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The evolutionary ecology of CRISPR-Cas adaptive immunity

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

Microbial communities play a vital role in shaping their local environment and provide many important ecosystem services. The structure and function of microbial communities is dependent on interactions with prokaryote-specific viruses and other mobile genetic elements (MGEs), but we know relatively little about these interactions in nature. The prokaryotic adaptive immune system CRISPR-Cas provides resistance to phage and other MGEs by inserting phage-derived sequences into CRISPR loci on the host genome to allow sequence specific immunological memory against re-infection. Compared to the specific mechanism of CRISPR-Cas, phage resistance via surface modification provides general defense against a range of phage by physically modifying the cell surface to prevent phage infection. CRISPR-Cas and surface modification have been shown to be the most common mechanisms for rapid evolution of de novo phage resistance and therefore likely play important roles in shaping microbial communities. It has been suggested that we may be able to manipulate CRISPR-Cas evolution to our advantage, but very little research has been done investigating the evolutionary outcome of such manipulation. In this thesis I investigate the importance of different ecological drivers on when CRISPR-Cas is favoured over phage resistance via surface modification. I find that increasing CRISPR allele diversity within a host population increases phage immunity at the population level. However, increasing genetic diversity within the phage population increases selection for generalist phage defence via surface modification over specific CRISPR-Cas resistance. I also attempt to investigate the importance of cell-cell communication in the evolution of bacterial resistance; however these experiments were hampered by secondary effects of inhibiting cell-cell communication. These results are discussed in context with recent findings with the aim of expanding our knowledge of CRISPR-Cas evolution and ecology and suggesting where further research would be beneficial.

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General Introduction

The importance of microbial communities

Microbial communities play a vital role in shaping their local environment and provide many important ecosystem services including the breaking down of pollutants, municipal waste and ingested food, and the recycling of organic matter to plants and other autotrophs (1). These services are often essential to the health of the ecosystem: Microbial community health has been shown to be linked to the health and survival of associated organisms and therefore there are cascading effects of disturbance of natural microbial community dynamics. With recent rapid advances in 'omics' techniques and technologies we are now able to analyse microbes and their communities in more detail than ever before advancing a wide range of fields of microbial study (1–6). Microbial community health and dynamics have been shown to be important in many ecological processes, for example, plant-associated microbes trigger essential functions in plants such as germination, growth, disease defence, stress resistance, and influence plant fitness with microbial dysbiosis correlating with disease prevalence (7, 8). Insect population health has also been shown to be dependent on a healthy microbiome (9), and in aquatic environments microbes are known to be fundamental to biogeochemical cycles, the disruption of which has been suggested to have negative global effects (10, 11). In humans it has been found that early life exposure to microbes is a key determinant of gut microbiota diversity throughout the individual's life. Studies have shown that humans living in Western cultures where exposure to diverse microbial populations is reduced due to the effects of urbanisation and agriculture have decreased gut microbiota diversity (12, 13). This has been linked with an increase in inflammatory bowel disease, diabetes, obesity, allergies and asthma (13, 14), which has led to the suggestion that soil biodiversity loss could pose public health threat (13).

Studies focusing on microbial community dynamics and health are revealing that many human activities have a drastic and long-lasting impact. For example crop production has been shown to alter microbial communities in soil for years after the land is no longer used, even when varying crop rotations were implemented, which we now understand may represent irreparable damage to the associated

ecosystem (15). This may be partly due to the majority of microbial diversity being found in the top layers of soil (16) which is frequently and radically disturbed by any agricultural activity. Furthermore, we have found that we have been inadvertently imposing selective pressures on microbial communities through activities such as antibiotic and pesticide treatment which have resulted in undesirable changes in local microbial communities in agricultural and clinical settings. These changes include introducing antibiotic (17–19) and pesticide resistance (20, 21) which are strong contributing factors to major global health concerns (17).

In addition to their importance in nature, many industrial processes rely on microbes. For example yoghurt and cheese production typically involve the use of “starter strains” of bacteria such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which are added to milk where natural bacterial activity converts the lactose sugar found in milk to lactic acid (22, 23). Spores of the bacterium *Bacillus thuringiensis* are produced in vats, known as bioreactors, via fermentation. *B. thuringiensis* spores are the active agent in more than 50% of microbial control agents sold as they are relatively cheap and easy to apply (24). Different subspecies of *B. thuringiensis* are effective against a range of species including within the orders of Lepidoptera, Diptera, and Coleoptera and some pest nematode species (25–27). The ability to target different pest species allows for organic targeted pest control, minimising harm to the surrounding environment and allowing for more responsible and sustainable pest control (28–30). Further research into microbial interactions will allow for advances in practice and policy in industry which minimise product loss and reduce the cost of these products.

Bacteria-phage interactions influence microbial communities

Bacteria-phage interactions are significant because they play a major role in shaping microbial communities (31, 32). Bacterial populations which are currently, or have recently had contact with phage usually undergo genomic changes as a result of bacteria-phage interactions (33). The key processes of bacteria-phage interactions are the ability of phage to infect hosts and the ability of bacteria to defend themselves from phage infection. Both phage and bacteria

are known to evolve different mechanisms of infectivity and resistance, respectively, with different traits being favoured in differing ecological settings (34–37). Co-evolution between phage and bacterial populations has been found to be associated with strong selective pressures, resulting in drastically altered phenotypes evolving in both bacteria and phage populations when compared with the ancestral populations (33). Therefore understanding bacteria-phage dynamics will allow us greater insight into how to predict evolutionary outcomes with many potential applications including vaccine development (38), ecosystem analysis (39), co-evolution studies (40), infection prevention in microbial industrial applications (41), phage based medical and industrial treatments and environmental prophylaxis (42).

Phage infection can have dramatic effects on bacterial host population genomes (32, 37, 43, 44). Phage infection can select for genome wide evolution in bacterial hosts (45) as well as direct phage-mediated transfer of genes between host cells (46, 47). Common mutations acquired by bacteria as a result of contact with phage include reduced expression of genes involved in type IV pilus (T4P) and lipopolysaccharide (LPS) biosynthesis (48). Reduced T4P and LPS production infers resistance to a range of phage by interfering with phage adsorption, however mutations in these regions are associated with a fitness cost in the absence of phage (48). The T4P is known to act as a receptor for conjugation which has been shown to be an important mechanism in the horizontal transfer of pathogenicity islands (49) while lipopolysaccharide production is associated with virulence and biofilm formation (50–52). Therefore controlled phage infection presents an opportunity to manipulate bacterial populations to our advantage, reducing or selecting against the spread of pathogenicity and virulence traits, however detailed understanding of the evolutionary outcomes of specific bacteria-phage interactions is required before these practices can be applied on a large scale.

The antibiotic crisis underlines the importance of understanding microbes and how their evolution may impact us and is driving a wealth of research into alternative treatments (42, 53–55). Hospitals are known to be sources of microbial diversity and our search for ways to treat multi drug resistant strains has led to the resurrection of research into phage therapy (42). During phage therapy a patient with a bacterial infection is treated with phage which infect the

virulent bacterial species, thereby eradicating the virulent bacteria without the use of antibiotics. There have been promising trials and reports of using phage-based treatments for bacterial infections (56–60), however phage therapy is not standardly available yet, a lack of knowledge of microbial interactions may prevent the advancement of this treatment since tailoring the phage “cocktail” correctly to each patient is vital (42).

Phage based treatments could also help reduce losses in industrial production processes such as bioreactors incubating the bacterium *B. thuringiensis* to phage infection (61) or vats fermenting dairy products which rely on bacterial activity (41). These processes are highly vulnerable to phage infection as they require the creation of high densities of bacteria (41, 62) so phage infection can lead to huge losses, increasing the prices of products. This has led to the suggestion of manipulating the bacteria to infer phage resistance however, bacterial resistance against phage infection typically carries a cost to the host which results in reduced growth, productivity or other undesirable effects (34, 63, 64). This has led to numerous studies attempting to produce starter strains which are phage resistant but with minimal loss of productivity in yoghurt and cheese production (22, 23, 65, 66), as well as biopesticide production (67). Given the current global antibiotic and food crisis studies into microbial community dynamics could now provide new data to help remedy or inform policy relating to the use of microbes in both natural and industrial applications helping to alleviate these issues.

Phage defence mechanisms in bacteria

Recent advances in ‘omics’ technologies have rapidly accelerated our ability to locate possible novel defence mechanisms resulting in a recent boom in the number of known defence mechanisms. Makarova *et al* (68) first analysed the distribution of defence genes on the bacterial genome and revealed they typically form clusters known as defence islands. Consequent studies have used similar methods and have identified and characterised numerous novel defence systems with great success (69, 70), reviewed in (70). Our mechanistic understanding of most of these putative systems is still in very early stages but many bacterial resistance mechanisms have been previously described and studied in more detail, such as Restriction-Modification (RM) systems, surface modification (sm),

Abortive Infection (Abi) (71) and prokaryotic argonaute proteins (pAgo) (72, 73), and these have been reviewed in (37, 74–76).

Based on their mode of action phage defense mechanisms of bacteria can be broadly classified into three groups: resistance via cell dormancy and induction of programmed cell death, resistance based on variation of virus receptors, or resistance via immunity (69, 77, 78). Different types of defense are associated with different fitness costs which are thought to be key drivers of when different modes of phage defence evolve (79). Induced cell dormancy or death in response to phage infection prevents or delays the replication of phage within the infected host (77, 80), it has been hypothesised that this may “buy time” for the remaining host population to evolve immunity (37).Phage resistance via immunity relies on the ability of the immune mechanism to discriminate self from non-self DNA and to target only foreign DNA. Immune systems can be loosely categorised into two groups: Relatively non-specific innate immune systems or specific adaptive immune systems. Non-specific innate immune systems attack non-self DNA mostly indiscriminately. pAgos are a more newly discovered group of innate immune system and further research is needed to clarify the mechanism, costs and benefits of carrying this system (79, 81, 82). RM systems are a more well characterised example of innate defense mechanism which provide defence against phage as well as other mobile genetic elements (MGEs) (83–85), however carrying an RM system is thought to cause high rates of auto-immunity thereby making the system costly in the absence of maladaptive MGEs (86–88). CRISPR-Cas is a specific adaptive immune system which enables cells to “remember” previously encountered foreign DNA such as phage, plasmids or other MGEs. As in RM systems CRIPSR-Cas adaptive immunity functions by cleaving invading MGEs, however in contrast to innate immune systems cleavage is specific and guided by the “memory” of the adaptive immune system (89–92). CRISPR-Cas has found to carry an inducible fitness cost, i.e. the cost of defence increases with infection frequency (34). In contrast to the inducible cost of CRISPR-Cas immunity phage resistance which is acquired via alteration of receptors to which phage bind, preventing phage adsorption or injection, such as sm resistance (37, 93, 94) is associated with constitutive (fixed) fitness costs due to secondary effects of the physiological changes which can affect motility and biofilm formation (95), as well as growth rates (34, 64).

In the following studies we use an established laboratory bacteria-phage system to focus on when adaptive immunity with an inducible fitness cost (CRISPR-Cas) is favoured over sm resistance which is associated with a constitutive (fixed) cost. CRISPR-Cas and sm are the most common mechanisms for rapid evolution of de novo phage resistance, and therefore present an ideal system to study the selective pressures which drive the evolution of one type of defence mechanism over the other. We expect systems with different fitness costs will be favoured in different environments associated with different selective pressures, therefore by understanding what drives the evolution of one defence over another we can increase our ability to predict and manipulate microbial evolution.

sm mediated resistance and CRISPR-Cas defence in microbial communities

sm resistance and CRISPR mediated defence have been shown to be important in microbial ecological interactions (76, 79, 96). This is due to the rapid rate of evolution, compared to RM and Abi systems, meaning sm and CRISPR mediated immunity respond much more rapidly to changes in environment, with CRISPR loci evolution patterns consistent with an important role in antagonistic co-evolution with sympatric viruses (36). Horizontal gene transfer is thought to be one of the major ways CRISPR-Cas defence is acquired (97–101) while sm resistance is acquired via mutation, downregulation, loss or masking of the receptor to which the phage binds (94). sm mutations have been associated with a fitness cost, or increased susceptibility to another virus (64). While carrying a CRISPR loci does not appear to have a significant fitness cost compared with a sensitive strain in the absence of phage (34), it does not provide resistance against the same range of phage genotypes as sm resistance. It also may be costly to carry a CRISPR-Cas system when beneficial plasmids are present, for example plasmids conferring antibiotic resistance in an environment where antibiotics are present may be targeted by CRISPR-Cas as invading DNA (102).

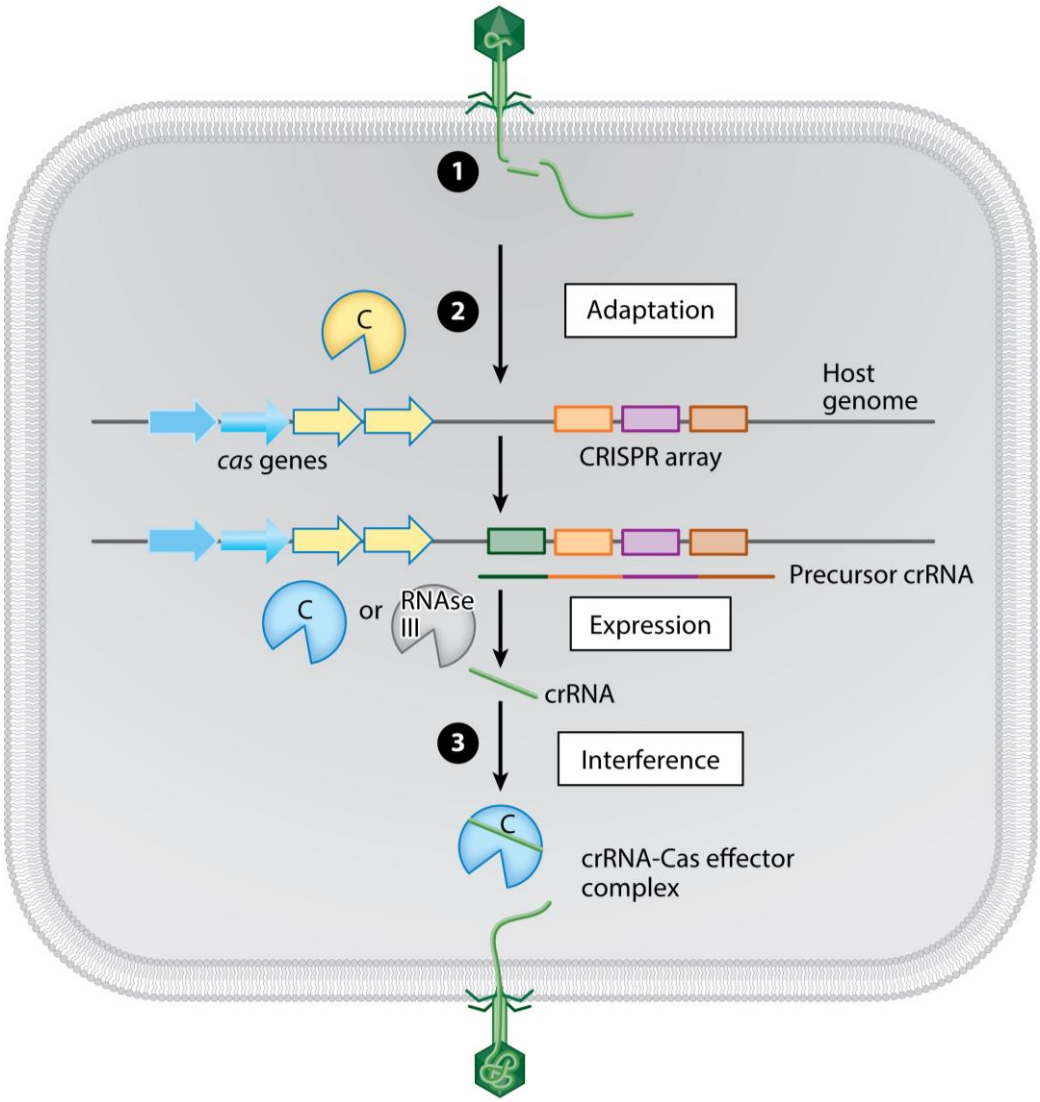
The evolution of bacterial immune defence drives diversification in phage populations resulting in phage which are able to overcome bacterial defences (37, 103). Phage have been found to evolve to overcome sm resistance using carbohydrate degrading enzymes (104) and overcome other types of receptor

modification by mutation within the phage genome which allows the phage to adsorb to the altered receptor (105). Comparatively, phage can evolve to overcome CRISPR-based resistance relatively easily by a single point mutation (106), in a specific region of the targeted sequence. These mutations arise frequently and are typically not costly to the phage, however the fitness cost associated with maintaining these mutations will likely depend on a range of variables, including the number of point mutations previously acquired and host diversity (103). When phage evade targeting by an existing spacer, bacteria are only able to re-gain resistance by acquiring a new spacer matching the new phage genotype (107).

Evolution of sm mediated phage defence has been associated with various traits of interest such as virulence factors, the ability to form biofilms (50–52), and transfer MGEs (49). The secondary effects of carrying a CRISPR-Cas system are less clear. Understanding when sm defence evolves over CRISPR-Cas resistance could have direct applications in medical or industrial settings. By comparing rates of evolution of a phage defence mechanism associated with a fixed fitness cost and broad range of phage resistance, such as sm, with a specific resistance mechanism with a varying fitness cost such as CRISPR-Cas we can gain insight into when one type of defence or resistance is likely to evolve over the other. This data will help us to predict and manipulate the evolution of microbial communities where these systems are present as well as contributing to our ecological understanding of how CRISPR-Cas systems function.

CRISPR Mechanism

Box 1



(Figure previously published in (36))

Box 1 continued

CRISPR-Cas Mechanism: **(1)** Adaptation takes place when a bacterial cell encounters a novel phage; Cas (CRISPR-associated) proteins detect and target the invading DNA via recognition of a specific sequence known as a PAM (Protospacer Adjacent Motif). **(2)** A portion of the phage genome (known as a protospacer) is then cleaved and incorporated into the CRISPR loci (107, 108) where it is known as a "spacer" in the CRISPR array (109). Expression happens when the CRISPR array is transcribed as a precursor transcript (pre-crRNA), which is processed and matured to produce CRISPR RNAs (crRNAs). **(3)** Finally, during interference processed CRISPR transcripts form a crRNA-effector complex which guide Cas proteins to detect and destroy re-infecting phage that carry the cognate target sequence (89–92, 110, 111).

CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR-associated) adaptive immune systems are found in approximately 30% of bacteria (78) and around 90% of archaea. In most CRISPR-Cas systems there are 3 main stages to acquisition of immunity; adaptation, expression and interference (see box 1). Phage can in turn evolve to overcome CRISPR-based resistance by point mutation in the PAM sequence on their genome (106), although the emergence and spread of such phage "escape" mutants is constrained by both the diversity of CRISPR resistance alleles in the bacterial population and the fitness costs associated with the phage mutations (35, 112–114). More detailed reviews of the intricacies of CRISPR-Cas defence can be found in the following (108, 115–120).

We are already aware of a wide range of CRISPR-Cas variants divided into 2 classes, 6 types and 33 subtypes. Classification of CRISPR-Cas systems is based on a range of factors including signature genes, gene repertoire comparison and genomic loci organizations (2). The 2 main classes are distinguished by the architecture of the proteins which locate and cleave invading target sequences, known as effector complexes. In class 1 systems the effector complex produced during the expression stage is made up of multiple Cas proteins forming a multisubunit to which the matured guide crRNAs bind. In Class 2 systems the guide crRNA binds with a single multidomain protein. Research

into Class 2 CRISPR-Cas systems has advanced rapidly in recent years driven by the desire for new genome editing tools (121–130), however class 1 systems are by far more prevalent in nature (100). While research into Class 2 systems has yielded vital and revolutionary laboratory techniques research into Class 1 systems is far more relevant to natural scenarios. It has also been suggested that some Class 1 derived variants of CRISPR-Cas may perform functions other than adaptive immunity (119, 131, 132) highlighting our lack of understanding of CRISPR-Cas function in nature and the need for further investigation into Class 1 CRISPR-Cas systems.

Understanding CRISPR-Cas in ecologically relevant contexts

Most laboratory experiments use clonal phage and bacterial cultures when investigating the evolution of CRISPR-Cas, which is not representative of typical natural scenarios. To date most studies have focused on either the evolution of CRISPR-Cas at the individual level or the evolution of CRISPR-Cas in isolation of other traits. It has been shown that diversity of a Class 1 CRISPR-Cas system at the population level was an important driver in the evolutionary outcome of phage infection on both the host and phage populations (35), and it has recently been shown that the evolution of CRISPR-Cas is influenced by the local microbial community (133). These insights are relatively new and highlight how the reductionist approach taken by many previous studies hampers our ability to understand the ecological relevance of CRISPR-Cas in nature.

Genetic diversity is likely an important determinate of CRISPR-Cas evolutionary dynamics

Despite advances in our mechanistic understanding of the different defence strategies of bacteria, the ecological conditions that favour one type of defence over another remain unclear (36, 79). High levels of within-population CRISPR spacer diversity have been found in nature (134–136) and it has been shown in laboratory experiments that bacterial population resistance increases with the number of unique spacers present in the population (35). High population level CRISPR-allele diversity results in increasingly rapid phage extinction (35), and

bacterial species diversity increases selection for CRISPR-Cas evolution (133). Intuitively, phage genetic diversity is likely to be an important determinant of when CRISPR-based resistance or sm based immunity evolves since the latter generally provides more broad-range resistance compared to the sequence-specific resistance of CRISPR-Cas. Because CRISPR-Cas needs a spacer targeting an infecting phage in order to recognise and destroy the phage each infection by a new genotype requires the acquisition of a new spacer to provide resistance.

Theoretical models support the idea that diversity is an important driver in the evolution of CRISPR-Cas resistance with increasing phage diversity increasing the cost of CRISPR-Cas resistance through cost of new spacer acquisition and system activation costs (137, 138). This idea is supported by correlational studies, which suggest that CRISPR-Cas immune systems are over-represented in thermophiles, which tend to have a lower mutation rates (137–140) and have lower host and parasite densities (137). Apart from accelerating the evolution of "escape" phages, high mutation rates will also increase the rate at which surface based resistance evolves (141). Empirical evidence is needed to determine the importance of phage diversity in the evolution of CRISPR-Cas.

