-well block was sealed and vortexed at high speed for 5 minutes and centrifuged at 3,000 × g for another 5 minutes. A silicon-A plate was mounted on a collection plate and 500 µL of lysed cells was transferred into the wells of the silicon-A plate. The assembly was centrifuged at 3,000 × g for 5 minutes and the flowthrough was discarded. This was repeated for the remaining 500 µL of lysed cells, followed by 200 µL of DNA pre-wash buffer and 500 µL of gDNA wash buffer. The collection plate was then replaced with an elution plate and the DNA was eluted into it with 50 µL of elution buffer. The silicon-A plate was discarded and the elution plate containing purified DNA was stored at -20 °C.

2.5.2. DNA Quantification via Quant-iT Fluorometry

Extracted DNA was quantified using a Quant-iT dsDNA high sensitivity assay kit (ThermoFisher Scientific, Basingstoke, UK). The Quant-iT working solution was made by diluting the Quant-iT reagent 1:200 into the Quant-iT buffer. A standard curve was generated by adding 10 µL of standard solution to 190 µL of working solution and 2 µL of extracted DNA was added to 198 µL of working solution. Fluorescent excitation and emission wavelengths were measured at 500/530 nm. Fluorescent readings for extracted DNA were interpolated into the standard curve to yield DNA concentrations.

2.5.3. Illumina NextSeq Short Read Sequencing

Whole genome sequencing was performed using the Illumina NextSeq 500 platform (Illumina, Cambridge, UK). Extracted gDNA was diluted to 5 µg/mL in ultrapure deionised water and sequencing libraries were prepared according to Illumina DNA Prep Reference Guide (Illumina, document #1000000025416 v10, 2020). Samples were first tagmented. A tagmentation master mix was created on ice containing 0.5 µL

bead linked transposomes, 0.5 µL tagmentation buffer 1 and 4 µL ultrapure water per sample. In a semi-skirted PCR plate, 5 µL of the tagmentation mix was added to 2 µL of 5 µL/ mL gDNA and vortexed gently. The gDNA was then tagmented at 55°C for 15 minutes and immediately indexed using unique dual barcoding. In a fresh PCR plate, 1 µL of different i5 and i7 primers were added to each row and column of the plate, respectively. With a different index primer combination in each well, 2 µL ultrapure water, 10 µL KAPA2G Fast ReadyMix (Roche, Welwyn Garden City, UK) and 7 µL of the tagmented gDNA was added. Barcoding was performed via PCR by initially denaturing at 95 ºC for 1 minute, followed by 14 cycles which denatured at 95 ºC for 10 seconds, annealed at 55 ºC for 20 seconds and extending at 72 ºC for 3 minutes.

The indexed sequencing libraries were purified using solid phase reversible immobilisation (SPRI) beads. Cells were pelleted by centrifugation at 4,500 rpm for ten minutes and the supernatant was discarded. The pellets were resuspended in 100 µL of lysing buffer, containing 10 mL 10 mM Tris-EDTA buffer, 100 µL lysozyme and 10 µL RNase A (QIAGEN Ltd, Manchester, UK) and were incubated at 37 °C, shaking at 1600 rpm on an Eppendorf ThermoMixer for 25 minutes. After incubation, the lysate was decanted into 100 µL volumes in a lo-bind PCR 96-well plate (Eppendorf UK Ltd, Stevenage, UK). Lysing additive was made, containing 528 µL Tris-Ethylenediaminetetraacetic acid (EDTA) buffer, 600 µL 10% sodium dodecyl sulphate, 12 µL RNase A and 60 µL proteinase K (QIAGEN Ltd, Manchester, UK), and 10 µL of the additive was added to the mix. The tubes were then incubated at 65 °C, shaking at 1600 rpm for 15 minutes. The lysate was then transferred to a fresh lo-bind PCR 96 well plate and 50 µL of AMPure XP SPRI beads (Sigma-Aldrich Corporation, Gillingham, UK) were added and mixed by pipetting. The beads were incubated at room temperature for five minutes and placed on a magnetic separation block (Fisher Scientific, Loughborough, UK). The liquid was discarded, and the beads were washed three times in freshly made 80% ethanol. The ethanol was removed, and the wells were air dried for five minutes. The plate was removed from the magnetic block and the beads were resuspended in 50 µL 10 mM Tris-EDTA buffer. The plate was placed back on the magnetic block and the gDNA was transferred to a fresh PCR plate and quantified via Qubit fluorometry.

The purified libraries were pooled into a single microcentrifuge tube, normalising to 50 ng per sample and quantified again using Qubit fluorometry. The pooled libraries were diluted to 2 ng/ µL and run on a 4200 TapeStation system (Agilent Technologies, Stockport, UK) using a high sensitivity D5000 ScreenTape reagent system (Agilent Technologies, Stockport, UK) to determine DNA concentration, the mean insert size

and the molarity at the 300-700 bp insert size region. Based on these values, the libraries were diluted to 20 pM with an equal volume of libraries, 2 M sodium hydroxide and 200 mM Tris-hydrochloric acid which is made up to 1 mL in hybridisation buffer. The same was performed to a PhiX control. The libraries were then diluted to 1.8 pM in hybridisation buffer to a final concentration of 1.3 mL with 1 µL of PhiX and were loaded into 300 cycle high-output NextSeq 500/550 kit for sequencing (Illumina, Cambridge, UK).

2.5.4. Bioinformatic Analysis

Genome sequencing data was analysed using the Integrated Rapid Infectious Disease Analysis (IRIDA) release 19.09.2 (Matthews et al., 2018) and Galaxy release 19.05 platforms (Afgan et al., 2018). Demultiplexing of sequencing libraries and generation of fastq files was carried out on BaseSpace v6.16. (Illumina, Cambridge, UK). Illumina adapters and low quality reads below a Phred score of 30, or a read length of 36 bp were trimmed using Trimmomatic v0.38.0 (Bolger et al., 2014). Reads were assembled into contigs with Shovill v1.1.0 (https://github.com/tseemann/shovill) using the SPAdes assembler and assembly quality was assessed using QUAST v5.0.2 (Gurevich et al., 2013). Draft genomes were annotated using Prokka v1.14.5 (Seemann, 2014). SNP calling and core genome alignment was performed using paired reads with snippy v4.4.3 (https://github.com/tseemann/snippy) against a circularised *P. aeruginosa* UCBPP-PA14 reference genome (GenBank accession GCF_000014625.1). Phylogenies were constructed through maximum likelihood inference with IQ-TREE v1.6.12 (Nguyen et al., 2015) and visualised in ITOL v6.4 (Letunic and Bork, 2007).

2.6. Data Visualisation and Statistical Analysis

Data were statistically analysed in RStudio v1.3.1073 (RStudio Team, 2020) and visualised using ggplot2 v3.3.5 (Wickham, 2016) and manually annotated in GIMP v2.10.20 (revision 1) (The GIMP Development Team, 2019). Growth curves were plotted and statistically analysed in GraphPad Prism v9.2.0 (GraphPad Software, 2021).

CHAPTER 3. VALIDATION OF AN EXPERIMENTAL EVOLUTION MODEL TO STUDY ADAPTATION TO SELECTIVE PRESSURES IN BIOFILMS

3.1. Introduction

Experimental evolution is a heuristic approach to studying the adaptation of model organisms to selective pressures under laboratory conditions. This closed system design can eliminate confounding variables encountered *in situ* and accelerate evolutionary processes to experimental timescales. Experimental evolution offers a platform to leverage control over the fundamental parameters underpinning natural selection (Elena and Lenski, 2003). Culture conditions, population bottlenecks and the selective environment can be manipulated by experimental design to test the impact of evolutionary forces (Barrick and Lenski, 2013). Microorganisms represent the principal reservoir of model organisms for experimental evolution studies due to their large population sizes, rapid asexual reproduction, and broad capacity for adaptation (Lenski et al., 1991). Beginning with an often clonal ancestor, experimental evolution is typically performed through serial passage via repeated subculture under a subinhibitory selective pressure (Barrick and Lenski, 2013). In passage experiments, when exponential growth ceases and the population achieves the maximum density its carriage medium can support, cultures are diluted into fresh media permitting re-entry into logarithmic phase. Consequently, serial passage artificially expands the proportion of time spent in exponential growth (Elena and Lenski, 2003). This vastly increases the number of selective events and opportunities for competitive exclusion, thus accelerating the adaptive process.

Experimental evolution as a laboratory model has a long history dating to the development of continuous culture apparatus in the 1950s, coinciding with the discovery of the genetic basis of heredity. Such studies demonstrated some of the earliest reproducible experimental evidence of natural selection in a field which previously relied almost exclusively on observation and theory (Atwood et al., 1951, Northrop, 1957, Novick and Szilard, 1950, Witkin, 1953). The wider adoption of experimental evolution in the following decades led to a number of pivotal investigations conducted by arguably the first generation of experimentalists studying microbial evolution. Using competitive fitness as the main metric by which evolutionary change was measured, increasing attention was made towards understanding evolutionary principles rather than simply observing selective consequences (Dykhuizen and Hartl, 1981, Hartl and Dykhuizen, 1979, Helling et al., 1987, Levin, 1972). Later, Richard Lenski introduced the concept of collateral trade-offs to experimental evolution in a series of studies investigating pleiotropic antagonism among *E. coli* adapted to bacteriophage T4 and thermal stress (Bennett et al., 1992, Lenski and Levin, 1985, Lenski, 1988). Additionally, he elaborated on this principle and

was the first to use experimental evolution to investigate how future adaptation becomes contingent on prior evolutionary outcome (Bennett and Lenski, 1993, Leroi et al., 1994). This work preceded arguably the most lauded evolution experiment in history, the long-term evolution experiment (LTEE), which has propagated twelve lineages of *E. coli* daily in glucose-limited minimal media since 1987 (Lenski et al., 1991). As of 2021, the lineages in the LTEE have undergone approximately 75,000 generations in monoculture without complex ecological niches and with glucose catabolism as the single limiting factor (Blount et al., 2018). Though there is no “stress”, the LTEE has demonstrated a dynamic selective landscape is intrinsic to even the most rudimentary ecological conditions (Grant et al., 2021).

Experimental evolution possesses several distinct advantages over investigating microbial evolution in the real world. Evolutionary hypotheses resigned to investigation by comparative or theoretical means using *in situ* approaches can often be studied directly in appropriately designed experimental systems (Hoang et al., 2016). Laboratory evolution benefits from the clarity provided by deconstructing evolutionary dynamics into easily modelled elements which allows adaptation to selective pressures to be distinguished from those selected by the background environment. The ability to design control conditions lacking the test pressure but otherwise identical to the basal selective conditions supplies the capacity to make such inferences. Potential confounders can be identified, and their effects subtracted from test regimes to distinguish true adaptive observations from those intrinsic to the experimental microcosm (Lenski, 2017). The selective environment in experimental systems can also be replicated in multiple identical conditions. Replicate lineages negotiating the same selective conditions in functionally independent environments provides insights into stochasticity and parallelism associated with evolutionary trajectories (McDonald, 2019). Moreover, the storage and recovery of archived strains allows the adaptive process to be reiterated from an earlier state facilitating investigation into evolutionary contingency (Blount et al., 2018). The repeatability of evolution is a fundamental question underpinning evolutionary biology and the power this yields to interrogate the selective process is enormous. Genetic parallelism between independently evolved lineages is strong evidence for a role in adaptation. Furthermore, much information regarding the fitness landscape of selective pressures can be inferred by the patterns of contingency and replicability arising in adapted populations. Selective pressures which have few pathways to adaptation are likely to demonstrate high degrees of repeatability with limited sensitivity to historical contingency (Turner et al., 2015). With little epistasis available to reveal new adaptive trajectories, unique mutations arising

between lineages are unlikely to give rise to alternative mechanistic outcomes. Conversely, selective pressures which have rugged fitness landscapes with multiple trajectories to fitness peaks select for varied genotypic and phenotypic consequences. Consequently, experimental evolution provides a strong framework to understand the genetic contingency arising from the pleiotropic properties of mutations and reveal novel evolutionary pathways associated with adaptation (Elena and Lenski, 2003).

In essence, an evolution experiment is a vehicle for selecting mutants in a system which preserves evolutionary continuity between the ancestor and its adapted descendants. A central feature of experimental evolution is the production of longitudinal archives of adapted lineages at intermittently-spaced timepoints (Kawecki et al., 2012). The close generational intervals at which lineages are sampled provides the means to investigate evolutionary trajectories at high resolution. Downstream analyses are used to characterise experimentally evolved strains for emergent properties selected as a result of adaptation. Phenotypic assays have long been the mainstay of laboratory evolution for their ease in characterising large numbers of mutants at scale (Elena and Lenski, 2003). Antimicrobial susceptibility, infection models and fitness profiling represent the most widely used assays, reflecting the hypotheses which experimental evolution is commonly used to investigate. However, in recent years, advances in whole genome sequencing have revealed new layers of granularity to understand evolution *in vitro*. The ability to interrogate evolutionary change at the nucleotide scale reveals new avenues to understand natural selection at an unprecedented fidelity (Lenski, 2017). Concepts which experimental evolution is particularly adept at tackling includes understanding the timing and distribution of genomic evolution, identifying parallel selective targets, and elucidating the adaptive role of mutations (Dettman et al., 2012).

The qualities which make evolution experiments highly replicable, interpretable and convenient, can also make them unrepresentative of evolution in nature which is inherently more complex (Hoang et al., 2016). Experimental platforms eliminate almost all complexity from the selective landscape in order to deconvolute the evolutionary process into resolvable parameters. Monoculture studies retain only the test pressure and those intrinsic to the experimental design, such as nutrient catabolism. The effect these disparities can have on selective outcome is significant. Mutations which confer strong phenotypic changes to the focal selective pressure will be rapidly selected in monoculture, irrespective of the fitness defects it may confer in its absence (Kawecki et al., 2012). However, communities *in situ* do not experience static stress; multiple selective pressures are in constant flux, most notably interspecific competition, nutrient

acquisition, and antimicrobial stress. Similarly, the homogeneity of laboratory culture renders superfluous many genes essential for central metabolism, stress responses and macromolecular synthesis (McDonald, 2019). A hallmark of laboratory-adapted mutants is high fitness in the presence of the selective pressure, this often results in punitive fitness defects in alternative environments (Cooper et al., 2001, Travisano et al., 1995). Conversely, natural selection in the real world often preferentially selects for mechanisms which show the fewest collateral effects on other phenotypic traits, even if fitness gains are modest.

Most microbial evolution experiments are performed in planktonic culture; however, microorganisms predominantly reside in biofilms in the natural world. This disparity may be a significant contributor to the variable representation of experimental evolution to real-world selective outcomes, especially at the genotypic level. Consequently, this chapter aims to independently validate and optimise the experimental evolution platform originally described by Poltak and Cooper (2011) for the use of understanding the selective dynamics of biofilm formation of *P. aeruginosa* PA14 adapted on bead substrata. With this, the tractability of the model for stratifying the selective potential of antimicrobial agents and how it interfaces with the evolutionary forces intrinsic to biofilm formation is determined.

3.2. Results

3.2.1. Biofilms can be cultivated at high productivity and propagated under antimicrobial stress on bead substrata

The culture of biofilms of *P. aeruginosa* was optimised to yield the most productive biofilms on three clinically- and industrially-relevant surfaces: glass, type-316 stainless steel and PVC (Figure 3.1.). There was no significant difference in productivity between biofilms cultivated on glass and PVC substrata ($p = 0.4159$), however biofilms formed on stainless-steel were significantly less productive than both other substrata ($p < 0.0001$, $p < 0.0001$). Glass and PVC substrata supported approximately 10^5 to 10^7 c.f.u. /mm^2 after 24 hours of growth, however stainless steel could only support 10^3 to 10^5 c.f.u. /mm^2. Furthermore, longer incubation durations reduced maximum productivity in all conditions ($p < 0.0001$). Biofilms grown for 24 hours were significantly more productive than later timepoints ($p < 0.0001$) by approximately an order of magnitude. There was no significant difference between productivity at 48 hours and any later timepoint (48 hrs vs 72 hrs: $p = 0.8497$, 48 hrs vs 96 hrs: $p = 0.0556$) or 72 hours and 96 hours ($p = 0.3117$). Biofilms grown on glass and PVC exhibited a 2-log reduction in productivity over time, however on stainless-steel, only a single log reduction was observed. The reduction in cell carriage over time remained generally consistent regardless of incubation temperature or medium. The culture medium generally did not have a significant impact on biofilm productivity on any substrate ($p = 0.2680$). However, considerable differences in cell viability were observed between biofilms grown at different temperatures. Biofilms grown at high temperatures (37 °C and 42 °C) were significantly less productive than biofilms grown at low temperatures (25 °C and 30 °C) ($p < 0.0001$). Biofilms grown at 25 °C were the most productive by approximately an order of magnitude (25 °C vs 30 °C: $p = 0.0038$, 25 °C vs 37 °C: $p < 0.0001$, 25 °C vs 42 °C: $p < 0.0001$) followed by biofilms grown at 30 °C (30 °C vs 37 °C: $p < 0.0001$, 30 °C vs 42 °C: $p = 0.0022$). There was no significant difference in productivity between biofilms grown at 37 °C or 42 °C ($p = 0.8555$).

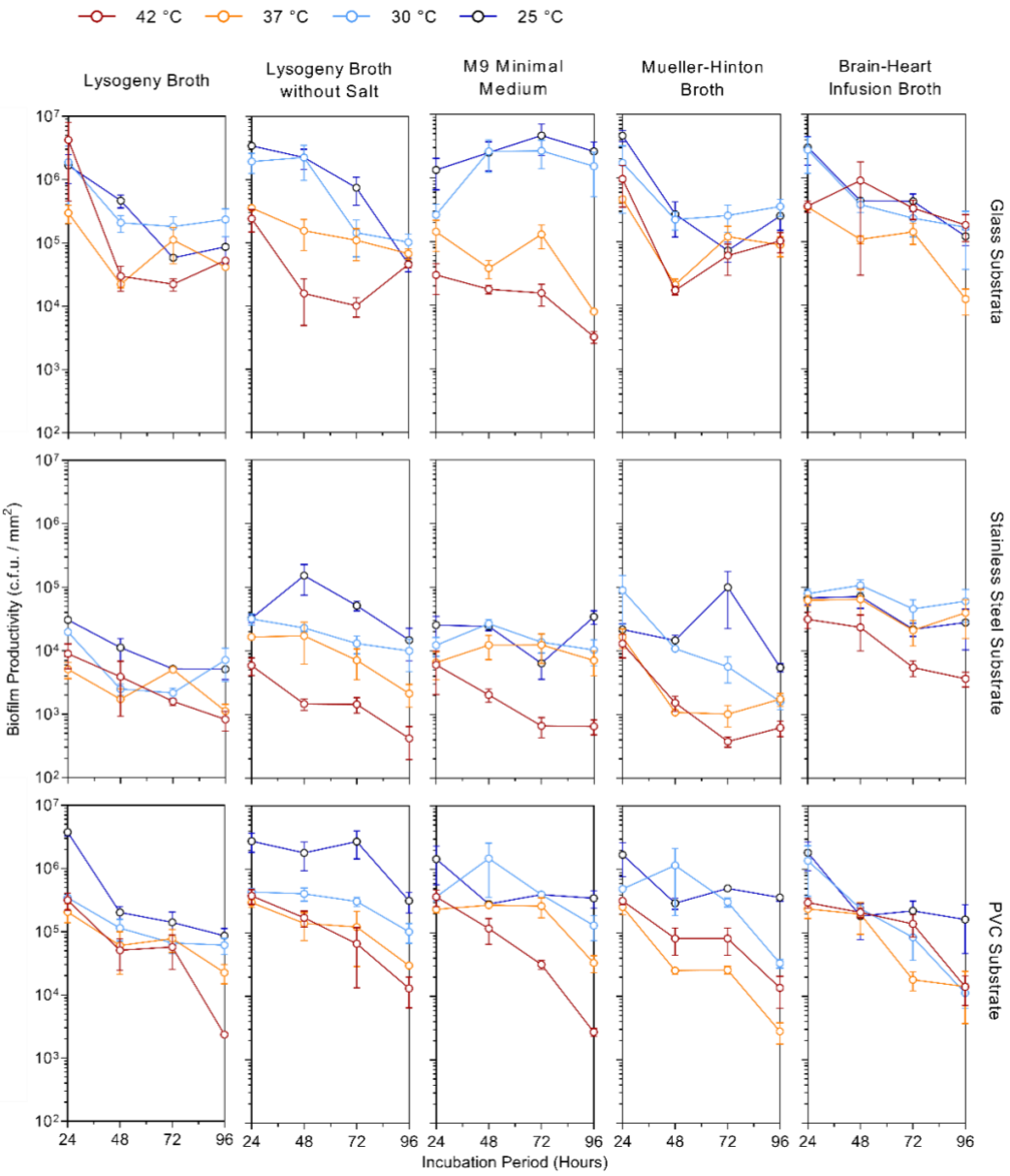

Figure 3.1. Productivity over time of *P. aeruginosa* biofilms grown on 5 mm diameter glass, PVC or stainless-steel bead substrata in 1 mL lysogeny broth, lysogeny broth without salt, M9 minimal medium, Mueller-Hinton broth or brain-heart infusion at either 25 °C, 30 °C, 37 °C or 42 °C. Statistical differences in biofilm productivity between culture medium, temperature and incubation period were detected via a two-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as mean c.f.u./ mm^2, ± standard error of the mean, n = 4.

The sustainability of passaging biofilms on bead substrata under subinhibitory stress from mechanistically diverse antimicrobial agents, including ciprofloxacin, zinc sulfate and benzalkonium chloride, was established (figure 3.2.). The concentrations at which biofilms experienced reductions in viability was consistently higher than planktonic cells. The planktonic MIC was not able to inhibit growth of biofilms exposed to any agent. Moreover, the concentration at which reductions in viability were experienced

was agent-specific. Biofilms did not encounter viability-reducing stress from ciprofloxacin until exposed to a 2-$\log_2$ fold increase in concentration from the planktonic MIC. Zinc sulfate conferred 2- and 3-log reduction in viability at 1- and 2- $\log_2$ fold MIC, respectively. Benzalkonium chloride conferred a 2-log reduction in viability at the MIC, with higher concentrations resulting in complete inhibition. At these concentrations where viability was attenuated, all biofilms were observed to increase in productivity gradually over the course of the experiment. Cell viability was recapitulated by approximately an order of magnitude against all agents, irrespective of the original reduction in viability. Generally, recovery in cell viability began early in the passage series but did not become significant until the later transfers. The biofilms adapted to 2-$\log_2$ fold MIC ciprofloxacin stress did not demonstrate a significant increase in viability until transfer 4 ($p = 0.0091$). This also applied for biofilms adapted to 1- $\log_2$ fold MIC zinc sulfate stress ($p = 0.0078$). Despite this, the viability of biofilms adapted to the highest concentration of zinc sulfate trended towards significance more quickly, reaching a significant increase by transfer 3 ($p = 0.0398$). Similarly, biofilms adapted to MIC benzalkonium chloride stress also experienced a significant increase in viability by transfer 3 ($p = 0.0004$). There were no other significant changes in biofilm productivity associated with any other challenge condition.

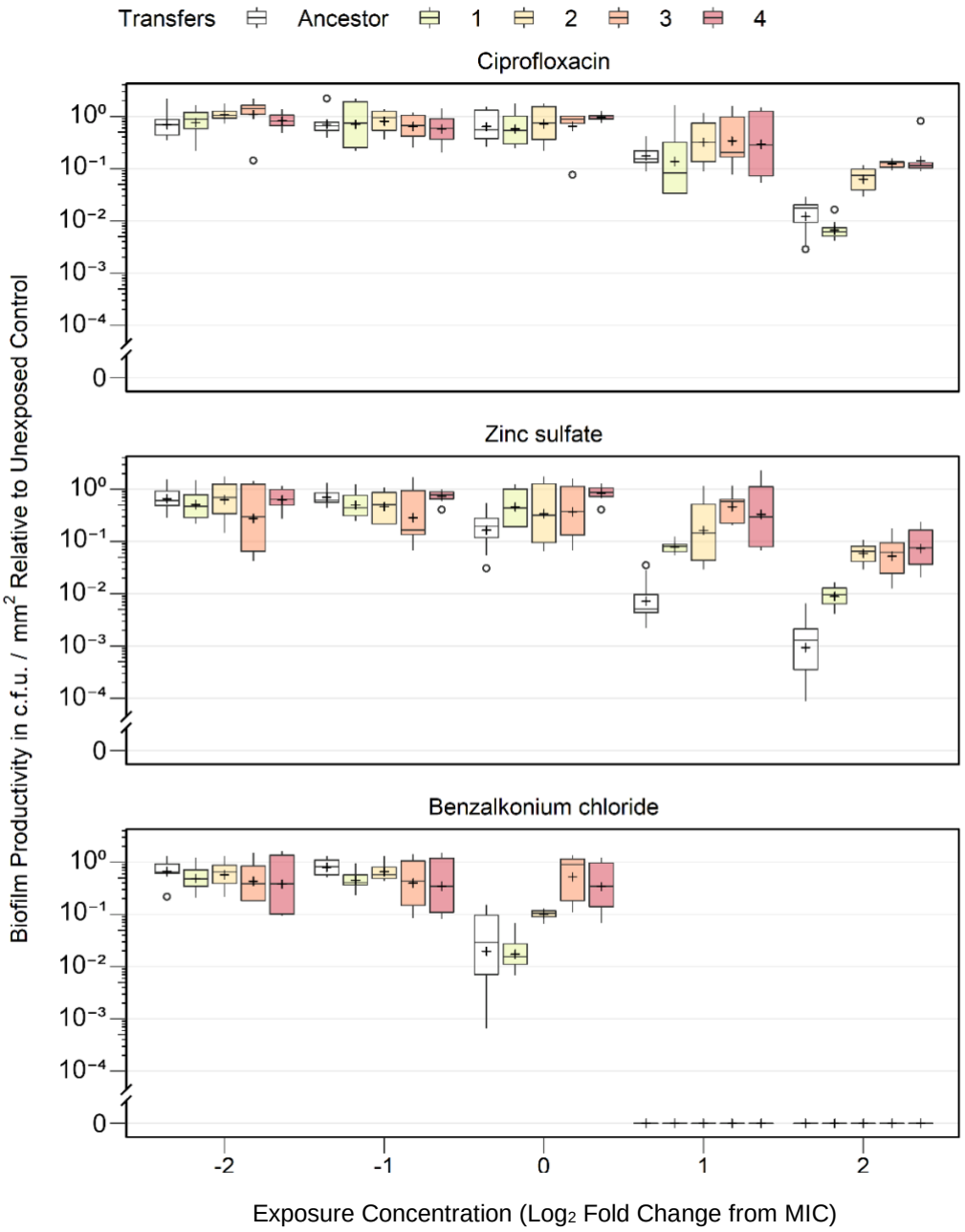

Figure 3.2. Relative productivity of *P. aeruginosa* biofilms grown on PVC beads in LB at 25 °C for 24 hours for four passages under antimicrobial stress from ciprofloxacin, zinc sulphate or benzalkonium chloride at a concentration of -2–, -1–, 0–, 1– and 2– log_2 fold MIC. Statistical differences in biofilm productivity between selective agent and concentration of stress over the passage series were detected via a one-way ANOVA with Dunnett's multiple comparison post-hoc test. Data shown as mean c.f.u./ mm^2 relative to unstressed control, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, free datapoints show outliers, n = 8.

3.2.2. Selection for biofilm hyperproduction through serial passage is growth medium-specific and evolution of antimicrobial resistance is dependent on exposure regime

The optimum propagation medium was determined by identifying the medium most proficient at selecting for increased biofilm formation in *P. aeruginosa* (figure 3.3.).

There was no significant difference in the intrinsic biofilm formation of biofilms cultivated in lysogeny broth, lysogeny broth without salt or M9 minimal (LB vs LB without salt: $p = 0.3979$, LB vs M9 minimal: $p = 0.9991$, LB without salt vs M9 minimal: $p = 0.5555$). However, biofilms of the *P. aeruginosa* ancestor grown in Mueller-Hinton broth or brain-heart infusion produced between approximately two- and three-times more biomass than the other propagation media (LB vs Mueller-Hinton: $p = 0.0026$, LB vs brain-heart infusion: $p = 0.0012$). Biofilms adapted in lysogeny broth, M9 minimal and brain-heart infusion were selected for significantly increased biofilm formation at all timepoints (ancestor vs transfer 10 in LB: $p = 0.0345$, ancestor vs transfer 20 in LB: $p < 0.0001$, ancestor vs transfer 30 in LB: $p < 0.0001$, ancestor vs transfer 10 in M9 minimal: $p = 0.0239$, ancestor vs transfer 20 in M9 minimal: $p = 0.0088$, ancestor vs transfer 30 in M9 minimal: $p = 0.0009$, ancestor vs transfer 10 in brain-heart infusion: $p = < 0.0001$, ancestor vs transfer 20 in brain-heart infusion: $p < 0.0001$, ancestor vs transfer 30 in brain-heart infusion: $p = 0.0187$). Biofilms adapted in lysogeny broth selected for successive increases in biofilm formation between the ancestor and transfer 10 ($p = 0.0345$) and between transfer 10 and 20 ($p = 0.0076$). There was no significant increase between transfer 20 and 30 ($p > 0.9999$) and biofilms produced at approximately 5-times more biomass than the ancestor. Despite biofilms adapted in M9 minimal and brain-heart infusion demonstrating increased biofilm formation relative to the ancestor at all timepoints, there were no successive increases after transfer 10 (transfer 10 vs transfer 20 in M9 minimal: $p < 0.9999$, transfer 10 vs transfer 30 in M9 minimal: $p > 0.9999$, transfer 10 vs transfer 20 in brain-heart infusion: $p > 0.9999$, transfer 10 vs transfer 30 in brain-heart infusion: $p = 0.9594$). Lysogeny broth without salt did not select for a significant increase until the final timepoint (ancestor vs transfer 10 in LB without salt: $p = 0.1261$, ancestor vs transfer 20 in LB without salt: $p = 0.1561$, ancestor vs transfer 30 in LB without salt: $p = 0.0004$) and biofilms adapted in Mueller-Hinton broth demonstrated a significant increase only at the first timepoint (ancestor vs transfer 10: $p = 0.0003$, ancestor vs transfer 20: $p = 0.9744$, ancestor vs transfer 10: $p = 0.1414$). Despite this, of the five media tested, with the exception of lysogeny broth and Mueller-Hinton, there were significant increases in biofilm formation when adapted planktonically at one or more timepoints (ancestor vs transfer 30 in LB without salt: $p = 0.0005$, ancestor vs transfer 30 in M9 minimal: $p < 0.0001$, ancestor vs transfer 10 in brain-heart infusion: $p = 0.0432$). Consequently, only lysogeny broth fulfilled the desired criteria as an optimal propagation medium by selecting for successive increases in biofilm formation when passaged as a biofilm, without selection on this phenotype when passaged planktonically.

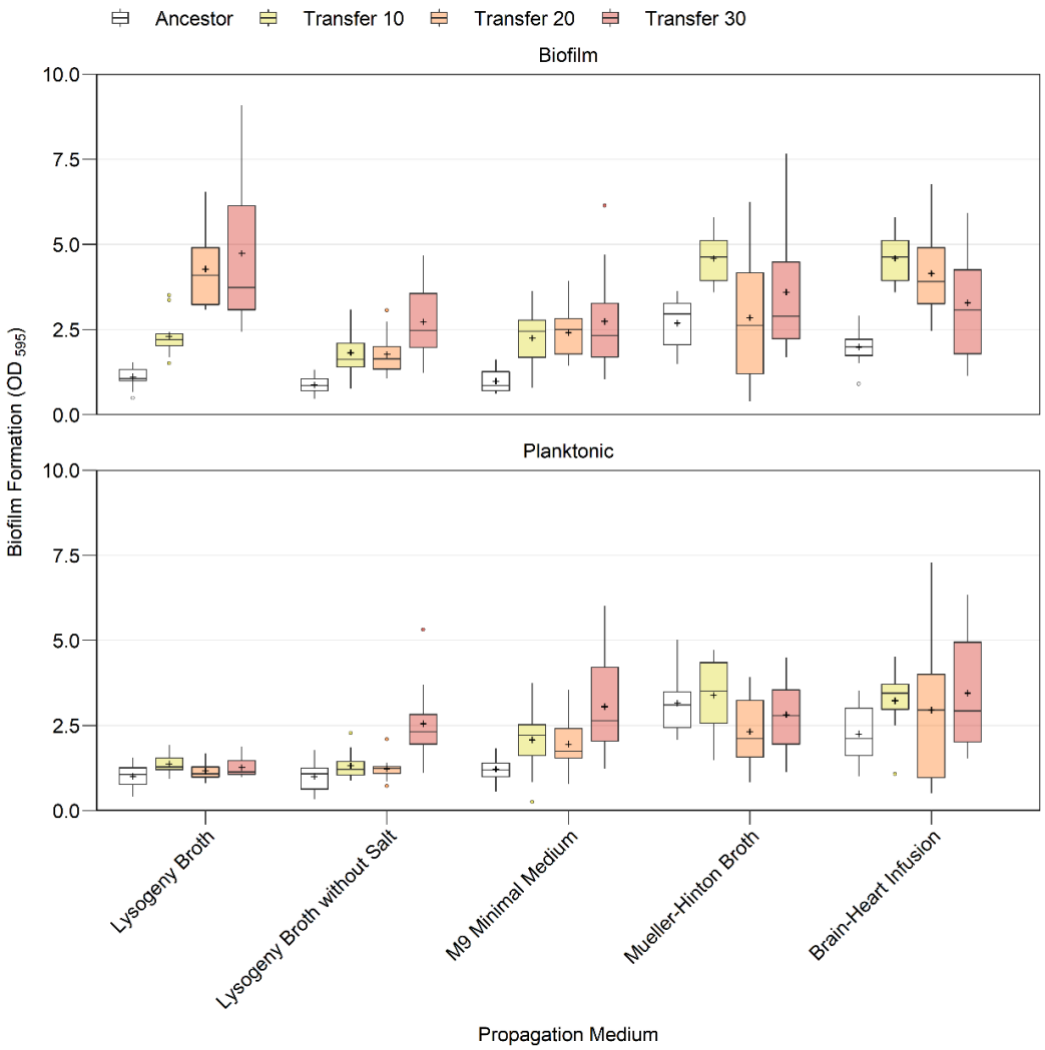

Figure 3.3. Biofilm formation as measured via crystal violet staining of *P. aeruginosa* adapted through serial passage in LB, LB without salt, M9 minimal, Mueller-Hinton broth and brain-heart infusion over 30 transfers in either a biofilm lifestyle on PVC beads or planktonically. The intrinsic biofilm formation in each propagation medium was determined in addition to the biofilm formation of lineages adapted to biofilm and planktonic growth every ten transfers. Statistical differences in biofilm formation between propagation medium and lifestyle over the passage series were detected via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as mean OD_{595}, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, free datapoints show outliers, n = 8.

The optimum experimental regime for selecting for biofilms with decreased susceptibility to antimicrobial agents was determined (Table 3.1.). Lineages were either adapted to a static subinhibitory -2–log_2 fold MIC stress or adapted in a stepwise manner which increased by a log_2 fold every three passages for 30 transfers. Selection for increased biofilm formation in the naïve lineages adapted without antimicrobial stress did not have any impact on intrinsic susceptibility to any agent. Stepwise adaptation selected for larger log_2-fold reductions in susceptibility than static adaptation. Ciprofloxacin-adapted strains were characterised by the largest log_2-fold

reductions in susceptibility out of all selective agents tested. After 30 transfers, the statically-adapted strains were between five- to seven-log_2 fold less susceptible to ciprofloxacin. However, all stepwise-adapted strains were over seven-log_2 fold less susceptible to ciprofloxacin which was the highest concentration assayed, and all survived to the end of the passage series. Static adaptation to colistin was only able to select for a two-log_2 fold reduction in susceptibility in a single lineage. Whereas all stepwise adapted strains demonstrated significant reductions in susceptibility between three- and five-log_2 fold. No significant changes in susceptibility to either metal compound was observed from either static- or stepwise-adaptation. However, in benzalkonium chloride-adapted lineages, stepwise adaptation was able to select for five-log_2 fold reductions in susceptibility whereas no change in susceptibility was observed in any of the statically-adapted lineages. Reductions in susceptibility in adapted strains were generally lower than the magnitude of stress at growth failure. For example, stepwise-adapted lineages selected under benzalkonium chloride stress were able to grow up to a four-log_2 fold above the ancestral MIC but were only two-log_2 fold less susceptible when tested. All stepwise-adapted lineages adapted to benzalkonium chloride or copper sulfate failed at the same transfer, when the magnitude of stress increased from four- to five-log_2 fold and one- to two-log_2 fold MIC, respectively. However, parallel lineages adapted to colistin and zinc sulfate showed disparate attrition concentrations. The two stepwise-adapted lineages with three-log_2 fold reduction in colistin susceptibility failed at the increase in stress to seven-log_2 fold MIC, whereas the lineages with five-log_2 fold reductions survived to the end of the experiment. Furthermore, though the magnitude of reduced susceptibility was correlated with duration of survival in colistin-adapted lineages, this was not the case in zinc sulfate-selected lineages. All stepwise-adapted lineages demonstrated a non-significant fold reduction in susceptibility however the growth failure varied between transfer 15 and 21.

Table 3.1. Susceptibility of experimentally evolved biofilms of *P. aeruginosa* adapted to a number of selective agents either at a static -1–fold MIC concentration or concentrations increasing in a stepwise fashion from -2– to 6– log_2 fold over 30 transfers or until growth failure.

Selective Agent	Lineage	Log_2 Fold Change in MIC from Ancestor After Adaptation*		
		Static Adaptation	Stepwise Adaptation†	Naïve
Ciprofloxacin	1	**5**	**> 7** (survived)	0
	2	**7**	**> 7** (survived)	0
	3	**7**	**> 7** (survived)	0
	4	**6**	**> 7** (survived)	0
Colistin	1	0	**3** (transfer 27)	0
	2	0	**3** (transfer 27)	0
	3	0	**5** (survived)	0
	4	**2**	**5** (survived)	0
Zinc sulfate	1	0	1 (transfer 18)	0
	2	0	1 (transfer 15)	0
	3	-1	1 (transfer 21)	0
	4	-1	1 (transfer 21)	0
Copper sulfate	1	0	0 (transfer 12)	0
	2	0	0 (transfer 12)	0
	3	0	0 (transfer 12)	0
	4	0	0 (transfer 12)	0
Benzalkonium chloride	1	0	**2** (transfer 21)	0
	2	0	**2** (transfer 21)	0
	3	0	**2** (transfer 21)	0
	4	0	**2** (transfer 21)	0

*Significant changes in susceptibility (≥2-log_2 fold change from ancestral MIC) shown in bold.

†Brackets show transfer at which growth failure occurred, ‘survived’ denotes populations which remained viable at the end of the passage series.

3.3. Discussion

To gain an understanding into how the selective forces intrinsic to the biofilm lifestyle can dictate the evolution of antimicrobial resistance, an experimental model was required where both selective pressures could interface through natural selection. To incorporate both of these elements, the biofilm evolution platform described here was adapted from an experimental evolution model originally devised by Poltak and Cooper (2011) to investigate ecological succession in *Burkholderia cenocepacia* biofilms. Poltak and Cooper (2011) observed that the serial passage of biofilms grown on bead substrata was able to select for increased biomass production, productivity and phenotypic diversification. Thus, by facilitating repeated colonisation, growth and dispersal, a selective pressure towards increased biofilm formation is conferred. We aimed to leverage this intrinsic selective pressure associated with serial passage of biofilms by introducing antimicrobial stress and observing the effect of selective pressures in competition on evolutionary outcome. This chapter aimed to establish the tractability of this experimental evolution platform for experimentally modelling such selective interactions. The platform must demonstrate a positive selective effect on biofilm formation in the model organism used in this project, *P. aeruginosa* UCBPP-PA14. Further to this, it must also be established that the acquired resistance to antimicrobials can be selected during serial passage of biofilms. Without the fulfilment of these criteria, the scope of the biofilm evolution platform is limited, and the selective dynamics between antimicrobial resistance and biofilm formation cannot be adequately assessed.

3.3.1. Biofilm Cultivation and Propagation

Several parameters of experimental design were systematically validated to demonstrate the tractability of the use of an experimental evolution platform for the adaptation of biofilms of *P. aeruginosa* to selective pressures. Firstly, the optimum culture conditions for the cultivation of biofilms on glass, PVC and type-316 clinical-grade stainless steel substrates were determined. Figure 3.1. establishes that the optimum incubation period to achieve mature, productive biofilms was 24 hours. Furthermore, longer incubation periods were generally associated with reductions in biofilm productivity. Biofilms were cultivated in 1 mL of liquid media in order to increase the surface area: volume ratio and maximise the vicinity of the biofilm-seeded bead to the air: liquid interface (Chang et al., 2015). This low volume likely leaves most nutrients in the media expended by 24 hours, leading to entry into death phase as toxic

growth byproducts accumulate, yielding successive decreases in biofilm productivity over time. Furthermore, figure 3.1. also demonstrates that lower temperatures were found to be universally superior in achieving greater biofilm productivity. Temperature is a key regulator of the motile-sessile switch. In human pathogens such as *S. aureus, Listeria monocytogenes* and *Enterobacteriaceae*, physiologically-relevant temperatures are a broadly conserved signal for the lifestyle transition to a motile, invasive phenotype (Lam et al., 2014). This involves activation of stress responses such as H-NS, the alternative σ-factor RpoH and heat-shock proteins GroEL which upregulate pathogenicity islands, flagellar biosynthesis, type III secretion and iron scavenging. Conversely, sub-physiological temperatures have been observed to stimulate formation of biofilms in *Vibrio cholerae*, *B. pseudomallei* and *S. epidermidis* (Fitzpatrick et al., 2005, Ramli et al., 2012, Römling et al., 2013). Room temperature was associated with an increase in expression of *algD, psl* and *pel* in *P. aeruginosa*, in addition to elevated levels of c-di-GMP relative to incubation at 37 °C (Kim et al., 2020). At sub-optimal temperatures, six GGDEF-domain containing diguanylate cyclases which synthesise c-di-GMP were found to upregulated in *V. cholerae*, *cdgA/H/K/L/M*, and *vpvC* (Townsley and Yildiz, 2015). It was hypothesised that the membrane-bound sensor domains of these enzymes could detect temperature-induced changes in phospholipid fluidity to activate c-di-GMP synthesis and induce biofilm formation at sub-physiological temperatures. Based on these findings, incubation at 25 °C for 24 hours were used as the optimum biofilm cultivation conditions.

There was little difference between the culture medium and productivity of the biofilm. However, intrinsic productivity is not the only consideration when deciding an optimal propagation medium for biofilm evolution experiments. The medium used must demonstrate that biofilm formation is under positive selection which manifests as the increased secretion of matrix biomass as determined via crystal violet staining. Mutants which overproduce biomass are termed ‘biofilm hyperproducers’ and biomass production is used as the main metric to quantify the degree of biofilm formation as used elsewhere (Cattelan et al., 2017, Deng et al., 2020, Gloag et al., 2019). Establishing the optimum conditions for selection of biofilm hyperproduction is necessary to determine the effect of antimicrobial stress on biofilm formation, and *vice versa*. Figure 3.3. shows some culture media demonstrably inferior for the selection of biofilm hyperproduction. Growth of the ancestral strain in Mueller-Hinton broth and brain-heart infusion broth supported significantly higher biofilm formation than LB, LB without salt or M9 minimal medium. Corroborating these observations, Wijesinghe et al. (2019) also observed that *P. aeruginosa* demonstrated higher biofilm formation in

brain-heart infusion than LB. A complex medium such as brain-heart infusion is rich in nitrogen, carbon and growth factors derived directly from calf brain and beef heart. In comparison, defined media such as LB and M9 minimal contain only a carbon source, salts and essential amino acids. Such stark nutritional differences can confer broad transcriptional changes affecting central physiological properties, including in biofilm formation (Blair et al., 2013, Folsom et al., 2010). Despite this, neither brain-heart infusion nor Mueller-Hinton broth were able to consistently select for increased biofilm formation. Therefore, there is a clear disparity between the ability to cultivate a biofilm and select for biofilm hyperproduction. It is possible that conditions which intrinsically support the production large amounts of biomass weaken selection for biofilm hyperproduction as mutants cannot outcompete the ancestor. Indeed, the greatest increases in biofilm formation were yielded from biofilm adapted in LB, which supported markedly lower intrinsic biofilm formation than Mueller-Hinton or brain-heart infusion.

Importantly, LB did not select for increased biofilm formation through serial passage in planktonic culture, unlike LB without salt and M9 minimal. The planktonic lineages were grown in 1 mL of media without any bead substrates and serially passaged via 1:100 dilution to fresh media. Significant increases in biofilm formation in the planktonically-adapted lineages could be a result of inadvertent passage of pellicle biofilms. Pellicles are biofilm flocs which form at the air: liquid interface mainly characterised in *Bacillus subtilis*, however, *P. aeruginosa* is also known to form pellicles of which the Pel polysaccharide is an essential constituent (Armitano et al., 2014). Therefore, due to the high surface area: volume ratio of culture, pellicles may have formed and been carried through the experiment when the surface tension of the broth was broken to transfer the culture. In light of these findings, figure 3.3. demonstrates that LB was the optimum biofilm propagation medium based on its strong capacity to select or biofilm hyperproduction exclusively in the biofilm lifestyle.

3.3.2. Adaptation of Biofilms to Antimicrobials

It is an essential criterion of the model for biofilms to be successfully passaged under an antimicrobial selective pressure with adherence, colonisation and dissemination achieved at high productivity during each transfer. As the population bottleneck is largely fixed by transfer of a biofilm-colonised bead, the main determinant which can be controlled to modulate the effective population size is antimicrobial stress. Therefore, the stress must not be too punitive to prevent the biofilm from being able to disseminate and recolonise a new environment, but also strong enough to confer a

selective pressure for development of resistance. In order to establish the sustainability of serially propagating biofilms under antimicrobial stress, the productivity of a biofilm was assessed over four transfers at antimicrobial concentrations ranging from sub-inhibitory to super-inhibitory. Three antimicrobials with different applications and modes of action were investigated, including ciprofloxacin, a DNA replication-inhibiting fluoroquinolone antibiotic with a highly specific mode of action via inhibition of DNA gyrase (Sanders, 1988). Non-therapeutic antimicrobials of which the adaptive capacity remains poorly characterised were also investigated. This included zinc sulfate, a porcine feed additive which exerts a non-specific mode of action characterised by induction of ROS and cytosolic coagulation, and benzalkonium chloride, a common non-specific membrane-active biocide (Merchel Piovesan Pereira and Tagkopoulos, 2019, Sirelkhatim et al., 2015). Figure 3.2. demonstrates a clear disparity in the relative concentration between the agents at which attenuation of productivity begins to occur which likely represents the different modes of action of each agent. Ciprofloxacin did not demonstrate a significant reduction in productivity except at 2-$\log_2$ fold MIC whereas zinc sulfate and benzalkonium chloride demonstrated comparable reductions in susceptibility at 1-$\log_2$ fold MIC and at the MIC, respectively. Antibiotics exhibit their antimicrobial action mainly through inhibition of a specific macromolecular target and such competitive binding can yield dose-dependent inhibitory effects (Eyler and Shvets, 2019). In contrast, biocides and metals confer gross cellular damage through membrane disruption, cytosolic coagulation and formation of reactive oxygen species (McDonnell and Russell, 1999). By virtue of their non-specific modes of action, such agents are often associated with inhibitory thresholds, where the transition from sub-inhibitory, to inhibitory and bactericidal concentrations can be observed in a non-linear fashion (Maillard, 2007). As a result, it is not surprising that zinc sulfate and benzalkonium chloride exerted greater bactericidal effects than ciprofloxacin. In spite of this, zinc sulfate was markedly better tolerated than benzalkonium chloride which rapidly went from no effect to complete eradication within two doubling dilutions. Zinc is an essential trace element in bacterial cells which acts as co-factors in many metabolic processes but is toxic in excess, therefore mechanisms to regulate its intracellular concentration are extensive (Gonzalez et al., 2019). In *P. aeruginosa*, the transcriptional repressors Zur and CzcR are the primary regulators of zinc uptake which de-repress the respective efflux pumps ZnuABC and CzcCBA to alleviate zinc toxicity (Ducret et al., 2020, Pederick et al., 2015). Consequently, such mechanisms may account for the difference in tolerance between zinc sulfate and benzalkonium chloride, a synthetic compound to which bacteria lack the benefits of billions of years of

evolution to tolerate (Harrison et al., 2007). This process likely also explains the rapid amelioration of productivity observed across such a short passage series. Within one or two transfers, all conditions which conferred a significant reduction in productivity demonstrated a significant increase in viability. Furthermore, this was generally a two-step process with an additional recovery of productivity occurring by transfer 4. Amelioration of antimicrobial stress can occur in such short timeframes via transcriptional activation of stress responses to confer transient phenotypic resistance. Such mechanisms are widespread in bacteria; in addition to the aforementioned metal export systems, multidrug transporters also exist which can be induced by ciprofloxacin and cationic surfactants such as benzalkonium chloride (Corona and Martinez, 2013). Moreover, these mechanisms are often targets for selection as constitutive de-repression can permit larger reductions in susceptibility than substrate-dependent induction. Acquisition of *de novo* resistance was only observed in biofilms adapted to ciprofloxacin, there was no significant change in MIC observed in zinc sulfate- or benzalkonium chloride-adapted lineages after four transfers (appendix 1.). Nevertheless, this demonstrates that biofilms can be successfully passaged under concentrations of antimicrobials which exhibit inhibitory action.

