The role of interspecific competition on the evolution of phage resistance in *Pseudomonas aeruginosa*

Submitted by

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Abstract

Pseudomonas aeruginosa is a pathogen of increasing medical concern due its inherent tolerance and ability to overcome antibiotics. Because of this, bacteriophages are increasingly being considered and applied as alternative therapeutics. *P. aeruginosa* however can evolve resistance to phages through a range of different means, including CRISPR-Cas adaptive immunity. Yet experimental studies on phage resistance evolution have almost exclusively been done using clonal bacterial populations. This, despite bacteria commonly thriving in complex microbial communities with potentially significant consequences for phage resistance and virulence evolution. Here, I first summarise the existing literature on *P. aeruginosa* ecology, virulence, antibiotic resistance, phage therapy and resistance, before I present experiments looking at how interspecific competition affects bacteria-phage co-evolution. I demonstrate how growth in the presence of other bacterial species (*Staphylococcus aureus*, *Burkholderia cenocepacia*, and *Acinetobacter baumannii*) causes *P. aeruginosa* to evolve higher levels of CRISPR-based immunity than when in monoculture. This again has important knock-on effects for *P. aeruginosa* virulence, which becomes attenuated if the bacterium evolves surface-based resistance. Next, to understand the causative mechanism(s) underlying the selection for CRISPR-based resistance in polyculture, I look at the evolution of phage resistance in conditioned media. I show that a greater proportion of *P. aeruginosa* clones evolve phage resistance through CRISPR-Cas when cultured in the conditioned media from *A. baumannii*. This suggest that the effect of this competitor species is caused by changes to the chemical environment, such as resource depletion and toxin secretion. Finally, I examine how phage and CRISPR-Cas immune systems shape microbial community structure. I find that *A. baumannii* takes over to become the dominant species in the presence of phage, regardless of the presence or absence of a CRISPR-Cas system in the *P. aeruginosa* genome. Additionally, phage has a diversity maintaining effect, with all four community members persisting for longer in the presence of phage. Collectively, this thesis sheds light on how interspecific competition shapes the evolution of phage resistance, and vice versa.

Covid-19 statement

The ongoing Covid-19 pandemic interrupted the contents of this thesis in the ways as outlined below.

From March 2020 till July 2020, I did not have University or equipment access due to complete building closure. After this, time allowed in the laboratory spaces was still strictly limited through heavily reduced capacity until early 2021. In this same time span, there were consumables and reagent shortages worldwide, including those used for DNA extractions, qPCR, and general evolution experiments (e.g. pipette tips). This severely delayed and impacted experiments, especially those planned for Chapter III.

For Chapter III, I intended to visit Prof. Whiteley's group at Georgia Tech, USA, for a month in November 2020 to utilise their facilities for molecular training and research to examine the interactions between *P. aeruginosa* and *A. baumannii*. This had to be cancelled because of travel restrictions and experimental delays. This work would have formed a significant part of Chapter III, and I had to adjust the approach to what was possible to do with limited time and resources, while also having newly developed equipment shipped over from the US in the time between February–June 2021.

Had my research activities not been curtailed by the pandemic, it is likely Chapter IV would have been sent out for review and perhaps publication. I also acknowledge that Chapter III would have been more complete in terms of experimental results and possible conclusions had I not been impacted by Covid-19.

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Thanks also go out to my supervisor Edze Westra, for always believing in me, supporting me, and for trusting me, as well as his contributions to the work that makes up this thesis. To my second supervisor, Angus Buckling, for being the one who introduced me to the field of experimental evolution: Thank you.

Til mamma, pappa, Sigrid, og Marit. Elsker dere.

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Author’s declaration

Chapter II, II and IV are the results of collaborative work, the majority of which is my own. Chapters I and V are entirely my own, with revisions offered by Prof. Edze Westra. Author contributions to published and unpublished papers are fully outlined in a dedicated section of Chapter I.

Abbreviations

ASM	Artificial sputum medium
BCC	*Burkholderia cepacia* complex
Cas	CRISPR associated
CF	Cystic fibrosis
CFU	Colony forming units
CRISPR	Clustered regularly interspaced short palindromic repeats
DPI	Days post infection
GLM	Generalised linear model
HAP	Hospital-acquired pneumonia
HGT	Horizontal gene transfer
LB	Lysogeny broth
LPS	Lipopolysaccharide
MDR	Multidrug resistant/resistance
MGE	Mobile genetic element
MIC	Minimal inhibitory concentration
OMV	Outer membrane vesicles
PFU	Plaque forming units
RM	Restriction modification
RPM	Rotations per minute
SM	Surface mutant/modification
T2SS	Type II secretion system
T3SS	Type III secretion system
T6SS	Type VI secretion system
CAUTI	Catheter-associated urinary tract infection
QS	Quorum sensing

Chapter I – Introduction

Literature review – *Pseudomonas aeruginosa*

Ecology

As bacteria go, *Pseudomonas aeruginosa* can in many ways be considered to be extraordinary. From harmless saprophyte and commensal organism, to model bacterium and deadly pathogen, this Gram-negative bacillus and opportunist has from first being described in 1882 shaped the fields of microbiology and experimental evolution today (Diggle & Whiteley, 2020). Its name is derived from the Greek *Pseudo* and *monas* meaning “false” and “single unit”, and the Latin word for “rusted copper”: aerūgō. The latter most likely being inspired by the striking cyan-green phenazine compound it produces – pyocyanin. With its properties as both a toxin and an antimicrobial (Lau et al., 2004), pyocyanin is however only part of what makes *P. aeruginosa* such a fascinating and important bacterium.

P. aeruginosa is a non-fermenter and facultative aerobe, and its large genome of between 5-7 million base pairs (one of the largest known bacterial genomes when it was first sequenced in 2000)(Stover et al., 2000) encodes a range of regulatory circuits that facilitate phenotypic plasticity and environmental adaptation (Grekov et al., 2021). Being fast-growing and thriving under nutrient rich conditions and at 37 °C (Yang et al., 2008) also makes for an ideal bacterium for studies on microbial ecology and evolution. Today, most experimental research involving *P. aeruginosa* is done utilising two specific strains: PAO1 (Stover et al., 2000) and PA14. The prior strain was originally isolated from a chronic wound in the 1950s and has become a staple in laboratories around the world. PA14 as well is equally common in experimental setting: Having been initially isolated from a burn wound, it was first sequenced in 2006 (Lee et al., 2006) and has since been isolated again numerous times from both clinical and aquatic environments (Wiehlmann et al., 2007). PA14 also has markedly higher virulence than strain PAO1 (Mikkelsen et al., 2011). In addition to thriving under nutrient rich conditions, *P. aeruginosa* can additionally utilise a wide variety of organic carbon

sources for growth in minimal salts media and survive at temperatures ranging from 4–42 °C (Diggle & Whiteley, 2020). This propensity for adaptation and resilience is most likely beneficial for growth in soil and polluted water-reservoirs, but has also in turn become devastating from a human health and infection control perspective (Breidenstein et al., 2011).

Most infections caused by *P. aeruginosa* are nosocomial, and – one way or another – associated with a compromised host immune system. Whether it be acute or chronic, *P. aeruginosa* infection is particularly prevalent and problematic in people with cystic fibrosis (CF), in whom it colonises the airway, causes progressive loss of lung function, and is the leading cause of morbidity and mortality (Elborn, 2016). Additionally, it is known to infect patients with catheter-associated urinary tract infections (CAUTI) (Mittal et al., 2009), hospital-acquired pneumonia (HAP) (Fujitani et al., 2011), burn wound trauma (Azzopardi et al., 2014), and to cause ulcerative keratitis of the cornea (Lyczak et al., 2000; Weissman et al., 1984). During the COVID-19 pandemic, there has also been reports of rising incidences of hospital-onset bacteraemia in England, caused by *P. aeruginosa* (Sloot et al., 2021). These infections may be endogenous or exogenous, however the abundance of *P. aeruginosa* in the environment, both in nature and hospitals, seems to be a potential source of infection. In environmental soil, for example, *P. aeruginosa* was detected in 24% of analysed samples (S. K. Green et al., 1974), however, whether these strains of *P. aeruginosa* found in soil are the same ones that may cause clinical infection is still somewhat uncertain. Yet strains such as PA14 and other clinical isolates have been found to be able to colonize plants under certain conditions (Schroth et al., 2018), as well as having been isolated from various natural habitats (Wiehlmann et al., 2007). In addition, *P. aeruginosa* is known to contaminate sinks, floors, showers, and bed rails in hospitals (Lyczak et al., 2000). It has even been cultured from endoscopes used for diagnostics, and from the hands of health care personnel (Lyczak et al., 2000). In most cases however, *P. aeruginosa* clinical isolates are indistinguishable from environmental isolates, with few endemic strains (Pirnay et al., 2009).

Yet, although it is relatively common in our surrounding environment, prevalence in humans increases sharply post hospitalisation. This change is assumed to be

driven by a compromised host immune system (Lyczak et al., 2000), but also at least partially by perturbation caused by antibiotic treatments on the human microbiome, as interspecific interactions and commensals may facilitate protection against pathogens (Blaser & Falkow, 2009; L. Wang et al., 2020). In cases of *P. aeruginosa* infection, this has for example been illustrated by Ewig *et al.*, who showed a significant increase in *P. aeruginosa* detection when comparing bacterial colonisation pre versus post hospital admission and intensive care treatment (Ewig et al., 1999). Another recent study, by Wang *et al.* found that antibiotics disrupted the composition of the intestinal commensal microbiota, to the point where it impaired the host immune system against *P. aeruginosa* (L. Wang et al., 2020). Yet, the true extent to which the microbiota might protect against *P. aeruginosa* infection is still not fully understood.

Additionally, there is the issue of cross-transmission. Cross-transmission can happen between patients and from healthy to health-compromised individuals, with healthy individuals working as environmental reservoirs for *P. aeruginosa*. One study for instance, applied molecular subtyping to show how cross-transmission occurred in between 29.5% to 52.6% of patients with nosocomial infections (Fujitani et al., 2011). Other research implies these numbers may be higher, with the impact of cross-transmission estimated to represent at least 59.5% of all colonisation or infection events (Agodi et al., 2007). This becomes particularly problematic in cases of multidrug resistance (MDR): In a report by Hsueh *et al.*, when tracking the spread of one single MDR strain of *P. aeruginosa*, they found that over a period of about a year, one *P. aeruginosa* strain was carried by a few asymptomatic patients through several rounds of antibiotic treatment (Hsueh et al., 1998). This one strain also infected other patients during this time, resulting in not just the spread of *P. aeruginosa*, but antibacterial resistance as well.

Co-infection

Historically, bacterial infections have been attributed to single pathogens (Kaufmann & Schaible, 2005). However, as technology and diagnostic tools have improved over the last couple of decades, so has our understanding of the importance of interspecific dynamics and polymicrobial infections (Smith, 1982). After all, bacteria rarely exist in isolation, a simple truth that also encompasses

P. aeruginosa. Instead, they are embedded in complex biotic networks with great implications for their ecology and evolution, and consequently, disease progression.

Interspecific interactions, be they symbiotic or antagonistic, are key to understanding the within-host selection pressures that in turn can shape the biogeography and morbidity of infection (Stacy et al., 2016). The more classic, culture-based approach to clinical microbiology long only allowed for identification of the major players in infection, failing to account for the full complexity of the microbiota (O'Toole, 2017). In the last few years meanwhile, more culture-independent approaches have started to take hold and are being increasingly applied to further examine the composition and role of interspecific dynamics in clinical infection. The advent of deep sequencing especially (be it examining the microbiomes of people considered healthy or otherwise), has drastically increased our knowledge of function, structure, and dynamics of microbial communities and their implications for disease (Byrd et al., 2018; Glassner et al., 2020; Lloyd-Price et al., 2017; Rogers et al., 2004; Sharma & Tripathi, 2019).

For *P. aeruginosa*, co-infection seems to be common, and in many cases the rule rather than the exception. In people with CF in particular, a wealth of studies on the lung microbiota have mapped the changes in species composition over time (Harrison, 2007; Fodor et al., 2012; Rudkjøbing et al., 2012). *P. aeruginosa* is one of four key bacterial species in these people, with the other three being *Staphylococcus aureus*, *Burkholderia cepacia* complex (BCC), and *Haemophilus influenzae* (Harrison, 2007). In addition, there are also interkingdom interactions with fungi, such as *Candida albicans* and *Aspergillus fumigatus* (Bisht et al., 2020; Harrison, 2007; O'Brien & Fothergill, 2017). The microbiota and the rate at which *P. aeruginosa* is detected is not consistent over time, however. Young children with CF usually suffer from intermittent, acute infections, involving a range of different bacteria typically acquired from environmental reservoirs (Gibson et al., 2003). The formation of biofilms then facilitates the establishment of chronic infection, with approximately 60-80% of young adults with CF being chronically infected with *P.* aeruginosa (Folkesson et al., 2012; Rudkjøbing et al., 2012). What causes this shift from acute to chronic lung infection is not fully understood, but seems to follow after a few phenotypic and genotypic changes

that may be linked to selection and decreasing diversity due to interspecific interactions and adaptation (O'Brien & Fothergill, 2017; Winstanley et al., 2016). While perhaps most studied and highly associated with lung infection, *P. aeruginosa* is also known to co-infect with a range of other pathogens in cases of chronic and surgical wound infections (Fazli et al., 2009; Giacometti et al., 2000), bloodstream infections (Archibald et al., 1998; Wang et al., 2020), and CAUTIs (Azevedo et al., 2017), all of which are associated with higher patient mortality.

P. aeruginosa is both shaped by and shapes its wider microbial community. People with CF and chronic *P. aeruginosa* infection for example, experience an increased risk of secondary infection by *B. cenocepacia* (part of the BCC), which again is linked to usually fatal necrotising pneumonia (known as cepacia syndrome)(O'Brien & Fothergill, 2017; Hansen et al., 2010; Bragonzi et al., 2012). When studied together, *P. aeruginosa* and *B. cenocepacia* have been found to engage in a network of interactions, forming complex mucoid biofilms together, and exchanging genetic material (Eberl & Tümmler, 2004). Competition between these two pathogens is also fierce. A study by Bakkal *et al.* screened 66 clinical isolates from CF samples, and found that over half of the screened *B. cenocepacia* and 81% of the *P. aeruginosa* isolates produced bacteriocin-like toxins specifically inhibiting the other species (Bakkal et al., 2010). *P. aeruginosa* is also capable of invading niches occupied by *B. cenocepacia* (Costello et al., 2014), and vice versa (O'Brien & Fothergill, 2017; Schwab et al., 2014), with the outcome of competition most likely context dependent. Regardless of outcome however, it is generally to the detriment of the human host. This is because of how the different molecules they produce to inhibit one another usually also serve secondary purposes as virulence factors, which may inadvertently aid in the other's survival by inhibiting the human immune system (Chattoraj et al., 2010).

S. aureus is another species known to co-infect with *P. aeruginosa*, both in chronic wounds and in CF, these two pathogens together are two of the most recognised causes of polymicrobial infection (Rudkjøbing et al., 2012; DeLeon et al., 2014). The *P. aeruginosa – S. aureus* dynamic is a well-studied one due to its commonality and host-impact: By producing a range of exoproducts targeting *S. aureus*, *P. aeruginosa* can efficiently outcompete this Gram-positive

competitor (Briaud et al., 2019; Cardozo et al., 2013; Hoffman et al., 2006; A. Korgaonkar et al., 2013; A. K. Korgaonkar & Whiteley, 2011; O'Brien & Fothergill, 2017), however it may also manipulate the hosts innate immune system to induce an immune response against *S. aureus*, facilitating its own survival (Pernet et al., 2014). There has also been reported synergy between the two species when co-infecting (DeLeon et al., 2014), with polymicrobial infections being reported as more virulent than monoculture infections. Using a wound model, DeLeon *et al.* showed how when grown together, both *P. aeruginosa* and *S. aureus* have increased survivability and tolerance when faced with antibiotics (DeLeon et al., 2014), implying situations where they may benefit from co-infection.

Perhaps no clearer has this issue of co-infection become than over the last couple of years during the COVID-19 pandemic. The SARS-Cov-2 virus, which first emerged December 2019, has led to more than 200 million cases and 4 million deaths around the world so far (*WHO Coronavirus (COVID-19) Dashboard*, 2021). Whereas most cases are mild or moderate in their severity, comorbidities may cause patients to require mechanical ventilation and intensive care, predisposing them to complicated secondary infections (Lansbury et al., 2020). These co-infections severely increase morbidity and mortality, and *P. aeruginosa* is commonly reported alongside *Acinetobacter baumannii*, *Staphylococcus aureus*, *Candida* non-*albicans*, and *Candida albicans* to be one of the worst perpetrators, with polybacterial co-infections being common (Fontana et al., 2021; Silva et al., 2021). What is of noticeable concern, is the seemingly growing incidence of reported *P. aeruginosa* and *A. baumannii* co-infection (Rangel et al., 2021), possibly now detectable due to improved diagnostic tools. Like *P. aeruginosa*, *A. baumannii* belongs to the ESKAPE pathogen group, where they are the current World Health Organisation's critical priority pathogens (Tacconelli et al., 2018). Both pathogens are also known for their ability to overcome antibiotic treatments, and when co-infections do occur, they are associated with higher mortality rates (Y. C. Wang et al., 2020). This is something that might become an increasing concern as *A. baumannii* is being reported to have emerged in some cases of lung infection such as in people with CF, usually alongside *P. aeruginosa* (Rocha et al., 2018; Jenkinson et al., 2021).

Virulence and antimicrobial resistance

What is it that has made *P. aeruginosa* such a notorious hospital pathogen in the first place? As an opportunistic pathogen commonly found in our surrounding environment, it is not necessarily a given that it should become a leading cause of nosocomial infections, with calls for intense research into new antibiotics and therapeutic alternatives (Tacconelli et al., 2018). However, ever since humans started developing clinical antibiotics, bacteria have evolved to overcome them, with reports of resistance in *P. aeruginosa* following not long after their introduction as common in clinical therapy (Finland, 1959). This has led to *P. aeruginosa* becoming a so-called 'superbug', and what initially made it a successful and fit environmental bacterium is now also what helps it adapt and thrive in clinical infection.

Virulence factors

On a cellular level, *P. aeruginosa* produces a range of exoproducts and secondary metabolites including pyocyanin, proteases, exotoxin A, and hydrogen cyanide. Pyocyanin is perhaps the most studied of these as an antimicrobial and toxin, the production of which is regulated by quorum sensing (Das et al., 2013; Dietrich et al., 2006; Lau et al., 2004). High concentrations of the strikingly cyan-green, redox-active phenazine can be found in the sputum of people with CF who are infected with *P. aeruginosa*, and the production of pyocyanin is thought to play a major role in acute and chronic lung infections, burn wounds in mammals, and the general pathogenicity of disease in invertebrates and plants (Winstanley & Fothergill, 2009). Pyocyanin's effects as a toxin are also well documented. It can for instance inactivate nitric oxide, which influences host blood flow, pressure, and immune functions, and negatively impact host ciliary function and T cell response (Winstanley & Fothergill, 2009). Being an antimicrobial, pyocyanin is also key in interspecific interactions and the killing of competitors, which might be one of the reasons why *P. aeruginosa* tends to dominate the infections it is present in. Additionally, *P. aeruginosa* produces two siderophores responsible for scavenging iron from iron-depleted environments: pyoverdine and pyochelin. These secondary metabolites play important roles in *P. aeruginosa* fitness across a range of environments, social behaviours, and biofilm development (Butaitė et al., 2018; Das et al., 2013; Diggle & Whiteley, 2020).

P. aeruginosa also possesses five recognised and described protein secretion systems (types I, II, III, V, and VI), each serving a different function. Yet not all systems are equal when it comes to their impact on virulence. The type II system (T2SS) for instance (also referred to as the 'general secretion pathway'), secretes the virulence factor LasB, responsible for elastin degradation and the cleavage of immune system proteins (Depluverez et al., 2016). In addition, the T2SS also secretes products that play part in targeting the host membrane, eukaryotic cell colonisation, protein synthesis inhibition and degradation, and cell death (Depluverez et al., 2016; Jyot et al., 2011). The type III system (T3SS) meanwhile, controls toxin protein injection into the cytoplasm or cell membrane of host cells. These proteins, called effectors, are ExoU, ExoT, ExoY, and ExoS, and once injected may protect *P. aeruginosa* from phagocytosis, and result in host cell death, acute cytotoxicity, and lung tissue damage (Depluverez et al., 2016; Diggle & Whiteley, 2020). The last secretion system key to *P. aeruginosa* virulence is the type VI system (T6SS). Effectively, the T6SS works by 'stabbing' other cells using a machinery composed of elements that are not unlike those of contractile phage tails (Basler et al., 2012). This 'stabbing' plays a crucial role in the killing of eukaryotic cells, but it has also been proposed to be used for interspecific competition during co-infection (Ho et al., 2014).

Moving outwards, the outer membrane of *P. aeruginosa* is composed of lipopolysaccharide (LPS), another virulence factor. LPS is a large molecule that consists of a lipid (lipid A), an O-antigen polysaccharide, and a branched oligosaccharide known as the core (King et al., 2009). By protecting the membrane from different chemicals, increasing the negative cell charge, and adding structural integrity, LPS is a key virulence factor, as well as playing part in how the host responds to *P. aeruginosa* infection (Pier, 2007). For instance, *P. aeruginosa* strains are known to produce two different types of O-antigen simultaneously in the same cell, called A and B bands (King et al., 2009), which affect the host immune response in markedly different ways. While the A band (the common polysaccharide antigen) causes a weak antibody response, the B band (the specific polysaccharide antigen) is highly immunogenic (Diggle & Whiteley, 2020). It is because of this immunogenic nature, its high molecular mass, and possible selection pressure from antibiotics that the B band is the one

commonly lost in chronic *P. aeruginosa* infections, like those of people with CF (King et al., 2009).

The cell surface with its associated structures also plays an integral part of infection through adhesion to epithelial cells and mucin. *P. aeruginosa* achieves this by using its pili and flagella for swimming, twitching, and swarming motility. Mutants without pili are selected for through various means, and exhibit reduced virulence compared to ancestral strains when tested in various animal infection models of burn wounds, intraperitoneal, and lung infections (Hahn, 1997; Persat et al., 2015). Particularly pili play an integral role in the first step of biofilm formation (Klausen et al., 2003; Talà et al., 2019), and the loss or modification of the Type IV pilus is especially costly because of its crucial role in *P. aeruginosa* adhesion to human lung cells (Farinha et al., 1994; Hahn, 1997). Similarly, flagella are important in the early stages of mucin adhesion. Mucin is a protein commonly found in the sputum of people with CF and is produced by epithelial tissues, and *P. aeruginosa* clones with flagella mutations have like those with pili mutations been shown to have reduced virulence in acute infection models (Montie et al., 1982; Rossez et al., 2015).

On a more population-wide scale, biofilm formation and its main regulators are considered to be signature characteristics of *P. aeruginosa* growth and pathogenicity. Being of paramount importance for clinical *P. aeruginosa* infections, this ability to develop multicellular biofilm communities, or aggregates, has been extensively studied. These biofilms and aggregates are bacterial populations enclosed in a matrix consisting mostly of exopolysaccharides, proteins, lipids, nucleic acids, cell debris and outer membrane vesicles (OMVs), and are important for surface attachment (Costerton et al., 1995; Diggle & Whiteley, 2020; Häussler & Parsek, 2010). The exopolysaccharides especially help in resisting stressors such as the host immune system and bacteriophages by protectively surrounding the bacterial cells (Lewis, 2001). This also facilitates *P. aeruginosa* persistence for longer periods of time in places such as the respiratory tract and paranasal sinuses. The three major polysaccharides produced by *P. aeruginosa* are Pel, Psl, and alginate, which have all been found to play important roles in *in vitro* biofilm formation (Colvin et al., 2012; Diggle & Whiteley, 2020). Alginate, for instance, is key in the adaptation and transition to

chronic lung infections, as it is over-produced in mucoid *P. aeruginosa* variants associated with CF (Govan & Deretic, 1996). Pel and Psl on the other hand are mostly utilised by non-mucoid strains of *P. aeruginosa*, such as PAO1 and PA14 – although the latter lacks Psl– that are more motile but still maintain some of the most crucial biofilm properties, such as increased drug resistance (Wozniak et al., 2003).

When growing in biofilms, bacteria display different phenotypes and gene expression than their planktonic counterparts, a change largely credited to cellular signalling systems collectively referred to as quorum sensing (QS). In *P. aeruginosa*, QS is used to modulate many of its virulence factors in a cell-density-dependent manner through two major quorum transcriptional factors: LasR and RhlR (Dekimpe & Déziel, 2009; Whiteley et al., 2017). Studies into these QS systems have found that *P. aeruginosa* LasR mutants are particularly common in many patients, but that these same mutants have instead evolved for the RhlR system to replace it (Whiteley et al., 2017). This emphasises how QS is used to coordinate gene expression to activate virulence genes during infection and group behaviours such as biofilm formation. QS is also a social trait, and can as such be exploited by social cheats, which again has downstream effects on virulence (Whiteley et al., 2017).

Antimicrobial resistance

P. aeruginosa's ability to readily evolve resistance to clinically applied antibiotics makes it as an opportunistic pathogen increasingly difficult to combat. If anything, few bacterial infections are as difficult to treat as those caused by *P. aeruginosa*. Multiple strains of *P. aeruginosa* possess several mechanisms to counter nearly every clinical antibiotic in use, making it a so-called 'superbug', and it is only predicted to get worse: By 2050, deaths attributed to antimicrobial resistance is expected to reach 10 million per year, surpassing all other major causes of death in humans (O'Neill, 2014). The mechanisms of antibiotic resistance can be categorised as intrinsic, acquired, or adaptive, and the distinction between them will be explained in detail below.

Intrinsic resistance refers to mechanisms naturally found in bacteria, and which predate the clinical use of antibiotics. These mechanisms are all part of the

genetic makeup of *P. aeruginosa*; the result of adaptation to various natural environments that now also renders many common clinical antibiotics ineffective. For example, *P. aeruginosa*'s outer membrane has a low permeability that naturally obstructs entry into the cell by antibiotics such as aminoglycosides, quinolones, and ß-lactams (Nicas & Hancock, 1983). These antibiotics need to penetrate the cell to work as intended, but the tightly controlled porins on the bacteria's outer membrane limit or completely hinder this penetration. Even if the antibiotic managed to enter the cell, the limited rate at which this happens would allow for acquired and adaptive resistance mechanisms to take full effect (Breidenstein et al., 2011; Pang et al., 2019).

Acquired resistance on the other hand encompasses the ways in which bacteria may become less susceptible to antibiotics through the acquisition of heritable traits conferring resistance. *P. aeruginosa* can for example acquire novel and heritable antibiotic resistance through mutations (Munita & Arias, 2016). The intrinsic mechanisms already mentioned above may for instance be altered by mutation (with the frequency of spontaneous mutations ranging between 10^{-6} to 10^{-9} for individual antibiotics), affecting the expression or activity of resistance genes and their products, to the point where most clinical antibiotics are rendered unusable (Breidenstein et al., 2011). Mutations may also increase the baseline minimal inhibitory concentrations (MIC) needed for these antibiotics to be effective against *P. aeruginosa*, something that may become especially problematic if mutations accumulate against multiple antibiotics. The rate of mutation has also been found to increase under certain conditions, such as when growing in a biofilm (Driffield et al., 2008) or when experiencing subinhibitory concentrations of certain antibiotics (i.e. DNA-damaging agents) (Tanimoto et al., 2008). Then, there is also the matter of hypermutator strains, which may exhibit 70-fold mutation frequency increases (Wiegand et al., 2008), and are commonly found in people with CF (e.g. mutL and mutS), facilitating rapid adaptation to overcome antibiotics (Oliver et al., 2000).