Cell-Cell communication may influence CRISPR-Cas evolution

There has been suggestion that CRISPR-Cas evolution could be manipulated via alternate methods, for example it has been shown that inhibiting Quorum Sensing (QS), a cell-cell communication system used by bacteria reduces the production of the Cas protein involved in DNA cleavage (142). Since CRISPR-Cas is selected for at low cell densities (under low risk of infection), it is unsurprising that it would be regulated by a mechanism which monitors cell density, however research has shown that QS is more important at high cell densities so the strength of interaction between QS and CRISPR-Cas remains unclear as we expect each system to be important in contrasting environments. Evolution experiments are required to directly test the outcome of QS inhibition on CRISPR-Cas resistance evolution to validate if this may be a viable approach to manipulate CRISPR-Cas evolution.

Can we manipulate CRISPR-Cas evolution to our advantage?

The CRISPR-Cas machinery has proved to be a powerful tool in genome editing as it is a specific, accurate and programmable mechanism and numerous methods of manipulating CRISPR-Cas to our advantage have been already been proposed (143). The presence of CRISPR-Cas in natural microbial communities could present an opportunity to manipulate the CRISPR-Cas system to target undesirable MGEs such as plasmids containing antimicrobial resistance genes (102). Some studies have already suggested that by inserting or manipulating a CRISPR-Cas system in bacteria used in industry involving high density cultures of bacteria which are susceptible to phage infection (for example in yoghurt and cheese production) we could reduce the volume of product which is lost to phage infection by providing resistance with minimal loss of productivity (144). This method may also be applicable to the production of *B. thuringiensis* spores which are fermented in high densities and so are also susceptible to phage infection. However, the presence of CRISPR-Cas resistance in a bacterial population would potentially be undesirable in alternate scenarios, for example in phage therapy where the presence and rapid adaptation of CRISPR-Cas could result in eradication or reduced efficacy of the applied phage cocktail and the evolution of phage resistant virulent cells.

Aims and Objectives

Here I explore the importance of CRISPR-Cas ecology investigating both within genome and within population outcomes of bacteria-phage interactions. I have specifically focused on two possibly influencing factors which have recently been indicated to be involved in CRISPR-Cas evolution: Firstly I investigate the role of genetic diversity within a phage population, which has been predicted to be an important influence of CRISPR-Cas evolution (137) and population level diversity in the CRISPR-Cas locus of a host population has also been shown to be an important driver of CRISPR-Cas evolution (35). Secondly, I investigate the importance of QS in the evolutionary outcome of phage infection on CRISPR-Cas since QS has been shown to affect the expression of a vital CRISPR-Cas component (53) but the importance of this in the evolution of CRISPR-Cas in response to phage infection is not known.

In this thesis I investigate the evolutionary and ecological importance of CRISPR-Cas evolution, specifically focusing on when the evolution of sm (fixed cost) resistance is favoured over the evolution of CRISPR-Cas (inducible cost) resistance. The data presented here provide insights into the ecological conditions which favour one type of defence over the other and can inform any studies investigating systems where CRISPR-Cas is present, including in clinical, natural and industrial settings.

Chapter 1: Phage adaptation in the context of diverse CRISPR resistance alleles

High within-population CRISPR-Cas allele diversity is often observed in natural systems but it is unclear why this is important. Here I use bacterial populations harbouring varying levels of CRISPR allele diversity to investigate:

1. The role of within-population CRISPR allele diversity in phage defence.
2. How CRISPR allele diversity affects the ability of phage to overcome targeting by CRISPR-Cas and how this affects local adaptation to hosts by phage.

Chapter 2: The Effect of Phage Genetic Diversity on Bacterial Resistance Evolution

Using an experimentally evolved phage population with increased within-population genetic diversity I test:

1. How within-population phage diversity affects when the evolution of CRISPR-Cas is favoured over sm in infected populations.
2. The effect of within-population phage diversity on the rate of CRISPR spacer acquisition
3. Differences in rates of phage extinction/persistence when phage populations differ in within-population diversity.

Chapter 3: The interaction between Quorum Sensing and CRISPR-Cas evolution

Based on findings that QS plays a role in activation of CRISPR-Cas I manipulate QS to test:

1. How inhibiting QS affects the evolutionary dynamics of CRISPR-Cas, specifically if manipulating QS favours CRSISPR-Cas over sm mediated phage defence.

2. If QS manipulation affects the fitness of bacteria in the presence of phage
3. For secondary phenotypic effects induced by QS inhibition which may influence bacterial fitness

Study system

Pseudomonas aeruginosa is a well-studied model organism, it is environmentally ubiquitous (145), known to cause infections in a range of hosts (146–148). Single strains of *P. aeruginosa* are capable of infecting multiple host species, it is prevalent in nosocomial infections and is capable of causing chronic and acute infections in humans (149, 150). It is the main pathogen causing chronic lung infections and leading cause of mortality in Cystic Fibrosis patients (151). Research into the development of new antimicrobials effective against *P. aeruginosa* is listed as of critical importance by the World Health Organisation (152) with multiple strains now found to be resistant to our last-line of defence antibiotics such as β-lactams and carbapenem (149). In our search for alternatives to antibiotic treatments research into phage therapy has seen a reawakening in recent years with *P. aeruginosa* frequently being the target species (153–155) with some reports of successful eradication of virulent strains via phage therapy (56).

P. aeruginosa strain PA14 and its phage DMS3vir (156) are an established laboratory model system for studying the evolution of CRISPR-Cas. *P. aeruginosa* PA14, originally isolated from a human infection (157) carries a type I-F CRISPR-Cas system, has a broad host range and is of primary concern in nosocomial infections (148). The phage DMS3 is a mu like phage, originally isolated from a pathogenic strain of PA14 and infects bacterial cell via the Type IV Pilus (158). Here we use an engineered obligately lytic version of DMS3, DMS3vir (156). Under laboratory conditions PA14 evolves resistance to DMS3vir through either CRISPR-Cas defence or sm resistance. Research using this organism then has direct applications to the management of problematic *P. aeruginosa* strains in clinical or natural environments but will also provide more general knowledge about the evolutionary dynamics which dictate when different types of resistance mechanism are likely to evolve.

Chapter 1

Phage adaptation in the context of diverse CRISPR resistance alleles

Abstract

The prokaryotic adaptive immune system CRISPR-Cas is known to play an important role in bacteria-phage dynamics. CRISPR-Cas provides sequence specific immunity by inserting sections of foreign DNA into the host genome as “spacers”, allowing hosts to detect and destroy invading DNA of viruses and other parasitic mobile genetic elements. CRISPR-Cas is a highly specific defense mechanism, and in theory this could lead to ongoing co-evolution and local adaptation. Host populations possessing CRISPR-Cas have been shown to develop high within- population spacer diversity, and increasing levels of CRISPR allele diversity in a host population can prevent phage from becoming locally adapted. However, increased levels of phage diversity can increase phage adaptation, and CRISPR-Cas diversity can drive the evolution of genetic diversity in phage populations, and vice-versa. The relationships between CRISPR-cas diversity and local adaptation may therefore be complex. Here I reanalyse data derived from previous work to investigate in more detail the relationship between host CRISPR allele diversity and the evolutionary effects that has on phage populations. Specifically, I quantify and look for relationships between CRISPR allele diversity, phage local adaptation, phage diversity and rates phage evolution (genetic distance from ancestor). I show that increasing CRISPR allele diversity in a host population reduces the evolutionary potential of phage to become locally adapted. This is a because increasing CRISPR allele diversity reduces the amount of genetic diversity which evolves in the phage population, and therefore the likelihood of local adaptation occurring. These new analyses help us understand in more detail the intricate balances in host-parasite evolution and indicate which variables are important for ongoing co-evolution.

Introduction

CRISPR-Cas is a prokaryotic adaptive defence mechanism which allows hosts to target previously encountered phages and other parasitic mobile genetic elements (109). The mechanism of CRISPR-Cas has been described in detail previously (108, 115, 118, 120). In brief, there are three stages to CRISPR-Cas defence: **1)** During *adaptation* a section of DNA is cleaved from the phage genome (where it is known as a protospacer) and integrated into the CRISPR

array as a spacer (107). **2)** *Expression* of the CRISPR array and subsequent cleaving of the array produces mature guide CRISPR-RNAs (crRNAs) complementary to single spacers acquired from previously encountered foreign DNA elements (159). **3)** *Interference* occurs when CRISPR-associated (*Cas*) proteins bind with the mature crRNAs which serve as guides for the *Cas* proteins to cleave foreign DNA complementary to the spacer, destroying the invasive DNA (160). Mature crRNAs detect foreign genetic elements via highly specific base-pairing with a short string of nucleotides within the protospacer known as the seed region, as well as a highly-conserved sequence directly adjacent to the protospacer on the phage genome termed the protospacer-adjacent motif (PAM; see (161)). It has been suggested that the absence of PAMs in the host CRISPR loci helps to prevent autoimmunity (162), however the high specificity of binding during interference by CRISPR-Cas means that phage are able to evade targeting via single point mutations in the PAM or protospacer regions (34, 106). High infection specificity can lead to ongoing coevolution (137, 163, 164). Indeed, phage have been reported to persist in wild-type populations of *Streptococcus thermophilus* for at least 30 days (136); although this length of co-evolution under laboratory conditions is unusual (34, 35, 165) and may be attributed to the lower rate of spacer acquisition and high number of duplicate spacers that *S. thermophilus* is known to acquire (135, 136) compared to other bacteria.

Most of our knowledge of the evolution of CRISPR-Cas comes from laboratory studies using clonal bacteria and phage stocks. In bacteria-phage interactions in nature we expect much higher levels of diversity in both bacteria and phage populations. While phage are able to escape CRISPR-Cas targeting relatively easily when there is only one spacer targeting them, as spacer diversity within a host population increases so does the number of mutations phage require to escape targeting making them less able to adapt to host populations (35). Under the assumptions that the infection process is specific, that a parasite genotype can infect a restricted number of host genotypes and that a failed infection results in parasite death, it is predicted that host genetic diversity will prevent parasites from spreading through the population (112, 137, 164, 166–171). Since this results in fewer successful infections the viral population size (166, 167), and therefore genetic diversity, is minimised, reducing the likelihood of the emergence

of phage which can escape CRISPR-Cas targeting. As spacer diversity within a host population increases so does the number of mutations required for phage to escape targeting (35), concurrently diverse phage are more able to infect host populations and are more likely to elicit the acquisition of multiple spacers in CRISPR-resistant hosts (43). This indicates a complex role of genetic diversity in bacteria-phage interactions which is currently poorly understood.

In a specific interaction such as that between CRISPR-Cas resistant hosts and infecting phage the ability of phage to become locally adapted is predicted to be affected by genetic diversity (172–174). Local adaptation can be said to have occurred when an organism is more adapted to one environment than another (for example, when a phage is more infective against sympatric than allopatric hosts) (174). Understanding how and when local adaptation occurs will help us to understand what traits are favoured in different environments as well as how natural selection functions relative to gene flow or other evolutionary drivers (174). Assuming a parasite genotype needs to match the host genotype for infection to occur, increasing the number (diversity) of host genotypes will reduce the potential for local adaptation (175) because it will reduce both between host population differentiation and mean infectivity across all populations. The reciprocal argument holds if resistance is determined by matching: more parasite genotypes (more diverse parasites) decrease differentiation between parasite populations and mean resistance. Given that host inter and intra population diversity affects the diversity of infecting parasites and vice versa, this can lead to complex relationships between genetic diversity and patterns of local adaptation.

Here I present data which is a collation and re-analysis of results from two collaborative projects I worked on. The first project investigated the effect of CRISPR allele diversity within a host population on the ability of phage to infect the population (35) and the second looked at the resultant impact of host CRISPR allele diversity on parasite local adaptation (165). During the first project we created host populations harbouring increasing levels of CRISPR allele diversity and then monitored phage infectivity over time to test the effect of CRISPR allele

diversity on phage persistence and evolution. We found phage are less able to infect a host population as host CRISPR allele diversity increases. The second project looked at phage local adaptation as a function of differing levels of CRISPR allele diversity using evolved phage and CRISPR host populations from the first project. We calculated the level of phage local adaptation to host populations by measuring phage infectivity against hosts which were or were not present in their host population. I extend this work in two key ways. Firstly, I determine accurate levels of host diversity; due to duplication of spacers within host populations used in these studies host diversity was overestimated. I correct for this and use the new data to investigate the role of host diversity in phage evolution in more detail. Secondly, I determine how genetic changes in the evolved phages resulting from the different host diversity treatments correlate with phage local adaptation. Specifically, I determine how phage genetic diversity and the extent to which phage have evolved (genetic distance from their ancestor) correlate with local adaptation. All things being equal, high phage diversity should lower local adaptation rates, while genetic distance from ancestor should increase, because populations will be more differentiated. However, the possible correlation between these two phage measures as well as host diversity, makes the outcomes unclear. My new analyses demonstrate that increasing host CRISPR allele diversity greatly impacts the amount of phage adaptation and that increasing CRISPR allele diversity limits the evolution of genetic diversity and the extent to which phage can become locally adapted.

Methods

The following experiments were performed by van Houte *et al* (35), I assisted in running and processing data from the competition experiments and extracting the evolved phage which were used in follow up work: Myself and Dan Morley tested each phage isolate for infectivity against sympatric and allopatric hosts (see (165) for full analyses). Consequent to these papers being published it was discovered that some of the CRISPR-resistance hosts used in these experiments harboured either multiple or duplicate spacers. In this chapter I use this data to investigate how variables such as phage genetic diversity and genetic distance from ancestor correlate with local adaptation and host population CRISPR allele diversity.

Below are brief methodologies of how data was generated, please see the original papers (35, 165) for full details and alternate analyses.

Previously published experimental work

Bacterial strains and viruses

P. aeruginosa UCBPP-PA14 (WT), *P. aeruginosa* UCBPP-PA14 *csy3*::LacZ (referred to as CRISPR KO, which carries a disruption of an essential cas gene and can therefore not evolve CRISPR immunity), the CRISPR KO-derived surface mutant and virus DMS3vir have all been described in (34) and references therein. Phage DMS3vir+*acrF1*, which carries the anti-CRISPR gene *acrF1* (formerly known as DMS3vir 30-35), was made by inserting *acrF1* into the DMS3vir genome using methods described in (176). *Streptococcus thermophilus* strain DGCC7710 and its virus 2972 have been described in (109).

Generation of populations with different levels of CRISPR diversity

48 individual clones that had acquired CRISPR immunity against virus DMS3vir were isolated from the 6 replicates of the coevolution experiment described in Fig. 1 in (35). It has previously shown that individual clones tend to have unique spacers (34). Using these 48 clones, populations with five different levels of CRISPR spacer (allele) diversity were generated. These populations consisted of: 1) 1 clone (a monoculture; a clonal population carrying a single spacer); equal mixtures of 2) 6 clones; 3) 12 clones; 4) 24 clones and 5) 48 clones. In total 48 different monocultures (48 x monocultures), 8 x 6-clone populations, 4 x 12-clone populations, 2 x 24-clone populations and 1 x 48-clone population were generated.

To ensure equal representation of each of the 48 clones across the different treatments, the number of replicate experiments for a given diversity treatment was adjusted accordingly, with a total number of replicates of at least six for sufficient statistical power. Hence, competition experiments with the one-clone (monoculture) populations were performed in 48 independent replicates, each

corresponding to a unique monoculture of a CRISPR clone (clones 1–48; each clone is equally represented). Competition experiments with the 6-clone populations were performed in eight independent replicates, each corresponding to a unique polyculture population (population 1: equal mixture of clones 1–6; population 2: clones 7– 12; population 3: clones 13–18; population 4: clones 19–24; population 5: clones 25–30; population 6: clones 31–36; population 7: clones 37–42; population 8: clones 43–48). Competition experiments with the 12-clone populations were also performed in eight replicates, corresponding to four unique polyculture populations (replicate 1 and 2: clones 1–12; replicate 3 and 4: clones 13–24; replicate 5 and 6: clones 25–36; replicate 7 and 8: clones 37–48). Competition experiments with the 24-clone populations were performed in six replicates, corresponding to two unique polyculture populations (replicate 1–3: clones 1–24; replicate 4–6: clones 25–48). Competition experiments with the 48-clone populations were performed in six replicates, each corresponding to the same polyculture population (replicate 1–6: clones 1–48).

Competition experiments in *P aeruginosa*

Competition experiments were done in glass microcosms in a total volume of 6 ml M9 supplemented with 0.2% glucose. Competition experiments were initiated by inoculating 1:100 from a 1:1 mixture (in M9 salts) of overnight cultures of the appropriate *P aeruginosa* CRISPR population and the surface mutant. At the start of each experiment 10^9 pfu of DMS3vir was added, unless indicated otherwise. Cultures were transferred daily 1:100 into fresh broth. At 0 and 72 hours post-infection (hpi) samples were taken and cells were serially diluted in M9 salts and plated on LB agar supplemented with 50 $\mu g.ml^{-1}$ X-gal (to allow discrimination between WT-derived CRISPR clones (white) and CRISPR KO or surface mutant (blue)).

Effect of spacer diversity in *Streptococcus thermophilus*

Streptococcus thermophilus DGCC7710 was grown in M17 medium supplemented with 0.5% α-lactose (LM17) at 42°C. Virus 2972 was used throughout the experiments. Virus infections were carried out using 10^6 pfus of

phage 2972 and 10mM $CaCl_2$ to facilitate the infection process. To obtain virus-resistant *S. thermophilus* clones, a sample of virus lysate at 24 hpi was plated on LM17 agar plates. Individual colonies were picked and PCR-screened for the acquisition of novel spacers in each of the 4 CRISPR loci, as described in (109). A total of 44 individual clones with a novel spacer in CRISPR1 (see (164) and references therein) were selected to generate 44 monocultures and a single polyculture comprised of a mix of 44 clones. These cultures were infected with 10^7 pfu of virus, and samples were taken after the indicated periods of time to isolate virus. We determined virus titres by spotting viral dilutions on lawns of ancestral bacteria, and the emergence of escape virus by spotting virus on lawns corresponding to each of the 44 CRISPR resistant clones.

Determination of escape virus emergence

To determine the emergence of escape virus during the competition experiments, every isolated virus sample was spotted onto 48 (*P aeruginosa*) or 44 (*S thermophilus*) different bacterial lawns, corresponding to each of the different CRISPR clones present in the competition experiments. This procedure was done for each of the seven time points (see above), to enable us to track the emergence of escape virus against every individual clone over the time course of the experiment.

Deep sequencing

Isolated phage samples from t=1 dpi of the competition experiment shown in Fig. 1 were used to perform deep sequencing of spacer target sites on the phage genomes. To obtain sufficient material, phage were amplified by plaque assay on the CRISPR KO strain. Viruses from all replicates within a single diversity treatment were pooled. As a control, ancestral virus and escape virus from competition between sm and monocultures of CRISPR clones 1-3 were processed in parallel. Virus genomic DNA extraction was performed from 5 ml sample at approximately 10^{10} pfu/ml using the Norgen phage DNA isolation kit, following the manufacturer's instructions. Barcoded Illumina Truseq Nano libraries were constructed from each DNA sample with an approximately 350bp insert size and 2x 250bp reads generated on an Illumina MiSeq platform. Reads

were trimmed using Cutadapt v1.2.1 and Sickle v1.200 and then overlapping reads merged using Flash v1.2.8 to create high quality sequence at approximately 8000x coverage of DMS3vir per sample. These reads were mapped to PA14 and DMS3vir genomes using bwa mem v0.7.12 and allele frequencies of Single Nucleotide Polymorphisms (SNPs) within viral target regions quantified using samtools mpileup v0.1.18. Further statistical analyses were performed in R v3.2.2. Sequence data are available from the European Nucleotide Archive under accession PRJEB12001 and analysis scripts are available from https://github.com/scottishwormboy/vanHoute.

Measuring local adaptation of phage to host populations competed with a phage-immune host

To investigate the extent of phage local adaptation to host populations which had occurred during the previously described experiment I conducted follow up experiments with Dan Morley (previously published (165). We took evolved phage from the experiment described above from 1,2 and 3 dpi and tested their ability to infect hosts which were in the host population which the phage had evolved with (allopatric hosts) or hosts which the phage had not been evolved with (sympatric hosts). To do this we performed spot assays on monoculture bacterial lawns of each of the host bacteria used in the mixed host populations so each phage population was tested for infectivity against both allopatric and sympatric hosts. Here phage "performance" was measured as the ability to infect a CRISPR clone. Phage local adaptation was analysed by subtracting the performance of allopatric phages from the performance of the sympatric phage for each host ('foreign vs. local') and by subtracting the performance of a phage population on allopatric hosts from the performance of that phage population on sympatric hosts ('home vs. away') (174) (Discussed further below).

New Analyses of Data

Quantifying SNP frequencies

Following evolution on host populations harbouring increasing levels of CRISPR allele diversity van Houte *et al* (35) performed deep sequencing on the regions of the phage genome targeted by each of the CRSIPR spacers (Fig. 2). In order to prepare this data (raw data available from https://github.com/scottishwormboy/vanHoute) I corrected for any selection bias that may have been introduced by duplicate spacers within a group as well as accounting for dilution effects which occurred during the sequencing process due to treatments being pooled. To do this the following formula was applied to each sequenced target region for each phage group:

SNP frequency x (number of groups in treatment/number of duplicate spacers at target site present in group)

This provided me with a corrected SNP frequency for each site on the phage genome targeted by one of the CRISPR resistant hosts in the set of 48 BIMs which made up the populations the phage were evolved on. I was then able to use this data to quantify phage diversity, and the difference between the evolved and ancestral phage at each target site (see below).