With the tractability of serial transfer of biofilms under antimicrobial stress established, selection for constitutive resistance over a longer passage series was attempted. In addition to ciprofloxacin, zinc sulfate and benzalkonium chloride, colistin, a membrane-active polymyxin antibiotic, and copper sulfate, another common feed additive, were also used. Two exposure regimes were used, a static subinhibitory stress 0.5× MIC and a stepwise training regime which increased from -2– to 6– $\log_2$ fold MIC every three passages or until growth failure. It has been documented the minimum concentration which can still exert a selective pressure can be orders of magnitude lower than the MIC (Gullberg et al., 2011, Stanton et al., 2020). However, much of this work has been conducted with antibiotics which possess highly specific modes of action and thus can achieve large reductions in susceptibility readily. It is not known if subinhibitory concentrations of non-therapeutic agents have the same selective impact given their differences in mode of action and mechanisms of resistance. Therefore, an exposure regime which trained lineages of biofilms to achieve decreases in susceptibility through stepwise increases in antimicrobial concentration at regular intervals was also used. Stepwise adaptation has been used extensively to determine the maximum adaptive capacities of biocides. (Gadea et al., 2017, Jahn et al., 2017, Kampf, 2018). Therefore, this protocol may be more appropriate to select for adaptation to agents with non-specific modes of action. Table 3.1. shows that there

was no change in susceptibility in any of the unstressed naïve lineages indicating that biofilm hyperproduction was not associated with intrinsic resistance to antimicrobials. The biofilm lifestyle is generally considered to be a state which protects bacteria from antimicrobial insults (Flemming et al., 2016). However, there is nuance to this observation which may explain why drug resistance was not recapitulated in the naïve lineages. The most important determinants of altered susceptibility in biofilms *in situ* appear to be a result of persisters and intrinsically resistant taxa in polymicrobial communities (Hall and Mah, 2017). The role of the EPS in reduced susceptibility to antimicrobials is now believed to be limited. Therefore, as this experiment was conducted in monoculture and if biofilm hyperproduction does not have an impact on persister fractions, changes in susceptibility would be limited, as observed here. Table 3.1. also demonstrated that stepwise adaptation to antimicrobials was associated with a greater capacity to selected for reduced susceptibility to antimicrobials than static, sublethal exposure. Static adaptation was only able to select for decreased susceptibility to ciprofloxacin and in one colistin-adapted lineage. No changes in susceptibility were observed in any zinc sulfate-, copper sulfate- or benzalkonium chloride-adapted lineages. It is possible that the subinhibitory concentration used in the static regime did not adequately capture the optimum selective window due to the non-linear inhibitory effect of antimicrobials which do not possess specific targets. In contrast, in addition to ciprofloxacin, stepwise adaptation selected for decreased susceptibility to colistin and benzalkonium chloride in all lineages. Furthermore, stepwise adaptation selected for larger reductions in susceptibility than the statically adapted counterparts, driven by the super-inhibitory stress toward the end of the experiment. On the basis of these strengths, table 3.1. established that the stepwise adaptation regime possessed a superior selective capacity for reduced susceptibility to antimicrobials, especially agents with non-specific modes of action such as benzalkonium chloride. Therefore, the stepwise regime was selected as the antimicrobial exposure regime for all future evolution experiments.

3.3.3. Conclusions

The experimental evolution platform described here offers a comprehensively validated model to explore the adaptation of biofilms under antimicrobial stress. The final parameters of the model were chosen as they yielded the most productive biofilms, conferred an inherent selective pressure for increased biofilm formation and possessed the capacity to select for antimicrobial resistance. These experiments also highlighted some noteworthy observations which should be investigated further. Most notably, this includes that biofilm hyperproduction can be readily selected but does not seem to be associated with altered antimicrobial susceptibility and that there is an adaptive disparity between non-therapeutic antimicrobials and antibiotics. These findings will be explored further in the coming chapters.

CHAPTER 4. ECOLOGICAL SUCCESSION AND SELF-GENERATED DIVERSITY IN EXPERIMENTALLY-EVOLVED BIOFILM HYPERPRODUCERS

4.1. Introduction

The evolution of free-living planktonic cells is defined by extraneous selective pressures exerted at the individual scale; it is generally more advantageous to selectively exclude competitors than to cooperate when members of a population are functionally independent (Martin et al., 2016). In contrast, members of a biofilm are localised within a community which share space, nutrients and adverse environments such as suboptimal growth conditions or antimicrobial insults. In such circumstances, it can be more beneficial to cooperate with other members of the community in order to achieve increases in fitness than to compete (Brockhurst et al., 2006). As a result, the emergent properties which distinguish biofilms from planktonic cells also elicit selective forces intrinsic to the biofilm lifestyle.

Biofilm specialisation carries two main selective hallmarks, ecological diversification and the development of sociality. Both of these characteristics are facilitated by the intrinsic physiological heterogeneity in biofilms given rise by gradients in solute chemistry and the partitioning of cells into microcolonies (Kierek-Pearson and Karatan, 2005). Microcolonies are the basic structural unit of the biofilm; they are generally clonal, founded by a single cell and segregated from neighbouring microcolonies by interstitial voids (Donlan, 2002). Microcolonies are subject to steep physicochemical gradients of gases, nutrients, metabolites, pH and water whereby reaction-diffusion interactions produce discrete microenvironments which can vary drastically over micrometre ranges (Stewart and Franklin, 2008). As a consequence, cells adaptively form transcriptionally -specialised subpopulations in order to acclimate to their specific niche. Across the community, this gives rise to a large repertoire of distinct phenotypes which can be phenotypically stratified based on growth rates, nutrient utilisation and metabolic output (Bisht and Wakeman, 2019). Strains adapted to form biofilms have been shown to exploit this emergent property to yield subpopulations which are constitutively specialised to their role, such as matrix production, motile colonisers or genetic competence (Lopez et al., 2009, Madsen et al., 2015, Zhang et al., 2016). Furthermore, phenotypic diversification of the biofilm also occurs as a corollary of greater complexity within the community, which yields insurance effects against environmental perturbations (Boles et al., 2004, Poltak and Cooper, 2011).

Social interactions in biofilms can be best characterised by the division of labour between subpopulations. This refers to the collective fitness benefits which cannot be achieved by generalists, realised through the allocation of essential tasks to specialists. Sharing of public goods are the main mechanism by which positive social interactions are elicited by the biofilm lifestyle as they alleviate the energy burden associated with

the individual responsibility of synthesising secreted products (Fredrickson, 2015). The most fundamental public good shared in a biofilm is the extracellular matrix, and mechanisms which divide matrix production to specialists to achieve higher-order fitness advantages have been widely observed (Dragoš et al., 2018b, Dragoš et al., 2018a, Klausen et al., 2003). For example, heterogeneity in c-di-GMP levels in *P. aeruginosa* biofilms generated by differential expression of the Wsp surface-sensing cascade yields distinct, spatially organised subpopulations. A high c-di-GMP subpopulation serves as the sessile matrix producer, whereas a low c-di-GMP population plays a role in motility-dependent exploration to promote the interstitial expansion of the biofilm (Armbruster et al., 2019). Elimination of either subpopulation possesses a deleterious effect on the capacity for colonisation of surfaces thus, both subpopulations exist in an equilibrium stabilised by the potentiated fitness gains they confer to the community at large. Mutations in the *wsp* system have been shown to be selected through serial passage of biofilms of *P. fluorescens* (Kim et al., 2016). This induces the constitutive formation of a specialised subpopulation which exists in a negative frequency-dependent equilibrium with the ancestor. The *wspA* mutant, being c-di-GMP signalling compromised, is characterised by weak EPS production but maintains motility. The two subpopulations are localised with the *wspA* mutant sitting on top of the matrix-producing ancestor. The *wspA* mutant supports biofilm formation, despite contributing little to the matrix ultrastructure, by mechanically pushing the ancestor outwards leading to faster expansion of the colony. The importance of this relationship is highlighted by the bidirectional selection for the two subpopulations. Serial passage of biofilms of the *wspA* mutant selects for revertants with restored c-di-GMP signalling functionality, which also enter a stable equilibrium with the *wspA* mutant.

Cooperative action in biofilms is not altruistic, the fitness gains achieved by concerted effort can only be selected if robust enough against displacement by selfish mutants known as ‘cheaters’ (Brockhurst et al., 2006). This is known as the public goods dilemma which posits that selfish matrix-nonproducing mutants could achieve the benefits of the biofilm lifestyle without the associated energy expenditure (Drescher et al., 2014). Despite this, matrix cheaters are not well represented in biofilms of *P. aeruginosa* as they cannot acquire oxygen as effectively as matrix producers (Nadell et al., 2015, Xavier and Foster, 2007). As cells are most active at the dynamic boundary of a biofilm, cheaters must be continuously replenished at the surface to avoid suffocation by deposited EPS. This bottleneck polices the fixation of cheaters within a

population as a mechanism to prevent establishment of parasitism within the community which would destabilise the biofilm (Dragoš et al., 2018b).

Understanding the evolutionary dynamics intrinsic to biofilms yields valuable insights into the pathophysiology of a number of clinically-relevant processes. Biofilm-associated infections are often characterised by long-term chronicity which facilitates evolutionary change over a single infectious period (Viberg et al., 2017). This is best characterised in the pathogenesis of *P. aeruginosa* infection in the cystic fibrosis lung. Approximately 70% of adult cystic fibrosis sufferers carry *P. aeruginosa* due to its unique capacity to survive the hostile environment of the cystic fibrosis lung (Winstanley et al., 2016). The initial acquisition of *P. aeruginosa* in cystic fibrosis sufferers typically occurs from an environmental reservoir during childhood, manifesting as a mild upper respiratory tract infection to moderate pneumonia (Zemanick et al., 2015). Failure to eradicate *P. aeruginosa* when symptomatic results in periodic exacerbations until a transition to a subclinical state achieved via the selection of mucoidy through constitutive overproduction of alginate (Govan and Deretic, 1996). Mucoidy confers the characteristic persistence of *P. aeruginosa* in the face of the host immune response, oxygen tension, osmotic stress and competition with respiratory commensals in the cystic fibrosis lung (Folkesson et al., 2012).

Mucoidal strains of *P. aeruginosa* do not form biofilms *per se* in anatomical settings and it is spurious reasoning to assume the prototypical biofilm lifecycle applies to *in vivo* communities (Bjarnsholt et al., 2013). The monospecies communities of *P. aeruginosa* in the cystic fibrosis lung are observed as aggregate microcolonies suspended in sputum. Indeed, mucoidal conversion is associated with selection directly on the AlgU alginate biosynthesis pathway, most notably *mucA*, rather than pleiotropic regulators of biofilm formation such as diguanylate cyclases, phosphodiesterases and c-di-GMP signalling cascades (Boucher et al., 1997, Martin et al., 1993, Martin et al., 1994). This indicates that some aspects of the biofilm lifestyle may be disadvantageous to survival *in vivo*, providing rationale that surface-associated biofilms and aggregate communities in the cystic fibrosis lung are functionally distinct. Nevertheless, these aggregates share conserved emergent properties with surface-associated biofilms, including a strong capacity for further evolutionary change (Bianconi et al., 2019). *P. aeruginosa* isolates from chronic infections of the cystic fibrosis lung possess staggering within-population diversity. This has been attributed to the complex and heterogenous ecological conditions of the cystic fibrosis lung which promotes opportunities for diversification to occupy vacant niches (Schick and Kassen, 2018). Such diversity has been shown to provide the evolutionary amplitude to adapt to

changes in environmental conditions, most notably antimicrobial chemotherapy (Kordes et al., 2019). Indeed, drug resistance can be generated from the pleiotropic effect of adaptation to a cystic fibrosis lung-like environment, even in absence of antibiotic selection (Schick and Kassen, 2018). The importance of phenotypic diversification in chronic infection is exemplified by the close association of long-term cystic fibrosis lung colonisers and acquisition of hypermutator phenotypes (Oliver et al., 2000). Hypermutators are associated with late-stage infection and may act as a mechanism to achieve continued diversification in spite of the diminishing returns on fitness associated with long-term adaptation (Ciofu et al., 2012, Elena and Lenski, 2003). Consequently, this clinically relevant biofilm-like phenotype facilitates self-generated diversity which reveals more trajectories to traverse the highly deformable fitness landscape associated with the cystic fibrosis lung.

Self-generated diversity has also been extensively observed in experimental populations of biofilm adapted to growth at a solid: liquid interface (Flynn et al., 2016, Kirisits et al., 2005, Penterman et al., 2014, Poltak and Cooper, 2011, Savage et al., 2013). However, despite surface-associated biofilms such as medical device-related infections possessing comparable chronicity and recalcitrance as cystic fibrosis lung infections, little work has been conducted investigating how evolved sociality contributes to pathogenesis. Similarly, the capacity for biofilm formation is broadly correlated with persistence of pathogens in clinical and industrial environments (Lehner et al., 2005, Heckman and Soto, 2021, Vestby et al., 2009). However, whether an *ex vivo* lifestyle selects for biofilm hyperproduction, or if persistent strains possess a biofilm hyperproducer phenotype beforehand remains unknown. This chapter aims to select for biofilm hyperproducers on bead substrates of various clinically- and industrially-relevant materials in order to identify the molecular mechanisms underpinning biofilm hyperproduction and self-generated diversity. The collateral phenotypic traits associated with adaptation to a biofilm lifestyle, including on fitness, antimicrobial susceptibility and stress tolerance are also investigated.

4.2. Results

4.2.1. Experimentally evolved biofilm hyperproducers form more biomass but do not become more productive or less fit in broth

The effect of serial subculture of biofilms on different bead substrata on biofilm formation was determined (figure 4.1.). None of the planktonically-adapted lineages demonstrated a significant increase in biofilm formation relative to the ancestor at any timepoint (ancestor vs planktonically-adapted transfer 10: $p = 0.9973$, ancestor vs planktonically-adapted transfer 20: $p > 0.9999$, ancestor vs planktonically-adapted transfer 30: $p = 0.9978$). The stainless steel-adapted lineages were significantly better at forming biofilms than the ancestor at all timepoints (ancestor vs stainless steel-adapted transfer 10: $p = 0.0326$, ancestor vs stainless steel-adapted transfer 20: $p < 0.0001$, ancestor vs stainless steel-adapted transfer 30: $p < 0.0001$). The glass- and PVC-adapted lineages were significantly better at forming biofilms by transfer 20 (ancestor vs glass-adapted transfer 20: $p = 0.0005$, ancestor vs PVC-adapted transfer 20: $p < 0.0001$) and 30 (ancestor vs glass-adapted transfer 30: $p = 0.0002$, ancestor vs PVC-adapted transfer 30: $p < 0.0001$) but not at transfer 10 (ancestor vs glass-adapted transfer 10: $p = 0.2461$, ancestor vs glass-adapted transfer 10: $p = 0.4624$). A significant increase in biofilm formation between transfer 10 and 20 was observed in PVC- and stainless steel-adapted lineages (PVC-adapted transfer 10 vs transfer 20: $p = 0.0003$, stainless steel-adapted transfer 10 vs transfer 20: $p = 0.0194$). No significant change in biofilm formation was observed between transfer 10 and 20 in glass-adapted lineages ($p = 0.1489$). There was no further increase in biofilm formation observed between transfer 20 and 30 in any selective condition (glass-adapted transfer 20 vs transfer 30: $p > 0.9999$, PVC-adapted transfer 20 vs transfer 30: $p > 0.9999$, stainless steel-adapted transfer 20 vs transfer 30: $p = 0.8798$). At transfer 30, the substrate-adapted biofilms produced on average 2× to 3× more biomass than the ancestor. There was no significant difference in biofilm formation between any of the selective substrates (glass-adapted transfer 30 vs PVC-adapted transfer 30: $p = 0.9894$, glass-adapted transfer 30 vs stainless steel-adapted transfer 30: $p = 0.0500$, PVC-adapted transfer 30 vs stainless steel-adapted transfer 30: $p = 0.6791$).

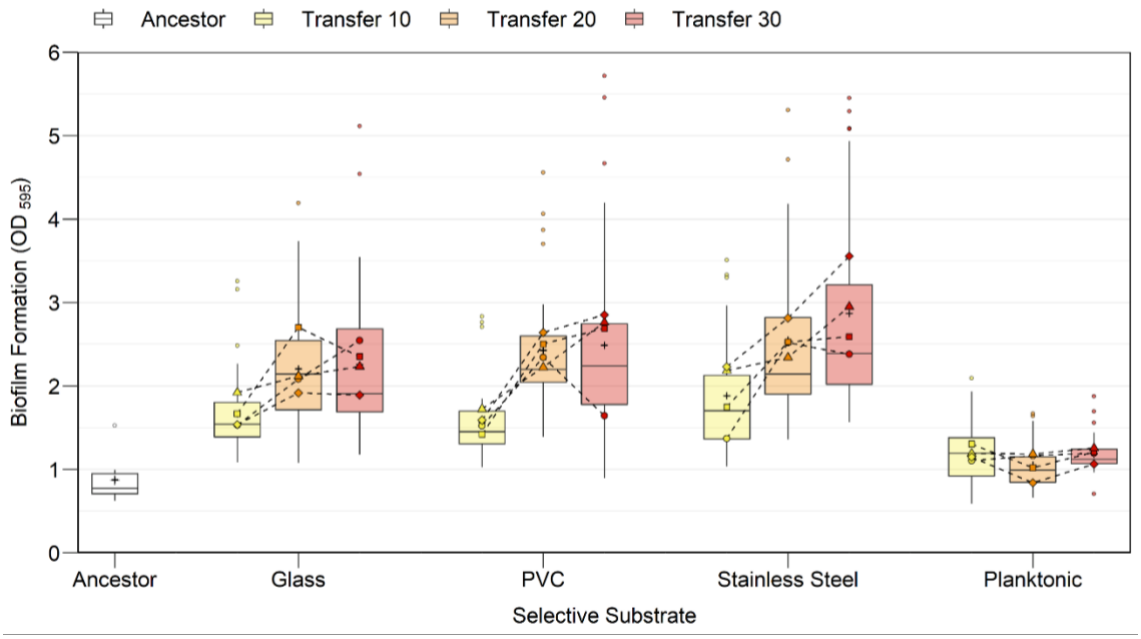

Figure 4.1. Biofilm formation as measured via crystal violet of experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates in LB for 30 transfers. Statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparisons post-hoc test. Data shown as mean OD_{595}, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 8 per lineage.

The effect of biofilm hyperproduction on biofilm productivity, as defined by cell density per unit area of substrate, was determined (figure 4.2.). Approximately 50,000 to 500,000 cells per mm^2 colonised the bead-cultivated biofilm. There was no significant difference between the ancestor and the planktonically-adapted lineages grown on any substrate at any timepoint (ancestor vs planktonically-adapted transfer 10: $p = 0.9619$, ancestor vs planktonically-adapted transfer 20: $p = 0.3215$, ancestor vs planktonically-adapted transfer 30: $p = 0.5957$). Biofilms grown on glass substrates were approximately 1-log_{10} more productive than PVC or stainless steel substrates (glass-adapted transfer 30 vs PVC-adapted: $p > 0.0001$, glass-adapted transfer 30 vs stainless steel-adapted $p > 0.0001$). However, there was no significant difference between the ancestor and biofilm lineages adapted to any selective substrate at any timepoint (ancestor on glass substrate vs glass-adapted across all timepoints: $p = 0.6268$, ancestor on PVC substrate vs PVC-adapted across all timepoints: $p > 0.9999$, ancestor on stainless steel substrate vs stainless steel-adapted across all timepoints: $p = 0.0842$).

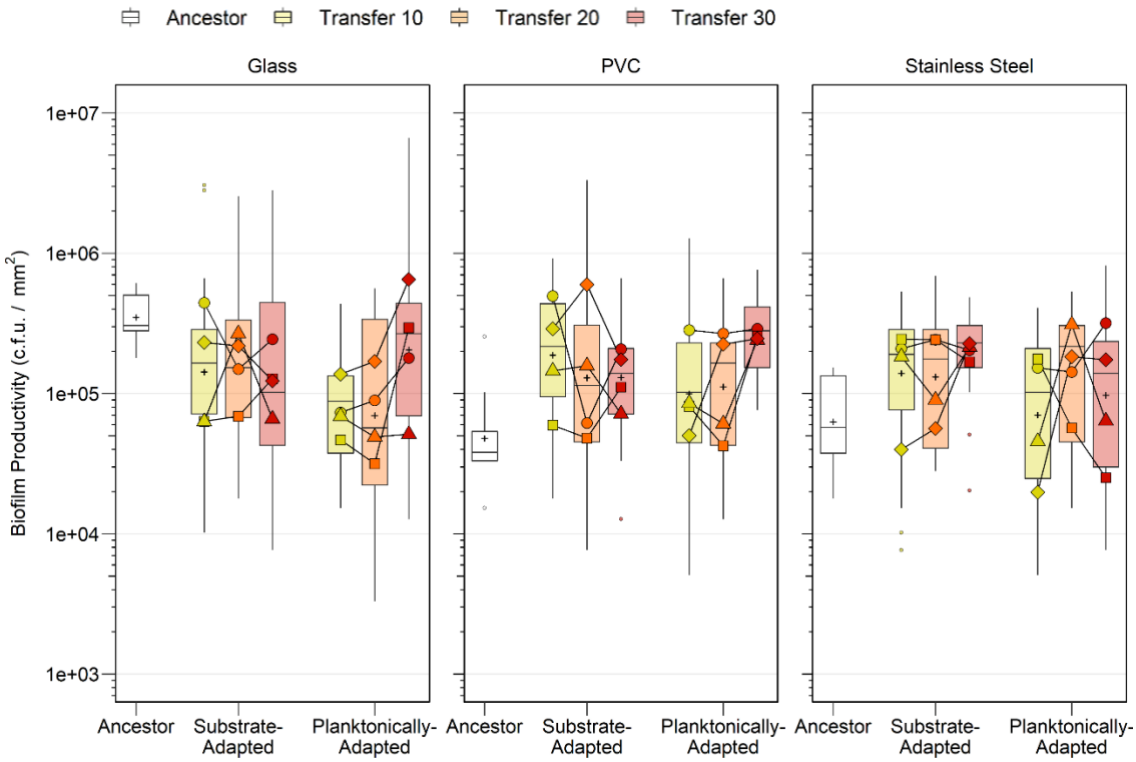

Figure 4.2. Biofilm productivity of experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates in LB for 30 transfers. Statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as mean c.f.u., box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

Gross fitness changes associated with biofilm hyperproduction were assessed by measuring the integral area under curve (AUC) of growth curves in broth (figure 4.3.). There was no significant change in AUC associated with adaptation to any of the selective substrates as a biofilm over the course of the passage series (planktonically-adapted vs glass-adapted: $p > 0.9999$, planktonically-adapted vs PVC-adapted: $p > 0.9999$, planktonically-adapted vs stainless steel-adapted: $p = 0.1218$). However, by transfer 30, the planktonically-adapted lineages became significantly more fit than at transfer 10 ($p = 0.0182$) and were significantly more fit than the biofilm-adapted lineages at transfer 30 (planktonically-adapted transfer 30 vs glass-adapted transfer 30: $p = 0.0047$, planktonically-adapted transfer 30 vs PVC-adapted transfer 30: 0.0056, planktonically-adapted transfer 30 vs stainless steel-adapted transfer 30: < 0.0001).

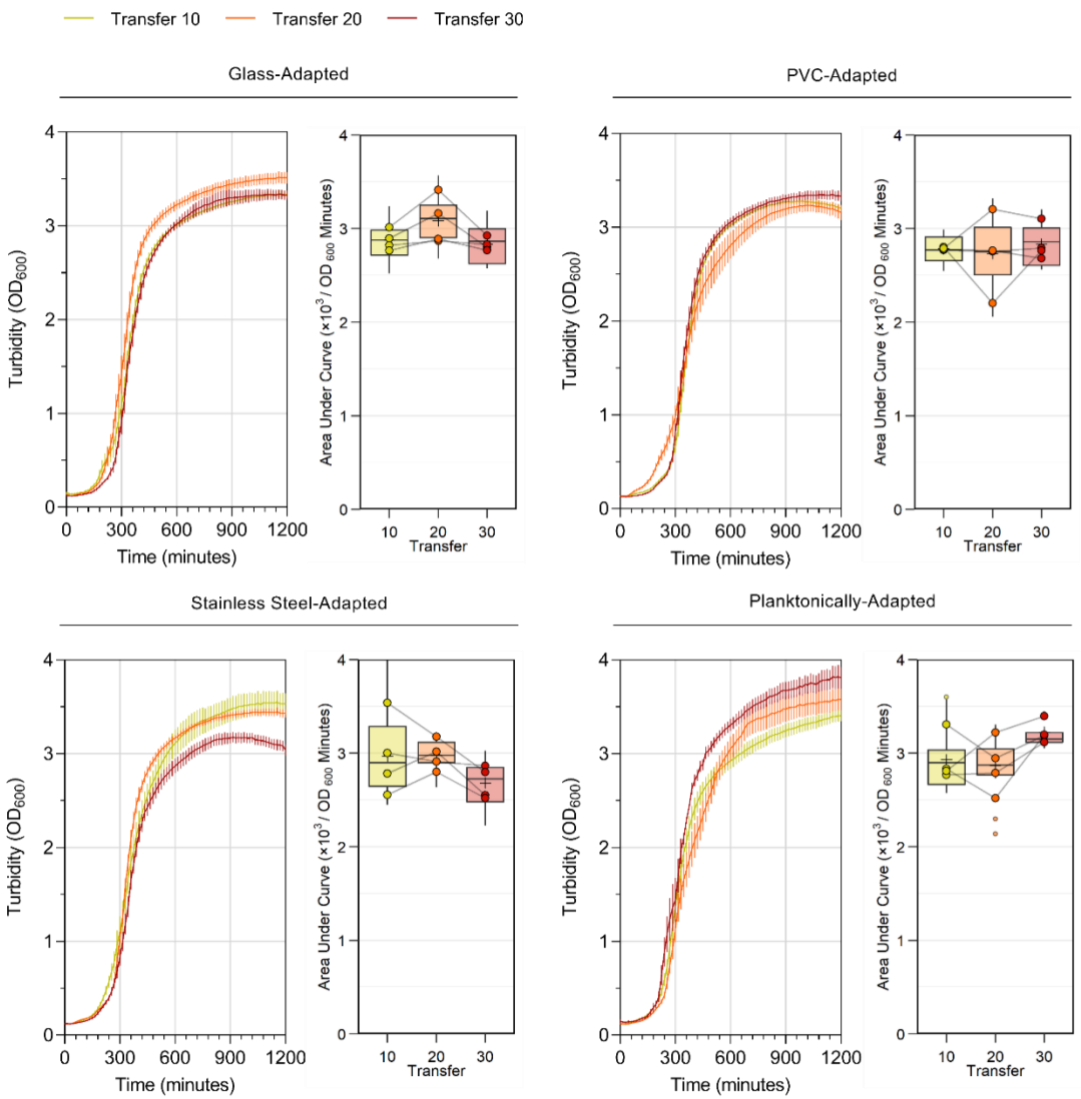

Figure 4.3. Growth kinetics of experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates in LB for 30 transfers. The area under the curve was calculated and statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparisons test. By transfer 30, the planktonically-adapted lineages became significantly more fit than they were at transfer 10 and were significantly more fit than the biofilm-adapted lineages at transfer 30. There was no significant change in AUC associated with adaptation to any of the selective substrates as a biofilm at any timepoint. Curves show mean OD_{600}, ± standard error of the mean, Boxplot shows area under curve, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

4.2.2. Biofilm hyperproducers do not demonstrate reduced susceptibility to antibiotics, but become more tolerant to salt stress and less tolerant to alkaline stress

There was no change in the minimum inhibitory concentration or biofilm eradication concentration of any agent observed in the experimentally evolved strains in any of the selective conditions (Table 4.1.).

Table 4.1. Drug susceptibility of experimentally evolved endpoint populations of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates.

Selective Substrate		Minimum Inhibitory Concentration (µg/ mL)							Minimum Biofilm Eradication Concentration (µg/ mL)						
		Piperacillin	Ceftazidime	Meropenem	Aztreonam	Ciprofloxacin	Tobramycin	Colistin	Piperacillin	Ceftazidime	Meropenem	Aztreonam	Ciprofloxacin	Tobramycin	Colistin
Ancestor		2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
Glass-adapted	Lineage 1	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 2	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 3	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 4	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
PVC-adapted	Lineage 1	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 2	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 3	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 4	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
Stainless Steel-adapted	Lineage 1	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 2	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 3	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 4	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
Planktonically-adapted	Lineage 1	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 2	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 3	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32
	Lineage 4	2	2	0.25	2	0.06	2	0.5	128	64	16	32	1	32	32

The effect of biofilm hyperproduction on tolerance to salt stress was determined (figure 4.4.). Supplementation of growth media with 9% w/v salt conferred an approximate 4-$\log_{10}$ reduction in viability in the ancestral strain. There was no significant change in salt tolerance in the planktonically-adapted lineages ($p = 0.1020$). However, all of the biofilm-selective conditions selected for an increase in salt tolerance after 30 transfers with salt stress conferring a 2- to 3-$\log_{10}$ reduction in viability (ancestor vs glass-adapted: $p < 0.0001$, ancestor vs PVC-adapted: $p < 0.0001$, ancestor vs stainless steel-adapted: $p < 0.0001$). There was no significant difference in salt tolerance selected between any of the biofilm-adapted conditions (glass-adapted vs PVC-adapted: $p = 0.9993$, glass-adapted vs stainless steel-adapted: $p = 0.6573$, PVC-adapted vs stainless steel-adapted: $p = 0.7983$).

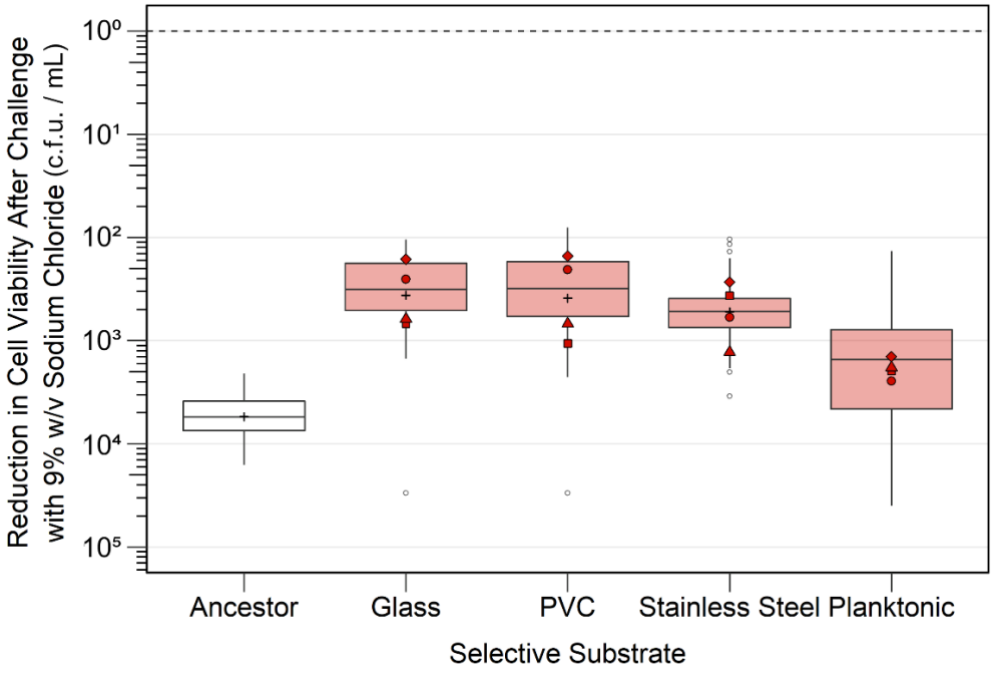

Figure 4.4. Tolerance of endpoint experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates to 9% w/v salt stress. Statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as reduction in viability in c.f.u., box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

The effect of biofilm hyperproduction on tolerance to acid and alkaline stress was determined by exposing endpoint populations to a sub-inhibitory concentration of stress at high and low pH (figure 4.5.). At pH 4, the ancestral strain was approximately 2-$\log_{10}$ less viable than in unstressed conditions. All selective conditions, including the

planktonically-adapted lineages, selected for a significant increase in acid tolerance after 30 transfers, with acidic conditions conferring less than a $\log_{10}$ reduction in cell viability (ancestor vs glass-adapted: $p < 0.0001$, ancestor vs PVC-adapted: $p < 0.0001$, ancestor vs stainless steel-adapted: $p < 0.0001$, ancestor vs planktonically-adapted: $p < 0.0001$). There was otherwise no significant difference in acid tolerance between any of the biofilm- or planktonically-adapted lineages (glass-adapted vs PVC-adapted: $p = 0.8787$, glass-adapted vs stainless steel-adapted: $p = 0.9818$, PVC-adapted vs stainless steel-adapted: $p = 0.8164$). At pH 12, the ancestral strain was between 5-and 6-$\log_{10}$ less viable than in unstressed conditions. The planktonically-adapted lineages were significantly less tolerant to alkaline stress than the ancestor with basic conditions conferring a 6- to 8-$\log_{10}$ reduction in viability ($p < 0.0001$). However, the biofilm-adapted lineages were significantly less tolerant to alkaline stress than the planktonic lineages, many of which were unable to grow entirely in basic conditions (planktonically-adapted vs glass-adapted: $p < 0.0001$, planktonically-adapted vs PVC-adapted: $p < 0.0001$, planktonically-adapted vs stainless steel-adapted: $p < 0.0001$). There was no significant difference in alkaline tolerance selected between any of the biofilm-adapted conditions (glass-adapted vs PVC-adapted: $p = 0.9882$, glass-adapted vs stainless steel-adapted: $p > 0.9999$, PVC-adapted vs stainless steel-adapted: $p = 0.9675$).

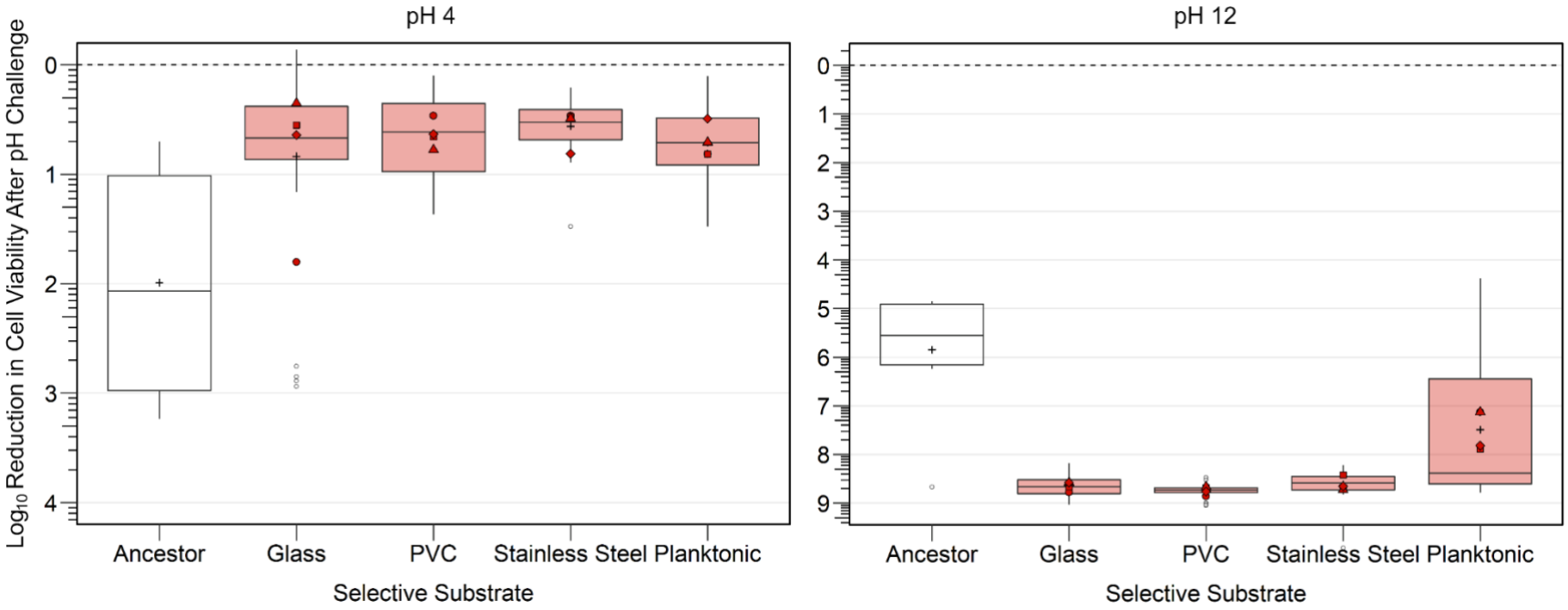

Figure 4.5. Tolerance of endpoint experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates to acid and alkali stress. Statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as $\log_{10}$ reduction in viability in c.f.u., box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

4.2.3. Biofilm hyperproduction is associated with dynamic switching between complex colony morphotypes which possess increased rugosity and decreased agar invasion

The colony morphologies of the biofilm hyperproducers were investigated by plating populations on bacteriological agar supplemented with tryptone, Coomassie brilliant blue and Congo red (figure 4.7.). After ten days of incubation at room temperature, the ancestor formed a planar colony which was surrounded by a large halo of bacterial growth invading the agar. However, a series of morphotypic variants were identified in the experimentally evolved biofilms which possessed distinct, complex colony architectures characterised by increased rugosity and decreased agar invasion. The filiform and circumscribed morphotypes were characterised by large central folds of rugosity with a clearly delineated colony wall bordering the colony. Whereas the colony wall of the filiform morphotype was composed of many filamentous folds, the wall of the circumscribed morphotype was an uninterrupted assemblage of biomass. Unlike the filiform and circumscribed morphotypes, the hyperrugose, radial and diffuse morphotypes did not possess a defined border distinguishing an internal and enclosing colony wall. Instead, folds tended to radiate outwards to the colony perimeter. The hyperrugose morphotype possessed many small folds evenly distributed across the colony, whereas the diffuse morphotype possessed few large folds across the radius of the colony. The radial morphotype appeared to be an intermediate state with moderate rugosity between the latter two morphotypes.

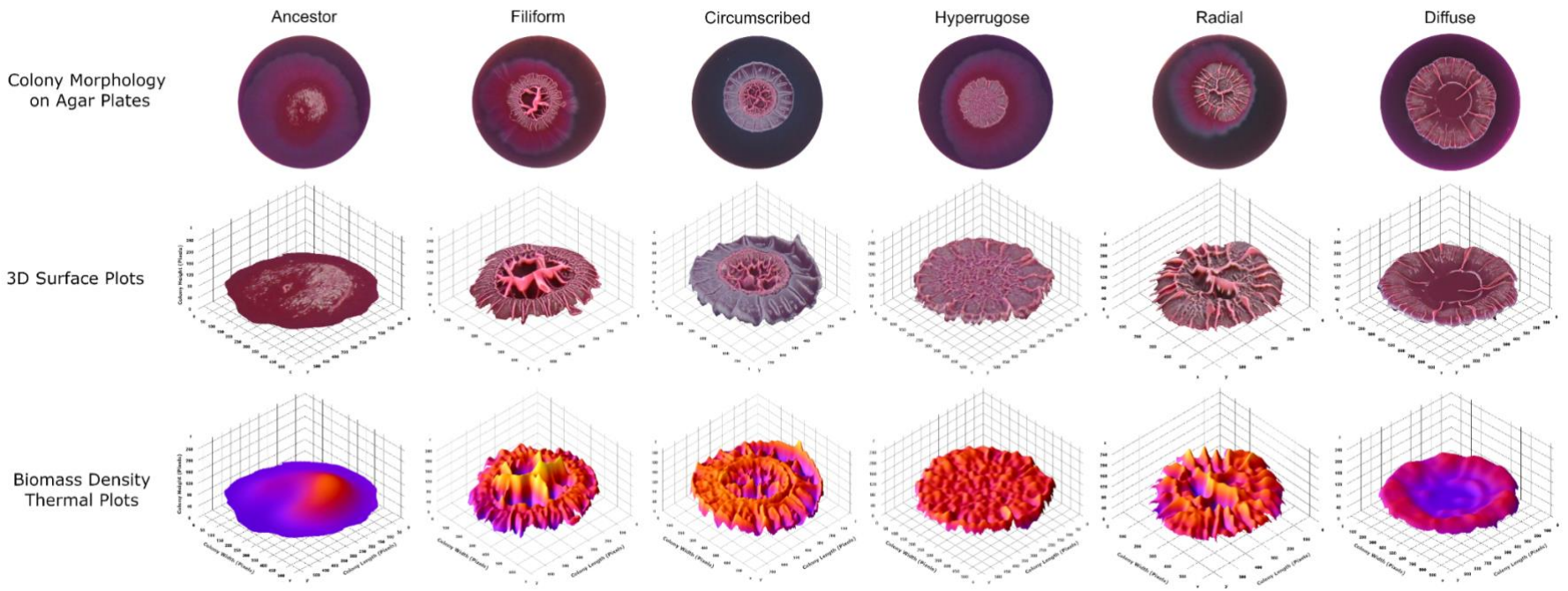

Figure 4.6. Colony morphotypes identified from experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates after a 10 day incubation at 20 °C on bacteriological agar supplemented with tryptone, Congo red and Coomassie brilliant blue. Data shown as colony dimensions in pixels, n = 4.

The distribution of colony morphotypes across the experiment was investigated (figure 4.7.). The planktonically-adapted lineages retained an ancestral morphotype throughout the experiment. The biofilm-adapted lineages did not demonstrate significant commitment to a single morphotype, instead lineages dynamically switched between morphotypes throughout the experiment. The only lineage to possess a single morphotype at all timepoints was one lineage of stainless steel-adapted biofilms. Evidence of a conserved morphotypic trajectory across the experiment was weak. Most lineages demonstrated a different morphotype at each transfer. Overall, the hyperrugose and filiform morphotypes were the most commonly observed morphotypes in the biofilm-adapted lineages with 13 and 10 unique observations, respectively. The diffuse, radial and circumscribed morphotypes were less common with 7, 4 and 2 unique observations, respectively. The hyperrugose morphotype was the most common morphotype in glass- and stainless steel-adapted lineages, constituting 5/12 and 8/12 assayed populations, respectively. The hyperrugose morphotype was rare in PVC-adapted lineages, only constituting 2/12 populations. However, the filiform morphotype was far more common at 6/12 populations in PVC-adapted lineages than lineages adapted to the other selective substrates at 2/12 populations. The hyperrugose morphotype was most common at transfer 10, became rare at transfer 20 but increased in proportion again at transfer 30. Conversely, the filiform morphotype was rare at transfer 10, became the most common at transfer 20 and reduced in proportion again at transfer 30. The proportion of diffuse and radial morphotypes varied little over time or substrate. The circumscribed morphotype was exclusively observed at transfer 20 constituting 1/12 populations in the glass- and PVC-adapted lineages.

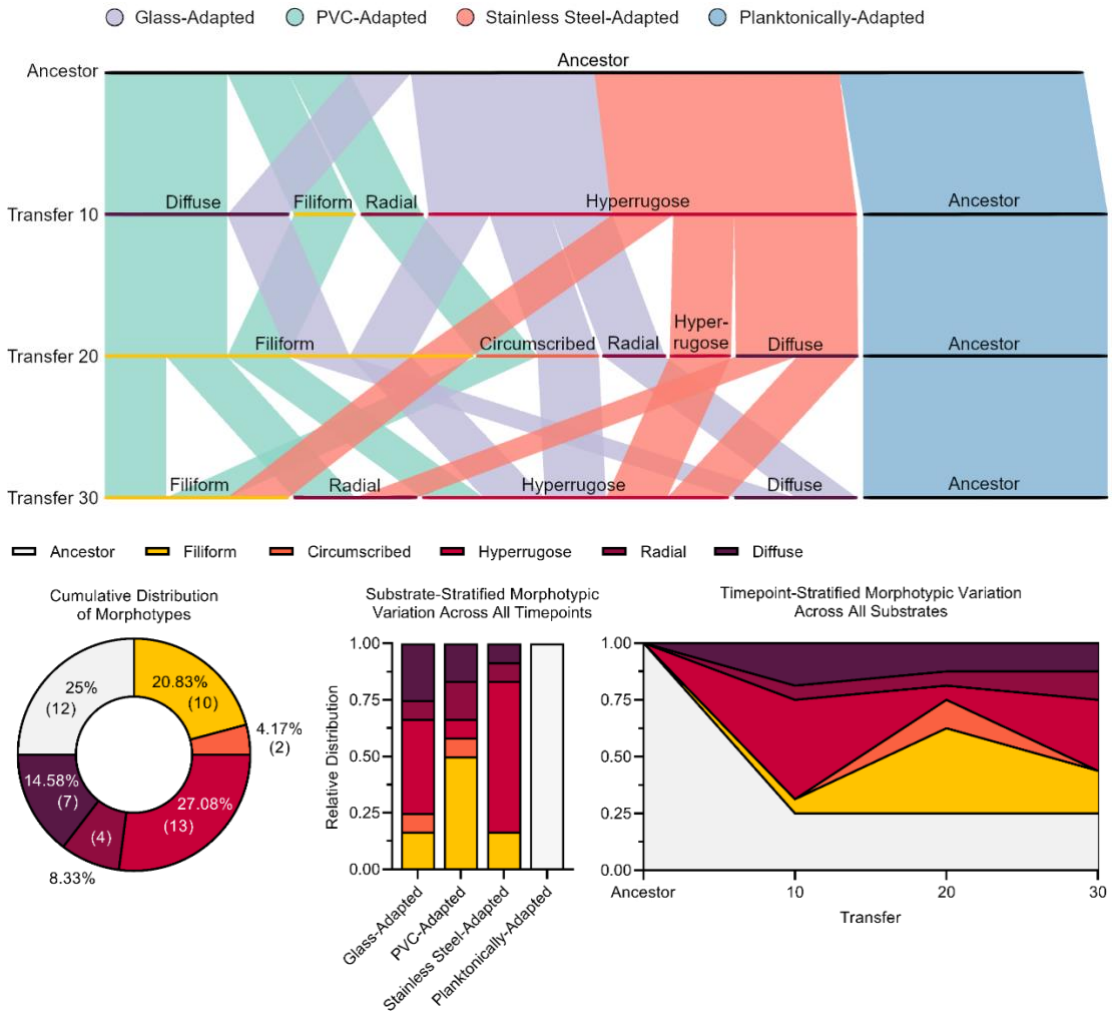

Figure 4.7. Succession and distribution of colony morphotypes in experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates. The morphotypic trajectory of each lineage at each timepoint was plotted using parallel sets v.2.1. The planktonically-adapted lineages retained an ancestral morphotype throughout the experiment. Data shown as proportional distribution of morphotypes, n = 4.

The rugosity properties of different experimentally evolved biofilm hyperproducer morphotypes were quantified using a linear threshold model in Fiji (figure 4.8.). The coverage of rugose folds across the ancestral colony was approximately 15% according to the model. None of the planktonically-adapted lineages demonstrated a significant change in colony rugosity relative to the ancestor at any timepoint (ancestor vs planktonically-adapted transfer 10: $p > 0.9999$, ancestor vs planktonically-adapted transfer 20: $p = 0.9993$, ancestor vs planktonically-adapted transfer 30: $p > 0.9999$). Biofilm-adapted lineages adapted to all substrates became significantly more rugose by transfer 20, doubling the coverage of rugosity relative to the ancestor (ancestor vs glass-adapted transfer 20: $p = 0.0130$, ancestor vs PVC-adapted transfer 20: $p =$

0.0235, ancestor vs stainless steel-adapted transfer 20: $p < 0.0001$). There was no significant difference in rugosity between any of the substrate-adapted biofilms at transfers 20 (ancestor vs glass-adapted transfer 20: $p > 0.9999$, ancestor vs PVC-adapted transfer 20: $p = 0.9988$, ancestor vs stainless steel-adapted transfer 20: $p > 0.9999$) or 30 (ancestor vs glass-adapted transfer 30: $p > 0.9999$, ancestor vs PVC-adapted transfer 30: $p = 0.9981$, ancestor vs stainless steel-adapted transfer 30: $p = 0.9891$). By transfer 30, the coverage of rugosity across biofilm-adapted lineages was approximately 40%. All morphotypes were significantly more rugose than the ancestor (ancestor vs filiform: $p < 0.0001$, ancestor vs circumscribed: $p = 0.0049$, ancestor vs hyperrugose: $p < 0.0001$, ancestor vs radial: $p < 0.0001$, ancestor vs diffuse: $p < 0.0001$). The hyperrugose lineages were significantly more rugose than any other morphotype, with approximately 50% coverage of rugosity (hyperrugose vs filiform: $p < 0.0001$, hyperrugose vs circumscribed: $p = 0.0330$, hyperrugose vs radial: $p < 0.0001$, hyperrugose vs diffuse: $p < 0.0001$). There was no significant difference in rugosity between the filiform, circumscribed and radial morphotypes (filiform vs circumscribed: $p = 0.9974$, filiform vs radial: $p = 0.8982$, circumscribed vs radial: $p = 0.9586$). The diffuse lineages were significantly less rugose than all other morphotypes (diffuse vs filiform: $p = 0.0073$, diffuse vs circumscribed: $p < 0.0001$, diffuse vs hyperrugose: $p < 0.0001$) except for the radial morphotype ($p = 0.0520$).