Antibiotic resistance genes can also be carried on mobile genetic elements (MGE), such as plasmids, transposons, integrons, integrative and conjugative elements (ICEs), and prophages, which *P. aeruginosa* is able to acquire through HGT (Breidenstein et al., 2011). HGT works through transformation, transduction,

or conjugation, and plays a key role in moving resistance genes between bacterial species and strains. This is known to impact clinical antibiotics such as aminoglycoside and ß-lactams, but also a range of other classes of antibiotics as well. The former (aminoglycosides) for instance, may be inactivated through modifying enzymes carried on mobile genetic elements, leading to chemical modifications and reduced affinity for the main antibiotic target (Breidenstein et al., 2011). The latter meanwhile (ß-lactams), is hydrolyzed by *P. aeruginosa* metallo-beta-lactamases (MBLs), and six MBL genes have been detected as being carried by MGEs, such as integrons and plasmids (Pang et al., 2019).

Adaptive antibiotic resistance, meanwhile, increases the resilience of *P. aeruginosa* against antibiotics and is dependent on continued exposure to antibiotic induced selection pressures or other environmental stimuli. Two of the most extensively studied and characterised mechanisms of adaptive resistance in *P. aeruginosa* are biofilm formation and persister cell generation (Pang et al., 2019). Biofilms, in addition to being a virulence factor as already discussed, protect bacteria from antibiotics through many means. For example, the polysaccharides inhibit penetration by beta-lactam antibiotics, while antibiotics such as ciprofloxacin and tobramycin are thwarted by limited oxygen and low metabolic activity (Gordon et al., 1988; Walters et al., 2003). In addition, biofilms can induce an adaptive stress response and persister cell differentiation (Stewart, 2002). Persister cells are also a major obstacle on their own accord. Defined as dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics (Lewis, 2010), *P. aeruginosa* persister cells are especially making the treatment of infections in people with CF more complicated (Drenkard & Ausubel, 2002; Pang et al., 2019). Comprising about 1% of biofilm cells, these persisters are slow growing, metabolically inactive, and highly tolerant to antibiotics. This to the point where they are able to remain viable and can re-populate the host post antibiotic treatment (Lewis, 2010).

Therapeutic alternatives

What then are the potential alternatives to antibiotics? With the rise of antibiotic resistance, we are in increasing need of new treatment methods against *P. aeruginosa* infections, the development of which has gained more attention over

the last decade or so. New therapeutics should ideally be able to act either alone or in combination with conventional antibiotics. To date, suggested alternatives or combination treatments include the inhibition of QS and bacterial lectins, and the use of iron chelation, vaccines, nanoparticles, antimicrobial peptides, electrochemical scaffolds, and phage therapy (Chattoraj et al., 2010; Pang et al., 2019). QS inhibitors (such as zeaxanthin, flavonoids, and N-decanoyl cyclopentylamide, which usually interfere with LasR and RhlR) for instance, have the potential to reduce or prevent biofilm formation, with downstream effects on virulence and the evolution of antibiotic resistance (Pang et al., 2019; Whiteley et al., 2017). On a similar note, researchers have suggested using bacterial social cheats as 'Trojan horses', with the possibility to invade populations carrying alleles for antibiotic sensitivity, or with lethal toxins (Brown et al., 2009). Both methods exploit *P. aeruginosa* social traits for medical intervention; social traits which are key in the ecology, co-infection, and pathogenicity of the bacterium.

Lectin inhibition on the other hand, targets outer membrane proteins (lectins) that facilitate adhesion to the epithelial cells of the lung. However, Diggle *et al.* also found lectins to be involved in biofilm production (Diggle et al., 2006), which might allow for reduced virulence and changes to social behaviours as well. The potential use of nanoparticles is also mostly seen in the context of combating biofilms, as they have higher penetrability into bacterial membranes, can disrupt biofilms, and possess and carry multiple antimicrobial mechanisms or antibiotics (Wang et al., 2017). Electrochemical scaffolds on the other hand produce low but constant concentrations of H_2O_2 that destroy biofilms, allowing for antibiotic penetration (Sultana et al., 2015), however this treatment has yet to be tested in patients. Meanwhile, iron chelation involves the limitation of extracellular iron or iron-uptake disruption (Smith et al., 2013), while antimicrobial peptides target the cytoplasmic membrane and causes pathogen cell death (Park et al., 2011). Another treatment option currently being re-considered is to utilise bacteriophages therapeutically, also known as phage therapy.

Phage therapy

Bacteriophages (simply referred to as phages) are viruses that work as bacteria's natural predators, and that can be used to treat various infections (Clokie et al., 2011). Today, we also know that phages are everywhere. After all, with a total

global estimate at around 10^{31}, they are the most abundant biological entities on the planet, even outnumbering bacteria tenfold in certain ecosystems (Dion et al., 2020; Wittebole et al., 2014). Found in every explored and described ecosystem, phages play major roles in aquatic biochemical cycling, and the physiology and metabolism in animal – including human – microbiomes, such as the gut through their effect on bacterial traits and abundance (Dion et al., 2020; Hsu et al., 2019; Shkoporov & Hill, 2019).

Phages were first discovered over a century ago, in two independent instances: First by Frederick Twort in 1915, and then by Felix d'Herelle in 1917, but it was the latter who first suggested phages as therapeuticals. However, after the advent of clinical antibiotics and World War II, and at least partially due to the following Cold War between the United States and the former USSR, phages were essentially disregarded in the West. In the USSR meanwhile, especially in what is now Georgia, they were and continue to be used to treat a number of bacterial infections (Chanishvili, 2012; Myelnikov, 2018). Phages have two main life cycles. The lytic cycle involves phages binding to receptors on the host cell surface, followed by infection by injecting their genome, before rapidly lysing their host, releasing progeny to repeat the cycle. The lysogenic cycle on the other hand, involves phages integrating into the host genome, often with a latent and induced lytic cell lysis later (Clokie et al., 2011). Whereas temperate phages can replicate through both life cycles, virulent phages can only replicate through the lytic cycle, which makes them more suitable for clinical treatment purposes.

There are several advantages to phage therapy that makes it such an enticing treatment alternative to antibiotics – especially with the increase of MDR *P. aeruginosa*. For example, phages are self-replicating, adaptive, highly specific, without a broader impact on the commensal microbiome of the human host, and generally come with fewer side-effects than other broad-spectrum antimicrobials (Maier et al., 2021; Roach & Debarbieux, 2017). With many phages being evolved to readily destroy the extracellular matrix that make up biofilms, phage therapy is also a prime candidate for combination therapy. By removing biofilms as an obstacle, antibiotics will have increased permeability, and if combined with QS inhibition it may additionally prevent biofilm formation from re-occurring. Furthermore, if combined with other alternative treatments that also target

biofilms (e.g. nanoparticles and lectin inhibition), biofilm eradication might become more efficient because of the various mechanisms at play, limiting the likelihood of bacterial evolution towards treatment resistance (Chegini et al., 2020).

The use of phages against *P. aeruginosa* infection in particular has been extensively studied, and new *P. aeruginosa* targeting phages are continuously being isolated and characterised (Aghaee et al., 2021; Chen et al., 2021). In addition, there has been an increasing number of studies over the past decade, both *in vitro* and *in vivo*, exploring the efficiency and potential of phage therapy for *P. aeruginosa* infection (Cafora et al., 2019; Morello et al., 2011; Vieira et al., 2012; Waters et al., 2017). We have also reached a stage where phages are being used in clinical trials in the West (Chan et al., 2018; Furfaro et al., 2018; A. Wright et al., 2009). For instance, Chan *et al.* reported how phage OMKO1 was used to treat a chronic infection of an aortic graft: After being admitted multiple times over several years for recurring *P. aeruginosa* infection and bacteraemia, the patient underwent phage therapy by choice, rather than be subject to indefinite antibiotic treatment. Following one application of phage in combination with the antibiotic ceftazidime, the *P. aeruginosa* infection appeared resolved as although the patient had to be re-admitted, the only pathogen of significance found was *C. albicans* (Chan et al., 2018). There have however been few randomised control trials of phage therapy in humans, and the results have been mixed (Fabijan et al., 2020). One trial for instance, compared the topical application of a phage cocktail to standard care against *P. aeruginosa* infection, and found that reductions in bacterial load took longer in the phage therapy group, with lower treatment success (Jault et al., 2019).

With this influx of research and case studies comes new insights into how phages may affect the evolution of *P. aeruginosa*. The aforementioned phage OMKO1 for instance, uses a porin associated with efflux systems that have known links to MDR as its binding site. This subsequently means that the bacteria is faced with an evolutionary trade-off: become immune to phage infection, or remain antibiotic resistant (Chan et al., 2016). Consequently, by utilising phages such as OMKO1 we may, through natural and strong selection pressures induced by phage, get a secondary benefit of phage therapy by causing MDR *P. aeruginosa*

to become re-sensitised to common antibiotics. Many other possible phage receptor sites also serve other functions, most commonly ones integral to virulence. *P. aeruginosa* for instance, has 306 described virulence factors, 45% of which are likely to be localised on the cell surface, making them potential phage receptors (Gurney et al., 2020; Yu et al., 2010). The Type IV pilus, LPS and siderophores/iron acquisition are all potential targets for phage resistance vs virulence trade-offs, as they are strongly associated with both *in vivo* virulence and bacterial membrane sites for phage attachment (Green & Mecsas, 2016; Kortright et al., 2019; León & Bastías, 2015; Petrova & Sauer, 2012; Skaar, 2010). Overall, this means we may one day be able to treat *P. aeruginosa* infections while also selecting for any surviving cells to have more compromised and less virulent phenotypes, and thus improve clinical treatment outcomes (Gurney et al., 2020).

Phage resistance

The most likely obstacle to phage therapy success, is the evolution of phage resistance (Torres-Barceló, 2018). Similar to its ability to evolve resistance antibiotics, *P. aeruginosa* has an arsenal of genes and systems that may provide innate and adaptive immunity to phages. These cover a range of different underlying molecular mechanisms that can act at different stages of phage infection (Rostøl & Marraffini, 2019). To disrupt the initial binding of phage by blocking phage entry into the cell, bacteria may for instance modify, mask, or completely lose the relevant surface receptor (Harvey et al., 2018). Superinfection exclusion (Sie) meanwhile, can block phage genome injection into the cytoplasm, and is encoded by infecting phage (i.e. prophages (Bondy-Denomy et al., 2016; van Houte, Buckling, et al., 2016)). Then, there are the mechanisms which may disrupt phage intracellular genome replication. These mechanisms include restriction modification (RM), prokaryotic Argonaute (pAgo), and CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated genes) (van Houte, Buckling, et al., 2016; Barrangou et al., 2007; Cady et al., 2012). Last, but not least, there are abortive infection (Abi) and toxin-antitoxin (TA) systems, which are last-resort systems that trigger “programmed cell death” or dormancy (Rostøl & Marraffini, 2019).

Research over the last few years has shown that although there is variation between strains, *P. aeruginosa* primarily utilises phage-resistance systems that inhibit initial phage adsorption, and CRISPR-Cas (Cady et al., 2012; Harvey et al., 2018). Phage adsorption in *P. aeruginosa* is commonly associated with Type IV pili, which can be glycosylated to provide resistance to pilus-specific phages (Harvey et al., 2018). Additionally, there are several LPS-binding phages targeting *P. aeruginosa* (Huszczynski et al., 2019). The modification or masking of these surface receptors comes with a fitness cost, yet the complete loss of the receptor – offering total resistance – is frequently reported under laboratory conditions (van Houte, Buckling, et al., 2016; Westra et al., 2015). CRISPR-Cas systems on the other hand, are found in approximately 36% of clinical *P. aeruginosa* isolates (Cady et al., 2012), and work by integrating small sequences (called spacers) of the infecting phage's genome into its own CRISPR loci (Barrangou et al., 2007). These spacers are heritable, and provides the host with an immunological memory, making the bacteria able to cleave phage genomes upon re-infection (Garneau et al., 2010; Westra et al., 2012).

Both systems of phage resistance are important in nature (Andersson & Banfield, 2008), and recently, researchers have started investigating what determines the natural selection of one mechanism over the other (Westra et al., 2015). Surface modification for instance, is associated with a fixed cost due to the many other key functions associated with the Type IV pili and LPS (Hahn, 1997; Huszczynski et al., 2019). Yet, the timing and order in which bacteria are exposed to phages can drastically alter this associated fitness cost (Wright et al., 2019). The costliness of inducible CRISPR-based phage resistance is less clear. Work by Meaden *et al.* found that high infection risk leads to a high fitness cost of CRISPR-based immunity, possibly because of the expression of toxic phage genes before CRISPR-mediated cleavage has time to take place (Meaden et al., 2020). Therefore, under conditions associated with a high infection risk, surface resistance is favoured over CRISPR immunity, and vice versa under conditions of low infection risk, where CRISPR immunity is almost cost free (Westra et al*.,* 2015). Additionally, a high mutation rate has been found to favour surface modification over CRISPR-Cas due to the higher frequency of mutator phenotypes, which rapidly become the dominant mode of phage resistance (Chevallereau et al., 2019). However, phage and host genetic diversity also

matters in shaping the evolution of phage resistance in *P. aeruginosa*. Both experimental and modelling research has shown how CRISPR-Cas may facilitate host diversity through distributed immunity (the coexistence of multiple and equally fit immune alleles in a population) (Childs et al., 2014). This in turn can lead to rapid phage extinction because of the lack of susceptible hosts (van Houte, Ekroth, et al., 2016), or phage diversification and red queen co-evolutionary dynamics (Pilosof et al., 2020).

Most of the research on phage resistance evolution to date has been done either through mathematical modelling or *in vitro* experiments in extremely simplified biotic environments, neither of which necessarily match within-patient abiotic and biotic complexity. This, despite CRISPR-Cas having been found to be utilised by *P. aeruginosa* in clinical infections such as those in people with CF, with potential consequences for clinical outcomes (England et al., 2018). While some research has started looking at potential synergy between phages and the human host immune system (Roach et al., 2017), the impact of bacterial biodiversity and interspecific competition on phage resistance evolution remains largely unexplored (Blazanin & Turner, 2021) – despite the importance and common nature of co-infections.

Aims & objectives

With this thesis, I aim to start to examine – for the first time – how interspecific interactions affect bacteria-phage co-evolution. To start bridging the gap between the lab and clinical environments, I am using an artificial microbial community consisting of other opportunistic pathogens that can co-infect with *P. aeruginosa* and apply a diverse range of wet-lab approaches to address questions related to evolution, ecology, and molecular microbiology. The main objectives will be addressed in the next three chapters as follows:

1. How does interspecific competition impact the evolution of phage resistance, and what are the consequences for *P. aeruginosa virulence*?
Although about half of all bacteria possess CRISPR-Cas systems that provide adaptive immunity to phages and other invasive DNA elements, bacteria such as *P. aeruginosa* typically evolve phage resistance through surface-modification

under laboratory conditions. One objective of this thesis is to explore this discrepancy between *in vitro* and natural environments by looking at the impact of interspecific competition on phage-resistance evolution. Having determined the effect of interspecific competition on the evolution of CRISPR-based immunity, I then determine how this in turn impacts the evolution of *P. aeruginosa* using an *in vivo* infection model.

2. What is the main underlying mechanism(s) driving the evolution of increased CRISPR-based phage resistance in *P. aeruginosa* as described in Chapter II?

The evolution of phage resistance is influenced by biotic factors, such as the presence of other bacterial species not targeted by a specific phage, but which compete with the phage's host. Trade-offs due to interspecific competition result in selective shifts from one phage-resistance mechanism to another, yet the molecular mechanism(s) that underpin this effect remains unclear. An objective of this thesis is to explore what the potential underlying mechanisms behind this shift may be.

3. How does phage affect polybacterial community dynamics and co-existence?

Microorganisms play key roles in human health and disease, yet little is known about how these microbial communities form and change over time. This is especially true when it comes to the role of phage in bacterial co-infection as potentially diversity maintaining agents. By using an artificial microbial community, an objective of this thesis is to study the impact of phage over time to explore the wider implications of bacteria-phage interactions on microbial community dynamics.

Results summary

Chapter II explores how interspecific competition affects the evolution of phage resistance against phage DMS3vir in *P. aeruginosa* PA14, and at the potential downstream effects this has on *in vivo* virulence. The data showed that competition with other bacteria amplifies the fitness cost associated with surface-based phage resistance, to the point where selection tips in the favour of

CRISPR-Cas. The strength of this shift depended on which species *P. aeruginosa* was co-inoculated with, with the strongest effect being observed in the presence of *A. baumannii* or all three other species (*S. aureus, A. baumannii,* and *B. cenocepacia*). Additionally, we infected *Galleria mellonella* wax-moth larvae with *P. aeruginosa* clones sampled post phage infection. This showed that while surface-modification of the Type IV pilus comes with an associated cost in terms of reduced virulence, this was not the case for clones that had remained sensitive or evolve phage resistance through CRISPR. This confirmed that microbial biodiversity can drive the evolution of different phage resistance mechanisms, with key implications for *in vivo* virulence.

Chapter III builds on Chapter II by starting to delve into the underlying mechanism(s) behind the main effects observed; namely the shift form surface- to CRISPR-based phage resistance caused by interspecific competition. This was done by growing *P. aeruginosa* in the conditioned media of the different microbial community members used throughout this thesis. The data showed how while conditioned media in general resulted in an increase in the evolution of CRISPR-based phage resistance, this effect was amplified if the conditioned media had been recovered from *A. baumannii*. Confirming this, experiments were run in a novel trans-well system to allow for bacterial exoproducts but not cells to pass between *P. aeruginosa* and *A. baumannii*, showing a similar increase in CRISPR-based resistance in the *P. aeruginosa* populations grown in proximity to *A. baumannii*. This study suggests that the effect of *A. baumannii* and other conditioned media may be caused by changes to the chemical environment.

Chapter IV looks at how bacteria-phage interactions can shape microbial community dynamics, and how this in turn may be affected by phage resistance evolution. Using the same microbial system and phage as in Chapter II, the results showed that while *P. aeruginosa* is the dominant species in the absence of phage, *A. baumannii* quickly takes over once a *P. aeruginosa* targeting phage is introduced. In addition to this competitive release of *A. baumannii*, the addition of phage also facilitated the maintenance of bacterial diversity as both *S. aureus* and *B. cenocepacia* also performed better in phage treatments. A *P. aeruginosa* strain with the ability to evolve CRISPR-based phage resistance did better in the presence of *A. baumannii* than an isogenic mutant lacking a functional CRISPR

system. However, this effect was lost as phage was driven extinct. These findings highlight how phages may cause competitive release and diversity maintenance, which may consequently lead to polybacterial infection.

Contribution of published papers

Chapter II – Alseth E.O., Pursey E., Luján A.M., McLeod I., Rollie C. & Westra E.R. (2019). Bacterial biodiversity drives the evolution of CRISPR-based phage resistance in Pseudomonas aeruginosa. *Nature* 574, 549-552. This study examines the natural selection of phage resistance mechanisms in the context of polybacterial competition and clinical infection for the first time.

Statement of contributions as co-author

My contributions to the published and unpublished papers in this thesis are as follows, and I thank the reviewers for their contributions towards Chapter II.

Chapter II – Alseth E.O., Pursey E., Luján A.M., McLeod I., Rollie C. & Westra E.R. Conceptualisation of the study was done by E.R.W. and myself. Experimental design was carried out by me, A.M.L., C.R. and E.R.W. All experiments were done by me, E.P., and I.M. Formal analysis of results was done by me, E.P., C.R. and E.R.W. The original draft was written by me, with later edits and reviews done by E.R.W. and myself.

Chapter III – Alseth E.O., Da Silva Custodio R., Gurney J., Brown S. & Westra E.R. Conceptualisation and experimental design was done by E.R.W., R.D.C. and me. The novel trans-well system, which was key to experimental findings, was developed by J.G. and S.B. Experiments and formal analyses were carried out by R.D.C. and me. Original manuscript draft was written by me, with comments and crucial revisions by E.R.W. and R.D.C.

Chapter IV – Alseth E.O., Brown S. & Westra E.R. The study was designed and conceptualised by E.R.W and myself, and I carried out all experiments and formal analyses. I wrote the original manuscript, with comments and critical revisions provided by S.B. and E.R.W.

Chapter II – Bacterial biodiversity drives the evolution of CRISPR-based phage resistance

Publication details

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The main text, references, figures, and supplemental information are the same as those that appear in the publication. They have however been formatted to follow submission requirements and to ensure thesis text consistencies.

Abstract

Approximately half of all bacterial species encode CRISPR-Cas adaptive immune systems (Grissa et al., 2007), which provide immunological memory by inserting short DNA sequences from phage and other parasitic DNA elements into CRISPR loci on the host genome (Barrangou et al., 2007). Whereas CRISPR loci evolve rapidly in natural environments (Andersson & Banfield, 2008; Laanto et al., 2017), bacterial species typically evolve phage resistance by the mutation or loss of phage receptors under laboratory conditions (Westra et al., 2015; van Houte, Buckling, et al., 2016). Here, we report how this discrepancy may in part be explained by differences in the biotic complexity of *in vitro* and natural environments (Hibbing et al., 2010; O'Toole, 2017). Specifically, using the opportunistic pathogen *Pseudomonas aeruginosa* and its phage DMS3*vir*, we show that coexistence with other human pathogens amplifies the fitness trade-offs associated with phage receptor mutation, and therefore tips the balance in favour of CRISPR-based resistance evolution. We also demonstrate that this has important knock-on effects for *P. aeruginosa* virulence, which became attenuated only if the bacteria evolved surface-based resistance. Our data reveal that the biotic complexity of microbial communities in natural environments is an important driver of the evolution of CRISPR-Cas adaptive immunity, with key implications for bacterial fitness and virulence.

Introduction

Pseudomonas aeruginosa is a widespread opportunistic pathogen that thrives in a range of different environments, including hospitals, where it is a common source of nosocomial infections. In particular, it frequently colonises the lungs of cystic fibrosis patients, in whom it is the leading cause of morbidity and mortality (Folkesson et al., 2012). In part fuelled by a renewed interest in the therapeutic use of bacteriophages as antimicrobials (phage therapy)(Roach & Debarbieux, 2017; Rossitto et al., 2018), many studies have examined if and how *P. aeruginosa* evolves resistance to phage (reviewed in (De Smet et al., 2017)). The clinical isolate *P. aeruginosa* strain PA14 has been reported to predominantly evolve resistance against its phage DMS3*vir* by the modification or complete loss of the phage receptor (Type IV pilus) when grown in nutrient-rich medium (Westra

et al., 2015), despite carrying an active CRISPR-Cas adaptive immune system (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR-associated). Conversely, under nutrient-limited conditions, the same strain relies on CRISPR-Cas to acquire phage resistance (Westra et al., 2015). These differences are due to higher phage densities during infections in nutrient-rich compared to nutrient-limited conditions, which in turn determines whether surface-based resistance (with a fixed cost of resistance) or CRISPR-based resistance (infection-induced cost) is favoured by natural selection (Chabas et al., 2016; Westra et al., 2015). While these observations suggest abiotic factors are critical determinants of the evolution of phage resistance strategies, the role of biotic factors has remained unclear, even though *P. aeruginosa* commonly co-exists with a range of other bacterial species in both natural and clinical settings (Harrison, 2007; O'Brien & Fothergill, 2017). We hypothesised that the presence of a bacterial community could drive increased levels of CRISPR-based resistance evolution for mainly two reasons. Firstly, reduced *P. aeruginosa* densities in the presence of competitors may limit phage amplification, favouring CRISPR-based resistance (Westra et al., 2015). Secondly, pleiotropic costs associated with phage receptor mutation may be amplified during interspecific competition.

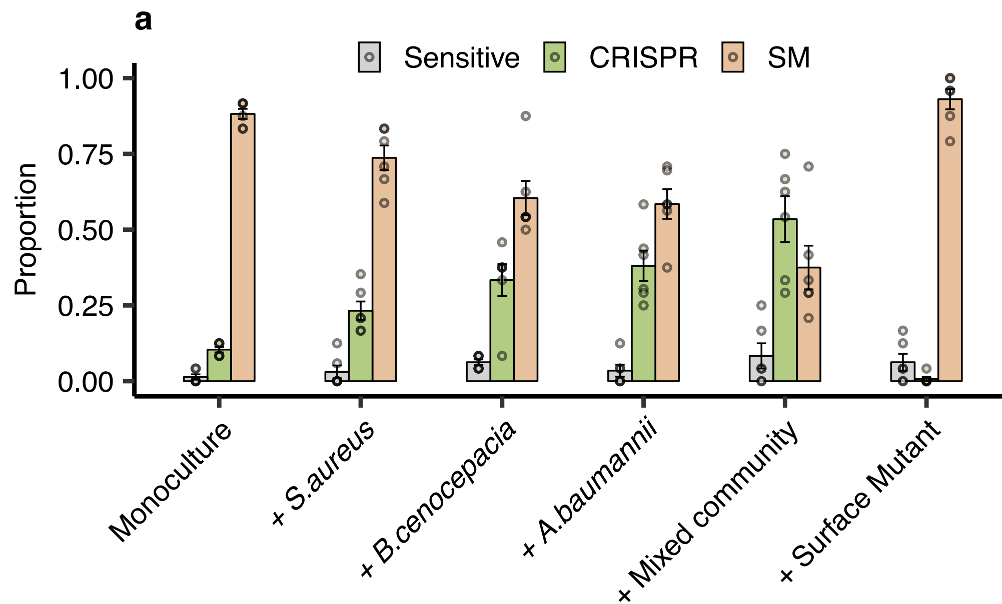
Results

Bacterial biodiversity drives CRISPR evolution

To explore these hypotheses, we co-cultured *P. aeruginosa* PA14 with three other clinically relevant opportunistic pathogens that can co-infect with *P. aeruginosa*, namely *Staphylococcus aureus*, *Burkholderia cenocepacia*, and *Acinetobacter baumannii* (Harrison, 2007; O'Brien & Fothergill, 2017; Bhargava et al., 2014; Rocha et al., 2018), none of which can be infected by or interact with phage DMS3*vir* (Fig. S2.1). We applied a "mark-recapture" approach using a *P. aeruginosa* PA14 mutant carrying streptomycin resistance in order to monitor the bacterial population dynamics and phage resistance evolution in the focal subpopulation at 3 days post infection (DPI). This revealed that in nutrient-rich Lysogeny Broth, PA14 evolved significantly higher levels of CRISPR-based resistance following infection with 10^6 plaque forming units (PFU) of phage DMS3*vir* when co-cultured with other bacterial species compared to when grown

in isolation or co-cultured with an isogenic surface mutant (Fig. 2.1a). Additionally, we found that these effects were dependent on the identity of the species that were present in the mixed culture, with the strongest effects being observed in the presence of *A. baumannii* or a mix of the three bacterial species, and an absence of any effect when PA14 was co-cultured with an isogenic surface mutant that lacked the phage receptor (Fig. 2.1a, Deviance test: Relationship between community composition and CRISPR; Residual deviance(30, n = 36) = 1.81, p = 2.2 x 10^{-16}; Tukey contrasts: Monoculture v Mixed; z = -5.99, p = 3.02 x 10^{-8}; Monoculture v *A. baumannii*; z = -4.33, p = 0.00023; Monoculture v *B. cenocepacia*; z = -3.76, p = 0.0026; Monoculture v *S. aureus*; z = -2.38, p = 0.26; Monoculture v surface mutant; z = 2.26, p = 0.35). Interestingly, *P. aeruginosa* densities were strongly reduced in the presence of *A. baumannii*, *B. cenocepacia* and the mixed community, while on the other hand it dominated the community during competition with *S. aureus* despite the presence of phage DMS3*vir* (Fig. 2.1b), suggesting a positive relationship between the strength of interspecific competition and the levels of CRISPR-based resistance evolution.