Quantifying Diversity

Using the phage SNP data described above and shown in Fig. 2 I applied a gini-simpson's diversity calculation to each sequenced target site. I used the formula, where P is the SNP frequency within a phage sample at a given target site:

(1-(P^2))+((1-P)^2))

Calculating genetic distance from ancestor

Using the phage SNP data described above and shown in Fig. 2 I calculated the genetic distance of the evolved phage from the ancestral phage (DMS3vir) using the formula:

SNP freq. of evolved phage at target site/SNP freq. of ancestral at same target site

Quantifying phage local adaptation

I initially applied both the 'foreign vs. local' and 'home vs. away' measures of local adaptation suggested by Kawecki and Ebert (2004) (174), however, as in the findings presented in Morley & Broniewski (2016) (165) the difference in the results of these two measures was negligible. I therefore used the 'foreign vs. local' formula to calculate levels of local adaptation for further analysis and collation with other data sets. The 'foreign vs. local' value is calculated by subtracting the performance of allopatric (foreign) phages from the performance of the sympatric (local) phage for each of the 48 clones from work with van Houte *et al* (35) where phage performance was defined as ability to infect a given host.

Results

Infectivity evolution and phage persistence

I assisted van Houte *et al* (35) in investigating the idea that increasing CRISPR-allele diversity within a host population would make phage less able to infect that population. To generate the data I have shown here in Figure 1 bacterial populations were generated in which the level of spacer diversity was manipulated. Using 48 individual clones with CRISPR-based immunity against virus DMS3vir bacterial populations with five distinct diversity levels were generated: monocultures or polycultures consisting of equal mixtures of either 6, 12, 24 or 48 clones. To allow for direct comparisons, each of the 48 clones was equally represented at each diversity level by adjusting the number of replicate experiments accordingly. Each population was competed against a previously described strain of *P aeruginosa* PA14 which has a mutation in the receptor where the phage bind with the host, preventing phage infection (34). This is known as surface mutant (sm) based resistance and provides defence against a range of phage genotypes as opposed to the very specific mechanism of CRISPR based immunity. The competitions were performed in the presence or absence of virus DMS3vir and the evolution of viral ability to overcome CRISPR resistance

alleles was monitored over time. To this end, virus isolated from each time point (0, 16, 24, 40, 48, 64 and 72 hours post-infection) was spotted onto lawns of each of the 48 CRISPR clones. To test that our results were not limited to the *P aeruginosa* PA14 Type I-F CRISPR-Cas system, we performed a similar experiment with *Streptococcus thermophilus* DGCC7710 clones that evolved resistance against virus 2972 using a Type II-A CRISPR-Cas system. As shown in Fig. 1B, we found a similar effect of CRISPR based resistance allele diversity on escape virus emergence.

Figure 1A

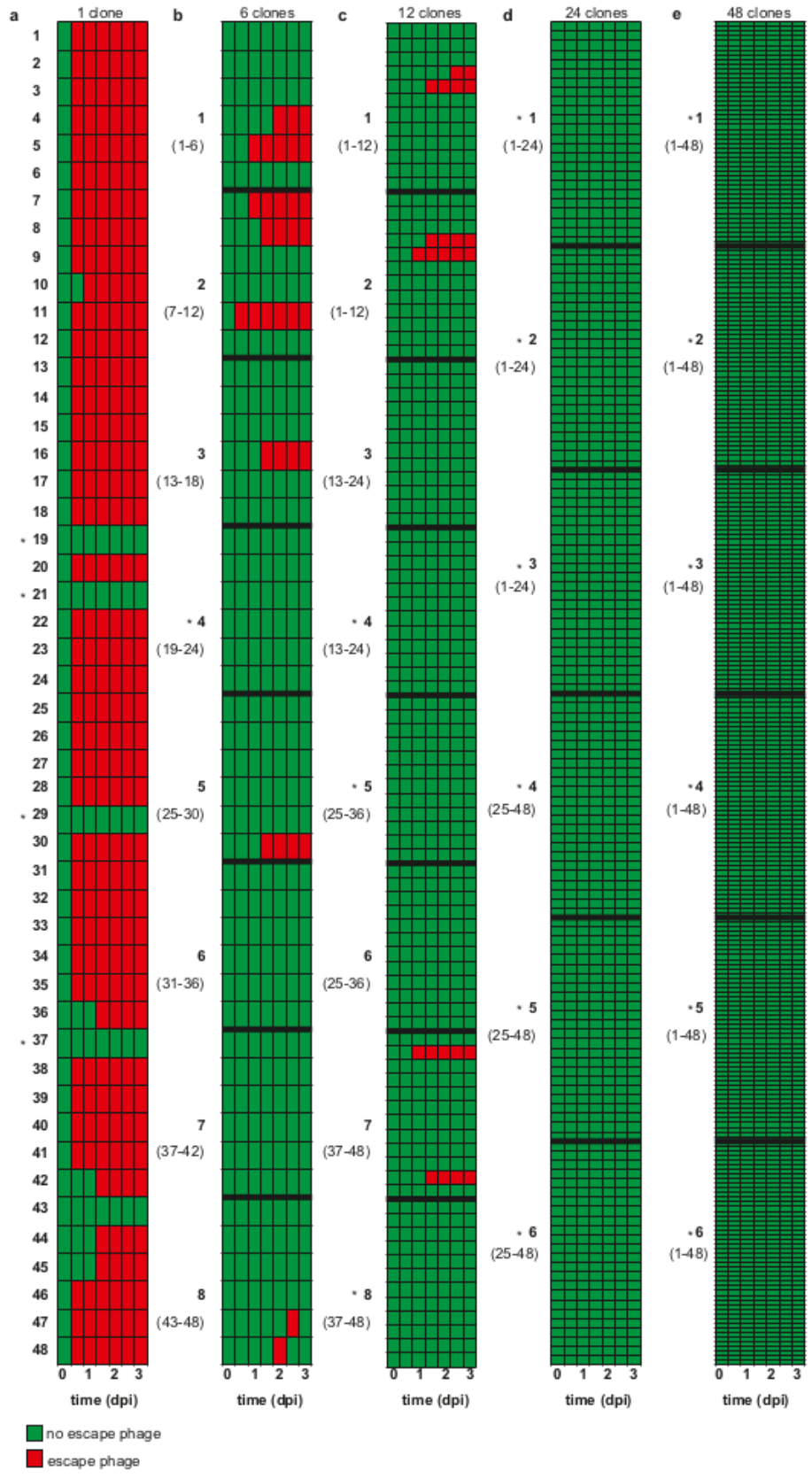
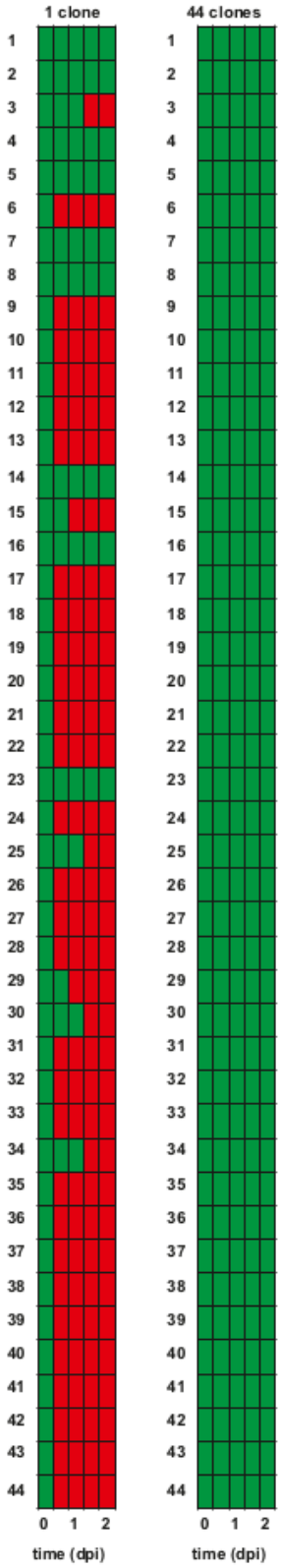


Figure 1B



Figures from van Houte *et al* (2016) (35)

Figure 1. Evolution of virus infectivity is constrained by spacer diversity

Figure 1A Emergence of virus that overcomes *P aeruginosa* CRISPR mediated immunity (escape virus) over time. Table columns correspond to time points where virus was isolated (0, 16, 24, 40, 48, 64 and 72 hours post-infection; indicated below the table in days post-infection). Green: no escape virus. Red: escape virus. Bold numbers indicate individual biological replicates, as detailed in the legend of Figure 2. In columns B-E replicates are separated by bold lines in the table. Numbers between parentheses refer to the clones in the CRISPR population. Asterisks indicate virus extinction. **Figure 1B** Emergence of virus mutants that overcome *S thermophilus* CRISPR-mediated immunity after 0, 16, 24, 40 and 48 hours post-infection. Green indicates no escape virus. Red indicates emergence of escape virus. Escape virus emerged in none of the polyculture experiments.

As expected, we could not detect escape virus in the ancestral virus (Fig. 1A and 1B; left column, indicated in green). However, in *P. aeruginosa* 43 of the 48 CRISPR monocultures, virus evolved within 2 days to overcome CRISPR based immunity (Fig. 1A; indicated in red). The emergence of escape virus decreased as diversity increased to 6, 12, 24 and 48 CRISPR alleles (Fig. 1A); in the latter two, no escape virus could be detected. These phenotypic data were supported by results of deep sequencing of virus genotypes isolated from 1 dpi: there was a significant inverse relationship between host diversity and the accumulation of viral mutations in the target sequences (Fig. 2). This is because virus needs to overcome multiple spacers in the diverse host population if it is to increase in frequency. These results are replicated in *S. thermophilus* where we see escape phage in 35 of the 44 monoculture treatments but no escape phage against the diverse treatments (Fig. 1B).

For 5 of the *P. aeruginosa* clones no escape virus could be detected, and virus went extinct in 4 of these instances (Fig. 1A, asterisks). Following sequencing of these clones it was discovered that three of these 5 clones carried multiple spacers targeting the virus, which limits the emergence of escape virus (164), furthermore, it was discovered that some spacers were duplicated within the set of 48 clones. Although this does not affect the results of this publication it provides

an opportunity to look at the role of diversity in the host population in more depth with new analyses. Data from this experiment was correlated with data from figure 2 and 3 in figures 6, 7 and 8 to further investigate the effect of CRISPR spacer diversity on infecting phage populations.

Phage evolution in response to varying host CRISPR allele diversity

van Houte *et al* (2016) (35) performed deep sequencing of the evolved phage populations to look for SNPs in the PAM or seed region of the phage genome following evolution on host populations carrying 1, 6, 12, 24, or 48 unique CRISPR alleles. After correcting for the increased selection on sites targeted by duplicate spacers I found that SNP frequency decreased as host population CRISPR allele diversity increased, in agreement with van Houte *et al*.

Figure 2

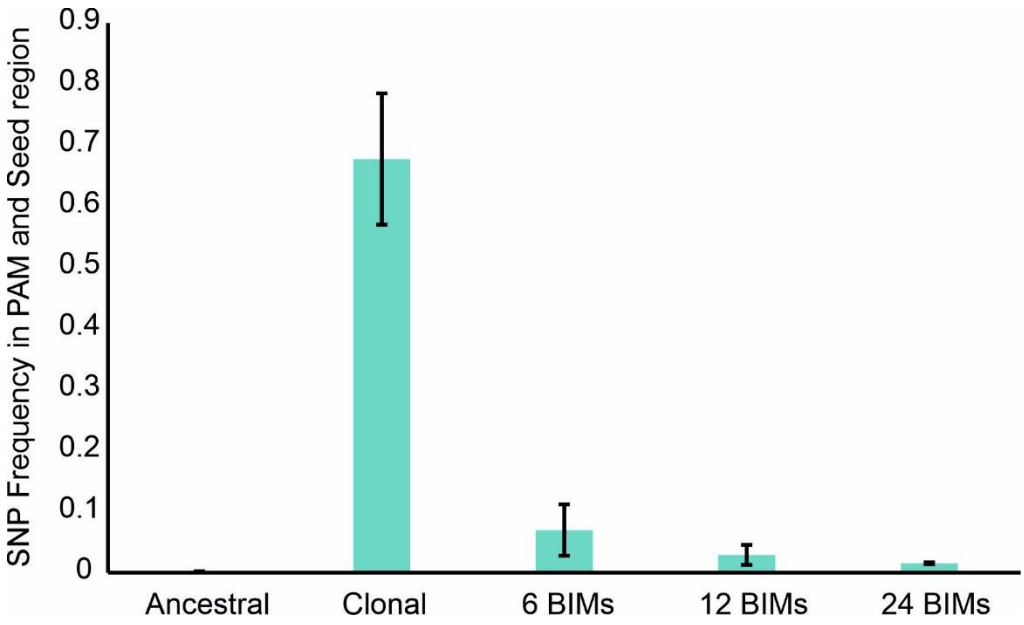


Figure 2. Spacer diversity reduces the frequency of SNPs in infecting phage

Deep sequencing analysis of the frequency of mutations in the seed region and PAM of the target sequence of virus isolated at t=1 from the experiment shown in Fig. 1 after correction for dilution effects and selection bias due to duplicate spacers within treatment groups**.** Bars show average frequency of point mutation across all target sites in the ancestral virus genome and in the genomes of virus from pooled samples of all biological replicates from a single diversity treatment, error bars show 95% confidence intervals (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

The data here show that when host CRISPR allele diversity is low parasites are able to rapidly overcome CRISPR based immunity in a host population (Fig. 1A, column a) but that when a host population harbours increasing levels of CRISPR diversity the parasites are less able to infect (Fig. 1A, columns b-d). The inverse relationship between host diversity and viral mutations in regions targeted by CRISPR and the rapid evolution within the CRISPR-phage interaction suggests that under the right conditions antagonistic evolution may occur. Using the corrected SNP frequency data I calculate levels of genetic diversity in evolved phage populations and how genetically different they are from the ancestral phage. I then correlate these variables with levels of host population CRISPR allele diversity (Fig. 1) and local adaptation (Fig. 3).

Local adaptation

Using the original data from the experiments presented in (165) I reanalysed the data to confirm I found the same trend. My results replicate that of Morley *et al*; I found that the ‘home vs away’ and ‘foreign vs ‘local’ metrics suggested by Kawecki *et al* (174) showed a significant negative effect of increasing CRISPR allele diversity within a host population and the amount of phage local adaptation. I found that both metrics gave the same values and there was <0.0001 difference in the 95% Confidence Intervals upon analyses so I have used the ‘foreign vs local’ to quantify local adaptation in the following figures and analyses.

Figure 3

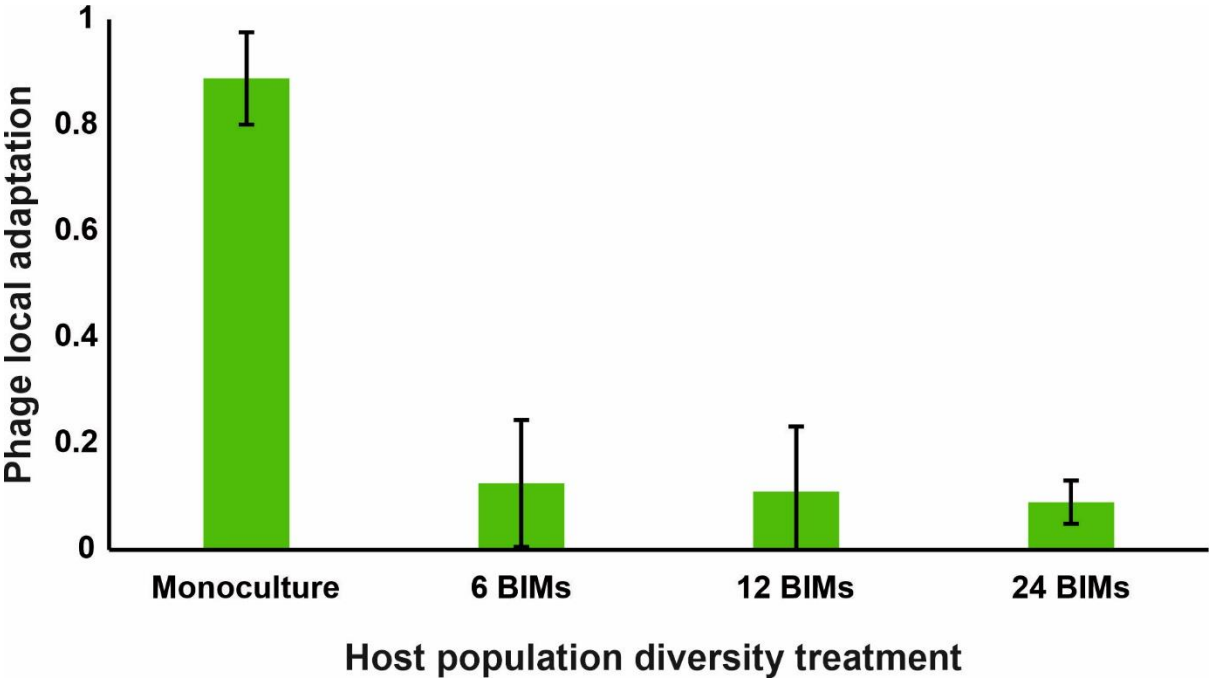


Figure 3. Phage local adaptation decreases as host spacer diversity increases

Phage local adaptation which occurred during the experiment shown in Fig. 1. Local adaptation was quantified using the 'foreign vs local' metric, bars show average values of local adaptation for each diversity treatment, error bars show 95% confidence intervals (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

As in (165) I found that phage evolved in monoculture were more locally adapted than when evolved on multiple hosts (Wilcoxon test: $p < 0.0001$, W = 616). Using this data combined with the phage diversity and ancestral distance data derived from the SNP frequency data shown in Fig. 2 I investigated the relationship between diversity and adaptation in further detail.

Quantifying evolved genetic change in phage after evolution on hosts with varying CRISPR allele diversity levels

Figure 4

One way of quantifying the effect of varying CRISPR allele diversity on a phage population is to compare the evolved phage with the ancestral phage. To this end I used the SNP frequency of each of the pooled samples at each CRISPR targeted site across the ancestral genome (Fig. 2) to calculate the genetic distance from the ancestor of each sample.

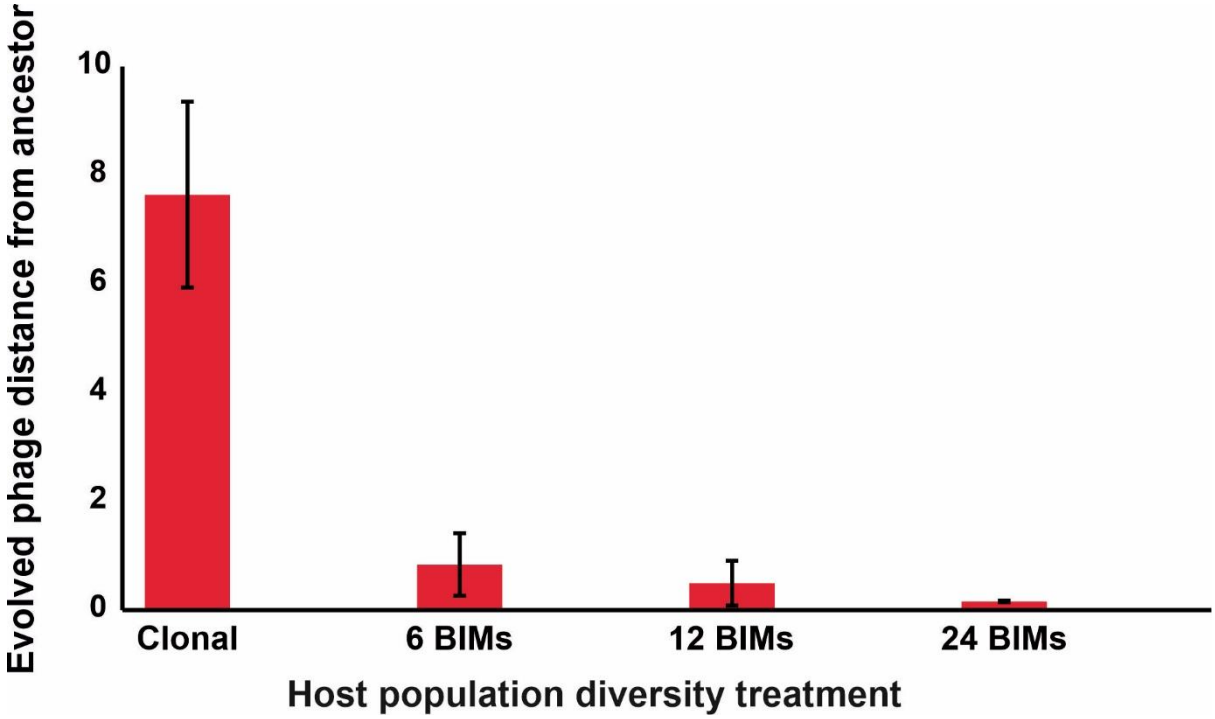


Figure 4. Phage evolved on less diverse host populations are more genetically distant from the ancestral phage

Bars show average change relative to the ancestral phage genome across all target sites in the ancestral virus genome and in the genomes of virus from pooled samples of all biological replicates from a single diversity treatment, error bars show 95% confidence intervals (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

In accordance with theory increasing the CRISPR allele diversity of a host population reduces the likelihood that phage will be able to become locally

adapted to that population (175), and that phage which do become locally adapted are likely to be more genetically diverse as a result of selection on their genomes through CRISPR-cas targeting. An ANOVA revealed an interaction between the number of unique hosts in the population and the amount of genetic change phage underwent during evolution on the different host populations ($F_{3,184} = 94.18$, $p < 0.0001$).

Another way to evaluate the evolutionary effect of varying CRISPR allele diversity in a host population on phage is to use a diversity metric to quantify levels of genetic diversity. Using a gini-Simpson's index on the phage SNP data shown in Fig. 2 I calculated levels of evolved phage diversity to allow me to compare them with host diversity data from Fig. 1 and local adaptation data from Fig. 3. Given that the SNP data comes from pooled samples we assume that there is no genetic drift and 100% linkage disequilibrium.