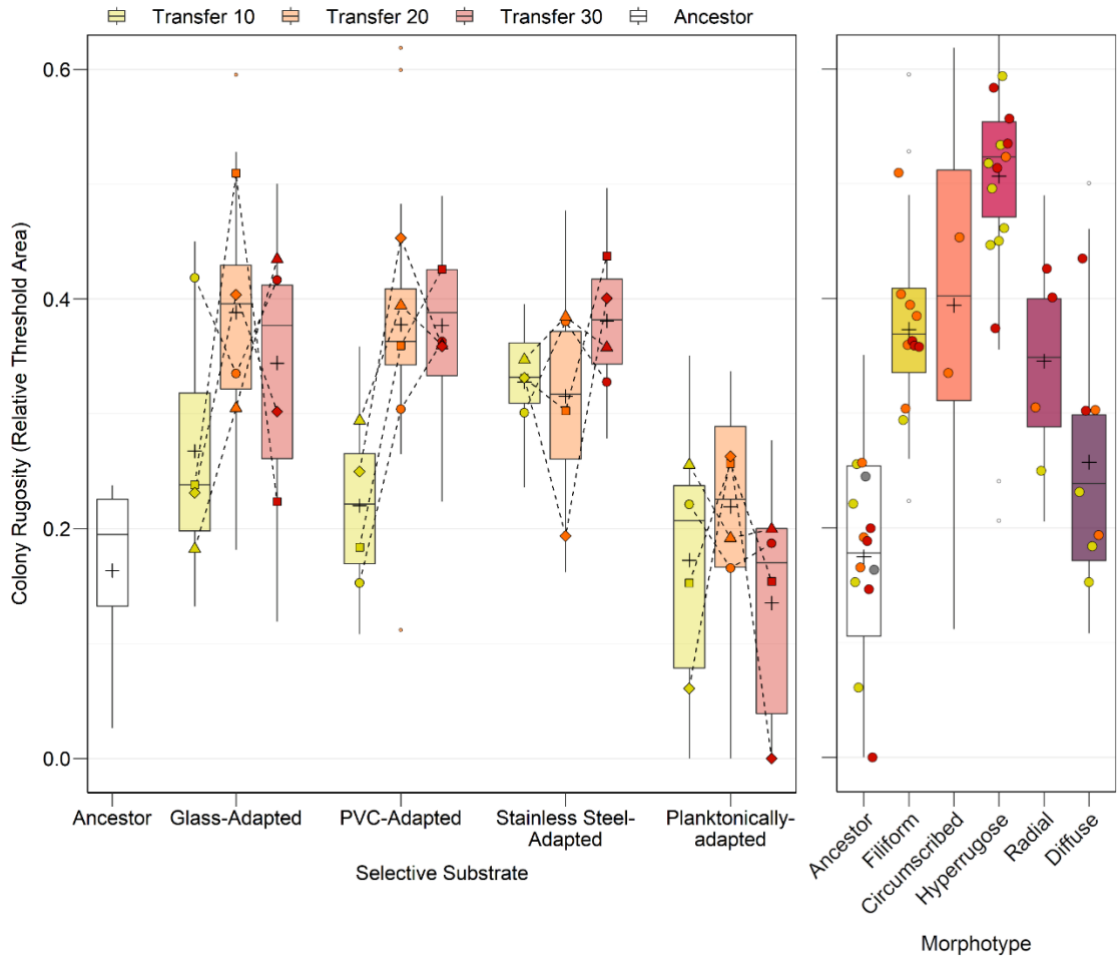

Figure 4.8. Colony rugosity morphometry of experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates. The colony rugosity of each lineage was determined by measuring the coverage of rugose folds using a linear threshold model in Fiji and plotting the threshold area relative to the total colony area. Statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparisons post-hoc test. Data shown as mean coverage of rugosity relative to colony area, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

The agar invasion properties of different experimentally evolved biofilm hyperproducer morphotypes were quantified in Fiji (figure 4.9.). The ancestor formed a halo of bacterial growth surrounding the colony which occupied approximately 65% of free space. The planktonically-adapted lineages did not demonstrate a significant change in agar invasion relative to the ancestor at any timepoint (ancestor vs planktonically-adapted transfer 10: $p > 0.9999$, ancestor vs planktonically-adapted transfer 20: $p = 0.9998$, ancestor vs planktonically-adapted transfer 30: $p > 0.9999$). Similarly, the biofilm-adapted lineages did not demonstrate a significant change in agar invasion relative to the ancestor at transfer 10 (ancestor vs glass-adapted transfer 10: p =

0.9967, ancestor vs PVC-adapted transfer 10: $p = 0.7504$, ancestor vs stainless steel-adapted transfer 10: $p = 0.9882$). However, by transfer 20 all biofilm-selective conditions selected for a significant reduction in agar invasion (ancestor vs glass-adapted transfer 20: $p = 0.0002$, ancestor vs PVC-adapted transfer 20: $p = 0.0021$, ancestor vs stainless steel-adapted transfer 20: $p = 0.0120$) resulting in a 15% to 40% reduction in area of agar invasion. There was no further significant change in agar invasion observed between transfer 20 and 30 (glass-adapted transfer 20 vs glass-adapted transfer 30: $p > 0.9999$, glass-adapted transfer 20 vs glass-adapted transfer 30: $p = 0.0502$, glass-adapted transfer 20 vs glass-adapted transfer 30: $p = 0.8002$). By transfer 30 all biofilm-selective conditions demonstrated a 25% area of agar invasion, with no significant difference between selective substrates (glass-adapted transfer 30 vs PVC-adapted transfer 30: $p > 0.9999$, glass-adapted transfer 30 vs stainless steel-adapted transfer 30: $p = 0.9994$, PVC-adapted transfer 30 vs stainless steel-adapted transfer 30: $p = 0.9953$). All morphotypes possessed significantly lower agar invasion than the ancestor (ancestor vs filiform: $p < 0.0001$, ancestor vs circumscribed: $p < 0.0001$, ancestor vs hyperrugose: $p < 0.0001$, ancestor vs radial: $p < 0.0001$, ancestor vs diffuse: $p = 0.0015$). The only significant differences in agar invasion between morphotypes observed was between the filiform and diffuse morphotypes ($p = 0.0445$), and the hyperrugose and diffuse morphotypes ($p = 0.0454$). The diffuse lineages possessed significantly lower agar invasion than the other morphotypes.

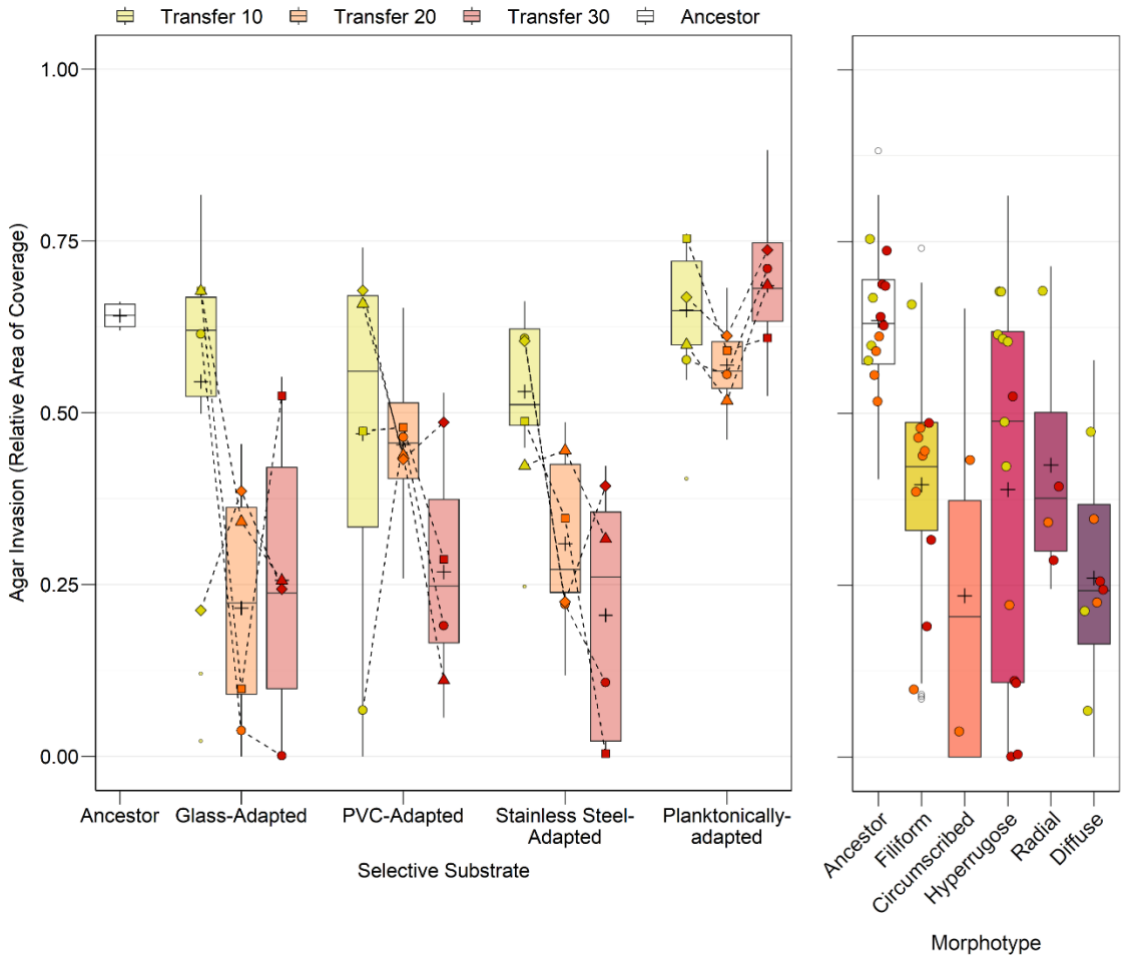

Figure 4.9. Agar invasion morphometry of experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates. The area of agar invasion was determined in Fiji by tracing the perimeter of the invasion halo, subtracting the colony area and plotting the invasion area relative to the total area of the agar plate. Statistical differences between groups were determined via a one-way ANOVA with Tukey's multiple comparisons post-hoc test. Data shown as mean area of agar invasion relative to agar plate area, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

4.2.4. The cyclic-di-GMP signalling network is the main nidus of selection in experimentally evolved biofilm hyperproducers

From the adapted lineages, mutations were identified in genes which were conserved between multiple lineages (figure 4.10.). Mutations found exclusively in the planktonically-adapted lineages included a T785C missense mutation in the PA14_RS16310 HlyD family efflux transporter periplasmic adaptor subunit in planktonic-1, -2 and -4 which resulted in an Ile262Thr substitution. Similarly, planktonic-2, -3 and -4 possessed a 6 bp inframe insertion at position 558 in the enoyl-coenzyme A hydratase/isomerase family protein PA14_RS22245 which resulted in an Asp_Leu insertion at Leu186_Ala187.

Gene targets which were conserved between the biofilm- and planktonically adapted lineages included the DNA-directed RNA polymerase *rpoB*, putative efflux RND transporter permease subunit PA14_RS13085 and quorum sensing regulator *lasR*. At transfer 30, glass-3 and planktonic-1 both possessed a G1562A missense mutation in *rpoB* which resulted in a Gly521Asp substitution. Planktonic-1 possessed a 15 bp indel at position 1006 in PA14_RS13085 at transfer 30 which resulted in a Met336 frameshift. In Glass-2, -4 at transfer 20 and PVC-3 at transfer 30, a A1006T missense mutation caused a Met336Leu substitution. Moreover, at transfer 20, PVC-2 possessed a T1007G missense mutation which resulted in a Met336Arg substitution.

Most genes demonstrating selective parallelism were shared across multiple selective conditions with little substrate-specific parallelism observed. The exception to this was a thymine deletion at position 774 in the site-specific integrase PA14_RS24540 which resulted in a Pro259 frameshift in PVC-1 and -4 at transfer 10. However, most mutations were in genes involved in regulation of c-di-GMP synthesis or transcriptionally regulated by c-di-GMP which were observed in two or more lineages adapted to different substrates. This included the Gac/Rsm cascade regulators *gacA* and *gacS*. At transfer 30 glass-1, -2 and PVC-2 possessed unique mutations in *gacA*, including a 14 bp deletion at position 323 conferring a Leu108, frameshift, C553T missense mutation resulting in an Arg185Cys and a cytosine deletion at position 438 conferring a Phe147 frameshift, respectively. Glass-2, -3 and steel-4 possessed unique mutations in *gacS*, including a CT deletion at position 166 resulting in a Leu56 frameshift, a guanine duplication at position 2607 resulting in a Val870 frameshift and a C2301G missense mutation resulting in gain of a stop codon at Tyr767, respectively.

Glass-1, PVC-4, steel-4 also possessed unique mutations in the diguanylate cyclase inhibitor *yfiR* at transfers 20 and 30 including a C533A missense mutation conferring a

Pro178Gln substitution, a T527G missense mutation conferring a Val176Gly substitution, and a TT deletion at position 484 resulting in Phe162 frameshift, respectively. At transfer 20, PVC-3 and steel-3 possessed a 12 bp inframe deletion at position 71 in the diguanylate cyclase *tpbB* which resulted in a Gly24_Leu27 deletion. At transfer 30, glass-3 and PVC-3 possessed mutations in the c-di-GMP receptor *morA* including a C2444A missense mutation resulting in a Asn1148Lys substitution and a A2866T missense mutation which resulted in a Ser956Cys substitution, respectively. At transfer 20, steel-1 possessed a C2923T missense mutation conferring a His975Tyr substitution. However, by transfer 30 this was replaced by a A3638G missense mutation conferring a Glu1213Gly substitution. At all timepoints, glass-1 and PVC-4 possessed a TAC infame deletion in the phosphodiesterase *dipA* at position 2623 resulting in a Tyr875 deletion. At transfer 10 PVC-4 also possessed a bp disruptive inframe deletion at position 1593 in PVC-4 which resulted in an Arg532_Ala545 deletion. Moreover, glass-3 at transfer 10, PVC-2 at transfer 20, and steel-2 at transfer 30 also possessed unique mutations in *dipA*. This included a A814C missense mutation in glass-3 which resulted in a Thr272Pro substitution, a thymine deletion at position 1754 in steel-2 which caused a Leu585 frameshift and a guanine duplication at position 2381 which resulted in a Ala794 frameshift.

Finally, at transfer 10, PVC-1, steel-3 and -4 possessed A440G missense mutations in the DNA polymerase Y family protein PA14_RS22670 which resulted in a Lys147Arg, in addition to a 35 bp indel at position 445 at transfer 10 in steel-4.

Mutations which did not demonstrate genotypic parallelism but nevertheless possessed hypothesised roles in the adaptive process based on broader phenotypic functions were identified (table 4.2.). Mutations in genes involved in the c-di-GMP-sensing Wsp signal transduction pathway involved in the motile-sessile switch were identified in glass-1 and PVC-2 including the glutamate methylesterase *wspF*, chemoreceptor *wspA* and two-component sensor *wspE.* Similarly, mutations in two putative c-di-GMP regulators PA14_RS18640 and PA14_RS22965 were identified in glass-3 and steel-3, respectively. In glass-4 and steel-2, mutations were identified in global regulators of flagellar motility including *fleQ* and *cheB*, respectively.

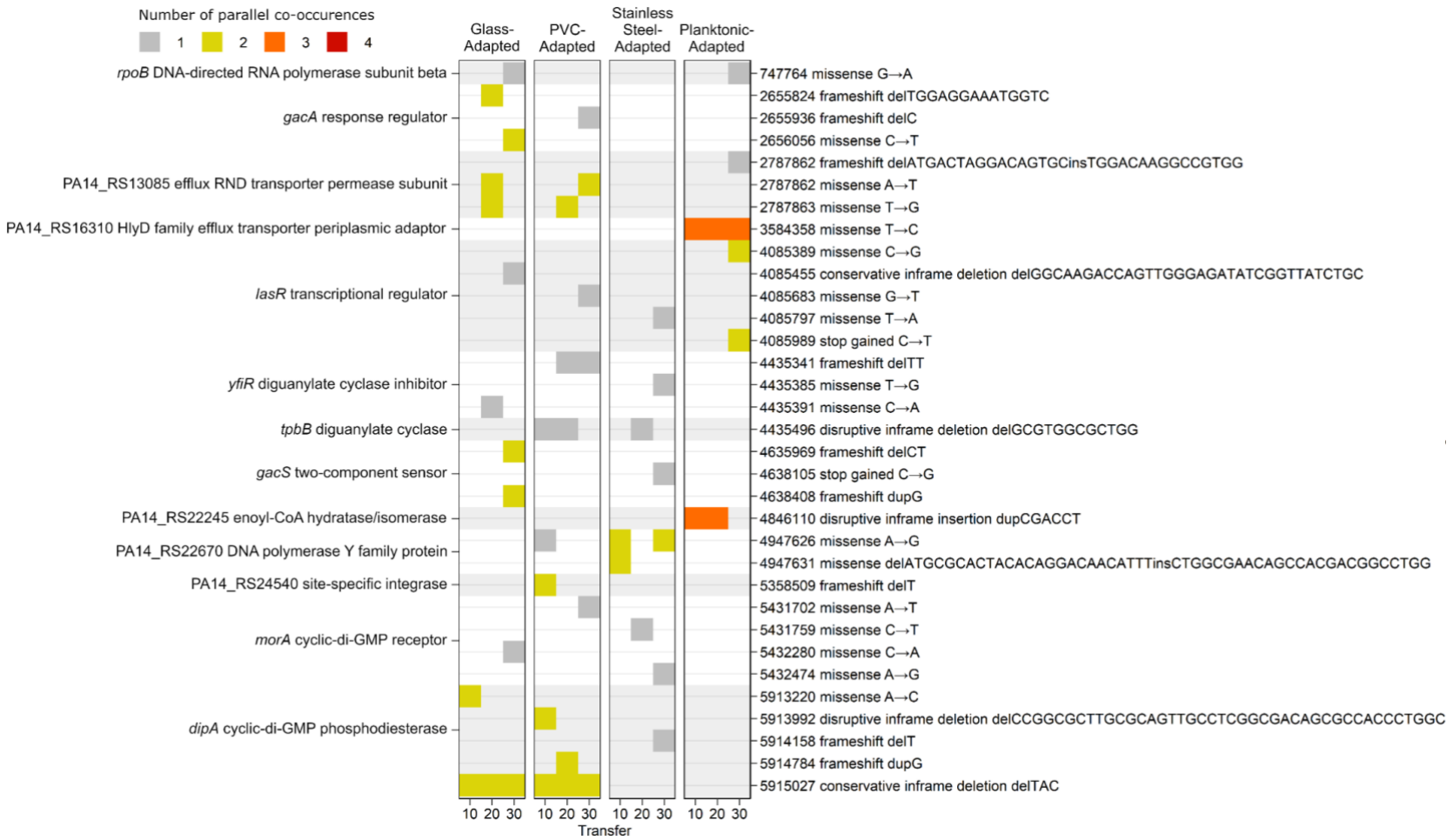

Figure 4.10. Gene targets under parallel selection in experimentally evolved lineages of *P. aeruginosa* adapted to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates identified from Illumina short-read whole genome sequencing data using snippy v 4.6.0. Data shown as number of times a mutation co-occurred in the four parallel lineages sequenced, n = 4.

Table 4.2. Non-synonymous substitutions in genes after substrate-specific adaptation which did not demonstrate genotypic parallelism but possess hypothesised roles in the adaptive process.

Lineage		Gene	Substitution	Transfer	Hypothesised Role
Glass-adapted					
	Lineage 1	*wspF* Wsp signal transduction glutamate methylesterase	1412807 frameshift delTT	30	Motile-sessile switch
	Lineage 3	PA14_RS18640 EAL domain-containing protein	4083502 stop gained C→T	20	c-di-GMP regulation
	Lineage 4	*fleQ* transcriptional regulator	4460924 missense A→G	30	Flagellar motility
PVC-adapted					
	Lineage 2	*wspA* Wsp signal transduction system chemoreceptor	1406486 conservative inframe deletion delTCCAAGCAGCAGCAGGCCACCGCCAC CGAAACCGCCGCGACC	30	Motile-sessile switch
		wspE Wsp signal transduction system sensor histidine kinase	1409866 stop gained G→T	30	Motile-sessile switch
Stainless steel-adapted					
	Lineage 2	*cheB* chemotaxis protein	476993 frameshift dupGA	30	Flagellar motility
	Lineage 3	PA14_RS22965 GGDEF domain-containing protein	5024358 missense G→T	30	c-di-GMP regulation

The phylogenetic relationship between the adapted lineages was inferred via maximum likelihood (Figure 4.11.). There was little phylogenetic association between the number of transfers a lineage had undergone and evolutionary distance from the ancestor. Moreover, populations sampled from the same lineage could demonstrate large differences in relatedness between consecutive timepoints. The biofilm-adapted lineages were largely interspersed indicating that there was little selection for substrate-specific evolutionary trajectories. However, half of the planktonically-adapted lineages clustered in a single clade, with another three planktonically-adapted lineages in a nearby clade. This may indicate that the planktonically-adapted lineages took specific evolutionary trajectories which were distinct from the biofilms.

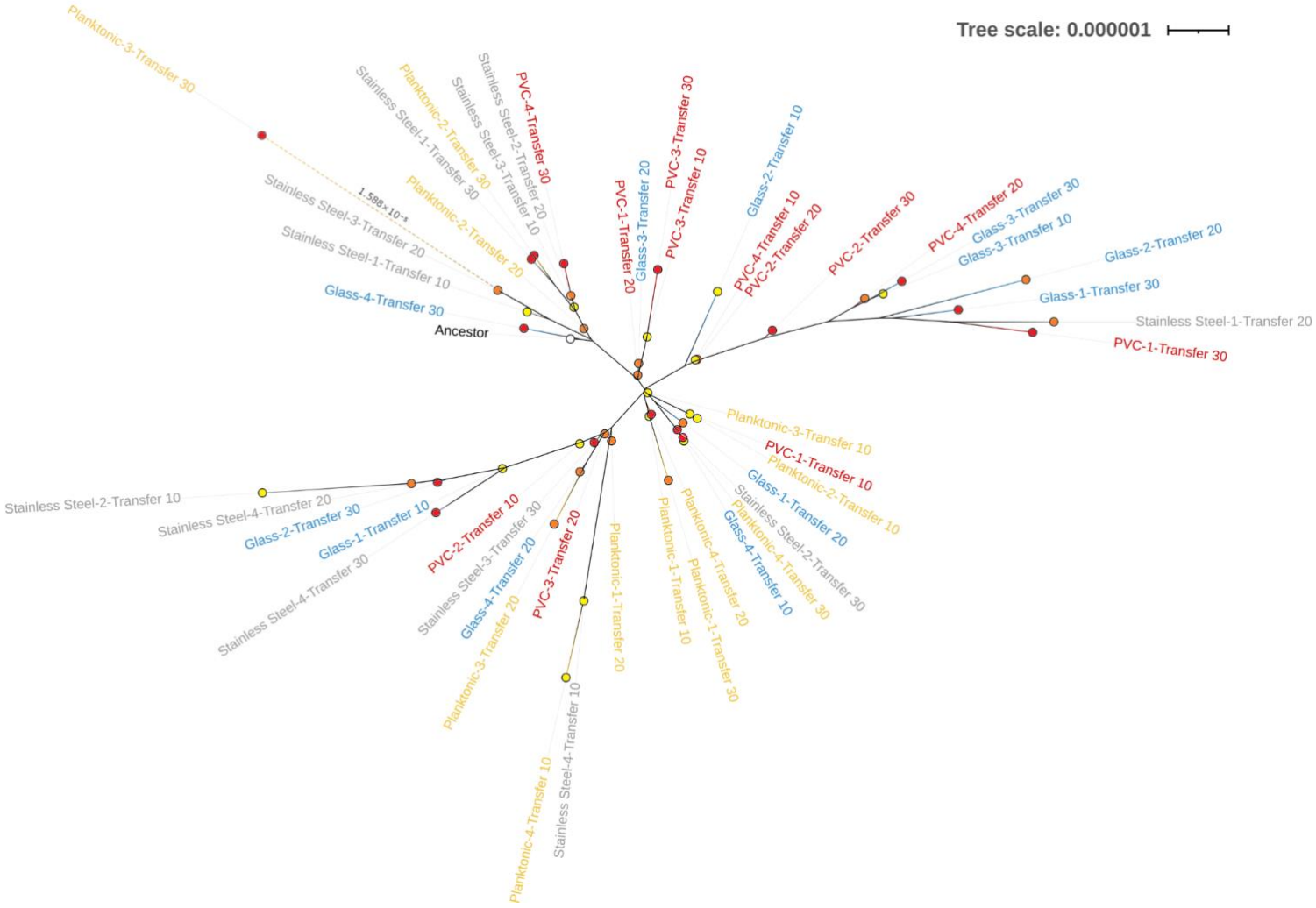
Tree scale: 0.000001
Planktonic-3-Transfer 30
Stainless Steel-1-Transfer 30
Planktonic-2-Transfer 30
Stainless Steel-2-Transfer 30
Stainless Steel-3-Transfer 10
PVC-4-Transfer 30
PVC-1-Transfer 20
Glass-3-Transfer 20
PVC-3-Transfer 30
PVC-3-Transfer 10
Glass-2-Transfer 10
PVC-4-Transfer 10
PVC-2-Transfer 20
PVC-2-Transfer 30
PVC-4-Transfer 20
Glass-3-Transfer 30
Glass-3-Transfer 10
Glass-2-Transfer 20
Glass-1-Transfer 30
Stainless Steel-1-Transfer 20
PVC-1-Transfer 30
1.588×10⁻¹
Stainless Steel-3-Transfer 20
Stainless Steel-1-Transfer 10
Planktonic-2-Transfer 20
Glass-4-Transfer 30
Ancestor
Planktonic-3-Transfer 10
PVC-1-Transfer 10
Planktonic-2-Transfer 10
Glass-1-Transfer 20
Stainless Steel-2-Transfer 30
Planktonic-4-Transfer 30
Glass-4-Transfer 10
Planktonic-4-Transfer 20
Planktonic-1-Transfer 30
Planktonic-1-Transfer 10
Planktonic-1-Transfer 20
Stainless Steel-4-Transfer 10
Planktonic-4-Transfer 10
Planktonic-3-Transfer 20
Glass-4-Transfer 20
Stainless Steel-3-Transfer 30
PVC-2-Transfer 10
PVC-3-Transfer 20
Glass-1-Transfer 10
Glass-2-Transfer 30
Stainless Steel-4-Transfer 20
Stainless Steel-2-Transfer 10
Stainless Steel-4-Transfer 30

Figure 4.11.

Figure 4.11. Phylogeny of experimentally evolved populations of P. aeruginosa lineages to growth planktonically or as a biofilm on glass, PVC or stainless steel substrates. Full-genome alignments were constructed with Snippy-core and a maximum-likelihood matrix was used to reconstruct the phylogeny of experimentally evolved lineages using IQ-TREE. The phylogenetic tree was arbitrarily rooted at the ancestral strain. Label colours show selective substrate, node colour shows timepoint. Broken line shows branches not to scale, in such cases evolutionary distances is displayed on the branch. Data shown as units of substitutions/site.

4.3. Discussion

Biofilm hyperproducers are found widely in clinical and industrial environments as persistent strains which are hard to decontaminate and tolerant to adverse conditions. *L. monocytogenes* and salmonellae in food processing environments, and *P. aeruginosa* and *Legionella pneumophila* in hospital plumbing systems are notable examples of surface-associated biofilms under selection in the real world (Bédard et al., 2016, Lee et al., 2019, Moloney et al., 2020). Exhaustive work has been undertaken characterising the phenotypic properties of strong biofilm-forming strains, however systematic investigation of the evolution of such phenotypes has been limited. Consequently, understanding the evolutionary trajectories when adapted to a surface-associated biofilm lifestyle can offer novel insights into the role of the environment in selection of high-risk persistent strains.

4.3.1. Phenotypic Properties

In this experiment, a selective environment was constructed which provided an advantage to individuals most proficient at repeatedly colonising and disseminating from bead substrata in liquid culture. It was observed in figure 4.1. that through the serial passage of biofilms, biofilm hyperproduction was readily selected via a multiple-step process. Biofilm hyperproducers formed approximately 1.5× to 2× more biomass than the ancestor after 10 transfers, which increased to 2× to 3× more biomass by transfer 30. In this regard, there was little disparity in the capacity for selection of biofilm hyperproduction on different surfaces. Consequently, biofilm hyperproduction appears to be a mechanism by which mutants can achieve fitness gains in an environment where surface-associated cells are artificially selected to populate the next transfer. The main limiting factor on biological success in this experiment is substratum space, therefore mutants which colonise the surface fastest will obtain the greatest representation in the next transfer. Biofilm hyperproduction likely facilitates this by increasing the mutant's ability to adhere to a surface, colonise the surface more quickly or mechanically disseminate to a new substrate. In order to be selected, biofilm hyperproducers must have outcompeted its ancestor, and this must have occurred at least one other time during the experiment to account for the stepwise increase in biofilm formation observed. This indicates that the degree of biofilm formation is correlated with fitness in the experimental environment and the more biomass that is produced, the greater selective advantage a mutant possesses. Increased biomass production was used as a marker for biofilm hyperproduction in this work. However,

biofilm hyperproduction is an aggregate output with huge transcriptional ramifications. Whether the physical properties of increased matrix production contribute to the selective advantage seen in biofilm hyperproducers, or if it is a pleiotropic consequence of adaptation to a biofilm lifestyle remains unknown.

Despite biofilm hyperproducers producing more biomass than the ancestor, cellular productivity was not significantly altered when cultivated on beads as shown in figure 4.2. These results are in line with observations from Jerdan et al. (2020) in which an experimentally-defined multispecies soil biofilm, including several species of pseudomonads, serially passaged daily demonstrated no significant change in total community productivity after 11 transfers. Furthermore, Jerdan et al. (2020) demonstrated that during serial transfer under 74 and 148 hour incubation periods, biofilms adapted to become less productive than the ancestor. Similarly Lee et al. (2019) observed little correlation between biomass production and cellular productivity in *L. monocytogenes* strains isolated from food processing environments. Therefore, these results indicate that increased matrix production is independent of productivity and does not provide more habitat for cells to reside in.

Fitness of the adapted lineages was measured through growth kinetics in planktonic culture. Figure 4.3. demonstrated that biofilm hyperproducers did not possess gross fitness defects relative to the ancestor based on doubling times or viability in broth. This indicates that although lineages were adapted to a biofilm lifestyle, the intrinsic capacity to grow as well as the ancestor was not compromised by the mutations selected to achieve biofilm hyperproduction. However, whilst gross fitness was not impinged by selection of biofilm hyperproduction, it is possible that the biofilm hyperproducers were still less competitively fit than their planktonic counterparts in a liquid environment. Competitive fitness refers to the ability of a strain selectively to exclude a competitor. Little work has been undertaken investigating the dynamics of competitive fitness of evolved biofilms. However, Madsen et al. (2015) demonstrated that experimentally evolved pellicle biofilms of *P. aeruginosa* were significantly more fit than the ancestor in a static microcosm than in conditions with orbital shaking. Therefore, though there was no observable change in the growth kinetics of the biofilm hyperproducers, this does not exclude environment-dependent fitness costs associated with adaptation to a surface niche.

Biofilm hyperproduction was not associated with a significant change in susceptibility to a panel of clinically-relevant antibiotics as shown in table 4.1. The MIC measured changes in the inhibitory concentration in a planktonic lifestyle, which would account for selection of *de novo* resistance, independent of the biofilm lifestyle-specific emergent

properties. Moreover, the MBEC was also measured to identify if the phenotypic consequences of biofilm hyperproduction attenuated antimicrobial activity. No changes in susceptibility were observed using either metric despite conventional dogma of the nature of drug tolerance in biofilms. It has been stated in the previous chapter that the biofilm matrix as a diffusion barrier is no longer believed to be a significant mechanism of non-specific antimicrobial tolerance. Indeed, Trampari et al. (2021) did not observe significant changes in intrinsic resistance in experimentally evolved biofilm hyperproducers of *Salmonella* sv. Typhimurium. Reports of disparate susceptibility to antibiotics between biofilm and planktonic lifestyles have been largely conducted in single-exposure formats and it is acknowledged that the stoichiometry of induced resistance can be lifestyle-specific (Brooun et al., 2000). However, this work demonstrates that such transient resistance mechanisms are selectively independent of biomass production indicating that intrinsic resistance is not an innate consequence of adaptation to a biofilm lifestyle.

Though no changes in drug susceptibility was observed, biofilm-specific altered responses to environmental insults such as salinity and alkali stress were identified. Figure 4.4. establishes that biofilm hyperproducers were associated with a significant increase in viability relative to the planktonic lineages under inhibitory concentrations of salt. Salinity tolerance has been observed to possess a close relationship with biofilm formation. Root-associated biofilms conferred increased salinity tolerance in plants (Qurashi and Sabri, 2012). Moreover, increased salt concentrations have been shown to induce biofilm formation. Chan et al. (1984) observed that supplementation of magnesium salts to culture media induced a mucoid phenotype in *P. aeruginosa.* Furthermore, Kim and Chong (2017) demonstrated that EPS production in a *Vibrio* sp. increased with sodium chloride concentration in a linear fashion. Given the evidence that salt tolerance can be induced by biofilm formation and *vice versa*, it is not surprising that constitutive salt tolerance was selected in biofilm hyperproducers. The EPS appears to have a direct role on salt tolerance, as experimental supplementation of xanthan gum, a diatom EPS constituent, reduced the degree of hypersaline shock in biofilms of the marine protist *Cylindrotheca closterium* (Steele et al., 2014). Therefore, it is conceivable that salt tolerance is collateral outcome of increased matrix production, whereby the matrix acts as a reaction-diffusion barrier by which ionic reactions protect cells from the inhibitory effect of salinity. Conversely, figure 4.5. shows that growth at alkaline pH was associated with a significant loss of viability relative to planktonic lineages. This indicates that though the matrix has been shown to possess a dampening effect on environmental perturbations, it possesses some specificity in the

ability to buffer suboptimal growth conditions. The observation that biofilms were more susceptible than the ancestor may be a corollary of being less susceptibility to acid stress. All lineages, including the planktonically-adapted lineages, were able to survive significantly better at inhibitory acidic pH. This may reflect adaptation to serial passage in which by the end of the incubation period, waste products and nutrient catabolism leads to an acidification of the media (Sánchez-Clemente et al., 2018). Therefore, tolerance to acidic conditions may have been selected by the culture conditions, of which collateral sensitivity to alkaline stress was also selected. Why biofilm-adapted lineages experienced greater susceptibility to alkaline stress than their planktonic counterparts is not known however this may indicate that the biofilm and planktonic lineages evolved different mechanisms for pH regulation.

4.3.2. Molecular Mechanisms of Biofilm Hyperproduction

No strong phylogenetic relationship between evolved lineages at different timepoints or selective conditions was observed from figure 4.11. At successive timepoints, mutants often had distinct repertoires of mutations which convoluted phylogenetic signals. As the evolutionary time elapsed was within an order of magnitude of 10^2 generations, it is likely there was still intraspecific competition occurring in the experiment which produced a significant amount of genetic diversity. This evolutionary noise made it hard to infer phylogenetic relationships as clonal interference had not yet led to the fixation of dominant polymorphisms required for cladogenesis. Nevertheless, the evolutionary trajectories to biofilm hyperproduction could be inferred by identifying mutations under purifying selection in multiple parallel lineages. Selective parallelism in independent lineages is a strong indication that an observed mutation is selectively advantageous and not a result of the random accumulation of neutral polymorphisms. In this regard, as shown in figure 4.10., mutations in the c-di-GMP signalling network were detected extensively in biofilm hyperproducers. This included both genes involved in c-di-GMP synthesis regulation and c-di-GMP-sensing transcriptional regulators. c-di-GMP is one of the foremost global regulators of the biofilm lifestyle in *P. aeruginosa* (Römling et al., 2013). Consequently, mutations in the c-di-GMP signalling network likely acted to constitutively express genes which support a sessile lifestyle.

4.3.2.1. Cyclic-di-GMP Regulation

Concomitant synthesis and hydrolysis of c-di-GMP by GGDEF domain-containing diguanylate cyclases and EAL or HD-GYP domain-containing phosphodiesterases,

respectively, tightly control intracellular concentrations of c-di-GMP (Galperin, 2005). In *P. aeruginosa*, 18 diguanylate cyclases are known, in addition to nine c-di-GMP-hydrolysing phoshodiesteases and 16 bifunctional enzymes with both GGDEF and EAL domains (Valentini and Filloux, 2016). Cellular perturbations affecting the expression and function of these enzymes act to enrich or deplete reserves of c-di-GMP which instigates pleiotropic changes to downstream gene expression. The motile-sessile switch is associated with an increase in the activity of membrane-bound diguanylate cyclases, sensing physicochemical cues associated with surface adhesion and enriching the c-di-GMP reservoir. During surface adhesion, intracellular c-di-GMP concentrations increase up to three-fold which regulates expression of secretion systems, flagellar motility and exopolysaccharide production to support biofilm formation (Chang, 2018). Consequently, mutations which constitutively activate diguanylate cyclase or inactivate phosphodiesterases also constitutively de-repress genes involved in biofilm formation. Furthermore, release from regulatory control can lead to degrees of overexpression greater than can be achieved under normal induction conditions.

Genes involved in c-di-GMP regulation were identified in multiple parallel lineages of biofilm hyperproducers. This included the phosphodiesterase *dipA,* the bifunctional diguanylate cyclase/ phosphodiesterase *morA,* the diguanylate cyclase *tpbB* (also known as *yfiN*) and the diguanylate cyclase inhibitor *yfiR*. *tpbB* and *yfiR* are encoded in an operon with the lipoprotein *yfiB* to express the YfiBNR tripartite complex which senses unknown periplasmic signals to modulate c-di-GMP levels (Giddens et al., 2007). During planktonic growth, YfiR represses TbpB to inhibit its diguanylate cyclase activity. However, during surface adhesion, the lipoprotein YfiB undergoes a conformational change which allows it to sequester YfiR, leading to TpbB activation. YfiBNR mutations have been detected in *P. aeruginosa* populations isolated from the cystic fibrosis lung where it conferred a c-di-GMP-dependent SCV phenotype with increased biofilm formation (Malone et al., 2010). In the experimentally evolved biofilm hyperproducers in this study, one lineage of glass-, PVC- and stainless steel-adapted biofilms each possessed unique substitutions in the putative TbpB binding site of YfiR hypothesised by Malone et al. (2012). Consequently, it is likely that these mutations result in impaired the capacity for TfiR to repress TbpB, thus increasing c-di-GMP synthesis. Similarly, one lineage of PVC- and stainless steel-adapted biofilms each possessed the same 12 bp disruptive inframe deletion at position 71 of *tpbB*. The YfiR binding site of TpbB is believed to be present within the periplasmic domain which spans residues 35 to 161, however the *tpbB* deletion mutation occurred in the first

transmembrane helix (Giardina et al., 2013). Whether a truncated transmembrane helix alters the conformation of the periplasmic domain of TpbB to release it from regulatory control is not known. Mutations in the transmembrane helix domains of TpbB which confer insensitivity to YfiR have been observed (Malone et al., 2012). However, this is the first documentation of a deletion polymorphism in *tpbB* with a hypothesised role in biofilm hyperproduction.

Mutations in the EAL domain-containing phosphodiesterase *dipA* were observed in two lineages of glass-adapted biofilms, two lineages of PVC-adapted and one lineage of steel-adapted biofilms. *dipA* mutations have a well-documented role in biofilm formation; deletion of *dipA* results in overexpression of *psl*, limits flagella rotation and reduces biofilm dispersal (Cai et al., 2020, Khong et al., 2021, Xin et al., 2019). Roy et al. (2012) conducted the most comprehensive analysis of the role of *dipA* to date and identified domain-dependent involvement in biofilm formation. However, the crystal structure of *dipA* has not yet been solved and the identity of specific residues involved in phosphodiesterase activity have not been defined. In experimentally-evolved biofilm hyperproducers, all substitutions in DipA were unique apart from a Tyr875 inframe deletion present in both glass-1 and PVC-4. Substitution sites were not clustered at any particular region of the protein, instead they were dispersed across several hundred amino acid residues. Four of the unique substitutions were located in the catalytic domain of DipA which spanned residues 460 to 885. The remaining substitution was present in the GAF module which Roy et al. (2012) observed did not affect phosphodiesterase activity but still modulated biofilm formation through an unknown mechanism.

One lineage of glass-, PVC- and stainless steel-adapted biofilm each possessed unique mutations in the c-di-GMP receptor *morA*. MorA possesses both a EAL and GGDEF domain. In *P. aeruginosa* PAO1, MorA possesses preferential diguanylate cyclase activity, and deletion of *morA* results in a compromised ability to form biofilms (Choy et al., 2004, Phippen et al., 2014). Despite this, mutations in *morA* were seen in three separate experimentally evolved biofilm hyperproducers, which may reflect strain variation if the *morA* allele possessed by *P. aeruginosa* PA14 favours phosphodiesterase activity. Despite this, two mutations were observed in the C-terminal region of the GGDEF domain. Therefore, more work is required to understand the selective advantage associated with *morA* mutations in these biofilm hyperproducers.

4.3.2.2. Cyclic-di-GMP Signalling Cascades

Translating extracellular signals into regulation of the c-di-GMP reservoir relies on intracellular signalling cascades which bridge the interface between environmental sensors and c-di-GMP regulators (Boyd and O'Toole, 2012). c-di-GMP signalling pathways are widespread and varied in regulatory mechanism and activation stoichiometry (Hengge, 2009, Whitney et al., 2015). One of the most important properties of the c-di-GMP network is the capacity for localised activation of signalling pathways. The intracellular c-di-GMP reservoir is not uniformly distributed across the cell, instead localised concentrations of c-di-GMP dictate activation of receptors (Hengge, 2009). Furthermore, signalling cascades are often associated with endogenous diguanylate cyclases or phosphodiesterases to create feedback systems (Valentini and Filloux, 2016). These processes facilitate the staggering complexity of the c-di-GMP signalling network and provide means for a single molecule to regulate expression of hundreds of genes in a highly specific manner. The pleiotropic mechanism by which c-di-GMP signalling pathways can regulate multiple phenotypes simultaneously lends it to regulating central cellular behaviours. Consequently, they are often a target of selection for lifestyle-specific adaptation such as hypervirulent and biofilm hyperproducing phenotypes (Blanka et al., 2015, Hall and Lee, 2018).

The Gac/Rsm cascade is a major regulator of the motile-sessile switch in *P. aeruginosa*. The GacSA two-component system is positively and negatively regulated by the hybrid sensors LadS and RetS, respectively (Valentini and Filloux, 2016). LadS is a calcium-response kinase, the activation of which has been reported to induce GacSA-dependent downregulation of virulence (Broder et al., 2016). The RetS kinase negatively regulates biofilm formation via dimerisation with GacS and inactivation of RetS confers biofilm hyperproduction (Bordi et al., 2010). RetS is in turn negatively regulated by the polygamous histidine phosphotransfer protein HptB using biofilm lifestyle-specific orphan sensors SagS, ErcS and PA1611 (Park and Sauer, 2021). Therefore, during biofilm growth, LadS is activated and RetS is inactivated by HptB to de-repress expression of GacS. The subsequent phosphorylation of GacA promotes the expression of the non-coding RNAs RsmYZ which sequesters the translational repressor RsmA. Titration of RsmA de-represses the diguanylate cyclase SadC leading to increased c-di-GMP production facilitating the motile-sessile switch. (Moscoso et al., 2014). Consequently, mutations which de-repress the Gac/Rsm cascade lead to biofilm hyperproduction. In the experimentally evolved biofilm hyperproducers, two lineages of glass- and one lineage of stainless steel-adapted biofilms possessed unique mutations in *gacA* and *gacS*. No mutations in the secondary regulators of the Gac/Rsm cascade

such as HptB, LadS or RetS were found. This seems paradoxical as expression of *gacSA* must be maintained in order to de-repress the Gac/Rsm cascade (Choi et al., 2007). All lineages with mutations in *gacSA* also possessed pre-existing mutations in the c-di-GMP synthesis r egulators *dipA* at some timepoint. It is possible that in these lineages, constitutive inactivation of Gac/Rsm occurred to ameliorate excessive c-di-GMP concentrations. Nevertheless, not all *dipA* mutants were Gac/Rsm-compromised, but DipA substitutions were highly heterogenous and likely have disparate impacts on phosphodiesterase activity. In this experiment, dissemination to new beads was a selective pressure as important as colonisation of the substrate. *dipA* is main regulator of biofilm dispersal in *P. aeruginosa* (Roy et al., 2012). Therefore, it is hypothesised that reducing c-di-GMP concentrations via inactivation of GacSA in dispersal-compromised biofilm hyperproducers may support transmission through the experiment.

Another major c-di-GMP-sensing regulator of the biofilm lifestyle in *P. aeruginosa* is the Wsp surface sensing system. It is able to sense surface adhesion via the chemoreceptor WspA which detects mechanical pressure on membranes (Valentini and Filloux, 2016). Subsequently, activation of WspA supports the autophosphorylation of the histidine kinase WspE which in turn, phosphorylates the response regulators WspR and WspF. WspR is a diguanylate cyclase whereas WspF is a methylesterase which negatively regulates expression of WspA (Hickman et al., 2005). In experimentally evolved biofilm hyperproducers, parallelism was not observed for individual genes in the *wsp* cascade, however mutations in *wspA, wspE* and *wspF* were observed across a lineage of glass- and PVC-adapted biofilms. Mutations in the *wsp* system which act to increase the expression of WspR constitutively increase biofilm formation. SCVs of *P. aeruginosa* isolated from a porcine wound infection model possessed mutations in *wspA* and *wspF* which conferred biofilm hyperproduction (Gloag et al., 2019). In both this study and Gloag et al. (2019), the same polymorphism in *wspA* was detected. A 42 bp deletion of residues 285 to 298 flanked by a direct repeat region. This region has been shown to contain one of two methylation sites through which WspF interacts with WspA, the deletion of which results in constitutive activation of WspA de-repressing WspR-mediated c-di-GMP synthesis (Xu et al., 2021). Whilst mutations in *wspF* were observed in both this study and Gloag et al. (2019), different polymorphisms were selected. A Phe257 frameshift facilitated by a TT deletion was associated with biofilm hyperproduction in experimentally evolved lineages. This is the first reported role of residue 257 changes in enzyme function of WspF. However, many mutations in the methylesterase domain of WspF which spans

from residue 154 to 330 have been previously identified to impair its interaction with WspA (Bantinaki et al., 2007). A mutation was identified which resulted in an early stop codon at residue 44, truncating WspE. Whilst mutations in *wspE* in biofilm hyperproducers have been selected, none were loss-of-function mutations given the essential role of WspE for activation of WspR (McDonald et al., 2009). Therefore, the selection of inactivation of WspE seems paradoxical. Hickman et al. (2005) did not observe compromised biofilm formation associated with deletion of *wspE*, in spite of its role, presumably as functional redundancy within the c-di-GMP signalling network can compensate for loss of Wsp expression. It is possible that like the mutations in GacSA, the strain with a truncated *wspE* gene may be compensating for reduced capacity for dissemination by virtue of mutations in *dipA.*

4.3.3. Morphotypic Diversification

In addition to increased biomass production, adaptation to a biofilm lifestyle was also associated with the acquisition of complex colony morphologies characterised by increased rugosity and decreased invasion through agar. The original investigation which used the biofilm evolution model by Poltak and Cooper (2011) explored ecological succession in *B. cenocepacia* lineages adapted to a biofilm lifestyle. It was observed that three distinct morphotypes which possessed biofilm hyperproducing phenotypes were selected: a studded variant demonstrating increased Congo red dye uptake, a hyperrugose SCV and a large, diffuse colony known as the ‘ruffled spreader’. Similarly, in this study, five morphotypes were identified in experimentally evolved biofilms of *P. aeruginosa* identified based on differences in rugosity patterns as shown in figure 4.6. Increased rugosity is a well characterised phenomenon in biofilm mutants of *Pseudomonas* spp., *Burkholderia* spp. and *Vibrio* spp. as a result of increased c-di-GMP synthesis, in line with the observations in this study (Beyhan and Yildiz, 2007, Gloag et al., 2019, Traverse et al., 2013). Rugosity is facilitated by c-di-GMP-dependent expression of exopolysaccharide production, however the mechanisms which regulate self-organisation into complex architectures are not well understood (Curtis et al., 2007, Lim et al., 2006). The mechanistic distinction between the morphotypic variants is even more opaque and this study did not detect any associations between particular polymorphisms and the manifested morphotype. Furthermore, the evolution of biofilm hyperproduction was not associated with commitment to a specific morphology, instead lineages underwent dynamic switching between morphotypes throughout the experiment as shown in figure 4.7. Evidently, biofilm hyperproduction appears to generate a conserved repertoire of potential

morphotypes which evolve in parallel in independent lineages. The selective tendency towards phenotypic diversity in inherent to the biofilm lifestyle has been well established as a bet-hedging mechanism (Flynn et al., 2016, Kirisits et al., 2005, Penterman et al., 2014, Savage et al., 2013). This experiment's relatively short evolutionary timescale may not have been sufficient for complete specialisation yielding multiple subpopulations in intraspecific competition. The isolation of a single mutant to sequence would therefore not capture this genotypic diversity. Therefore, the morphotypes may be the result of the interaction between multiple subpopulations contributing to biofilm formation. The associated switching between morphotypes could be a function of the relative abundance of multiple genotypes changing due to the action of competition. Further work investigating within-population heterogeneity is required to investigate this hypothesis.