Next, to explore the clinical relevance of this observation, we performed a similar experiment in artificial sputum medium (ASM), which is a nutrient rich medium that mimics the abiotic environment of sputum from cystic fibrosis patients (Sriramulu, 2010). This revealed a similar pattern as that observed in Lysogeny Broth, with *A. baumannii* and the community as a whole resulting in a drastic increase in CRISPR-based resistance (Fig. 2.2). To further explore the generality of these findings, we also manipulated the microbial community composition by varying the proportion of *P. aeruginosa* versus the other pathogens. This revealed that increased CRISPR-based resistance evolution occurred across a wide range of microbial community compositions, with a maximum effect size when *P. aeruginosa* made up 50% of the initial mixture (Fig. S2.2). An exception to this trend was when the *P. aeruginosa* subpopulation made up only 1% of the total community; in this case sensitive bacteria persisted alongside resistant bacteria because of the reduced size of the phage epidemic and hence relaxed selection for resistance (Fig. S2.2). Collectively, these data suggest that greater levels of interspecific competition contribute to the evolution of CRISPR-based resistance.



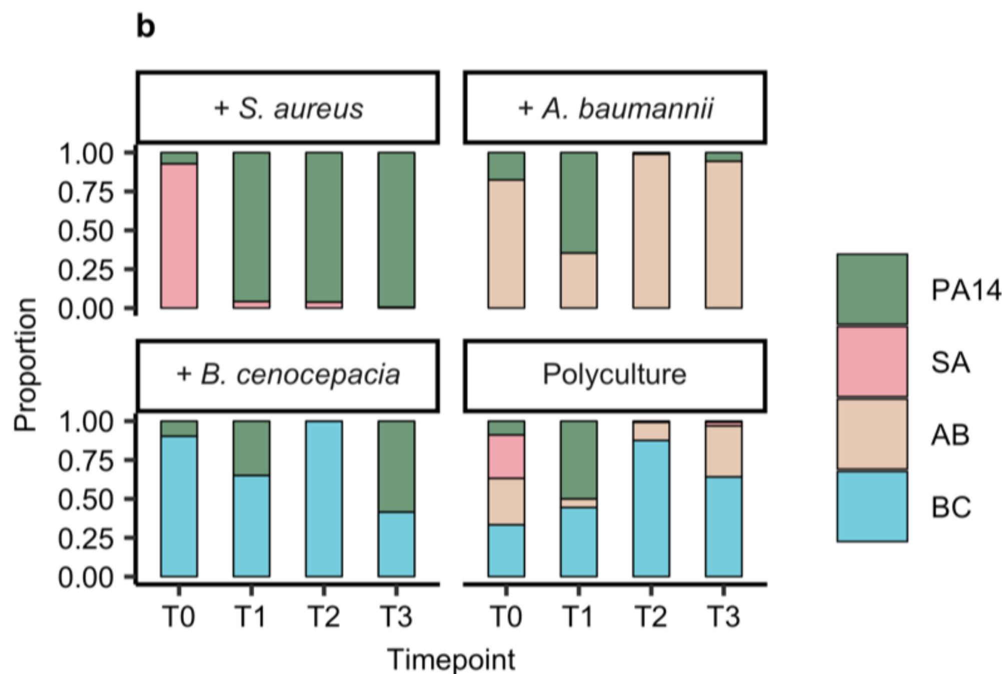


Figure 2. 1 | Biodiversity affects the evolution of phage resistance. (**a**) Proportion of *P. aeruginosa* that acquired surface- (SM) or CRISPR-based resistance, or remained sensitive at 3 d.p.i. with phage DMS3*vir* in LB medium when grown in monoculture or polycultures, or with an isogenic surface mutant (6 replicates per treatment, with 24 colonies per replicate, n = 36 biologically independent replicates). Error bars ± one SE, with the mean as centre. (**b**) Microbial community composition over time for the mixed-species infection experiments. Legend abbreviations: PA14 = *P. aeruginosa*, SA = *S. aureus*, AB = *A. baumannii*, and BC = *B. cenocepacia*.

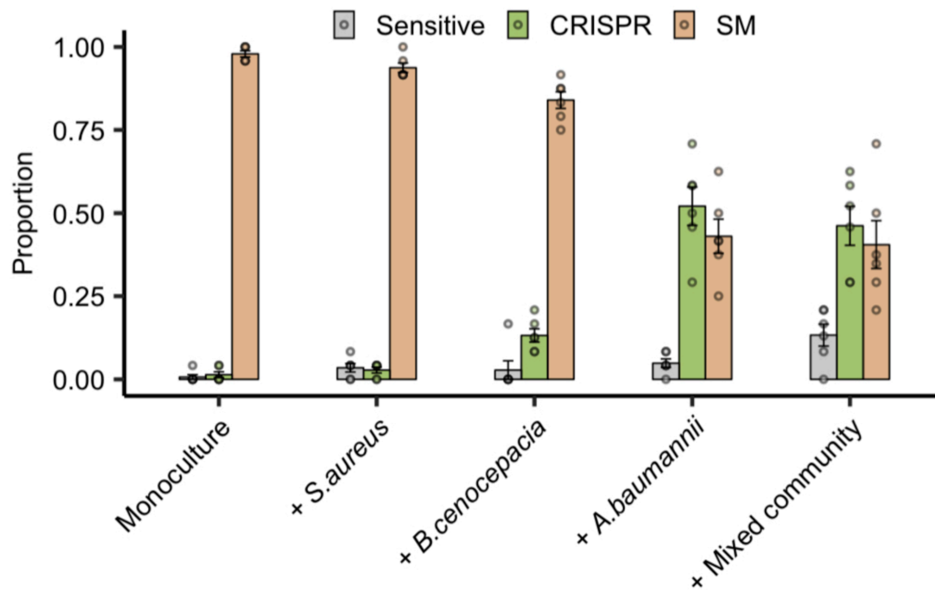


Figure 2.2 | Enhanced CRISPR resistance evolution in artificial sputum medium. Proportion of *P. aeruginosa* that acquired surface modification (SM) or CRISPR-based immunity (or remained sensitive) at 3 days post infection with phage DMS3*vir* when grown in artificial sputum medium (6 replicates per treatment, with 24 colonies screened from each replicate, n = 30 biologically independent replicates). Deviance test: Relationship between community composition and CRISPR; Residual deviance(25, n = 30) = 1.26, $p = 2.2 \times 10^{-16}$; Tukey contrasts: Monoculture v Mixed; z = -5.30, $p = 1 \times 10^{-4}$; Monoculture v *A. baumannii*; z = -5.60, $p = 1 \times 10^{-4}$; Monoculture v *B. cenocepacia*; z = -2.80, p = 0.02; Monoculture v *S. aureus*; z = -0.76, p = 0.93. Error bars correspond to ± one standard error, with the mean as the measure of centre.

Biodiversity amplifies costs of surface-based resistance

We hypothesised that reduced *P. aeruginosa* population sizes in the presence of competitors might explain the increased evolution of CRISPR-based resistance, as this leads to smaller phage epidemics, which is known to favour CRISPR over surface-based resistance (Westra et al., 2015). However, variation in the force of infection did not seem to play a strong role in the observed effects, since even though phage epidemic sizes varied depending on the microbial community composition (Fig. S2.3), this did not correlate with the levels of evolved CRISPR-resistance (Fig. S2.4). Moreover, when manipulating the DMS3*vir* starting phage titres, we observed no differences in the levels of evolved CRISPR-based resistance when *P. aeruginosa* was co-cultured in the presence of the microbial community (Fig. S2.5). An alternative explanation for the observed effects may therefore be that the fitness cost of surface-based resistance is amplified in the presence of other bacterial species, for example due to cell surface molecules playing a part in interspecific competition (An et al., 2006), which again would result in stronger selection towards bacteria with CRISPR-based resistance. To test this hypothesis, we competed the two phage resistant phenotypes (i.e. CRISPR-resistant and surface mutant) in the presence or absence of the microbial community, and across a range of phage titres. In the absence of the microbial community and phage, CRISPR-resistant bacteria had a small fitness advantage over bacteria with surface-based resistance, but this advantage disappeared when phage was added and as titres increased (Fig. 2.3a, and ref. 5). In the presence of the biodiverse microbial community however, the relative fitness of bacteria with CRISPR-based resistance was consistently higher, demonstrating that mutation of the Type IV pilus is more costly when bacteria compete with other bacterial species (Fig. 2.3a, Linear model: Effect of community absence; t = -5.54, p = 1.49 x 10^{-7}; Effect of increasing phage titre; t = -2.41, p = 0.017; Overall model fit; Adjusted R^2 = 0.41, $F_{4,139}$ = 25.48, p = 7.65 x 10^{-16}). The increased fitness trade-off associated with surface-based resistance was also observed when the CRISPR- and surface-resistant phenotypes competed in the presence of only a single additional species (Fig. 2.3b, Two-way ANOVA with Tukey contrasts: Overall difference in fitness; $F_{4,2}$ = 8.151 p = 6.31 x 10^{-6}; Monoculture v Mixed; p = 0.011; Monoculture v *A. baumannii*; p = 0.016; Monoculture v *B. cenocepacia*; p = 0.022), with the exception of

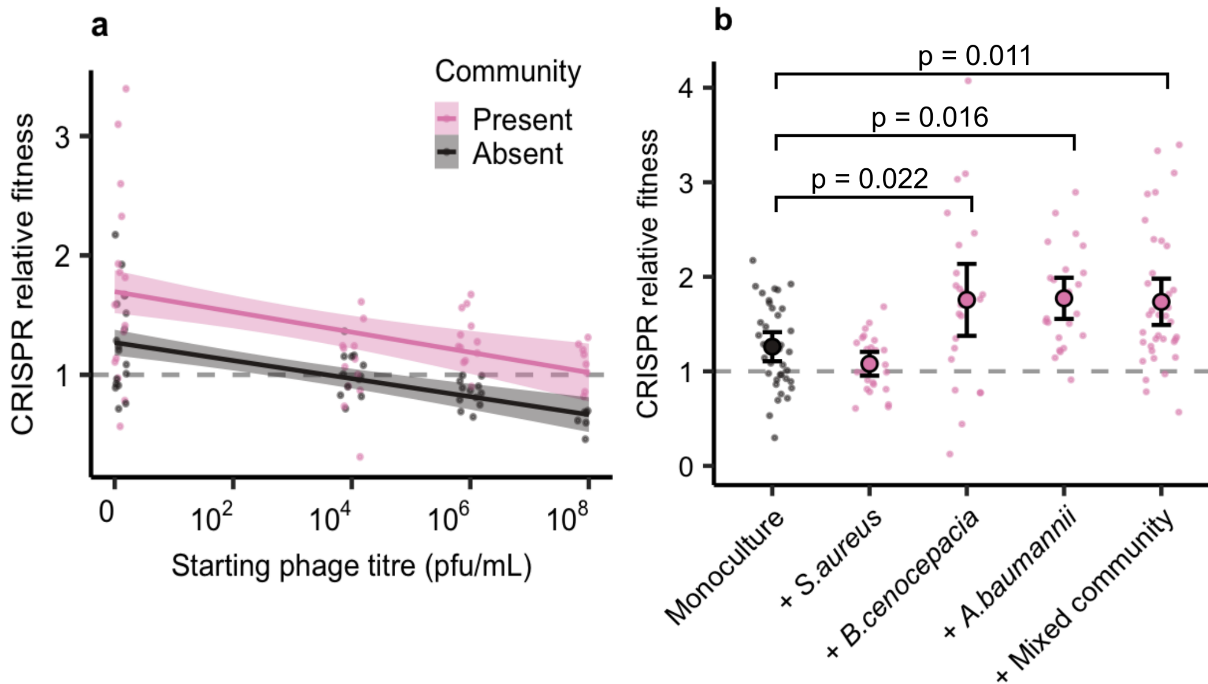


Figure 2.3 | Biodiversity amplifies fitness costs associated with surface-based resistance. Relative fitness of a *P. aeruginosa* clone with CRISPR-based resistance after competing for 24h against a surface modification clone at (**a**) varying levels of phage DMS3*vir* in the presence or absence of a mixed microbial community. Regression slopes with shaded areas corresponding to 95% CI (n = 144 biologically independent samples). (**b**) Relative fitness after competition in the absence of phage, but in the presence of other bacterial species individually or as a mixture. Error bars 95% CI and the mean as centre (n = 144 biologically independent samples).

S. aureus (Fig. 2.2b. Monoculture v *S. aureus*; p = 0.80), concordant with this species being the weakest competitor and inducing the lowest levels of CRISPR-based resistance (Fig. 2.1). These fitness trade-offs therefore explain why *P. aeruginosa* evolved greater levels of CRISPR-based resistance in the presence of the other pathogens, and why this varied depending on the competing species (Fig. 2.1).

Type of phage-resistance affects *P. aeruginosa* virulence

Evolution of phage resistance by bacterial pathogens is often associated with virulence trade-offs when surface structures are modified (León & Bastías, 2015), whereas similar trade-offs have not yet been reported in the literature for CRISPR-based resistance. We therefore hypothesised that the community context in which phage resistance evolves may have important knock-on effects for *P. aeruginosa* virulence. To test this, we used a *Galleria mellonella* infection model, which is commonly used to evaluate virulence of human pathogens (Hernandez et al., 2019; Kavanagh & Reeves, 2004). We compared *in vivo* virulence of *P. aeruginosa* clones that evolved phage resistance against phage DMS3*vir* in different community contexts by injecting larvae with a mixture of clones that had evolved phage-resistance in either the presence or absence of the mixed bacterial community (Fig. S2.4c). Taking time to death as a proxy for virulence, we found that evolution of phage resistance in the presence of a microbial community was associated with greater levels of *P. aeruginosa* virulence compared to when phage-resistance evolved in monoculture, and remained similar to that of the ancestral PA14 strain (Fig. 2.4a, Cox proportional hazards model with Tukey contrasts: Community present v absent; z = 5.85, p = 1×10^{-4}; ancestral PA14 v community absent; z = 4.42, p = 1×10^{-4}; ancestral PA14 v community present; z = -1.30, p = 0.38. Overall model fit; LRT_3 = 51.03, n = 376, p = 5×10^{-11}). These data, in combination with the fact that the Type IV pilus is a well-known virulence factor (Craig et al., 2004), are consistent with the notion that the mechanism by which bacteria evolve phage resistance has important implications for bacterial virulence. To more directly test this, we next infected larvae with each individual *P. aeruginosa* clone for which we had previously determined the mechanism underlying evolved phage resistance (Fig. S2.4c), again using time to death as a measure of virulence. This showed that

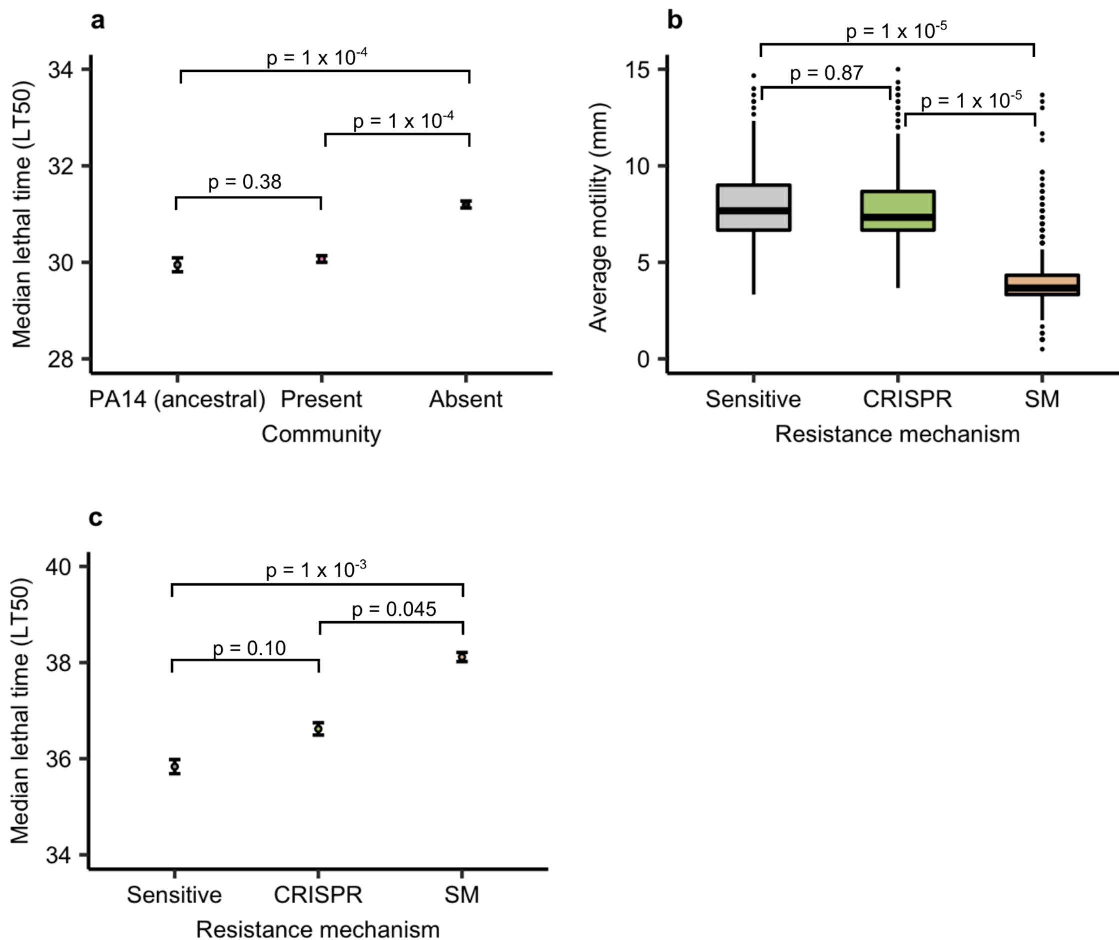


Figure 2.4 | Evolution of phage resistance affects *in vivo* virulence. (**a**) Time to death (given as the median ± one standard error) following infection with PA14 clones that evolved phage resistance either in the presence or absence of a mixed microbial community (n = 376 biologically independent samples, analysed using a Cox proportional hazards model with Tukey contrasts). Type of evolved phage resistance (CRISPR- or surface-based (SM)) drastically impacted (**b**) bacterial motility (n = 981 biologically independent samples). Boxplots show the median with the upper and lower 25th and 75th percentiles, the inter-quartile range, and outliers shown as dots. (**c**) Type of resistance also affected *in vivo* virulence (time to death, given as the median ± one standard error, n = 981, analysed using a Cox proportional hazards model with Tukey contrasts).

bacterial clones with surface-based resistance – unlike those with CRISPR-based resistance - both had drastically reduced swarming motility (as expected for mutations in the Type IV pilus[23]) (Fig. 2.4b, One-way ANOVA with Tukey contrasts: Overall effect; $F_{2,977} = 472.5$, $p = 2.2 \times 10^{-16}$; Sensitive v CRISPR; $p = 0.87$; CRISPR v Surface mutant ; $p = 1 \times 10^{-5}$) and impaired virulence compared to phage sensitive bacteria (Fig. 2.4c, Cox proportional hazards model with Tukey contrasts: Surface mutant v CRISPR; $z = -2.37$, $p = 0.045$; Sensitive v CRISPR; $z = 2.10$, $p = 0.10$; Surface mutant v Sensitive; $z = -4.23$, $p = 1 \times 10^{-3}$. Overall model fit; $LRT_3 = 48.66$, $n = 981$, $p = 2 \times 10^{-10}$). Similar virulence trade-offs were also observed when larvae were injected with *P. aeruginosa* PA14 clones that had evolved surface-based resistance against phage LMA2, which uses LPS (lipopolysaccharide) as a receptor (Fig. S2.6).

Discussion

We have shown that the evolutionary outcome of bacteria-phage interactions can be fundamentally altered by the microbial community context. While traditionally studied in isolation, these interactions are usually embedded in complex biotic networks of multiple species, and it is becoming increasingly clear that this can have key implications for the evolutionary epidemiology of infectious disease (Alizon et al., 2013; Benmayor et al., 2009; Chabas et al., 2018; Johnson et al., 2015; Keesing et al., 2010). Our work shows that the community context can also shape the evolution of different host resistance strategies. Specifically, we find that the interspecific interactions between four bacterial species in a synthetic microbial community can have a large impact on the evolution of phage resistance mechanisms by amplifying the constitutive fitness cost of surface-based resistance (Westra et al., 2015). The finding that biotic complexity matters complements previous work on the effect of abiotic variables and force of infection on phage resistance evolution (Westra et al., 2015). The data presented here suggests that the impact of biotic complexity on the evolution of CRISPR-based resistance is stronger than that of variation in phage abundance, which is consistent with the observation that in the presence of the polymicrobial community, bacteria with CRISPR-based resistance outcompeted bacteria with surface-based resistance at all phage titres (Fig. 2.3). The amplified fitness cost of surface mutation also suggests that the type Type IV pilus plays an important

role in interspecific competition. While future work will be critical to understand the detailed molecular mechanism that underpins these effects, and to further generalise the findings described here to other bacterial species and strains, we speculate that the way in which the microbial community composition drives the evolution of phage resistance strategies may be important in the context of phage therapy. Primarily, the absence of detectable trade-offs between CRISPR-based resistance and virulence, as opposed to when bacteria evolve surface-based resistance, suggests that evolution of CRISPR-based resistance can ultimately influence the severity of disease. Moreover, evolution of CRISPR-based resistance can drive more rapid phage extinction (van Houte, Ekroth, et al., 2016), and may in a multi-phage environment result in altered patterns of cross-resistance evolution compared to surface-based resistance (Wright et al., 2018). The identification of the drivers and consequences of CRISPR-resistance evolution might help to improve our ability to predict and manipulate the outcome of bacteria-phage interactions in both natural and clinical settings.

Material and Methods

Bacterial strains and viruses

We used a marked *P. aeruginosa* UCBPP-PA14 mutant carrying a streptomycin resistant gene inserted into the genome using pBAM1 (Martínez-García et al., 2011) (referred to as the ancestral PA14 strain). The WT PA14 bacteriophage-insensitive mutant with 2 CRISPR spacers (BIM2), the surface mutant derived from the PA14 *csy3::LacZ* strain, and phage DMS3*vir* and DMS3*vir+acrF1*(carrying an anti-CRISPR gene) have all been previously described (refs. 5 and 29 and references therein). The bacteria used as the microbial community were *Staphylococcus aureus* strain 13 S44 S9, *Acinetobacter baumannii* clinical isolate FZ21 and *Burkholderia cenocepacia* J2315, and were all isolated from patients at Queen Astrid Military Hospital, Brussels, Belgium.

Adsorption and infection assays

Phage infectivity against each of the bacterial species used in this study was assessed by spotting serial dilutions of virus DMS3*vir* on lawns of the individual community bacteria, followed by checking for any plaque formation after 24 hours

of growth at 37ºC. Adsorption assays (as shown in Supplemental Fig. 2.1) were performed by monitoring phage titres over time, for up to an hour (At 0, 2, 4, 6, 8, 10, 15 and 20 minutes post infection for PA14, and at 0, 5, 10, 20, 40 and 60 minutes post infection for the other bacteria species. For the no-bacterial control, sampling was done at 0 and 60 minutes post infection), after inoculating the individual bacteria in mid-log phase at approximately 2 x 10^8 c.f.u. with phage DMS3*vir* at 2 x 10^6 PFU (final MOI = 0.001). Adsorption assays were carried out in falcon tubes containing 15mL LB medium, incubated at 37ºC while shaking at 180 RPM (three independent replicates per species). At each timepoint, 50µl of sample was transferred to pre-cooled eppendorfs on ice, containing 900µl LB medium and 50µl chloroform, before vortexing for 10 seconds. After sampling was completed, all eppendorfs were centrifuged at full speed at 4 ºC for >5 minutes after which 300µl of the supernatant was extracted, diluted and spotted onto lawns of *P. aeruginosa* before checking for plaque formation after 24h of growth at 37 ºC.

Coevolution experiments

The streptomycin resistant mutant of the ancestral strain of *P. aeruginosa* was used for all coevolution experiments. Evolution experiments (shown in Fig. 2.1, and Figs. S2.2 and S3) were performed by inoculating 60µl from overnight cultures (containing approximately 10^6 colony-forming units (CFU)) into glass microcosms containing 6mL LB medium (Fig. 2.1 and Fig. S3), or artificial sputum medium[18] (ASM) (Fig. S2.2). 1 litre of ASM was made by mixing 5g mucin from porcine stomach (Sigma), 4g low molecular-weight salmon sperm DNA (Sigma), 5.9mg diethylene triamine pentaacetic acid (DTPA) (Sigma), 5g NaCL (Sigma), 2.2g KCl (Sigma), 1.81g Tris base (Thermo Fisher Scientific), 5mL egg yolk emulsion (Sigma), and 250mg of each of 20 amino acids (Sigma), as described in ref. 18. Inoculation was followed by incubation at 37ºC while shaking at 180 RPM (n = 6 per treatment). The polyculture mixes either consisted of approximately equal amounts of all four bacterial species or mixes of *P. aeruginosa* with just one additional species where *P. aeruginosa* made up 25% of the total volume used for inoculation (i.e. 15 µl of 60µl), unless otherwise indicated (i.e. Supplemental Fig. 2.3). Before inoculation, phage DMS3*vir* was added at 10^6 PFU (Fig. 2.1 and Fig. S2.2), or at 10^4 PFU (Fig. S2.3). Transfers of 1:100 into fresh broth were done daily for a total of three days. Additionally,

phage titres were monitored daily by spotting chlorophorm-treated lysate dilutions on a lawn of *P. aeruginosa csy3::LacZ*. Downstream analysis to determine if and how bacteria evolved phage resistance was done by cross-streak assays and PCR on 24 randomly selected clones per replicate experiment, as described in ref. 5.

DNA extraction and qPCR

For the experiment shown in Fig. 2.1, the densities of the different bacterial species in the microbial communities over time were determined using qPCR. DNA was extracted from all replicas using the Dneasy UltraClean Microbial Kit (Qiagen), following the manufacturer instructions. Prior to DNA extraction, to ensure lysis of *S. aureus*, 15µl lysostaphin (Sigma) at 0.1 mg/mL was added to 500µl of sample followed by incubation at 37ºC for at least one hour. For *P. aeruginosa*, *A. baumannii*, and *B. cenocepacia*, the 16S gene was chosen as the target for the qPCR primers and were as follows: the PA14 forward primer (PA14-16s-F), AGTTGGGAGGAAGGGCAGTA; the PA14 reverse primer (PA14-16s-R), GCTTGCTGAACCACTTACGC; the *A. baumannii* forward primer (AB-16s-F), ATCAGAATGCCGCGGTGAAT; the *A. baumannii* reverse primer (AB-16s-R), ACCGCCCTCTTTGCAGTTAG; the *B. cenocepacia* forward primer (BC-16s-F), ATACAGTCGGGGGATGACGG; the *B. cenocepacia* reverse primer (BC-16s-R), TCACCAATGCAGTTCCCAGG. For *S. aureus*, we used qPCR primers previously described in (Goto et al., 2007). The amplification reactions were performed in triplicates, with Brilliant SYBR Green reagents (Agilent) in 20µl reactions made up of 10µl master mix, 2µl primer pair, 0.4µl dye, and sterile nuclease free water to a total volume of 15µl before adding 5µl diluted DNA sample. The qPCR program was as follows: 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds and 60°C for 30 seconds. All qPCR's and results were analysed using the Applied Biosystems QuantStudio 7 Flex Real-Time PCR system, before adjusting output for gene copy number (4, 1, 6, 6, for *P. aeruginosa*, *S. aureus*, *A. baumannii*, and *B. cenocepacia* respectively).