Figure 5

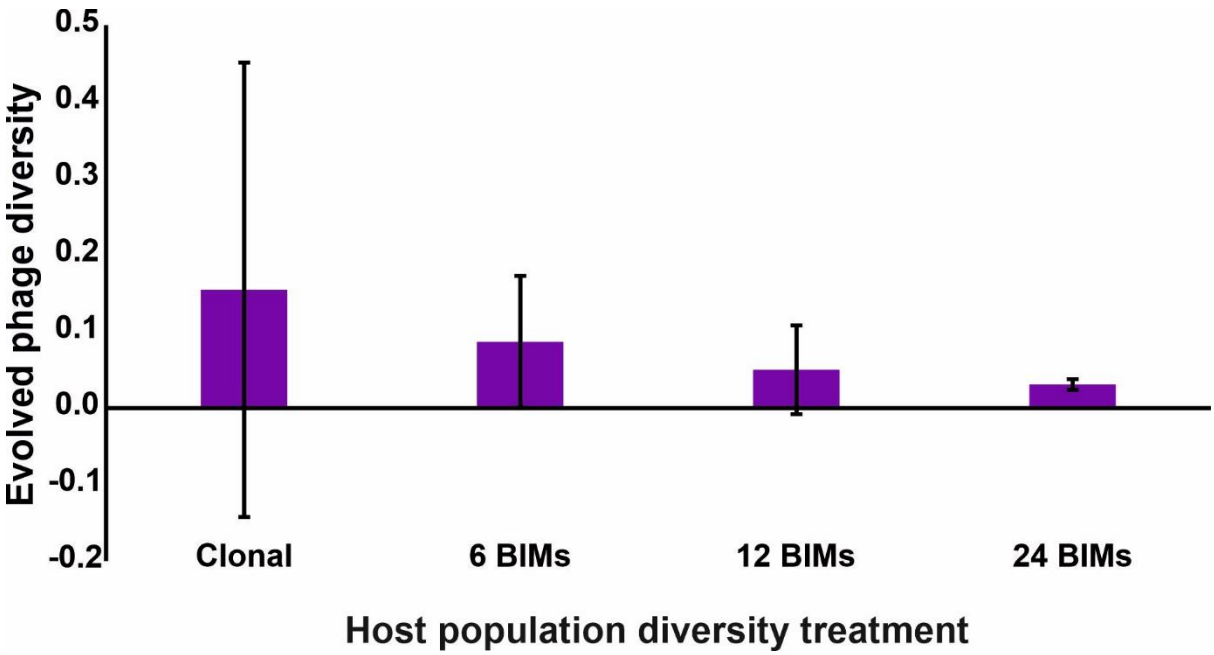


Figure 5. Phage evolved on less diverse host populations are more genetically diverse

Bars show average gini-simpson's diversity score of evolved phage SNP frequencies shown in Fig. 2 across all target sites in the genomes of virus from pooled samples of all biological replicates from a single diversity treatment, error bars show 95% confidence intervals (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

Using the gini-simpson's diversity metric I found that there was much more variation in the samples than when measuring change in phage populations as the genetic distance from ancestor (Fig. 4), this is likely due to significant polymorphisms in a few samples. However, the number of unique hosts in the population still has an effect on the amount of genetic diversity in evolved phage (ANOVA, $F_{3,184} = 81.99$, $p < 0.0001$). Since the two measures of change in evolved phage (distance from ancestor and genetic diversity) show the same trend but with different patterns of variation I use both of these metrics in the following figures where I investigate the relationship between changes in evolved phage and other variables.

CRISPR allele diversity effects on local adaptation

In the first of my comparisons of the new data I have generated I used the reanalysed data from my work with van Houte *et al* (35), which accounted for duplicate BIMs in the population, and plotted this against my re-analysis of the prevalence of local adaptation from my work with Morley (165). This allowed me to investigate the relationship between host population CRISPR allele diversity and phage local adaptation to host populations.

Figure 6

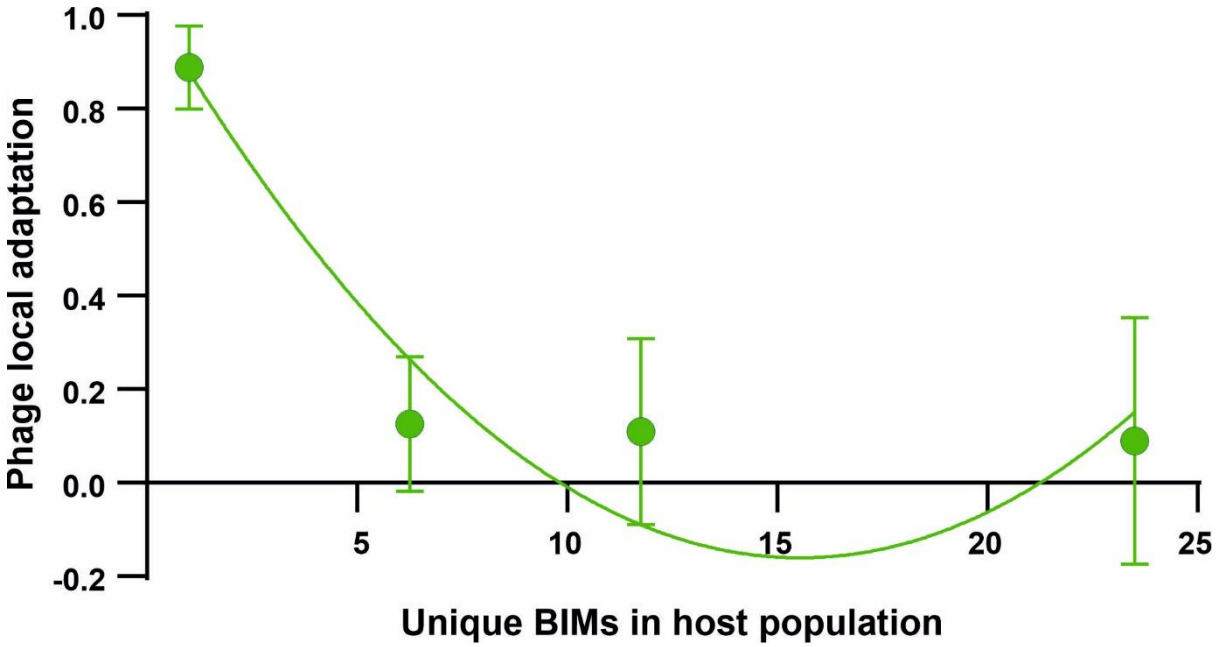


Figure 6. Local adaptation decreases as host diversity increases

Nonlinear regression: Line shows second order polynomial, points show mean number of BIMs in host population plotted against mean local adaptation score for each SNP site (Fig. 2), error bars show 95% confidence interval. (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

In agreement with previous work (165) I find that as the number of unique spacers in the host population increases the rate of phage local adaptation decreases (Nonlinear regression: $p < 0.001$, $F_{1,59} = 362.9$, $R^2 = 0.5526$). I next wanted to investigate how becoming locally adapted affected the phage populations on a genetic level in order to understand the relationship between genetic change and local adaptation.

The importance of host diversity in phage adaptation

In the original data from van Houte *et al* (35) host diversity treatments were split categorically into 1, 6, 12, 24 and 48 unique CRISPR spacers. It was assumed that all spacers were unique, however sequencing revealed that some duplicate spacers were present. Here I use data in which I have corrected for the selection bias on protospacers in phage genomes caused by duplicate spacers within diversity treatments and used the average numbers of unique spacers in each group to quantify host diversity. To compare how genetic change in phage was affected by the number of CRISPR alleles in a host population I plotted phage adaptation (as measured by distance from ancestor (Fig. 7A) or by phage genetic diversity (Fig. 7B)) against the average number of unique CRISPR alleles in host populations.

Figure 7

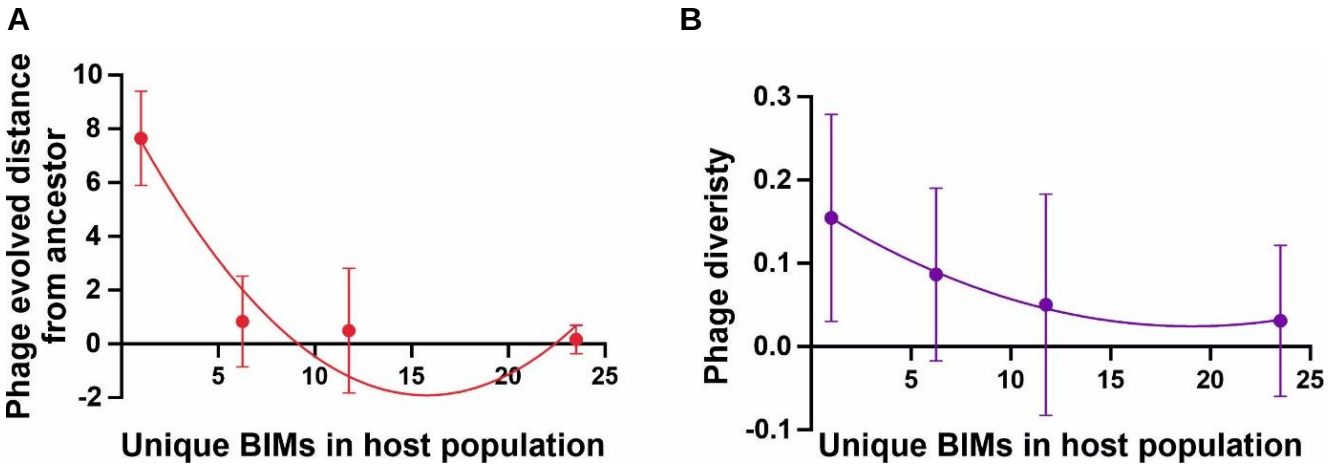


Figure 7A. Phage become more genetically distant from the ancestral phage as local adaptation increases

Nonlinear regression: Line shows second order polynomial, points show mean number of BIMs in host population plotted against mean genetic distance from ancestor, error bars show 95% confidence interval. (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

Figure 7B. Phage adaptation decreases with increasing host diversity

Nonlinear regression: Line shows second order polynomial, points show mean number of BIMs in host population plotted against mean evolved phage diversity at each SNP site, error bars show 95% confidence interval. (monocultures: n=48; 6-clone: n=8; 12-clone: n=8; 24-clone: n=6).

Supporting the findings of van Houte *et al* (35) I find that phage which were evolved with fewer unique CRISPR alleles in the host population are more genetically distant from the ancestral phage (Fig. 7A, non-linear regression, $F_{1,59} = 76.95$, $R^2 = 0.2224$, $p < 0.001$) and that evolved phage diversity decreases with increasing host diversity (Fig. 7B, non-linear regression $F_{1,59} = 5.64$, $R^2 = 0.00987$, $p = 0.0208$). This fits with the findings of Morley *et al* (165) who showed phage are more able to become locally adapted to host populations containing fewer CRISPR alleles and highlights the importance of both host and parasite diversity in local adaptation.

Figure 8

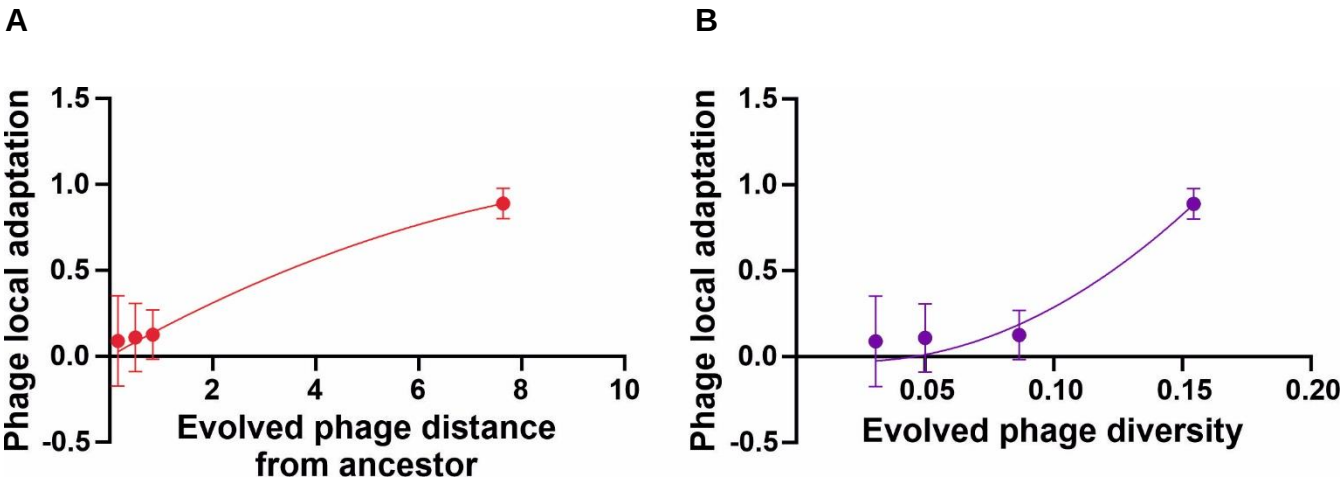


Figure 8A.

Nonlinear regression: Line shows second order polynomial, points show mean phage genetic distance from ancestor for each SNP site plotted against the mean local adaptation, error bars show 95% confidence interval.

Figure 8B.

Nonlinear regression: Line shows second order polynomial, points show mean phage genetic diversity score for each SNP site plotted against the mean local adaptation, error bars show 95% confidence interval.

I found that the relationship between local adaptation was not significant with phage genetic change measured either by distance from ancestor (Fig. 8A, non-linear regression, $F_{1,59} = 0.94$, $R^2 = 0.58$, $p = 0.3356$) or by phage diversity score (non-linear regression, $F_{1,59} = 0.1028$, $R^2 = 0.58$, $p = 0.7497$). This is surprising since earlier analyses show a relationship between host diversity and the resulting diversity in evolved phage populations (Fig. 7), and that phage local adaptation correlates with host diversity (Fig. 6).

Discussion

Here I investigate how host diversity affects phage evolution and local adaptation, van Houte *et al* (35) showed that the ability of CRIPSR-Cas to generate host genetic diversity limits the emergence of escape virus and present data which indicate population-level selection may have contributed to the evolution of CRISPR-Cas. In agreement, Morely *et al* (165) examined the effect of host diversity on the likelihood of infecting phage populations becoming locally adapted and found that increasing CRISPR allele diversity reduced the ability of phage populations to successfully infect and become locally adapted to host populations. In this chapter I have collated and reanalysed data from both these papers in order to investigate the relationship between host CRISPR allele diversity and phage adaptation in more detail. My novel analyses confirmed that the previous findings of van Houte *et al* (35) and Morley *et al* (165) were robust to the changes in host diversity controlled for here. Additionally, I show that evolution on host populations containing increasing CRISPR allele diversity results in lower phage genetic diversity and fewer mutations acquired when compared with the ancestral virus in evolved phage populations following exposure to host populations with increasing CRISPR allele diversity. I found that the rate of phage local adaptation did not correlate with the amount of evolved phage diversity, or distance from the ancestral phage.

Theoretical studies predict that when infections are specific, increased host genetic diversity reduces the severity of an epidemic by reducing the frequency of susceptible hosts to a phage genotype (166). Because infections of a non-matching host genotype will result in parasite death parasite population sizes, and therefore evolutionary potential, will be reduced, limiting parasite adaptation (35). van Houte *et al* (35) showed that increasing CRISPR allele diversity reduced the ability of phage to replicate on host populations, this likely explains my finding that increasing host CRISPR allele diversity decreases the ability of phage populations to evolve (measured as the distance of the evolved phage from the ancestral phage), limiting the diversity of the phage population, and therefore the evolutionary potential. It is often assumed that specificity in host-parasite interactions results in local adaptation. This is because parasites can

drive diversification of host genotypes across time and space, which in turn selects for different parasite genotypes (177, 178) and the degree of local adaptation is expected to increase with the strength of selection imposed by the parasite (172). While I was unable to detect a significant relationship between phage genetic diversity and rates of local adaptation the analyses presented here show a strong relationship between host diversity and phage adaptation. Increasing host diversity decreases the ability of phage to evolve, and therefore the evolutionary potential of the phage population to become locally adapted. Previous work has shown that infection with diverse phage can drive the evolution of diversification of CRISPR alleles within host populations (43). The experiments described above used clonal phage stocks in order to specifically examine the effects of host diversity, it is possible that if the infecting phage population harboured pre-existing diversity within the protospacer or PAM sequences targeted by the host populations CRISPR-Cas system then successful infections are more likely, increasing parasite evolutionary potential, and promoting ongoing co-evolution (173). In combination with previous co-evolution data (136) and work investigating the effect of phage genetic diversity on the evolution of CRISPR-Cas (43) the data presented here suggest that local adaptation may occur between phage and host populations with CRISPR-Cas under the right conditions, and genetic diversity of both host and parasite populations will be important factors in dictating whether or not local adaptation occurs and the outcome of such interactions, however further studies are required to clarify this.

The speed at which CRISPR based defence and escape phage can evolve under the right conditions indicates the potential for rapid local adaptation. A mechanism known as ‘priming’, whereby CRISPR-Cas systems can rapidly evolve high levels of CRISPR allele diversity, but only if the CRISPR-Cas system possesses a pre-existing spacer in the CRISPR array which has partial complementarity to the phage (179–181) may indicate that local adaptation occurs in natural interactions between phage and hosts possessing CRISPR based immunity. Priming greatly accelerates the addition of novel spacers into the hosts CRISPR-array and therefore the number of phage genotypes which CRISPR-Cas can recognise and defend against. Because of the requirement for priming, CRISPR-Cas systems are much better in dealing with phages that are similar to those that have infected

before compared to novel phages. This suggests that in nature CRISPR-Cas systems may cause local adaptation of bacterial hosts (and therefore maladaptation of phage). Several metagenomic studies have shown that CRISPR spacers tend to match sympatric phage genomes rather than phages from foreign environments (182–186). Reinfection with these phages or genetically similar phages will trigger a strong CRISPR immune response to which the phage cannot adapt, whereas infection with phage from a foreign environment will not trigger a strong CRISPR immune reaction. The importance of CRISPR-Cas in such inter-specific local adaptation awaits further experimental study.

Chapter 2

The Effect of Phage Genetic Diversity on Bacterial Resistance Evolution

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Abstract

CRISPR-Cas adaptive immune systems are found in bacteria and archaea and provide defense against phage by inserting phage-derived sequences into CRISPR loci on the host genome to provide sequence specific immunological memory against re-infection. Under laboratory conditions the bacterium *Pseudomonas aeruginosa* readily evolves high levels of CRISPR-based immunity against clonal populations of its phage DMS3vir, which in turn causes rapid extinction of the phage. However, in nature phage populations are likely to be more genetically diverse, which could theoretically impact the frequency at which CRISPR-based immunity evolves which in turn can alter phage persistence over time. Here we experimentally test these ideas and found that a smaller proportion of infected bacterial populations evolved CRISPR-based immunity against more genetically diverse phage populations, with the majority of the population evolving a surface modification preventing phage adsorption and providing generalised defence against a broader range of phage genotypes. However, those cells that do evolve CRISPR-based immunity in response to infection with more genetically diverse phage acquire greater numbers of CRISPR memory sequences in order to resist a wider range of phage genotypes. Despite differences in bacterial resistance evolution, the rates of phage extinction were similar in the context of clonal and diverse phage infections suggesting selection for CRISPR-based immunity or sm-based resistance plays relatively minor role in ecological dynamics in this study. Collectively, these data help to understand the drivers of CRISPR-based immunity and their consequences for bacteria-phage coexistence, and, more broadly, when generalised defences will be favoured over more specific defences.

Introduction

Bacterial and archaeal viruses can influence microbial communities both through direct interactions with their hosts as well as indirect effects on non-host populations (46, 47, 187, 188). The selective pressure of viral predation has resulted in the evolution of a range of archaeal and bacterial defence mechanisms, which includes CRISPR-Cas systems, prokaryotic argonautes, Restriction-Modification (R-M) systems, surface modification (sm), toxin-antitoxin

and Abortive Infection systems (69). These defences differ in multiple ways including the range of phage to which they provide resistance (generalist versus specialist) and in their fitness costs. (79). In bacteria two of these mechanisms, sm and CRISPR-Cas systems, are often found to be important for rapid evolution of phage resistance, with sm perhaps representing the simplest, and most common, line of defence by mutation of the bacterial cell surface, obscuring or altering the receptor that is used by the phage to attach to the cell (37). Alternatively, bacteria may employ their CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR-associated) adaptive immune systems, which occur in approximately 30% of the bacterial genomes sequenced to date (78). Initially naïve bacteria acquire CRISPR-based immunity by incorporating nucleic acids from phage genomes into CRISPR loci on their own genome (107, 108), where they are known as “spacers” (109). Processed CRISPR transcripts guide Cas proteins to detect and destroy re-infecting phage that carry the cognate target sequence (110, 111). Phage can in turn overcome CRISPR-based immunity by acquiring a point mutation in the target sequence on their genome (106), although the emergence and spread of such phage “escape” mutants is constrained by both the diversity of CRISPR immunity alleles in the bacterial population and the fitness costs associated with the phage mutations (35, 112–114).

While our mechanistic understanding of the different defence strategies of bacteria has raced ahead, the ecological conditions that favour one type of defence over another, and their consequences for the ensuing bacteria-phage population dynamics remain unclear (36, 79). Previous work has shown that the inducible cost of CRISPR-Cas means CRISPR-based immunity will typically be favoured over more costly constitutive defences, such as sm, under relatively low forces of infection (34). However, because the cost of sm-based resistance is fixed, it may provide a selective advantage when the force of infection is high. Given that the cost of sm-based resistance can increase in the presence of bacterial competitors, CRISPR-based immunity evolution may generally be more important in a microbial community context (133) .

Here, we focus on another key distinction between the different types of defence: their different levels of specificity. CRISPR-Cas mediated defence is necessarily fairly specific, with a single spacer unlikely to be able to target all phage

genotypes within a population. While spacer diversity within bacterial populations provides an additional population level resistance by increasing the chance of phage extinction, this benefit may be reduced if phage populations are sufficiently diverse and hence there are variants that can escape targeting by many spacers. By contrast, sm-based resistance provides resistance against a broad range of phage genotypes, and typically requires multiple mutations for phage to overcome (104, 105, 189). We therefore hypothesised that increasing the diversity of infecting phage populations would increase selection for sm-based resistance.