Other than the hyperrugose morphotype possessing the greatest rugosity, figure 4.8. demonstrates that timepoint was a better correlate than morphotype for the extent of rugosity. Similarly, rugosity was inversely correlated with agar invasion independently of morphotype. The ancestor was able to colonise the agar surface forming a translucent halo of growth surrounding the colony. As the experiment progressed, this capacity to invade agar was diminished as shown in figure 4.9. *P. aeruginosa* possesses three mechanisms of motility, type IV pili-mediated twitching, flagella-mediated swarming and swimming (Badal et al., 2021). Increased c-di-GMP production associated with biofilm hyperproduction reduces the expression and activity of the flagellum. This limits swimming and swarming to promote a sessile lifestyle, however the pattern of agar invasion observed in this experiment lacks the radiated hallmark of flagellar motility (McCarter et al., 2015). However, type IV pili are conspicuously absent from the c-di-GMP regulatory network of *P. aeruginosa*, and their loss-of-function would seem counterproductive given their role in facilitating cell-matrix interactions (Nolan et al., 2020, Ribbe et al., 2017). Ribbe et al. (2017) observed that the presence of Pel increased the strength of interaction between type IV pili and the surface. Therefore, the increased matrix production associated with biofilm hyperproduction may impair the capacity for twitching motility if type IV pili are occupied with matrix binding.

4.3.4. Conclusions

In conclusion, experimental evolution of biofilms was able to select for biofilm hyperproduction on beads of three clinically- and industrially-relevant surfaces, glass, PVC and type-316 stainless steel. Biofilm hyperproduction was not associated with

intrinsic resistance to antibiotics however they did demonstrate a significant reduction in susceptibility to salinity stress and increased susceptibility to alkaline stress. The c-di-GMP signalling network was the main target for selection for biofilm hyperproduction likely as it facilitates the constitutive transition to a sessile lifestyle in a single selective step. Lineages took diverse c-di-GMP-dependent pathways to biofilm hyperproduction independent of selective substrate, which acted to increase the c-di-GMP content of the cell, including the *dipA* phosphodiesterase, *yfiBNR* complex, *morA* c-di-GMP receptor and *wsp* surface sensing cascade. Self-generated diversity was intrinsic to selection for biofilm hyperproduction and yielded several morphologically distinct rugose colony morphotypes and switched dynamically between them, although the molecular basis for this could not be determined.

CHAPTER 5. EVOLUTIONARY TRAJECTORIES TO ANTIBIOTIC RESISTANCE SELECTED IN BIOFILM AND PLANKTONIC LIFESTYLES

5.1. Introduction

Antibiotics are a staple of modern medicine. As immunocompromised populations grow due to an aging population and better clinical outcomes for the immunosuppressed, efficacious antimicrobial chemotherapy becomes ever more necessary (Beckett et al., 2015). Despite the increasing reliance on antibiotics, they are becoming less effective as resistance escalates. To overcome the current pace of antimicrobial resistance, it has been recommended that nineteen new antibiotics are required per decade, with two novel classes of both empiric and targeted agents necessary (The Review on Antimicrobial Resistance, 2015). However, in the last 40 years only two new classes of antibiotics, the oxazolidinones and lipopeptides, have been introduced into widespread clinical practice. Decades of divestment from multinational pharmaceutical companies due to diminishing returns from traditional discovery platforms and commercial challenges associated with restrictive, short-time therapy have left antibiotic discovery largely to academic and small biopharmaceutical enterprises (Rahman et al., 2021). This has enabled the progression of drug resistance to escalate rapidly, as resistance cannot be offset by a steady supply of now agents, which now causes approximately 50,000 deaths per year in the United States and Europe (Murray et al., 2022). Extrapolating from current trends, it has been estimated that antibiotic-resistant infections could contribute to ten million deaths per annum by 2050 (The Review on Antimicrobial Resistance, 2016).

With a weak market for antibacterial drugs stifling the antibiotic development pipeline, preserving the efficacy of existing agents is becoming ever more important to stem the clinical impact of antimicrobial resistance (Ventola, 2015). Systemic issues associated with administration of antibiotics have made this difficult to maintain. Failure to achieve empiric coverage upon disease presentation is associated with a significant increase in incidence of negative clinical outcome. Therefore, antibiotics must be frequently prescribed without prior knowledge of aetiology or susceptibility which takes days to obtain. Such a necessity confounds the success of prescription guidelines to mitigate the selection of resistance without compromising patient care. In this regard, understanding the evolutionary dynamics of drug resistance can yield insights into mitigating its clinical impact (MacLean and Millan, 2019). The fidelity with which the molecular mechanisms of drug resistance can now be studied has been tremendously improved thanks to the increasing accessibility of whole genome sequencing. *In vitro* evolution has been used extensively to study the evolutionary trajectories of drug resistance by exploring the molecular targets of natural selection, compensation of fitness costs and collateral selective effects (Brockhurst, 2015). Understanding the

breadth and intricacies of the evolutionary processes which underpin the selection of drug resistance could make a significant contribution to preserving the continued efficacy of antibiotics in spite of resistance (Kurt Yilmaz and Schiffer, 2021). Rationally identifying selectively neutral treatment regimes, resistance-silencing drug targets and predicting the evolution of high-risk clones is fertile ground for experimental evolution to contribute to allaying the antibiotic resistance crisis.

In its simplest form, antimicrobial resistance is the action of Darwinian natural selection driving evolution on the timescale of a human lifespan. Chromosomal mutations occur randomly throughout bacterial genomes and in the presence of antibiotics, mutants with increased fitness, manifesting as reduced susceptibility, are selected by outgrowing competitors (Levy and Marshall, 2004). This basic principle broadly underpins most adaptive phenomena including resistance, and any complexity associated with adaptation, such as linkage disequilibrium, epistasis or genetic drift are subject to this selective criterion. Whether a mutation can rise to fixation within a population and how quickly is described by the selection co-efficient, a metric gauging relative fitness between a mutant and its derived wild-type (Day et al., 2015). It is an overgeneralisation to suggest that mutants with larger decreases in susceptibility are inherently more fit in the presence of antimicrobial stress. Antibiotics often target macromolecular processes such as cell wall biosynthesis, protein synthesis and DNA replication. These essential cellular processes are highly optimised by selection, and mutations which allow escape from inhibition can result in growth defects due to the introduction of deficiencies in enzyme activity (Lenormand et al., 2018). Even resistance mechanisms which do not directly involve macromolecular activity, such as multidrug efflux or porin loss, can result in growth defects attributable to the energetic costs of impaired nutrient acquisition. Consequently, fitness costs to the organism associated with antimicrobial resistance must not exceed the inhibitory effect of the drug in order to enter positive selection (MacLean et al., 2010). In this way, the strength of the selective pressure dictates the genotypes under selection based on the distribution of the fitness effects of available mutations.

At sub-inhibitory concentrations, resistance mechanisms with low fitness costs will have greater selection coefficients than mutations which confer high level resistance but possess punitive fitness defects (Andersson and Hughes, 2012). As the strength of selection increases as a function of antibiotic concentration, the magnitude reduction in susceptibility becomes the dominant factor determining the selective coefficient of individual mutations (MacLean et al., 2010). The threshold at which this occurs is dependent on additional population genetic factors which alter the accessibility of

particular trajectories across the fitness landscape, such as standing genetic variation, effective population size and mutation supply rate (Pennings, 2012, Raynes et al., 2018, Chevereau et al., 2015). Moreover, though genes are considered discrete selective units, a mutation's genomic background can have a profound effect on its impact on fitness through higher-order selective events (Gagneux et al., 2006, Card et al., 2019). Card et al. (2021) demonstrated that small genotypic variations with no direct impact on antibiotic susceptibility can alter trajectories to resistance by virtue of their epistatic interactions with target site mutations to ameliorate fitness costs. Moreover, this can also occur *a posteriori* due to downstream compensatory adaptation which acts to deform the distribution of mutational fitness effects (Durão et al., 2018). Gene amplification, metabolic remodelling and secondary catalytic domain mutations are mechanisms by which growth defects can be compensated without compromising acquired resistance (Davis et al., 2009, Sun et al., 2009, Händel et al., 2013). Mutations which act to ameliorate fitness defects are central to allowing drug resistance to be maintained even after cessation of selection (Schulz zur Wiesch et al., 2010).

Whilst the evolutionary dynamics of resistance-associated fitness costs have been predominantly studied in a drug-versus-no drug context, there are pleiotropic effects of mutations which extend into additional selective dimensions. Evolution in the natural world is rarely associated with a single selective pressure. The diversity of selective pressures encountered *in situ* are emphasized by the plethora of mechanisms which have evolved to utilise nutrients, adapt to environmental insults and kill competitors (Cooper et al., 2020). Mutations which confer increased fitness to one may have downstream effects which compromise fitness to others (Trampari et al., 2021). A hallmark of laboratory-adapted mutants is high fitness in the presence of a focal pressure, the antagonistic pleiotropic effects of which causes punitive fitness defects in alternative environments (Travisano et al., 1995, Cooper et al., 2001). For example, lineages of *E. coli* adapted to become resistant to bacteriophage λ by loss-of-function of the phage's internalisation receptor, the maltoporin LamB which conferred the collateral effect of impaired maltose utilisation (Lenski, 1988). In the glucose-rich experimental conditions, impaired utilisation of maltose was not detrimental to fitness. However, in a complex selective environment, for a mutation to be positively selected, it must not possess collateral trade-offs which diminish fitness in other contexts (MacLean and Millan, 2019). Indeed, natural selection often preferentially selects for mutations which show the fewest collateral effects on other phenotypic traits, even if fitness gains are modest (Kawecki et al., 2012). Therefore, reconciling fitness costs

with the multifaceted selective pressures of the natural world often impact further limitations on what genotypes can be selected.

It has been established in the previous chapter and in other work that experimental evolution of biofilms on bead substrata can select for biofilm hyperproduction (Trampari et al., 2021, Poltak and Cooper, 2011). Therefore, there are selective pressures intrinsic to the biofilm lifestyle adaptation to which cannot be compromised without introducing fitness defects. Though it was previously demonstrated that biofilm hyperproduction did not confer intrinsic resistance to antibiotics, it is not known if the biofilm lifestyle can alter selection for acquired resistance. If there are collateral trade-offs between selection of resistance and biofilm hyperproduction, this may deform the fitness landscape such as to alter adaptive trajectories. This chapter aims to investigate the mechanisms of drug resistance and collateral trade-offs selected by biofilm- and planktonically-adapted lineages exposed to three antibiotics with distinct modes of action, ciprofloxacin, ceftazidime and tobramycin. The effect of progressively increasing drug stress over the course of the experiment on relative fitness, drug susceptibility and biofilm formation was investigated. The molecular mechanisms underpinning the associated evolutionary trajectories was determined by genome sequencing evolved lineages over the course of the experiment. Following this, lineages from each investigated timepoint were re-adapted to a static concentration of drug representing the previous maximum tolerated concentration. The phenotypic effects on static re-adaptation between the lifestyles were then determined.

5.2. Results

5.2.1. Stepwise adaptation to drugs is associated with compromised selection for biofilm hyperproduction and lifestyle-specific modulation of antibiotic susceptibility

Lineages adapted to ciprofloxacin, ceftazidime and tobramycin in a stepwise manner in biofilm and planktonic lifestyles were assessed for altered susceptibility to a panel of antipseudomonal drugs over the course of the passage series (figure 5.1.). No significant changes in susceptibility (≥2-log_2 fold) were observed in either the unstressed naïve planktonic or biofilm lineages. The largest changes in susceptibility in ciprofloxacin-adapted lineages were observed against the selective agent. The mean ciprofloxacin MIC surpassed the EUCAST clinical breakpoint of 0.5 µg/ mL by the first assayed timepoint in both lifestyles. Further decreases in ciprofloxacin susceptibility were observed at each successive timepoint. Adaptation in the planktonic lifestyle tended to select for a broader susceptibility range than the biofilm-adapted lineages, particularly at earlier timepoints. At transfer 6, the planktonically-adapted lineages were between 1- to 5-log_2 fold less susceptible ciprofloxacin than the ancestor and the biofilms were 2- to 3-log_2 fold less susceptible. At transfer 12, the planktonically-adapted lineages were between 2- and 6- log_2 fold less susceptible to ciprofloxacin and the biofilms were 3- to 4-log_2 fold less susceptible. At the final timepoint, both the biofilm- and planktonically-adapted lineages achieved a maximum 6-log_2 fold reduction in ciprofloxacin susceptibility. Decreased susceptibility to other assayed agents in ciprofloxacin-adapted lineages did not reach clinical breakpoints, nevertheless significant reductions in susceptibility were still observed. Modest reductions in susceptibility to β-lactams were observed in both biofilm and planktonic lifestyles. Reductions in β-lactam susceptibility did not progressively increase with time; transfer 6 generally presented with the highest mean β-lactam MIC in both lifestyles. Biofilm-adapted lineages generally possessed larger decreases in β-lactam susceptibility than planktonically-adapted lineages. Only two planktonically-adapted lineages demonstrated a significant decrease in piperacillin susceptibility at transfer 6, whereas 10 of 12 biofilm-adapted lineages possessed a significant decrease. This trend was also conserved across ceftazidime, meropenem and aztreonam. No significant change in colistin susceptibility was observed in ciprofloxacin-adapted biofilms, however, up to 2-log_2 fold increases in susceptibility to colistin was observed in planktonically-adapted lineages. No change in susceptibility to tobramycin was observed to be selected in either lifestyle.

The largest changes in susceptibility in ceftazidime-adapted lineages were observed against ceftazidime and piperacillin. Significant reductions in susceptibility to both

agents were selected by the first assayed timepoint in both lifestyles and increased at each successive timepoint. The mean ceftazidime susceptibility decreased from 2- to 4- to 5-log_2 fold at each timepoint in the biofilm-adapted lineages and 3- to 5- to 5-log_2 fold in the planktonic lineages. Similarly, piperacillin susceptibility decreased from 3- to 4- to 7-log_2 fold in the biofilm-adapted lineages at transfers 6, 12 and 18, respectively and from 2- to 5- to 7-log_2 fold in the planktonic lineages. The mean susceptibility to ceftazidime and piperacillin in both lifestyles exceeded the clinical breakpoints of 8 µg/ mL and 16 µg/ mL, respectively, at transfer 12. Meropenem and aztreonam susceptibility significantly decreased between transfer 6 and 12 in both lifestyles, but not between transfer 12 and 18 and never surpassed the clinical breakpoint of 8 µg/ mL and 16 µg/ mL, respectively. Ceftazidime adaptation in the planktonic lifestyle was associated with greater decreases in β-lactam susceptibility than biofilm-adapted lineages. The mean log_2 fold change in ceftazidime susceptibility after 18 transfers in the biofilm-adapted lineages was 5 whereas the planktonic lineages possessed a 6-log_2 fold change (1 s.f.). Similarly, the planktonic lineages possessed MICs 1-, 2- and 2-log_2 fold greater than biofilms to piperacillin, meropenem and aztreonam, respectively. No significant change in susceptibility to tobramycin was observed in the planktonically-adapted lineages, however five lineages of biofilms possessed a 2-log_2 fold reduction in tobramycin susceptibility by transfer 12. No significant change in susceptibility to colistin was observed in the biofilm-adapted lineages, however planktonic lineages developed collateral sensitivity to colistin and became up to 3-log_2 fold more susceptible by transfer 18. Both biofilm and planktonic lineages developed 2-log_2 fold decreases in susceptibility to ciprofloxacin by transfer 12.

The largest susceptibility changes in tobramycin-adapted lineages were also observed against the selective agent. At transfers 6 and 12, the tobramycin-adapted planktonic lineages were respectively 3- and 4-log_2 fold less susceptible to tobramycin than the ancestor which surpassed the clinical breakpoint of 2 µg/ mL. However, the biofilm lineages were 1- to 2-log_2 fold less susceptible which did not surpass the clinical breakpoint. Moreover, at transfer 12, the biofilms lineages were on average 3-log_2 fold less susceptible which surpassed the clinical breakpoint. However, by transfer 18, similar degrees of reduced tobramycin susceptibility were observed both the biofilm- and planktonically-adapted lineages, both achieving a maximum 6-log_2 fold change. No significant change in piperacillin, ceftazidime or aztreonam susceptibility relative to naïve lineages was observed to be selected in either biofilm or planktonic lifestyles. However, increasing number of planktonic-adapted lineages achieved 2-log_2 fold reductions in meropenem susceptibility with increasing timepoints. By transfer 18, six

planktonic lineages demonstrated modest reductions in meropenem susceptibility, whereas only one biofilm-adapted lineage did. No change in susceptibility to colistin was observed in planktonic lineages, however by transfer 6, the biofilm-adapted lineages demonstrated collateral sensitivity to colistin with a 3-log_2 fold increase in susceptibility. Both lifestyles were able to select for 2-log_2 fold reductions in ciprofloxacin susceptibility, with increasing number of lineages achieved susceptibility reductions with increasing timepoints. By transfer 18, all 12 planktonically-adapted lineages and 10 biofilm-adapted lineages possessed significant reductions in ciprofloxacin susceptibility.

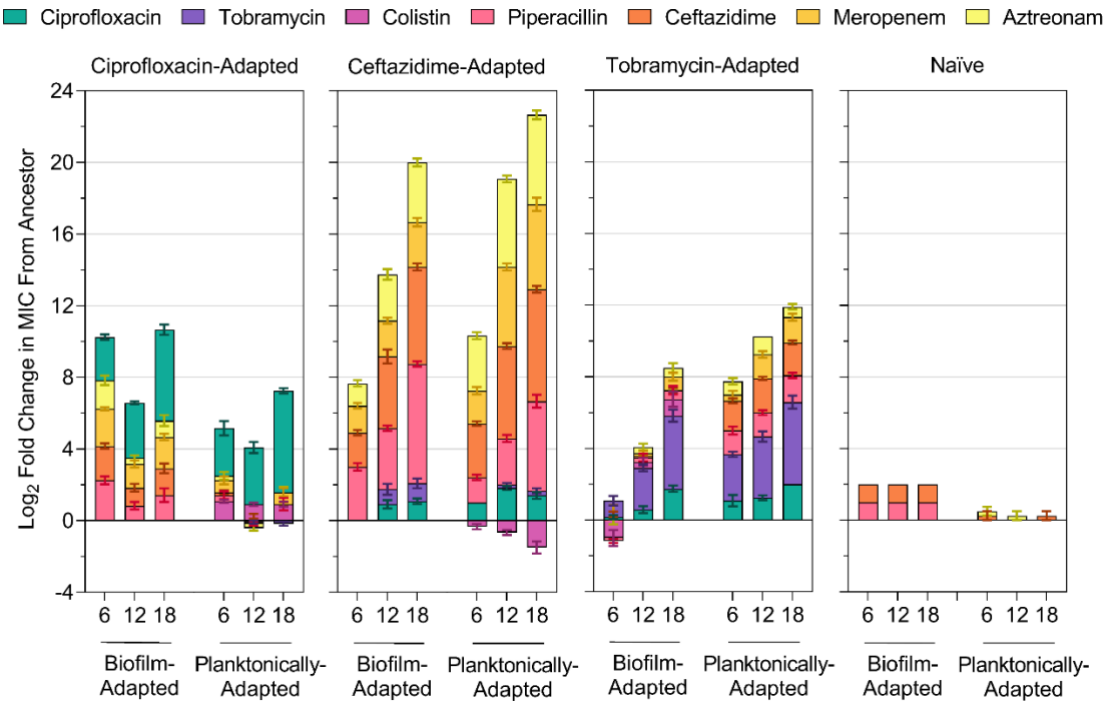

Figure 5.1. Antibiotic susceptibility to a panel of antipseudomonal antibiotics determined via agar dilution MIC of experimentally evolved lineages of *P. aeruginosa* adapted in a stepwise fashion in biofilm or planktonic lifestyles to the antibiotics: ciprofloxacin, ceftazidime or tobramycin Twelve replicate lineages were evolved under antibiotic stress which doubled in concentration every three passages from 0.25× to 8× MIC over 18 passages. Significant changes in susceptibility were defined as a >1-log_2 fold change. Data shown as cumulative log_2 fold change from ancestor, errors bars show ± standard error of the mean, n = 4 per lineage.

The capacity for experimentally evolved lineages to form biofilms after drug adaptation was determined (figure 5.2.). There was no significant difference in biofilm formation between the ancestor and the naïve planktonically-adapted lineages at any timepoint (ancestor vs naïve planktonically-adapted transfer 6: $p > 0.9999$, ancestor vs naïve planktonically-adapted transfer 12: $p > 0.9999$, ancestor vs naïve planktonically-adapted transfer 18: $p > 0.9999$). The naïve biofilm-adapted lineages formed approximately 1.5× to 2× more biomass than the ancestor by transfer 18 ($p = 0.0493$). Significant changes in biofilm formation were not observed in naïve biofilms at earlier timepoints (ancestor vs naïve biofilm-adapted transfer 6: $p = 0.9994$, ancestor vs naïve biofilm-adapted transfer 12: $p = 0.9991$). There was no significant different between the ancestor and biofilm lineages adapted to any selective agent at any timepoint (ancestor vs biofilm ciprofloxacin-adapted transfer 6: $p > 0.9999$, ancestor vs biofilm ciprofloxacin-adapted transfer 12: $p = 0.8282$, ancestor vs biofilm ciprofloxacin-adapted transfer 18: $p = 0.9376$, ancestor vs biofilm ceftazidime-adapted transfer 6: $p > 0.9999$, ancestor vs biofilm ceftazidime-adapted transfer 12: $p > 0.9999$, ancestor vs biofilm ceftazidime-adapted transfer 18: $p > 0.9999$, ancestor vs biofilm tobramycin-adapted transfer 6: $p > 0.9999$, ancestor vs biofilm tobramycin-adapted transfer 12: $p = 0.9887$, ancestor vs biofilm tobramycin-adapted transfer 18: $p = 0.7360$). Conversely, all ciprofloxacin-adapted planktonic lineages formed significantly less biofilm than the ancestor (ancestor vs planktonic ciprofloxacin-adapted transfer 6: $p < 0.0001$, ancestor vs planktonic ciprofloxacin-adapted transfer 12: $p = 0.0353$, ancestor vs planktonic ciprofloxacin-adapted transfer 18: $p = 0.0008$). Moreover, in ceftazidime-adapted planktonic lineages, transfer 12 demonstrated a significant reduction in biofilm formation ($p = 0.0211$) and in tobramycin-adapted planktonic lineages, transfers 12 and 18 demonstrated a significant reduction in biofilm formation (ancestor vs planktonic tobramycin-adapted transfer 12: $p = 0.0010$, ancestor vs planktonic tobramycin-adapted transfer 18: $p = 0.0007$).

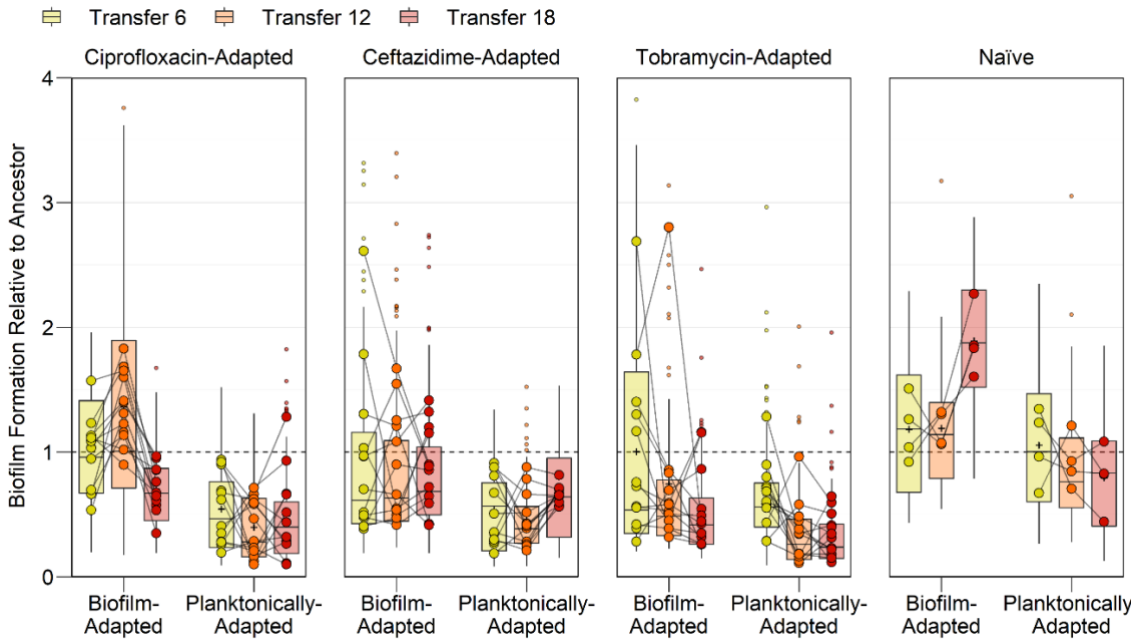

Figure 5.2. Biofilm formation as determined via crystal violet staining of experimentally evolved lineages of *P. aeruginosa* adapted in biofilm or planktonic lifestyles to ciprofloxacin, ceftazidime or tobramycin in a stepwise fashion. Statistical differences in biofilm formation between exposure condition and lifestyle over the passage series were detected via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as mean biofilm formation relative to ancestor, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 8 per lineage.

Gross fitness changes associated with drug adaptation in biofilm and planktonic lifestyles were assessed by measuring the AUC of growth curves in broth (figure 5.3.). No change in fitness was detected in the naïve biofilm lineages (naïve biofilm transfer 6 vs naïve biofilm transfer 12: $p = 0.9999$, naïve biofilm transfer 6 vs naïve biofilm transfer 18: $p = 0.9971$, naïve biofilm transfer 12 vs naïve biofilm transfer 18: $p > 0.9999$) or naïve planktonic lineages (naïve planktonic transfer 6 vs naïve planktonic transfer 12: $p = 0.9999$, naïve planktonic transfer 6 vs naïve planktonic transfer 18: $p = 0.9958$, naïve planktonic transfer 12 vs naïve planktonic transfer 18: $p = 0.9999$) at any timepoint. Ciprofloxacin adaptation was associated with fitness defects exclusively in the biofilm-adapted lineages. Transfers 12 and 18 of the ciprofloxacin-adapted biofilm lineages possessed significantly lower AUCs than transfer 6 (biofilm ciprofloxacin-adapted transfer 6 vs biofilm ciprofloxacin-adapted transfer 12: $p = 0.0027$, biofilm ciprofloxacin-adapted transfer 6 vs biofilm ciprofloxacin-adapted transfer 18: $p = 0.0008$), however no significant decrease in AUC was detected between transfer 12 and 18 ($p > 0.9999$). The ciprofloxacin-adapted planktonic lineages did not demonstrate a significant change in AUC at any timepoint ($p = 0.9985$, $p = 0.9467$, $p = 0.3918$). Ceftazidime adaptation was associated with fitness defects in

both biofilm and planktonic lifestyles. Ceftazidime-adapted biofilm lineages demonstrated significantly lower AUCs at transfers 12 and 18 than transfer 6 ($p = 0.0057$, $p = 0.0067$), however no significant decrease in AUC was detected between transfer 12 and 18 ($p > 0.9999$). The ceftazidime-adapted planktonic lineages did not demonstrate a significant change in AUC between transfer 6 and 12 ($p > 0.9999$) or transfer 12 and 18 ($p = 0.1842$). However, there was a significant reduction in AUC between transfer 6 and 18 ($p = 0.0365$). There was no significant difference between the fitness defects selected by ceftazidime adaptation between the biofilm or planktonic lifestyles at any timepoint (biofilm cefazidime-adapted transfer 6 vs planktonic ceftazidime-adapted transfer 6: $p = 0.9620$, biofilm cefazidime-adapted transfer 12 vs planktonic ceftazidime-adapted transfer 12: $p = 0.7400$, biofilm cefazidime-adapted transfer 18 vs planktonic ceftazidime-adapted transfer 18: $p = 0.9990$). Tobramycin-adapted biofilm lineages demonstrated significantly lower AUCs at transfers 12 and 18 than transfer 6 (biofilm tobramycin-adapted transfer 6 vs biofilm tobramycin-adapted transfer 12: $p < 0.0001$, biofilm tobramycin-adapted transfer 6 vs biofilm tobramycin-adapted transfer 18: $p < 0.0001$), however no further decrease in AUC was detected between transfer 12 and 18 ($p > 0.9999$). The tobramycin-adapted planktonic lineages did not demonstrate a significant change in AUC between transfer 6 and 12 ($p = 0.9958$), however significant reductions in were observed between transfers 6 and 12 ($p < 0.0001$) and transfers 12 and 18 ($p < 0.0001$). There was no significant difference between fitness defects between the biofilm or planktonic lifestyles at transfers 6 or 18 (biofilm tobramycin-adapted transfer 6 vs planktonic tobramycin-adapted transfer 6: $p = 0.1293$, biofilm tobramycin-adapted transfer 12 vs planktonic tobramycin-adapted transfer 12: $p = 0.6696$). However, tobramycin-adapted biofilms possessed significantly larger fitness defects than tobramycin-adapted planktonic lineages at transfer 12 ($p = 0.0025$).

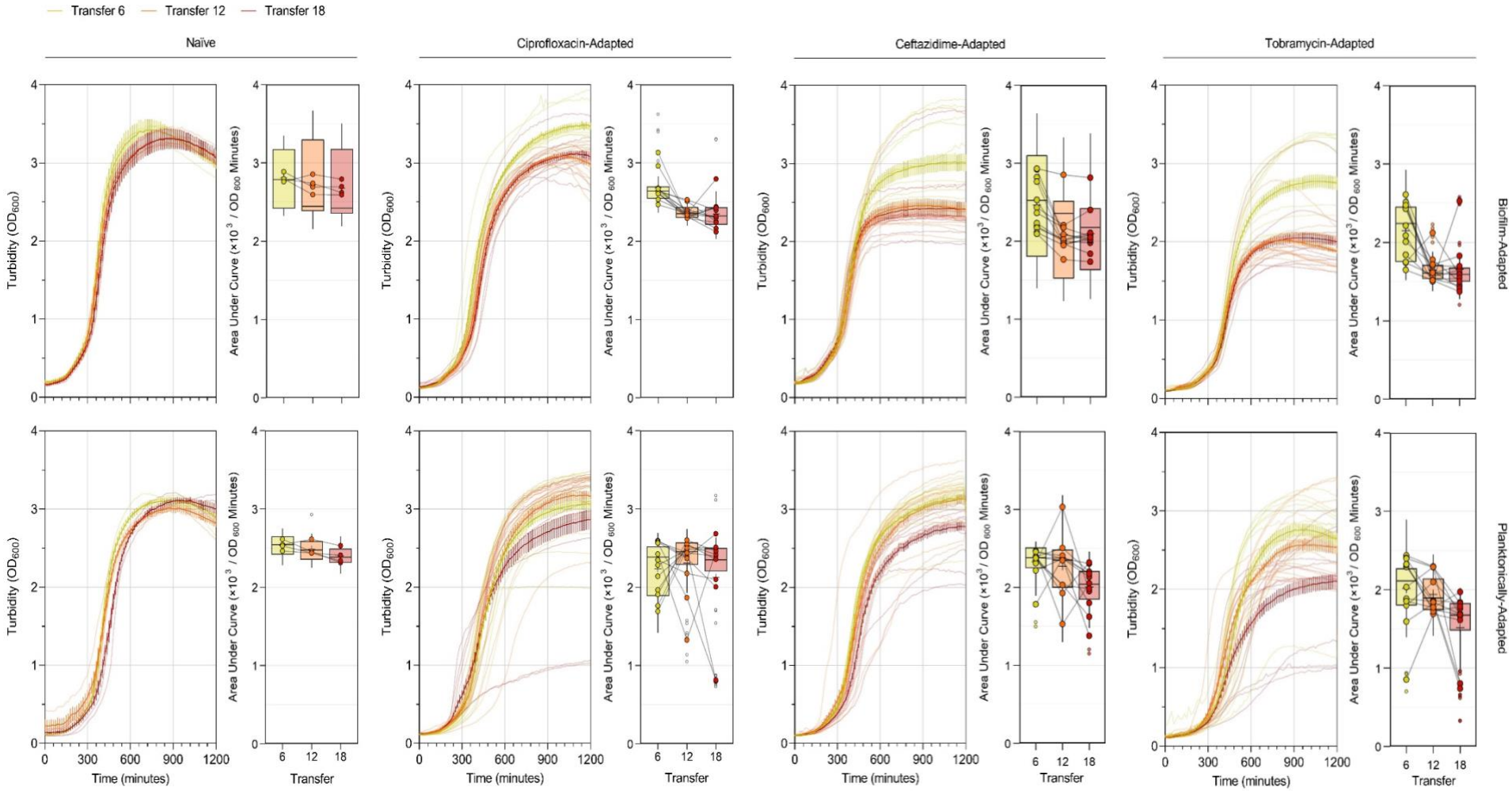
Transfer 6
Transfer 12
Transfer 18
Naïve
Ciprofloxacin-Adapted
Ceftazidime-Adapted
Tobramycin-Adapted
Biofilm-Adapted
Planktonically-Adapted
Turbidity (OD600)
Area Under Curve (×10^3 / OD600 Minutes)
Time (minutes)
Transfer
0
300
600
900
1200
6
12
18

Figure 5.3.

Figure 5.3. Growth kinetics of experimentally evolved lineages of *P. aeruginosa* adapted in biofilm or planktonic lifestyles to ciprofloxacin, ceftazidime or tobramycin in a stepwise fashion. The area under the curve was calculated and statistical differences between exposure condition and lifestyle over the passage series were detected via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Curves show mean OD_{600}, ± standard error of the mean, Boxplot shows area under curve, box limits show ± 1.5× interquartile range, whiskers show minimum and maximum, large free datapoints show lineage mean, small free datapoints show outliers, n = 4 per lineage.

Correlations between changes in phenotypic traits associated with drug adaptation in biofilm and planktonic lifestyles were investigated via linear regression analysis (figure 5.4.). There was no correlation detected between AUC and biofilm formation in naïve biofilm (R^2 = 0.0198, F = 0.7868, p = 0.3959) or planktonic lineages (R^2 = 0.1745, F = 3.325, p = 0.0982). No significant correlations were detected in ciprofloxacin-adapted planktonic lineages between biofilm formation and ciprofloxacin susceptibility (R^2 = 0.0235, F = 0.1954, p = 0.6613), fitness as measured by AUC and ciprofloxacin susceptibility (R^2 = 0.0250,1 F = 0.1462, p = 0.7046) or fitness and biofilm formation (R^2 = -0.0276, F = 0.0594, p = 0.8089). Similarly, no significant correlations were detected in ceftazidime-adapted planktonic lineages between biofilm formation and ceftazidime susceptibility (R^2 = 0.0170, F = 1.6050, p = 0.2137), fitness and ceftazidime susceptibility (R^2 = -0.0004, F = 0.9856, p = 0.3278) or fitness and biofilm formation (R^2 = -0.0442, F = 2.6160, p = 0.1150). Furthermore, no significant correlations were detected between fitness and biofilm formation susceptibility in biofilm lineages adapted to ceftazidime (R^2 = -0.0206, F = 1.7350, p = 0.1966) or ciprofloxacin (R^2 = 0.0274, F = 0. 0676, p = 0.7964). A statistically significant negative correlation between biofilm formation and ciprofloxacin susceptibility (R^2 = -0.1480, F = 7.0820, p = 0. 0118), and between fitness and ciprofloxacin susceptibility (R^2 = -0.0398, F = 2.4520, p = 0.1266) was detected in the biofilm-adapted lineages. Similarly, a statistically significant negative correlation between fitness and ceftazidime susceptibility was detected in the ceftazidime-adapted biofilm lineages (R^2 = -0.1966, F = 15.2200, p = 0. 0004). However, a significant positive correlation was observed between biofilm formation and fitness in ceftazidime-adapted biofilms (R^2 = 0.1153, F = 5.5630, p = 0. 0242). Statistically significant negative correlations between biofilm formation and tobramycin susceptibility were detected in both tobramycin-adapted biofilm (R^2 = -0.1106, F = 5.3520, p = 0. 0269) and planktonic lineages (R^2 = -0.1065, F = 5.1730, p = 0. 0294). Significant negative correlations between biofilm formation and tobramycin susceptibility were also detected in tobramycin-adapted biofilms (R^2 = -0.5970, F = 52.8600, p < 0.0001) and planktonic lineages (R^2 = -0.1473, F = 7.0480, p = 0. 0120).

Furthermore, significant positive correlations between fitness and biofilm formation were also detected in tobramycin-adapted biofilm (R^2 = 0.1070, F = 5.1930, p = 0. 0291) and planktonic lineages (R^2 = 0.0631, F = 3.3570, p = 0. 0757). Biofilm lineages which possessed reduced susceptibility to drugs tended towards reduced biofilm formation which was not observed in the planktonic lineages. Despite this, the planktonic lineages consistently possessed lower biofilm formation than the biofilm lineages regardless of their susceptibility to the selective agent. Consequently, though the drug-adapted biofilms became progressively worse at forming biofilms, they remained consistently higher than the planktonic lineages which were biofilm-compromised from the first assayed timepoint. Furthermore, fitness defects associated with drug adaptation appeared to be more punitive in biofilms than planktonic lineages. Fitness had little effect on biofilm formation in the planktonic lineages, however, biofilm-adapted lineages which possessed fitness defects tended to form significantly less biofilm.

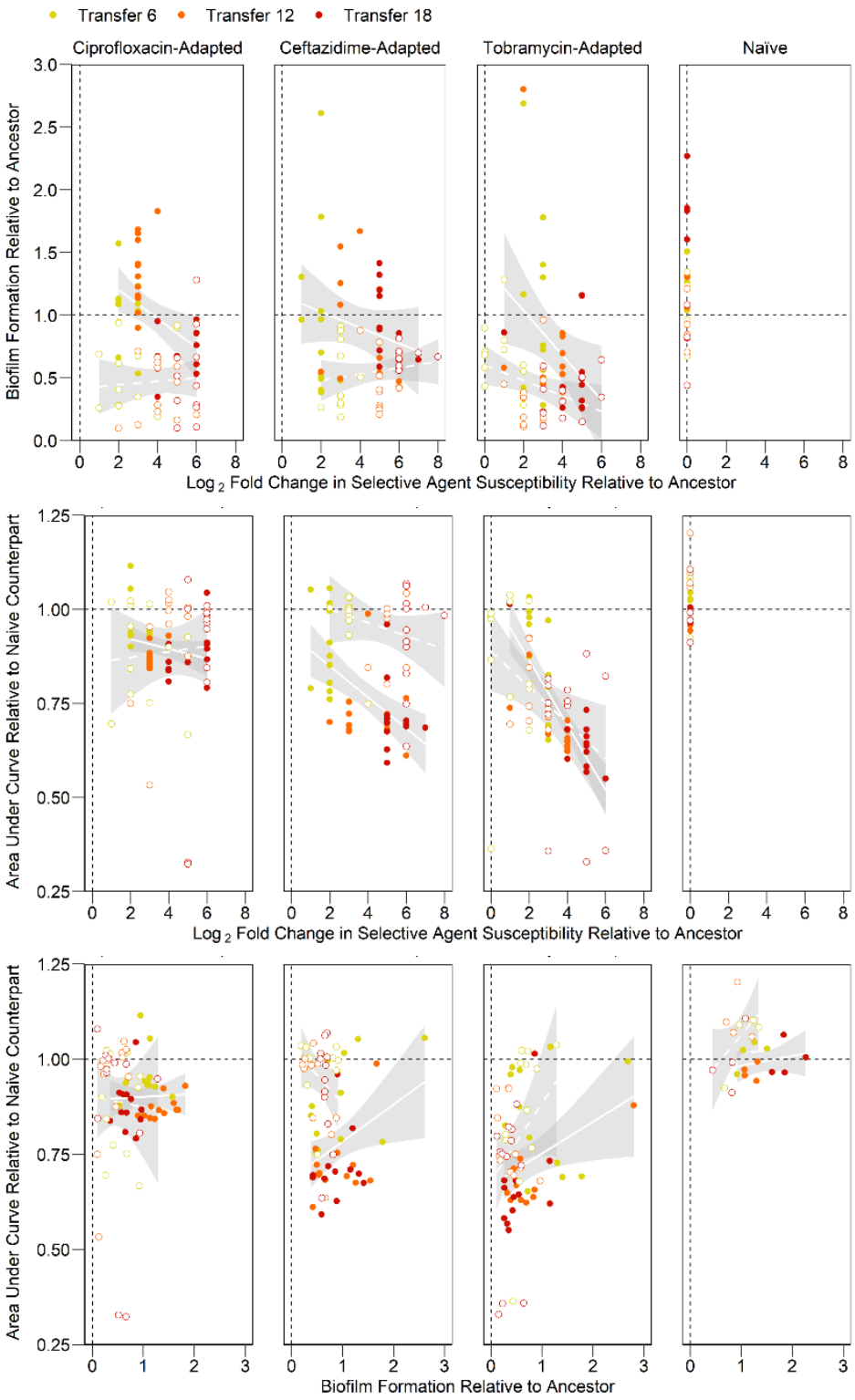

Figure 5.4. Regression analysis of independent phenotypic properties in experimentally evolved populations of *P. aeruginosa* adapted to antibiotic stress increasing in a stepwise fashion in a biofilm or planktonic lifestyles. Correlations between biofilm formation, fitness and drug susceptibility across the passage series were calculated by generating a linear regression model from each permutation of phenotypes in RStudio v 2021.09.0. Data shown as regression trendline ± 95% confidence intervals, biofilm trendline shown as solid line, planktonic trendline shown as broken line, n = 4 per lineage.

5.2.2. Evolutionary trajectories to drug resistance are characterised by lifestyle-specific selective targets

Four lineages from each exposure condition and lifestyle were randomly selected for whole genome sequencing and sequenced at each assayed timepoint. SNPs were identified which were conserved between multiple parallel lineages but not found in naïve counterparts (figure 5.5.). Two lineages of naïve biofilms possessed a mutation in the EAL-domain containing c-di-GMP phosphodiesterase *dipA*. At transfers 6, 12 and 18, the naïve biofilm-1 possessed a 3 bp inframe deletion at position 2623 resulting in a Tyr875 deletion. Moreover, naïve biofilm-2 possessed a guanine duplication at position 2381 resulting in an Ala794 frameshift. At all timepoints, all naïve planktonic lineages possessed a T785C missense mutation in the PA14_RS16310 HlyD family efflux transporter periplasmic adaptor subunit resulting in a Ile262Thr substitution which was not observed in the naïve biofilms. All ciprofloxacin-adapted lineages in both lifestyles possessed mutations in *gyrA* DNA gyrase subunit A exclusively at transfer 18. Ciprofloxacin-adapted biofilm-1, -2 and -4 and planktonic-1, -3 and -4 possessed a C248T missense mutation resulting in a Thr83Ile substitution. Biofilm-3 possessed a A260G missense mutation resulting in a Asp87Gly substitution. Planktonic-2 possessed a GCC conservative inframe insertion at position 394 resulting in an Ala131 duplication.

Seven unique substitutions were identified in *nfxB*, the transcriptional repressor of MexCD-OprJ in all ciprofloxacin-adapted planktonic lineages and two biofilm lineages. Planktonic-1 demonstrated a 10 bp deletion at position 543 at transfer 6 and a 20 bp deletion at position 544 at transfer 18, both resulting in a Ser182 frameshift. Planktonic-2 possessed an A564C stop codon loss at transfer 6. Planktonic-3 possessed a C434T missense mutation resulting in a Thr145Ile substitution at transfer 12. Planktonic-4 possessed a T544C missense mutation resulting in a Ser182Pro substitution at transfers 6 and 18. At transfer 12, planktonic-4 possessed a stop codon loss. Ciprofloxacin-adapted biofilms-1 and -3 also possessed a stop codon loss and biofilm-3 also possessed a 12 bp inframe insertion at position 469 resulting in a Tyr153_Val156 duplication.

Mutations in the transcriptional repressors of MexAB-OprM, *mexR* and *nalC* were exclusively seen in ciprofloxacin-adapted biofilm lineages. Biofilm-1 and -2 possessed *mexR* mutations and biofilm-3 and -4 possessed *nalC* mutations. Biofilm-1 possessed a G196A missense mutation in *mexR* which resulted in a Ala66Thr substitution at transfer 6. At transfer 18 biofilm-1 also possessed an ACC inframe insertion resulting in a His insertion at Asn53_Leu54. At transfer 6 biofilm-2 possessed an 11 bp deletion at

position 174 in *mexR* resulting in a Gln55 frameshift. Biofilm-3 possessed a cytosine duplication in *nalC* at position 448 at transfer 6 and a GGCCG duplication at position 339 at transfer 18 which resulted in a His150 and Gln134 frameshift, respectively. Biofilm-4 possessed a 14 bp deletion at position 609 in *nalC* at transfers 6 and 12 which resulted in Ile199 frameshift.

Ciprofloxacin-adapted planktonic lineages also possessed mutations in several genes involved in type IV pili biogenesis which were not observed in ciprofloxacin-adapted biofilms. Ciprofloxacin-adapted planktonic-1 and -4 possessed mutations in the type IV pilus secretin *pilQ*. Planktonic-1 possessed a C1805T missense mutation in *pilQ* which resulted in a Pro602Leu substitution at transfer 18. Planktonic-4 possessed a 16 bp duplication in *pilQ* at position 1388 at transfer 12 and an adenine duplication at position 1680 at transfer 18 resulting in frameshifts in Val561 and Asn458, respectively. Similarly, planktonic-1 and -2 possessed mutations in the type IV pilus biogenesis protein *pilQ*. Planktonic-1 possessed an A365G missense mutation in *pilQ* resulting in Glu122Gly substitution at transfer 18 and planktonic-2 possessed a 12 bp inframe deletion at position 554 resulting in a Leu185_Glu188 deletion at transfer 6.

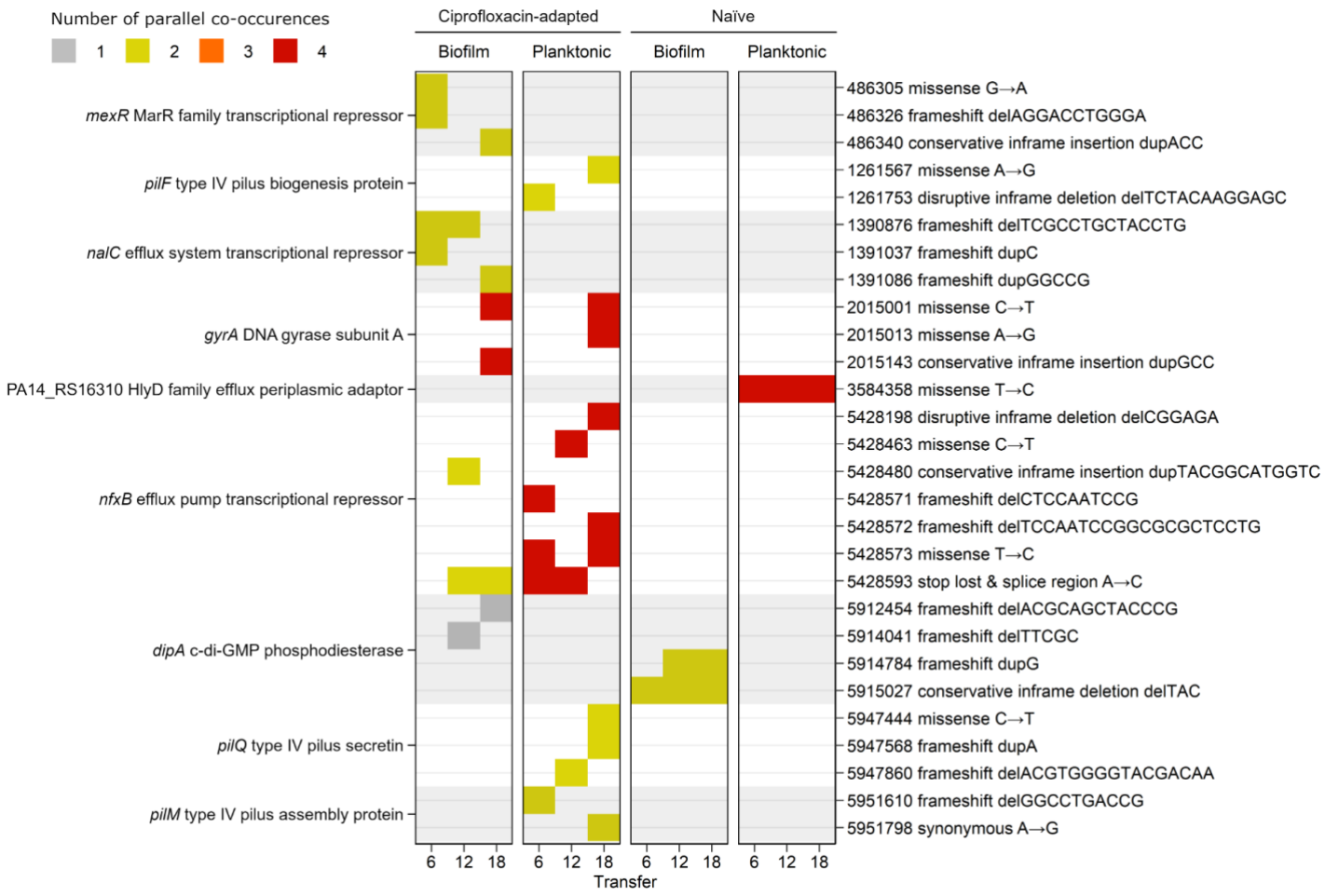

Figure 5.5. Gene targets under parallel selection in experimentally evolved lineages of *P. aeruginosa* adapted to ciprofloxacin stress increasing in a stepwise fashion in biofilm or planktonic lifestyles identified from Illumina short-read whole genome sequencing data using snippy v 4.6.0. Data shown as number of times a mutation co-occurred in the four parallel lineages sequenced, n = 4.