Competition experiments

For both competition experiments shown in Fig. 2.2, the BIM2 clone was competed against the surface mutant derived from the PA14 *csy3::LacZ* strain[5]. Bacteria were grown for 24 hours in glass microcosms containing 6mL LB

medium, in a shaking incubator at 180 RPM and at 37°C. For the experiment shown in Fig. 2.2a, the two phenotypes were competed in the presence or absence of the mixed microbial community, either without the addition of phage (n = 36), or infected with phage DMS3*vir* at 10^4, 10^6, and 10^8 PFU (n = 12 per treatment). For the experiment shown in Fig. 2b, the two phage resistant phenotypes were again competed either in the presence or absence individual bacterial species or a mixed community of all species. *P. aeruginosa* made up 25% of the total volume of 60µl that was used to inoculate the 6mL of LB medium (n = 24 per treatment). Samples were taken at 0 and 24 hours post infection., and the cells were serial diluted in M9 salts and plated on cetrimide agar (Sigma) supplemented with ca. 50µg ml^{-1} X-gal (to select for *P. aeruginosa*, while also differentiating between the CRISPR-resistant clones (white) and the surface mutant (blue)). Relative fitness was calculated as described in refs. 5 and 29.

Virulence assays

All infection experiments were done using *Galleria mellonella* larvae (UK WaxWorm Ltd). Throughout the experiments, the larvae were stored in 12-well plates, with one larva per well, and were all checked for mortality and melanisation before injection. Bacterial inoculums were prepared depending on experiment, and were as follows; For the experiment shown in Fig. 2.3a, all 24 evolved clones from each replicate from the 25% (community present) and 100% (community absent) treatments (Fig. S2.3) were pooled together by replica (n = 6 per treatment) and mixed in 6mL of LB medium. Each mixture of clones was injected into ten individual larvae, with time to death measured as a proxy for virulence. This procedure was performed in three independent repeats by injecting the same mixtures of bacterial clones into independent batches of larvae in separate experiments (total no. of larvae = 420). To assess virulence of all evolved clones (Fig. 2.3c), infections were done independently using all the individual PA14 clones from 3 DPI from the experiment shown in Fig. S2.3 (n = 1008). Here (Fig. 2.3c), the bacterial inoculums were prepared individually for each clone by inoculating 200µl LB medium with 5µl bacterial sample from freezer stock, repeated for all individual clones in 96-well plates. Finally, to measure whether surface-based resistance against an LPS-specific phage was associated with similar virulence trade-offs (Fig. S2.7), we isolated *P. aeruginosa* clones from 6 independent infection experiments with phage LMA2. A total of 10 clones per

replicate experiment, isolated from 3 DPI, were phenotypically characterised to confirm resistance, and examined by PCR to exclude that resistance was CRISPR-based. All 10 clones with LPS-based resistance from the same replicate experiment were pooled together in 6mL of LB medium (n = 6), and infections of *G. mellonella* larvae were carried out as described above, with each mixture of clones injected into ten individual larvae, performed in three independent repeats (total no. of larvae = 240). Prior to infection, all bacterial inoculums were grown overnight at 37ºC on an orbital shaker (180 RPM) before being diluted by adding 20µl to 180µl of M9 salts. Cell density was then assayed by measuring OD_{600} absorbance, with 0.1OD being ~1 x 10^8 CFU/mL, before being further diluted down to approximately 10^4 CFU/mL, which was subsequently used for infection by injecting 10µl into the rear proleg of individual *G. mellonella* using a sterile syringe as further described in ref. 22. OD measurements and experimental repeats were taken into account during formal data analysis. Following infection, larvae were incubated at 28ºC, with mortality monitored hourly for up to 48 hours. For all independent experiments, a control where larvae were injected with just M9 salts was included. All work conforms to ethical regulations regarding the use of invertebrates, with approval from The University of Exeter ethics committee.

Motility assays

Swarming motility of all evolved bacterial clones from the experiment shown in Supplemental Fig. 2.3c (n = 1008) was assayed by using a 96-well microplate pin replicator to stamp the individual clones on 1% agar before overnight growth at 37ºC. The diameters of the individual clones were then taken as a measure of motility (three replicas per clone).

Statistical analyses

To test the effects of interspecific interactions on the evolution of CRISPR-based phage resistance (Fig. 2.1a and Fig. 2.2), we used quasibinomial generalised linear models (GLM) with proportion of *P. aeruginosa* CRISPR clones as the response variable, and treatment and replica as explanatory variables.

When looking at relative fitness of CRISPR clones and how this is influenced by phage titre and the presence of a microbial community, we used linear models to test the effects of increasing phage titres (Fig. 2.3a) in the presence of all community members, or the effect of various community species on relative

fitness in the absence of phage (Fig. 2.3b). The response variable in both cases was relative fitness, with treatment, replica, and experimental repeat included as explanatory variables, as well as starting phage titre where applicable.

Galleria mellonella mortality analyses were done using the Survival package version 2.38 (Therneau, 2021). Cox proportional hazards models were used, with the response variable derived from the Surv function, which takes start time, end time, and larvae survival status into consideration. The explanatory variables were either the treatment conditions PA14 evolved in (Fig. 2.4a) or type of phage resistance of the infection *P. aeruginosa* clone (Fig. 2.4c). Additionally, OD600 measurements done prior to infection were also included as a possible explanatory variable, to control for varying starting densities. Differences in motility (Fig. 2.4b) were assessed by a linear model with motility as response variable, and type of phage resistance evolved and treatment the clones were from as explanatory variables.

For all data analyses, additional post-hoc pairwise comparisons were done using the Multcomp package version 1.4-13 (Hothorn et al., 2008), and were all Bonferroni adjusted where appropriate. All model fits were assessed using Chi-squared tests and by comparing Akaike information criterion (AIC) values, as well as plotting residuals and probability distributions using histograms and quantile-quantile plots (Q-Q plots) respectively. All statistical analyses were done using R version 3.5.1. (R Core Team, 2020), and the Tidyverse package version 1.2.1. (Wickham et al., 2019).

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Chapter III – Increased evolution of CRISPR-based phage resistance in *Pseudomonas aeruginosa* in the presence of *Acinetobacter baumannii* is independent of cell-to-cell contact

Abstract

Bacteria often thrive in complex microbial communities where they influence the growth and evolution of one another through either physical or chemical interactions, or both. We previously reported that competition between *Pseudomonas aeruginosa* and *Acinetobacter baumannii* results in increased levels of CRISPR-based phage resistance in the opportunistic pathogen *P. aeruginosa* in response to its phage DMS3vir. However, the molecular mechanisms driving this effect remain largely unexplored. Here, we report how growth of *P. aeruginosa* in conditioned media from *Acinetobacter baumannii* without direct cell-to-cell contact results in a similar shift towards CRISPR-based phage resistance as the one observed in the physical presence of this competitor species. Our results suggest that the eco-evolutionary dynamics of bacteria-phage interactions are driven by contact independent factors. Identification of potential chemical changes or signals involved would offer ways to manipulate the evolution of phage resistance using small molecules in clinical contexts.

Introduction

Since their discovery, bacteria were long assumed to primarily exist, and consequently studied, in isolation (Kaufmann & Schaible, 2005). However, over the last couple of decades, researchers have become increasingly interested in the complex biotic networks bacteria are part of, and subsequently have started studying the implications of interspecific interactions on a multitude of eco-evolutionary dynamics. Be they synergistic or antagonistic, these interactions can for instance affect community formation and spatial organisation (Coyte et al., 2021; Stacy et al., 2016), gene role conversion (Ibberson et al., 2017), and virulence evolution (Gospodarek et al., 2009), with consequences for disease progression and morbidity in humans (Bisht et al., 2020; Lloyd-Price et al., 2017).

However, we still lack knowledge when it comes to how bacteria-phage interactions are affected by interspecific dynamics, especially those found in polymicrobial infections. Still, few studies on bacteria-phage ecology and evolution consider polymicrobial interactions, and those that do commonly use highly simplified two-species model communities or extremely complex study

systems, with very little in-between (Blazanin & Turner, 2021). This makes untangling polymicrobial interactions, and their underlying drivers and downstream effects a challenge. Yet understanding these dynamics is of particular importance due to the renewed interest in bacteriophages (phages) as antimicrobials (Kortright et al., 2019; Petrovic Fabijan et al., 2020). There is especially a gap in the literature regarding how bacteria evolve to overcome phages *in vivo*, what selection mechanisms are at play and whether we can use them to our benefit, which will be key in making phage therapy a success (Gurney et al., 2020).

Bacteria-phage dynamics are complicated by the many different means by which bacteria can evolve phage resistance. These mechanisms may act at various stages of phage infection, and be innate or adaptive (Rostøl & Marraffini, 2019). In the opportunistic pathogen *Pseudomonas aeruginosa*, phage resistance is primarily acquired through systems that either inhibit initial phage adsorption, or CRISPR-Cas (Cady et al., 2012; Harvey et al., 2018). Initial phage adsorption is hindered by surface modification of the receptor, which for many *P. aeruginosa* phages is either LPS or the Type IV pilus (Harvey et al., 2018; Huszczynski et al., 2019), whereas CRISPR-Cas works by integrating a small DNA sequence from phage and other parasitic DNA elements into the CRISPR loci for later immunological memory (Barrangou et al., 2007). While surface modification is often associated with costs due to loss of functions carried by the receptor (Anyan et al., 2014; Burrows, 2012; Hahn, 1997), the costliness of CRISPR-based phage resistance is considered to be largely inducible and associated with high infection risk from phage (Meaden et al., 2020; Westra et al., 2015).

In addition, the presence of other bacterial species can amplify the fitness cost associated with surface modification, to the point where selection tips the balance in favour of CRISPR-Cas in *P. aeruginosa* (Alseth et al., 2019). Biotic factors like the presence of a microbial community can thus shape the ecological and evolutionary effects of phage (Blazanin & Turner, 2021), however we still do not know the underlying molecular mechanism(s) that underpin these effects. The increase in CRISPR-based phage resistance in *P. aeruginosa* that we previously reported (Alseth et al., 2019) could be driven by contact dependent or independent effects, or a mixture of both, for instance through the competition for

shared nutrients, quorum sensing, production of antimicrobials, and direct interference through the Type IV Secretion System (Ghoul & Mitri, 2016; Hibbing et al., 2010). Understanding these mechanisms of phage resistance evolution is becoming of increasing importance in the light of novel therapeutics involving phages, largely because of the risk of resistance and the potential to manipulate evolutionary trade-offs to our benefit.

Results

Conditioned media from *A. baumannii* culture affects phage resistance evolution in *P. aeruginosa*

Our previous experiments on the evolution of phage resistance were all done in liquid cultures being continuously mixed at 180 rotations per minute (RPM). Under these conditions, direct cell-to-cell contact between bacterial species is expected to be limited. We therefore hypothesised that the increase in the evolution of *P. aeruginosa* CRISPR-based immunity in the presence of *A. baumannii* is caused by *A. baumannii*-induced changes to the chemical environment. To test this hypothesis, we co-cultured the PA14 WT strain with its lytic phage DMS3vir for three days; either in lysogeny broth (LB) or in cell-fee conditioned media from the PA14 WT strain, *Staphylococcus aureus*, *Acinetobacter baumannii*, or *Burkholderia cenocepacia*: all three of which *P. aeruginosa* is known to co-infect and compete with (Bhargava et al., 2014; Harrison, 2007; O’Brien & Fothergill, 2017; Rocha et al., 2018). Conditioned media was prepared by inoculating bacteria in LB broth for 24h, before centrifugation and fine filtering to eliminate remaining bacterial cells from the media, while retaining small molecules. The experiment was carried out until three days post infection (DPI) with 10^6 plaque forming units of phage DMS3vir, with daily transfers into freshly prepared conditioned media, or LB for the control groups. Additionally, *P. aeruginosa* was inoculated in the conditioned media from *P. aeruginosa* and *A. baumannii* that had been grown together for 24h. This enabled testing of whether compounds that are specifically released by *A. baumannii* during pairwise competition, such as toxins and/or signalling molecules, are involved in the shift towards CRISPR-based phage resistance.

In our control experiment, where we co-cultured *P. aeruginosa* in the same way as in our previous study (Alseth et al., 2019), we observed a significant increase in the proportion of bacteria that acquired CRISPR-based resistance against phage DMS3vir when co-culturing *P. aeruginosa* with *A. baumannii*. Specifically: when compared to *P. aeruginosa* in monoculture, we observed an increase in the proportion of CRISPR-based resistance that was acquired in response to phage DMS3vir (Fig. 3.1: Wilcoxon rank sum test; $p = 0.029$), which was in concordance with our previous findings (Alseth et al., 2019). Interestingly, when we infected PA14 with DMS3vir in *P. aeruginosa* conditioned media, this also resulted in a shift towards CRISPR-based phage resistance when compared to the PA14 control in LB (Fig. 3.1: Generalised linear model (GLM), quasibinomial; $t = -2.172$, $p = 0.0379$; residual deviance(30, n = 41) = 3.29). In conditioned media from *A. baumannii* alone or *A. baumannii* co-cultured with *P. aeruginosa*, we found an even further increase in the observed proportion *P. aeruginosa* clones that had evolved CRISPR-based immunity (Fig. 3.1: GLM, quasibinomial; PA14 v *A. baumannii* conditioned media; $t = 2.317$, $p = 0.027$; PA14 v PA14 + *A. baumannii* conditioned media; $t = 2.757$, $p = 0.0098$). This shows that *A. baumannii* can drive a shift from surface- to CRISPR-based phage resistance, whether it was physically present in the population or not (Fig. 3.1). Hence, these results indicate that changes in the chemical environment were solely or partially responsible for triggering the observed evolutionary responses.

Contact independent selection for CRISPR-based phage resistance by *A. baumannii*

The results from the conditioned media experiments show that *A. baumannii* impacts the evolution of phage resistance in *P. aeruginosa* through mechanisms that do not require direct cell-to-cell contact. To confirm this under conditions more closely mimicking the co-culture experiments in LB, we developed a trans-well system to permit for two bacteria to grow in near proximity, allowing for adaptive responses to one another, but without direct, physical contact. These trans-wells fit inside a 24-well plate, with a filter running vertically across the centre to permit for small molecules to pass through, but not bacterial cells. Using this system, we carried out infection experiments with *P. aeruginosa* and phage DMS3vir, in either the presence or absence of *A. baumannii*. Bacteria were transferred daily, into new, sterile wells with fresh LB media, until 3 DPI, with

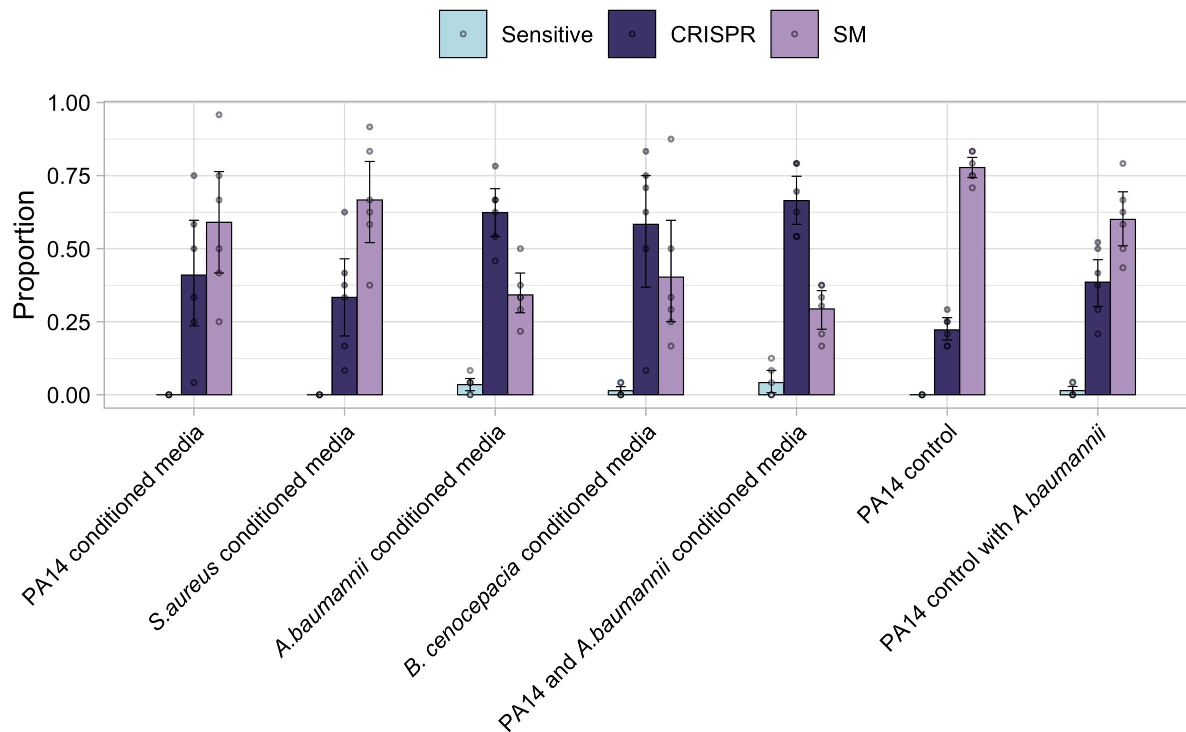

Figure 3.1 | The evolution of phage resistance is affected by conditioned media from *A. baumannii*. Proportion of *P. aeruginosa* clones that evolved phage resistance through either surface modification (SM) or CRISPR-Cas, or remained sensitive to phage DMS3vir at 3 DPI in conditioned media or LB controls (6 replicates per treatment, with 24 colonies per replicate, n = 42 biologically independent replicates). Error bars ± 95% CI, with the mean as centre. Asterisks indicate statistically significant effect of treatments compared to the PA14 conditioned media (GLM: * $p < 0.05$; ** $p < 0.01$), or a significant difference between control groups (Wilcoxon signed rank exact test: * $p < 0.05$).

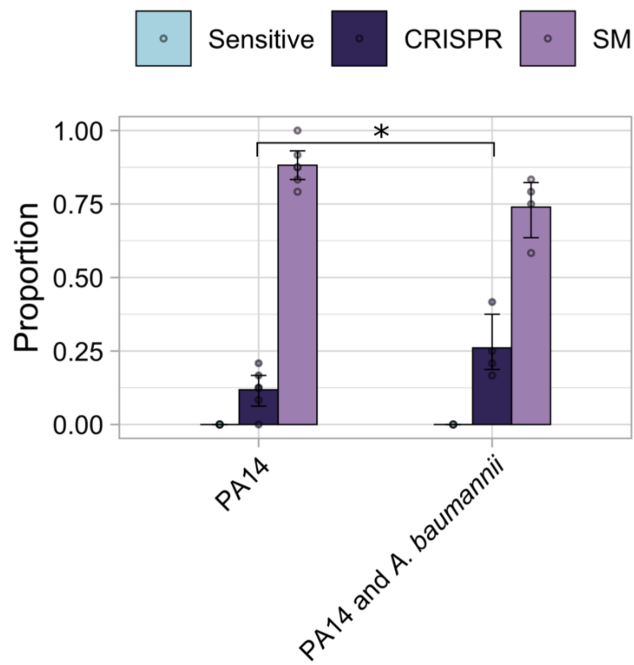

Figure 3.2 | The evolution of CRISPR-based immunity in the presence of *A. baumannii* is contact independent. Proportion of *P. aeruginosa* clones that evolved phage resistance in a trans-well system through either surface modification (SM) or CRISPR-Cas, or remained sensitive to phage DMS3vir at 3 DPI (n = 6 replicates for the PA14 treatment, and n = 4 replicates for the PA14 and *A. baumannii* treatment, with 24 colonies per replicate, n = 10 biologically independent replicates). Error bars ± 95% CI, with the mean as centre. Asterisks indicate statistically significant difference in CRISPR-based resistance between treatments (Wilcoxon rank sum test; * $p < 0.05$).

bacteria and phage populations monitored daily. This experiment revealed a significant increase the acquisition of CRISPR-based phage resistance when *P. aeruginosa* was grown in the proximity of *A. baumannii* (Fig. 3.2: Wilcoxon rank sum test; p = 0.04), confirming that *A. baumannii* can shape the evolution of phage resistance in *P. aeruginosa* through mechanisms that are independent of direct cell-to-cell contact.

Increased evolution of CRISPR-based immunity is unrelated to bacterial and phage dynamics

We considered two possible reasons why *P. aeruginosa* evolves higher levels of CRISPR immunity against phage DMS3vir in these conditioned media compared to fresh LB. First, it is possible that the chemical environment of conditioned media causes phage to amplify less efficiently, for example due to reduced phage adsorption rates. Previous studies have shown that selection favours CRISPR- over surface-based resistance when phage infection rates are reduced, and vice versa when they are increased (Alseth et al., 2019; Chabas et al., 2016; Meaden et al., 2020; Westra et al., 2015). Second, it may be that changes to the chemical environment cause physiological changes to the bacteria that favour acquisition of and/or selection for CRISPR-based resistance, for example by triggering elevated *cas* gene expression levels or slowing of the phage replication cycle. While our previous work has demonstrated that bacteria with immunity through CRISPR-Cas have a greater fitness advantage over bacteria with mutations in the phage receptor in the presence of *A. baumannii* (Alseth et al., 2019), this does not rule out that the presence of *A. baumannii* may also impact the rates at which CRISPR-based resistance is acquired. Such an effect would be analogous to those of bacteriostatic antibiotics, which have been reported to increase acquisition rate of immunity through CRISPR-Cas by slowing down the phage replication cycle (Dimitriu et al., 2021).

To explore this first hypothesis (i.e. that changes in the chemical environment affect phage amplification), we measured *P. aeruginosa* and phage DMS3vir densities over time in the various conditioned media. This revealed no clear difference in bacteria and phage population dynamics between the conditioned media treatments that could potentially explain why *A. baumannii* conditioned media results in increased CRISPR-based immunity (Fig. S3.1 and Fig. S3.2).

However, there was an overall effect of phage on bacterial densities when *P. aeruginosa* was cultured in the conditioned media from PA14 or *S. aureus*, but for none of the conditioned media from *A. baumannii* (Fig. S3.1). Yet, the largest impacts on PA14 densities were seen in the control experiment where *P. aeruginosa* was co-cultured with *A. baumannii* in LB media: Here, PA14 densities dropped both in the absence and presence of phage DMS3vir. This is likely to have be due to direct competition between *P. aeruginosa* and *A. baumannii* (Fig. S3.1).

We similarly examined the bacteria-phage dynamics for the trans-well experiment in Fig. 3.2, to see if indirect contact with *A. baumannii* influenced *P. aeruginosa* and DMS3vir densities over time. This showed that while phage caused a drop in *P. aeruginosa* densities at 1 DPI similar to that observed in the conditioned media experiment (Fig S3.1), there was no effect of being in near proximity of *A. baumannii* on the focal PA14 subpopulations (Fig. 3.3a: Linear model; No phage; effect of treatment; $t = 0.08$, $p = 0.94$; effect of time; $t = 1.37$, $p = 0.18$; overall model fit; $F_{8,24}= 1.09$, adjusted $R^2 = 0.02$, $p = 0.40$; With phage; effect of treatment; $t = -1.45$, $p = 0.16$; effect of time; $t = 4.59$, $p = 0.00016$; overall model fit; $F_{8,21}= 6.23$, $R^2 = 0.59$, $p = 0.0003$), or on phage DMS3vir (Fig. 3.3b: Linear model; effect of treatment; $t = -1.57$, $p = 0.13$; effect of time; $t = -27.87$, $p < 2 \times 10^{-16}$; overall model fit; $F_{8,27}= 175.5$, adjusted $R^2 = 0.97$, $p < 2.2 \times 10^{-16}$). Similarly, the *A. baumannii* densities were not influenced by indirect contact with *P. aeruginosa* (Fig. 3.3c: Linear model; effect of phage; $t = 1.32$, $p = 0.20$; effect of time; $t = 0.101$, $p = 0.92$; overall model fit; $F_{7,19}= 1.65$, adjusted $R^2 = 0.15$, $p = 0.18$). Overall, there was no clear correlation between the *P. aeruginosa* or phage DMS3vir population dynamics, and the levels of CRISPR-based immunity that evolved in these experimental populations, similar to what we previously observed (Alseth et al., 2019). There results therefore suggest that the increase in the evolution of CRISPR-based resistance in *A. baumannii*-conditioned media are caused by changes to the physiology of the bacteria rather than changes in the rates of phage infection

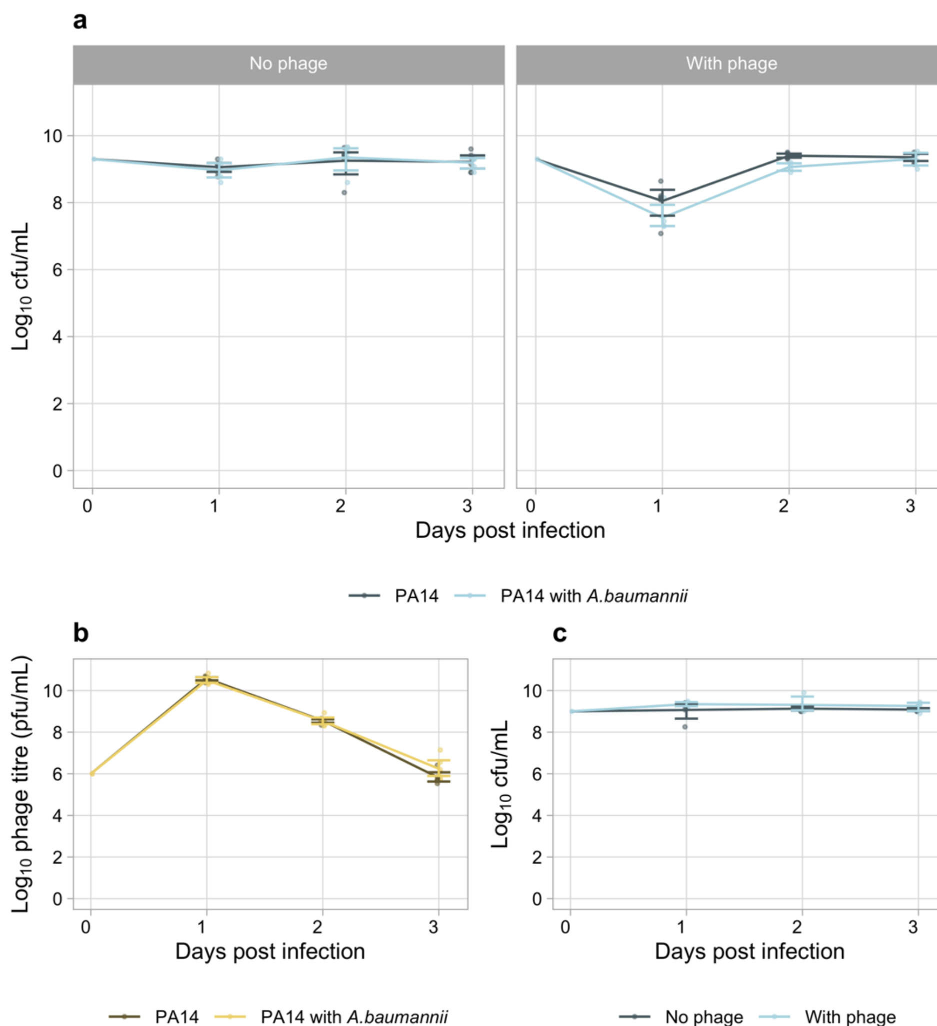

Figure. 3.3 | Bacteria and phage dynamics are not affected by indirect interspecific competition. The **(a)** *P. aeruginosa* densities in CFU/mL over time in the absence and presence of phage DMS3vir, when growing opposite either LB or *A. baumannii* in a trans-well. (**b**) DMS3*vir* phage titres (in plaque-forming units (PFU) per millilitre) over time up until 3 DPI of *P. aeruginosa* growing opposite either LB or *A. baumannii* in a trans-well. (**c**) *A. baumannii* densities in CFU/mL over time when growing opposite *P. aeruginosa* in a trans-well, while the latter bacteria is being either infected with phage DMS3vir or not. Data are mean ± 95% CI.