Here we test the effect of phage genetic diversity using *P. aeruginosa* PA14 and its phage DMS3vir. *P. aeruginosa* is an opportunistic human pathogen and a model organism for studying the mechanism and evolutionary ecology of CRISPR-Cas systems. *P. aeruginosa* PA14 can evolve resistance to the phage DMS3vir through either surface modification or CRISPR-Cas (34, 156). In order to directly test the impact of phage genetic diversity on the frequency at which CRISPR-based immunity evolves, we generated phage DMS3vir populations with increased genetic diversity by serial passage of the phage on a phage-sensitive *Pseudomonas aeruginosa* PA14 derived strain. We then exposed the bacterium *Pseudomonas aeruginosa* strain PA14 to the genetically more diverse populations of the phage DMS3vir, or to a clonal phage DMS3vir population. Following infection with genetically diverse phage populations, a smaller proportion of the initially phage-sensitive bacterial populations relied on CRISPR-based immunity. Moreover, those bacteria that relied on CRISPR-based immunity in the face of diverse phage populations acquired more spacers per individual compared to those that were exposed to clonal phage populations, presumably because this allows them to resist a wider range of phage genotypes (35, 164). The ultimate effect of the frequency of CRISPR-based immunity on the rate of phage extinction was however independent of the levels of standing genetic variation in the phage populations.

Methods

Bacterial strains and phages

P. aeruginosa UCBPP-PA14 (referred to as WT, carrying no spacers with a perfect match to the DMS3vir genome), *P. aeruginosa* UCBPP-PA14 *csy3::LacZ* (156) (referred to as CRISPR-KO, since it carries a disruption of an essential *cas* gene that causes the CRISPR-Cas system to be non-functional), and *P. aeruginosa* UCBPP-PA14 *mutS*::MAR2xT7 (190), which was kindly provided by Alexandro Rodriguez Rojas (below this strain is also referred to as a mutator strain of PA14), and the CRISPR-KO-derived surface mutant (sm) (described previously in ref. (34)), were used in all experiments. The obligately lytic phage DMS3*vir* was used in all experiments, and has previously been described in (156). All statistical analyses were carried out in R version 3.4.4.

Generating Diversity within Phage Populations

To generate genetically diverse populations of phage DMS3vir, the phage was amplified on *P. aeruginosa* UCBPP-PA14 *mutS*::MAR2xT7, a transposon mutant of *P. aeruginosa* that lacks the *mutS* gene. No DNA-polymerase has been characterized on the DMS3vir genome so we hypothesized that replication on a mutator strain of its host would result in increased levels of genetic variation within the phage population. Bacteria and phage were grown in 10ml LB media, by inoculating 1:100 from an overnight culture of *P. aeruginosa* UCBPP-PA14 *mutS*::MAR2xT7 and subsequent infection with 10^6 pfu (plaque forming units) of DMS3vir. 12 independent cultures were inoculated, followed by incubation at 37^{o}C while shaking at 180 rpm. After 24h phage were sampled via chloroform extraction by mixing the overnight cultures 2:1 with chloroform followed by vortexing and centrifugation at 3500 rpm to pellet bacterial debris. Supernatant containing phage was stored at 4^{o}C and used to infect naïve *P. aeruginosa* UCBPP-PA14 *mutS*::MAR2xT7 cells in fresh media, as described above. This procedure was repeated daily for 17 days, resulting in phage populations with increased levels of genetic diversity. We then generated derived clonal populations by isolating a single phage clone from each of the twelve diverse populations, using double plaque purification, followed by a single round of amplification on *P. aeruginosa* UCBPP-PA14 *csy3::LacZ* bacteria.

Deep sequencing of phage populations

To measure the levels of standing genetic variation in the diverse and derived clonal phage populations that were generated as described above, we extracted phage DNA from approximately 10^8 - 10^9 pfu using the Norgen phage DNA isolation kit, following the manufacturer's instructions. After QC with Nanodrop, Qubit and electrophoresis to quantify the amount and quality of extracted material, the extracted DNA was sequenced using MiSeq by the Liverpool Center for Genomic Research, using previously described protocols (35). Ancestral virus was processed in parallel as a control for SNP calling. Barcoded Illumina Truseq Nano libraries were constructed from each DNA sample with an approximately 350 base insert size and 2×250 base reads generated on an Illumina MiSeq platform. Reads were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 and Sickle version 1.200 with a minimum window quality score of 20. After trimming most reads were around 250 bases and reads shorter than 10 bases were removed. Overlapping reads were joined using Flash version 1.2.8 to create high quality sequence at approximately 8,000x coverage of DMS3vir per sample. Reads from evolved phage populations were mapped to the ancestral DMS3vir genomes using bwa mem version 0.7.12. Sites which had coverage lower than 100 and an alternate allele frequency of >0.005 in the ancestor were filtered out to minimise sequencing error and noise. Data for SNPs which were fixed (>0.95 frequency) in any of the clonal population samples was extracted. No fixed mutations were found in the diverse populations that were not present in one of the clonal populations. Sequence data have been deposited in the European Nucleotide Archive under accession number ENA: PRJEB31472

Infectivity of genetically diverse phage on CRISPR resistant bacteria

We investigated the effect of phage genetic diversity on the ability of the phage population to overcome CRISPR targeting by testing the 12 experimentally evolved phage populations for infectivity against 12 CRISPR-immune bacteria. The bacteria used for infectivity tests were obtained from previous co-evolution experiments with DMS3vir. The selected isolates have been sequenced to ensure each was carrying a unique single spacer sequence (35). The infectivity of phage was determined by spot assay on a lawn of each of the CRISPR-

immune bacteria and the CRISPR-KO strain (which carries an inactive CRISPR-Cas system and is therefore sensitive to phage infection). Infectivity is expressed as the Efficiency of Plaquing (EOP) on the CRISPR immune host, which was calculated by dividing the PFU/ml formed by the phage population on the relevant CRISPR-immune bacteria by the PFU/ml formed on the CRISPR-KO strain. Additionally, we tested all phage populations' infectivity against 6 CRISPR-immune bacterial strains each carrying 2 spacers, and against one strain with sm-based resistance.

Measuring the Effect of Diversity on Phage Persistence

To investigate how genetic diversity within a phage population affects the phage population dynamics we inoculated 6ml M9 media supplemented with 0.2% glucose 1:100 with WT cells and added 10^4 pfus of either clonal or diverse phage. Phage titers were measured after 1, 3 and 5 days: Phage were extracted using chloroform as described above and a dilution series was spotted on a lawn of soft LB (0.5% agar) inoculated with *P. aeruginosa* PA14 *csy3::LacZ* to quantify phage titers.

Effect of phage diversity on CRISPR-based immunity evolution

To investigate how the diversity of an infecting phage population affects the evolution of resistance mechanisms in the host we co-cultured WT *P. aeruginosa* UCBPP-PA14 with either clonal or diverse phage for 3 days. Glass vials were filled with 6 ml of M9 media supplemented with 0.2% glucose and inoculated with 10^6 cfu/ml of *P. aeruginosa* and 10^4 pfu of phage from one of either the diverse or clonal populations (n=8) then incubated for 24h (+/-3h) at 37ºC while shaking at 180 rpm. Transfers to fresh media were performed daily at a concentration of 1:100. The bacterial populations were sampled at 3 dpi (days post infection) and resistance profiles of the bacterial isolates from the experimental populations were determined via streak assays against either ancestral phage (DMS3*vir*) or the isogenic phage DMS3*vir*-AcrIF1, which carries an antiCRISPR (Acr) gene that blocks the CRISPR-Cas system of *P. aeruginosa* UCBPP-PA14 (176). Lines of phage were applied to agar plates and allowed to dry for 20 minutes. Bacterial

colonies were streaked across the lines of phage and plates were incubated overnight at 37ºC. Bacteria were scored to be CRISPR immune when they were resistant to DMS3*vir*, but not to DMS3*vir*-AcrIF1 (also associated with a motile, swarming phenotype) and surface-based resistance was scored when they were resistant to both phages (also associated with a smooth, non-motile phenotype). Bacteria were scored to be sensitive when they displayed resistance to neither phage. Additionally, evolution of CRISPR-based immunity was further confirmed by PCR of both CRISPR loci, using primer pairs 5'-CTAAGCCTTGTACGAAGTCTC-3' and 5'-CGCCGAAGGCCAGCGCGCCGGTG-3' to determine spacer acquisition at the CRISPR 1 locus, and primer pairs 5'-GCCGTCCAGAAGTCACCACCCG-3' and 5'-TCAGCAAGTTACGAGACCTCG-3' to determine spacer acquisition at the CRISPR 2 locus.

Results

Diverse phage populations harbour more SNPs

Given the sequence specificity of CRISPR-based immunity against phage, we hypothesised that phage genetic diversity will constrain the evolution of CRISPR-based immunity relative to sm-based resistance, with potential knock-on effects for phage persistence. To test this, we amplified phage DMS3vir on a mutator strain of *P. aeruginosa* PA14 with the aim of increasing phage genetic diversity. After 17 passages of DMS3vir on the mutator strain, we performed deep sequencing analysis on the phage populations to examine if the standing levels of genetic diversity (i.e. SNPs frequency) were increased relative to paired clonal control populations that were derived from each of the diverse populations.

Although the sensitivity of deep sequencing is insufficient to identify rare genotypes, it revealed 40 SNPs across the 16 phage populations (8 "clonal" and 8 "diverse") which were present at 0.01% frequency or higher after processing to remove sequencing noise and error. All of the SNPs found in clonal populations were also present in the paired diverse population. Based on this analysis, diverse populations were found to contain an average of 9.2 SNPs (ranging from 5-13 in each population), while clonal populations were found to only have an average of 3 SNPs (ranging from 1-4). However, SNP frequencies based on deep

sequencing analysis inevitably underestimate the true level of genetic variation in the populations as rare alleles are filtered out to limit the noise from sequencing errors. Nonetheless, a Kruskal-Wallis test confirmed that the diversified phage populations had a higher average SNP frequency than the paired clonal strains (Figure 1A) ($x^2(2) = 11.94$, $p<0.001$), and most SNPs were located close to gene 42, which contains a CRISPR priming site (34), which suggests that the observed SNP clustering may result from acquisition of spacers from this area of the phage genome and subsequent evolution of escape by the phage during the passaging on the mutator strain.

Genetically diverse phage are more infective on hosts with CRISPR-based immunity

Previous work has shown that point mutations in the phage genome sequence that is targeted by the CRISPR-Cas immune system often allows the phage to by-pass this defence (35, 106, 161, 165). We therefore predicted that the genetically more diverse phage populations would have greater infectivity on bacteria with CRISPR-based immunity compared to their paired clonal phage populations or the ancestral population. Given that it is much harder for phage to evolve to recognize a novel receptor (189), we expected that the levels of infectivity of the diversified and clonal phage populations would be the same on a bacterium with sm-based resistance. To test these ideas, we examined the levels of infectivity for each of our diversified and clonal populations against 12 clones with CRISPR-based immunity of *P. aeruginosa* PA14, each possessing a single unique spacer, and against 6 clones with CRISPR-based immunity each carrying two spacers targeting DMS3vir, which is harder to overcome by point mutation (35, 164), as well as a PA14 strain with sm-based resistance. As expected, we found that infectivity of genetically more diverse phage populations was higher on bacteria with CRISPR-based immunity than that of clonal phage populations (Wilcoxon Signed Rank Test, W=1359, Z=-9.5082, $p<0.0001$), but neither of the phage populations were infective against hosts with 2 spacers or sm-based resistance (Figure 1B). Consistent with the idea that SNPs are present at a low frequency in the phage population, the EOP of more genetically diverse phage was typically around 10^{-5} for each spacer tested, an increase of around 10-200 fold compared with the clonal phage populations. Collectively, these data

support the idea that phage diversified during the repeated amplification on the mutator strain, resulting in greater infectivity of CRISPR-resistant bacteria.

Figure 1

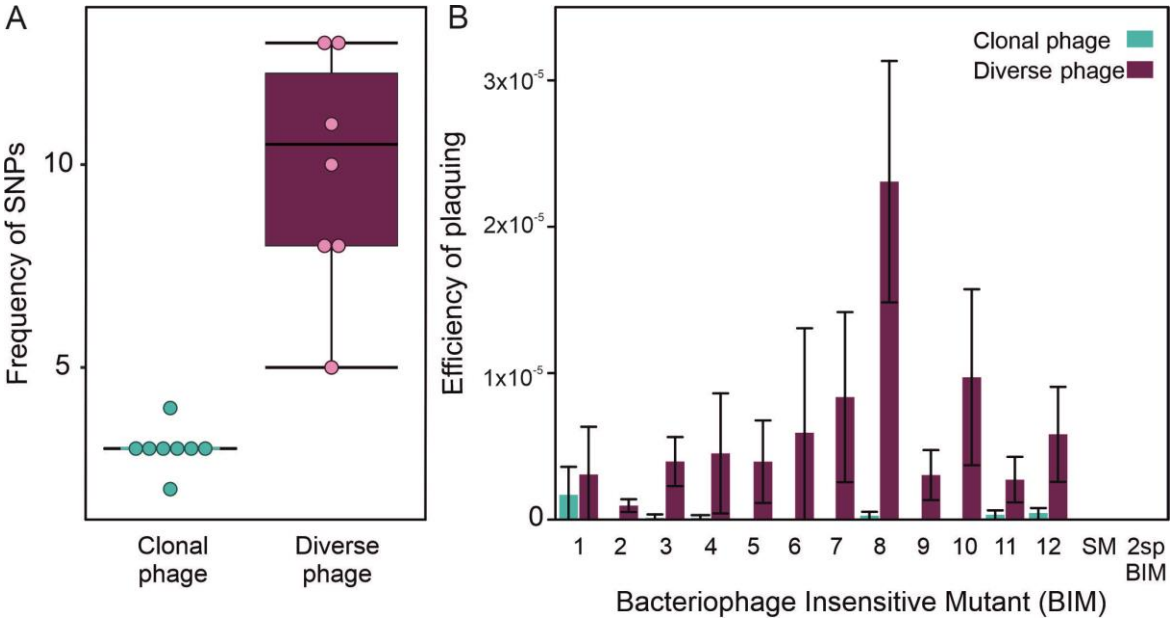

Figure 1. Our 12 evolved phage populations harbour greater genetic variation than the derived clonal populations**. A)** The frequency of SNPs in diverse (purple) phage populations (N=12) and clonal (blue) (N=12) populations. **B)** The average efficiency of plaquing of our 12 experimentally evolved diverse phage populations, or the 12 derived clonal phage populations on each of 12 CRISPR-resistant Bacteriophage Insensitive Mutants (BIMs). Each BIM carries a single unique spacer targeting the ancestral phage. infectivity of phage populations was also tested against 6 BIMs carrying 2 spacers targeting the ancestral phage (this data is shown as one bar as no escape mutants were identified against any of the 6 clones possessing 2 spacers each) and a surface mutant (sm). All graphs show averages and error bars represent 95% confidence intervals.

Type of evolved host resistance depends on the levels of phage genetic diversity

In order to study if and how phage genetic diversity impacts the frequencies of CRISPR and sm-based resistance that evolve in the bacterial population following infection, we performed an evolution experiment and monitored the phage population dynamics as well as the levels of CRISPR-based immunity observed following infection of WT PA14 with the clonal or more genetically

diverse phage DMS3vir populations. Consistent with previous studies (34, 35), we found that phage titres increased in all replicates following infection of the WT strain, which is expected given that bacteria are initially sensitive to the phage. However, from 1 days post infection (dpi) onwards, phage titres started to decline until complete extinction at 6 dpi, with no clear difference between the diversified and paired clonal phage populations (Figure 2A, ANOVA, $F_{(1,22)}$=0.02, p>0.8). However, analysis of individual bacterial clones that were isolated at 3 dpi revealed clear differences in the frequencies of CRISPR-based immunity and sm-based resistance that had evolved following infection with genetically diverse or clonal phage populations (Figure 2B, ANOVA, $F_{(2,1)}$= 54.72, p<0.0001). Specifically, we found that the frequency of sm-based resistance was higher in populations challenged with a genetically more diverse phage population than in populations challenged with the paired clonal population (Post hoc analysis with Tukey HSD, p<0.0001), and this was associated with a corresponding decrease in the frequency of CRISPR-based immunity (p<0.0001) (Figure 2B). These data therefore show that increasing phage genetic diversity can cause a decrease in the frequency at which CRISPR-based immunity evolves and a corresponding increase in the frequency at which sm-based resistance evolves.

Figure 2

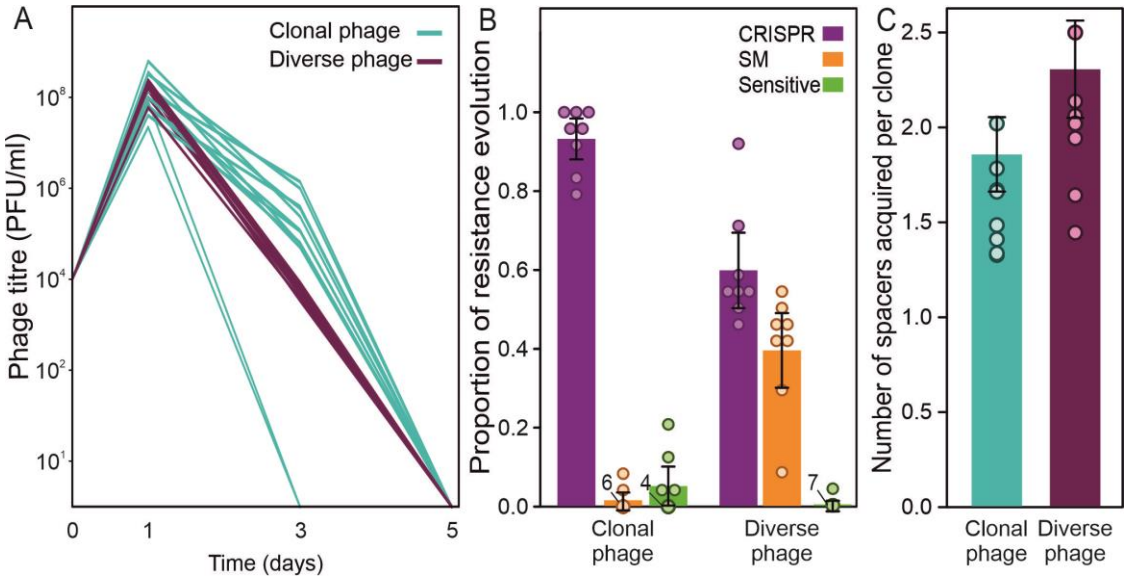

Figure 2. No effect of phage genetic diversity on phage persistence. **A)** Phage titers (PFU/ml) over time following infection of WT bacteria with a genetically diverse or a derived clonal phage population. Each line represents a single replicate. **B)** The average proportion of cells at 3 dpi that evolved CRISPR-based immunity (purple), surface modification (sm) based resistance (orange), or that remained sensitive (green) and **C)** the average number of spacers acquired by each CRISPR clone in response to infection with clonal (blue) or diverse (purple) phage. All graphs show averages and error bars represent 95% confidence intervals. Individual data points are indicated with dots, and numbers next to the dots indicate the number of replicates in which the same value was observed.

Bacteria evolving CRISPR-based immunity against genetically diverse phage carry more spacers

Given that the number of spacers targeting a phage within a single host influences the propensity of phage to overcome CRISPR-based immunity (Figure 1B and (35, 164)), we also examined how genetic diversity within infecting phage populations affects the number of spacers that individual bacterial clones acquire. PCR analysis of the CRISPR loci of bacterial clones following exposure to either the genetically more diverse phage DMS3vir populations or the paired clonal controls revealed that the patterns of spacer acquisition in cells using CRISPR-based immunity differed depending on the genetic diversity of the phage they were infected with (Figure 3B). Specifically, upon infection with clonal phage the majority of cells which had acquired CRISPR-based immunity had acquired only one spacer (Figure 3A, Tukey HSD, $p<0.005$), whereas upon infection with genetically more diverse phage the majority of cells which had acquired CRISPR-based immunity had acquired multiple spacers (Tukey HSD, $p<0.05$). Despite differences in the patterns of spacer acquisition, analysis with ANOVA revealed the average total number of spacers per clone gained was not affected ($F_{(1,14)}=7.42$, $p>0.1$) (Figure 2C). Collectively, the higher proportion of bacteria with sm-based resistance or CRISPR-based immunity with multiple new spacers in response to infection with genetically more diverse phage suggests selection for more generalist defence mechanisms under those conditions.

Figure 3

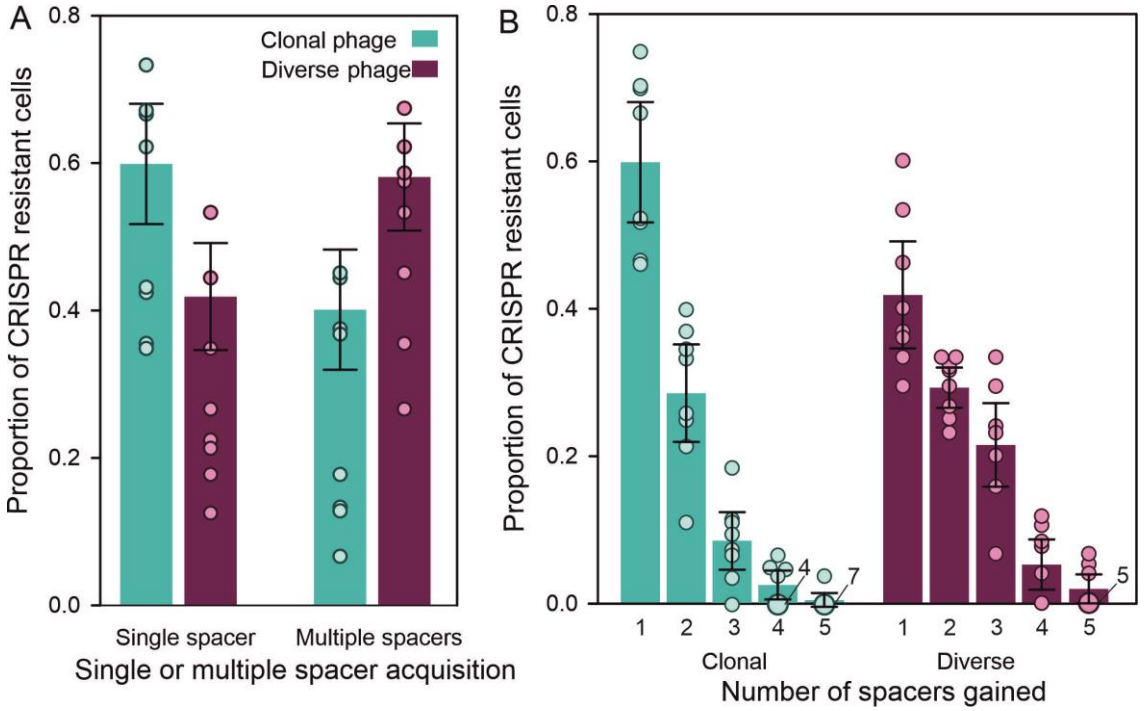

Figure 3. Patterns of spacer acquisition differ in response to infection with clonal or diverse phage. **A)** Average proportion of CRISPR clones in a population which acquired single or multiple spacers when infected with clonal (blue) or diverse (purple) phage. **B)** Average proportion of CRISPR clones in a population which acquired 1,2,3,4 or 5 spacers when infected with clonal or diverse phage. All graphs show averages and error bars represent 95% confidence intervals. Individual data points are indicated with dots, and numbers next to the dots indicate the number of replicates in which the same value was observed.