From the ceftazidime-adapted lineages, SNPs were identified which were conserved between multiple parallel lineages but not found in naïve counterparts (figure 5.6.). All ceftazidime-adapted biofilm lineages and three planktonic lineage possessed mutations in *mutL*. At transfers 12 and 18, ceftazidime-adapted biofilm-1 and -4 possessed a 22 bp deletion at position 1164 resulting in a Val383 frameshift. Biofilm-2 and -3 possessed the same mutation at transfer 18 exclusively. At transfer 12 and 18, ceftazidime-adapted planktonic-2 and -3 possessed a TG duplication at position 552 in *mutL* resulting in an Ala185 frameshift. Planktonic-4 possessed a thymine deletion at position 837 which resulted in a Arg280 frameshift

Three lineages of ceftazidime-adapted biofilms and all ceftazidime-adapted planktonic lineages demonstrated mutations in *ampR,* the transcriptional repressor of the chromosomal β-lactamase AmpC. All ceftazidime-adapted planktonic lineages possessed an A404G missense mutation resulting in an Asp135Gly substitution at transfer 12 in planktonic-1 and transfer 18 in planktonic-2 and -3. At transfers 12 and 18, respectively, biofilm-1 and -2 possessed a C640T missense mutation resulting in an Arg214Trp substitution. At transfer 6, biofilm-4 demonstrated a G403T missense mutation resulting in an Asp135Tyr substitution, and also possessed a G268A missense mutation resulting in a Gly90Ser substitution at transfer 12.

The ceftazidime-adapted biofilms possessed a number of parallel mutations absent in planktonic lineages. Three of the ceftazidime-adapted biofilm lineages possessed mutations in the peptidoglycan recycler *mpl*. At transfer 6, biofilm-1 possessed a G991C missense mutation resulting in an Ala331Pro substitution. Biofilm-2 demonstrated a A236G missense mutation resulting in a Glu79Gly substitution at transfer 18. Biofilm-4 demonstrated a C395T missense mutation resulting in a Pro132Leu at transfer 18. At transfers 12 and 18, respectively, biofilm-1 and -2 possessed T664C missense mutations in PA14_RS01115 Coenzyme A transferase subunit A resulting in a Cys222Arg substitution. In the same lineages, a guanine duplication at position 143 causing a Val48 frameshift in the putative LysE-family transporter PA14_RS10610, an A226G missense mutation in the multidrug efflux pump *mexK* resulting in a Thr76Ala substitution, an A154G missense mutation resulting in a Thr52Ala substitution in the thioesterase II family protein PA14_RS13660 and a T359C missense mutation in the *thrB* homoserine kinase resulting in a Val120Ala substitution were also observed. At transfer 18, both biofilm-1 and -2 demonstrated mutations in the 2-oxoisovalerate dehydrogenase subunit beta *bkdA2*. Biofilm-1 possessed a G880A

missense mutation resulting in a Gly294Arg substitution whereas lineage 2 possessed a cytosine duplication at position 972 resulting in aTyr325 frameshift. At transfers 12 and 18, biofilm-1 and -3 possessed an A383G missense mutation resulting in a Asn128Ser substitution in the sterol desaturase family protein PA14_RS16205. Biofilm-1 and -4 possessed mutations in the two copies of the phosphotransferase *ptsP* at transfers 12 and 18. Lineage 1 possessed an A226G missense mutation in the allele at genomic position 393035 resulting in an Asn89Ser substitution and lineage 4 possessed a T557C missense mutation in the allele at position 1564275 resulting in a Val186Ala substitution. At transfer 18, mutations in the *dacB* carboxy/ endopeptidase were observed in biofilm-1 and -4 with a G1342A missense mutation causing a Val448Met substitution and a T32C missense mutation causing a Leu13Pro substitution, respectively. Biofilm-2 and -4 both possessed a guanine duplication at position 1797 in the lipase *tle1* causing a Val600 frameshift at transfer 12 and 18, respectively.

The ceftazidime-adapted planktonic lineages also possessed several parallel mutations absent in biofilm lineages. At transfer 6, all ceftazidime-adapted planktonic linages possessed mutations in the phosphomannomutase *algC*. Planktonic-1 possessed a T2498A missense mutation in *algC* at transfer 6 resulting in a Leu833Gln substitution. Pltnaktonic-2 and -3 possessed a T2261C missense mutation at transfer 6 resulting in a Leu754Pro substitution. At transfer 6 and 12, biofilm-4 demonstrated an A2071C missense mutation in *algC* resulting in a Thr691Pro substitution. Planktonic-3 and -4 also possessed mutations in the purine biosynthesis gene *purL.* At transfer 12 and 18, planktonic-3 possessed an A3224G missense mutation which conferred a His1148Arg substitution. Planktonic-4, one the other hand, possessed an A259G missense mutation which resulted in a Ser87Pro substitution. At transfer 18, biofilm-2 possessed a G1222A missense mutation in *leuC* which resulted in an Ala408Thr substitution. Moreover, at transfer 12 and 18, biofilm-3 possessed a G2506A missense mutation which resulted in a Glu836Lys substitution in *leuC*. Mutations in *nalC* were identified in planktonic biofilm-3 and -4 at transfers 12 and 18. Biofilm-3 possessed a cytosine duplication a position 448 resulting in a His150 frameshift and biofilm-4 possessed a A268C missense mutation causing a Thr90Pro substitution.

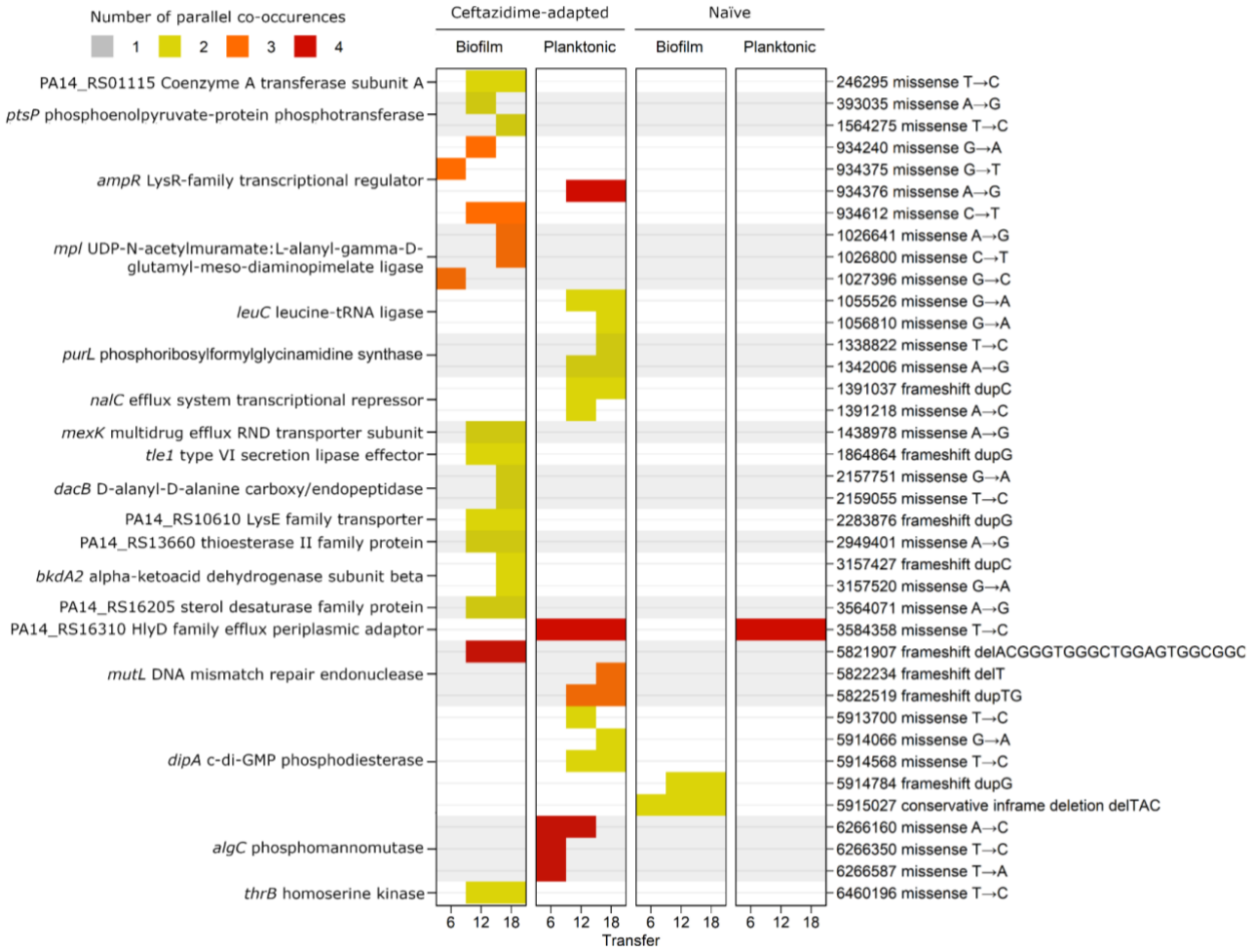

Figure 5.6. Gene targets under parallel selection in experimentally evolved lineages of *P. aeruginosa* adapted to ceftazidime stress increasing in a stepwise fashion in biofilm or planktonic lifestyles identified from Illumina short-read whole genome sequencing data using snippy v 4.6.0. Data shown as number of times a mutation co-occurred in the four parallel lineages sequenced, n = 4.

From the tobramycin adapted lineages, SNPs were identified which were conserved between multiple parallel lineages but not found in naïve counterparts (figure 5.7.). All tobramycin-adapted biofilm and planktonic lineages possessed mutations in *fusA* encoding elongation factor G. In total, ten different mutations were identified in *fusA,* none of which were identified in both biofilm and planktonic lifestyles. An A2011G missense mutation resulting in a Thr671Ala substitution was identified in biofilm-1, -3 and -4 at transfers 6 to 18, 6 to 12, and 12, respectively. Biofilm-4 also possessed a T1786C missense mutation resulting in a Phe596Leu substitution. Biofilm-2 possessed A286G, A1366G and A2033G missense mutations in *fusA* resulting in Thr96Ala, Thr456Ala and Gln678Arg substitutions at transfers 12, 6 and 18 and 18, respectively. Tobramycin-adapted planktonic-1, -2 and -4 possessed a C2038T missense mutation which resulted in a Arg680Cys substitution at transfers 6, 6 to 18, and 12, respectively. Planktonic-1 and -3 possessed a G61A missense mutation resulting in an Ala21Thr substitution at transfer 18 and 12 to 18, respectively. Planktonic-2 possessed an A299G missense mutation resulting in a Glu100Gly substitution at transfer 6. Planktonic-4 possessed an A678T missense mutation resulting in a Gln678Leu substitution at transfer 6.

Two planktonic lineages and one biofilm lineage possessed mutations in the two-component sensor *amgS*. Planktonic-1 and -3 possessed a G55C missense mutation resulting in a Val19Leu at transfer 18 and 12, respectively. At transfer 18, biofilm-1 possessed a T130A missense mutation in *amgS* which resulted in a Trp130Arg substitution. At transfer 12, planktonic-1 and -2 possessed mutations in the two-component sensor *pmrB* including Tl260A and T842C missense mutations resulting in Leu87Gln and Val281Ala substitutions, respectively. Planktonic-1 and -2 possessed mutations in another two-component response regulator *parR* at transfer 18 including G177A and G259A missense mutations which resulted in Met59Ile and Glu87Lys substitutions.

Three planktonic lineages possessed mutations in the large-conductance mechanosensitive channel protein *mscL* at transfer 12*.* Planktonic-1 possessed a guanine deletion at position 113 resulting in a Gly38 frameshift. Planktonic-2 possessed a T103C missense mutation which resulted in a Ser35Pro substitution and planktonic-4 possessed a T218 missense mutation which resulted in a Leu73Gln substitution.

Two lineages of tobramycin-adapted biofilms possessed mutations in the c-di-GMP receptor *morA* which were absent in the planktonic lineages. Biofilm-1 and -4 possessed a A2866T missense mutation resulting in a Ser956Cys substitution.

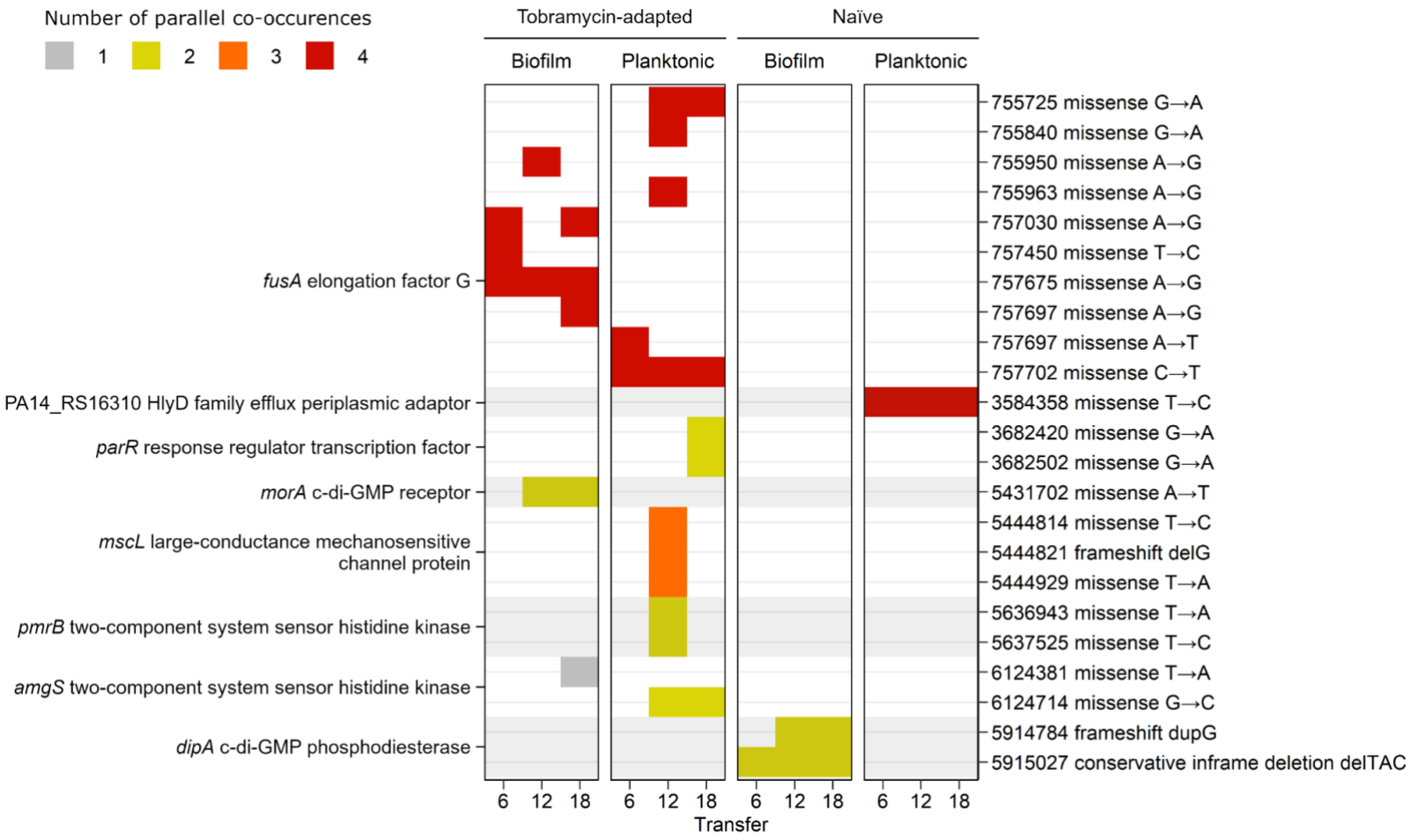

Figure 5.7. Gene targets under parallel selection in experimentally evolved lineages of *P. aeruginosa* adapted to tobramycin stress increasing in a stepwise fashion in biofilm or planktonic lifestyles identified from Illumina short-read whole genome sequencing data using snippy v 4.6.0. Data shown as number of times a mutation co-occurred in the four parallel lineages sequenced, n = 4.

Mutations which did not demonstrate genotypic parallelism but nevertheless possessed hypothesised roles in the adaptive process based on known phenotypic functions were identified (table 5.1.). At transfers 12 and 18, naïve biofilm-3 possessed a 12 bp inframe deletion at position 71 in the GGDEF-domain containing c-di-GMP diguanylate cyclase *tpbB* resulting in a Gly24_Leu27 deletion. Moreover, naïve biofilm-4 possessed a TT deletion at position 484 in the diguanylate cyclase inhibitor *yfiR* resulting in a Phe162 frameshift.

Across both ciprofloxacin-adapted biofilm and planktonic lineages, mutations in genes involved in type III and type IV secretion were observed. Biofilm-1 and planktonic-1 possessed mutations in the type VI secretion system tip protein *vgrG*. Planktonic-1 also possessed mutations in the type III secretion system export apparatus subunit *pscU*, and type VI secretion system effector protein *tseT*. Biofilm-4 and planktonic-4 possessed mutations in the type VI secretion system FHA domain protein *tagH*. Moreover, both ciprofloxacin-adapted biofilm and planktonic lineages possessed mutations in genes involved in the branched-chain alpha-keto acid dehydrogenase pathway. Biofilm-2 possessed a mutation in the branched-chain alpha-keto acid dehydrogenase subunit B *bkdB*, biofilm-4 and planktonic-2 possessed mutations in the 2-oxoisovalerate dehydrogenase subunit beta *bkdA2* and planktonic-1 possessed a mutation in the 2-oxoisovalerate dehydrogenase subunit alpha *bkdA1*. Ciprofloxacin-adapted planktonic lineages also possessed additional mutations in genes involved in type IV pili biogenesis including type IV pilus biogenesis protein *pilB* and type IV pilus assembly ATPase *pilN* in planktonic-1, type IV pilus biogenesis protein *pilC* in planktonic-2 and type IV pilus assembly protein *pilM* in planktonic-3. Ciprofloxacin-adapted planktonic-1 and -2 also possessed mutations in regulators of quorum sensing systems. Planktonic-1 possessed a mutation in the transcriptional regulator of the Pqs system *mvfR* and planktonic-2 in the transcriptional repressor of the Las system *lasR.* Finally, planktonic-1 also possessed a number of distinguishing mutations including the DNA mismatch repair protein *mutS*, conferring a hypermutator phenotype, the σ-factor *rpoC* and flagellar biosynthesis genes PA14_RS03255 tail length determinator protein, *fleQ* master transcriptional regulator of flagellar motility and *fliJ* flagella biosynthesis chaperone.

Genes which demonstrated parallelism in single lineages across lifestyles were also identified. This included the putative GNAT family N-acetyltransferase PA14_RS20560 in biofilm-1 and planktonic-2, the type I methionyl aminopeptidase *map* in biofilm-4 and planktonic-2, the adenosylcobinamide-phosphate synthase *cbiB* in biofilm-4 and planktonic-3 and the denitrification protein *norD* in biofilm-1 and planktonic-2. Mutations

in genes involved in c-di-GMP signalling were also identified in lineages both lifestyles including *dipA* in planktonic-3, the repressor of the cyclic diguanylase TpbB, *tpbA* in biofilm-1 and the EAL domain-containing phosphodiesterase PA14_RS12830 in biofilm-4.

Mutations in the group 4 glycosyl transferase *orfN* were observed in both ceftazidime-adapted biofilm-2 and planktonic-1. Mutations in genes involved in protein synthesis were also observed including 30S ribosomal protein S12 *rpsL* in biofilm-1, 50S ribosomal protein L2 *rplB* in planktonic-3 and translation initiation factor IF-2 *infB* in planktonic-4. Similarly, two lineages possessed mutations in the peptidoglycan biosynthesis genes *wecB* in planktonic-2 and *wecC* in planktonic-4. Planktonic-1 and -4 also possesses mutations in genes involved in type IV pilus biogenesis including *pilQ, pilY* and *chpA*. Planktonic-2 and -4 possessed mutations in cytochrome biosynthesis genes *ccoN* and *ccmA*. Planktonic-4 acquired a hypermutator phenotype at transfer 12 due to a *mutL* DNA mismatch repair protein mutation. Planktonic-4 also possessed mutations in the oxidative stress response regulators *soxR* and *oxyR*.

Table 5.1. Non-synonymous substitutions in genes after drug adaptation which did not demonstrate genotypic parallelism but possess hypothesised roles in the adaptive process based on known phenotypic functions.

Lineage	Gene	Substitution	Transfer	Hypothesised Role
Ciprofloxacin-adapted				
Biofilm lineage 1	*vgrG* type VI secretion system tip protein	2545634 missense C→T	12	Secretion
Biofilm lineage 2	*bkdB* branched-chain alpha-keto acid dehydrogenase subunit	3156123 frameshift delACGGGATGGACGC	18	Metabolism
Biofilm lineage 4	*bkdA2* 2-oxoisovalerate dehydrogenase subunit beta	3157646 frameshift delCGCAGCCGAC	18	Metabolism
	tagH type VI secretion system FHA domain protein	96353 missense A→C	18	Secretion
		96356 missense T→G	18	
Planktonic lineage 1*	*pilN* type IV pilus biogenesis protein	5950878 frameshift delGCAA	6	Twitching motility
	pilB type IV pilus assembly ATPase	5235104 missense A→C	12	Twitching motility
	bkdA1 2-oxoisovalerate dehydrogenase subunit alpha	3159535 frameshift dupC	18	Metabolism
	mvfR virulence factor transcriptional regulator	4563039 missense T→C	18	Quorum sensing
	vgrG type VI secretion system tip protein	6003007 frameshift delC	18	Secretion
	mutS DNA mismatch repair protein	1502037 disruptive inframe deletion delGGGTGCAGGCCGAAC	18	Hypermutation
	rpoC DNA-directed RNA polymerase subunit beta	753798 missense C→T	18	Stress response
Planktonic lineage 2	*bkdA2* 2-oxoisovalerate dehydrogenase subunit beta	3157945 frameshift delGTTCACCC	6	Metabolism
	lasR transcriptional regulator	4085422 stop gained G→T	12	Quorum sensing
	pilC type IV pilus biogenesis protein	5236526 missense G→C	12	Twitching motility

Planktonic lineage 4	*tagH* type VI secretion system FHA domain protein	96353 missense A→C	18	Secretion
Ceftazidime-adapted				
Biofilm lineage 1*	PA14_RS20560 GNAT family N-acetyltransferase	4499871 frameshift dupG	12	Metabolism
	PA14_RS22265 β-lactamase	4849927 missense G→A	12	Drug inactivation
	tpbA tyrosine phosphatase	1174642 missense T→C	12	Biofilm formation
	norD denitrification protein	594980 missense C→T	18	Metabolism
Biofilm lineage 2*	*mutS* DNA mismatch repair protein	1502008 missense A→C	12	Hypermutation
	mexB Multidrug efflux RND transporter permease	488209 missense A→G	18	Membrane transport
Biofilm lineage 4*	*map* type I methionyl aminopeptidase	1462514 missense T→C	18	Metabolism
	cbiB adenosylcobinamide-phosphate synthase	4247537 missense C→T	18	Metabolism
Planktonic lineage 1	*mexB* Multidrug efflux RND transporter permease	488468 missense A→G	18	Membrane transport
Planktonic lineage 2*	*pilN* type IV pilus biogenesis protein	5950878 frameshift delGCAA	12	Twitching motility
	norD denitrification protein	595490 missense A→G	18	Metabolism
	nalD efflux system transcriptional repressor	1551471 missense A→G	18	Membrane transport
	map type I methionyl aminopeptidase	2464199 missense C→T	18	Metabolism
	PA14_RS16855 nitrate reductase	3711600 frameshift delC	18	Metabolism
	PA14_RS20560 GNAT family N-acetyltransferase	4499871 frameshift delG	12	Metabolism
Planktonic lineage 3*	*cbiB* adenosylcobinamide-phosphate synthase	4247900 missense A→G	12, 18	Metabolism
	triC triclosan efflux RND transporter permease subunit	179480 missense T→C	12	Membrane transport

	ohrR organic hydroperoxide resistance protein	2365094 missense G→A	18	Stress tolerance
Planktonic lineage 4*	PA14_RS22190 OprD family porin	4834911 missense T→C	18	Membrane transport
Tobramycin-adapted				
Biofilm lineage 2	*rpsL* 30S ribosomal protein S12	754922 missense A→G	12	Protein synthesis
	orfN group 4 glycosyl transferase	2041037 frameshift delG	18	Flagellar motility
Biofilm lineage 3	*tpbB* diguanylate cyclase	4435496 disruptive inframe deletion delGCGTGGCGCTGG	18	Biofilm formation
Planktonic lineage 1	*pilQ* type IV pilus secretin	5948806 frameshift delG	12	Twitching motility
Planktonic lineage 2	*ccoN* cytochrome-c oxidase, cbb3-type subunit I	3950801 frameshift delT	18	Metabolism
	wecB UDP-N-acetylglucosamine 2-epimerase	2028245 frameshift dupA	18	Cell wall biosynthesis
Planktonic lineage 3	*orfN* group 4 glycosyl transferase	2040286 frameshift delG	12	Flagellar motility
	rplB 50S ribosomal protein L2	761529 missense G→A	6	Protein synthesis
Planktonic lineage 4*	*pilY* type IV pilus biogenesis factor	5374338 stop gained G→A	12	Twitching motility
	oxyR oxidative stress transcriptional regulator	6285390 missense A→G	12	Oxidative stress response
	ccmA cytochrome c biogenesis heme-transporting ATPase	4042506 missense T→C	12	Respiration
	chpA chemotaxis signal transduction system protein	472401 missense C→T	12	Twitching motility
	infB translation initiation factor IF-2	5601277 missense A→G	12	Protein synthesis
	mutL DNA mismatch repair endonuclease	5822864 frameshift delC	12	Hypermutation

		soxR redox-sensitive transcriptional activator	3129432 missense C→T	12	Oxidative stress response
		wecC UDP-N-acetyl-D-mannosamine dehydrogenase	2030085 frameshift dupG	12	Cell wall biosynthesis
Naïve					
	Biofilm lineage 3	*tpbB* diguanylate cyclase	4435496 disruptive inframe deletion delGCGTGGCGCTGG	12, 18	Biofilm formation
	Biofilm lineage 4	*yfiR* diguanylate cyclase inhibitor	4435341 frameshift delTT	18	Biofilm formation

*Lineage gained a hypermutator phenotype

The distribution of mutations in experimentally evolved populations was determined (figure 5.8.). The mean number of SNPs in naïve biofilms was 6 at all timepoints and in planktonic lineages was 8, 6 and 7 at transfer 6, 12 and 18, respectively. The mean number of SNPs in ciprofloxacin-adapted biofilm lineages was 7, 8 and 10 at transfer 6, 12 and 18, respectively. The mean number of SNPs in ciprofloxacin-adapted planktonic lineages was 11, 8 and 7 ([1 s.f.] excluding hypermutator) at transfer 6, 12 and 18, respectively. Ciprofloxacin-adapted planktonic-1 gained a hypermutator phenotype at transfer 18 and possessed 66 unique mutations. There was no significant change in the number of SNPs between naïve and ciprofloxacin-adapted biofilm or planktonic lineages ($p = 0.3178$). Similarly, there was no significant change in the number of SNPs between timepoints ($p = 0.1649$).

The mean number of SNPs in ceftazidime-adapted planktonic lineages was 10, 20 and 37 respectively. The mean number of SNPs in ceftazidime-adapted biofilm lineages was 8, 28 and 36, respectively. All ceftazidime-adapted biofilm lineages and three planktonic lineages became hypermutators which acquired the mutator phenotype by transfer 12 or 18. There was no significant change in the number of SNPs between the ciprofloxacin-adapted biofilms (naïve biofilm transfer 6 vs biofilm ciprofloxacin-adapted transfer 6: $p = 0.9909$, naïve biofilm transfer 12 vs biofilm ciprofloxacin-adapted transfer 12: $p = 0.0587$) or the ciprofloxacin-adapted planktonic lineages and their naïve counterparts at transfers 6 or 12 (naïve planktonic transfer 6 vs planktonic ciprofloxacin-adapted transfer 6: $p = 0.9968$, naïve planktonic transfer 12 vs planktonic ciprofloxacin-adapted transfer 12: $p = 0.3644$). However, at transfer 18, significantly less SNPs were observed in the naïve lineages than the ceftazidime-adapted biofilm ($p = 0.0060$) and planktonic counterparts ($p = 0.0316$). There was no significant difference in the number of SNPs in the ciprofloxacin-adapted biofilm or planktonic lineages at any timepoint (biofilm ciprofloxacin-adapted transfer 6 vs planktonic ciprofloxacin-adapted transfer 6: $p = 0.9968$, biofilm ciprofloxacin-adapted transfer 12 vs planktonic ciprofloxacin-adapted transfer 12: 0.7820, biofilm ciprofloxacin-adapted transfer 18 vs planktonic ciprofloxacin-adapted transfer 18: $p = 0.9511$). There was no significant difference between the number of SNPs in transfer 6 or 12 in the ciprofloxacin-adapted biofilm ($p = 0.0646$) or planktonic lineages ($p = 0.4733$). Similarly, there was no significant difference between transfer 12 or 18 in the ciprofloxacin-adapted biofilm ($p = 0.5804$) or planktonic lineages ($p = 0.3443$). Despite this, significantly more SNPs were observed at transfer 18 than transfer 6 in both biofilm ($p = 0.0056$) and planktonic lineages ($p = 0.0356$).

The mean number of SNPs in tobramycin-adapted biofilm lineages was 9, 8 and 9 at transfer 6, 12 and 18, respectively. The mean number of SNPs in tobramycin-adapted planktonic lineages was 9, 10 and 12 ([1 s.f.] excluding hypermutator) at transfer 6, 12 and 18, respectively. Tobramycin-adapted planktonic lineage 4 gained a hypermutator phenotype at transfer 12 and possessed 45 unique mutations. There was no significant change in the number of SNPs between naïve and ciprofloxacin-adapted biofilm or planktonic lineages (p = 0. 0.0593). Similarly, there was no significant change in the number of SNPs between timepoints (p = 0. 6551).

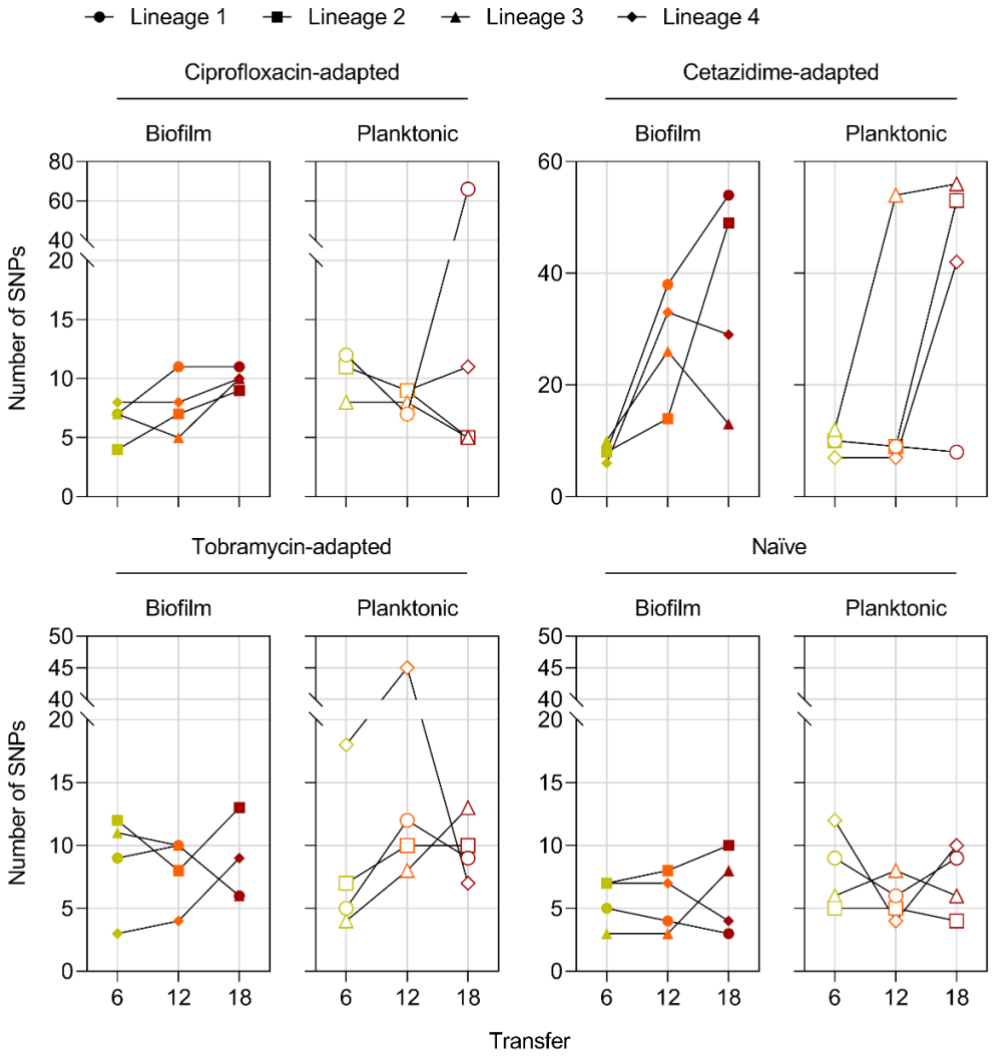

Figure 5.8. Distribution of mutations in experimentally evolved lineages of *P. aeruginosa* adapted to ciprofloxacin, ceftazidime or tobramycin stress increasing in a stepwise fashion in biofilm or planktonic lifestyles. Statistical differences in mutational load between exposure condition and lifestyle over the passage series were detected via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as number of mutations per lineage, n = 1.

The phylogenetic relationship between the ciprofloxacin-adapted biofilm and planktonic lineages was reconstructed via maximum likelihood (Figure 5.9.). Ciprofloxacin- and ceftazidime-adapted lineages did not bifurcate according to lifestyle; position within the phylogeny was largely independent of lifestyle. However, the tobramycin-adapted biofilm lineages generally clustered away from the planktonic lineages. Furthermore, there was no correlation identified in the ciprofloxacin- and tobramycin-adapted lineages between the number of transfers and evolutionary distance from the ancestral strain. Despite this, ceftazidime-adapted lineages were more evolutionarily distant from the ancestor at transfer 18 than transfer 6 or 12, due to the presence of a large number of hypermutators. Similarly, ceftazidime-adapted lineages became more divergent from each other due to the action of hypermutators.

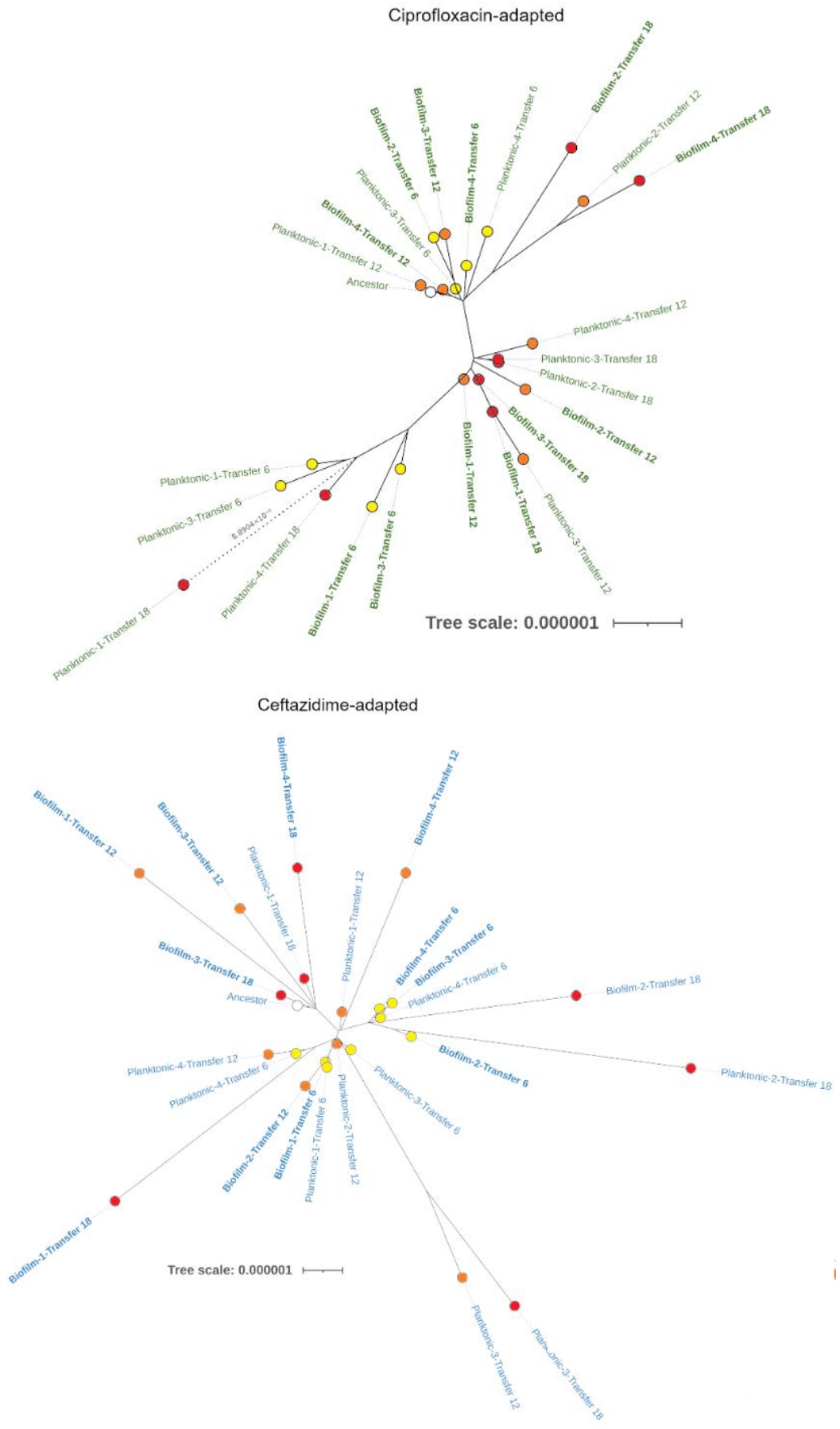
Ciprofloxacin-adapted
Biofilm-2-Transfer 18
Biofilm-3-Transfer 12
Biofilm-2-Transfer 6
Biofilm-4-Transfer 6
Planktonic-4-Transfer 6
Planktonic-2-Transfer 12
Biofilm-4-Transfer 18
Planktonic-3-Transfer 6
Biofilm-4-Transfer 12
Planktonic-1-Transfer 12
Ancestor
Planktonic-4-Transfer 12
Planktonic-3-Transfer 18
Planktonic-2-Transfer 18
Biofilm-2-Transfer 12
Biofilm-3-Transfer 18
Biofilm-1-Transfer 12
Biofilm-1-Transfer 18
Planktonic-3-Transfer 12
Planktonic-1-Transfer 6
Planktonic-3-Transfer 6
6.8904×10⁻⁷
Planktonic-4-Transfer 18
Biofilm-1-Transfer 6
Biofilm-3-Transfer 6
Planktonic-1-Transfer 18
Tree scale: 0.000001
Ceftazidime-adapted
Biofilm-4-Transfer 18
Biofilm-4-Transfer 12
Biofilm-1-Transfer 12
Biofilm-2-Transfer 12
Planktonic-1-Transfer 18
Planktonic-1-Transfer 12
Biofilm-4-Transfer 6
Biofilm-3-Transfer 6
Biofilm-3-Transfer 18
Ancestor
Planktonic-4-Transfer 6
Biofilm-2-Transfer 18
Planktonic-4-Transfer 12
Biofilm-2-Transfer 6
Planktonic-2-Transfer 18
Planktonic-4-Transfer 6
Planktonic-3-Transfer 6
Biofilm-2-Transfer 12
Biofilm-1-Transfer 6
Planktonic-1-Transfer 6
Planktonic-2-Transfer 12
Biofilm-1-Transfer 18
Tree scale: 0.000001
Planktonic-3-Transfer 12
Planktonic-3-Transfer 18

Figure 5.9.

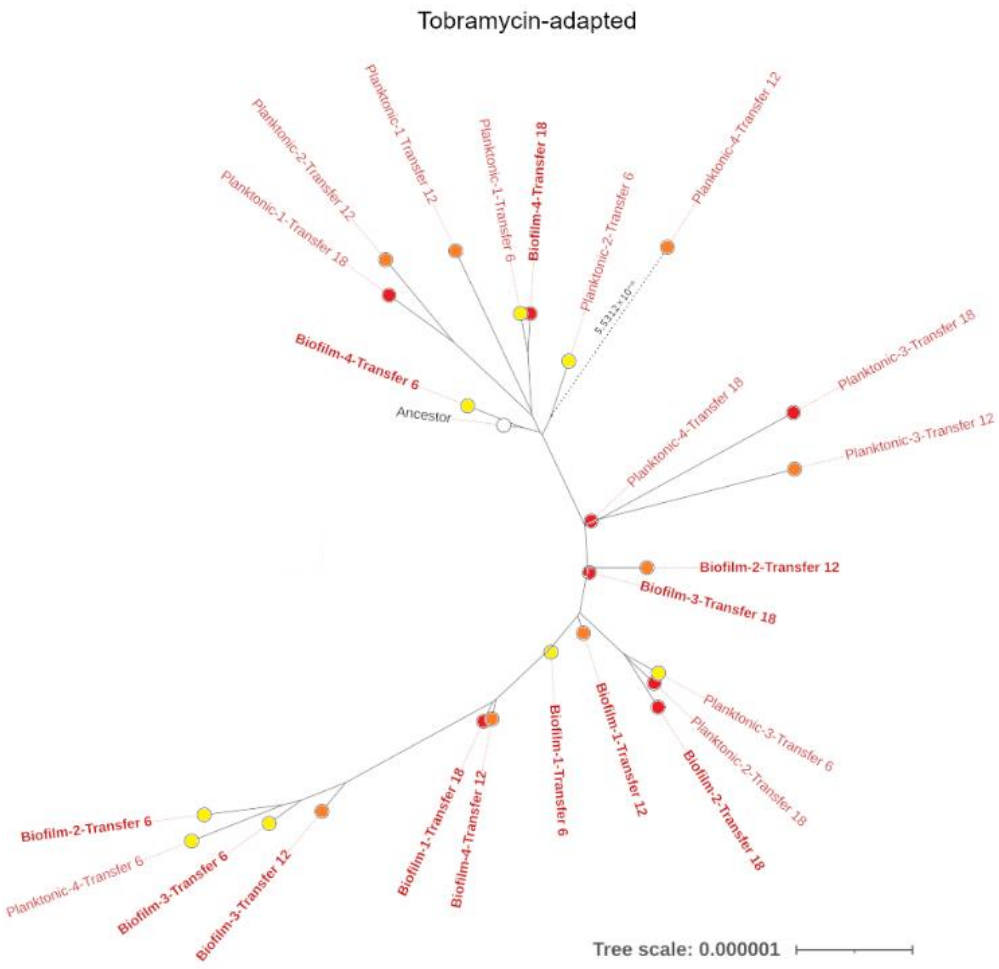

Figure 5.9. Phylogeny of experimentally evolved lineages of *P. aeruginosa* adapted to tobramycin stress increasing in a stepwise fashion in biofilm or planktonic lifestyles. Whole genome alignments were constructed with Snippy-core and a maximum-likelihood matrix was used to reconstruct the phylogeny of experimentally evolved lineages using IQ-TREE. The phylogenetic tree was arbitrarily rooted at the ancestral strain. Label colours show selective agent, node colour shows timepoint. Broken line shows branches not to scale, in such cases evolutionary distances is displayed on the branch. Data shown as units of substitutions/site.

5.2.3. Re-adaptation to a static stress is associated with contraction of multidrug resistance and agent-specific modulation of biofilm competency

Four stepwise-adapted lineages were selected from each assayed timepoint, lifestyle and exposure condition and re-adapted to the maximum antibiotic concentration they were previously exposed to for an additional ten transfers. The capacity for amelioration of fitness defects, compromised biofilm formation and patterns of altered susceptibility was assessed. Each starting timepoint represents lineages habituated to a subinhibitory, inhibitory or super-inhibitory stress. The effect of static re-adaptation on susceptibility to a panel of antipseudomonal drugs was then determined (figure 5.10.). No significant change in susceptibility to any agent was observed in naïve lineages of either lifestyle at any timepoint. In drug-readapted lineages, altered susceptibility to individual antibiotics relative to the stepwise adapted ancestor was generally small, rarely exceeding one- to two-$\log_2$ fold changes. Trends towards decreased susceptibility to the selective agent, but loss of multidrug resistance were broadly observed.

Lineages of ciprofloxacin-readapted biofilms were characterised by the loss of cross-resistance to β-lactams and a further one- to two-$\log_2$ fold decrease in ciprofloxacin susceptibility in lineages re-adapted from all timepoints. Ciprofloxacin-readapted planktonic lineages were also associated with further modest reductions in ciprofloxacin susceptibility, however, increases in β-lactam susceptibility were not observed due to the lack of β-lactam cross-resistance in the stepwise-adapted ancestor. Instead, ciprofloxacin-readapted planktonic lineages exhibited increases in colistin susceptibility. These increases in susceptibility relative to the stepwise-adapted ancestor largely resulted in the restoration of pre-adapted ancestral susceptibility.

Both the ceftazidime-readapted biofilm and planktonic lineages were associated with increases in susceptibility to meropenem. Later starting timepoints were associated with larger increases in meropenem susceptibility which resulted in restoration of pre-adaptation ancestral meropenem susceptibility. One- to two-$\log_2$ fold decrease reductions in ceftazidime and piperacillin susceptibility were broadly observed in both biofilm and planktonic lineages re-adapted to ceftazidime. This resulted in the surpassing of the ceftazidime breakpoint in lineages which did not start the re-adaptation regime with it.

Tobramycin readaptation was associated with further reductions in tobramycin susceptibility in both biofilm and planktonic lineages. In general, the lineages which started with the lowest tobramycin susceptibility achieved the greatest susceptibility

reductions. This also resulted in the surpassing of the tobramycin breakpoint in lineages which did not start the re-adaptation regime with it. Tobramycin-readaptation in biofilm lineages was associated with increases in susceptibility to ciprofloxacin across all starting timepoints. No increases in susceptibility were observed against any agent in tobramycin-readapted planktonic lineages. The tobramycin-readapted planktonic lineages demonstrated starting transfer-specific reductions in susceptibility to other agents. Transfer 18 re-adapted lineages exhibited no additional changes in susceptibility other than tobramycin, whereas transfer 12 exhibited reductions in susceptibility to colistin and transfer 6 to colistin, piperacillin and aztreonam.

The effect of static re-adaptation on biofilm formation was determined (figure 5.11.). Adaptation of naïve biofilms for an additional ten transfers resulted in selection for significant increases in biofilm formation at starting transfers 6 and 12 (naïve biofilm transfer 6 vs re-adapted naïve biofilm transfer 6: $p < 0.0001$, naïve biofilm transfer 12 vs re-adapted naïve biofilm transfer 12: $p < 0.0001$) but not transfer 18 ($p = 0.8669$). Re-adaptation of naïve planktonic lineages was not associated with a significant change in biofilm formation at starting transfers 6 and 18 (naïve planktonic transfer 6 vs re-adapted planktonic biofilm transfer 6: $p = 0.6613$, naïve planktonic transfer 18 vs re-adapted naïve planktonic transfer 18: $p > 0.9999$) however starting transfer 12 lineages became significantly worse at forming biofilms ($p = 0.0003$). Static re-adaptation to ciprofloxacin in planktonic lineages was associated with significant increases in biofilm formation after exposure to sub-inhibitory and inhibitory stress (planktonic ciprofloxacin-adapted transfer 6 vs re-adapted planktonic ciprofloxacin-adapted transfer 6: $p > 0.0001$, planktonic ciprofloxacin-adapted transfer 12 vs re-adapted planktonic ciprofloxacin-adapted transfer 12: $p = 0.0065$), not after super-inhibitory stress ($p = 0.0705$). Ciprofloxacin-readapted biofilms did not exhibit significant changes in biofilm formation after sub- or super-inhibitory exposure to ciprofloxacin (biofilm ciprofloxacin-adapted transfer 6 vs re-adapted biofilm ciprofloxacin-adapted transfer 6: $p = 0.2382$, biofilm ciprofloxacin-adapted transfer 18 vs re-adapted biofilm ciprofloxacin-adapted transfer 18: $p = 0.9982$), however inhibitory exposure resulted in a significant reduction in biofilm formation ($p < 0.0001$). Ceftazidime re-adaptation was associated with significant increases in biofilm formation in planktonic lineages after sub-inhibitory exposure to ceftazidime ($p = 0.0030$), and in biofilm lineages after sub-inhibitory and inhibitory exposure (biofilm ceftazidime-adapted transfer 6 vs re-adapted biofilm ceftazidime-adapted transfer 6: $p < 0.0001$, biofilm ceftazidime-adapted transfer 12 vs re-adapted biofilm ceftazidime-adapted transfer 12: $p = 0.0122$). No significant changes were observed in planktonic lineages after inhibitory readaptation to ceftazidime ($p =$

0.9974) or after super-inhibitory readaptation in either the biofilm or planktonic lifestyle (biofilm ceftazidime-adapted transfer 18 vs re-adapted biofilm ceftazidime-adapted transfer 18: $p > 0.9999$, planktonic ceftazidime-adapted transfer 18 vs re-adapted planktonic ceftazidime-adapted transfer 18: $p = 0.2129$). No significant change in biofilm formation was observed after tobramycin-readaptation in any of the planktonic lineages at any degree of stress (planktonic tobramycin-adapted transfer 6 vs re-adapted planktonic tobramycin-adapted transfer 6: $p = 0.1158$, planktonic tobramycin-adapted transfer 12 vs re-adapted planktonic tobramycin-adapted transfer 12: $p > 0.9999$, planktonic tobramycin-adapted transfer 18 vs re-adapted planktonic tobramycin-adapted transfer 18: $p = 0.0905$). However significant increases in biofilm formation were observed in biofilm lineages after tobramycin-readaptation at sub-inhibitory, inhibitory and super-inhibitory concentrations (biofilm tobramycin-adapted transfer 6 vs re-adapted biofilm tobramycin-adapted transfer 6: $p < 0.0001$, biofilm tobramycin-adapted transfer 12 vs re-adapted biofilm tobramycin-adapted transfer 12: $p = 0.0071$, biofilm tobramycin-adapted transfer 18 vs re-adapted biofilm tobramycin-adapted transfer 18: $p < 0.0001$).