The evolution of resistance through CRISPR-Cas may be impacted by changes to the chemical environment

We reasoned that the increase in the evolution of CRISPR-based resistance in *A. baumannii*-conditioned media could have been caused by the depletion of key nutrients. Low nutrient availability has previously been found to favour inducible (CRISPR-Cas) over constitutive (surface modification) defence mechanisms (Westra et al., 2015). However, we also considered the alternative possibility that *A. baumannii* may secrete molecules affect the physiology and/or growth of *P. aeruginosa* in a way that leads to higher rates of acquisition of CRISPR-based resistance, analogous to the effects of bacteriostatic antibiotics (Dimitriu et al., 2021). To distinguish between these two possibilities, we first looked at *P. aeruginosa* growth in conditioned media with or without added nutrients, to examine if conditioned media from *A. baumannii* negatively affected *P. aeruginosa* growth, and whether this is due to nutrient depletion. Extra nutrients were introduced by adding one tenth of the total volume of a 10 x concentrated LB solution to the conditioned media prior to infection and inoculation.

Looking at *P. aeruginosa* growth in conditioned media with and without extra nutrients, we found that conditioned media overall negatively affected *P. aeruginosa* growth, regardless of which bacteria the conditioned media had been recovered from (Fig. 3.4: No extra nutrients: Linear model with Tukey contrasts; LB control v *S. aureus* conditioned media; $t = 9.06$, $p < 0.001$; LB control v *A. baumannii* conditioned media; $t = 8.26$, $p < 0.001$; LB control v *B. cenocepacia* conditioned media; $t = 7.06$, $p < 0.001$; LB control v PA14 + *A. baumannii* conditioned media; $t = 7.21$, $p < 0.001$; overall model fit; $F_{10,889} = 12.07$, adjusted $R^2 = 0.11$, $p < 2.2 \times 10^{-16}$). This was markedly different once resource availability was restored however: Whereas all conditioned media resulted in limited growth compared to the LB control, this effect was completely lost in the same treatments with added nutrients (Fig. 3.4: With extra nutrients: Linear model with Tukey contrasts; LB control v *S. aureus* conditioned media; $t = 2.38$, $p = 0.16$; LB control v *A. baumannii* conditioned media; $t = -2.65$, $p = 0.09$; LB control v *B. cenocepacia* conditioned media; $t = -2.50$, $p = 0.13$; LB control v PA14 + *A. baumannii* conditioned media; $t = -0.50$, $p = 0.99$; overall model fit; $F_{10,889} = 5.31$, adjusted $R^2 = 0.05$, $p = 1.25 \times 10^{-7}$). Additionally, large amounts of variation within the

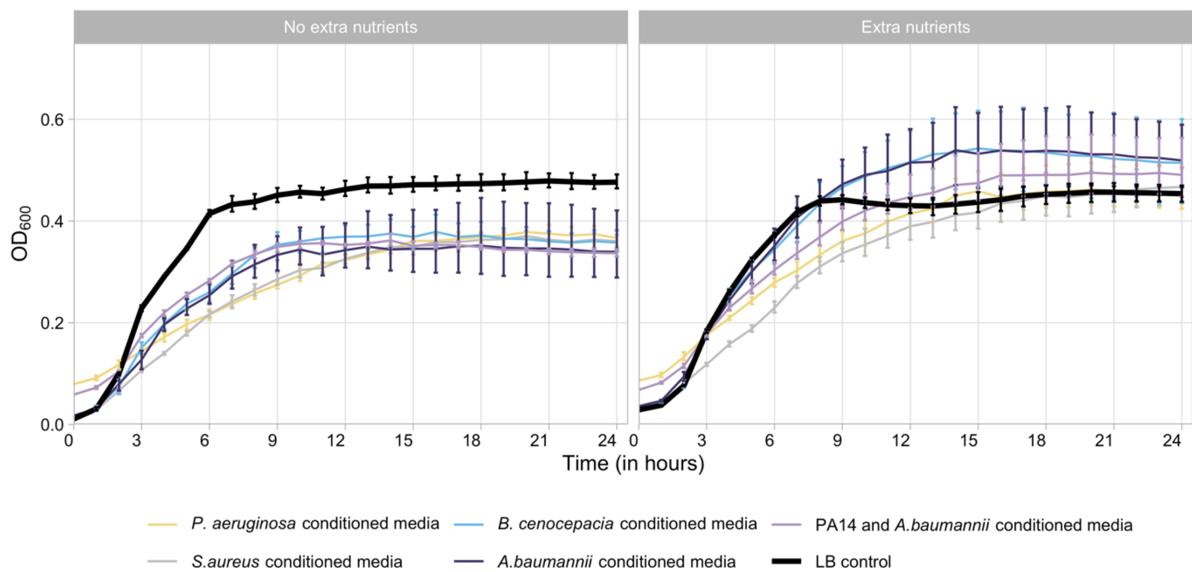

Figure 3.4 | *P. aeruginosa* growth curves are affected by conditioned media without restored resource conditions. The growth curves of *P. aeruginosa* in control LB and in conditioned media from different bacteria, either without or with added nutrients. Data points are the mean ± 95% CI (n = 6 replicates per treatment).

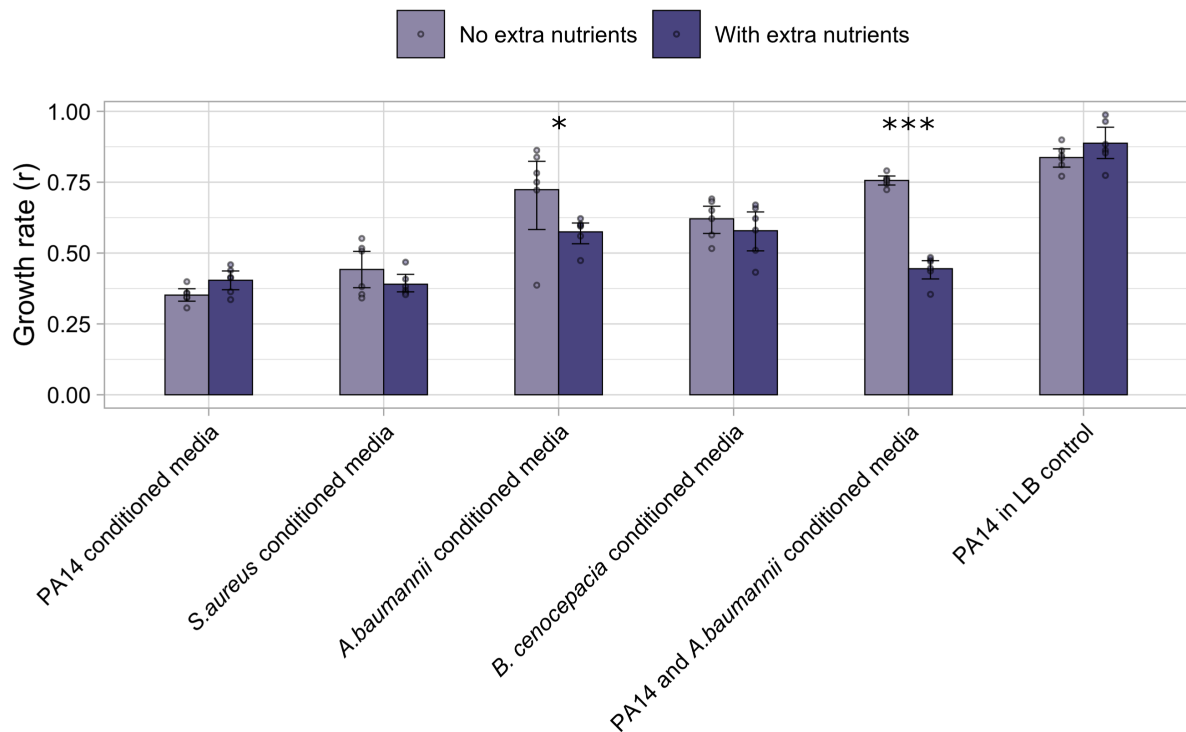

Figure 3.5 | *P. aeruginosa* growth rate is affected by conditioned media, and to some extent nutrient availability. The growth rates of *P. aeruginosa* in conditioned media from different bacteria and in control LB, either without or with added nutrients. Data points are the mean ± 95% CI (n = 6 replicates per treatment). Asterisks indicate a statistically significant difference when comparing treatments with different resource availability (GLM with Tukey Contrasts: * $p < 0.05$, *** $p < 0.001$; overall model fit; $F_{16,55} = 22.25$, adjusted $R^2 = 0.83$, $p < 2.2$ x 10-16).

treatments with resource restoration made any clear effect of conditioned media from different competitors on overall growth near undetectable.

When looking at *P. aeruginosa* exponential growth rates however, we found that they were drastically reduced in conditioned media from PA14 and *S. aureus*, both with and without extra nutrients (Fig. 3.5: Linear model with Tukey contrasts; No extra nutrients; LB control v *P. aeruginosa* conditioned media; $t = -10.68$, $p < 0.0001$; LB control v *S. aureus* conditioned media; $t = -8.69$, $p < 0.0001$; With extra nutrients; LB control v *P. aeruginosa* conditioned media; $t = -10,64$, $p < 0.0001$; LB control v *S. aureus* conditioned media; $t = -10.95$, $p < 0.0001$; overall model fit; overall model fit; $F_{16,55} = 22.25$, adjusted $R^2 = 0.83$, $p < 2.2 \times 10\text{-}16$). This was also true when compared to all other conditioned media, both with and without restored nutrients, apart from conditioned media with extra nutrients from co-cultured *P. aeruginosa* and *A. baumannii* (Fig. 3.5: Linear model with Tukey contrasts; With extra nutrients; LB control v PA14 + *A. baumannii* conditioned media; $t = -1.20$, $p = 0.99$). We speculate this may be due to molecules secreted by the bacteria into the conditioned media that negatively affect growth rates of *P. aeruginosa.* Surprisingly, when comparing growth rates between conditioned media with and without extra nutrients, we observed reduced exponential growth rates under restored nutrient conditions in *A. baumannii* conditioned media, and conditioned media from co-cultured PA14 and *A. baumannii* (Fig. 3.5). Collectively, these results suggest that *A. baumannii* and *P. aeruginosa* compete for resources and that *A. baumannii* secretes toxins, both of which could potentially drive the increase in CRISPR-based phage resistance in *P. aeruginosa* (Fig. 3.1 and 3.2).

To test if resource competition indeed could underpin the shift to CRISPR-based immunity, we performed the same experiment as before (Fig. 3.1), but with conditioned media supplemented with nutrients. We monitored phage resistance evolution in conditioned media with restored resources, to see if there would be a quantitative difference compared to conditioned media with no extra nutrients. Doing this, we found no significant differences in the evolution of phage resistance through CRISPR-Cas between the conditioned medias with or without restored resource conditions (Fig. 3.6: GLM with Tukey contrasts; PA14 conditioned media without v with extra nutrients; $z = -1.23$, $p = 0.22$;

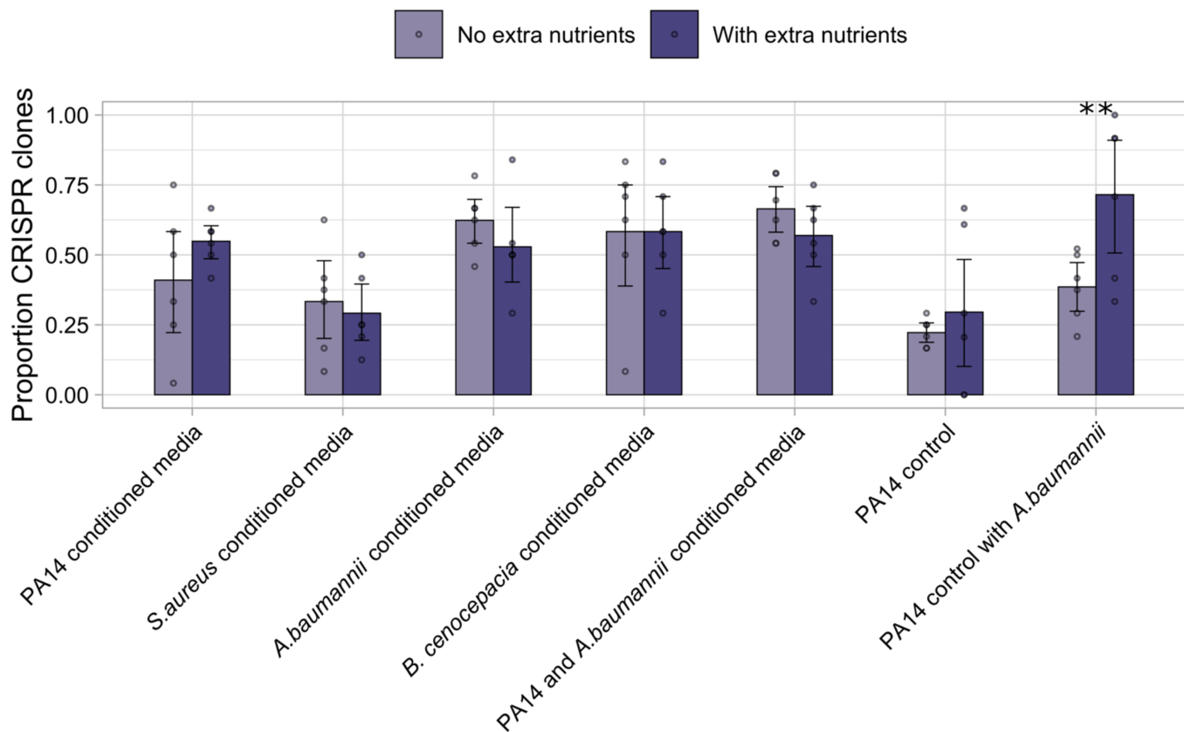

Figure 3.6 | Restoring nutrients results in similar proportions of evolved CRISPR-based immunity. Proportion of *P. aeruginosa* clones that evolved phage resistance against phage DMS3vir through CRISPR-Cas at 3 DPI in conditioned media with or without extra nutrients (6 replicates per treatment, with 24 colonies per replicate, n = 48 biologically independent replicates). Error bars ± 95% CI, with the mean as centre. Asterisks indicate a statistically significant difference when comparing treatments with different resource availability (GLM with Tukey Contrasts: ** $p < 0.01$).

S. aureus conditioned media without v with extra nutrients; z = 0.40, p = 0.69; *A. baumannii* conditioned media without v with extra nutrients; z = 0.85, p = 0.40; *B. cenocepacia* conditioned media without v with extra nutrients; z = 0.001, p = 0.99; PA14 + A. *baumannii* conditioned media without v with extra nutrients; z = -0.74, p = 0.39; residual deviance(65, n = 83) = 10.90). The only treatment affected by the addition of nutrients was the polybacterial control in LB with *P. aeruginosa* and *A. baumannii*, where we saw an increase in CRISPR-based immunity in the conditions with extra nutrients (Fig. 3.6: GLM with Tukey contrasts; z = -2.88, p = 0.004). Overall, however, the data indicated that restoring resources caused a similar outcome to that observed in regular conditioned media in terms of resulting in an increase in CRISPR-based phage resistance (Fig. 3.6). This implies that resource competition is not the main driver behind the effects observed in conditioned media, and the increase in the evolution of CRISPR-based immunity is instead most likely caused by molecules secreted into the growth media.

Discussion

Here, we studied whether the ways interspecific interactions can shape the eco-evolutionary outcome of bacteria-phage interactions (Alseth et al., 2019) are contact dependent or independent. Interspecific interactions between microorganisms are key determinants of several bacterial traits with detrimental consequences for human health (Azevedo et al., 2017; Bottery et al., 2021; Giacometti et al., 2000; Glassner et al., 2020; Gospodarek et al., 2009; Y. C. Wang et al., 2020). Yet, many aspects of the downstream consequences of polybacterial interactions remain poorly understood. This is especially true for bacteria-phage co-evolution. Several mechanisms of phage resistance are associated with potential virulence trade-offs (Hahn, 1997; León & Bastías, 2015; Persat et al., 2015; Pier, 2007), and yet little experimental research has been done on bacteria-phage interactions in a biotic context (Blazanin & Turner, 2021). Our work shows how indirect contact with a competitor can affect the evolution of phage resistance in bacteria through the production of exoproducts and consequent changes to the wider chemical environment.

Specifically, we find that *P. aeruginosa* grown in conditioned media recovered from various bacterial species generally evolve towards utilising its CRISPR-Cas system for phage resistance (Fig. 3.1). This effect was amplified further if that conditioned media was recovered from the other opportunistic pathogen *A. baumannii,* or *P. aeruginosa* that had been co-cultured with *A. baumannii*. To further examine that this effect of *A. baumannii* was due to indirect contact, we also ran an experiment using a newly developed trans-well system: By growing both pathogens together in LB rather than conditioned media, but separated by a filter allowing only small molecules to pass through, we further confirm that direct contact is not necessary for triggering the increase in CRISPR-based resistance in *P. aeruginosa* (Alseth et al., 2019)(Fig. 3.2). We hypothesised that the results could in part be explained by resource competition, but experimental tests of this hypothesis reveal similar levels of CRISPR-based immunity under in conditioned media with and without added nutrients. Together, these results imply bacterial secreted exoproducts result in chemical changes to the environment, which causes an evolutionary shift from surface- to CRISPR-based phage resistance.

Overall, these results suggest the production of exoproducts by competing bacteria, resulting in changes to the chemical environment, may play a crucial role in bacteria-phage interactions. Which molecule or molecules are produced by the various bacteria, and in particular *A. baumannii*, that cause this shift still remains unclear however. Future research would benefit from looking more in depth at this, by for instance exploring chemical compound changes between LB and conditioned media from different bacteria. Applying techniques such as two-dimensional electrophoresis, mass spectrometry, and fractionation experiments, along with monitoring pH changes between the different media, could allow us to start untangle these chemical changes between LB and conditioned media, and their role in shaping *P. aeruginosa* evolution in response to phage. Additionally, RNA-seq could be applied for the examination of *P. aeruginosa* transcriptional changes as a response to the different growth conditions. Identification of the molecules responsible for shaping the evolution of phage resistance in *P. aeruginosa* could pave the way for novel therapeutics modalities that could be used in combination with phage, to shape the eco-evolutionary outcomes of phage applications in clinical settings.

There may be many different types of small molecules that can affect the evolution of phage resistance, and these are likely to vary between different competitor species and strains. Identifying these molecules, and their genetic basis, can help in predicting how *P. aeruginosa* ecology and evolution is both shaped by and shapes its wider microbial community. Understanding the bacterial exoproducts that may inform microbial eco-evolution is important if we wish to further understand the selection pressures that shape microbial community assembly and change over time. This again has the potential to affect the development of polymicrobial co-infections and their eventual outcome in terms of human host health (Chattoraj et al., 2010; DeLeon et al., 2014; Gospodarek et al., 2009; Y. C. Wang et al., 2020). Untangling these underlying mechanisms in bacterial interspecific competition is also key in that we need to understand the ways to overcome and potentially manipulate the selective trade-offs that shape bacteria-phage interactions (Gurney et al., 2020; Sánchez et al., 2021). By doing so, we are much better positioned to develop ways to manipulate the evolution of phage resistance through small molecules, which will aid us in avoiding the biggest hurdle with phage therapy: resistance.

Material and Methods

Bacteria and phages

Throughout these experiments, the bacterial strain P. aeruginosa UCBPP-PA14 marked with streptomycin resistance, the PA14 *csy3::LacZ* strain (CRISPR-KO), and phages DMS3vir and DMS3vir+acrF1 were used. Additionally, the bacteria *S. aureus* 13 S44 S9, *A. baumannii* FZ21, and *B. cenocepacia* J2315 were used to generate spent medium or act as microbial competitors (all isolated at Queen Astrid Military Hospital, Brussels, Belgium).

Conditioned media experiments

The conditioned media experiments shown in Fig. 3.1 and Fig. 3.3 were performed by inoculating 60µL *P. aeruginosa* PA14 marked with streptomycin resistance from an overnight culture into glass microcosms that contained 6mL of cell-free conditioned media or lysogeny broth (LB). For the conditioned media experiment with extra nutrients shown in Fig. 3.6, 600µL of LB x 10 was added

to all treatments before inoculation and the following 1:100 transfers. A control without extra nutrients was also included. Cell-free conditioned media was generated by inoculating bacteria in LB medium for 24 hours in falcon tubes containing 25mL fresh LB. After inculcation the bacteria were spun down, before extracting and filter-sterilising the conditioned media (carefully avoiding the bacterial pellet) using 0.22μm filter units (Merck Millipore, Ireland). This was done daily, prior to experimental 1:100 transfers into a newly prepared batch of conditioned media. Phage DMS3vir was added at 10^6 PFU/mL at the start of the experiment, before inoculation. Inoculation was done at 37°C, rotating at 180 RPM, with phage titres monitored daily by spot assays onto *P. aeruginosa csy::LacZ* lawns. Additionally, *P. aeruginosa* CFU/mL was monitored daily by spotting serial dilutions onto square plates of LB agar. Evolved phage resistance was assessed by cross-streak assays at 3 days post infection (DPI) on 24 randomly selected clones per replicate experiment, with downstream analyses to determine whether and how bacteria evolved phage resistance as previously described (Westra et al., 2015).

Growth curve experiment

P. aeruginosa growth curves, shown in Fig. 3.4, were measured by taking overnight cultures of PA14 and diluting 100-fold into fresh LB media or bacterial conditioned media, recovered as mentioned above. For the conditioned media experiment with extra nutrients, a 10-fold dilution of LB x 10 was added to all treatments before inoculation. Growth of 200μL of *P. aeruginosa* PA14 culture in LB/conditioned media was measured in a 96-well plate by measuring optical density at λ = 600nm (OD600) for 24h at 37°C with 180 RPM in a BioTek Synergy 2 plate reader. All growth curves were performed on two independent occasions, with three technical replicates each time. Exponential growth rate (r) (Fig. 3.5) was determined using GraphPad Prism 9.2 software (San Diego, CA).

Trans-well experiment

For the experiments shown in Figures 3.2 and 3.3, 24-well trans-well inserts were 3D printed at Georgia Tech (Atlanta, USA) in nylon before being sent to and assembled by researchers at The University of Exeter following already established protocols. Shortly put, a 0.1μm membrane filter (Merck Millipore, Ireland) was cut to size before being carefully fitted vertically across each well,

while Sylgard™ 184 elastomer (Electron Microscopy Sciences, USA) was used to seal the filter and the trans-wells in place before autoclaving to ensure the system was sterile. After autoclaving, each side of the well was filled with 300µL of LB medium, before one side being inoculated with 3µL of *P. aeruginosa* PA14 from an overnight culture. The other side was either left free of bacteria, or inoculated with 3µL of *A. baumannii*, also from an overnight culture. In addition, phage DMS3vir was added at 10^6 PFU/mL to the *P. aeruginosa* side of the well for the phage treatments, before inoculation at 37°C while rotating at 180 RPM. *P. aeruginosa* and *A. baumannii* population densities were monitored daily, as were DMS3vir phage titres, by either doing spot assays on LB agar for the bacteria, or on P. *aeruginosa csy::LacZ* lawns for phage. We checked for possible contamination across wells by spotting the *A. baumannii* side of the trans-wells onto cetrimide agar, which is *P. aeruginosa* selective. Evolved phage resistance was assessed at 3 DPI, in the same way as for the conditioned media experiments as described above.

Statistical analyses

To explore the effects of the various conditioned media on the evolution of CRISPR-based phage resistance (Fig. 3.1), we used a quasibinomial generalised linear model (GLM) where the proportion of evolved *P. aeruginosa* CRISPR clones was the response variable, with treatment and replica as explanatory variables. Comparison of the proportion of evolved CRISPR-clones in conditioned media with or without restored nutrient conditions (Fig. 3.6), were similarly analysed through a GLM. Here too, the proportion of CRISPR clones was the response variable, while treatment, the addition of extra nutrients, and experimental repeat were set as explanatory variables, after which pairwise comparisons were computed using the Emmeans package version 1.5.0 (Lenth, 2016).

Statistically testing the differences in evolved CRISPR-based phage resistance in the absence or near proximity to *A. baumannii* in a novel trans-well system (Fig. 3.2) was done using a Wilcox rank sum test. A Wilcox test was also performed to directly compare the control groups in Fig. 3.1 for easier direct comparison to prior results in Alseth et. al 2019. In both cases, a non-parametric test was chosen after performing a Shapiro-Wilk test for normality.

Bacteria-phage dynamics (Fig. 3.3) were analysed using linear models, with the log10 CFU or PFU as appropriate being the response variable, and treatment, timepoint, experimental replica, and phage (where applicable) as explanatory variables. Growth curves (Fig. 3.4) were also analysed using a linear model, with OD600 measurements as response variable, with treatment, time, and experimental replica and repeats as explanatory variables. Similarly, linear models were used on the exponential growth rates (r) (Fig. 3.5), where r was the response variable and treatment, added nutrients, and experimental repeat were set as explanatory variables. For the growth curve analyses, post-hoc pairwise comparisons were done using the Multcomp package version 1.4-13 (Hothorn et al., 2008), whereas growth rate pairwise comparisons were computed using the Emmeans package version 1.5.0 (Lenth, 2016).

Throughout the paper, all model fits were assessed using Chi-squared tests and Akaike information criterion (AIC) values, as well as by plotting residuals and probability distributions using histograms and quantile-quantile plots (Q-Q plots) respectively. All statistical analyses were done using R v.4.1.0 (R Core Team, 2020), and the Tidyverse package v.1.3.1 (Wickham et al., 2019), as well as GraphPad Prism 9.2 software for growth rate calculations (San Diego, CA).

Acknowledgements

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Chapter IV – The impact of a phage and phage resistance on microbial community dynamics

Abstract

Bacteria possess a range of distinct immune mechanisms that provide protection against bacteriophages, including the mutation or complete loss of the phage receptor, and CRISPR-Cas adaptive immunity. Yet little is known about how phages and these different phage resistance mechanisms affect the wider microbial community in which they are embedded. Here, we apply experimental evolution to examine how phage impacts the structure of an artificial bacterial community that includes either *Pseudomonas aeruginosa* WT or an isogenic mutant unable to evolve phage resistance through CRISPR-Cas. Our results show that the microbial community structure is drastically altered by the addition of phage, with *Acinetobacter baumannii* becoming the dominant species and *P. aeruginosa* being driven nearly extinct, whereas *P. aeruginosa* outcompetes the other species in the absence of phage. Moreover, we find that a *P. aeruginosa* strain with the ability to evolve CRISPR-based resistance generally does better when in the presence of *A. baumannii*, but that this benefit is largely lost over time as phage is driven extinct. Our data highlight how competitive release by phage may facilitate secondary infection by other pathogenic species, and underline the importance of mapping community composition before therapeutically applying phage.

Introduction

Humans are colonised by a large number of microorganisms that play key roles in health and disease. For example, deep sequencing of the microbiomes of individuals, healthy and otherwise, has dramatically increased our knowledge of the composition and function of these microbial communities, such as the microbiomes of the nasal passage, the oral cavity, the gastrointestinal and urogenital tracts, and skin, and more recently cohorts of pregnant women (vaginal microbiomes), people suffering from inflammatory bowel disease (gut microbiome), and Type 2 diabetes (gut microbiome) (Byrd et al., 2018; Escapa et al., 2018; Freitas et al., 2017; Glassner et al., 2020; Lloyd-Price et al., 2017; Thursby & Juge, 2017; Sharma & Tripathi, 2019). These studies have firmly established a direct link between the microbial community composition, and human health, but there are still questions that need answering.

A key knowledge gap of human-microbiome interactions concerns the role of bacteriophages (viruses that infect bacteria). Bacteriophages, or simply phages, are thought to play key roles in shaping both the taxonomic and functional composition of microbial communities as well as their stability and evolution (Fazzino et al., 2020; Sullivan et al., 2017; Chevallereau et al., 2021). They are also increasingly linked to the health status of humans, with people suffering from inflammatory bowel disease (Clooney et al., 2019; Norman et al., 2015; Zuo et al., 2019), diabetes (Ma et al., 2018), Parkinson's disease (Tetz et al., 2018), and malnourished "stunted" children (Mirzaei et al., 2020) having all been reported to have abnormal phage community compositions compared to healthy individuals. Yet only a very limited number of experimental studies have explored the ecology and evolution of bacteria-phage interactions in a microbial community context (Blazanin & Turner, 2021), often using highly simplified model communities (Tecon et al., 2019). Consequently, we lack the steppingstones for a deeper understanding of how naturally occurring and therapeutically applied phages shape clinical microbial community dynamics (reviewed in Chevallereau et al., 2021).