Discussion

Here, we tested whether increasing genetic diversity within a parasite population will increase selection for generalist over specific immune defences. We find that infecting *P. aeruginosa* with experimentally evolved populations of phage DMS3vir that have elevated levels of standing genetic diversity results in, first, higher frequencies of sm-based resistance compared with CRISPR-based immunity and that, second, cells with CRISPR-based immunity are more likely to acquire multiple spacers. Interestingly no difference in phage persistence between populations that were infected with clonal or genetically more diverse

phage populations were observed. These data show that genetic diversity has an important effect on the frequencies of CRISPR-based immunity (specialist defence) and sm-based resistance (generalist defence) in this system. While the elevated genetic variation of phage DMS3vir is presumably more reflective of natural environments compared to the clonal phage populations that are typically used in experimental evolution studies, it should be noted that our experiments do not capture the complexity of natural environments where different phages with overlapping host ranges tend to coexist, which can further impact the evolution of CRISPR-based immunity (191).

CRISPR-Cas is a specific and adaptive immune defence that is widespread, yet not equally abundant in different ecosystems. Although CRISPR-Cas systems are found in a diverse range of habitats, there is a highly uneven distribution across different environments. Metagenomic sequence analyses have shown that the frequency of the evolution and maintenance of CRISPR-Cas systems in bacterial and archaeal communities may be linked to environmental factors as higher frequencies of CRISPR-Cas are often found in extreme environments (137–140). This suggests that there are environmental attributes that constrain the fitness advantages (or ecological success) of this form of phage defence. Some of the earliest studies that looked at the ecological distributions of CRISPR-Cas immune systems observed that these systems are overrepresented in high-temperature environments, with thermophiles typically possessing more and longer CRISPR arrays (139, 192). Indeed, over 90% of hyperthermophilic archaea harbor CRISPR-Cas, and these environments are generally throught to be associated with moderate virus diversity (193). The ancestral primitive CRISPR-Cas systems likely evolved in archaea and were subsequently acquired by bacteria through horizontal transfer (194). It has been suggested that the prevalence of CRISPR-Cas in hyperthermophilic conditions may be due to lower mutation rates in those environments, which is supported by correlational and theoretical studies (137–140, 195). However, apart from accelerating the evolution of “escape” phage, high mutation rates will also increase the rate at which sm-based resistance evolves in the bacterial population, hence reducing the relative benefit of CRISPR-Cas immune systems (141). Here we teased these two effects apart by exposing bacterial populations to experimentally diversified or clonal phage populations of the phage DMS3vir. Our experimental data

support the idea that phage genetic diversity can tip the balance in the evolution of different defence strategies that bacteria employ to combat phage infections. Given that CRISPR-Cas immune systems are highly sensitive to the evolution of “escape” phage that overcome CRISPR immunity through mutation of their target sequences, greater levels of phage genetic diversity are likely to favour generalist defences that are more robust to phage evolution. Indeed, our experiments show that in this empirical system phage was unable to overcome sm-based resistance. Evolution of a novel receptor specificity would presumably require multiple adaptations in the phage tail fibres (189), and while this will occur in certain environments, the rate at which this happens will in most cases be low relative to the rate of CRISPR escape mutation.

The results of this study highlight how ecological variables can drive the evolution of bacterial defence strategies and it will likely be important to consider phage genetic diversity when studying natural systems. For example, we may expect to find higher levels of viral genetic diversity, and therefore lower levels of CRISPR-based immunity, in well-mixed environments such as in aquatic ecosystems, but the opposite may be true in systems with less migration, and therefore lower viral diversity, such as in soil. Environments associated with low viral mutation rates, as is often found in thermophilic microbial communities, will likely harbour higher levels of CRISPR-based immunity as generalist defences with a constitutive cost such as sm will have a selective disadvantage under these conditions. Biotic complexity is frequently ignored in laboratory evolution studies. It is also important to consider that in bacteria-phage interactions in natural ecosystems there will usually be a range of different phage types able to infect each host, and further work is needed to clarify how the presence of multiple phage would affect the evolution of bacterial resistance.

Here we have shown that diversity in an infecting parasite population can impact the outcome of host-parasite interactions. In the data presented here our experimentally evolved phage were passaged on a mutant genotype of the same host used in our experiments. Therefore, the mutations acquired may not be as varied as when phage evolves on a range of hosts as will typically be the case in nature. The observed effects of intraspecific diversity (i.e. genetic diversity within the same species of phage) have been previously predicted by studies that modelled CRISPR-phage interactions (137),but as far as we are aware this is the

first work to demonstrate this experimentally. This work further contributes to our understand of the role of the CRISPR-Cas adaptive immune system in a microbial community context, and complements previous work on the role of bacterial diversity (133). Furthermore, this study suggests that genetic diversity is an important driver of when specific defences are favoured over generalist defences and should therefore be considered when investigating any host-parasite interaction where generalist or specific defences can evolve.

Data Accessibility

Raw data files from the experiments have been uploaded to Dryad (https://doi.org/10.5061/dryad.6djh9w0x7). Sequence data are available on the ENA PRJEB31472

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Authors' Contributions

ERW and JMB conceived and design the study. JMB and SM performed the experiments. JMB, SM and ERW analysed and interpreted the data. SP analysed sequence data. JMB and ERW drafted the article, which was further edited by SM and AB. All authors approved the version to be published.

Competing Interests

We have no competing interests.

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Chapter 3

The Role of Quorum Sensing in Bacterial Resistance Evolution

Abstract

Quorum sensing controls expression of a wide range of important traits in the opportunistic pathogen *Pseudomonas aeruginosa*, including the expression of its CRISPR-Cas immune system. This finding has led to the speculation that synthetic QS inhibitors could be used to limit the evolution of CRISPR immunity during phage therapy. Here we use experimental evolution to explore if and how a quorum sensing inhibitor influences the population and evolutionary dynamics of *P. aeruginosa* upon phage infection. We find that chemical inhibition of quorum sensing decreases phage adsorption rates due to downregulation of the Type IV pilus, which causes delayed lysis of bacterial cultures and favours the evolution of CRISPR immunity. Hence, our data show that inhibiting quorum sensing may reduce rather than improve the therapeutic efficacy of pilus-specific phages, and this is likely a general feature when phage receptors are controlled by quorum sensing.

Introduction

The increase in antimicrobial resistance has led to a resurgence of interest in phage therapy, where bacterial viruses (phage) are used to treat bacterial infections (196, 197). *Pseudomonas aeruginosa* is an important source of nosocomial infections and potential target for phage therapy (198). In the lab, bacteria tend to rapidly evolve resistance to phage (199), which in a clinical setting might limit the efficacy of the therapeutic application (42). Such rapid resistance evolution is mostly due to cell surface alterations that interfere with phage adsorption or due to the acquisition of CRISPR-based immunity, where bacteria insert phage-derived sequences into CRISPR loci on the host genome, which are used to detect and destroy the same phage during future infections. In the case of *P. aeruginosa* PA14, both these mechanisms of phage resistance have been shown to evolve at high frequencies in the lab, but the dominant mechanism depends on environmental variables, such as the microbial community context in which infections occur (133), the level of phage genetic variation (43), and the force of infection (43, 113, 133). The force of infection is an important determinant of CRISPR immunity evolution because unlike surface-based resistance, which carries a fixed fitness cost, CRISPR immunity is

associated with an infection-induced fitness cost (34). Hence, in environments with a high force of infection, surface-based resistance is favoured by natural selection over CRISPR immunity and vice versa (34, 43, 113, 133). Because evolution of surface-based resistance, but not CRISPR immunity, is associated with virulence trade-offs (133), being able to manipulate which type of phage resistance evolves could have important clinical impact.

One way to manipulate this in a clinical setting may be by applying Quorum Sensing (QS) inhibitors, since it was recently demonstrated that QS controls expression of the CRISPR-Cas immune system in *P. aeruginosa* (53) and in *Serratia* (195). QS is a well-studied bacterial communication system which allows bacteria to monitor local cell densities. In *P. aeruginosa* PA14 QS is carried out by two separate but interrelated pathways, known as *lasR-lasI* and *rhlR-rhlI* (200, 201). Each system is comprised of a receptor and an autoinducer (AI); LasI produces the AI 3-oxo-C12-homoserine lactone (3OC12-HSL) which is bound by the LasR receptor. The LasR-3OC12-HSL complex activates expression of a wide range of target genes, including those required for virulence and biofilm formation. The LasR-3OC12-HSL complex also causes RhlI to produce the AI C4-homoserine lactone (C4-HSL), which binds to the receptor RhlR and activates a second wave of QS-induced gene expression (202, 203) (reviewed in (204, 205)). Because QS controls such a broad range of genes, many of which may impact phage infection dynamics, it remains unclear what the net effect of QS inhibitors is on the efficacy of phage therapy. For example, the QS system of *P. aeruginosa* controls expression of both the CRISPR-Cas immune system and its Type IV pili, which serve as receptors for many *Pseudomonas* phages (206–211). We therefore tested how inhibition of QS in *P. aeruginosa* PA14 impacts the ecological and evolutionary interaction with a pilus-specific phage, DMS3vir. Our data show that QS inhibition triggers phenotypic changes in the bacterial population that result in partial immunity to phage due to reduced adsorption to the host cells, and the resulting decrease in phage infection frequencies favours the evolution of CRISPR immunity over surface-based resistance. These data help to predict how manipulation of QS can impact the outcome of bacteria-phage interactions in a clinical context.

Methods

Bacterial Strains and Phage

We used *P. aeruginosa* UCBPP-PA14 (referred to as WT), *P. aeruginosa* UCBPP-PA14 *cas7::LacZ* (referred to as CRISPR-KO, since it carries a non-functional CRISPR-Cas system) (156), a previously described CRISPR-KO-derived surface mutant (referred to as sm) (34), and *P. aeruginosa Tn::pilA* (referred to as *pilA* KO) (190), a CRISPR-Cas resistant isolate of *P. aeruginosa* UCBPP-PA14 possessing two spacers targeting the phage DMS3vir (referred to as BIM2 – Bacteriophage insensitive Mutant, previously described in (34)), as well as double synthase mutant strain of *P. aeruginosa* UCBPP-PA14, Δ*lasI* Δ*rhlI*, (referred to as QS mutant) (53). For all infection experiments we used the previously described mu-like phage, DMS3vir (156).

Elastin Congo Red assay

Elastase production is a well-established method for measuring the levels of quorum sensing via elastase production since elastase is a public good and the production of elastase is known to be controlled by quorum sensing (212). WT or the QS mutant were grown in growth media as indicated with or without QS AIs. Samples were transferred to fresh media at a 1:100 dilution every 24 hours for 3 days. On the third day samples were taken, the OD_{600} was measured and samples were then centrifuged at 15,000xg for 15 minutes. Elastin Congo Red (ECR) buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) was pre-warmed to 37 °C before adding ECR powder 1:10 (w/v). For each sample 900µl ECR solution was added to a fresh 1.5ml Eppendorf, then 100µl of sample supernatant was added. Samples were incubated at 37 °C for 4 hours. Eppendorfs were centrifuged at 15,000xg to remove debris, and OD_{495} of the supernatant was measured. Elastase production per capita was calculated as OD_{495}/ OD_{600}.

Motility Assays

P. aeruginosa UCBPP-PA14 WT or the Δ*lasI* Δ*rhlI* QS mutant were streaked onto 1.5% LB agar plates and grown overnight at 37ºC. Liquid cultures were created by inoculating 6ml M9 media supplemented with 0.2% glucose and inoculated with either the WT or QS mutant and grown for 24h at 37ºC, 180rpm. Swimming

ability was determined by touching a sterile tip to a streak of the WT or QS mutant then inserting the tip halfway through the agar of a 0.3% LB agar plate. Swarming assays were performed by spotting 5µl of undiluted liquid culture onto 0.5% agar. All motility assay plates were incubated for 24h at 37ºC. Motility was measured as the diameter of bacterial growth in mm at the widest point which intersected the point of inoculation.

Evolution experiments

Infection experiments were carried out in glass microcosms containing 6ml of M9 media with 0.2% glucose supplemented with 100µM Baicalein (Cayman Chemical) dissolved in DMSO, and 10^6 cfu/ml *P. aeruginosa* PA14 WT. The same experiment was carried out concurrently with the QS mutant in the presence or absence of 2 µM $3OC_{12}$-HSL and 10 µM C_4-HSL (Sigma) dissolved in DMSO. Control treatments received the same amount of DMSO. Total amounts $1x10^4$ pfu of phage (low phage), $1x10^7$ pfu (mid phage) or $1x10^9$ pfu (high phage) were added at the same time as the bacteria. Microcosms were incubated at 37 ºC while shaking at 180 rpm. Every 24 hours microcosms were sampled and transferred to fresh media at a 1:100 dilution. Samples from the infected cultures were taken prior to each transfer and chloroform treated to kill bacteria as described previously (35). 5µl of a serial dilution of chloroform treated sample was spotted onto a lawn of sensitive bacteria (PA14 CRISPR-KO strain). Plaques were counted and densities were calculated as plaque forming units/ml (pfu/ml). Bacteria were sampled at 3 days post infection to analyse the resistance mechanisms that evolved in response to phage infection.

Resistance mechanism analysis

To determine whether bacteria had evolved resistance, and whether this was surface-based resistance or CRISPR-based immunity, we performed cross-streak assays as described previously (34). Briefly, clonal bacterial colonies were isolated by plating on LB agar, 24 colonies per replicate were picked at random per replicate, grown overnight in M9 media supplemented with 0.2% glucose and then streaked across either the ancestral phage (DMS3vir), or DMS3*vir-acrIF1* described in (35), which carries the anti-CRISPR gene *acrIF1* that effectively

blocks the CRISPR-Cas immune systems of *P. aeruginosa* PA14 (35, 213, 214). Colonies which were resistant to the ancestral phage but not the phage carrying an anti-CRISPR gene were classed as CRISPR immune, whereas colonies that were resistant against both DMS3vir and DMS3*vir-acrIF1* were classed as having sm (surface mutation) resistance. Colonies that were not resistant to either phage were classed as sensitive. Results were confirmed with PCR of both CRISPR loci to check for spacer acquisition as described previously (34).

Competition experiments

We measured fitness by competing the BIM2 targeting DMS3vir against either the phage-sensitive CRISPR-KO strain or against the phage-resistant surface mutant (the *pilA* KO or sm strain). A CRISPR immune strain with 2 phage-targeting spacers was used to avoid confounding effects from the evolution of "escape phage" that carry mutations in their target sequences (35, 164). Competitions were performed in glass microcosms containing 6ml of M9 media with 0.2% glucose, and containing either the inhibitor (100µM Baicalein), the autoinducers (2 µM $3OC_{12}$-HSL + 10 µM C_4-HSL), or both the QS inhibitor and inducer at these same concentrations. Controls contained the same volume of DMSO only. Microcosms were then inoculated with 10^6 cfu/ml of a 1:1 mix of the relevant bacteria. Cells were infected with 10^6 pfu/ml of phage DMS3vir. Vials were incubated at 37 ºC while shaking at 180 rpm for 24 hours and then sampled. The relative frequencies of each strain were determined via serial dilution and plating on 1.5% LB agar plates containing 50 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Relative fitness was calculated as (rel. fitness = [(fraction strain A at t=x) * (1 – (fraction stain A at t=0))] / [(fraction strain A at t=0) * (1 – (fraction strain A at t=x)]).

Phage adsorption

WT *P. aeruginosa* was grown for 24 h in media supplemented with either DMSO (control) or the QS inhibitor. Cultures were centrifuged at 3500 rpm to form a pellet. The supernatant was discarded and the pellets were resuspended in 5ml of pre-heated (37 ºC) medium with or without the QS inhibitor to OD_{600} 0.2. Cultures were incubated at 37 ºC, 180 rpm for 3 hours to achieve mid-log phase and then diluted to a final volume of 15ml. 6 ml of each sample was taken and

stored on ice. The remaining 9 ml was put back in the incubator at 37 ºC, 180 rpm. One tube of plain growth media was also processed as a control. Cultures were incubated for 5 minutes to allow for temperature equilibration. 1 ml of pre-warmed phage in growth media was added to each culture with a final concentration of 2 x 10^5 pfu/ml. Cultures were mixed and a sample taken and added to Eppendorf vials containing 100µl of chloroform, and stored on ice in an insulated box. This process was repeated at 3, 6, 9, 12, 15, 20, 25, 30, 40 and 50 minutes post infection. Phage densities were calculated via chloroform extraction and by spotting virus samples isolated by chloroform extraction on a lawn of CRISPR KO bacteria. Bacterial densities were determined by plating dilutions of the original cultures on LB Agar plates and counting CFUs. The fraction of free phage particles at each time point were calculated relative to the phage titre at T0 by taking the average PFU/ml of 3 replicates at each time point and calculating the % of remaining phage this value represented relative to it's starting frequency using the formula:

((Average PFU/ml at time X) x 100 / Average PFU/ml at T0)

Phage removal assays

To measure CRISPR-mediated phage removal over longer timespans, we mixed the BIM (BIM2) targeting DMS3vir with DMS3vir in glass microcosms containing 6ml of M9 media supplemented with 0.2% glucose or DMSO with or without a QS inhibitor (100µM Baicalein) or QS AIs (2 µM 3OC12-HSL + 10 µM C4-HSL) and inoculated with 10^6 cfu/ml of the BIM. Cells were then infected with 10^6 pfu/ml of phage DMS3vir. Vials were incubated at 37 ºC while shaking at 180 rpm for 24 hours and then sampled. Phage titres were determined via chloroform extraction and spot assay, as described above.

Results

The finding that QS controls expression of the Type IF CRISPR-Cas immune system of *P. aeruginosa* PA14 has led to the suggestion that QS inhibitors and phage may provide synergistic efficacies when applied together in a clinical context, due to more limited evolution of CRISPR immunity against the phage (53). To measure whether chemical QS inhibitors can limit the evolution of

CRISPR-based immunity against a pilus-specific phage, we exposed WT PA14 to either 10^4, 10^7 or 10^9 pfu of phage DMS3vir in the presence or absence of Baicalein, a potent QS inhibitor (215) (Fig. 1).

Figure 1

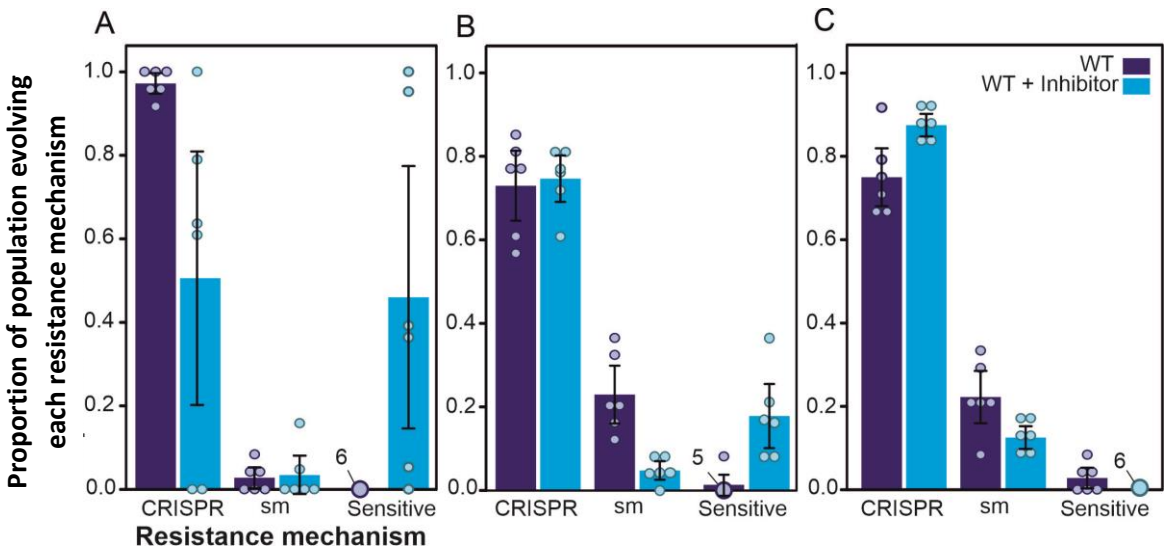


Figure 1. Inhibiting QS results in reduced phage sensitivity and prolonged phage persistence at low phage densities. **A-C)** Observed resistance mechanism evolution in *P. aeruginosa* PA14 WT supplemented with DMSO (dark blue bars/points) or a QS inhibitor (light blue bars/points) and at 3 days post infection with **A)** 10^4 pfu **B)** 10^7 pfu **C)** 10^9 pfu of phage DMS3vir. Bars represent mean proportion of resistance mechanism evolution, error bars indicate 95% confidence interval, points represent individual replicates, N = 6.