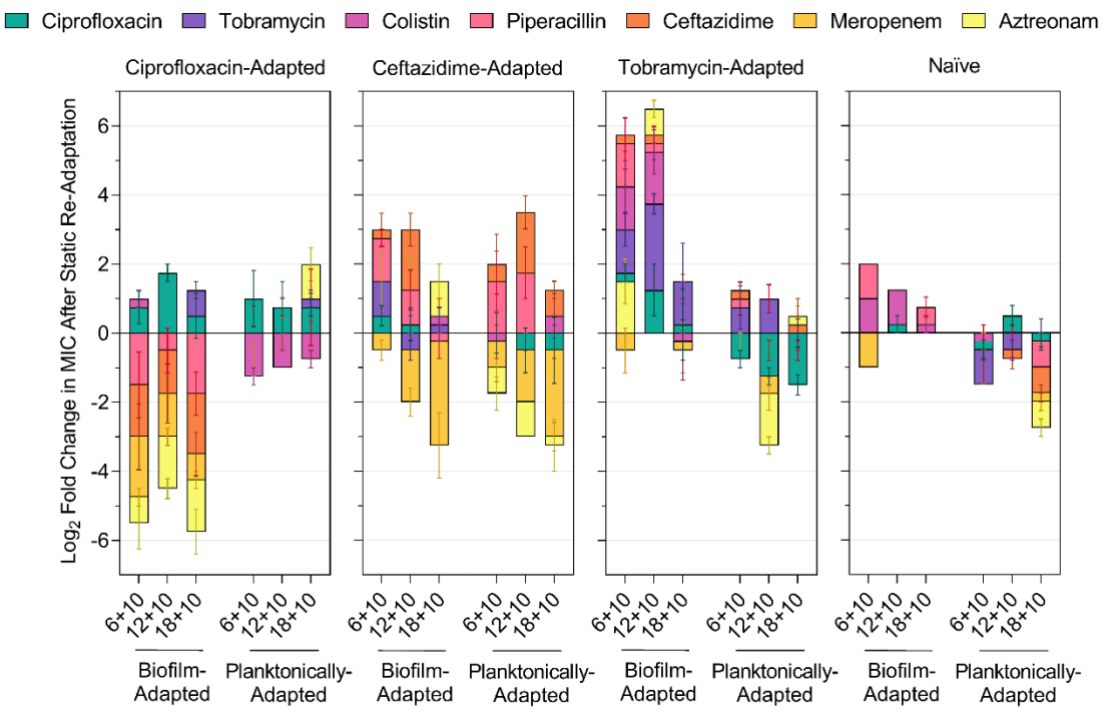

Figure 5.10. Changes in antibiotic susceptibility to a panel of antipseudomonal antibiotics determined via agar dilution MIC of experimentally evolved lineages of *P. aeruginosa* re-adapted ciprofloxacin, ceftazidime or tobramycin after a period of stepwise adaptation in biofilm or planktonic lifestyles. Four replicate lineages from each combination of exposure condition and lifestyle were taken at each assayed timepoint and re-adapted to the maximum concentration of the selective agent they previously tolerated for an additional ten transfers. Significant changes in susceptibility were defined as a >1-$\log_2$ fold change. Data shown as mean $\log_2$ fold change in MIC from stepwise-adapted ancestor ± standard error of the mean, n = 4 per lineage.

The effect of static re-adaptation on fitness was determined (figure 5.12.). There were no significant fitness changes observed at any starting timepoint in the naïve biofilms (naïve biofilm transfer 6 vs re-adapted naïve biofilm transfer 6: $p > 0.9999$, naïve biofilm transfer 12 vs re-adapted naïve biofilm transfer 12: $p > 0.9999$, naïve biofilm transfer 18 vs re-adapted naïve biofilm transfer 18: $p = 0.9998$) or planktonic lineages (naïve planktonic transfer 6 vs re-adapted naïve planktonic transfer 6: $p = 0.8793$, naïve planktonic transfer 12 vs re-adapted naïve planktonic transfer 12: $p = 0.9936$, naïve planktonic transfer 18 vs re-adapted naïve planktonic transfer 18: $p = 0.9543$). Static re-adaptation of biofilm-adapted lineages to ciprofloxacin was not associated with any significant change in fitness at any stress level (biofilm ciprofloxacin-adapted transfer 6 vs re-adapted biofilm ciprofloxacin-adapted transfer 6: $p > 0.9999$, biofilm ciprofloxacin-adapted transfer 12 vs re-adapted biofilm ciprofloxacin-adapted transfer 12: $p > 0.9999$, biofilm ciprofloxacin-adapted transfer 18 vs re-adapted biofilm ciprofloxacin-adapted transfer 18: $p > 0.9999$). Static re-adaptation of planktonically-

adapted lineages to sub-inhibitory and inhibitory concentrations of ciprofloxacin were also not associated with a significant change in fitness (planktonic ciprofloxacin-adapted transfer 6 vs re-adapted planktonic ciprofloxacin-adapted transfer 6: $p = 0.7882$, planktonic ciprofloxacin-adapted transfer 12 vs re-adapted planktonic ciprofloxacin-adapted transfer 12: $p = 0.9630$). However, re-adaptation to super-inhibitory concentrations of ciprofloxacin selected for a significant decrease in fitness in planktonic lineages ($p = 0.0025$). Biofilm lineages re-adapted to super-inhibitory ceftazidime stress demonstrated a significant increase in fitness after static re-adaptation ($p = 0.0263$). No significant change in fitness was observed in ceftazidime-readapted lineages adapted to sub-inhibitory or inhibitory ceftazidime stress (biofilm ceftazidime-adapted transfer 6 vs re-adapted biofilm ceftazidime-adapted transfer 6: $p = 0.8765$, biofilm ceftazidime-adapted transfer 12 vs re-adapted biofilm ceftazidime-adapted transfer 12: $p = 0.9262$). Ceftazidime-readapted planktonic lineages were not associated with significant changes in fitness after adaptation to sub- or super-inhibitory stress (planktonic ceftazidime-adapted transfer 6 vs re-adapted planktonic ceftazidime-adapted transfer 6: $p = 0.2528$, planktonic ceftazidime-adapted transfer 18 vs re-adapted planktonic ceftazidime-adapted transfer 18: $p = 0.9985$). However, inhibitory ceftazidime stress selected for a significant increase in fitness after static re-adaptation ($p = 0.0021$). Static re-adaptation was not associated with a significant effect on fitness in tobramycin adapted lineages at sub-inhibitory, inhibitory or super-inhibitory concentrations in biofilms (biofilm tobramycin-adapted transfer 6 vs re-adapted biofilm tobramycin-adapted transfer 6: $p = 0.9993$, biofilm tobramycin-adapted transfer 12 vs re-adapted biofilm tobramycin-adapted transfer 12: $p = 0.2269$, biofilm tobramycin-adapted transfer 18 vs re-adapted biofilm tobramycin-adapted transfer 18: $p = 0.9999$) or planktonic lineages (planktonic tobramycin-adapted transfer 6 vs re-adapted planktonic tobramycin-adapted transfer 6: $p > 0.9999$, planktonic tobramycin-adapted transfer 12 vs re-adapted planktonic tobramycin-adapted transfer 12: $p = 0.6928$, planktonic tobramycin-adapted transfer 18 vs re-adapted planktonic tobramycin-adapted transfer 18: $p = 0.9328$).

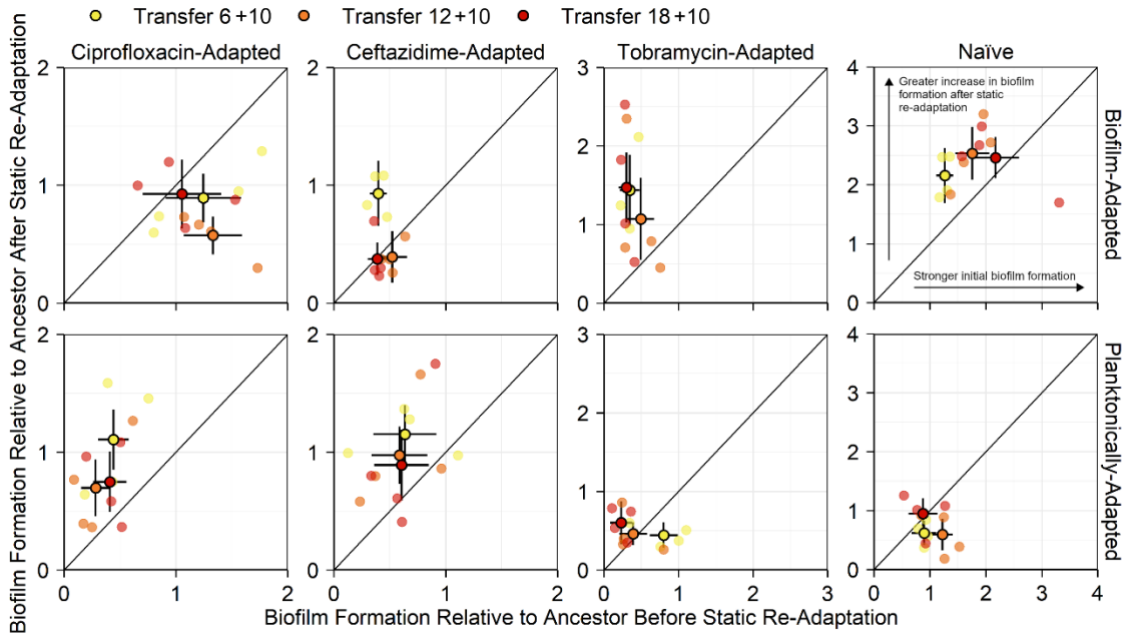

Figure 5.11. Changes in biofilm formation as determined via crystal violet staining of experimentally evolved lineages of *P. aeruginosa* re-adapted ciprofloxacin, ceftazidime or tobramycin after a period of stepwise adaptation in biofilm or planktonic lifestyles. Statistical differences in biofilm formation between the stepwise adapted ancestor and statically re-adapted lineages were detected via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as biofilm formation to stepwise adapted ancestor, ± standard error of the mean, n = 8 per lineage.

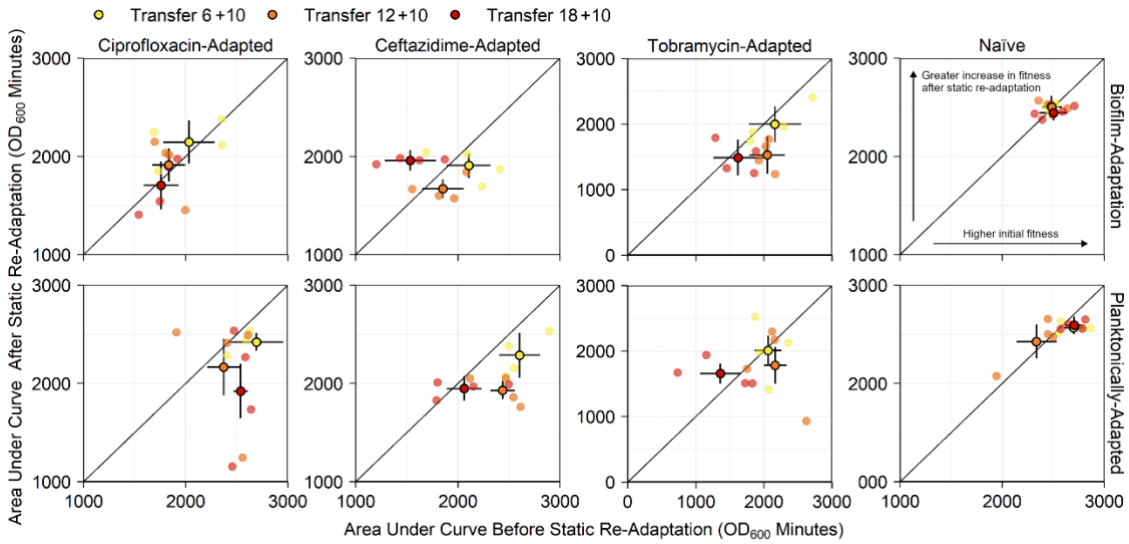

Figure 5.12. Changes in relative fitness of experimentally evolved lineages of *P. aeruginosa* re-adapted ciprofloxacin, ceftazidime or tobramycin after a period of stepwise adaptation in biofilm or planktonic lifestyles. The area under the curve was calculated and statistical differences in growth fitness between the stepwise adapted ancestor and statically re-adapted lineages were detected via a one-way ANOVA with Tukey's multiple comparison post-hoc test. Data shown as area under curve relative to stepwise adapted ancestor, ± standard error of the mean, n = 4 per lineage.

5.3. Discussion

Understanding the fundamentals of how antimicrobial resistance evolves is central to rationally using existing antibiotics, in addition to developing new ones. Therefore, characterising the molecular mechanisms underpinning lifestyle-specific evolutionary trajectories to resistance and the collateral trade-offs of adaptation, offers novel insights for limiting the selection of multidrug resistance. Antimicrobial resistance was readily selected in experimentally evolved lineages adapted to stepwise stress which doubled every three transfers. Significant reductions in susceptibility were observed by the first assayed timepoint even though the lineages had only been exposed to sub-inhibitory concentrations of drug. Indeed, concentrations far below the MIC can still exert strong selective effects, and it has been suggested that subinhibitory concentrations constitute the majority of the mutant selection window for antimicrobial resistance (Drlica and Zhao, 2007, Gullberg et al., 2011). As the antibiotic concentration increased, reductions in susceptibility also increased in a stepwise manner. At the subinhibitory concentrations, low-cost mutations which decrease susceptibility are often under greater selection than large-effect mutations as the fitness defects they confer can be more detrimental than the antimicrobial stress (MacLean et al., 2010). As the stress increases, the fitness landscape shifts, and previously selected mutations no longer confer a selective advantage. This leads to selective sweeps which optimise populations to be most fit in their selective environment according to the optimum trade-off between susceptibility and relative fitness. Supporting this, lineages broadly demonstrated an inverse relationship between drug susceptibility and relative fitness in a drug-free environment.

Like in the previous chapter, adaptation to a biofilm lifestyle in the absence of antibiotics (naïve) selected for biofilm hyperproduction. Identical mutations were identified in *dipA* and the *yfiBNR* complex as observed in the previous chapter. Despite this, adaptation to all selective agents in a biofilm lifestyle failed to select for biofilm hyperproduction. Mutations in c-di-GMP regulators were broadly absent from drug-adapted lineages. Nevertheless, some exceptions existed, one lineage of ciprofloxacin-adapted biofilms, two ceftazidime-adapted planktonic lineages and one tobramycin-adapted biofilm lineages possessed mutations in previously hypothesised regulators of biofilm hyperproduction. However, none of these mutations were able to confer the biofilm hyperproducing phenotype as shown in figure 5.2. Suppressed selection for biomass production associated with adaptation of *Salmonella* sv. Typhimurium to antibiotics has been previously observed (Trampari et al., 2021). Trampari et al. (2021) observed that ciprofloxacin was able to select for a modest increase in biofilm

formation in *Salmonella* lineages however was not able to recapitulate the biofilm hyperproducing phenotype observed in the naïve lineages. Cefotaxime, on the other hand, selected for severely compromised biofilm formation. in this study, no significant change in biofilm formation from the ancestor was associated with any selective agent. The mechanism by which adaptation to drugs suppresses selection for biomass hyperproduction is not well understood. However, Trampari et al. (2021) observed that de-repression of multidrug efflux via the global regulators Mar, Ram and Sox was associated with reduced biofilm formation. Therefore, compromised biofilm formation may arise as a collateral effect of fitness defects conferred by drug resistance, or biofilm hyperproduction may be maladaptive to the evolution of resistance. However, it is unlikely that mutations in the c-di-GMP signalling pathways have deleterious effects on drug susceptibility. High levels of c-di-GMP have been shown to reduce susceptibility to imipenem and heavy metals by negatively regulating porins (Chua et al., 2015, Nicastro et al., 2014). A more likely scenario is that the stepwise exposure regime used in this experiment exacerbates the effect of clonal interference (Crozat et al., 2005). When there are multiple genotypes available to acquire fitness gains, evolution tends towards selection of the ones with the greatest phenotypic advantage. This is because the smaller the selective advantage, the longer the time required for fixation. Therefore, mutants which possess the largest fitness gains will outcompete other coexisting individuals even if those are more fit than the ancestor. In the context of this experiment, though biofilm hyperproduction is evidently selectively advantageous, adaptation to the inhibitory antibiotic stress likely confers the greatest fitness gains. As the stress is constantly increases, mutations are preferentially selected for continued drug adaptation, at the detriment to selection for increased biofilm formation.

5.3.1. Ciprofloxacin Adaptation

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic which binds to the topoisomerase DNA gyrase in Gram-negative organisms, inhibiting regulation of negative chromosomal supercoiling and producing stalled DNA-drug-Gyrase complexes which result in double stranded breaks in DNA (Sharma et al., 2010). Chromosomally-encoded resistance to ciprofloxacin is mediated by two main mechanisms in *P. aeruginosa*, target site alteration in the DNA gyrase subunit A *gyrA* and constitutive overexpression of multidrug efflux pumps including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Rehman et al., 2019). In experimentally evolved lineages, mutations in local repressors of multidrug efflux pumps were

extensively observed by transfer 6, however there was a difference in the repertoires of pumps de-repressed between lifestyles as shown in figure 5.5. MexAB-OprM is the most clinically-relevant of the five RND efflux pumps in *P. aeruginosa* (Issa et al., 2018). It plays an instrumental role in the intrinsic resistance possessed by *P. aeruginosa.* It possesses two transcriptional repressors, MexR and NalD. NalD is controlled by an antirepressor, ArmR which is in turn repressed by NalC. Loss of function of MexR, NalC or NalD results in constitutive overexpression of MexAB-OprM. Two lineages of ciprofloxacin-adapted biofilms possessed mutations in *mexR,* and the other two in *nalC*. No mutations were identified in any MexAB-OprM regulator in the planktonic lineages. Instead, all ciprofloxacin-adapted planktonic lineages possessed mutations in the repressor of MexCD-OprJ, *nfxB*. MexCD-OprJ mutants were not exclusive to the planktonic lineages however, ciprofloxacin-adapted biofilm-1 and -3 also possessed mutations in *nfxB*. Therefore, two lineages of ciprofloxacin-adapted biofilms became MexAB-OprM mutants, two became MexAB-OprM/ MexCD-OprJ mutants and all planktonic lineages became MexCD-OprJ mutants. This demonstrates a lifestyle-specific repertoire of de-repressed efflux pumps selected by ciprofloxacin. All of the substitutions identified here were selectively novel. Despite this, Monti et al. (2013) demonstrated that amino acid substitutions at almost every residue within *nfxB* could confer reduced susceptibility to ciprofloxacin. Constitutive overexpression of MexAB-OprM, the pump which possesses the greatest phenotypic effect on susceptibility, combined with de-repression of MexCD-OprJ would explain the larger decreases in susceptibility to β-lactams collaterally selected in biofilm lineages observed in figure 5.1.

There was a strong evolutionary signal for loss of type IV pili biogenesis seen exclusively in ciprofloxacin-adapted planktonic lineages through mutations in *pilB/C/F/M/N/Q*. The role loss of type IV pili function may have in ciprofloxacin resistance is not understood but has been observed by other workers (Ahmed et al., 2018, Wardell et al., 2019, Ahmed et al., 2020). Type IV pili are essential for biofilm formation; they play an important role in early colonisation of surfaces through twitching motility and mediating cell-matrix interactions (Maldarelli et al., 2016). Consequently, it is not surprising that the loss of type IV pili biogenesis is not under positive selection in biofilm lineages. The *pil* genes under selection are structural or assembly proteins, therefore it is unlikely they possess pleiotropic regulatory roles. However, loss of twitching motility or acquisition of eDNA mediated by type IV pili may activate stress responses which ameliorate ciprofloxacin stress. Indeed, accumulation of

misassembled pilin subunits in the periplasm has been shown to activate the Cpx membrane stress response pathway (Nevesinjac and Raivio, 2005).

By transfer 18, all experimentally evolved ciprofloxacin-adapted lineages of both lifestyles possessed mutations in *gyrA*. Selection for target site mutations after acquiring constitutive de-repression of multidrug efflux likely explains the stepwise decrease in ciprofloxacin susceptibility observed in these lineages. Two of the *gyrA* mutants identified here are well characterised (Rehman et al., 2019). Three biofilm and three planktonic lineages possessed a Thr83Ile substitution in *gyrA*, one biofilm lineage possessed an Asp87Gly substitution, and one planktonic lineage possessed a conservative inframe insertion resulting in an Ala131 duplication. The former two mutations have been well characterised previously as the residues 51 to 106 of GyrA is known as the quinolone resistance determining region (QRDR) which determines quinolone binding (Jedrey et al., 2018). Missense mutations at the Ala131 residue have been implicated in quinolone resistance in *Salmonella* sp. but, until now, not been observed in *P. aeruginosa* or mediated by a duplication polymorphism (Eaves et al., 2002, Ricci et al., 2006). Extra-QRDR mutations in *gyrA* conferring quinolone resistance are uncommon but not unprecedented (Maruri et al., 2012). The topoisomerase domain of *gyrA* runs from residue 11 to 500. Therefore, it is likely that the Ala131 was selected to ameliorate reduced DNA binding when bound to ciprofloxacin. No mutations in other topoisomerase subunits such as *parC, parE* or *gyrB* which act as secondary targets for ciprofloxacin were detected. However, *gyrA* mutants only presented at the last assayed timepoint. If the experiment continued it would be plausible that they would be next selected after de-repressed multidrug efflux and *gyrA* target alteration based on their role as secondary mutations which exert epistatic action with GyrA (Pan et al., 2001).

5.3.2. Ceftazidime Adaptation

Ceftazidime is a third-generation cephalosporin in the β-lactam class of antibiotics. Like all β-lactams, ceftazidime inhibits cell wall synthesis by acetylating the active site of D-alanyl-D-alanine-transpeptidases which catalyse the terminal steps of peptidoglycan crosslinking (Bush and Bradford, 2016). The accumulation of peptidoglycan precursors also triggers the expression of autolytic hydrolases leading to bactericidal cell wall deficiency. Resistance to β-lactams such as ceftazidime in clinical environments is principally mediated by plasmid-encoded hydrolytic β-lactamases, however, *P. aeruginosa* also possesses important chromosomally-encoded resistance mechanisms

(Bush, 2018). Multidrug efflux is a significant contributor to β-lactam resistance and MexAB-OprM, MexCD-OprJ, and MexXY-OprM possess substrate repertoires which include ceftazidime (Poole, 2004). *P. aeruginosa* also possesses two characterised chromosomal β-lactamases, the cephalosporinase AmpC and oxacillinase PoxB, which are induced during arrest of peptidoglycan biosynthesis. Constitutive de-repression of AmpC is the most common mechanism of resistance to non-carbapenem β-lactams in *P. aeruginosa* (Poole, 2011).

In the experimentally evolved lineages, figure 5.6. shows that ceftazidime adaptation selected for mutations in regulators of AmpC in both biofilm and planktonic lifestyles. All planktonic lineages and three biofilm lineages possessed mutations in *ampR,* the transcriptional repressor of AmpC, within the muropeptide effector binding domain spanning residues 83 to 296 (Dik et al., 2017). The planktonic lineages all possessed a single conserved SNP resulting in an Asp135Gly substitution. Substitutions at the Asp135 residue have been observed to result in constitutive overexpression of AmpC in *Citrobacter freundii* and *Enterobacter cloacae* (Nakano et al., 2017, Kuga et al., 2000). In the biofilm lineages, a missense mutation at the Asp135 residue was also observed in biofilm-4. Biofilm-4 also possessed a Gly90Ser substitution and biofilm-1 and -2 both possessed a Arg214Trp substitution which have previously not been previously implicated in ceftazidime resistance. In addition to AmpR, AmpC is also regulated by the peptidoglycan recycler Mpl and carboxy/endopeptidase DacB which have both been shown to be under selection by ceftazidime exposure in *P. aeruginosa in vitro* (Cabot et al., 2018). β-lactam resistant Mpl mutants have been observed in clinical isolates of *Stenotrophomonas maltophilia* (Calvopiña and Avison, 2018). Similarly, DacB mutants of *P. aeruginosa* isolated from bacteraemia have been shown to be β-lactam resistant (Barbosa et al., 2020). Mpl and DacB both act to directly resolve stalled peptidoglycan biosynthesis, but in the presence of ceftazidime, this is a futile effort. Therefore, their loss-of-function results in increased expression of AmpC which is expressed when AmpR senses an accumulation of depolymerised muropeptides (Moya et al., 2009). Though *ampR* mutations were present in ceftazidime-adapted lineages of both lifestyles, *mpl* and *dacB* mutations were exclusively seen in biofilm lineages. Moreover, mutations *mpl* and *dacB* were generally selected after acquisition of *ampR* mutations. An *mpl* and *dacB* mutation was observed in biofilm-4 which possessed a substitution at Asp135 in AmpR, the same location as the planktonic lineages which did not select for *mpl* or *dacB*. Therefore, whilst the role of loss-of-function of the Mpl and DacB peptidases in AmpC de-repression is well characterised, why they conferred a biofilm-specific selective advantage during

ceftazidime exposure requires further investigation. Furthermore, despite AmpC de-repression appearing to be the main mechanism of β-lactam resistance, it is a cephalosporinase which lacks hydrolysis activity against carbapenems and has limited activity against monobactams (Jacoby, 2009). Therefore, the reduced susceptibility to meropenem and aztreonam shown in figure 5.1. must be mediated by another mechanism. The primary mechanism of meropenem resistance in *P. aeruginosa* is porin loss, most notably OprD, however there were no major porins identified in this study. Mutations in the *nalC* regulator of MexAB-OprM were identified in planktonic lineages in addition to a mutation in the MexJK-OprM transporter *mexK*. Biofilm-1 and -2 possessed a Thr76Ala substitution in MexK substitution which is outside of the MexJK repressor binding site upstream of *mexJ.* Moreover, MexJK has previously only been reported to export tetracycline, erythromycin and triclosan making the role of the *mexK* mutations cryptic (Depardieu et al., 2007). OprD has been shown to be regulated by AmpR with loss of AmpR resulting in reduced OprD expression (Balasubramanian et al., 2012). It is possible that the molecular basis of carbapenem resistance is AmpR-dependent but AmpC-independent by virtue of the pleiotropic regulatory network of AmpR.

Figure 5.8. demonstrates that all ceftazidime-adapted biofilm lineages and three planktonic lineages became hypermutators shown in figure 5.5. to be mediated by mutations in *mutL*. MutL is part of the methyl-directed mismatch repair system, a mechanism for post-replicative DNA proofreading and repair (Shaver and Sniegowski, 2003). Loss of DNA mismatch repair results in a one to three orders of magnitude increase in the mutation frequency. Long-term hypermutation is generally considered maladaptive in recombination-deficient environments due to the action of Muller's ratchet which results in the accumulation mildly deleterious alleles (Chu et al., 2017). Despite this, in environments which are undergoing rapid change, hypermutation can accelerate the adaptive process by increasing the mutation supply rate. Indeed, hypermutators in *P. aeruginosa* are commonly associated with adaptation to the diverse environment of the cystic fibrosis lung (Poole, 2011). Therefore, in experimentally-evolved lineages it is likely that hypermutation accelerated the development of resistance to ceftazidime. A single lineage of biofilms adapted to ciprofloxacin and tobramycin also became hypermutator, but these drugs did not select for hypermutation as broadly as ceftazidime. Ceftazidime/avibactam-resistant hypermutators have been observed to be selected in bacteraemia isolates of *P. aeruginosa* (Khil et al., 2019). The factors which determine the selective advantage of hypermutation are not well understood, however hypermutator alleles generally rise to

fixation by hitchhiking with the selective advantageously mutations which they originate (Eliopoulos and Blázquez, 2003). Therefore, low-monotonicity fitness landscapes which possess high fitness optima unconstrained by collateral fitness defects are generally more amenable to selecting for hypermutators (Belavkin et al., 2016). Such fitness landscapes are more likely to occur from a focal source of selection characteristic of super-inhibitory antimicrobial stress. Indeed, hypermutators were generally selected towards the end of the experiment at stress, however, why ceftazidime generates a fitness landscape more amenable for hypermutation than the other selective agents requires further investigation.

Hypermutation adds a confounding variable to the experiment as the ceftazidime-adapted hypermutators acquired mutations so much faster than the naïve lineages. Therefore, it is difficult to discriminate between mutations associated with drug-specific adaptation and adaptation to the selective background, such as nutrient catabolism. Indeed, figure 5.5. shows mutations in several genes were selected in parallel in ceftazidime-adapted lineages which possess cryptic roles in ceftazidime adaptation. The planktonic lineages possessed mutations in the *leuC* leucine tRNA-ligase and *purL* purine biosynthesis gene. Moreover, possessed mutations in coenzyme A subunit PA14_RS01115, *ptsP* phosphotransferase, *tle1* lipase, PA14_RS13660 thioesterase, *bkdA2* ketoacid dehydrogenase, PA14_RS16205 sterol desaturase and *thrB* homoserine kinase. These genes do not possess characterised roles in β-lactam resistance, and it is hard to conclude that they are involved specifically in ceftazidime adaptation from this experiment. Even though they are exclusively present in ceftazidime-adapted lineages, it may simply be that these mutations would also be selected in naïve lineages given enough evolutionary time had elapsed. Nevertheless, this still indicates that there is likely a trend towards lifestyle-specific adaptation as they were selected in parallel lineages but excluded from the alternate lifestyle. Indeed, all planktonic lineages possessed mutations in the alginate biosynthesis gene *algC* by transfer 6. AlgC is a phosphomannomutase which catalyses the formation of mannose-mannose-1-phosphate, an essential alginate monomer which is also important in rhamnolipid production (Olvera et al., 1999). Consequently, AlgC expression is essential for biofilm formation in *P. aeruginosa*. Transcription of the *alg* operon is under control from the AlgR repressor and transcribed polycistronically from the *algD* promoter with the exception of *algC*. Instead *algC* is transcribed from its own promoter which has been shown to be de-repressed due to exposure to cephalosporins (Zielinski et al., 1992, Hagras et al., 2021). Consequently, loss-of-function of AlgC may rescue planktonically-adapted lineages from ceftazidime-induced rhamnolipid and alginate

production which may incur a fitness defect in a selective environment which favours rapid planktonic growth. Furthermore, selection acted exclusive on *algC* with the rest of the *alg* operon preserved. Expression of *alg* is also dependent on the AlgU alternative σ-factor, an RpoE homologue which plays an important role in the envelope stress response (Fraud et al., 2008, Damron and Goldberg, 2012). Expression of *alg* also results in de-repression of MexCD-OprJ and MexEF-OprN via the AlgU regulon (Kohler et al., 1997). Consequently, inactivation of *algC* but retained activity of the *algD*-dependent operon allows maintenance of the drug-detoxifying action of the AlgU stress response.

5.3.3. Tobramycin Adaptation

Tobramycin is an aminoglycoside antibiotic which inhibits protein synthesis by binding to the 16S rRNA A-site of the 30S ribosomal subunit, arresting polypeptide chain elongation and interfering with tRNA binding to induce codon mistranslation (Krause et al., 2016). High-level resistance to tobramycin is predominantly mediated by plasmid-encoded aminoglycoside modifying enzymes and post-translational modifiers of ribosomal binding sites, however chromosomally-encoded mechanisms of resistance also exist. Aminoglycosides are not exported by most multidrug efflux pumps such as ArcAB-TolC, however some there are some exceptions. MexAB-OprM is a weak exporter of tobramycin with most aminoglycoside export in *P. aeruginosa* being performed by MexXY (Seupt et al., 2020). Moreover, cell envelope barrier functions including lipid decoration are also important mechanisms for reducing import of aminoglycoside molecules. Target site alterations in ribosomal rRNA genes are generally not observed in tobramycin-resistant organisms as tobramycin can bind to multiple sites on the 30S ribosomal subunit (Kondo and Koganei, 2019). Nevertheless, mutations in ribosomal proteins and peptide elongation factors have been observed to confer resistance without the lethality of rRNA mutations. All tobramycin-adapted biofilm and planktonic lineages possessed mutations in the elongation factor G gene *fusA* by transfer 12 as shown in figure 5.7. FusA catalyses the GTP-dependent translocation of the peptidyl-tRNA from the ribosomal A-site to the P-site, freeing the A-site for the next round of aminoacyl-tRNA binding during peptide elongation (Andersen and Nyborg, 2001). In total, ten unique mutations were identified in *fusA,* none of which were conserved between both biofilm and planktonic lifestyles. Substitutions were not localised to any particular domain or motif in FusA, four mutations were present in the GTPase domain I, four in translocase domain V and two in superdomain II which undergoes conformational change during ribosome binding (Salsi et al., 2015). The

mechanism by which such broad changes to elongation factor G confer tobramycin resistance is not known but *fusA* mutations have also been observed in both clinical and experimentally evolved strains (Sanz-García et al., 2018, Bolard et al., 2018). Mutations in *fusA* are commonly associated with aminoglycoside-resistant SCVs of *P. aeruginosa* isolated from the cystic fibrosis lung (López-Causapé et al., 2018). In SCVs, it was hypothesised that as aminoglycosides are dependent on active transport to enter cells, reduced translational capacity would impede drug uptake.

The tobramycin-adapted planktonic lineages possessed some distinct differences from the biofilm lineages. Most notably, adaptation to tobramycin in a planktonic lifestyle collaterally selected for larger decreases in β-lactam susceptibility than in biofilms. Planktonic-2 and -3 possessed mutations in the CheY-receiver domain of *parR,* a transcriptional regulator of multidrug efflux and lipid homeostasis. ParR is the response regulator of the two-component system ParRS which impacts expression of MexXY, PmrAB and PhoPQ (Muller et al., 2011). Constitutive activation of ParR leads to overexpression of MexXY-OprM, of which tobramycin is a substrate, in addition to β-lactams and ciprofloxacin. Moreover, expression of PmrAB and PhoPQ results in transcription of the *pmrFHIJKLM* operon which synthesises the cationic sugar 4-amino-4-deoxy-L-arabinose and catalyses its transfer to phosphate groups on nascent lipid A molecules (Aghapour et al., 2019). An decrease in the net negative charge of the outer membrane confers reduced activity of positive charged molecules, including tobramycin (Purdy-Drew et al., 2009). Lipid A modification is also a mechanism of polymyxin resistance to which no change in susceptibility was observed, this is in spite of planktonic-1 and -2 also possessing mutations in *pmrB* (Phan et al., 2017).

Mutations in another regulator of MexXY were also identified in planktonic-1 and -4, in addition to biofilm-3. *amgS* is the histidine kinase of the AmgRS system, homologous in function to ParRS (Lau et al., 2013). The two unique missense mutations in *amgS* were not in catalytic domains, instead they were present in transmembrane helix I and II, respectively. Mutations in *amgS* conferring aminoglycoside resistance have been previously identified, however they were localised within the histidine kinase domain to confer to constitutive activation of AmgR. How extra-catalytic substitutions in AmgS contribute to activation of AmgR are not known and require further investigation. It was hypothesised that all tobramycin-adapted planktonic lineages possessed constitutive de-repression of MexXY resulting from *parR* mutations in two lineages and *amgS* mutations in the other two. However, in three planktonic lineages, mutations were also present in the large-conductance mechanosensitive channel *mscL*, a gated porin which senses membrane tension to resolve osmotic stress (Iscla et al., 2014). MscL has both

a positive and negative effect on aminoglycoside susceptibility. Aminoglycosides have been shown to enter cells via MscL, however upon sufficient cellular perturbation, MscL can be opened to expel aminoglycosides from the cell (Zhao et al., 2020, Bruni and Kralj, 2020). Constitutively opened MscL may act to increase tobramycin export offseting MscL-dependent aminoglycoside entry, however the crystal structure of MscL has not yet been solved, therefore this remains hypothetical. Nevertheless, this is the first report of mutations in *mscL* being selected by tobramycin exposure.

Tobramycin resistance in biofilm lineages appeared largely independent of multidrug efflux and porins. Though it is now generally accepted that the biofilm matrix as a non-specific diffusion barrier is not a major contributor to resistance to many antimicrobials, some examples of drugs where the matrix contributes to antimicrobial tolerance have been demonstrated (Sharma et al., 2019). Most notably alginate has been shown to bind tobramycin and reduce susceptibility in a concentration-dependent fashion (Nichols et al., 1988, Cao et al., 2015). Moreover, modelling suggests that as a polyanion, alginate possesses sufficient ligand-binding capacity to attenuate inhibitory concentrations of positively-charged aminoglycosides (Cao et al., 2016). Consequently, matrix components appear to contribute to insusceptibility to some antimicrobials in specific circumstances through a mechanism of ligand-specific diffusion-reaction inhibition. Therefore, this may alter the strength of selection of resistance if tobramycin was sequestered in the matrix and was slower to exert inhibitory action. Supporting this, mutations in *fusA* were selected early and conferred modest reductions in susceptibility. However later timepoints resulted in planktonic lineages accumulating mutations and further decreases in susceptibility which did not appear in the biofilm-adapted lineages.

5.3.4. Static Re-Adaptation

The sequenced lineages were re-adapted for an additional ten transfers to a static stress which represented the maximum concentration of drug they were exposed to during stepwise adaptation. The effects of static re-adaptation in different lifestyles, antibiotics and concentrations on drug susceptibility, biofilm formation and fitness were examined. Figure 5.10. demonstrates a broad trend towards further reductions in susceptibility to the selective agent during static re-adaptation in addition to contraction of collaterally selected resistance to non-selective agents. Ciprofloxacin-readapted lineages of both lifestyles demonstrated further reductions in susceptibility to ciprofloxacin, however biofilm lineages were characterised by broad increases in β-

lactam susceptibility at each timepoint. In contrast, ciprofloxacin-readapted planktonic lineages were associated with modest increases in colistin susceptibility. As stepwise adaptation to ciprofloxacin was associated with modest cross-resistance to β-lactams and colistin in biofilm and planktonic lineages, respectively, these increases in susceptibility restored ancestral susceptibility to non-selective drugs. Similarly, during static re-adaptation to ceftazidime, lineages from both lifestyles demonstrated further reductions in susceptibility to piperacillin and ceftazidime, however this was accompanied by increases in susceptibility to meropenem which restored ancestral susceptibility. In tobramycin-readapted planktonic lineages, there was a trend restored ancestral ciprofloxacin susceptibility, however, in the biofilm lineages, decreased ciprofloxacin was maintained. Furthermore, the biofilm lineages also acquired reduced colistin susceptibility as a result of tobramycin readaptation. This could reflect specialisation to the drug-exposed niche which no longer needs some of the multiplicity of mechanisms accumulated during stepwise exposure from sub- to super-inhibitory stress. The effect of these changes in susceptibility on fitness was highly condition-specific. Though the lineages appeared to negatively select for multidrug resistance, this did not result in increased fitness in an unstressed environment. Furthermore, there were not any strongly conserved trends in fitness or biofilm formation within lifestyles, selective agent or timepoint as shown in figures 5.11. and 5.12. This may indicate that the lineages are undergoing evolutionary divergence due to different magnitudes of stress. Alternatively, if conserved mechanisms of resistance are being selected, this indicates that the fitness costs they incur are dependent on genomic background. Moreover, changes in biofilm formation were independent of lifestyle, with readaptation rescuing biofilm formation in some planktonic lineages and compromising it in some biofilm lineages. This provides a further indication that biofilm competency is somehow broadly influenced by changes in susceptibility.

In the stepwise ciprofloxacin-adapted lineages, it was hypothesised that collateral selection of reduced susceptibility to β-lactams and colistin was due to constitutively de-repressed multidrug efflux. Therefore, in an environment where lineages already possessed sufficient decreases in susceptibility to tolerate the stress through *gyrA* mutations, efflux revertants could be selected to ameliorate fitness defects associated with unnecessary expression. This could not be the only adaptation as these lineages underwent further reductions in ciprofloxacin susceptibility. Therefore, a ciprofloxacin-specific resistance mechanism must have been selected in order not to yield cross-resistance to other agents, such as the secondary topoisomerase targets *parCE* or *gyrB*. Such mutations are often selected consequently to *gyrA* mutations due to the

action of clonal interference as they possess equivalent fitness costs (Pan et al., 2001). Similarly, in the ceftazidime-readapted lineages, it is possible that, as meropenem is an efflux substrate, the loss of de-repressed multidrug efflux contributed to loss of meropenem resistance. Despite this only two planktonic lineages possessed mutations in known efflux repressors, and only two biofilm lineages possessed mutations in the MexJK subunit *mexK* which has an ambiguous substrate repertoire. Therefore, it is hard to hypothesise how increased susceptibility to carbapenems was selected as the mechanisms which selected reduced susceptibility previously could not be identified clearly. Nevertheless, reduced susceptibility specifically to piperacillin and ceftazidime is highly indicative of further increases in AmpC expression. Tobramycin-readapted planktonic lineages were associated with the loss of reduced susceptibility to ciprofloxacin which is also likely a result of reversion of efflux de-repression. Mutations in *pmrB* were selected by stepwise adaptation to tobramycin, therefore the reduced susceptibility to colistin observed in tobramycin-readapted biofilm lineages could recapitulate this genotype as there is evidence it is under selection by tobramycin exposure. A comprehensive genomic analysis of the molecular basis which underpins readaptation is currently underway to investigate these hypotheses.

5.3.5. Conclusions

In conclusion, though drug resistance is not inherent to biofilm hyperproduction and biofilm hyperproduction is suppressed by drug exposure, evolutionary trajectories to resistance are heavily influenced by the biofilm lifestyle. In this experiment, both lifestyles typically selected for the same ‘driver’ mutation, although there were often lifestyle-specific polymorphisms selected. In ciprofloxacin, ceftazidime and tobramycin, the driver gene target was *gyrA, ampR* and *fusA* respectively. However, patterns of secondary mutations were distinct between lifestyles. The repertoire of de-repressed efflux pumps was the most salient mechanistic difference, however other selective targets, including type IV pili, alginate biosynthesis and porins were also differentially selected between lifestyles. This manifests phenotypically as altered patterns of cross-resistance and the fitness costs incurred by resistance. Consequently, the biofilm lifestyle is an important property defining the selection of particular mechanisms of resistance and should be carefully considered when investigating the evolution of antimicrobial resistance.

CHAPTER 6. COLLATERAL MODULATION OF ANTIBIOTIC SUSCEPTIBILITY SELECTED BY NON-THERAPEUTIC ANTIMICROBIALS

6.1. Introduction

Non-therapeutic antimicrobials are grouped loosely based on their applications as antimicrobial agents useful for anthropogenic purposes but lack the selective toxicity to be used in antimicrobial chemotherapy (Jones and Joshi, 2021). Whereas antibiotics are typically naturally derived, or semi-synthetic modifications of natural products isolated from soil microbes, non-therapeutic antimicrobials are often synthetic or inorganic molecules, produced independently of microorganisms. Non-therapeutic antimicrobials are classified based on functional group, and in this regard, there are at least 20 distinct chemistries in use for biocidal purposes (Gnanadhas et al., 2012). Notable examples include aldehydes such as glutaraldehyde, commonly used in washer-disinfectors for the decontamination of medical devices such as endoscopes (Manzoor et al., 1999). Moreover, aromatic alcohols and phenolics are a very broad class of biocides which have been in use for hundreds of years. Phenoxyethanol, triclosan and chloroxylenol are among the most commonly used, particularly for the preservation of consumer goods (Gadea et al., 2016). Another important class are cationic surfactants, a diverse subgroup characterised by their net positive charge and ability to dissociate cell membranes (Fox et al., 2021). Quaternary ammonium compounds such as benzalkonium chloride and bisbiguanides such as chlorhexidine are used extensive in clinical environments as topical antiseptics. Other examples include anilides, diamidines and isothiazolinones, common preservative of consumer hygiene products, oxidising agents such as domestic bleach and hydrogen peroxide and peracetic acid which act to release reactive oxygen species (McDonnell and Russell, 1999).

Several order of magnitudes more non-therapeutic antimicrobials are consumed than antibiotics (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). Antisepsis and regular execution of disinfection regimes is central to infection prevention protocols in healthcare and veterinary environments. In addition to this cornerstone role, they have also become omnipresent in domestic environments (Harbarth et al., 2014). The proliferation of antimicrobial hygiene products such as toothpaste, antiperspirants and laundry detergents have become indispensable to consumers over the last century (Gilbert and McBain, 2003). It is hard to overstate the ubiquity of biocidal agents in the domestic environment. In addition to products designed to kill microorganisms, consumer goods which possess a biocide component as a means to promote longevity are even more common (Maillard, 2005). Many staple products including processed foods, cosmetics and pharmaceuticals contain a plethora of antimicrobial preservatives to increase shelf life. Furthermore, impregnation of

biocides into materials such as textiles, leather and plastics to retard microbial growth has become increasingly common. Moreover, non-therapeutic antimicrobials have widespread use in animal husbandry as adjuvants for growth promotion (Phillips et al., 2004). Growth promotion is the principle of administering broad-spectrum antimicrobials into animal feed to increase growth yields of livestock, particularly poultry and swine. By using antimicrobials prophylactically, the risk of infection impeding development is minimised and the resulting gut dysbiosis also increases the efficiency of calorie assimilation, resulting in faster growth (Visek, 1978). Feed supplementation of organic acids and heavy metals have been used for decades in swine to reduce post-weaning infection for mature piglets as an adjutant to antibiotic growth promotion (Yazdankhah et al., 2014). However even in the absence of antibiotics, dietary zinc and copper can decrease diarrhoea in piglets transitioning onto a solid diet and possess a growth promotion effect in mature pigs (Olukosi et al., 2018). With the recent paradigm shift prohibiting the use of antibiotics for the growth promotion of livestock across the Western world, there has been a proliferation of alternative growth promoters based on non-therapeutic antimicrobials (Niewold, 2007).

The modes of action of non-therapeutic antimicrobials have not been investigated to the same degree as antibiotics, partly due to the lack of burden of resistance to these agents (Russell, 2002). However, they generally operate though non-specific mechanisms of action which exerts antimicrobial effects through gross cellular damage (Walsh et al., 2003). This is achieved primarily through membrane disruption or coagulation of cytosolic constituents (Ashraf et al., 2014, Russell, 2003). These mechanisms confer a broader spectrum of activity than most antibiotics, but often exhibit cytotoxicity, hence a lack of therapeutic value. Their non-specific mechanisms of action and capacity to be employed at concentrations unconstrained by *in vivo* tolerability confers a low mutant prevention concentration which is readily attained during *in situ* use (McDonnell and Russell, 1999). Despite this, non-therapeutic antimicrobials are not selectively neutral. In practice, sub-inhibitory concentrations of non-therapeutic antimicrobials are more likely to be encountered than equivalent concentrations of antibiotics by virtue of the larger volumes consumed and routes of application (Maillard et al., 2013). Selection for reduced susceptibility for non-therapeutic antimicrobials rarely yields mutants with high-level resistance. A notable exception to this rule is triclosan, which unusually acts upon the FabI component of type II fatty acid biosynthesis as a defined molecular target (Zhu et al., 2010). In most cases however, reduced susceptibility generally manifests as low-level ≤ 2 $\log_2$ fold changes. Such modest changes can be selectively advantageous as fitness in

environments which experience subinhibitory stress will be increased, but strains will nonetheless remain susceptible to in-use concentrations.

As non-therapeutic antimicrobials are generally synthetic or inorganic, microbes have not evolved mechanisms to enzymatically modify them to inactive intermediates. Moreover, as non-therapeutic antimicrobials generally possess a mode of action which acts upon multiple physiological processes simultaneously, the required target modifications are probabilistically negligible to be selected. Therefore, the main mechanisms by which reduced susceptibility to biocides is achieved is preventing molecules from entering the cell or evacuating them upon entry (McBain and Gilbert, 2001). These generic mechanisms have polygamous phenotypic effects on susceptibility to other antimicrobial agents, including antibiotics (White and McDermott, 2001). Consequently, despite the change in susceptibility associated with these mechanisms being small, they can possess large collateral effects on a broad range of chemically-diverse agents which also lie within their repertoire (Oggioni et al., 2013). The specific instances and mechanisms by which this occurs has been discussed exhaustively in Section 1.4. In brief, selection of constitutively de-repressed multidrug efflux, porin loss, envelope charge modification and stress response-mediated respiratory depression can confer decreased susceptibility to non-therapeutic antimicrobials and clinical resistance to antibiotics simultaneously (Wales and Davies, 2015). This phenomenon is known as cross-resistance and has elicited concerns particularly regarding the possibility that non-therapeutic antimicrobials could contribute to the antibiotic resistance crisis. However, there is a significant amount of ambiguity regarding how and when selection of cross-resistance occurs due to conflicting evidence, poorly standardised assays and a lack of evolutionary understanding at the genotypic level (Fox et al., 2021). If exposure to biocides is a contributing factor to the emergence of antibiotic resistance this information would establish an evidence base to evoke policy changes to biocide utilisation. Using the biofilm evolution model, this chapter aims to identify, from a panel of 48 non-therapeutic antimicrobials of diverse classes and antipseudomonal antibiotics not previously investigated, agents which possess the capacity to collaterally select for reduced susceptibility to drugs. The effect of adaptation to these agents on relative fitness and biofilm formation was also determined. Qualitative assessment of the selective pressures of these compounds possess will allow production of a cross-sectional narrative for which agents possess high selection coefficients on mechanisms which contribute to reduced susceptibility to drugs. Therefore, this may be used as a tool for developing improved the design and utilisation selectively-neutral biocidal products.