Phages are highly diverse in terms of their morphology, genetics, and life histories (Chevallereau et al., 2021), with lytic phages being of particular interest for clinical application due to being obligate killers of their host. They can also regulate host densities through predator-prey dynamics, which again may facilitate coexistence of two bacterial competitor species (Brockhurst et al., 2006), but it is less clear how interactions between different species in more complex communities shape the effects that lytic phages have on microbial community dynamics. Furthermore, it has not been explored if and how this depends on the phage resistance strategies that bacteria evolve in response to phage.

Bacteria can overcome phage infection through a range of different means (van Houte, Buckling, et al., 2016), both innate and adaptive, with varied underlying molecular mechanisms and which can act during different stages of phage infection (Labrie et al., 2010; Bikard & Marraffini, 2012; Rostøl & Marraffini, 2019; Dimitriu et al., 2020). Through the modification, masking or complete loss of phage-binding surface receptors for example, bacteria can prevent phage

adsorption and injection (Rostøl & Marraffini, 2019; Harvey et al., 2018). Systems such as CRISPR-Cas on the other hand work by inserting short DNA sequences from phage and other invasive mobile genetic elements into the host genome to provide future immunological memory (Barrangou et al., 2007). Unlike CRISPR-based immunity (Alseth et al., 2019), phage resistance through receptor mutation can be associated with fitness trade-offs, such as virulence (Laanto et al., 2012; León & Bastías, 2015), biofilm formation (Hosseinidoust et al., 2013), and antibiotic resistance (Chan et al., 2016).

Strains of the opportunistic pathogen *Pseudomonas aeruginosa* can evolve phage resistance to phage DMS3vir using its CRISPR-Cas immune system, or by mutation of the phage receptor; the type IV pilus. We previously demonstrated that *P. aeruginosa* evolves increased levels of CRISPR-based immunity in the presence of an artificial community consisting of *Staphylococcus aureus, Acinetobacter baumannii*, and *Burkholderia cenocepacia*, all of which are opportunistic pathogens that cause severe infection (Bhargava et al., 2014; Drevinek & Mahenthiralingam, 2010; Pendleton et al., 2013; Stacy et al., 2016). We found that this is because of the fitness cost associated with surface modification, which is amplified in the presence of these bacterial species (Alseth et al., 2019). Here, we use this same empirical system to examine how phage and the type of phage resistance that evolves shapes the wider community structure.

Results

We carried out a fully factorial 10-day *in vitro* evolution experiment using *Pseudomonas aeruginosa* PA14 and its lytic phage DMS3vir in the presence or absence of competitor bacterial species (*S. aureus*, *A. baumannii*, and *B. cenocepacia*) to measure the effect of phage on microbial community dynamics. Firstly, we hypothesised that the addition of a *P. aeruginosa* specific phage would be a key determinant in the outcome of interspecific competition between multiple bacterial species. Secondly, we also hypothesised that the evolution of different phage resistance mechanisms by *P. aeruginosa* could affect the microbial community dynamics. This latter hypothesis we examined by using both a *P. aeruginosa* strain with the ability to evolve CRISPR-based phage resistance, and

an isogenic mutant lacking a functional CRISPR system throughout the study. Following inoculation, we tracked the microbial community dynamics for all experimental treatments both in the presence and absence of the *Pseudomonas*-specific phage at regular intervals over a period of 10 days.

***P. aeruginosa* dominates in the absence of phage**

In the absence of phage, *P. aeruginosa* quickly became the dominant species in the microbial community, regardless of the competitor species and the *P. aeruginosa* genotype (PA14 WT vs CRISPR-KO) (Fig. 4.1a). Consistent with this, the densities of the competitor species rapidly declined during these co-culture experiments (Fig. 4.1b). However, in the pairwise co-culture experiments, there were clear differences in the rate at which competitor species declined in frequency, which was highest for *S. aureus* and lowest for *A. baumannii*. In the treatments with additional species, the population dynamics were qualitatively similar to that during the pairwise competitions (Fig. 4.1b, ANOVA: effect of treatment on *S. aureus*; $F = 2.2$, $p = 0.09$; overall model fit; adjusted $R^2 = 0.60$, $F_{20,171} = 15.45$, $p < 2.2 \times 10^{-16}$: effect of treatment on *A. baumannii*; $F = 0.52$, $p = 0.67$; overall model fit; adjusted $R^2 = 0.66$, $F_{20,171} = 19.89$, $p < 2.2 \times 10^{-16}$: effect of treatment on *B. cenocepacia*; $F = 1.36$, $p = 0.26$; overall model fit; adjusted $R^2 = 0.69$, $F_{20,171} = 22.45$, $p < 2.2 \times 10^{-16}$), suggesting no detectable higher-order effects in this model community.

While the microbial community dynamics were relatively similar for the WT and CRISPR-KO strains, some significant differences were observed. For example, the densities of the CRISPR-KO strain were slightly lower in the presence compared to the absence of only *S. aureus* (Fig. 4.1a, linear model: $t = 2.048$, $p = 0.0413$; overall model fit; adjusted $R^2 = 0.21$, $F_{36,345} = 3.77$, $p < 6.03 \times 10^{-11}$). Moreover, *S. aureus* and *A. baumannii* reached higher densities in the presence of the PA14 WT compared to the CRISPR-KO strain, particularly at the earlier timepoints (Fig. 4.1b). In contrast to this, densities of *B. cenocepacia* over time were similar in the presence of both *P. aeruginosa* genotypes (Fig. 4.1b). Despite these differences, *P. aeruginosa* consistently and readily outcompeted the other community members in the absence of phage, with all three being extinct or close to extinction by day 10 (Fig. 4.1).

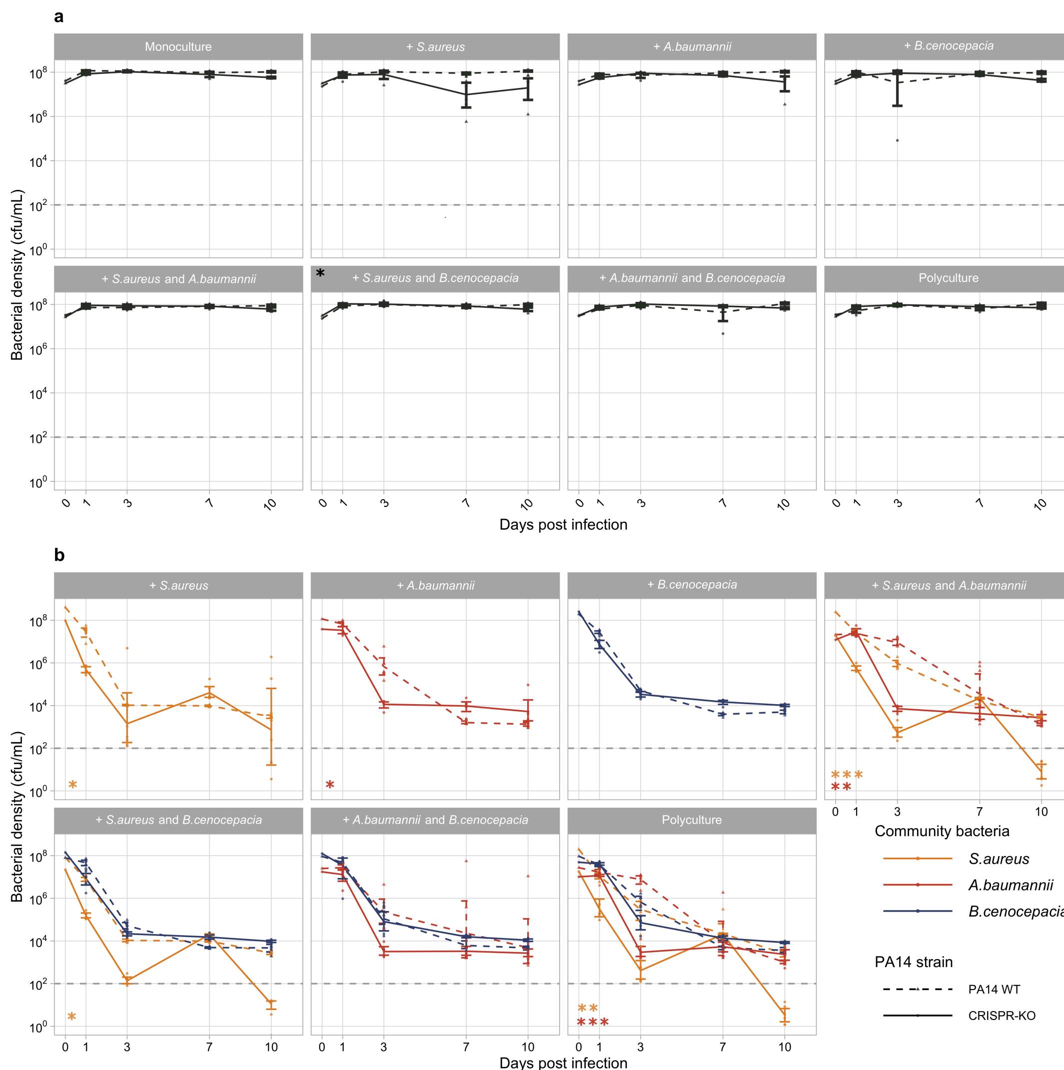

Figure 4.1 | *Pseudomonas aeruginosa* becomes the dominant species in the absence of phage. Showing the bacterial densities in CFU/mL over time for **a** the PA14 WT and CRISPR-KO *P. aeruginosa* strains, and **b** the other microbial community species in the absence of phage DMS3vir. Dashed horizontal line at 10^2 CFU/mL marks the threshold of reliable detection where the qPCR results indicate the bacteria has gone or is close to extinction from a population. Data are mean ± 95% CI, and asterisks indicate an effect on bacterial density when comparing treatments with either the PA14 WT (n = 12 per timepoint) or CRISPR-KO clone (n = 6 per timepoint) (effect of *P. aeruginosa* clone; linear models: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Phage affects microbial community dynamics

Whereas *P. aeruginosa* dominated in the absence of phage, we hypothesised this would change once a PA14 targeting phage (DMS3vir) was introduced, largely through "kill the winner" dynamics where a virulent phage reduces the susceptible host population, facilitating either co-existence or competitive release (Thingstad, 2000). As expected, phage DMS3vir initially reached high titres due to replication in sensitive *P. aeruginosa* hosts, followed by a rapid decline in phage densities due to the evolution of phage resistance, regardless of whether the host had a functional CRISPR-Cas system or not (Fig. S4.1). Crucially however, the presence of phage caused microbial communities to no longer be dominated by *P. aeruginosa*, as when compared to the no phage treatments, very few to none of the experimental repeats had one or more bacterial species go extinct, with *A. baumannii* reaching particularly high densities (Fig. 4.2).

Interestingly, the PA14 WT generally reached higher densities than the CRISPR-KO strain in the presence of *A. baumannii* (Fig. 4.2a), consistently doing so early in the experiment when phage remained in the population (Fig. 4.2a and Fig. S4.1). This was in concordance with *P. aeruginosa* evolving higher levels of CRISPR-based immunity against phage DMS3vir in treatments including *A. baumannii* due to the increased fitness cost of surface modification (Fig. 4.3 and (Alseth et al., 2019)): At 3 days post infection, there was a significant effect of all treatments on CRISPR immunity compared to the PA14 monoculture, but which was strongest for *A. baumannii*. At timepoint 10 we only found an increased proportion of *P. aeruginosa* clones immune through CRISPR-Cas when the treatment included *A. baumannii* (Fig. 4.3, GLM; *A. baumannii*; $t = 2.637$, $p = 0.01$; *S. aureus* and *A. baumannii*, $t = 2.283$, $p = 0.025$; A. *baumannii* and *B. cenocepacia*, $t = 2.689$, $p = 0.0087$; polyculture, $t = 2.141$, $p = 0.035$).

We have previously sown that the evolution of phage resistance by mutation of the Type IV pilus (the phage receptor) is associated with large fitness trade-offs in a microbial community context, whereas evolution of CRISPR-based immunity is not associated with any detectable trade-offs (Alseth et al., 2019). We therefore predicted that the ability to evolve phage resistance through CRISPR-Cas would also have knock-on effects for the microbial community dynamics. However, measurement of the densities of the competitors revealed that these were largely

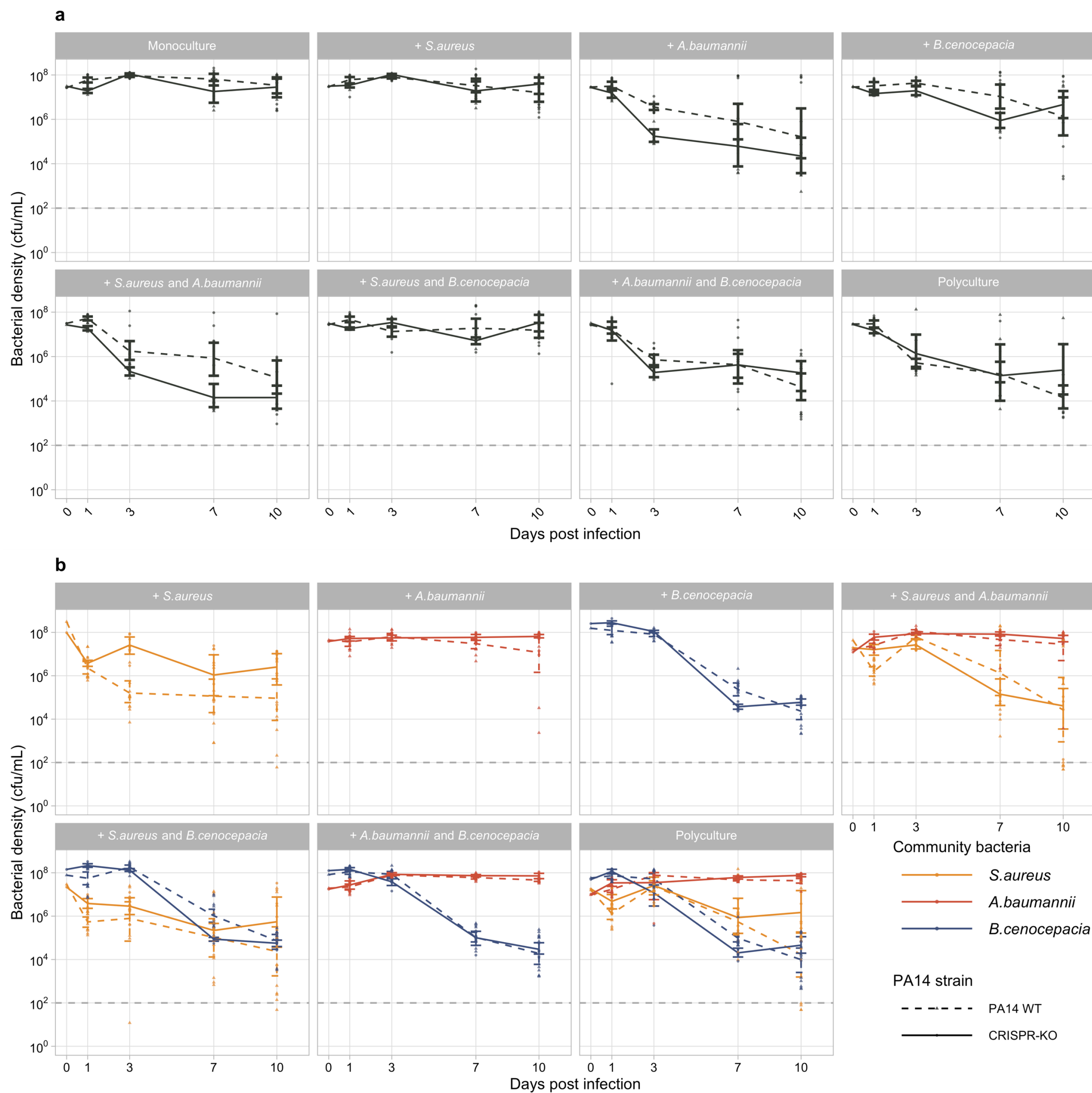

Figure 4.2 | Phage allows for the maintenance of all microbial community members, with *A. baumannii* becoming the new dominant species. Showing the bacterial densities in CFU/mL over time for **a** the PA14 WT and CRISPR-KO *P. aeruginosa* strains, and **b** the other microbial community species in the presence of phage DMS3vir. Dashed horizontal line at 10^2 CFU/mL marks the threshold of reliable detection where the qPCR results indicate the bacteria has gone or is close to extinction from a population. Data are mean ± 95% CI, and asterisks indicate a significant difference in bacterial density between treatments with either the PA14 WT (n = 12 per timepoint) or CRISPR-KO clone (n = 6 per timepoint) (effect of *P. aeruginosa* clone; **a** linear model with Tukey contrast per timepoint; **b** linear models: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

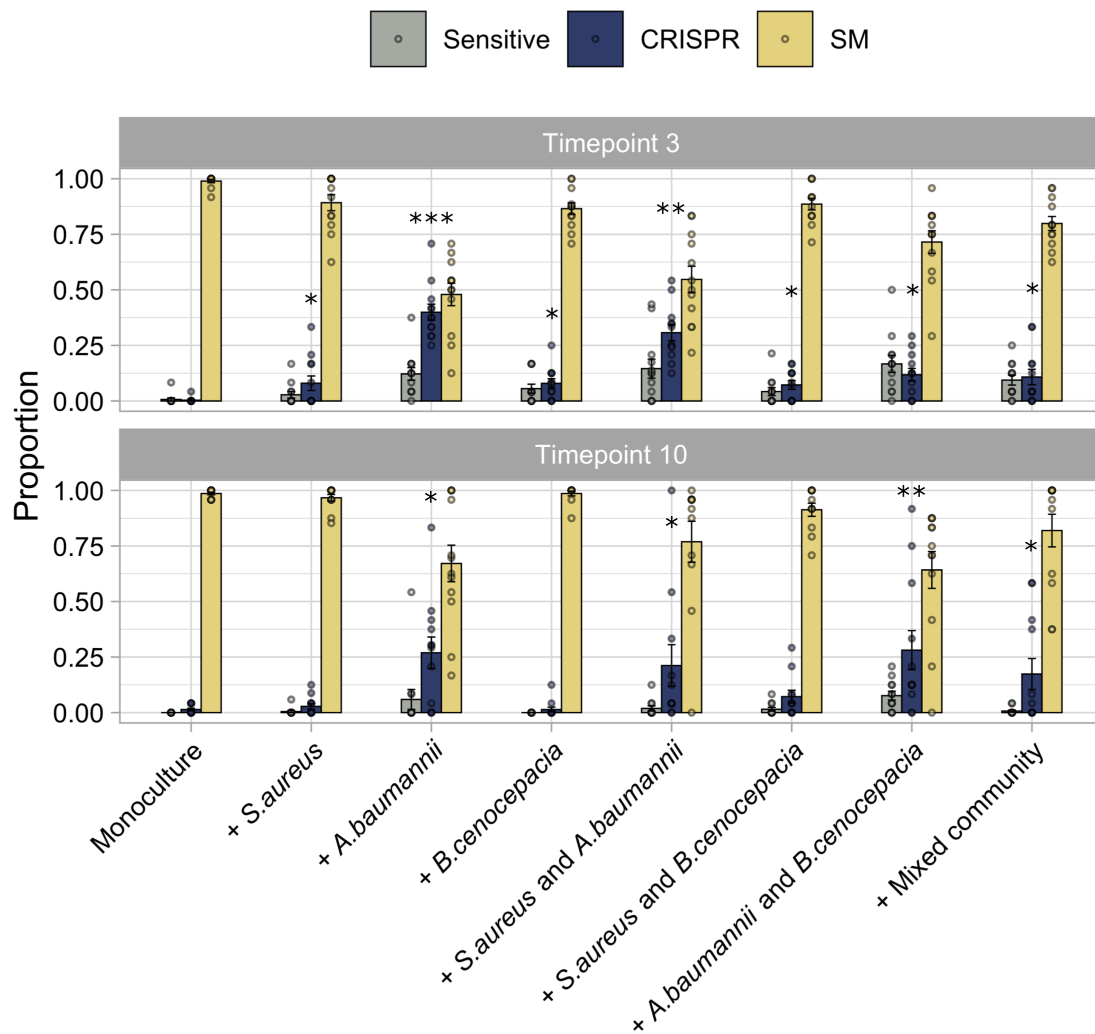

Figure 4.3 | Interspecific competition affects the proportion of evolved CRISPR-based phage resistance. Proportion of *P. aeruginosa* PA14 WT at timepoints 3 and 10 that evolved phage-resistance either through surface modification (SM) or CRISPR immunity, or which remained sensitive to phage DMS3vir when grown in monoculture or different polycultures. Data are mean ± SE. Asterisks indicate a significant difference in proportion of CRISPR immunity evolved when compared to the PA14 monoculture within each timepoint (n = 12 per treatment) (generalised linear model, quasibinomial: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

unaffected by the presence of a functional CRISPR-Cas immune system in *P. aeruginosa* (Fig. 4.2b), with the exception of *S. aureus*: In the presence of the *P. aeruginosa* WT strain, *S. aureus* densities were significantly lower in two of the microbial communities compared to the same co-culture experiments with CRISPR-KO strain (Effect of *P. aeruginosa* clone on *S. aureus* densities, linear model: Treatment *S. aureus*; $t = -2.363$, $p = 0.0216$, adjusted $R^2 = 0.2659$, $F_{14,57} = 2.837$, $p = 0.002786$; Treatment *S. aureus* and *A. baumannii*; $t = -2.043$, $p = 0.0457$, adjusted $R^2 = 0.3867$, $F_{14,57} = 4.198$, $p = 5.3 \times 10^{-5}$).

The addition of a *P. aeruginosa* targeting phage results in the competitive release of *A. baumannii*

We hypothesised that the effect of phage on microbial community structure could largely be explained by the competitive release (increase in absolute abundance, following removal of competitor) of *A. baumannii*, which then takes over to become the dominant species (Lloyd-Smith, 2013). To assess this, we examined the fold change difference for the final densities of all three community members in the presence versus absence of phage (Fig. 4.4). Crucially, this revealed a strong increase in *A. baumannii* densities in the presence of phage, supporting the idea that it becomes the dominant and determinant community member when *P. aeruginosa* is inhibited by phage (Fig. 4.2). By contrast, when phage was added, *S. aureus* only experienced a clear fold change increase if it was co-cultured with the CRISPR-KO strain, along with an additional competitor species. *B. cenocepacia* meanwhile seemed to be the species with the least benefit of phage, but still with a small fold change increase for some treatments (Fig. 4.4).

The effects of phage on microbial community diversity

Finally, we also hypothesised that the addition of phage not only results in competitive release (Fig. 4.4), but also results in the general maintenance of microbial diversity due to it controlling the population following a "kill the winner" model (Thingstad, 2000). To quantitatively assess this, we calculated Shannon diversity indexes for all experimental treatments. Plotting these diversity scores over time shows that without phage there is a rapid loss of diversity over time, whereas community complexity persists in the presence of phage

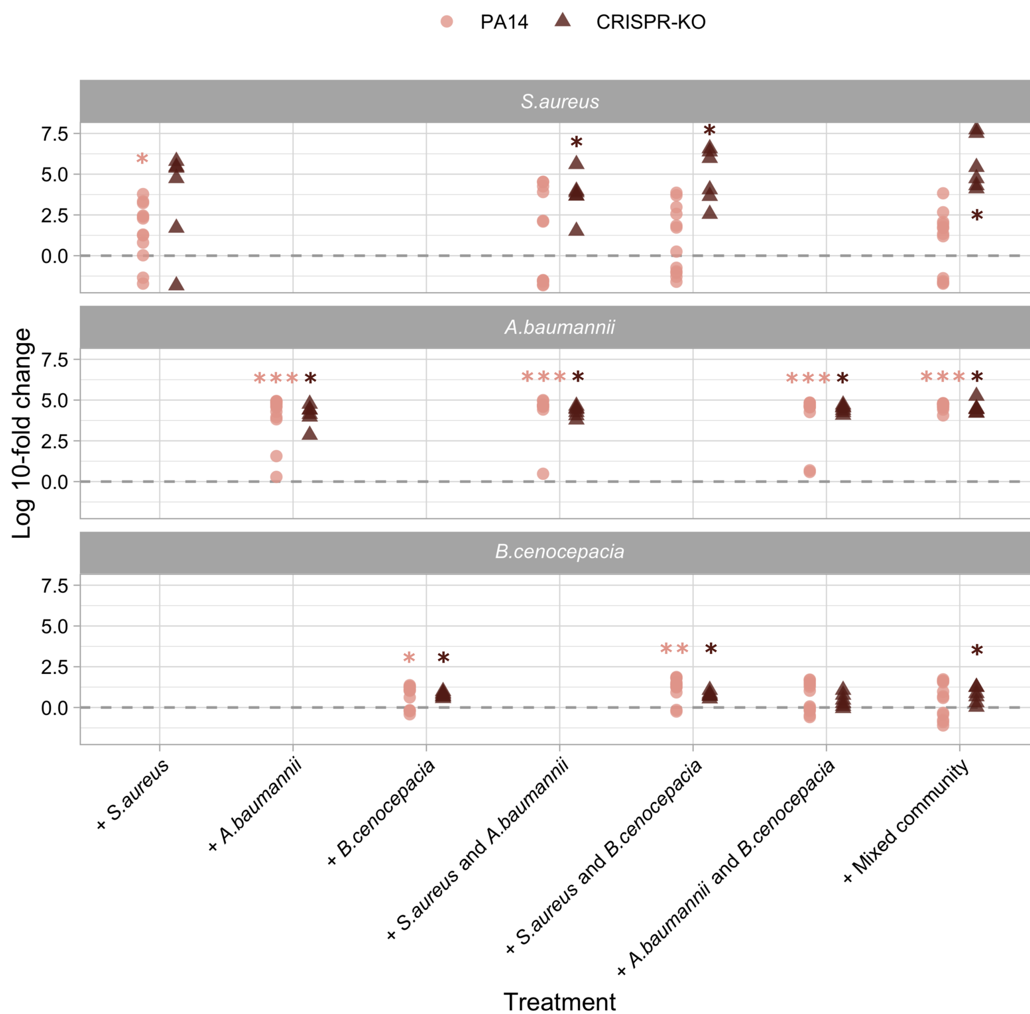

Figure 4.4 | Fold change between no phage and phage treatments at the end of the experiment. The fold change difference of the individual community species not targeted by phage when comparing absolute densities in the presence of phage to the absence at the final experimental timepoint. Colour of datapoints indicate which PA14 clone was present in the treatment (n = 12 for PA14 WT per treatment, n = 6 for CRISPR-KO). Asterisks indicate higher final densities in the presence of phage (Wilcoxon signed rank exact test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