In our control (WT supplemented with DMSO) evolution of CRISPR-based immunity decreased (and resistance via surface modification (sm) increased) with increasing phage titer, as reported previously (34). However, when QS inhibitors were added, we observed changes in the patterns of phage resistance evolution that were contrary to our expectation of a general reduction in CRISPR-mediated immunity.

Specifically, there was a significant increase in the persistence of phage-sensitive bacteria during infection with 10^4 pfu ($U = 3$, $p < 0.05$) or 10^7 pfu ($U = 1$, $p < 0.01$) of DMS3vir. This was associated with a reduction in the evolution of CRISPR immunity upon infection with 10^4 pfu of phage ($U = 0$, $p < 0.01$), or a reduction in

sm resistance upon infection with 10^7 pfu of phage (U = 0, $p < 0.01$). The persistence of sensitive bacteria was no longer observed when bacteria were infected with 10^9 pfu of phage DMS3vir. Under those conditions, levels of CRISPR immunity were slightly higher compared to the control, and levels of sm resistance were slightly reduced. Further analyses revealed a significant interaction between QS inhibition and the evolution of CRISPR when infected with 10^4 ($Z_{35,30} = -7$, $p < 0.0001$), 10^7 ($Z_{35,30} = 5.95$, $p < 0.0001$), or 10^9 phage ($Z_{35,30} = 7$, $p < 0.0001$). In accordance with our finding that sm resistance is most affected at intermediate (10^7) levels of infection we find an interaction between QS inhibition and sm evolution when infected with 10^7 phage ($Z_{35,30} = -2.02$, $p < 0.05$) but not the lower (10^4) or higher (10^9) phage treatments. We were unable to detect any significant interaction between QS inhibition and the persistence of sensitive bacteria, consistent with the observed variance . As a control, we performed the same experiment using the QS mutant in the presence or absence of AIs and observed a similar but weaker trend (Fig. 2).

Figure 2

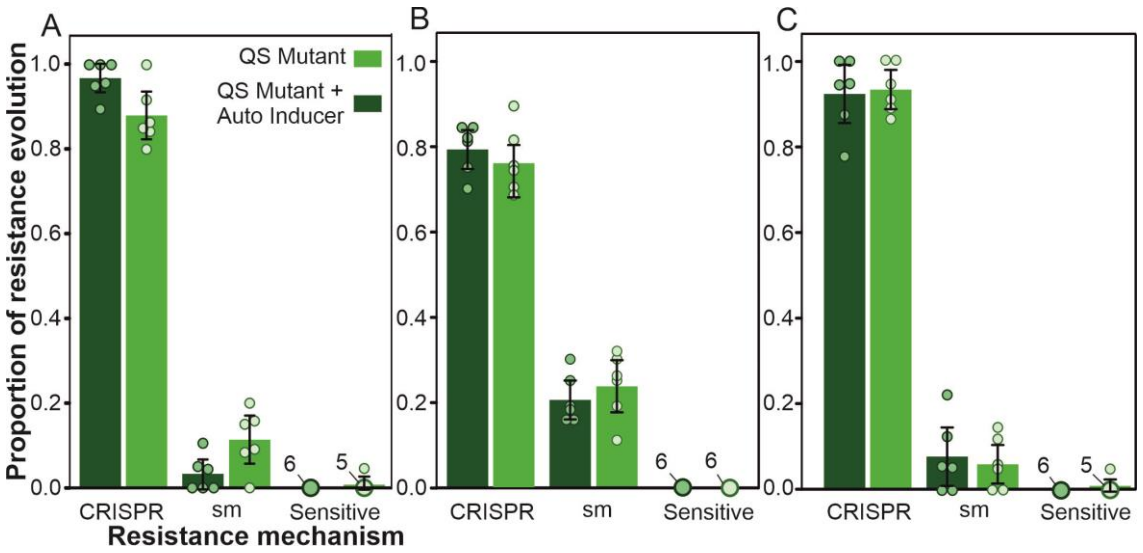


Figure 2. No significant difference is found in resistance mechanism evolution or phage persistence between a QS mutant which is unable to produce QS autoinducers and the same QS mutant supplemented with artificial autoinducers **A-C)** Observed resistance mechanism evolution in *P. aeruginosa* QS mutant when supplemented with DMSO (light green bars/points) or a QS autoinducer (dark green bars/points) and at 3 days post infection with **A)** 10^4 pfu **B)** 10^7 pfu **C)** 10^9 pfu of phage DMS3vir. Bars represent mean proportion of resistance mechanism evolution, error bars indicate 95% confidence interval, points represent individual replicates, N = 6.

A well-established method for quantifying QS activity is to measure elastase production since elastase production is known to be regulated by QS (212), to do this we used the Elastin Congo Red assay (212). This showed that the QS mutant produced significantly less elastase per capita than WT cells ($U = 0$, $p < 0.0001$), as expected, and addition of QS AIs reinstated elastase production to WT levels in the QS mutant ($U = 30$, $p > 0.3$, Fig. 3). This shows that QS controls previously described target genes but has counterintuitive effects on the evolution of phage resistance.

Figure 3

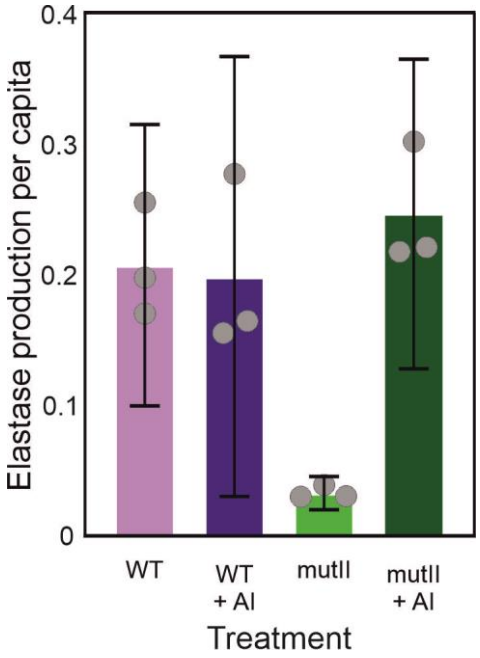


Figure 3. Addition of QS AI to media reinstates QS mutant to WT levels of elastase production. Bars represent mean elastase production per capita, error bars indicate 95% confidence interval, points represent individual replicates, N = 3.

We envisaged two possible reasons why in the presence of Baicalein the evolution of phage resistance might be reduced upon infection with 10^4 or 10^7 pfu of DMS3vir. First, it may be the case that phage amplification is less efficient in the presence of the QS inhibitor, resulting in weaker selection for resistance. Second, QS inhibition may cause cells to become intrinsically more resistant to phage, as a result of reduced expression of phage receptors if they are also regulated by QS (216, 217). Indeed, the T4P, which is the receptor of many Pseudomonas phages including DMS3vir, is known to be regulated by QS, and manipulation of QS can therefore affect phage sensitivity (206–211).

To investigate the idea that QS inhibition reduced the rate of phage amplification we monitored the phage densities throughout the evolution experiment described above. This showed that in the presence of the QS inhibitor, phage amplification was higher than or equal to that observed in the control (Fig. 4), and reduced phage amplification can therefore not explain the results of this experiment.

Figure 4

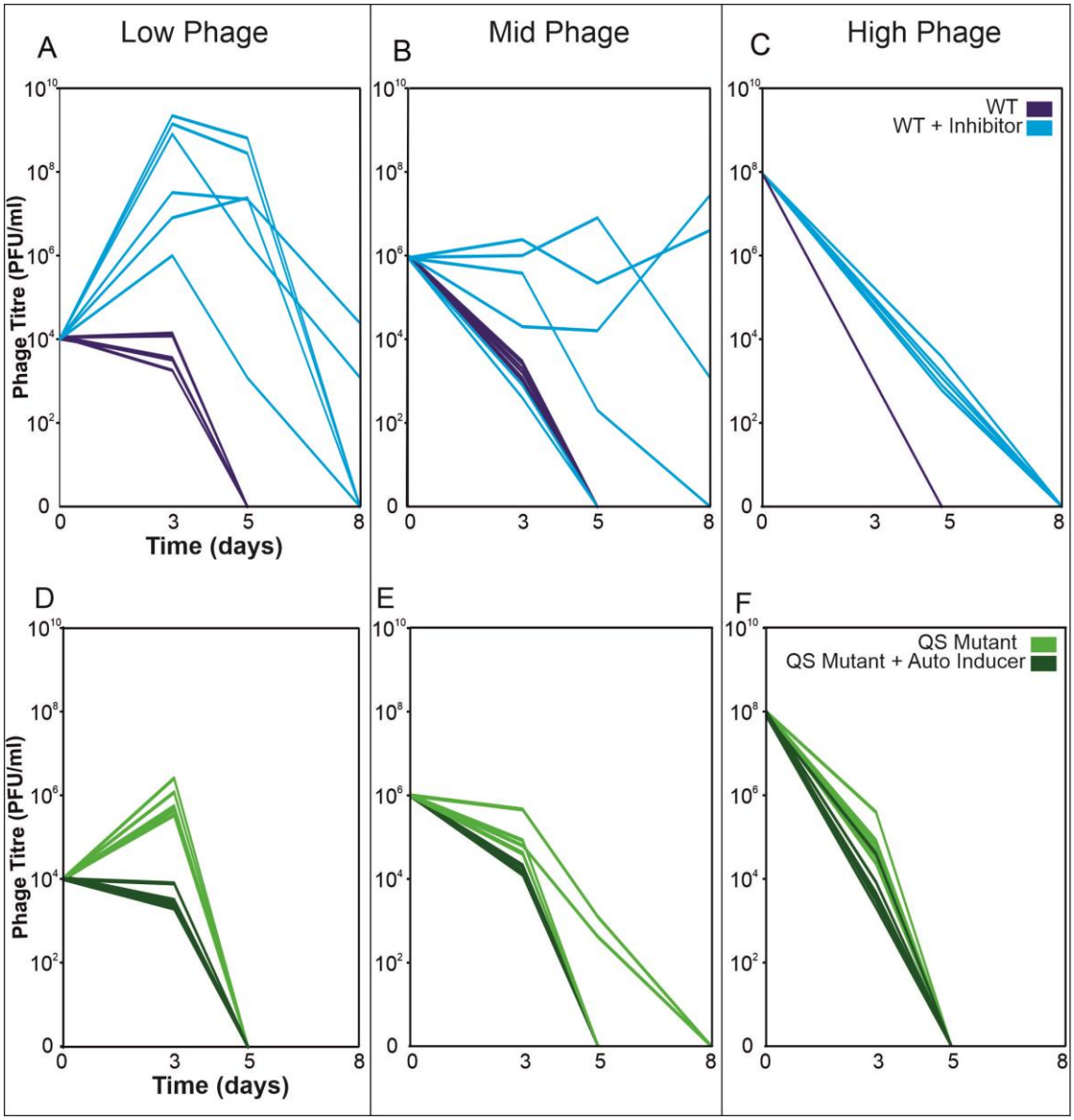


Figure 4 Phage replicate more efficiently when QS is inhibited and force of infection is low. Phage densities were monitored during the evolution experiment and quantified at 3, 5 and 8dpi when initial infections were with **A+D)** 10^4 pfu **B+E)** 10^7 pfu **C+F)** 10^9 pfu of phage DMS3vir. Phage persistence data was taken from the same experimental replicates as were analysed for the resistance mechanism graphs at the corresponding phage start density. Lines show individual replicates, N = 6.

Further analyses showed that addition of a QS inhibitor to WT PA14 cultures made a significant difference to phage dynamics when cultures were infected with

10^4 phage ($F_{3,42} = 3.72$, $p < 0.05$), but we could not detect a significant difference as phage titer increased to 10^7 ($p > 0.1$), or 10^9 PFUs ($p > 0.9$). Similarly we found an effect on phage dynamics of adding a QS autoinducer to cultures of the QS mutant when infected with 10^4 PFUs ($F_{3,42} = 3.52$, $p < 0.05$), but not when infected with 10^7 ($p > 0.9$), or 10^9 PFUs ($p > 0.9$). Since we are able to detect differences in both resistance mechanism evolution (Fig. 1) and phage dynamics (Fig. 4) when QS is manipulated only when phage density is low supports the hypothesis that inhibiting QS causes bacterial cells to become intrinsically more resistant to phage infection.

We next looked for phenotypic effects of QS inhibition which may help us to understand the mechanism responsible for the observed changes in resistance mechanism evolution (Fig. 1) and phage dynamics (Fig. 4). First we measured the impact of Baicalein on *P. aeruginosa* PA14 swarming motility, which is a Type IV pilus (T4P)-dependent and QS-regulated group behaviour (218–221). This analysis showed that the QS mutant was less able to swarm compared to WT cells ($p < 0.0001$) and that WT cells grown in the presence of the QS inhibitor Baicalein were no different in swarming ability to that of the QS mutants ($p > 0.9$) (Fig. 5A). As a control, we also measured swimming motility of the WT strain, which is known to be an individual behaviour not reliant on the T4P and QS (222). As expected, there was no significant effect of Baicalein on swimming motility in the WT and QS mutant strains ($p > 0.1$ in all cases) (Fig. 5B). Collectively, these data therefore confirm that QS inhibition using Baicalein inhibits a QS-controlled T4P-dependent group behaviour in PA14.

Figure 5

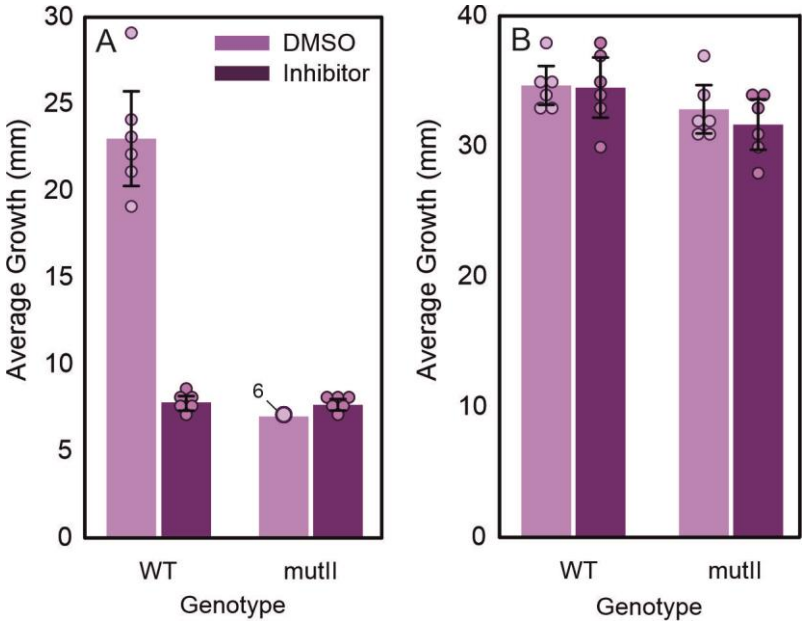


Figure 5 QS inhibition reduces swarming but not swimming ability **A)** QS inhibition reduces swarming motility. **B)** QS inhibition has no effect on swimming motility. Bars represent mean growth in mm, error bars indicate 95% confidence interval, points represent individual replicates, N = 6.

Next, we measured whether Baicalein affected phage adsorption. To this end, we infected WT *P. aeruginosa* PA14 cells in exponential growth phase with phage DMS3vir at an MOI of 0.01 and measured phage titers over 50 minutes in the presence or absence of Baicalein (Fig. 6A). This revealed a significant reduction in the rate of phage adsorption when the QS inhibitor was added to the media ($T = 2.548$, $DF = 13.78$, $p > 0.05$) supporting the hypothesis that QS inhibition leads to a reduction in phage adsorption.

Based on previous work showing the CRISPR activity reduces phage densities because of the ability to continually remove phages from the population, we hypothesized that the intrinsic increase in resistance and associated reduction in CRISPR activity would result in higher phage titres. To test this hypothesis, we also measured phage titers after 24h of infection of CRISPR immune bacteria with 10^9 pfu of DMS3vir in the presence or absence of Baicalein or synthetic autoinducers (Fig. 6B). This revealed a significant decrease in the amount of

phage removed in the presence of the QS inhibitor (U = 0, p <0.01) or the inhibitor and AIs combined (U = 0, p <0.01).

Figure 6

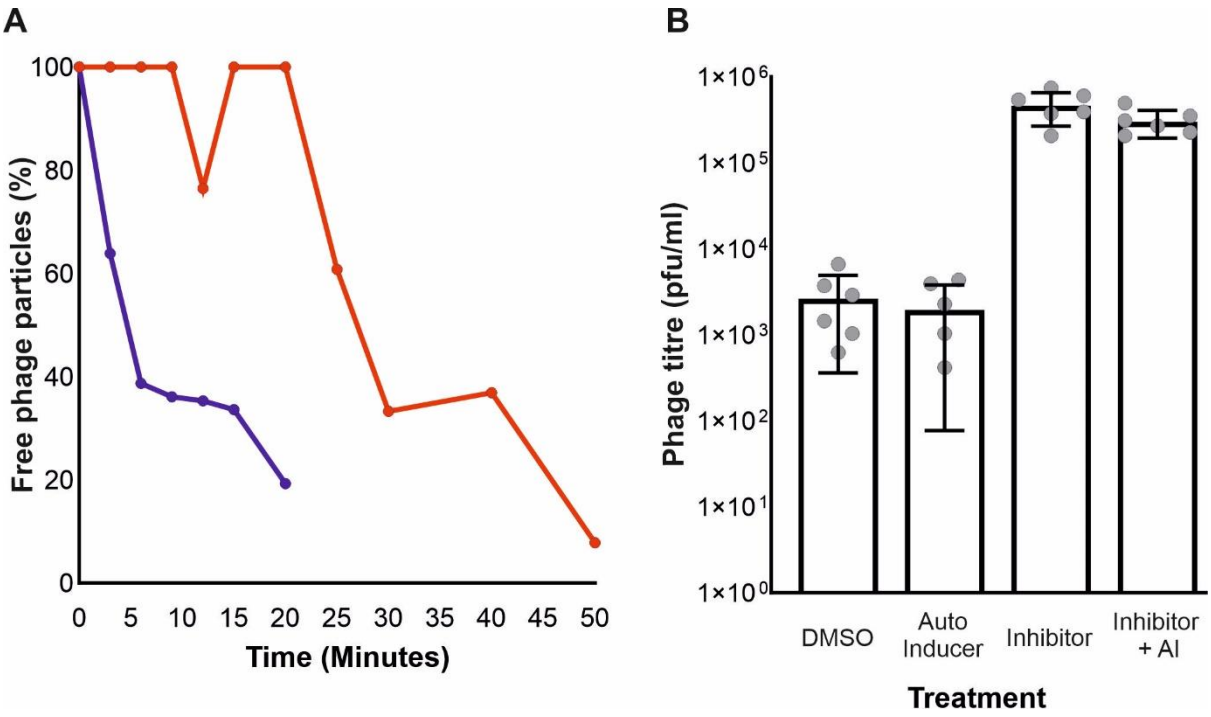


Figure 6. Phage are less able to infect hosts when QS is inhibited. **A)** Phage adsorption over time (3-50 minutes) when WT *P. aeruginosa* is infected with phage when supplemented with DMSO (control) (blue line) or the QS inhibitor (red line). Samples were taken at 3,6,9,12,15,20,25,30,40 and 50 minutes post infection. Lines represent % of free phage particles detected at each time point **B)** Titre of phage remaining at 24hpi of a CRISPR resistant host possessing two unique spacers targeting the infecting phage when initially infected with 10^9 phage. Bars represent mean titre of phage present at 24hpi, error bars indicate 95% confidence interval, points represent individual replicates, N = 6, Y axis is $\log^{10}$ scale.

Collectively, these data led us to predict that applying Baicalein may reduce the efficacy of phage-mediated killing by enhancing the intrinsic levels of phage resistance of the bacteria. To test this, we measured how Baicalein affects the fitness of phage sensitive bacteria in the presence of phage, by competing sensitive bacteria lacking a functional CRISPR-Cas immune system (CRISPR

KO strain) against a pilus deletion mutant (a *pilA* KO strain) that lacks the T4P providing complete surface modification (sm) mediated resistance against the phage DMS3vir. This showed that, sensitive bacteria had a significantly higher fitness when Baicalein was added to those competition experiments ($U = 0$, $p < 0.005$) (Fig. 3A). In this experiment we are competing a completely phage-sensitive strain (CRISPR-KO) against a fully resistant strain (sm). The resistant sm strain always has a higher fitness so the relative fitness of the sensitive cells does not exceed 1 (where a value of 1 indicates equal relative fitness). The addition of baicalein does increase the fitness of sensitive cells but does not provide complete immunity as sm resistance does. These data therefore confirm that Baicalein enhances phage resistance, hence lowering the efficacy of the phage.

We speculated that the reduced phage adsorption rates of phage in the presence of Baicalein may also explain the high levels of CRISPR immunity and low levels of sm resistance that evolve under the highest phage exposure levels (Fig. 1). CRISPR immunity is associated with an infection-induced fitness cost, whereas sm is associated with a fixed cost (34). As a consequence, inhibition of QS and phage adsorption could lead to an enhanced fitness of CRISPR immune bacteria relative to surface mutants. Indeed, direct competition between a CRISPR immune clone (BIM2) and a bacterium with surface-based resistance (sm) revealed that the relative fitness of BIM2 increased in the presence of Baicalein ($U = 0$, $p < 0.005$), and was not affected by the addition of AIs ($U = 17$, $p > 0.9$) (Fig. 7B). Since both the sm strain and BIM2 possess complete immunity against the infecting phage their relative fitness is equal in the absence of QS manipulation, however when QS is inhibited the relative fitness of BIM2 increases to around 10 times that of the sm strain. This can likely be explained by the relative costs of CRISPR-Cas compared with sm mediated resistance: Because sm resistance requires a fixed cost the cells pay this cost regardless of the frequency of infection, however CRISPR-Cas carries a induced cost which is dependent on the frequency of infection (34), therefore the increase of fitness we see here indicates that fewer infections occur when QS in inhibited.