6.2. Results

The transfer at which lineages could no longer survive from exposure to their selective agent and its relationship with the MIC and MBEC of the selective agent was determined (figure 6.1.). All lineages adapted to sodium hypochlorite, magnesium chloride, sodium benzoate, citric acid, copper sulfate, phenoxyethanol, benzoic acid and sorbic acid were unable to be passaged at more than one-log_2 fold the MIC. Most lineages, however, were able to survive well past the MIC as the planktonic inhibitory threshold during serial passage as a biofilm. The attrition transfer of oxidising agents, acids, salts, phenolic and aldehyde-releasing antimicrobials were generally low and rarely exceeded three-log_2 fold past the MIC. Metal- and isothiazolinones-adapted lineages demonstrated higher attrition transfers up to six-log_2 fold past the MIC. Despite this, lineages up to this point rarely exceed the MBEC, generally reaching the bactericidal concentration and failing to adapt further. Only 14 antimicrobials selected for lineages able to be passaged past the MBEC of the selective agent, most of which were cationic surfactants and antibiotics. Cationic surfactant-adapted lineages grew between five- and seven-log_2 fold past the MIC and up to four-log_2 fold past the MBEC. Antibiotic-adapted lineages dominated the end-stage of the experiment at ancestrally bactericidal concentrations. Lineages adapted to piperacillin, meropenem and fosfomycin were able to survive at 10-log_2 fold past the MIC and between three- to six-log_2 fold past the MBEC.

The attrition transfers of each lineage were stratified according to type of selective agent: antibiotic, metal or biocide (figure 6.2.). Lineages adapted to antibiotics did not reach the mean survival transfer (50% lineage attrition) until transfer 63. All biocide- and metal-adapted lineages completely failed to grow by transfer 51 and 48, respectively. The mean survival transfer of biocide-adapted lineages was transfer 15, whereas for metal-adapted lineages it was transfer 21. Acids and phenolics were the least tolerated classes of biocides which both reached mean survival at transfer 12. Salt-adapted lineages possessed a mean survival of 15 transfers and reached complete attrition at transfer 30. All but one aldehyde-adapted lineages failed at transfer 18 and the remaining lineage failed to grow at transfer 21. Isothiazolinones and oxidising agent-adapted lineages both reached mean survival at transfer 21. However, isothiazolinone-adapted lineages completely failed to grow by transfer 33 whereas oxidisers lasted until transfer 51, largely due to the high tolerance of lineages adapted to paraquat. Cationic surfactants were the most well tolerated of all classes of biocide. Surfactant-adapted lineages reached mean survival at transfer 45 and complete attrition at transfer 48.

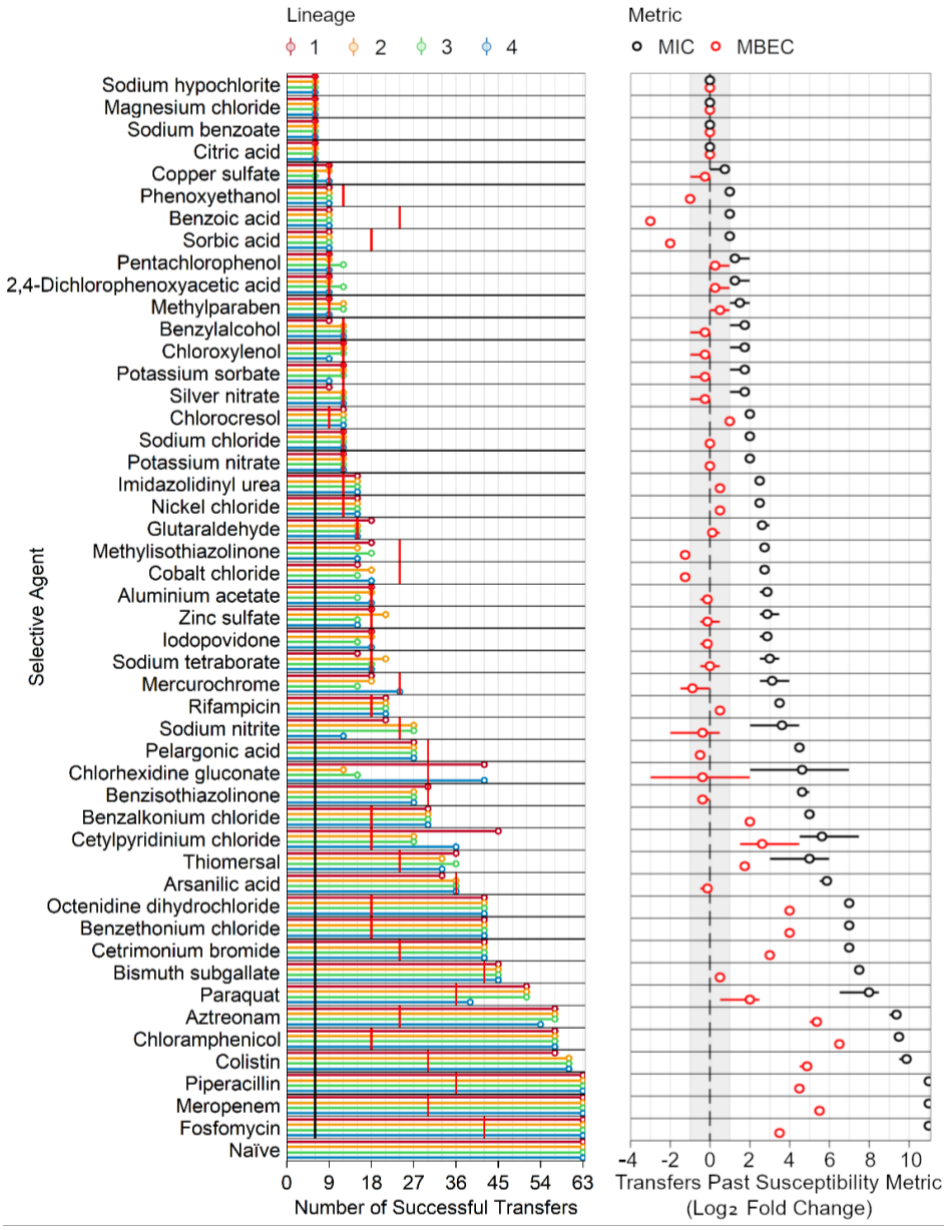

Figure 6.1. Survival of experimentally evolved biofilms adapted through serial passage to a panel of 48 antimicrobials in a stepwise fashion for up to 63 transfers from -2– to 10–log_2 fold MIC until growth failure. When a bead could no longer turn the growth medium turbid after 24 hours the last successful transfer was recorded. Left panel: Data shown as number of successful transfers performed until lineage was unable to be propagated to a new bead, black bar shows MIC, red bar shows MBEC, n = 1. Right panel: Data shown as mean log_2 fold change past susceptibility metric of final successful, error bars show range, n = 4.

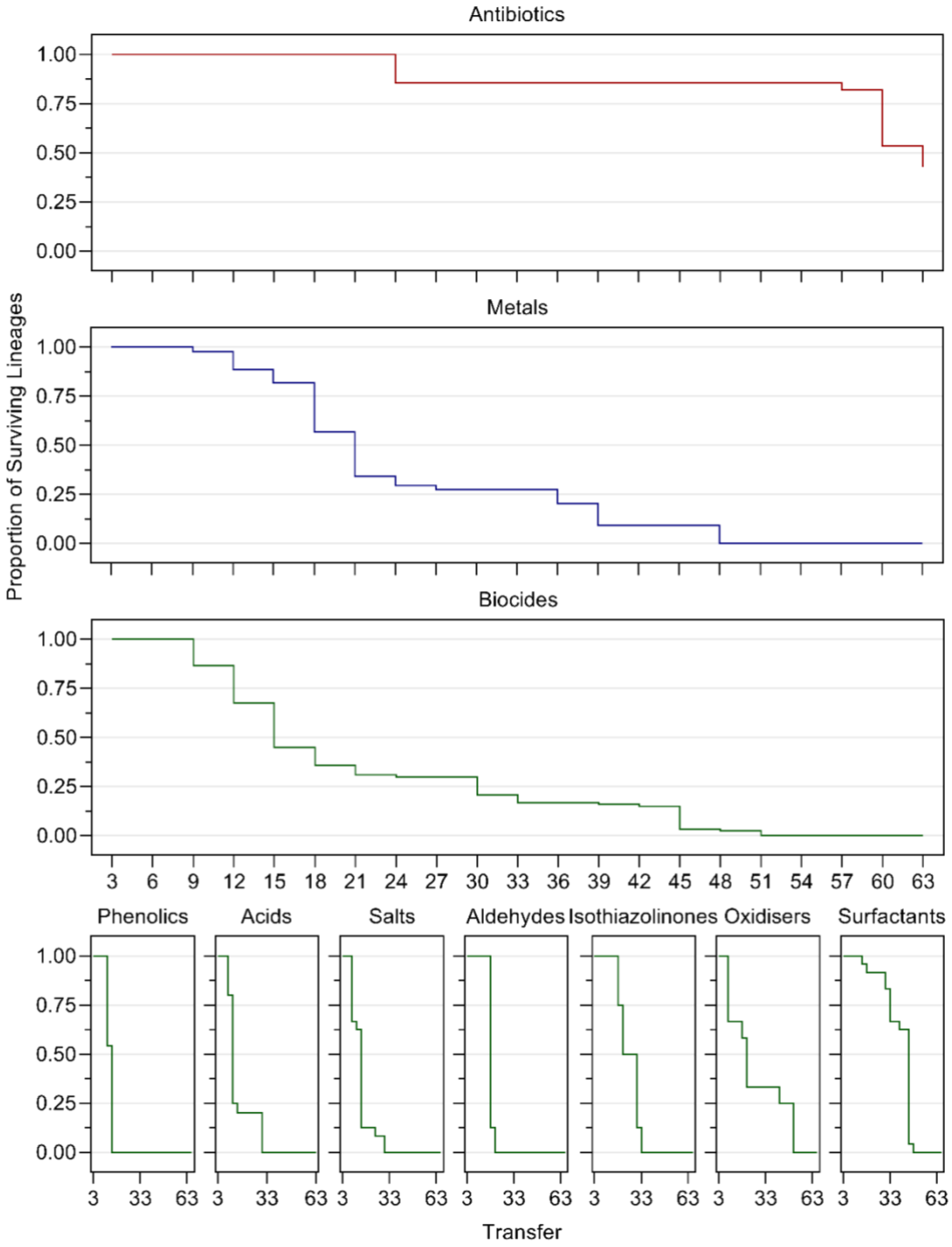

Figure 6.2. Attrition dynamics of experimentally evolved biofilms adapted through serial passage to a panel of 48 antimicrobials in a stepwise fashion for up to 63 transfers until growth failure. The last successful transfer from each lineage was stratified by the type of antimicrobial to which they were adapted: antibiotic, metal or biocide, generating an aggregate pattern of growth failure. Data shown as proportion of surviving lineages, n = 4

Endpoint populations of biofilms adapted to a panel of antimicrobials in a stepwise manner were assessed for altered susceptibility to a panel of antipseudomonal drugs (figure 6.3.). Of the 48 conditions tested, 21 were able to select for reduced

susceptibility to the selective agent. All antibiotics, except from rifampicin, were able to select for between six- and nine-log_2 fold reductions susceptibility to themselves which all exceeded EUCAST breakpoints. The only oxidising agent able to select for reduced susceptibility to itself was paraquat which ranged from a three- to nine-log_2 fold reduction. Most of the cationic surfactants were also able to select for reduced susceptibility to themselves. Cetylpyridinium chloride, cetrimonium bromide and chlorhexidine gluconate selected for a two- to three-log_2 fold reduction in susceptibility, whereas octenidine dihydrochloride and benzethonum chloride selected for a four- to five-log_2 fold reduction. All thiomersal- and bismuth subgallate-adapted lineages possessed two- to four-log_2 fold reductions in susceptibility to the selective agents. The phenolic biocides pentachlorophenol, chloroxylenol and chrlorocresol also selected for a two-log_2 fold reduction in susceptibility. Potassium sorbate and methylisothiazolinone were also able to select for two-log_2 fold reductions in susceptibility to themselves.

Collaterally altered susceptibility to tobramycin was broadly observed. Nine antimicrobials were able to select for up to a three-log_2 fold reduction in tobramycin including pelargonic acid, various metals, benzothiazolinone, sodium nitrite, chlorhexidine and cetylpyridinium chloride, but did not exceed clinical breakpoints. Conversely, eight agents were able to select for up to a three-log_2 fold increase in tobramycin susceptibility including piperacillin, colistin, potassium nitrate, potassium sorbate, zinc sulfate, imazolidinyl urea and methylisothiazolinone. Altered β-lactam susceptibility was also observed. β-lactam selective agents selected for the largest reductions in susceptibility to all other β-lactams. Octenidine dihydrochloride and arsanilic acid were selected for up to a two-log_2 fold reduction to all β-lactam agents. Moreover, cobalt chloride, chloroxylenol and thiomersal were able to select for up to a three-log_2 fold reduction in susceptibility to piperacillin and meropenem but did not exceed clinical breakpoints. Similarly, rifampicin selected for up to a two-log_2 fold reduction in susceptibility to piperacillin and ceftazidime. Between two- to three-log_2 fold increases in susceptibility to all β-lactam antibiotics was observed in cetrimonium bromide- and benzethonium chloride-adapted lineages. Moreover, colistin and chloramphenicol selected for between a one to four-log_2 fold increase in susceptibility to β-lactam except from meropenem. Chloramphenicol selected for a five-log_2 fold decrease in susceptibility to ciprofloxacin which surpassed the breakpoint. Furthermore, thiomersal-adapted lineages demonstrated a one- to four-log_2 fold reduction in susceptibility and meropenem and octenidine dihydrochloride selected for up to a two-log_2 fold reduction in ciprofloxacin susceptibility. No agent was able to select for altered susceptibility to colistin other than the selective agent itself.

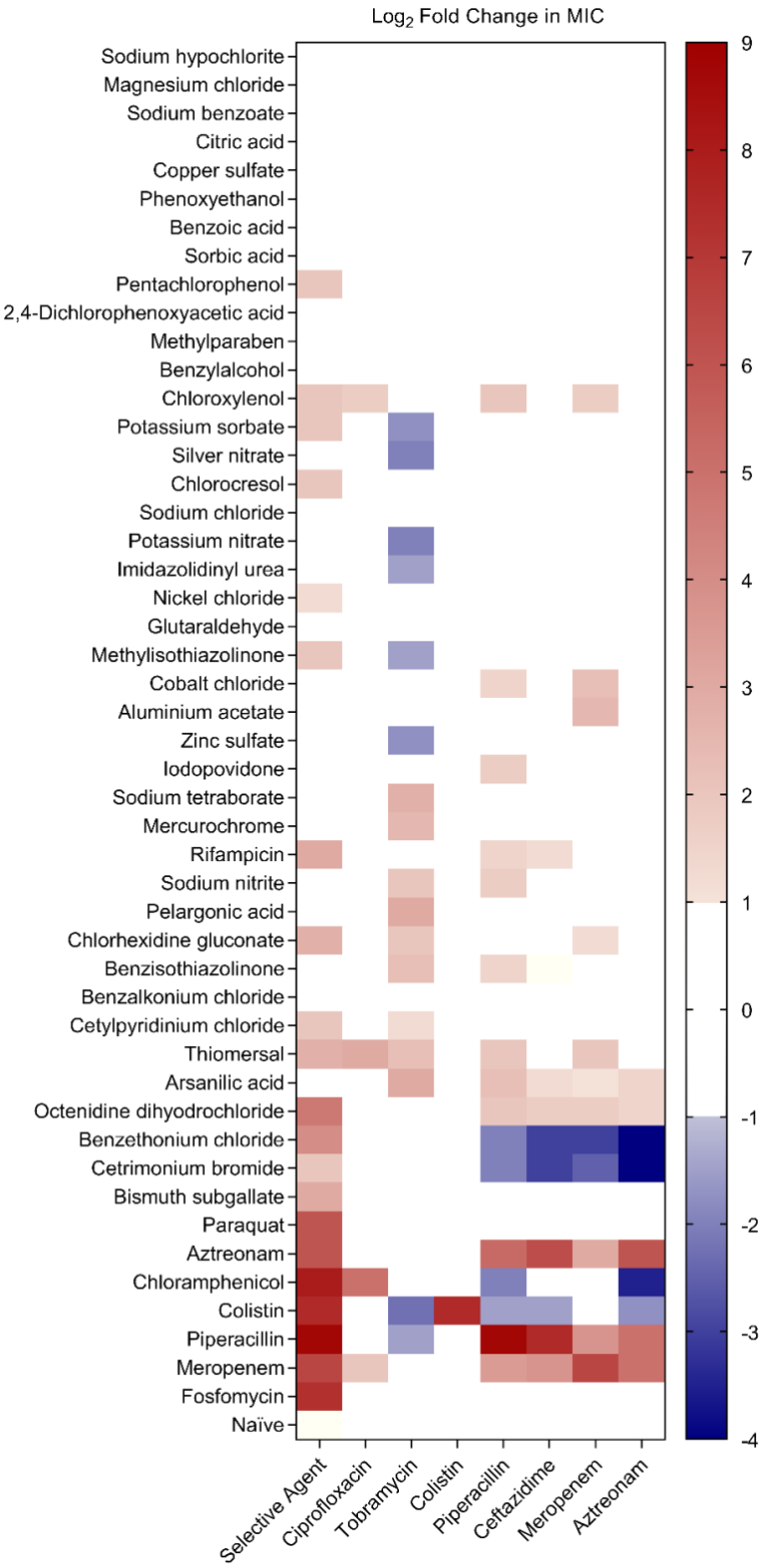

Figure 6.3. Antimicrobial susceptibility to a panel of antipseudomonal antibiotics determined via agar dilution MIC of experimentally evolved lineages of *P. aeruginosa* adapted through serial passage to a panel of 48 antimicrobials in a stepwise fashion for up to 63 transfers until growth failure. Significant changes in susceptibility were defined as a >1-log_2 fold change. Data shown as mean log_2 fold change in MIC from ancestor, n = 4.

The biofilm formation of experimentally evolved lineages adapted to a panel of antimicrobials was determined (figure 6.4.). The naïve biofilms formed approximately twice as much biomass than the ancestor by transfer 21 ($p < 0.0001$). By transfer 39, the naïve lineages again became significantly better at forming biofilms than they were at transfer 21 ($p = 0.0015$), forming approximately three-times as much biofilm as the ancestor. No further significant increases in biofilm formation were observed between transfer 39 and 63 ($p = 1.0000$). In addition to the naïve biofilm lineages, lineages adapted to eight antimicrobials possessed significantly increased biofilm formation relative to the ancestor including cobalt chloride ($p = 0.0368$), sodium chloride ($p = 0.0041$), cetylpyridinium chloride ($p = 0.0284$), glutaraldehyde ($p = 0.0006$), iodopovidone ($p < 0.0001$), rifampicin ($p = 0.0225$), bismuth subgallate ($p = 0.0019$) and chloramphenicol ($p = 0.0211$). No lineage demonstrated a significant reduction in biofilm formation relative to the ancestor. However, lineages adapted to 33 agents were associated with a significant reduction in biofilm formation relative to the naïve biofilms at an equivalent timepoint. Lineages adapted to 12 agents did not demonstrate a significant difference in biofilm formation. The only lineages to possess significantly greater biofilm formation than their naïve counterparts were those adapted to cobalt chloride ($p < 0.0001$), iodopovidone ($p < 0.0001$) and glutaraldehyde ($p < 0.0001$).

A.

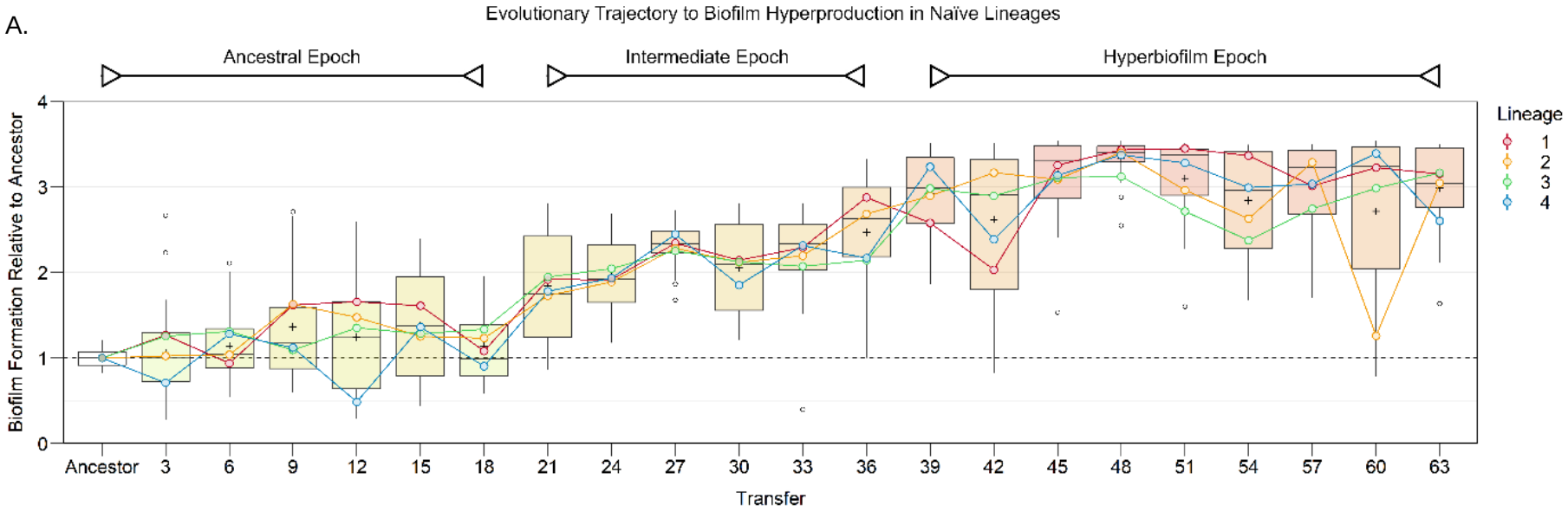
Evolutionary Trajectory to Biofilm Hyperproduction in Naïve Lineages
Ancestral Epoch
Intermediate Epoch
Hyperbiofilm Epoch
Biofilm Formation Relative to Ancestor
0
1
2
3
4
Ancestor
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54
57
60
63
Transfer
Lineage
1
2
3
4

Figure 6.4.

B.

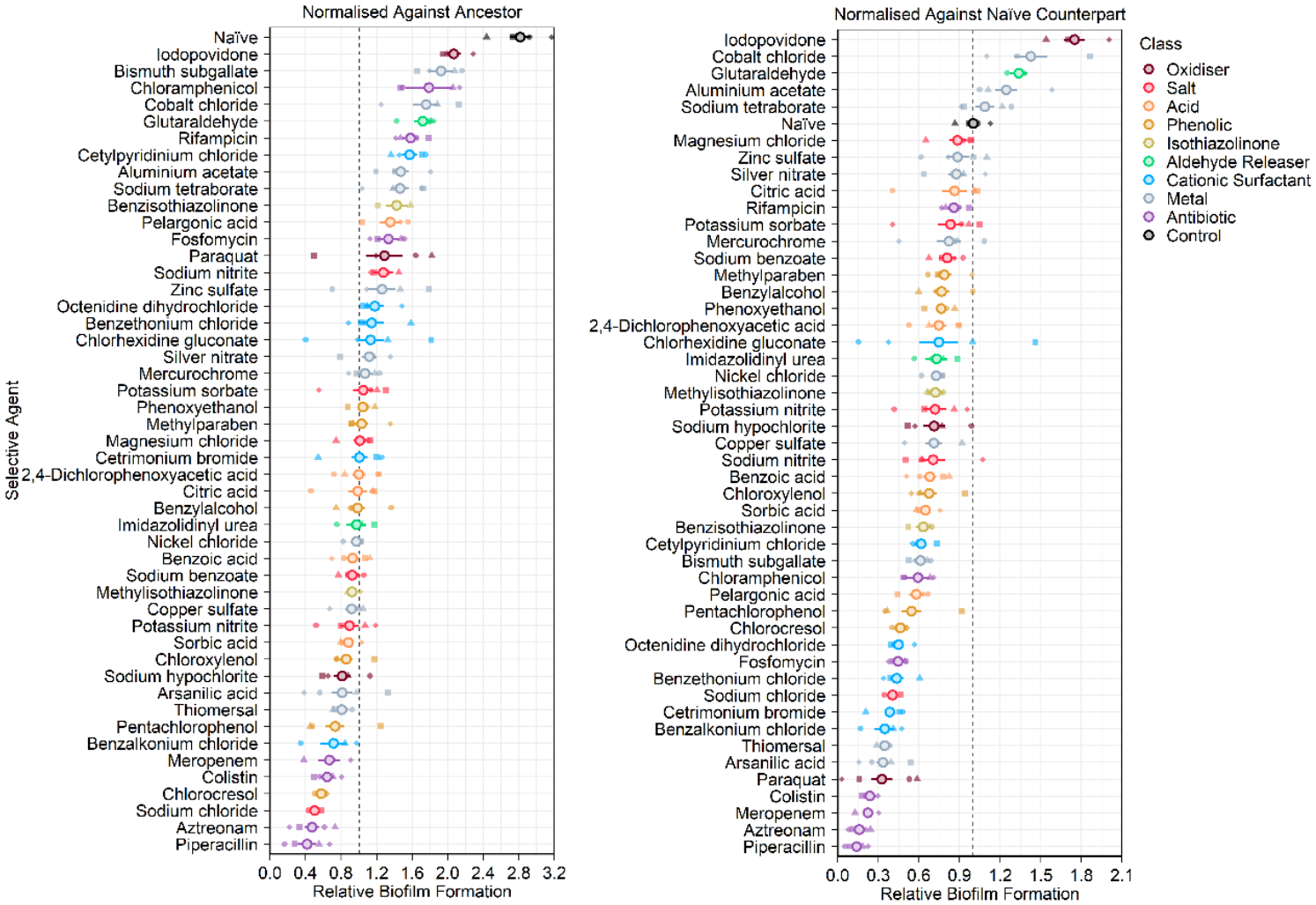
Normalised Against Ancestor
Selective Agent
Naïve
Iodopovidone
Bismuth subgallate
Chloramphenicol
Cobalt chloride
Glutaraldehyde
Rifampicin
Cetylpyridinium chloride
Aluminium acetate
Sodium tetraborate
Benzisothiazolinone
Pelargonic acid
Fosfomycin
Paraquat
Sodium nitrite
Zinc sulfate
Octenidine dihydrochloride
Benzethonium chloride
Chlorhexidine gluconate
Silver nitrate
Mercurochrome
Potassium sorbate
Phenoxyethanol
Methylparaben
Magnesium chloride
Cetrimonium bromide
2,4-Dichlorophenoxyacetic acid
Citric acid
Benzylalcohol
Imidazolidinyl urea
Nickel chloride
Benzoic acid
Sodium benzoate
Methylisothiazolinone
Copper sulfate
Potassium nitrite
Sorbic acid
Chloroxylenol
Sodium hypochlorite
Arsanilic acid
Thiomersal
Pentachlorophenol
Benzalkonium chloride
Meropenem
Colistin
Chlorocresol
Sodium chloride
Aztreonam
Piperacillin
0.0 0.4 0.8 1.2 1.6 2.0 2.4 2.8 3.2
Relative Biofilm Formation
Normalised Against Naïve Counterpart
Iodopovidone
Cobalt chloride
Glutaraldehyde
Aluminium acetate
Sodium tetraborate
Naïve
Magnesium chloride
Zinc sulfate
Silver nitrate
Citric acid
Rifampicin
Potassium sorbate
Mercurochrome
Sodium benzoate
Methylparaben
Benzylalcohol
Phenoxyethanol
2,4-Dichlorophenoxyacetic acid
Chlorhexidine gluconate
Imidazolidinyl urea
Nickel chloride
Methylisothiazolinone
Potassium nitrite
Sodium hypochlorite
Copper sulfate
Sodium nitrite
Benzoic acid
Chloroxylenol
Sorbic acid
Benzisothiazolinone
Cetylpyridinium chloride
Bismuth subgallate
Chloramphenicol
Pelargonic acid
Pentachlorophenol
Chlorocresol
Octenidine dihydrochloride
Fosfomycin
Benzethonium chloride
Sodium chloride
Cetrimonium bromide
Benzalkonium chloride
Thiomersal
Arsanilic acid
Paraquat
Colistin
Meropenem
Aztreonam
Piperacillin
0.0 0.3 0.6 0.9 1.2 1.5 1.8 2.1
Relative Biofilm Formation
Class
Oxidiser
Salt
Acid
Phenolic
Isothiazolinone
Aldehyde Releaser
Cationic Surfactant
Metal
Antibiotic
Control

Figure 6.4.

Figure 6.4. Biofilm formation of experimentally evolved biofilms adapted through serial passage to a panel of 48 antimicrobials in a stepwise fashion for up to 63 transfers or until growth failure. Endpoint populations exposed to each agent were resuscitated and the biofilm formation of each strain was determined via crystal violet staining. Statistical differences between selective conditions over the passage series were detected via a one-way ANOVA with Dunnett's multiple comparison post-hoc test. Panel A shows biofilm formation over time of the naïve lineages relative to the ancestral strain. Panel B shows biofilm formation of the endpoint populations adapted to antimicrobials relative to the ancestor or the naïve lineages at the equivalent timepoint. Data shown as mean biofilm formation relative to control, ± standard error of the mean, n = 8 per lineage.

The fitness of experimentally evolved lineages adapted to a panel of antimicrobials was determined through growth kinetics in broth (figure 6.4.). There was no significant change in area under curve between the naïve biofilm lineages and ancestor over the course of the passage series ($p = 0.9945$). Only pelargonic acid-adapted lineages possessed greater fitness than their naïve counterparts ($p = 0.0004$) which were approximately 10% more fit than the ancestor. Adaptation to benzethonium chloride, cetrimonium bromide, magnesium chloride, meropenem, paraquat and piperacillin were associated with significant fitness defects (naïve vs benzethonium chloride: $p = 0.0133$, naïve vs cetrimonium bromide: $p < 0.0001$, naïve vs magnesium chloride: $p = 0.0022$, naïve vs meropenem: $p = 0.0393$, naïve vs paraquat: $p = 0.0043$, naïve vs piperacillin: $p = 0.0280$). Most fitness-defective lineages possessed a relative fitness 20% to 30% worse than the ancestor, however, cetrimonium bromide-adapted lineages were associated with extremely punitive fitness defects, growing approximately 65% worse. No other significant change in fitness was observed after selection to any other agent.

A.

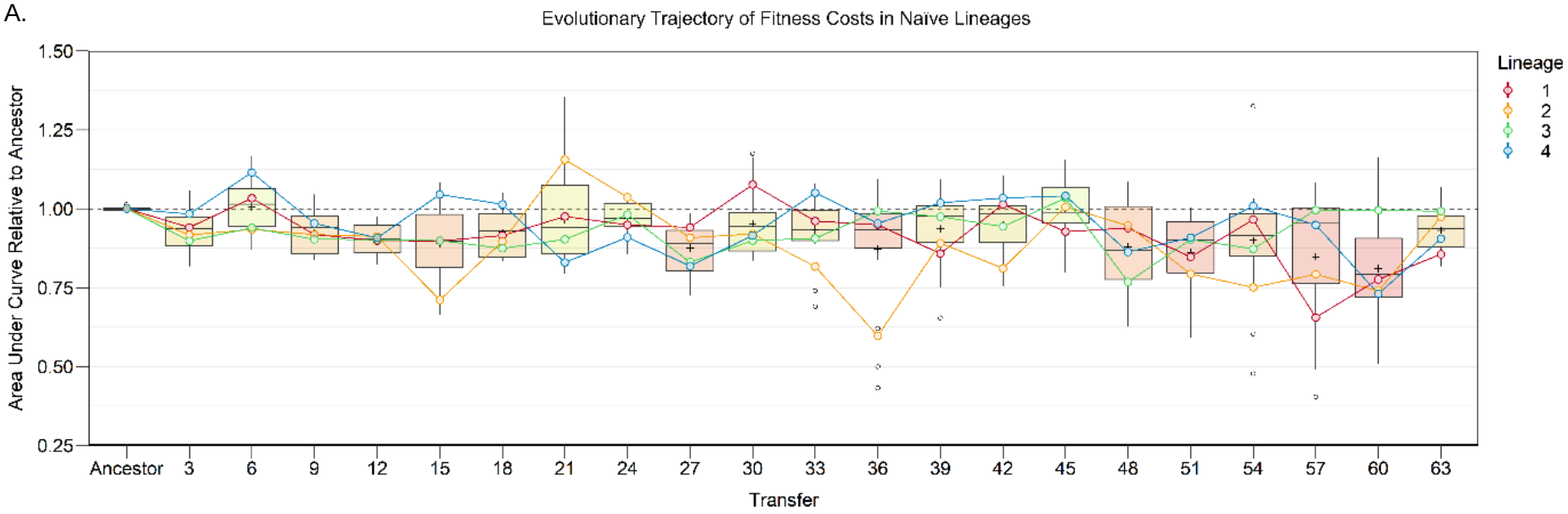
Evolutionary Trajectory of Fitness Costs in Naïve Lineages
Area Under Curve Relative to Ancestor
1.50
1.25
1.00
0.75
0.50
0.25
Ancestor
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54
57
60
63
Transfer
Lineage
1
2
3
4

Figure 6.5.

B.

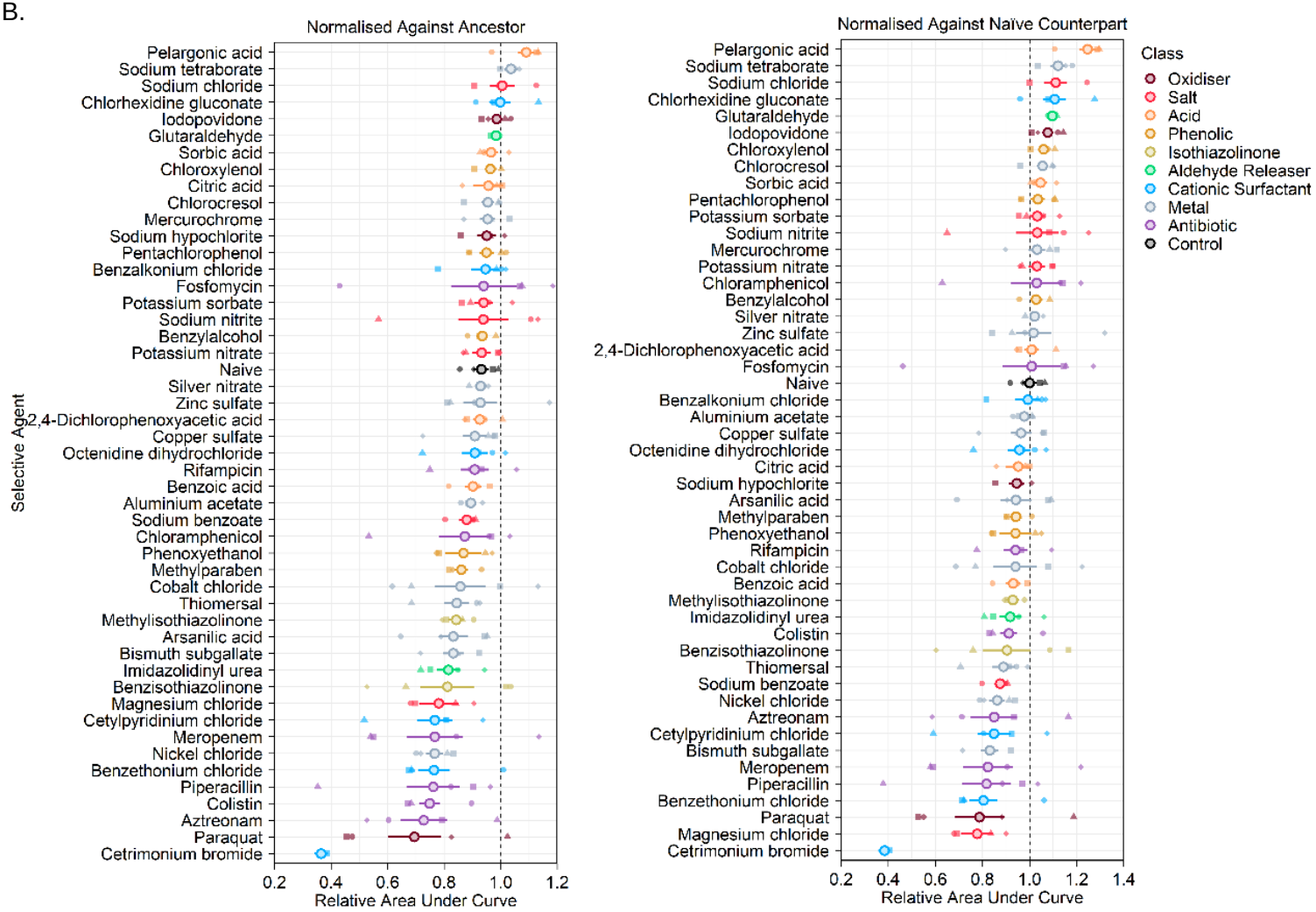
Normalised Against Ancestor
Pelargonic acid
Sodium tetraborate
Sodium chloride
Chlorhexidine gluconate
Iodopovidone
Glutaraldehyde
Sorbic acid
Chloroxylenol
Citric acid
Chlorocresol
Mercurochrome
Sodium hypochlorite
Pentachlorophenol
Benzalkonium chloride
Fosfomycin
Potassium sorbate
Sodium nitrite
Benzylalcohol
Potassium nitrate
Naive
Silver nitrate
Zinc sulfate
2,4-Dichlorophenoxyacetic acid
Copper sulfate
Octenidine dihydrochloride
Rifampicin
Benzoic acid
Aluminium acetate
Sodium benzoate
Chloramphenicol
Phenoxyethanol
Methylparaben
Cobalt chloride
Thiomersal
Methylisothiazolinone
Arsanilic acid
Bismuth subgallate
Imidazolidinyl urea
Benzisothiazolinone
Magnesium chloride
Cetylpyridinium chloride
Meropenem
Nickel chloride
Benzethonium chloride
Piperacillin
Colistin
Aztreonam
Paraquat
Cetrimonium bromide
Selective Agent
0.2
0.4
0.6
0.8
1.0
1.2
Relative Area Under Curve
Normalised Against Naïve Counterpart
Pelargonic acid
Sodium tetraborate
Sodium chloride
Chlorhexidine gluconate
Glutaraldehyde
Iodopovidone
Chloroxylenol
Chlorocresol
Sorbic acid
Pentachlorophenol
Potassium sorbate
Sodium nitrite
Mercurochrome
Potassium nitrate
Chloramphenicol
Benzylalcohol
Silver nitrate
Zinc sulfate
2,4-Dichlorophenoxyacetic acid
Fosfomycin
Naive
Benzalkonium chloride
Aluminium acetate
Copper sulfate
Octenidine dihydrochloride
Citric acid
Sodium hypochlorite
Arsanilic acid
Methylparaben
Phenoxyethanol
Rifampicin
Cobalt chloride
Benzoic acid
Methylisothiazolinone
Imidazolidinyl urea
Colistin
Benzisothiazolinone
Thiomersal
Sodium benzoate
Nickel chloride
Aztreonam
Cetylpyridinium chloride
Bismuth subgallate
Meropenem
Piperacillin
Benzethonium chloride
Paraquat
Magnesium chloride
Cetrimonium bromide
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Relative Area Under Curve
Class
Oxidiser
Salt
Acid
Phenolic
Isothiazolinone
Aldehyde Releaser
Cationic Surfactant
Metal
Antibiotic
Control

Figure 6.5.

Figure 6.5. Relative fitness as measured by area under the curve of experimentally evolved biofilms adapted through serial passage to a panel of 48 antimicrobials in a stepwise fashion for up to 63 transfers or until growth failure. Endpoint populations exposed to each agent were resuscitated and the growth kinetics of each strain were determined. The area under the curve was calculated and statistical differences between lineage and selective condition over the passage series were detected via a one-way ANOVA with Dunnett's multiple comparison post-hoc test. Panel A shows relative fitness of the naïve lineages relative to the ancestral strain. Panel B shows relative fitness of the endpoint populations adapted to antimicrobials relative to the ancestor or the naïve lineages at the equivalent timepoint. Data shown as mean area under curve relative to control, ± standard error of the mean, n = 8 per lineage.

From the sequenced lineages, mutations were identified in genes which were conserved between multiple parallel lineages (figure 6.6.). All naïve lineages possessed a A3638G missense mutation in the bifunctional diguanylate cyclase/ phosphodiesterase *morA* resulting in a Glu1213Gly substitution described in Chapter 4.

Adaptation to meropenem resulted for selection for mutations in the *ftsI* transpeptidase in all lineages. Meropenem-1 and -2 possessed C1510T missense mutations resulting in Arg504Cys substitutions. Meropenem-3 and -4 possessed G1393C and T1394 missense mutations at Val465 resulting in Leu and Ala substitutions, respectively. Meropenem-3 also possessed a T1568G missense mutation resulting in a Val523Gly substitution. Meropenem-2, -3 and -4 also possessed mutations in the porin *oprD* resulting in an early stop codon at position 830, position 1017 and a 27 bp inframe deletion from Tyr399_Lys407, respectively. Meropenem-2, -3 and -4 also possessed unique mutations in the ABC transporter substrate-binding protein *dppA3* including a thymine duplication at position 6 resulting in a Lys3 frameshift, a missense mutation at A722C resulting in a Lys308Gln substitution and a CC deletion at position 1154 resulting in a Pro385 frameshift. Additionally, meropenem-2 and -3 possessed missense mutations at G302A and T203C in *mexR,* respectively, resulting in Gly101Glu and Ile68Thr substitutions.

All aztreonam adapted lineages also possessed mutations in *ftsI.* Aztreonam-1 possessed a A725T missense mutation resulting in an Asn242Ile substitution. Aztreonam-2, -3, -4 all possessed a C737T missense mutation resulting in a Ala246Val substitution. All aztreonam-adapted lineages possessed a T716C missense mutation in *ampC* resulting in a Val239Ala substitution. Moreover, Aztreonam-1 and -4 also respectively possessed A125G and A404G missense mutations in *ampR* which resulted in Lys42Arg and Asp135Gly substitutions. Aztreonam-2, -3 and -4 also possessed a A1142A missense mutation in *dacB* resulting in a Ala381Glu substitution,

a 9 bp inframe insertion at position 1314 in *mpl* resulting a Phe438_Gly440 duplication. Aztreonam-1 possessed a G1280A missense mutation in *dacB* resulting in a Gly427Asp substitution. Aztreonam-2, -3 and -4 also possessed a G391A missense mutation in the LPS biosynthesis gene *galU* resulting in a Asp131Asn substitution and a C840A missense mutation in the hypothetical protein PA14_69510 resulting in a Phe280Leu substitution.

All piperacillin-adapted lineages possessed mutations in the AmpC regulator *ampD*, or its homologue, *ampDh3*. Piperacillin-1 and -2 possessed a A236T and a T493C missense mutation in *ampDh3*, respectively, which resulted in His79Leu and Ser147Pro substitutions. Piperacillin-3 possessed a A448C missense mutation in *ampD* resulting in a Thr150Pro substitution. Piperacillin-4 possessed both an A302T missense mutation in *ampD* conferring a Val101Gly substitution and an 18 bp inframe deletion at position 579 of *ampDh3* resulting in a Pro193_Leu198 deletion. Piperacillin-3 and -4 possessed a thymine deletion at position 817 and a cytosine duplication at position 177 in *dacB*, respectively, which resulted in Trp273 and Thr60 frameshifts. Piperacillin-2 and -4 possessed G1394A and C1755G missense mutations in *algC* which resulted in Gly465Asp and Asn585Lys substitutions.

All chloramphenicol-adapted lineages possessed mutations in the 50S ribosomal protein *rplD* and the MexEF-OprN efflux pump subunit *mexF*. Chloramphenicol-1, -2 and -3 possessed G185A missense mutations in *rplD* which resulted in a Lys62Arg substitution and chloramphenicol-4 possessed a G186T substitution which conferred a Lys62Asn substitution. Chloramphenicol-1, -2 and -3 possessed unique mutations in *mexB*. Chloramphenicol-1 possessed a G535A missense mutation which conferred a Gly179Ser substitution, Chloramphenicol-2 possessed a TGG deletion at position 1772 resulting in a Val591 deletion and Chloramphenicol-3 possessed a G2953A missense mutation in *mexB,* resulting in a Val985Ile substitution. Chloramphenicol-1 and -3 also possessed mutations in the peptidoglycan biosynthesis gene PA14_11960 and MexEF-OprN regulator *mexS*. Chloramphenicol-1 acquired a A961T missense mutation conferring a Ser321Cys substitution and Chloramphenicol-3 acquired a guanine deletion at position 1156 which resulted in a Val386 frameshift. Chloramphenicol-1 also possesses a 9 bp inframe deletion in *mexS* at position 733 which resulted in a Cys245_Gly247 deletion and chloramphenicol-4 possessed a G823T missense mutation which resulted in a Gly275Cys substitution. Both Chloramphenicol-2 and -4 possessed an A839C missense mutation in the LPS biosynthesis gene PA14_66100 which resulted in a His280Pro substitution.

Three fosfomycin adapted lineages possessed mutations in the ABC phosphonate transporter subunit *phnC,* Fosfomycin-2 possessed a C674T missense mutation in *phnC* which resulted in a Ala225Val, Fosfomycin-3 possessed a 21 bp inframe deletion which resulted in a Ser78_Leu84 deletion and Fosfmycin-4 possessed a 39 bp deletion at position 215 which resulted in a Gln72_Leu84 deletion. Three lineages also possessed mutations in regulator of the Pqs quorum sensing system *mvfR*. Fosfomycin-1, -2 and -3 possessed a A41C, G667A and T707G missense mutation, respectively, which conferred Gln14Pro, Asp223Asn and Ile236Ser substitutions. Moreover, Fosfomycin-2 and -4 also possessed mutations in the phosphonate transporter *glpT*. Fosfomycin-2 possessed an C938A mutation resulting early stop codon at Ser313, and Fosfomycin-4 possessed a 6 bp inframe deletion at position 220 which resulted in a Ile74_Ala75 deletion.

Two lineages adapted to thiomersal possessed mutations in *mutL* or *mutS*. In *mutL,* Thiomersal-1 and -4 possessed a TG duplication at position 551, respectively, which resulted in an Ala185 frameshift. In *mutS,* Thiomersal-1 and -2 all possessed a G1848C missense mutation which resulted in a Met616Ile substitution. Arsanilic acid-1 and -4, possessed T1402C missense mutations in *mexF*, which resulted in Phe468Leu substitutions. Thiomersal also selected for mutations in *mexS*, the regulator of the MexEF-OprN repressor MexT. Thiomersal-2 and -4 possessed a T29G missense mutation resulting in a Phe10Cys substitution, Thiomersal-3 possessed a T757C missense mutation resulting Phe253Leu substitution. Thiomersal-2 and -3 also possessed mutations in the anaerobic transcriptional regulator *anr* via a T68C missense mutation resulting in a Leu23Pro substitution and a TGTC deletion at position 467 resulting in a Leu156 frameshift.