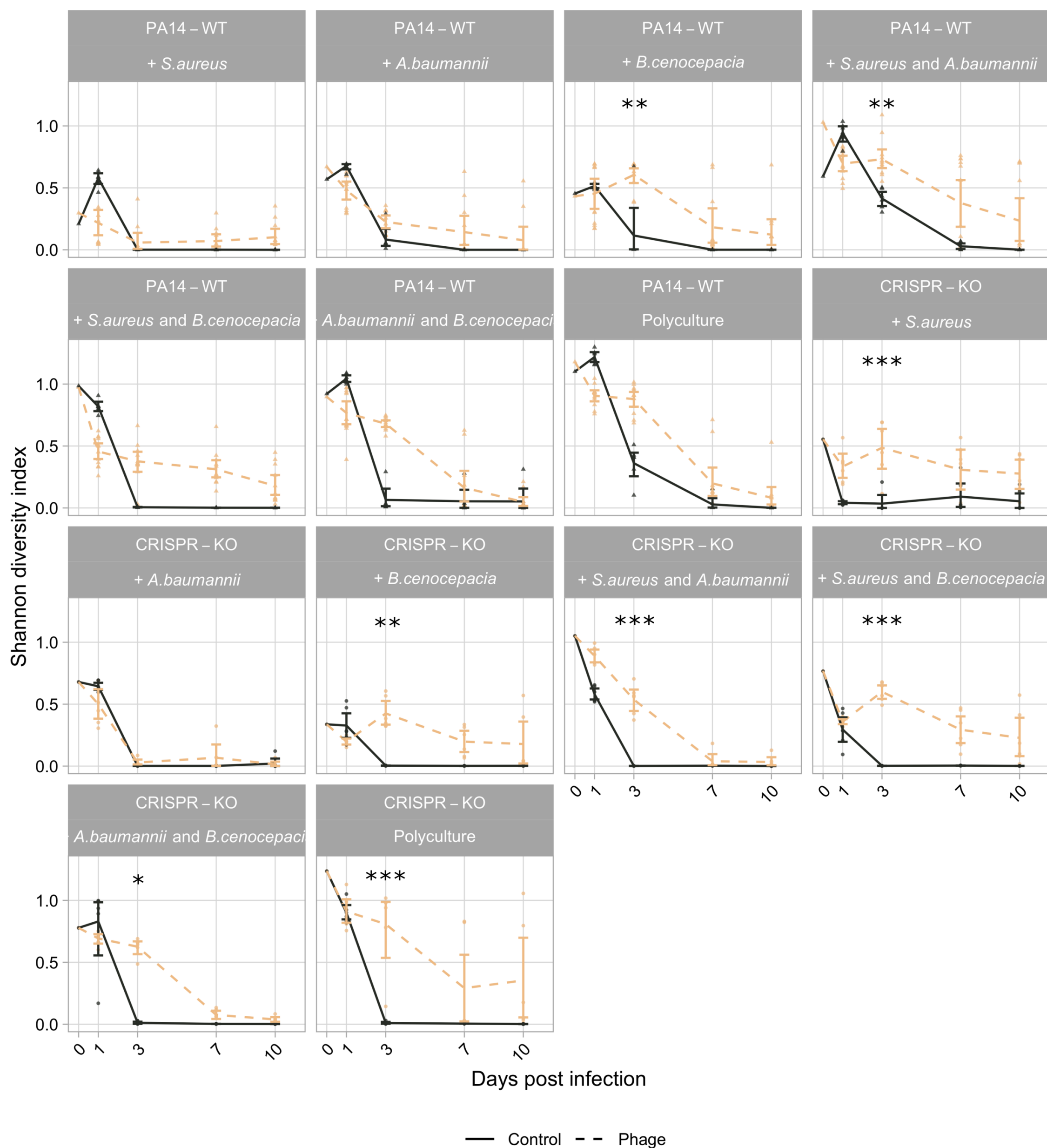

Figure 4.5 | Shannon diversity over time illustrate the diversity maintaining effects of phage. The change in diversity over time, illustrated using Shannon diversity indexes, for both the PA14 WT and CRISPR-KO strains. Data are mean ± 95% CI, and asterisks indicate a significant difference in Shannon diversity between treatments with either the phage (n = 6 per timepoint for all expect the PA14 WT with phage treatments, where n = 12) (effect of *P. aeruginosa* clone; linear models with Tukey contrasts: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(Fig. 4.5: ANOVA: PA14 WT effect of phage; $F = 27.57$, $p = 2.3 \times 10^{-7}$; CRISPR-KO effect of phage; $F = 89.19$, $p < 2.2 \times 10^{-16}$; Overall model fit for PA14 WT: adjusted $R^2 = 0.64$, $F_{38,465} = 24.87$, $p < 2.2 \times 10^{-16}$; Overall model fit for CRISPR-KO: adjusted $R^2 = 0.56$, $F_{32,303} = 14.56$, $p < 2.2 \times 10^{-16}$). This was true for treatments for both *P. aeruginosa* genotypes, but the trend became most pronounced for the CRISPR-KO strain when applying direct comparisons using Tukey contrasts, in which case we found phage to significantly increase diversity over time in nearly all treatments (Fig. 4.5, indicated by asterisks).

Discussion

It is still unclear what determines the domination of one or multiple pathogens in infection, as well as what may cause transitions between infecting bacteria (Smith et al., 2014; Taccetti et al., 2012; Welp & Bomberger, 2020). The natural host virome is increasingly being revealed as playing a crucial role in human health (Clooney et al., 2019; Zuo et al., 2019), and understanding these dynamics of infection transition will be of particular importance if phage therapy is to become a successful treatment option. Whereas antibiotic treatments can have broad-spectrum impacts that shift community structure (Langdon et al., 2016; Nelson et al., 2020; Smith et al., 2014), phages are perceived to be less disruptive due to their narrow host spectrum (Galtier et al., 2016; Lin et al., 2017). Still, the specific removal of a dominant competitor can in principle generate large community effects through processes such as competitive release (Lloyd-Smith, 2013). Previous work has indicated how phage may impact and shape microbial communities in a range of ways (Brockhurst et al., 2006; Guerrero et al., 2020; Mumford & Friman, 2017), and that bacterial biodiversity can affect the mechanism by which bacteria evolve resistance to phage (Alseth et al., 2019). Here, we study these interactions further and in combination by exploring how phage can shape microbial community dynamics, and how this again may be impacted by phage resistance evolution.

We show that in the absence of phage, *P. aeruginosa* quickly becomes the dominant species in the community, driving the other three pathogens to or close to extinction (Fig. 4.1). This is true for both the *P. aeruginosa* WT strain – which can evolve phage resistance through CRISPR-Cas – and an isogenic mutant

lacking a functional CRISPR system (Fig. 4.1). We find some variation in how the community bacteria respond to the different *P. aeruginosa* genotypes however. Specifically, both *S. aureus* and *A. baumannii* remain at higher densities for longer when competing against the PA14 WT, with a spike in *S. aureus* densities at timepoint 7 when grown with the CRISPR-KO clone. Still, the end points remain similar regardless of starting composition (Fig. 4.1), making the true extent of the cost of having a CRISPR-system in the absence of phage and the cause of these trends difficult to determine.

Once a *P. aeruginosa*-targeting phage is added on the other hand, we observe a drastic shift in the community structure, with *A. baumannii* becoming the dominant species and readily outcompeting *P. aeruginosa* (Fig. 4.2 and Fig. 4.4). This competitive release also occurs despite the fitness benefit provided by evolving CRISPR-based phage resistance over surface modification (Alseth et al., 2019). As although we see that the PA14 WT largely does better when compared to the CRISPR-KO strain in the presence of *A. baumannii* (Fig. 4.2), this effect disappears once phage is driven extinct, indicating it is not enough when faced with the challenge of both interspecific competition and phage infection. However, and of particular interest, we do not just see a complete takeover by *A. baumannii*, instead observing how the addition of a phage targeting the most dominant species facilitates – to varying degrees – the maintenance of all three other pathogens in the population in nearly all treatments (Fig. 4.2, Fig. 4.4 and Fig. 4.5).

Our results highlight the role phage, and by extent, the natural virome might play in microbial community assembly. By testing this experimentally, we show that while one species would normally dominate due to an imbalance in the strength of competition (Coyte et al., 2021) (Fig. 4.1), this dynamic is drastically altered by phage impacting community dynamics, facilitating the maintenance of bacterial diversity (Fig. 4.5). This supports the increasing understanding of the role of the natural virome, and how it plays a key role in the assembly and regulation of the complex microbial networks found within humans (Shkoporov & Hill, 2019).

Moreover, our data emphasises the importance of knowing which other pathogens are present in an infection before commencing phage therapy, as it

may have severe consequences for the patient treatment and infection management. For example, maintained polymicrobial infection might result in a more severe disease prognosis, through the adverse selection of increased virulence (Korgaonkar et al., 2013; Riedel et al., 2001; Stacy et al., 2016) and persister cells with heightened tolerance to antibiotics (Bhargava et al., 2014). We also speculate that a clinical community context like the one used here will constrain the antagonistic (co)evolution between *P. aeruginosa* and its targeting phage (Johnke et al., 2017; Mumford & Friman, 2017), partially by reducing host densities, but also because adaptation in general has been found to be constrained in more complex communities (Scheuerl et al., 2020). And while this may limit the likelihood of *P. aeruginosa* overcoming phage therapy through evolved resistance, it can, as shown here, facilitate competitive release, diversity maintenance, and consequently polymicrobial infections. It also supports the notion of carefully taking the microbiome into consideration when designing phage cocktails, to target not just the dominant pathogen, but also other pathogens that may be found in the same infection to avoid secondary infections (Divya Ganeshan & Hosseinidoust, 2019).

Materials & Methods

Bacteria and phages

The bacteria *P. aeruginosa* UCBPP-PA14 strain marked with streptomycin resistance, the PA14 *csy3::LacZ* strain (CRISPR-KO), and phages DMS3vir and DMS3vir+acrF1 were used throughout this study and have all been previously described (van Houte, Ekroth, et al., 2016; Westra et al., 2015). The microbial community consisted of *S. aureus* strain 13 S44 S9, *A. baumannii* clinical isolate FZ21, and *B. cenocepacia* J2315, and were all isolated at Queen Astrid Military Hospital, Brussels, Belgium.

Evolution experiment

The evolution experiment was performed by inoculating 60 µl from overnight cultures, that were grown for 24 hours, into glass microcosms containing 6 ml fresh LB medium (60 µl of culture containing ca. 10^6 CFU). All polyculture mixes were prepared so that *P. aeruginosa* made up approximately 25% of the total inoculation volume (15 µl of 60 µl), with the rest being made up of one or equal

amounts of the microbial community bacteria. In all monoculture controls, *P. aeruginosa* was diluted in LB medium to adjust starting densities for consistency across all treatments (*n* = 6 per treatment, unless indicated otherwise). Phage DMS3vir was added at 10^6 p.f.u. prior to inoculation. The experiment ran for ten days, with transfers of 1:100 into fresh LB medium being done every 24 hours. Throughout the experiment, the bacterial mixtures were grown at 37°C and shaking at 180 r.p.m. Phage titres were monitored daily, and were determined using chloroform-treated lysate dilutions which were spotted onto lawns of *P. aeruginosa csy::LacZ*. To determine which mechanism of phage-resistance had evolved, 24 randomly selected clones per treatment replica from timepoints 3 and 10 were analysed using methods as detailed in Westra *et al*. 2015 (Westra et al., 2015).

DNA extraction and qPCR

Bacterial densities, for both PA14 strains and the other individual microbial community bacteria, were determined using DNA extractions followed by qPCR analyses. DNA extractions were done using the DNeasy UltraClean Microbial Kit (Qiagen), following instructions from the manufacturer, but with an additional pre-extraction step where samples were treated with 15 µl lysostaphin (Sigma) at 0.1 mg ml^{-1} as previously described(Alseth et al., 2019) to ensure lysis of *S. aureus*. The qPCR primers for *P. aeruginosa*, *A. baumannii*, and *B. cenocepacia* were the same as in Alseth *et al*. (Alseth et al., 2019), whereas the *S. aureus* primers used are previously described (Goto et al., 2007). All reactions were done in triplicates, using Brilliant SYBR Green reagents (Agilent) and the Applied Biosystems QuantStudio 7 Flex Real-Time PCR system. For reaction mixture and details on PCR programme, see ref. (Alseth et al., 2019). Bacterial CFU/ mL were calculated from the quantities offered by the standard curve method, adjusting for gene copy number (4, 1, 6, 6, for *P. aeruginosa*, *S. aureus*, *A. baumannii*, and *B. cenocepacia* respectively).

Statistical analyses

Analysis of the effects of the various species compositions on *P. aeruginosa* densities in the presence (Fig. 4.1a) or absence (Fig. 4.2a) of phage were done using generalised linear models (GLM), with log10 CFU set as the response variable. The same type of analysis was done per treatment for the other species

that made up the microbial community (Fig. 4.1b and 4.2b). The explanatory variables used in the analyses were type of PA14 strain (PA14 WT or CRISPR-KO), treatment, timepoint, replica, and experimental repeat.

To explore the impact of interspecific competition on the evolution of phage resistance at timepoints 3 and 10 (Fig. 4.3), we used quasibinomial GLMs where the proportion of evolved CRISPR-based phage resistance was the response variable, and treatment, replica, and experimental repeat were the explanatory variables.

The analyses of fold-changes to assess competitive release by comparing absolute density differences of the individual community members (*S. aureus*, *A. baumannii*, and *B. cenocepacia*) in the absence v presence of phage (Fig. 4.4) was done through Wilcox signed rank exact tests. A non-parametric test was chosen after performing a Shapiro-Wilk test for normality.

Lastly, the diversity maintaining effects of phage were examined through assessing the impact of phage DMS3vir on Shannon diversity index scores over time (Fig. 4.5). This was done through linear models, where the Shannon diversity index score (H) was the response variable, and treatment, timepoint, the presence of phage, PA14 strain (PA14WT or CRISPR-KO), experimental repeat, and replica were the explanatory variables. Shannon diversity (H), was calculated as $H = -\Sigma p_i * \ln(p_i)$, where Σ is the sum and p_i is the proportion of the entire community made up of species *i*.

Throughout the paper, pairwise comparison of means were done using the Emmeans package (Lenth, 2016), and model fits were assessed using Chi-squared tests and Akaike information criterion (AIC) values, as well as plotting residuals and probability distributions using histograms and quantile-quantile plots (Q-Q plots) respectively. All statistical analyses were done using R version 4.0.3. (R Core Team, 2020), its built-in methods, and the Tidyverse package version 1.3.0 (Wickham et al., 2019).

Acknowledgements

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Chapter V – General discussion

"We fail to control infectious disease because of evolution, not because of lack of pharmacological options."
– Janis Antonovics

General remarks

In this thesis, I have aimed to start exploring the role of interspecific competition on the evolution of bacteriophage resistance in the opportunistic pathogen *Pseudomonas aeruginosa*. To briefly summarise; I have provided novel insights into how bacterial biodiversity can affect the evolution of phage resistance, how phage may impact microbial community dynamics, and some of the mechanisms that underpin the selection for different phage-resistance systems. Firstly, the presence of competitors can amplify fitness trade-offs associated with surface modification, tipping the balance in favour of CRISPR-based phage resistance. Secondly, I start to examine how phage resistance evolution is shaped by contact independent interspecific competition. Finally, I look at how the presence of a lytic phage can drastically alter community structure through competitive release and diversity maintenance.

Interspecific competition and CRISPR-based phage resistance

Through this work, I have shown how the presence of other bacteria may shape the evolution of phage resistance in *P. aeruginosa*, with fitness trade-offs resulting in a shift from surface- to CRISPR-based immunity. These results and their impact on *in vivo* virulence however, are novel findings that need to be further explored and generalised across systems. As highlighted in Chapter II; taking biotic community context into consideration is key when studying bacteria-phage interactions. Yet while studies on bacteria-phage dynamics to date have found consistent ecological effects of interspecific interactions, the evolutionary effects tend to vary and with little molecular detail (Blazanin & Turner, 2021). Additionally, the results presented in this thesis are done using an artificial community that while relatively complex for experimental evolution *in vitro*, still does not replicate the natural biotic complexity and composition of clinical *P.*

aeruginosa infections. Further research could build on this by utilising novel approaches such as the porcine cystic fibrosis lung model recently developed for exploring *in vivo* biofilm formation (Harrington et al., 2020). This model can also be applied for studying interspecific dynamics, with the potential to expand on experimental work on bacteria-phage interactions like that described in this thesis.

Similarly, these experiments do not account for potential host immune system effects and abiotic factors. To this end, I have established a collaboration between The University of Exeter and The University of Liverpool (through Professor Aras Kadioglu), to explore if our main finding of increased CRISPR-based phage resistance is replicable in a murine lung infection model. While still a work in progress, pilot data indicate *P. aeruginosa* can evolve phage resistance through CRISPR-Cas during infection (Fig. 5.1). Further work on this project aims on introducing a co-infecting competitor (such as *A. baumannii*), as well as looking more closely at the downstream effects of phage resistance on virulence evolution and host immune system response.

Furthermore, approximately 36% of clinical *P. aeruginosa* strains possess CRISPR-Cas systems, and around half of all bacteria (Cady et al., 2012; Grissa et al., 2007). By more broadly exploring the associated costs and mechanisms that drive the selection for different phage resistance systems this may also aid us in further understanding why CRISPR-Cas distribution is relatively patchy across the phylogenetic trees of bacteria and archaea.

The chemical environment and CRISPR-Cas

While the work presented in Chapter III starts exploring the molecular mechanisms that underpin the selective shift from surface modification to CRISPR-based immunity, more work is needed to fully understand the evolution of phage resistance systems as reported in Chapter II. Further research should carefully explore signalling molecules produced during interspecific competition, and how they may elicit a response with consequences for bacteria-phage co-evolution. Additionally, while some effects may be contact independent, effects of direct contact through – for example – the Type VI secretion system still cannot be completely ruled out. By combining the classic experimental evolution

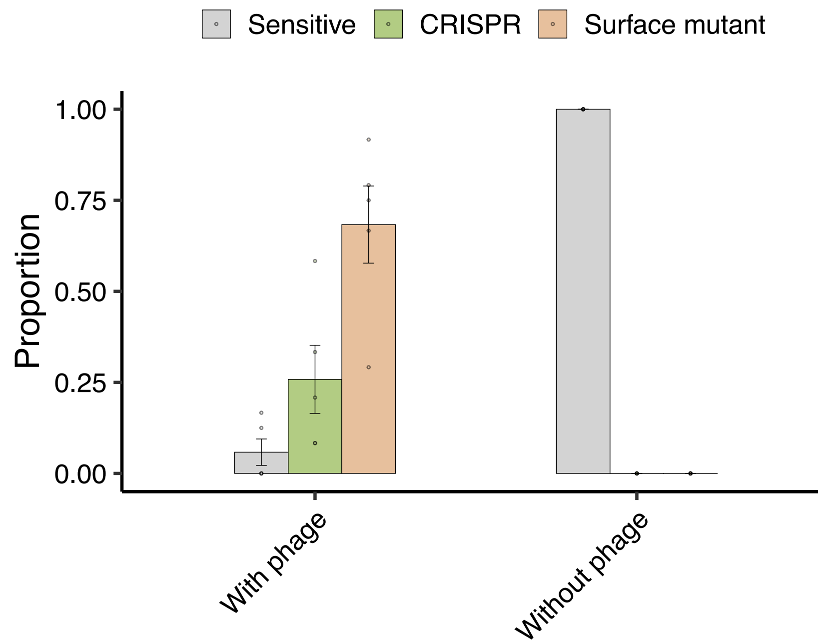


Figure 5.1 | *P. aeruginosa* can evolve phage resistance through CRISPR-Cas in a murine model. Proportion of *P. aeruginosa* that acquired surface- (surface mutant) or CRISPR-based immunity, or remained sensitive in a murine lung infection model after 24h either with or without phage DMS3vir (5 replicates per treatment, with 24 colonies screened from each replicate, n = 10 biologically independent replicates).

approach with molecular biology and biochemistry, we may start addressing these complex questions. It will be especially important to do so in the context of specific bacterial interactions and clinical polybacterial infection, where understanding the evolution of phage resistance will be of fundamental importance for phage therapy success. Specifically, as further study on the chemical signals involved would potentially indicate ways in which phage resistance evolution may be manipulated to our benefit.

Phage and microbial population dynamics

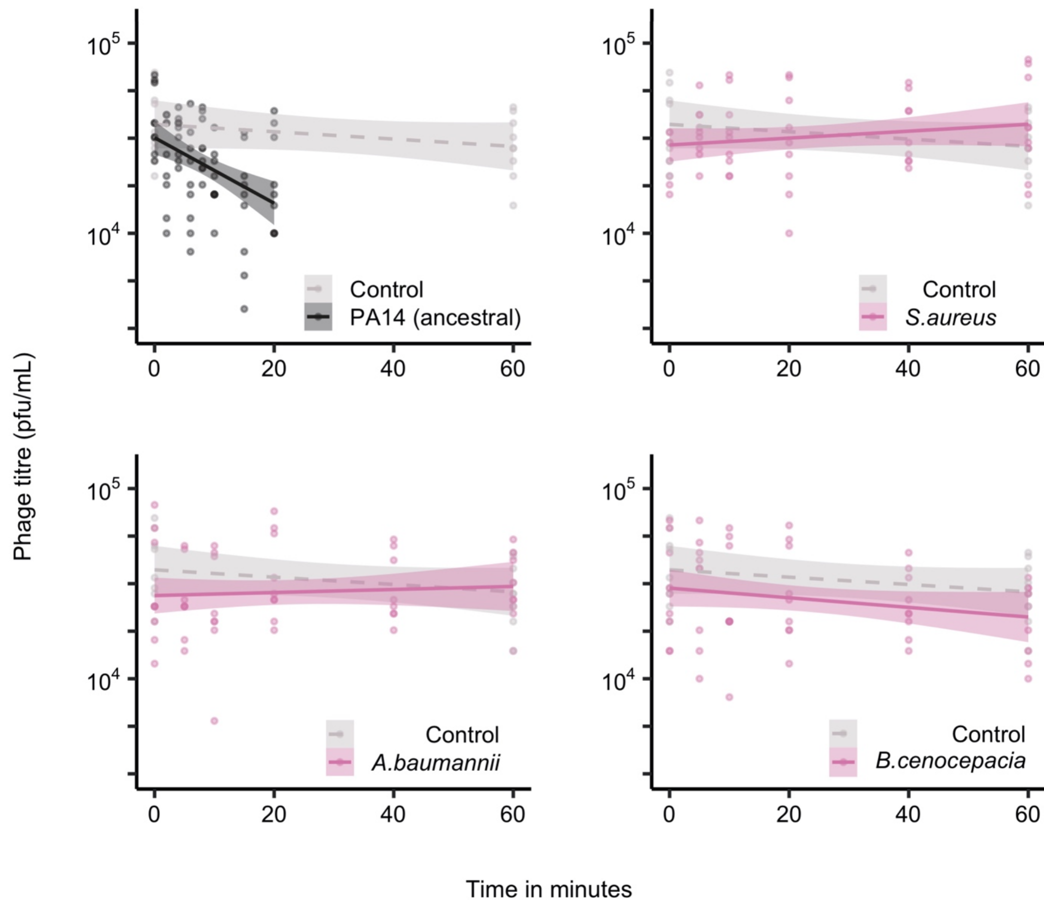
Phages may influence and shape microbial communities in a multitude of ways (Brockhurst et al., 2006; Guerrero et al., 2020; Mumford & Friman, 2017), just as microbial communities can shape bacteria-phage dynamics as shown in Chapter II. In Chapter IV, I study this further by showing how through "kill the winner" dynamics (Thingstad, 2000), a phage may facilitate competitive release and bacterial co-existence over a longer period of time. Further work could be undertaken to look more closely at the community dynamics in the absence of *P. aeruginosa* however. Additionally, it would be interesting to extend the use of phages to encompass ones that target the other members of the community, which is likely to significantly alter competitive balance and co-existence (Brockhurst et al., 2006).

Many mathematical models exist that explore how microbial communities form and fluctuate over time. From considering environmental harshness (Shibasaki et al., 2021) and resource diversity (Dal Bello et al., 2021), to higher level microbe-microbe interactions (Qian et al., 2021) and ecological dependencies (Coyte et al., 2021), these models have provided invaluable insight into the lives of microbes. However, more experimental work is needed to shape a more unified understanding of microbial community assembly, and while multiple studies have explored assembly in the absence of phage (Dal Bello et al., 2021; Goldford et al., 2018; Gralka et al., 2020), little work has been done that take bacteria-phage dynamics into consideration. The results from Chapter III start to address this by experimentally testing how phage impact a relatively complex microbial community. Further research could benefit from more experimental data, as well as mathematical models exploring the impact of phage on the formation of co-infections and transition of pathogens.

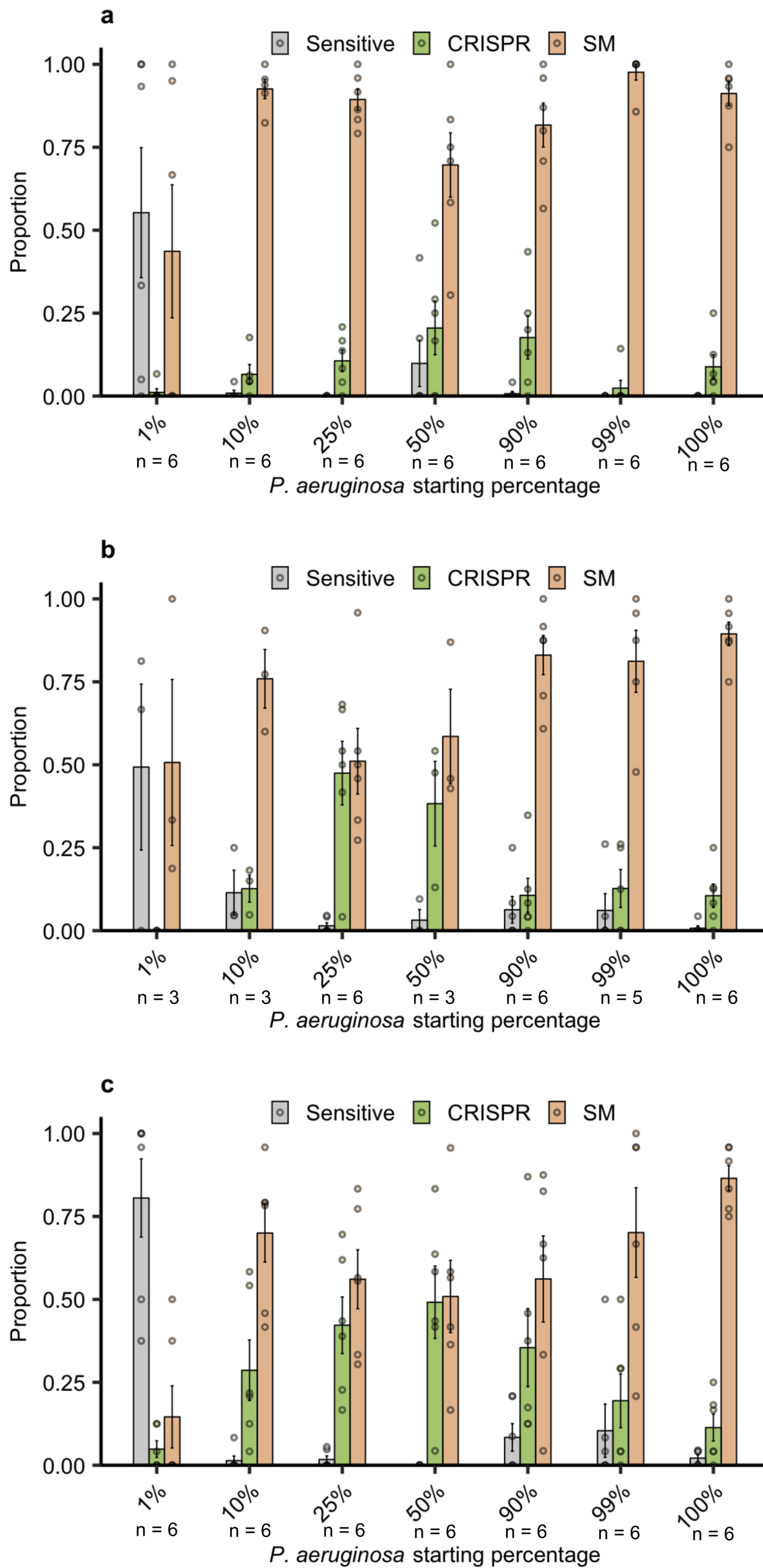
Concluding remarks

The field of microbiology has a long history of studying *P. aeruginosa*, and overall, we now have extensive knowledge of many individual (and often mechanistic) aspects of *P. aeruginosa* clinical infection. However, there is a need for more emphasis to be placed on the ecology and evolution of *P. aeruginosa* in complex clinical environments, particularly in response to potential antimicrobial treatments. This thesis has explored how interspecific interactions can impact phage resistance in *P. aeruginosa*, and what the potential consequences and benefits might be in the context of phage therapy. Still, there is a need to further examine how these effects are both influenced by and may influence other eco-evolutionary dynamics – both *in vitro* and *in vivo*. Spatial structure, host immune response, the metabolome, and wider metapopulation dynamics can all play part in bacteria-phage co-evolution. Yet to more thoroughly bridge the gap between the laboratory and the clinic, research combining theoretical models with experimental data is needed to form a more unified understanding of these complex eco-evolutionary dynamics. Increased knowledge of these bacteria-phage interactions is important. It highlights the need to unify ecological, evolutionary, and molecular approaches in microbiology, considering the whole image as well as its individual pieces. This will be especially important in the context of a renewed interest in phage therapy and the possibility of using the evolution of phage resistance to 'steer' pathogens such as *P. aeruginosa* towards reduced virulence and antibiotic susceptibility (Gurney et al., 2020), and understanding the potential role of bacteria-phage interactions in altering microbial communities, co-infection, and human health. Failing to do so may lead us down the same path as antibiotics; with all roads leading to resistance.