Finally, given that CRISPR immune bacteria are less efficient in removing phage in the presence of Baicalein (Fig. 6), we hypothesized the outcome of direct competition between CRISPR immune bacteria and sensitive bacteria is likely to

be also affected by Baicalein. Direct competition between CRISPR immune bacteria and sensitive bacteria showed that while QS AIs had no significant impact on the relative fitness of the CRISPR immune bacteria (U = 11, $p > 0.9$), their relative fitness increased in the presence of Baicalein (U = 2, $p < 0.05$) (Fig. 7C). BIM2 (the CRISPR immune bacteria) has a higher fitness compared with the sensitive CRISPR-KO in the absence of QS manipulation simply because BIM2 possesses phage resistance and the CRISPR-KO does not. Therefore, we expect very high values of relative fitness for BIM2 in competition with the CRISPR-KO. This is exactly what we see and, in agreement with the other competition experiments, we see that the addition of a QS inhibitor increases the relative fitness of BIM2.

Figure 7

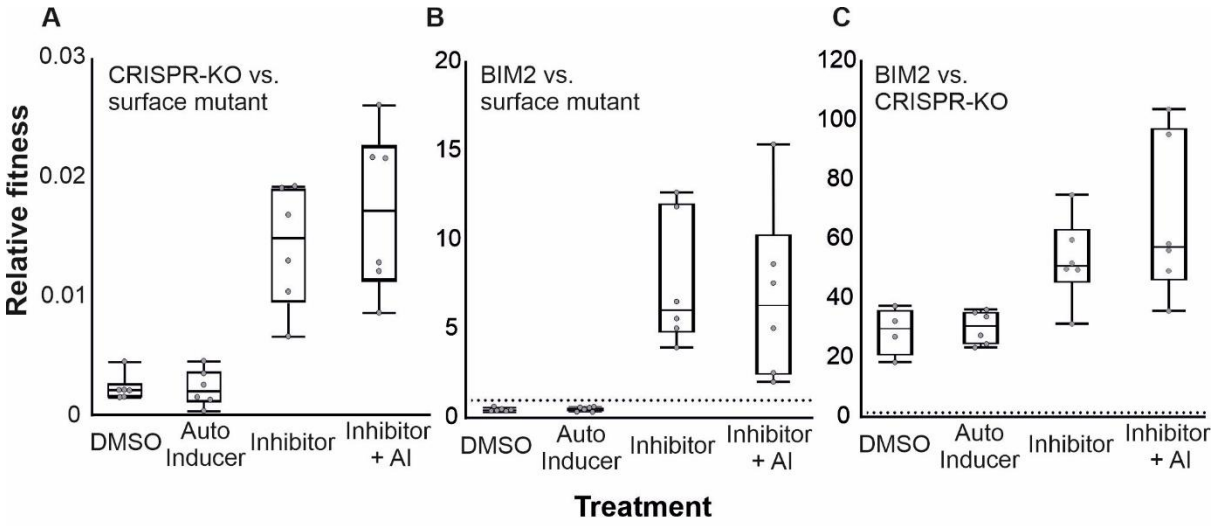


Figure 7. Inhibiting QS increases the fitness of *P. aeruginosa* in the presence of phage **A)** Relative fitness of sensitive CRISPR-KO strain with the phage resistant surface mutant (sm) **B)** Relative fitness of BIM2 competed with a phage resistant strain (sm) **C)** Relative fitness of BIM2 competed with a sensitive CRISPR-KO strain. Boxes show mean, minimum and maximum values, error bars show 95% confidence interval and points represent individual replicates, N = 6.

Dotted line in panels **B** and **C** indicates the point at which competing strains are equally fit (when relative fitness = 1). This is not present in panel **A** due to the very low relative fitness of the CRISPR-KO when competed with sm.

Collectively, these data show that inhibition of QS using Baicalein reduces the rate of phage adsorption, which in turn significantly changes the way selection acts on sensitive bacteria and those with CRISPR immunity or surface-based resistance.

Discussion

Being able to manipulate phage resistance evolution may have important clinical impact, since bacteria that remain phage sensitive or evolve CRISPR immunity are more likely to retain their virulence levels compared to bacteria that evolved surface-based resistance (133, 223, 224). In the experiments presented here we tested the idea that, because QS regulates CRISPR-Cas expression, chemical inhibition of QS might enhance the efficacy of phage-mediated killing of bacteria and steer evolution of phage resistance away from CRISPR immunity and towards surface-based resistance. Surprisingly, we found that Baicalein enhanced the fitness of sensitive and CRISPR immune bacteria relative to surface mutants and failed to reduce the evolution of CRISPR immunity. This is because QS also regulates expression of the T4P, which is used as a receptor by many *Pseudomonas* phages, including phage DMS3vir (158, 206–211). As a consequence, the rate of phage adsorption is reduced in the presence of Baicalein, which favours CRISPR immune bacteria over sm because of their induced and fixed costs of resistance, respectively (34). These findings are important in the context of phage therapy where the evolution of CRISPR immunity is undesirable, and show that QS inhibition may have unanticipated effects on the evolutionary outcome of bacteria-phage interactions.

QS has been studied extensively in *Vibrio harveyi* and *P. aeruginosa;* in *V. harveyi* the QS regulators AphA and LuxR control 167, and 625 genes respectively, and they co-regulate a further 77 genes (225) and at least 616 genes have been identified as being controlled by QS in *P aeruginosa* (226). QS systems have been shown to control expression of genes in accordance with both inter and intra specific densities and species specific QS molecules can induce changes in associated microorganisms (227–230), plants and eukaryotic cells (231, 232). Furthermore it has been shown that QS autoinducers influence symbiotic and pathogenic relationships between microbial, mammalian and plant

hosts (228–233). A family of QS compounds known as AI-2 (234) are synthesised by the *LuxS* enzyme (235). The *LuxS* gene is widespread throughout gram-positive and gram-negative bacteria (235), which has led to the suggestion that AI-2 is a “universal communicator” influencing behaviour of multiple species of bacteria (236). The potential of QS systems to influence such diverse behaviours and interactions has been led to them being referred to as “master regulators”. This makes manipulating QS to our advantage even more complex with many variables to account for (237). The data presented here provide an insight into how QS manipulation affects *P. aeruginosa* evolution in the presence of phage. This information will help to inform further investigations into how and why QS systems function in the manner they do and serve to help in predicting outcomes of manipulating QS. This is beneficial in medical settings where the ability to control commonly QS controlled traits such as virulence would be highly desirable, particularly in a clinically relevant strain such as *P. aeruginosa*.

QS systems most commonly control genes which underpin traits that confer fitness benefits when bacteria reach high densities. These include clinically important traits and group behaviours such as the expression of virulence determinants (238), toxin production (239), biofilm formation (240), and swarming motility (218). Both biofilm formation and swarming motility require T4P expression and the presence of high numbers of individual bacteria, hence explaining why T4P expression is commonly found to be controlled by QS (218, 221). In general, however, selection would be expected to drive gene regulatory networks that lead to the downregulation of phage receptors at high bacterial densities, since phages spread most effectively when their host bacteria are present at high densities. Indeed, experimental work has demonstrated that *Escherichia coli* downregulates receptors used by its phages Lambda and Chi when it reaches high cell densities, due to QS-mediated regulation of these genes (217). A similar finding was made for *Vibrio anguillarium*, which was more resistant to infection by its phage KVP40 at high cell densities due to QS-mediated downregulation of the phage receptor OmpK (216).

Some phages and other mobile genetic elements have evolved mechanisms that allow them to obtain information about the availability of hosts in the environment. For example, some temperate phages carry QS receptors (229) that can bind the autoinducers produced by the host they infect (228). A prophage of *Vibrio cholera*

that carries the QS receptor VqmA uses the autoinducers produced by the host as a cue to induce when cell densities of the host are high (233). Other temperate phages encode their own QS systems, known as Arbitrium systems, and preferentially enter the prophage state once the signal concentration becomes high, presumably because this indicates that available hosts are running out (241). Likewise, some plasmids that infect *Enterococcus faecalis* encode sex pheromones that are involved in the regulation of horizontal spread of these plasmids (242). These examples highlight the importance of host cell density and signaling amongst hosts and their mobile genetic elements in shaping the epidemiology of parasitic DNA.

Given that infection risk increases with cell density, it makes sense why CRISPR immune systems are positively regulated by QS (53, 195). At high cell densities it is important that hosts are able to target and remove non-adaptive MGEs that would otherwise spread rapidly through the population. However, whether or not these regulatory circuits can be leveraged in clinical settings will depend not only on regulation of CRISPR-Cas, but also on the way phenotypic phage resistance against MGEs is regulated. When phage receptors are negatively regulated by QS, as in the *E. coli* and *V. anguillarium* examples outlined above, QS inhibitors and phage may provide a synergistic efficacy. In the case of *P. aeruginosa*, the vast majority of phages characterized to date appear to either use LPS or the T4P as their receptor. This work shows that synergy is unlikely between pilus-specific phages and QS inhibitors. However, whether there is synergy between Baicalein and LPS-specific phage remains an exciting possibility that needs to be examined experimentally in future studies. Future work should also consider the possibility that QS and CRISPR may interact differently under different conditions. For example recent findings that the evolution of CRISPR-Cas is influenced by local microbial communities (133), that QS is more important in the evolution of CRISPR-Cas at environmental temperatures (243), and that QS signals can have inter-species effects (228–233) highlight a need to consider the importance of QS and CRISPR-Cas evolution in environmental studies where these factors will be more important.

General Discussion

In this thesis I present data which provides valuable insights into the evolutionary and ecological importance of CRISPR-Cas. Using a study system in which phage infection results in the evolution of either CRISPR-Cas resistance (associated with a constitutive fitness cost) or sm defence (associated with a fixed fitness cost), I investigate when one type of defence is favoured over another. The findings presented here may have implications in clinical, natural and industrial settings as well as informing further studies into the evolution and ecology of bacterial defence against phage and the evolutionary outcomes of bacteria-phage interactions.

Increasing within population CRISPR-Cas allele diversity decreases phage persistence and potential for local adaptation

High within-population CRISPR-Cas allele diversity is often observed in natural systems, but it is unclear why this is important (34, 134–136). Using bacterial populations harbouring varying levels of CRISPR allele diversity I found that phage were less able to infect host populations possessing high levels of CRISPR allele diversity and that as CRISPR allele diversity increased, infecting phage populations were eradicated more quickly (35). This demonstrates that population level CRISPR-Cas allele diversity is an important factor in bacteria-phage interactions, consistent with the idea of “herd immunity” whereby hosts are not resistant against all phage but sufficient resistance allele diversity exists at the population level to prevent viral epidemics (244). We also found that phage are less likely to become locally adapted to hosts when the host population possesses higher levels of CRISPR allele diversity (165). This could be due to the number of susceptible hosts decreasing as resistance allele diversity increases preventing sufficient replication of phage for the emergence of CRISPR-Cas escape mutants and ongoing co-evolution. This may help to explain why local adaptation is not commonly observed in natural systems (245, 246), although it is more common in systems where parasite migration is more frequent than host migration. In these scenarios the parasite population will likely be more

genetically diverse (246), which is predicted to reduce the benefit of CRISPR-Cas (137).

Increasing within-population phage diversity decreases the frequency of CRISPR-Cas evolution

Increasing within-population phage genetic diversity is predicted to increase the cost of CRISPR-Cas resistance through cost of spacer acquisition as a new spacer is required to provide immunity against each novel phage genotype encountered. If individual cells within the host population do not possess complete immunity they are sensitive to phage infection, allowing phage to replicate and diversify (137, 138). Phage isolated from natural populations are often found to be highly diverse (247–249), however many laboratory experiments use clonal phage populations. By evolving a population of phage with increased genetic diversity, I was able to investigate how genetic diversity within an infecting phage population affects the evolution of bacterial immune defence. In keeping with previous theoretical models and correlational studies (137–140), I found that bacteria infected with the evolved genetically diverse phage were more likely to evolve sm defence over CRISPR-Cas resistance. This is because sm defence protects the host against a range of phage genotypes as opposed to the genotype specific mechanism of CRISPR-Cas. I also found that hosts which had evolved CRISPR-Cas resistance against diverse phage populations were more likely to have acquired multiple spacers than those infected by clonal phage. Interestingly, despite a significant decrease in the proportion of the population which evolved CRISPR-Cas resistance in response to diverse phage, I found no difference in the total number of spacers gained at the population level, or the rate of phage extinction. This supports the previous finding that herd immunity can play an important role in the evolution of CRISPR-Cas (244), and is likely an important factor under the conditions tested here. Theoretical models predict that there will be a point at which the population level herd immunity effect is no longer effective if the genetic diversity of infecting phage population increases (137). As well as providing insight into the evolution and ecology of phage defence systems in bacteria, these findings may be of relevance to phage therapy, a possible alternative treatment for bacterial infections whereby a dose of phage is administered to eradicate pathogenic

bacteria (42, 57). In such scenarios it is important that the administered cocktail is tailored to the specific infection, therefore diversity is an important variable to be considered both in the pathogen population and the phage cocktail.

Inhibiting cell-cell communication can select for CRISPR-Cas evolution

Recent findings that the cell-cell communication system known as Quorum Sensing (QS) can affect the expression of CRISPR-Cas has led to the suggestion that we may be able to manipulate the evolution of CRISPR-Cas via QS to enhance phage therapy cocktails (53, 195). Since CRISPR-Cas has the ability to eradicate phage, its presence in a bacterial population could reduce the efficacy of phage therapy treatments. In addition, bacteria which remain phage sensitive or evolve CRISPR-Cas are more likely to retain virulence traits than bacteria which evolve resistance via sm (133, 223, 224). Hence the ability to predict and manipulate bacterial response to phage infection could be a valuable tool in treating problematic clinical infections. In this chapter I presented the first data on the evolutionary outcome of QS inhibition on the evolution of CRISPR-Cas immunity. I found that under certain conditions QS inhibition may increase selection for CRISPR-Cas and infer a partial phage resistance to host cells. This would be highly problematic in the context of phage therapy given that these cells are less likely to be infected by phage and therefore are more likely to survive rounds of phage therapy treatment. My findings show that inhibiting QS interferes with expression of the Type IV Pilus (T4P), this pilus is required for many *Pseudomonas* phage to adsorb to their hosts (210, 250), and so these findings may be applicable to other bacteria-phage interactions in which the T4P is the site of phage adsorption. However, the effect of QS inhibition on the expression of other phage binding sites, such as LPS binding page, were not tested here and therefore it is possible that QS inhibition may increase LPS binding phage sensitivity in bacteria but specific research into such a system would be required to confirm this. These findings highlight the importance of understanding the evolutionary outcome of manipulating QS in microbes as QS is a complex system controlling many regulatory pathways, therefore any manipulation may result in unexpected secondary phenotypic outcomes.

Conclusions

Diversity varies greatly in nature but how and why genetic diversity is generated and maintained is often not known. In host-pathogen relationships we know that diversity is an important determinant of the outcome of interactions. For example, in the monoculture effect in agriculture where less diverse crops are associated with a higher risk of disease infection and spread (251). Or in animals with low genetic diversity (for example inbred or endangered animals (252, 253)) which are also associated with increased susceptibility to pathogen infection. By understanding the evolutionary drivers and outcomes of host-pathogen interactions, we are more able to understand natural environmental interactions as well as potentially manipulate evolution in these systems to our advantage. Studies looking at the role of genetic diversity in evolution have the potential to influence many fields of study including conservation, medicine, epidemiology and evolution/co-evolution studies.

Genetic diversity, both standing and in-situ generated, is an important and frequently overlooked variable in bacteria-phage dynamics. CRISPR-Cas is an important resistance mechanism in host-pathogen interactions in nature. The specific targeting mechanism of CRISPR-Cas is known to drive rapid evolution of genetic diversity at the individual and population levels in both host and parasite species (35, 43, 165) compared to other known diversity generating mechanisms such as germline mutation and sexual reproduction. This is because CIRSPR-Cas can generate variation within a generation (i.e. by acquiring a new spacer), whereas diversity generated through sexual reproduction can only be created between generations (i.e during recombination stage of meiosis) (254). Bacteria carrying CRISPR-Cas systems are often associated with higher pathogenicity than other resistance mechanisms (133), so understanding the evolutionary drivers of CRISPR-Cas would allow us to predict and manipulate problematic pathogens carrying the system. Despite this we know relatively little about how and why CRISPR-Cas evolves and is maintained in nature. My experiments focusing on diversity show that within-population genetic diversity is an important factor in the evolution of bacterial immune defence and phage response with high CRISPR-Cas allele diversity associated with increased phage extinction, reduced local adaptation of phage, and therefore reduced likelihood of ongoing coevolution. Increased within-population phage diversity results in increasing

selection for generalist phage defence (sm) over specific phage defence (CRISPR-Cas).

QS systems are thought to be widespread in nature (235) and are known to be master regulators, influencing the expression of a wide range of genes within species (225, 226), between different bacterial species (236), other microorganisms (227–230) and even plants and eukaryotic cells (231, 232). Furthermore it has been shown that QS autoinducers influence symbiotic and pathogenic relationships between microbial, mammalian and plant hosts (228–233). This makes manipulating QS to our advantage incredibly complex with many variables to account for (237). While QS manipulation is a potentially powerful tool for improving the efficacy of phage therapy treatments the data I present here indicate that the complexity of this system means manipulation may have undesirable secondary effects. Through the experiments presented I was able to determine that in the study system I used QS inhibition was interfering with production of the Type IV pilus, which is required for binding by the phage (158). Further research is required into the bacterial species commonly targeted by phage therapy and their phage in order to determine if QS inhibition may be more effective in different systems. It has recently been shown that CRISPR-Cas is more active at environmental temperatures compared with warmer temperatures such as when infecting hosts (243). Bacteria occupying colder environmental niches grow more slowly than those occupying warmer within-host niches. The slower growth rate provides more time for the expression of CRISPR-Cas complexes within cells, increasing the chance of infecting phage being detected and destroyed by increasing the frequency of possible adaptation and interference events (243). This suggests that the link between QS and CRISPR-Cas may be important at environmental temperatures and it has been suggested that under these conditions QS and CRISPR-Cas work synergistically to enhance CRISPR-Cas resistance (243). This may explain why I was unable to detect a direct effect of QS on CRISPR-Cas activity as these experiments were carried out at temperatures representative of within-host conditions. It is therefore possible that under environmental conditions the inhibition of QS would have stronger effects on the evolution of CRISPR-Cas. Future experiments should focus on how QS manipulation affects the evolution of CRISPR-Cas in

environmental conditions to add further insight into the link between these mechanisms.

Studies which consider both the individual and group effects of evolution must be considered in order to gain a clear insight into the complexities of what drives and maintains CRISPR-Cas evolution. Experiments focusing on spatial structure may be of particular help when investigating the function and evolution of CRISPR-Cas in environmental settings, spatial structure is predicted to be important in the evolutionary outcome of bacteria-phage interactions (79, 255) and this has been shown to be true for CRISPR-Cas systems (244, 256, 257). Furthermore structured environments such as biolfilms and soil have been found to be important environmental reservoirs for multi drug resistant human pathogens (258). Increasing spatial structure decreases mobility of both phage and bacteria, affecting both the frequency of bacteria-phage encounters and the likelihood of encountering novel genotypes (79, 244). My data show that CRISPR-Cas is favoured when phage genetic diversity is low, therefore in spatially structured environments both QS and phage diversity are likely to be important factors in CRISPR-Cas evolution.

Applications of findings

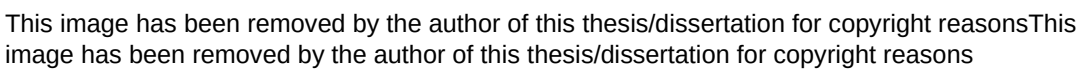
Any novel understanding or method of manipulation of CRISPR-Cas will help to inform studies investigating microbial systems where CRISPR-Cas is present as well as advancing our ability to utilise phage derived antimicrobial strategies or bio-technological tools. Phage based treatments have shown promise in the control the growth of *Campylobacter* and *Salmonella* on chicken skin (259), *Salmonella enteritidis* in cheese (260), *Listeria monocytogenes* on meat (261) and on fresh-cut fruit (262), and *Salmonella* also on fresh-cut fruit (262). In humans phage therapy has also shown promise, one study reports complete eradication of a multi drug resistant strain of *Acinetobacter baumannii* after isolation and administration of infective phage (263) while another reports clinical improvement of a *Mycobacterium abscessus* infection following treatment with a cocktail of engineered phage (60) as well as several other promising trials (56–60). In all applications of phage treatments it is vital that we have the necessary information to predict the evolutionary outcome of bacteria-phage interactions to

ensure efficacy of treatment and avoid inadvertently selecting for undesirable traits such as phage resistance and bacterial virulence, the findings presented here provide insight which may be of importance when predicting these interactions.

In industrial processes where phage infection can result in product loss and therefore increased product price it has been suggested that bacteria may be engineered to possess CRISPR-Cas defence against invading phage. Indeed, the discovery that commercially successful dairy “starter strains” (bacteria which are added to milk to produce products such as yoghurt and cheese) of *Streptococcus thermophilus* possessed CRISPR-Cas resistance against common phage (109) has led to research focussing on engineering the CRISPR-Cas systems of bacteria to further increase productivity and minimise product loss (66, 264). In addition to yoghurt and cheese production phage resistance via CRISPR-Cas would be applicable to many diverse industrial processes where microbial fermentations are vulnerable to infection. For example, *B. thuringiensis* is a commonly used organic pesticide which is present in 90% of all pesticides sold globally (265) and, as in yoghurt and cheese production, vats are often lost to phage infections. If we can reduce the cost of production of *B. thuringiensis* pesticides the cost of producing organic crops will be reduced promoting the use of these products over more harmful, but currently less expensive pesticide products at the production level and reducing the price of organic products at the consumer level making organic food a more affordable choice.

Overall, I hope that the findings presented here will help to inform and guide future research into microbial interactions, CRISPR-Cas evolution and may have applications in clinical, laboratory or field experiments working with microbial populations where CRISPR is present. Additionally, I hope that future clinical and industrial applications will consider these findings to help predict, manipulate and respond to the evolution of CRISPR-Cas to help us utilise this tool to our advantage.

Appendices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