Other than arsanilic acid and thiomersal, the non-therapeutic antimicrobials acquired significantly less parallel SNPs than the antibiotics. Benzisothiazolinone-adapted lineages possessed mutations in the peptidoglycan biosynthesis gene PA14_11960 and in glycosyl transferase *orfN.* Benzisothiazolinone-1 and -4 possessed C1125G and C1093T missense mutations in PA14_11960, respectively, which resulted in Asp375Glu and Arg365Trp substitutions. Benzisothiazolinone-2 and -3 both possessed a C917A missense mutation in *orfN* which conferred a Ala306Asp substitution. Two pelargonic acid-adapted lineages also possessed mutations in *orfN,* possessing a A1801G missense mutation resulting in a Thr601Ala substitution. Three chloroxylenol-adapted lineages possessed a A839C missense mutation in the LPS biosynthesis gene PA14_11960 which conferred a His280Pro substitution. In three lineages, paraquat selected for mutations in the methyl viologen resistance protein PA14_47640 and

transcriptional regulator PA14_47610. Paraquat-2, -3 and -4 all possessed a 12 bp inframe deletion at position 318 in PA14_47640 which conferred a Met106_Leu109 deletion. Moreover, Paraquat-2 also possessed A58C and T455C missense mutations in PA14_47610 which resulted in Ile20Val and Val152Ala substitutions. Paraquat-3 and -4 possessed C104T missense mutations which resulted in a Thr35Ile substitution and Paraquat-3 possessed an additional C350G missense mutation which conferred a Thr117Ser substitution. Three cobalt chloride adapted lineages possessed unique mutations in the putative two-component sensor PA14_31950. Cobalt chloride-1, -2 and -3 possessed T113G, T511C and G580T missense mutations in PA14_31950, respectively, which resulted in Leu38Arg, Phe171Leu and Val194Leu substitutions. Finally, adaptation to bismuth subgallate, was associated with mutations in the putative DNA polymerase Y family protein PA14_55600 and the putative two-component response regulator PA14_56950. In the DNA polymerase Y family protein PA14_55600, Bismuth subgallate-2 and -4 possessed A445C missense mutations resulting in a Met149Leu substitution. Bismuth subgallate-2 possessed an additional mutation in PA14_55600, with a A440G missense mutation conferring a Lys147Arg substitution. Bismuth subgallate-2 possessed T161A missense mutation in PA14_56950 resulting in a Leu54Gln and Bismuth subgallate-3 and -4 possessed T629C missense mutations resulting in a Leu210Pro substitution

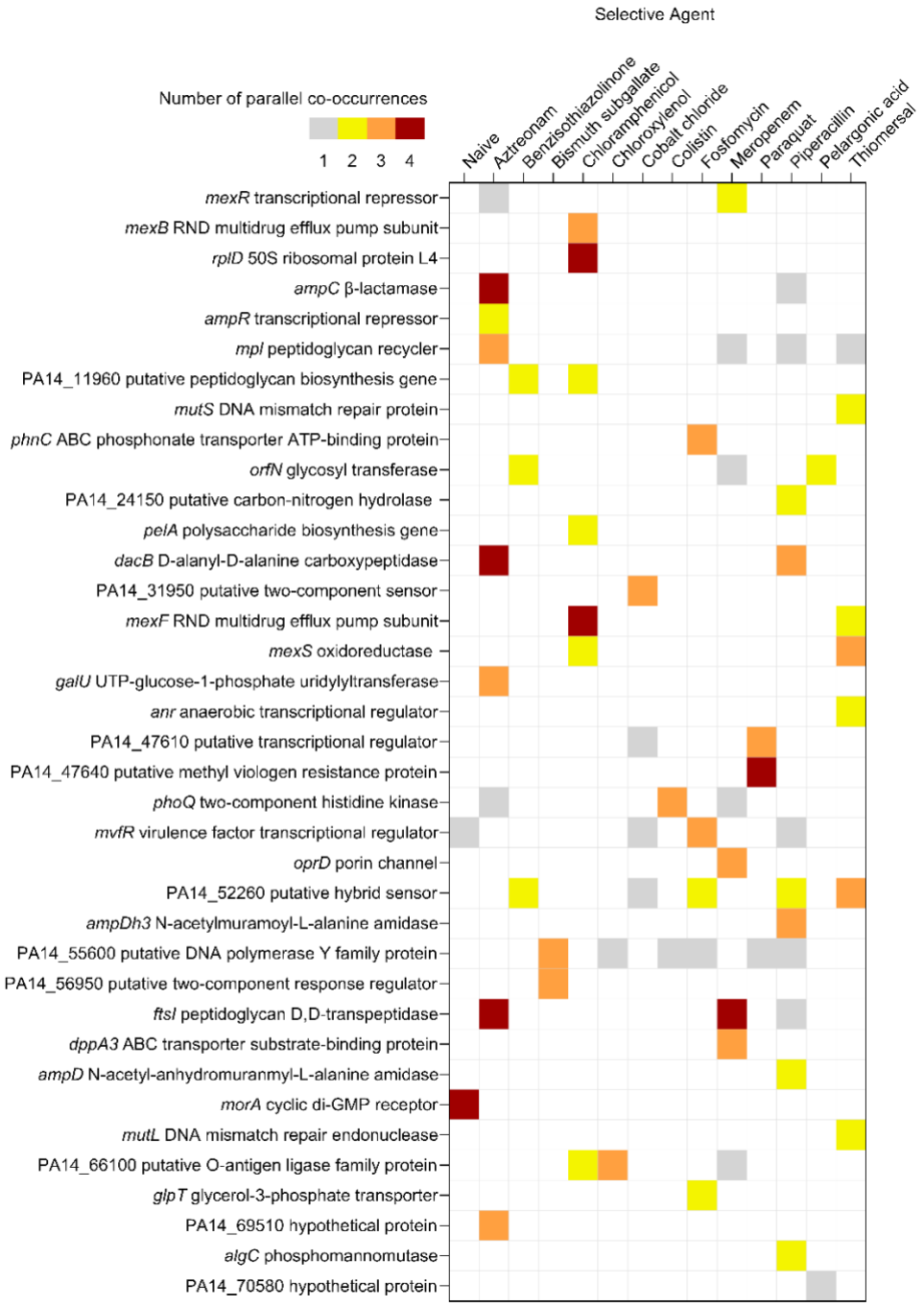

Figure 6.6. Gene targets under parallel selection in experimentally evolved biofilms of *P. aeruginosa* adapted to a panel of 48 antimicrobials in a stepwise fashion for up to 63 transfers or until growth failure. Endpoint populations which demonstrated altered susceptibility to the selective agent or antibiotics were chosen for Illumina short-read whole genome sequencing and SNPs were identified using snippy v 4.6.0. Data shown as number of times a mutation co-occurred in the four parallel lineages sequenced, n = 4.

6.3. Discussion

Consumption of non-therapeutic antimicrobials is at least an order of magnitude greater than antibiotics (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). Despite this, comparatively little is known regarding the selective potential of such agents. It has been observed that non-therapeutic antimicrobials can select for generic mechanisms of resistance which also confer reduced susceptibility to chemically unrelated compounds, including antibiotics (Wales and Davies, 2015). Therefore, exposure to non-therapeutic antimicrobials may act as a selective pressure for the development and maintenance of antibiotic resistance. Generating a cross-sectional hierarchy of the selective capacities of various classes of commonly used antimicrobials would help assess the role of non-therapeutic antimicrobials as non-canonical drivers of antibiotic resistance.

From the 48 selective conditions investigated, 21 were identified in figure 6.3. as able select for significant reductions in susceptibility to the selective agent. Moreover, 26 agents were able to select for reduced susceptibility to one or more antibiotics, half of which did not possess altered susceptibility to the selective agent in spite of this. This is in line with previous work investigating adaptation to non-therapeutic antimicrobials. The mechanisms selected during adaptation may have sub-$\log_2$ fold effects on susceptibility to non-therapeutic antimicrobials due to non-specific modes of action, instead manifesting as modest competitive fitness gains under stress (Maillard, 2018). However, these mechanisms can possess larger phenotypic effects on agents with highly defined molecular targets such as antibiotics (Russell, 2004). Ergo, adaptation to non-therapeutic antimicrobials may present with larger collaterally-selected phenotypic changes than to the selective pressure itself. Antibiotics yielded the greatest capacity for adaptation. Figure 6.1. demonstrated that antibiotic-adapted lineages were able to be passaged several $\log_2$ fold change past the MBEC and acquired changes in MICs greater than that selected by any other class of antimicrobials. Cationic surfactants were observed in figure 6.2. to be the most well tolerated of all classes of biocide, and acids, salts and phenolics were broadly poor at selecting for adaptation.

As observed in earlier chapters, antimicrobial exposure suppressed the selection of biofilm hyperproduction in almost all cases with the notable exceptions of cobalt chloride, cetylpyridinium chloride, glutaraldehyde, bismuth subgallate and chloramphenicol, as shown in figure 6.4. These lineages recapitulated selection for biofilm hyperproduction and possessed significantly greater biofilm formation than the ancestor, albeit at a slower rate than the naïve lineages. This may indicate that these lineages had exhausted the mechanism of achieving fitness gains and by virtue of

clonal interference, biofilm hyperproduction became the most selectively advantageous adaptation. However, some lineages, including β-lactam antibiotics and colistin produced less biomass than the ancestor, which may be a collateral effect of resistance mechanisms they evolved or the gross fitness defects it accrued. It was observed in figure 6.6. that all naïve lineages demonstrated parallel selection of a A3638G missense mutation resulting in a Glu1213Gly substitution in the EAL domain of *morA*. This mutation in the bifunctional diguanylate cyclase/ phosphodiesterase *morA* was also observed in Chapter 2. No other mutations were observed to be selected in parallel between naïve lineages. The naïve lineages in this experiment were passaged for over twice as many transfers as previous evolution experiments. The evolutionary parallelism at the nucleotide scale may indicate that it confers the greatest single-step fitness advantage out of all the possible hyperbiofilm-conferring mutations previously observed.

In total, lineages adapted to 20 selective agents were selected for whole genome sequencing based on altered patterns of susceptibility to the selective agent or antibiotics. From these lineages, hypotheses regarding the mechanisms of reduced susceptibility were generated for 14 agents. Of the remaining six selective agents, lineages lacked parallel SNPs selected to infer adaptive mechanisms. These lineages included pentachlorophenol, potassium sorbate, chlorocresol and methylisothiazolinone, which failed early in the experiment and manifested with modest reductions in susceptibility. It is possible that these observations were not constitutive and instead reflect induced transcriptional reorganisation carried over from experimental evolution during susceptibility testing. Nevertheless, figure 6.6. identifies highly parallel selective processes which were hypothesised to confer collaterally modulated antibiotic susceptibility and high-level resistance to non-therapeutic antimicrobials were observed in lineages adapted to the remaining agents.

6.3.1. Antibiotic Adaptation

6.3.1.1. β-lactams

Experimentally evolved lineages were adapted to the antipseudomonal β-lactams, piperacillin, meropenem and aztreonam. As described in the previous chapter, β-lactams are cell wall inhibitors which specifically bind to transpeptidase PBPs which crosslink peptidoglycan, however they differ in their affinities for PBP substrates and hydrolytic enzymes (Fernandes et al., 2013). In figure 6.5., adaptation to β-lactams was broadly associated with comparable fitness defects and suppressed selection for

biofilm hyperproduction. Reduced susceptibility to one β-lactam selected for the acquisition of cross-resistance to the other agents but differed in their patterns of susceptibility to non-β-lactams. Piperacillin selected for reduced susceptibility to ciprofloxacin, meropenem selected for collateral sensitivity to tobramycin and aztreonam did not alter susceptibility to non-β-lactam agents. Moreover, both conserved and agent-specific mutations were identified in lineages adapted to β-lactams antibiotics.

In piperacillin-adapted lineages, mutations in the N-acetylmuramyl-l-alanine amidase *ampD* or its homologue *ampDh3* were observed. AmpD regulates muropeptide processing into peptidoglycan precursors, therefore negatively regulates expression of AmpC via its muropeptide-sensing repressor AmpR (Schmidtke and Hanson, 2006). AmpD and its homologues act in concert to control AmpR and thus AmpC expression, therefore loss-of-function of the three AmpD alleles of *P. aeruginosa* results in stepwise increases in constitutive overexpression of AmpC (Juan et al., 2006). Despite this, Piperacillin-4 which possessed both *ampD* and *ampD2* mutations was not less susceptible to piperacillin although was one-$\log_2$ fold less susceptible to ceftazidime and meropenem than the other piperacillin-adapted lineages. Adaptation to aztreonam was also associated with mutations in regulators of AmpC, however they occurred exclusively in *ampR*. In the previous chapter, *ampR* mutations were also identified in ceftazidime-adapted lineages without *ampD* mutations. It has been observed that *ampD* mutations generally confer lower AmpC expression than *ampR* expression. AmpC is a cephalosporinase which readily hydrolyses penicillins. Therefore, it is possible that *ampR* mutations were required to achieve appropriate expression for drug inactivation of weaker AmpC ligands which would have incurred unnecessary fitness costs in penicillin-adapted lineages. Aztreonam is a weak substrate of AmpC and all lineages possessed a Val239Ala substitution in the omega-loop of the AmpC β-lactamase. This substitution is within the enzyme active site and has been observed previously to confer decreased susceptibility to aztreonam in experimentally evolved mutants and clinical isolates of P. *aeruginosa* (McLean et al., 2019). Adaptation to piperacillin and aztreonam was associated with selection of mutations in the AmpC regulator *dacB* which was also observed in ceftazidime-adapted lineages in the previous chapter. DacB is a peptidase involved in resolving stalled peptidoglycan synthesis through D-ala-D-ala-carboxy and -endopeptidase reactions. Its muropeptide substrates are ligands for AmpR. Therefore, DacB inactivation induces repression of AmpR and overexpression of AmpC (Cabot et al., 2018). Similarly, three aztreonam-adapted lineages possessed mutations in the peptidoglycan recycler *mpl* which

reutilises intact muropeptides by linking them to UDP-N-acetylmuramate. Therefore, its loss-of-function also contributes to repression of AmpR and overexpression of AmpC. Three aztreonam-adapted lineages possessed mutations in the UTP-glucose-1-phosphate uridylyltransferase *galU* required for LPS core biosynthesis. Transposon mutants of GalU has been shown to possess reduced β-lactam susceptibility in *P. aeruginosa* however it is not known how *galU* mutations achieve this (Dötsch et al., 2009, Alvarez-Ortega et al., 2010). Kurushima et al. (2021) observed that *galU*-deficient *Enterococcus faecalis* possessed abnormal cell morphology with impaired polarity during replication. This led to them hypothesising that this prohibited the accumulation of peptidoglycan for cell division which deprived the ability for β-lactam molecules to exert a bactericidal effect through osmotic pressure. However, this work appears to be the first report of *galU* mutations selected by a β-lactam.

All aztreonam- and meropenem-adapted lineages possessed mutations in the *ftsI* transpeptidase. FtsI, also known as PbpB, is a PBP and the main target for inhibition of monobactams and carbapenems, excluding imipenem (Clark et al., 2019). Despite their importance in the antimicrobial activity of β-lactam, mutations in PBPs are generally considered a minor mechanism of resistance in resistance in Gram-negative organisms due to the fitness defects they confer (Zapun et al., 2008). Despite this, observations of mutations in clinical isolates of *P. aeruginosa* have been made which conferred resistance to meropenem and aztreonam (Liao and Hancock, 1997, Clark et al., 2019). The Arg504Cys substitution selected in Meropenem-1 and -2 was the most common mutant *ftsI* polymorphism identified by Clark et al. (2019) in β-lactam-resistant *P. aeruginosa* isolated from the cystic fibrosis lung. However, the remaining mutations identified here appear to be novel, including meropenem-selected Val465Lue, Val465Ala and Val523Gly substitutions and aztreonam-selected Asn242Ile and Ala246Val substitutions. Other than *ftsI*, the repertoire of mutations possessed by meropenem-adapted lineages was more distinct than the other β-lactams investigated, attributable to its low affinity for AmpC and reliance on porin channels to enter cells. Indeed meropenem-adapted lineages possessed mutations in the porin *oprD* including two mutations resulting in truncation from the carbapenem-binding site in loop 8 onwards (Li et al., 2012). Furthermore, an inframe deletion was detected in loop 9, the loss of which Li et al. (2012) has also been observed to confer reduced carbapenem susceptibility. Moreover, three meropenem-adapted lineages possessed mutation in *dppA3*, a paralogue of the *dppA* component of the DppABCDF ABC transporter involved in the uptake of oligopeptides (Lee et al., 2018). Loss of its homologue OppA in *Enterobacteriaceae* has been implicated in aminoglycoside resistance (Acosta et al.,

2000, Ge et al., 2018). However, meropenem selected for collateral sensitivity to tobramycin. Despite this, loss of *dppA3* would not abolish expression of the Dpp system due to the remaining DppA paralogues. In fact, the Dpp paralogues possess different substrate specificities and if DppA3 possesses lower affinity for tobramycin, its inactivation could increase aminoglycoside import by virtue of increased representation of the DppA allele in the Dpp system (Pletzer et al., 2014). This may contribute to the observed collateral sensitivity to tobramycin. However, why this mutation would be selected by meropenem remains unclear. Whether DppA3 is a meropenem importer requires further investigation.

6.3.1.2. Fosfomycin

Fosfomycin is a cell wall synthesis inhibitor which possesses a distinct mode of action that acts at an earlier step in peptidoglycan biosynthesis than transpeptidation (Michalopoulos et al., 2011). Fosfomycin possesses homology to organic phosphonate compounds and enters the periplasm through the catabolically repressed glycerophosphate permease GlpT. A second phosphonate transporter, the hexose-monophosphate permease UhpT, which is present in *Enterobacteriaceae,* is absent in *P. aeruginosa* (Castañeda-García et al., 2009). Fosfomycin inhibits the peptidoglycan biosynthesis gene MurA which catalyses the formation of N-acetylmuramic acid, thus arresting cell wall synthesis. Fosfomycin is seldom used therapeutically in humans, however it has seen increasing clinical interest particularly for the treatment of MDR Gram-negative pathogens causative of upper UTIs (Falagas et al., 2008).

In this investigation, lineages adapted to fosfomycin became seven to eight-$\log_2$ fold less susceptible to the selective agent than the ancestor. Despite this, altered susceptibility to any other antibiotic was not observed indicating that fosfomycin shows promise for limiting the selection of cross-resistance. Two fosfomycin-adapted lineages possessed mutations in the phosphonate transporter *glpT* resulting in an early stop codon at Ser313, truncating α-helices 9 to 12, and an Ile74_Ala75 inframe deletion within helix 2. The fosfomycin-binding site of GlpT in *P. aeruginosa* is not known however, loss-of-function of GlpT is a well-characterised mechanism of fosfomycin resistance (Castañeda-García et al., 2013). Three lineages also possessed mutations in the ABC phosphonate transporter subunit *phnC,* The Phn phosphonate uptake and catabolism system has been implicated in reduced susceptibility to fosfomycin in transposon mutants of *E. coli* (Turner et al., 2020). Turner et al. (2020) suggested that selection for loss of the Phn system could indicate it is a route for fosfomycin entry.

Moreover, at subinhibitory concentrations, inactivation of the *phnCDE* transporter was selected by fosfomycin, but the remaining operon was preserved, which suggests that Phn system could contribute to the degradation of fosfomycin. Three lineages also possessed mutations in the repressor of the Pqs quorum sensing circuit *mvfR*. Fosfomycin exposure has been shown to activate of the Pqs system to induce swarming motility and the oxidative stress response (Bru et al., 2019). Therefore, the mutations in *mvfR* likely act to constitutively activate Pqs. How this specifically contributes to fosfomycin resistance without inducing cross-resistance is not known, however the selective parallelism observed suggests that it possesses an important role.

6.3.1.3. Chloramphenicol

Chloramphenicol is a bacteriostatic protein synthesis inhibitor which prevents polypeptide elongation by binding to the 23S rRNA of the 50S ribosomal subunit (Schwarz et al., 2004). Chloramphenicol is not frequently indicated for *P. aeruginosa* infection due to inferior activity, however *P. aeruginosa* can encounter chloramphenicol as a common contaminant of medicated eye drops (Bourkiza et al., 2013). Adaptation to chloramphenicol was associated with a seven- to eight-$\log_2$ fold reduction in chloramphenicol susceptibility, cross-resistance to ciprofloxacin and collateral sensitivity to piperacillin and aztreonam. In all experimentally evolved lineages, a target site mutation in the 50S ribosomal protein *rplD* at the Lys62 residue was selected. Moreover, mutations in the *mexB* and the *mexF* efflux pump subunits were observed across the chloramphenicol-adapted lineages. Chloramphenicol is a ligand for both the MexAB-OprM and MexEF-OprN pumps (Fetar et al., 2011, Li et al., 1995). Three lineages possessed substitutions at the Val636 residue and one lineage at Asp87 in MexF, in addition to Gly179Ser, Val985Ile substitutions and a Val591 deletion in MexB. It is possible that the MexF substitutions act to increase chloramphenicol binding. However, it is unlikely the diverse mutations seen in *mexB* also fulfil this role. Reduced of MexAB-OprM expression and collateral sensitivity to β-lactams is a common observation of MexEF-OprN mutants (Uwate et al., 2013). The transcriptional activator of MexEF-OprN, MexT also represses expression of MexAB-OprM through an unknown mechanism (Horna et al., 2018). The relative importance of MexEF-OprN in chloramphenicol adaptation is highlighted by mutations observed in the regulator *mexS* in three lineages. MexS is the divergently transcribed repressor of MexT and inactivation of MexS is the main mechanism by which constitutive overexpression of MexEF-OprN occurs (Richardot et al., 2016). However, why loss-of-function MexAB-

OprM was selected in MexEF-OprN mutants remains unclear. Horna et al. (2018) suggested that the antagonistic relationship between MexEF-OprN and MexAB-OprM may be bidirectional. Therefore, loss of MexAB-OprM may increase MexEF-OprN expression, however this remains hypothetical. Chloramphenicol-adapted lineages demonstrated significantly increased biofilm formation relative to the ancestor. Despite this, no mutations in the c-di-GMP signalling network or other known biofilm regulators were identified. Therefore, biofilm hyperproduction in these strains is likely a collateral result of the selected resistance mechanisms. Indeed, it is well known than expression of multidrug efflux pumps possesses phenotypic effects on biofilm formation (Baugh et al., 2014). Moreover, reduced expression of MexEF-OprN has been observed to increase virulence and motility, therefore it is feasible that overexpression of MexEF-OprN may promote biofilm formation (Vaillancourt et al., 2021). The effect of efflux pump mutations on biofilm formation should be investigated further.

6.3.2. Biocide Adaptation Conferring High-Level Resistance

Paraquat is a redox-active viologen and one of the common herbicides used in agriculture for the control of perennial weeds (Huang et al., 2019). Its use is banned in the European Union and in liquid form in China but is still widely used elsewhere in the world. It is not used as an antimicrobial, but by virtue of its oxidative mode of action, exerts an off-target bactericidal effect on bacteria. In this investigation, *P. aeruginosa* acquired reduced susceptibility to paraquat comparable in $\log_2$ fold change to resistance to antibiotics. One lineage was unable to adapted past one-$\log_2$ fold past the ancestral MBEC, however the remaining three lineages tolerated concentrations up to 2.5-$\log_2$ fold past the MBEC and eight-$\log_2$ fold past the ancestral MIC. This resulted in selection for a six- to eight-$\log_2$ fold reduction in susceptibility to the selective agent, without change in antibiotic susceptibility. These lineages possessed mutations in PA14_47640, an MFS transporter with homology to the SmvA methyl viologen resistance protein in *Enterobacteriaceae.* SmvA is involved in export of cationic molecules including paraquat, QACs and the DNA-intercalating dye acriflavine (Wand et al., 2019). Paraquat-2, -3 and -4 all possessed a Met106_Leu109 inframe deletion in PA14_47640, the role this has is unknown, although the paraquat-adapted lineages possess susceptibility far lower than is observed from de-repression of SmvA in *Salmonella* sv. Typhimurium (Santiviago et al., 2002). Therefore, this polymorphism may assist with paraquat export. Moreover, the paraquat-adapted lineages also possessed mutations in PA14_47610, a putative transcriptional regulator immediately upstream of PA14_47640. It is known that in *P. aeruginosa* PAO1, PA1283-PA1282

has been suggested to be the *smvAR* paralogue (Bock et al., 2021). Therefore in *P. aeruginosa* PA14, *smvAR* is likely the divergently transcribed PA14_47640-PA14_47610 operon. Whilst previously inferred by homology, this work offers the first experimental evidence that PA14_47640-PA14_47610 are both involved in reduced paraquat susceptibility in *P. aeruginosa*.

In addition to paraquat selecting for large reductions in susceptibility to itself, the internal deodorant bismuth subgallate was able to as well. Bismuth subgallate is primarily used to treat malodour after gastrointestinal surgery, however it also elicits antimicrobial effects by disrupting essential respiratory activities (Han et al., 2018). Adaptation of *P. aeruginosa* to bismuth subgallate was similarly not associated with altered drug susceptibility but resulted in a three- to four-$\log_2$ fold reduction in susceptibility to the selective agent. Mutations in the hypothetical proteins PA14_55600 and PA14_ 56950 were observed in bismuth subgallate-adapted lineages. PA14_ 56950 is a hypothesised two-component response regulator which shares homology with the *colR* gene of *P. aeruginosa* PAO1 (Vitale et al., 2020). The ColRS two-component system is involved in regulation of the lipid A charge modifier PhoPQ and *colR* mutations have been shown to confer reduced polymyxin susceptibility (Gutu et al., 2013). Despite this, bismuth subgallate-adapted strains did not possess altered colistin susceptibility and the selective advantage of PhoPQ activation is not known as bismuth subgallate possesses a net neutral charge. A second mutation was identified in PA14_55600, a hypothesised DNA repair protein in an operon with the error-prone DNA polymerase *dnaE2*. Loss-of-function of PA14_55600 may result in an increase in the mutation supply rate to accelerate adaptation. Why mutation rate modifiers are selected with parallelism is dependent on the fitness landscape, as described in Chapter 5., the properties of which remain broadly unknown. Therefore, further investigation is required to substantiate the selective role of these mutations in reduced susceptibility to bismuth subgallate.

6.3.3. Biocide Adaptation Conferring Cross-Resistance to Antibiotics

6.3.3.1. Thiomersal

Thiomersal is an antimicrobial preservative extensively used in the pharmaceutical industry. Adaptation to thiomersal was associated with reduced susceptibility to ciprofloxacin and β-lactams excluding aztreonam and lineages were also three-$\log_2$ fold less susceptibility to the selective agent. Two lineages possessed mutations in the *mutS* or *mutL* DNA mismatch repair proteins, however there was no difference in

susceptibility between the thiomersal-adapted hypermutators and non-mutator lineages. The observation that hypermutators could be selected by non-therapeutic antimicrobials indicates that they may be more proficient at developing antibiotic resistance at a later date (Kurenbach et al., 2018). Moreover, both hypermutators possessed mutations in *mexS,* the repressor of the MexEF-OprN transcriptional activator MexT. Despite this, unlike chloramphenicol which also selected for *mexS* mutants, increased susceptibility to β-lactams was not observed, indicating that MexAB-OprM inactivation was the main determinant of collateral sensitivity in chloramphenicol-adapted lineages.

6.3.3.2. Pelargonic Acid, Benzisothiazolinone and Chloroxylenol

Pelargonic acid is a common domestic herbicide, it is not an antimicrobial but nevertheless possesses off-target antimicrobial activity. Adaptation to pelargonic acid was associated with a three-log_2 fold reduction in tobramycin susceptibility. Moreover, benzisothiazolinone is an antimicrobial preservative used commonly in consumer goods and adaptation was associated with a two- to three-log_2 fold reduction in tobramycin and piperacillin susceptibility. There are very little known structural or mechanistic similarities between pelargonic acid and benzisothiazolinone, however both agents selected for mutations in the glycosyl transferase *orfN.* Mutations in *orfN* have been previously implicated in tobramycin resistance via a mechanism of decreased tobramycin binding due to loss of LPS O-antigen glycosylation (Scribner et al., 2020). Benzisothiazolinone also selected for mutations in the putative peptidoglycan biosynthesis gene PA14_11960. The characterisation of PA14_11960 conducted by Yang et al. (2021) suggested that it was a paralogue of the peptidoglycan recycler Mpl under regulatory control from the PhoPQ two-component system. If this is the case, loss-of-function of this Mpl paralogue may lead to low-level de-repression of AmpC which would confer the reduction in piperacillin susceptibility observed. Supporting this, the phenolic biocide chloroxylenol also selected for mutations in PA14_11960, and also possessed reduced susceptibility to piperacillin. However, the chloroxylenol-adapted lineages also possessed reduced susceptibility to ciprofloxacin and meropenem which would not be conferred if AmpC de-repression was the sole mechanism of resistance. Moreover, the selective advantage loss-of-function of PA14_11960 would confer against chloroxylenol and benzisothiazolinone is not known. Therefore, it seems likely that PA14_11960 possesses additional mechanistic roles which require further investigation.

6.3.3.3. Cobalt Chloride

Cobalt chloride is a toxic metal salt associated with contaminated agricultural soil from historical fertilisers, industrial effluent and quarrying activities (Mahey et al., 2020). Its antimicrobial activity has not been investigated; however, it is presumed like most heavy metal compounds, that it exerts a bactericidal effect through a strong affinity for thiol groups resulting in protein denaturation (Yazdankhah et al., 2018). Lineages adapted to cobalt chloride did not possess altered susceptibility to selective agent. Nevertheless, Cobalt chloride-1, -2 and -3 possessed a three-$\log_2$ fold reduction in susceptibility to meropenem exclusively. These lineages also possessed mutations in the heavy metal histidine kinase PA14_31950, the homologue of *czcS* from *P. aeruginosa* PAO1 (Elmassry et al., 2019). CzcS is in a two-component system with CzcR and is able to sense copper, cobalt, zinc and cadmium to activate a signal transduction cascade involved in metal detoxification via expression of the CzcCBA efflux pump (Wang et al., 2017). It has also been observed that CzcRS negatively regulates OprD. Therefore, constitutive activation of the response regulator PA14_31960 would lead to constitutive repression of OprD to confer reduced meropenem susceptibility (Perron et al., 2004). Indeed, clinical isolates of *P. aeruginosa* possessing decreased carbapenem susceptibility due to mutations in *czcS* have been observed (Fournier et al., 2013). Notably however, lineages were also adapted to zinc and copper sulfate in this experiment, also ligands of CzcRS, but altered carbapenem susceptibility was not selected. This may indicate that CzcRS possesses a stronger affinity for cobalt than zinc or copper.

Conclusions

This work demonstrates that there a complex selective landscape associated with adaptation to antimicrobials. Collateral selective effects on biofilm formation, fitness and susceptibility to chemically unrelated agents are common. Though cross-resistance was widespread, it generally manifested below clinical breakpoints. Nevertheless, the capacity for cross-resistance should be an important consideration when utilising antimicrobials as even small reductions in susceptibility can confer large fitness gains under appropriate selection. Therefore, cross-resistance may act as a foundation to acquire stepwise decreases in susceptibility to high-level resistance. Antibiotics were most proficient at selecting for resistance, including cross-resistance, which may diminish the effectiveness of resistance-curtailing initiatives such as

antibiotic stewardship. However, fosfomycin was a rare example of an antibiotic which did not select for cross-resistance to the antipseudomonal drugs investigated here.

Non-therapeutic antimicrobials were also able to broadly select for reduced antibiotic susceptibility indicating that the widespread use of these agents may act as a driver of clinically-relevant drug resistance. This was achieved this through a variety of mechanisms including constitutive expression of multidrug efflux, hypermutation, cell wall remodelling and porin loss. Furthermore, this often manifested without significant changes in susceptibility to the selective agent. Therefore, when developing antimicrobial compounds, it would be prudent to consider their collateral effects on antibiotics, even if they appear selectively neutral to themselves.

CHAPTER 7. GENERAL DISCUSSION

7.1. Experimental evolution is a powerful platform for understanding adaptation to selective pressures in biofilms

This work has presented a comprehensively validated experimental evolution model for studying the evolution of antimicrobial resistance in biofilms which offers some evident advantages over existing platforms. By cultivating biofilms on beads and serially passaging them under a selective pressure, biofilms must resolve adaptation with completing the biofilm lifecycle in order to be selected. Adaptation of biofilms to antimicrobials imparts a selective pressure not frequently accounted for in studies investigating the evolution of antimicrobial resistance. This may offer novel insights into the selective dynamics of antimicrobial resistance to translate findings from laboratory evolution to real-world environments.

In this thesis, experimental evolution was used as a vehicle to study the molecular mechanisms which underpin selection of biofilm hyperproduction and antimicrobial resistance in biofilms. Biofilms were grown and adapted on bead substrates of glass, PVC and type-316 stainless steel, materials frequently encountered as objects and surfaces in clinical and industrial environments. It was envisioned that adaptation of biofilms to these surfaces would offer a degree of representation of adaptation to conditions where biofilms are frequently encountered in the real world. It was demonstrated in figure 3.1. that biofilms can be cultivated on relevant bead substrates at the order of 10^5 to 10^6 cells/ mm^2. Such high productivities are essential to achieving a mutational supply rate for rapid evolutionary change. Biofilm productivities on beads in this experimental system were most productive at lower temperatures and shorter incubation periods, irrespective of culture medium. However, a medium-specific capacity to select for biofilm hyperproduction was observed in figure 3.3. Some media such as LB without salt and M9 minimal medium selected for increases in biofilm formation in planktonically-adapted lineages, hypothesised to be a result of pellicle formation. Furthermore, media which intrinsically sustained strong biomass production, such as brain-heart infusion and Mueller-Hinton broth generally demonstrated limited scope to select for further increases in biofilm formation. Therefore, only LB was able to successfully select for biofilm hyperproduction exclusively in biofilm adapted lineages allowing the impact of changed biofilm competency to be studied. Using this model, biofilms can be passaged at inhibitory concentrations of many mechanistically diverse antimicrobials to select for constitutive resistance dependent on exposure regime. Progressively increasing stress from subinhibitory to superinhibitory concentrations was more successful at selecting for decreased susceptibility to antimicrobials than a static sublethal stress as observed in table 3.1.

This work contributes to resolving a systemic limitation in experimental evolution which is an overreliance on homogenous planktonic culture with poor representation of nature to investigate adaptation to selective pressures (MacLean and Millan, 2019). It has demonstrated that the experimental evolution of biofilms is a viable model to investigate adaptation to a number of selective pressures and greatly expands the potential throughput of lineages evolved in parallel relative to previous work. The versatility of the biofilm evolution model enables its use to study a number of adaptive processes in addition to antimicrobial resistance, such as virulence, competition and horizontal gene transfer in biofilms. Consequently, further experiments using this model should be designed to continue to incrementally increase representation of experimental microcosms to real world conditions. In this regard, the monospecies biofilm cultivated in a defined medium in an HGT-deficient environment used in this investigation lacks the dynamism associated with real-world selective pressures. The nutritional environment, standing genetic diversity and capacity for recombination can fundamentally alter evolutionary trajectories (Cooper, 2018). Therefore, future work should attempt to incorporate these elements to increase the representation of a specific environment. A possible area which could be highly productive for experimental evolution would be to investigate evolution on indwelling medical devices prone to contamination. Biofilm growth on urinary, pleural or central venous catheters are a significant cause of morbidity and mortality. Using the catheter lumen as a substrate and anatomical fluid, such as urine, as the propagation medium would provide a highly clinically-representative environment to study evolution *in vitro.* Therefore, this work substantiates the use of experimentally evolution to investigate adaptation to selective pressures in biofilms and provide a foundation through which to design future experiments which simulate evolution in relevant natural conditions.

7.2. Mutations in the c-di-GMP signalling pathway confer biofilm hyperproduction which is associated with phenotypic diversification

Figure 4.1. demonstrates that experimentally evolved biofilm hyperproducers could be selected by serial passage on bead substrates. They were phenotypically characterised by a 3-fold increase in biomass production, salt tolerance and alkaline hypersusceptibility but possessed no change in cellular productivity, relative fitness and antibiotic susceptibility. The evolutionary trajectories to biofilm hyperproduction were largely independent of selective substrate and selected altered c-di-GMP synthesis. Figure 4.10. shows most common targets of selection were the DipA phosphodiesterase, components of the YfiBNR signalling complex and MorA

bifunctional diguanylate cyclase/phosphodiesterase. Such mutations act to inhibit c-di-GMP hydrolysis, constitutively increasing the intracellular c-di-GMP reservoir to support biofilm formation. However, a subset of lineages also counterintuitively possessed mutations in the Wsp and Gac/Rsm c-di-GMP signalling components which activate c-di-GMP synthesis. This may indicate that in this selective environment, which requires completion of the biofilm lifestyle from adhesion to dispersal, c-di-GMP concentrations are optimised to support a sessile lifestyle with the prerequisite of dispersal competency. Furthermore, biofilm hyperproduction was associated with the emergence of self-generated diversity characterised in figure 4.6. by the formation of complex, structured colony morphologies. Five distinct morphotypic variants were observed based on their patterns of increased rugosity and impaired capacity to invade the agar surface through twitching motility described in figures 4.8. and 4.9. Lineages did not commit to a specific morphotype as a result of biofilm hyperproduction, instead they resulted in rapid switching between a 'library' of morphotypes which arose in parallel lineages independently, as shown in figure 4.7. The molecular basis of morphotypic diversity could not be identified from the sequencing effort, perhaps as dictated by the pleiotropic, genomic background-dependent transcriptional effects of c-di-GMP signalling.

This work challenges the dogma that antimicrobial resistance is an intrinsic property of biofilm-adapted strains, however, it does provide further rationale for the role of biofilms in surviving environmental stress in specific conditions. Moreover, this study provides the most comprehensive evidence to date for the role of the c-di-GMP signalling network as the main nidus of selection for the adaptation of *P. aeruginosa* to a sessile lifestyle. This supports current molecular and biochemical understanding of regulation of the motile-sessile switch in an evolutionary context. Furthermore, this work also corroborates observations that biofilm adaptation is associated with phenotypic diversification (Poltak and Cooper, 2011). However, this phenomenon appears to be more complex than previously understood as biofilm adaptation is not associated just with morphological complexity, but hyperproducers appear to enter a morphotypically hypervariable state.

Understanding the mechanisms by which bacteria adapt to form biofilms can offer novel insights into the rational design of antibiofilm measures including selectively-neutral surfaces and antimicrobials. Genes in the c-di-GMP signalling network are highly pleiotropic regulators of up to hundreds of other genes. In order to identify the specific determinants that facilitate biofilm hyperproduction, mechanistic confirmation of the role of these mutations, followed by comprehensive transcriptomic analysis is

required. This would also help elucidate mechanistic differences between morphotypes. Similarly, differences in biofilm formation and relative fitness were not identified between morphotypes, those these traits were assayed out of context in a microtiter tray assay. Competition assays between morphotypes and the ancestor should be performed on beads in order to gain a better understanding of the niches in which the morphotypes reside and if morphotypes have any specific role in the community. Therefore, this work contributes to understanding the selective pressures inherent to biofilm formation with future work aimed at elucidating specific mechanisms and evolutionary roles of within-population variation and diversification.

7.3. Biofilm hyperproduction is not selected during adaptation to antibiotics but the biofilm lifestyle modulates evolutionary trajectories to antibiotic resistance

Figure 5.2. demonstrates that adaptation to the mechanistically diverse antibiotics, ciprofloxacin, ceftazidime and tobramycin in a stepwise manner constrained selection for biofilm hyperproduction in even when adapted as a biofilm. Stepwise adaptation to drugs was associated with successive decreases in susceptibility to the selective agent and collaterally modulated susceptibility to other classes of antimicrobial in a lifestyle-specific manner, as shown in figure 5.1. Moreover, figure 5.3. reveals successive decreases in relative fitness associated with the acquisition of resistance observed in both lifestyles, the cost of which manifested in an agent-dependent manner. Tobramycin was associated with the most punitive fitness defects whereas ciprofloxacin selected for the mildest, and these fitness costs were generally greater in biofilms than planktonic lineages. Biofilm lineages adapted to ciprofloxacin possessed greater decreases in susceptibility to β-lactams and ciprofloxacin than planktonic lineages. However, the opposite was the case in tobramycin-adapted lineages. Adaptation to ceftazidime was associated with broad reductions in β-lactam resistance in both lifestyles, however collateral sensitivity was observed exclusively in planktonically. Conversely, ciprofloxacin-adapted planktonic lineages demonstrated decreased susceptibility to colistin. Decreased susceptibility to non-selective agents rarely surpassed clinical breakpoints, nevertheless this work indicates that acquisition of resistance may prime strains for development of further resistance to functionally independent antibiotics, particularly β-lactams.

The mechanistic basis for these phenotypic observations were revealed in figures 5.5, 5.6 and 5.7. Irrespective of lifestyle, each antibiotic selected for conserved ‘driver’ mutations which were observed across most, if not all, parallel lineages and likely

facilitated the large reductions in susceptibility observed to the selective agent. The genes with which mutations were *gyrA, ampR* and *fusA* in ciprofloxacin-, ceftazidime- and tobramycin-adapted lineages, respectively. Mechanistic differences identified between lifestyles generally manifested in the patterns of secondary mutations. This included genes which are known to contribute to antimicrobial resistance, such as multidrug efflux and porins, in addition to ones known to contribute to biofilm formation, including type IV pili biogenesis and alginate production. In ciprofloxacin-adapted biofilm lineages, all lineages were hypothesised to become MexAB-OprM by virtue of mutations in the repressors *mexR* and *nalC*. Moreover, two lineages of ciprofloxacin-adapted biofilms were hypothesised to also acquire de-repressed MexCD-OprJ expression by virtue of mutations in *nfxB*. In contrast, mutations in MexAB-OprM regulators were entirely absent in planktonic lineages, which instead all possessed mutations in *nfxB*. Moreover ciprofloxacin-adapted planktonic lineages were also hypothesised to possess impaired type IV pili biogenesis through mutations in the *pilB/C/F/M/N/Q* which were not observed in naïve planktonic lineages.

Ceftazidime-adapted biofilm lineages possessed mutation in the transpeptidases *mpl* and *dacB*, the inactivation of which has been shown to increase *ampR* expression, which were not present in planktonic lineages. However, all planktonic lineages possessed inactivation of the alginate biosynthesis gene *algC* which is transcribed divergently of the rest of the *alg* operon. This was not observed in any biofilm lineage and was hypothesised to repress alginate biosynthesis maladaptive in a planktonic lifestyle, whilst maintaining the detoxification benefits of the wider drug-induced AlgU regulon. In addition to *ampR* mutations ceftazidime-adapted lineages of both lifestyles were also characterised acquisition of *mutL* mutation-dependent hypermutator phenotypes. Whilst the selective advantage of hypermutators in a rapidly changing fitness landscape such as stepwise adaptation to drugs has been documented, their disproportionate selection by ceftazidime relative to other drugs is less clear (Oliver et al., 2000). Moreover, hypermutation resulted in the acquisition significantly more SNPs than the naïve lineages by the end of the some of which cryptically demonstrated parallel selection. This included the leucine tRNA-ligase leuC and purine biosynthesis gene *purL* in planktonic lineages and PA14_RS01115 coenzyme A subunit, *ptsP* phosphotransferase, *tle1* lipase, PA14_RS13660 thioesterase, *bkdA2* ketoacid dehydrogenase, PA14_RS16205 sterol desaturase and *thrB* homoserine kinase. It is possible that these genes involved in respiration and metabolism represent lifestyle-specific niche adaptation independent of ceftazidime susceptibility. As the ceftazidime-adapted lineages became hypermutators, it is possible that these genes are also under

selection in naïve lineages but have not had sufficient mutational supply to occur and be selected. Therefore, these genes require further investigation to characterise their role in ceftazidime and lifestyle-specific niche adaptation.

In addition to *fusA*, adaptation of biofilms tobramycin only resulted in selection of the bifunctional diguanylate cyclase/ phosphodiesterase *morA*, however, did not result in increased biofilm formation. In contrast, tobramycin-adapted planktonic lineages possessed mutations in the efflux and lipid biosynthesis regulators *parR* and *amgS* which were hypothesised to result in de-repression of the MexXY-OprM efflux pump and activation of PmrAB and PhoPQ lipid A charge modifiers.

Exposing evolved lineages to static stress representing the maximum concentration to which they were previously exposed for an additional ten transfers resulted in broadly conserved further reductions in susceptibility to the selective agent but contraction of collaterally-selected drug resistance to non-selective agents, as shown in figure 5.10. Given this specialisation towards reduced susceptibility to the selective agent, it was anticipated that static re-adaptation would also result in selection of compensatory mutants for compromised fitness and biofilm formation. However, figures 5.11. and 5.12. suggested that this was not the case, instead static re-adaptation resulted in highly divergent, drug-, lifestyle- and concentration-dependent responses on fitness and biofilm formation which requires further mechanistic investigation.

This work demonstrates that drug resistance is not inherent to biofilm hyperproduction and biofilm hyperproduction is not selected by drug exposure. However, in spite of this evolutionary trajectories to resistance are heavily influenced by the biofilm lifestyle. Whilst both lifestyles typically selected for the same ‘driver’ mutation, there was lifestyle-specific secondary mutations which resulted in altered patterns of cross-resistance and fitness costs. This included a different repertoire of de-repressed efflux pumps and in planktonic lineages, the loss-of-function of pathways essential biofilm formation including type IV pili and alginate biosynthesis. Therefore, this provides important insights into the importance of bacterial lifestyle in defining evolutionary trajectories towards resistance. Future work should focus on confirming the hypothesised roles of these candidate genes in evolution of drug resistance, most notably the role loss of type IV pili biogenesis and *algC* mutations in ciprofloxacin and ceftazidime resistance, respectively. Furthermore, why DNA mismatch repair mutations are readily selected by ceftazidime adaptation, and the cryptic mutations demonstrating parallelism that hypermutation elicited, should also be investigated. Moreover, such mechanistic differences were selected by a stepwise exposure regime which ‘trains’ lineages to become resistant, therefore these observations require corroboration in a

selective regime representative of real-world exposure conditions. Nevertheless, this work demonstrates the important role of lifestyle in defining the selective landscape for adaptation to antibiotics with contrasting modes of action.

7.4. The collateral selective landscape of non-therapeutic antimicrobials on antibiotic susceptibility is highly diverse.

Figures 6.3., 6.4. and 6.5. reveal a complex selective landscape associated with adaptation to antimicrobials, with extensive collateral effects on biofilm formation, fitness and susceptibility to chemically unrelated agents. Antibiotics were most proficient at selecting for resistance, including cross-resistance, which may diminish the effectiveness of resistance-curtailing initiatives such as antibiotic stewardship. Alternatively, knowledge regarding conserved patterns of collateral sensitivity could be exploited to make better evidence-based decisions on antimicrobial utilisation. Chloramphenicol selected for cross-resistance to ciprofloxacin but was associated with collateral sensitivity to β-lactams. Chloramphenicol-adapted lineages possessed mutations in the 50S ribosomal protein *rplD* in addition to overexpression of MexEF-OprN and inactivation of MexAB-OprM which was hypothesised to support MexEF expression. Selection for resistance to one β-lactam agent also selected for resistance to the others. Piperacillin and aztreonam selected for de-repressed AmpC activity via several mechanisms involving the regulators AmpR, AmpD(h3), Mpl and DacB. Aztreonam and meropenem possessed mutations in the transpeptidase FtsI. Meropenem was exclusively associated with loss of OprD and mutations in the oligopeptide transporter DppA3, which was hypothesised to confer collateral sensitivity to tobramycin. Aztreonam was also exclusively associated with mutations in the LPS core antigen biosynthesis gene GalU.

Non-therapeutic antimicrobials were also able to broadly select for reduced antibiotic susceptibility indicating that the widespread use of these agents may act as a driver of clinically-relevant drug resistance. This was achieved this through a variety of mechanisms including overexpression of multidrug efflux, hypermutation, cell wall remodelling and porin loss, as shown in figure 6.5. The pharmaceutical preservative thiomersal selected for cross-resistance to ciprofloxacin and β-lactams via constitutive de-repression of the MexEF-OprN efflux pump. The biocides pelargonic acid, benzisothiazolinone and chloroxylenol selected for cross-resistance to tobramycin and piperacillin hypothesised to be a result of mutations in the LPS glycosylase *orfN* which impaired aminoglycoside uptake and in a putative *mpl* paralogue to activate AmpC.

Moreover, cobalt chloride selected for carbapenem resistance due to repression of *oprD* via constitutive activation of the CzcRS system. Therefore, there is a diverse repertoire of resistance mechanisms which confer collateral effects on susceptibility of unrelated agents. Though cross-resistance was widespread, it generally manifested below clinical breakpoints. Nevertheless, the capacity for cross-resistance should be an important consideration when utilising antimicrobials as even small reductions in susceptibility can confer large fitness gains under appropriate selection. Therefore, cross-resistance may act as a foundation to acquire stepwise decreases in susceptibility to high-level resistance. Furthermore, this often manifested without significant changes in susceptibility to the selective agent. Therefore, when developing antimicrobial compounds, it would be prudent to consider their collateral effects on antibiotics, even if they appear selectively neutral to themselves. Cross-resistance was not unilaterally observed however, fosfomycin was a rare example of an antibiotic which did not select for cross-resistance to the antipseudomonal drugs investigated here. The mechanisms by which fosfomycin resistance was attained was via mutations in the phosphonate transporters *glpT* and *phnC* in addition to mutations in the Pqs quorum sensing regulator *mvfR*.

This work has demonstrated the importance and prevalence of collateral selective effects in the evolution of antimicrobial resistance. It has identified a number of novel evolutionary targets hypothesised to be involved in the evolution of antimicrobial resistance which require mechanistic investigation. This includes the role of MexEF-OprN in β-lactam collateral sensitivity selected by chloramphenicol, DppA3 in meropenem resistance, MvfR in fosfomycin resistance, and OrfN in reduced benzisothiazolinone and pelargonic acid susceptibility. Future work should build on this foundation to investigate the selective potential of agents in an experimental environment more representative of the conditions in which they would be employed in the real-world. With this, the biofilm evolution model employed here could be used as a tool to qualify the selective capacity of new antimicrobial agents as part of the research development process.

7.5. Conclusion

In conclusion, this work demonstrates that experimental evolution is a useful vehicle to study the molecular mechanisms underpinning adaptive processes such as selection of biofilm hyperproduction and antimicrobial resistance. In this study, the experimental evolution model devised by Poltak and Cooper (2011) was systematically validated and

optimised to investigate the evolution of antimicrobial resistance in biofilms of *P. aeruginosa*. The serial passage of biofilms on beads of clinically- and industrially-relevant substrates selected biofilm hyperproduction characterised by increased biomass deposition and phenotypic diversification facilitated by constitutive increase of intracellular c-di-GMP concentration. Furthermore, adaptation to antibiotics broadly suppressed selection for biofilm hyperproduction, however biofilm-specific mechanistic distinctions were identified based on the repertoire of secondary mutations they selected. Finally, an extensive and highly diverse selective landscape associated with collateral selection of drug resistance exists which is mechanistically conserved across chemically unrelated antimicrobials. Therefore, this work provides novel insights into the role of lifestyle in adaptation to selective pressures and has demonstrated the importance of collateral selective effects in the evolution of antimicrobial resistance.

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Appendix

Appendix 1. Susceptibility of *P. aeruginosa* biofilms adapted to antimicrobials agents at various concentrations for four transfers.

Selective Agent	Selective Concentration (log_2 fold change from planktonic MIC)	Minimum Inhibitory Concentration (µg/ mL)*		
		Ciprofloxacin	Zinc sulfate	Benzalkonium chloride†
Ancestor		0.06	1024	2
Ciprofloxacin	-2	0.06		
	-1	**0.25**		
	0	**0.25**		
	1	**0.5**		
	2	**0.5**		
Zinc sulfate	-2		1024	
	-1		1024	
	0		1024	
	1		1024	
	2		1024	
Benzalkonium chloride	-2			2
	-1			2
	0			2
	1			n/a
	2			n/a

*Significant changes in susceptibility (≥2-log_2 fold change from ancestral MIC) shown in bold.

†n/a denotes concentrations unable to be assayed as selective concentrations are bactericidal