Appendix to Chapter II – Supplementary material

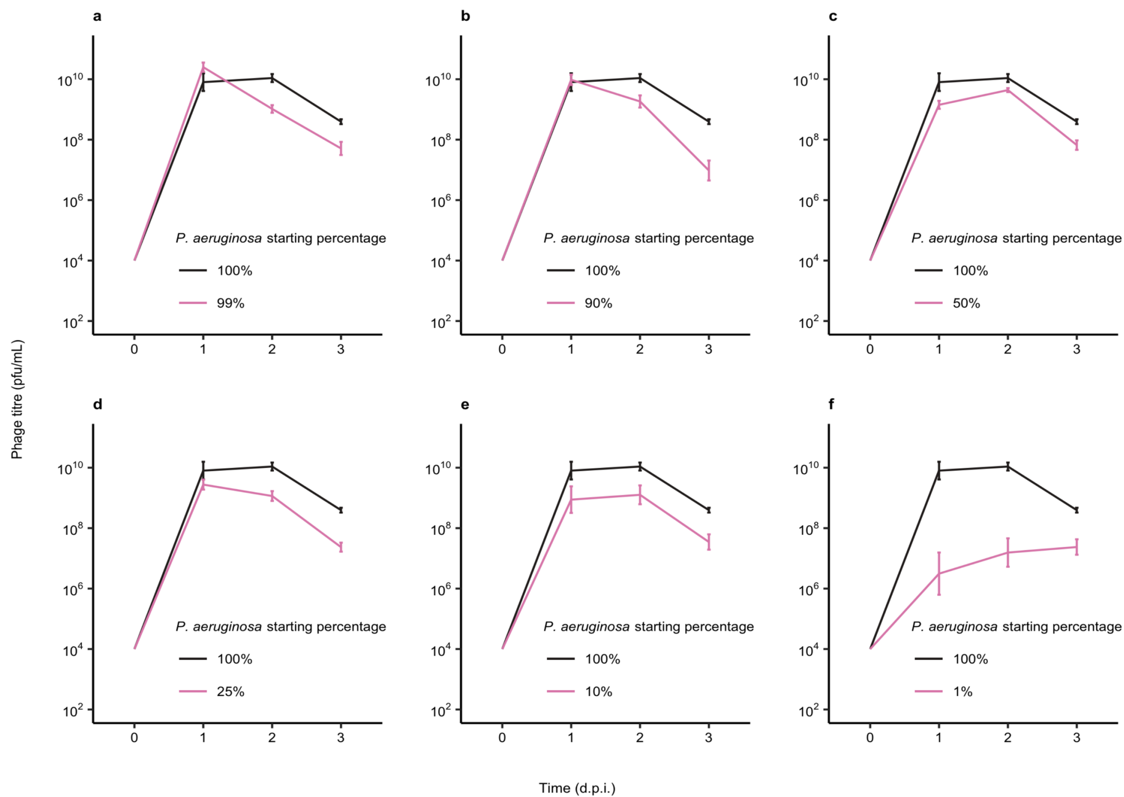


Supplemental Figure 2.1 | Only *P. aeruginosa* adsorbs phage DMS3*vir*. Phage levels, given in plaque-forming units per millilitre, in minutes post infection of *P. aeruginosa* PA14 and three other bacterial species (n = 84 biologically independent samples). Controls were carried out in the absence of bacteria. Here, the lines are regression slopes with shaded areas corresponding to 95% confidence intervals. Linear model: Effect of *P. aeruginosa* on phage titre over time; t = -3.37, p = 0.0009; *S. aureus*; t = 1.63, p = 0.11; *A. baumannii*; t = 1.20, p = 0.23; *B. cenocepacia*; t = -0.27, p = 0.79; Overall model fit; $F_{9,235} = 4.33$, adjusted $R^2 = 0.11$, $p = 3.17 \times 10^{-5}$.

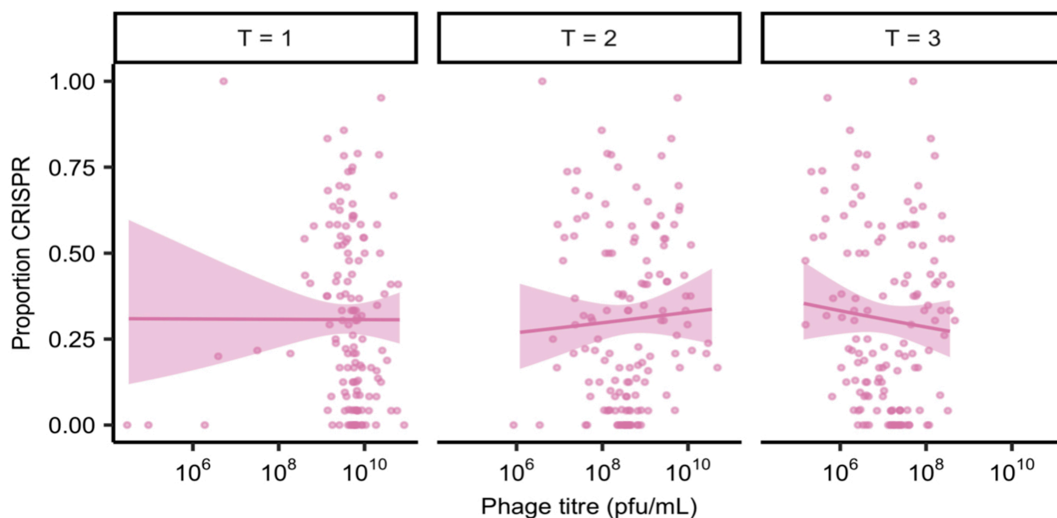


Supplemental Figure 2.2 | Increased CRISPR-based resistance evolution across a range of microbial community compositions over time. Proportion of *P. aeruginosa* that acquired surface modification (SM) or CRISPR-based

immunity (or remained sensitive) at up to 3 days post infection (d.p.i.) with phage DMS3*vir* when grown either in monoculture (100%), or in polyculture mixtures consisting of the mixed microbial community but with varying starting percentages of *P. aeruginosa* based on volume. (6 replicates for most samples, with 24 colonies per replicate, $n = 42$ biologically independent replicates for (**a**), $n = 32$ biologically independent replicates for (**b**), and $n = 42$ biologically independent replicates for (**c**)). (**a**) Resistance evolution at 1 d.p.i. Error bars correspond to ± one standard error, with the mean as the measure of centre. Deviance test: Relationship between CRISPR and *P. aeruginosa* starting percentage at timepoint 1; Residual deviance(34, n = 41) = 4.42, $p = 0.004$; 1%; $z = -3.27$, $p = 0.002$; 10%; $z = 1.21$, $p = 0.23$; 25%; $z = 1.62$, $p = 0.11$; 50%; $z = 2.20$, $p = 0.034$; 90%; $z = 2.07$, $p = 0.046$; 99%; $z = 0.47$, $p = 0.65$; 100%; $z = 1.47$, $p = 0.15$. (**b**) Resistance evolution at 2 d.p.i. Error bars correspond to ± one standard error, with the mean as the measure of centre. Deviance test: Relationship between CRISPR and *P. aeruginosa* starting percentage at timepoint 2; Residual deviance(25, n = 32) = 3.86, $p = 2.51 \times 10^{-6}$; 1%; $z = -2.14$, $p = 0.04$; 10%; $z = 1.19$, $p = 0.25$; 25%; $z = 2.07$, $p = 0.049$; 50%; $z = 1.89$, $p = 0.07$; 90%; $z = 1.12$, $p = 0.27$; 99%; $z = 1.21$, $p = 0.24$; 100%; $z = 1.11$, $p = 0.28$. (**c**) Resistance evolution at 3 d.p.i. Error bars correspond to ± one standard error, with the mean as the measure of centre. Deviance test: Relationship between CRISPR and *P. aeruginosa* starting percentage at Timepoint 3; Residual deviance(35, n = 42) = 8.24, $p = 0.0004$; 1%; $z = -3.38$, $p = 0.002$; 10%; $z = 2.12$, $p = 0.04$; 25%; $z = 2.77$, $p = 0.009$; 50%; $z = 3.07$, $p = 0.004$; 90%; $z = 2.46$, $p = 0.019$; 99%; $z = 1.55$, $p = 0.13$; 100%; $z = 0.87$, $p = 0.39$.

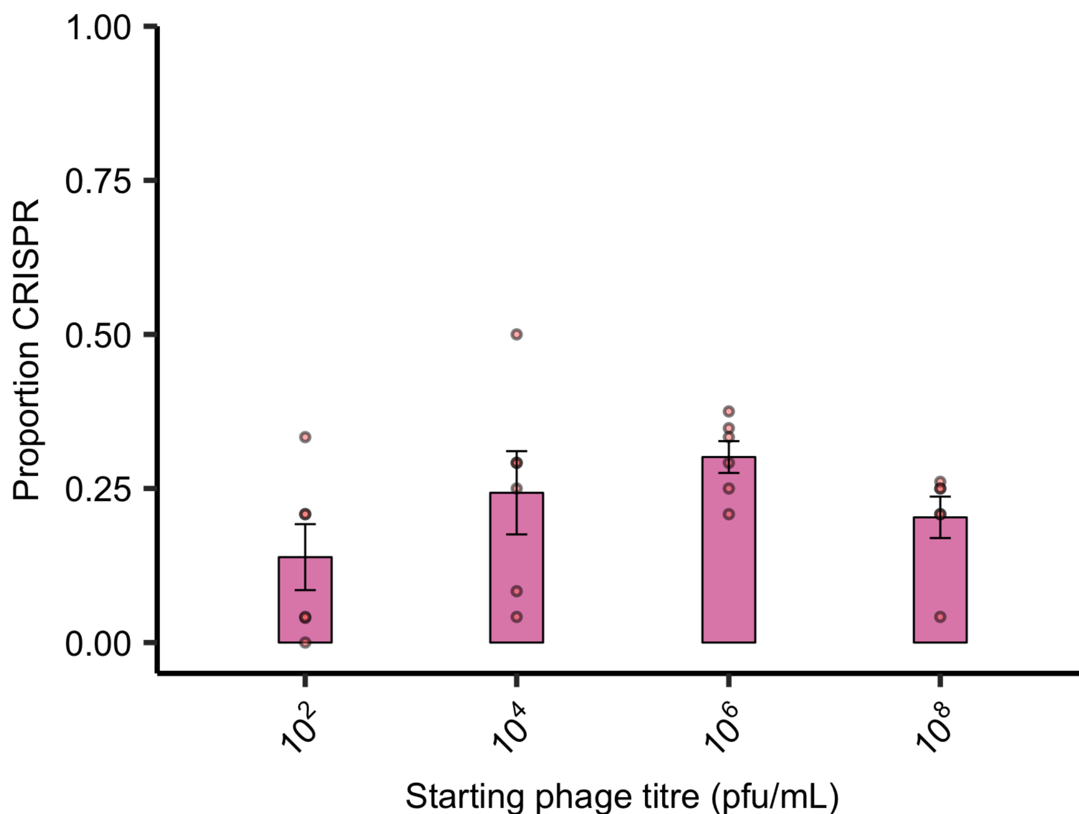


Supplemental Figure 2.3 | Microbial community composition impacts phage epidemic size. The DMS3*vir* phage titres (in plaque-forming units per millilitre) over time up to 3 days post infection of *P. aeruginosa* grown either in monoculture (100%), or in polyculture mixtures as shown in Supplemental Fig. 2.3. Each data point represents the mean, with error bars corresponding to ± one standard error (n = 171 independent biological samples). Two-way ANOVA: Overall effect of *P. aeruginosa* starting percentage on phage titre; $F_{6,105} = 14.84$, $p = 1.1 \times 10^{-12}$.



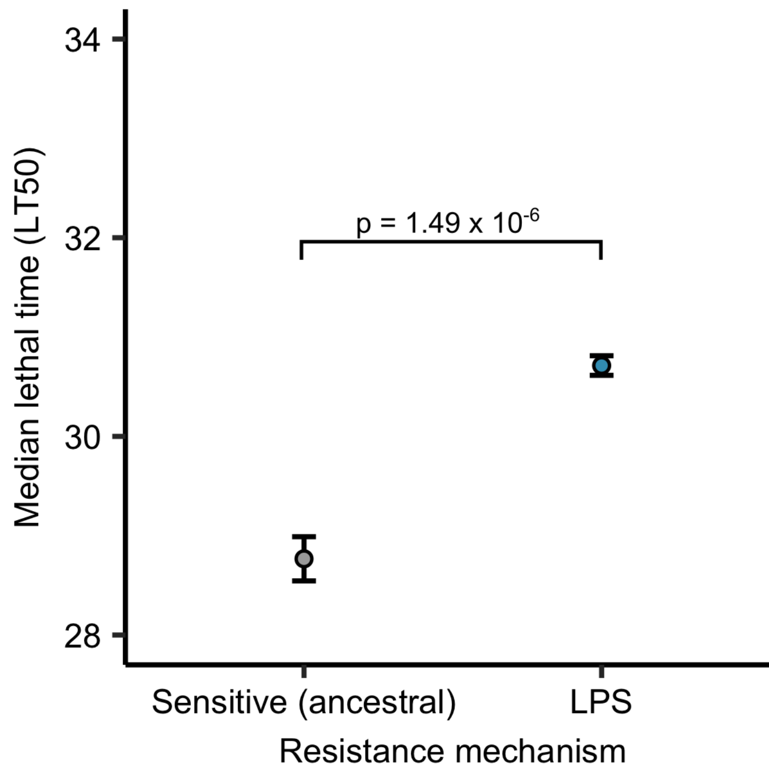
Supplemental Figure 2.4 | No correlation between phage epidemic size and evolution of CRISPR resistance. The correlation between the proportion

evolved phage resistant clones with CRISPR-based resistance and the phage epidemic sizes (in plaque-forming units per millilitre) in the presence of other bacterial species, using data taken from experiments shown in Fig. 2.1, Supplemental Fig. 2.2, Supplemental Fig. 2.3c and Supplemental Fig. 2.6 (n = 137 biologically independent samples per timepoint). Correlations are separated by day, as phage titres were measured daily. Here, the lines are regression slopes, with shaded areas corresponding to 95% confidence intervals. Pearson's Product-Moment Correlation tests between phage titres (at each day post infection) and levels of CRISPR-based resistance: T = 1; t_{136} = -0.02, p = 0.98, R2 = -0.002; T = 2; t_{136} = 0.59, p = 0.55, R2 = 0.05; T = 3; t_{136} = -0.90, p = 0.37, R2 = -0.08.



Supplemental Figure 2.5 | Starting phage titre does not affect CRISPR evolution in the presence of a microbial community. Proportion of *P. aeruginosa* that acquired CRISPR-based resistance at 3 days post infection with varying starting titres of phage DMS3*vir* when grown in polyculture (6 replicates per treatment, with 24 colonies per replicate, *n* = 24 biologically independent replicates). Deviance test: Start phage and CRISPR; Residual deviance(20, n = 24) = 2.00, p = 0.13; Tukey contrasts: 10^2 v 10^4; z = -1.52, p = 0.42; 10^4 v 10^6; z

= -0.76, p = 0.87; 10^6 v 10^8; z = 1.31, p = 0.56; 10^2 v 10^6; z = -2.24, p = 0.11; 10^2 v 10^8; z = -0.99, p = 0.75; 10^4 v 10^8; z = 0.56, p = 0.94. Error bars correspond to ± one standard error, with the mean as the measure of centre.



Supplemental Figure 2.6 | LPS-based phage resistance also affects in vivo virulence. Time to death (given as the median ± one standard error) for *Galleria mellonella* larvae infected with PA14 clones that evolved phage resistance through LPS modification, compared to the phage-sensitive ancestral (n = 209 biologically independent samples). Cox proportional hazards model with Tukey contrasts: Sensitive (ancestral) v LPS ; z = 4.81, p = 1.49 x 10^{-6}. Overall model fit; LRT_3 = 44.94, p = 1 x 10^{-9}.

Appendix to Chapter III – Supplementary material

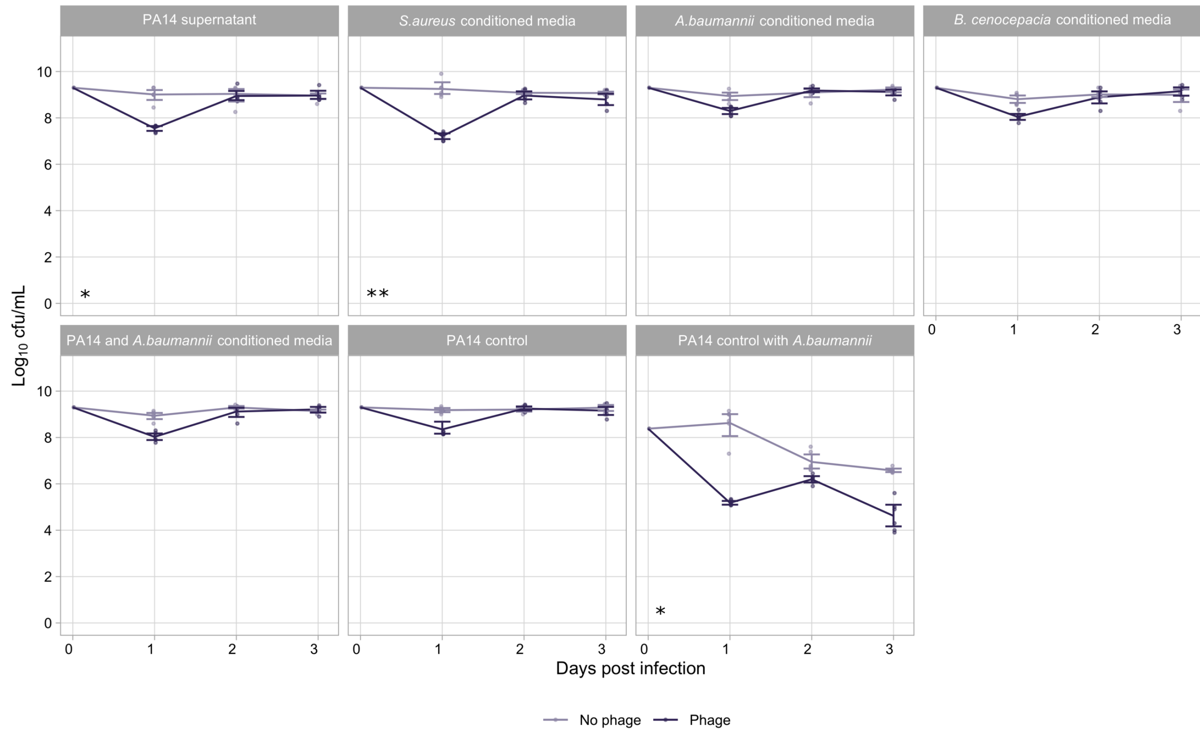


Figure S3.1 | *P. aeruginosa* growth densities in conditioned media is affected by phage. Showing *P. aeruginosa* densities in CFU/mL over time in the presence of absence of phage DMS3vir, in conditioned media from different bacteria and in LB controls (n = 12 per timepoint per treatment). Data are mean ± 95% CI, and asterisks indicate a significant difference in bacterial density over time between the no phage and phage treatments (GLM, effect of phage over time: PA14 conditioned media; t = 2.06, p = 0.041; *S. aureus* conditioned media; t = 2.72, p = 0.007; Control with *A. baumannii*; t = 2.12, p = 0.035; residual deviance(219, n = 251) = 32.79).

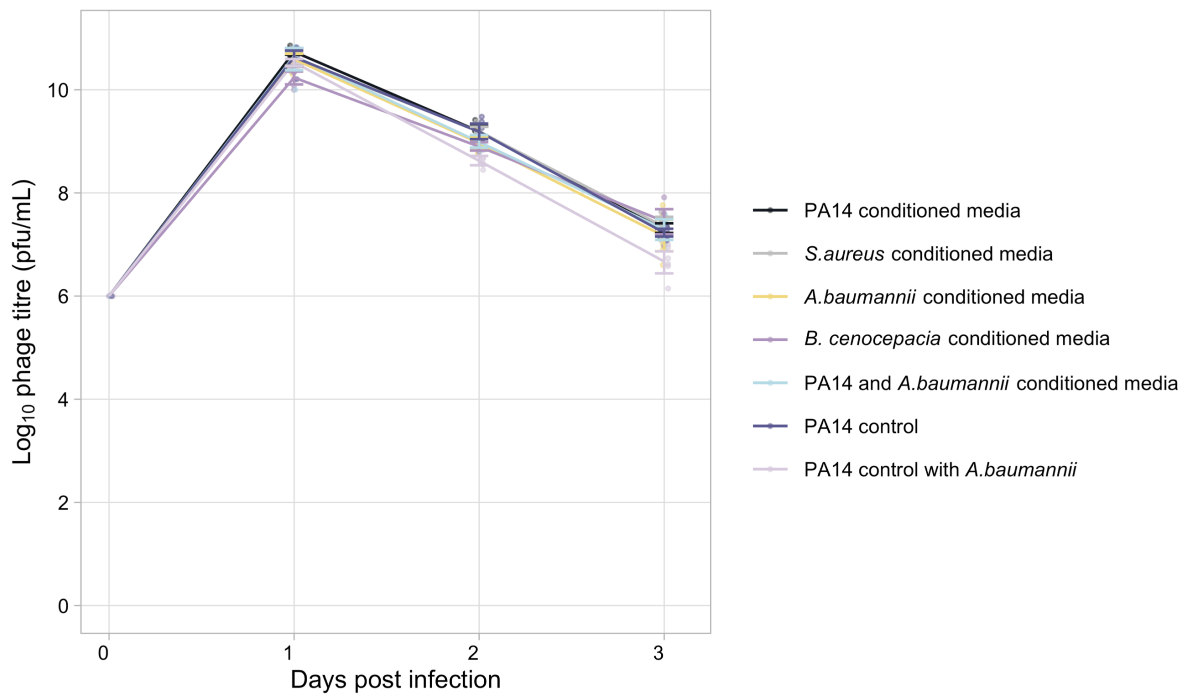
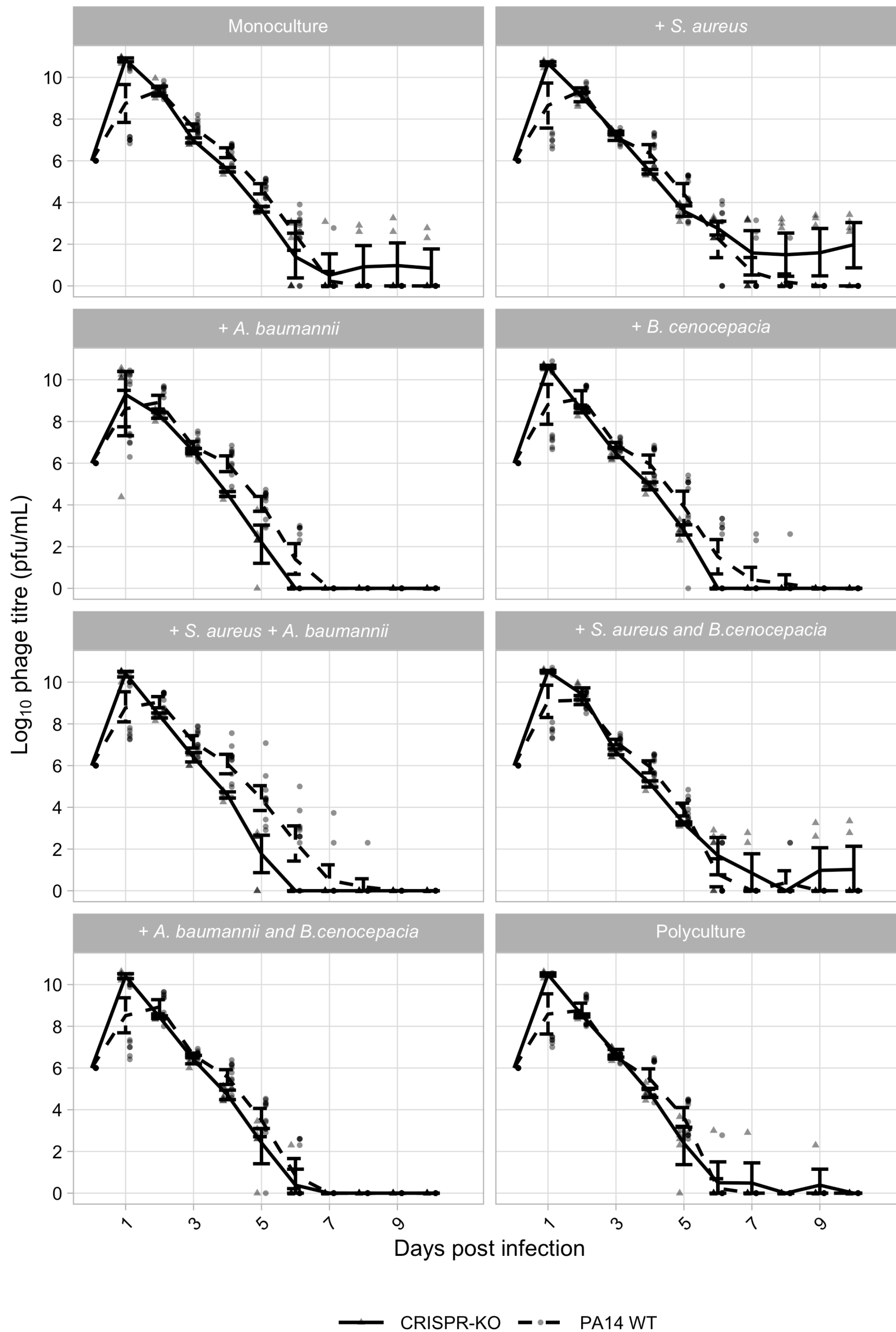


Figure S3.2 | Phage DMS3vir is not affected by conditioned media. DMS3*vir* phage titres in PFU/mL over time up until 3 DPI of its host *P. aeruginosa* in conditioned media from different bacteria and in LB controls (n = 12 per timepoint per treatment). Data are mean ± 95% CI.

Appendix to Chapter IV – Supplementary material



Supplemental Figure 4.1 | Phage titres over time for each experimental treatment. Phage titres for phage DMS3vir over time across all experimental treatments, infecting either the PA14 WT or the CRISPR-KO strain as indicated by colour. Each data point represents a replicate, with lines following the mean and the error bars denoting 95% CI. Asterisks indicate a significant overall difference in phage density between the PA14 WT (n = 12 per timepoint) or CRISPR-KO clone (n = 6 per timepoint) (effect of *P. aeruginosa* clone; linear models: * $p < 0.05$).

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