An investigation into the effects of phage diversity on the evolution of bacterial resistance mechanisms

Submitted by

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to the University of Exeter as a thesis for the degree of

Masters by Research in Biological Sciences

April 2016

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

Bacteria possess multiple resistance mechanisms, but little is known why one mechanism can be favoured over others. With a focus on two resistance mechanisms of the pathogenic bacteria *Pseudomonas aeruginosa*, I expand on present knowledge by looking at ecological and genetic selection pressures that drive the adaptive resistance mechanism of the CRISPR-Cas (Clustered Regular Interspaced Short Palindromic Repeats – CRISPR associated) system compared with general resistance through cell surface modification. Specifically, I show that 1) the evolution of CRISPR-Cas immunity is not general across all phage species. 2) I examine how adaptive evolution is affected when a phage species, known to elicit CRISPR-Cas evolution, is mixed with novel phage species and demonstrate that the resistance mechanism is switched in combination with multiple phages, to surface modification. 3) I show how priming is important for continued resistance when phage have escaped the CRISPR-Cas system. However, significant detection of priming may vary between different host-phage interactions. 4) I then show how primed bacterial strains fail to evolve CRISPR-Cas resistance when infected with phage mixtures, even though prior spacer acquisition exists. 5) Finally, the benefit of the CRISPR-Cas system in generating genetic diversity is shown to rapidly clear phage from the environment. Combined, these results show that, even though there are substantial fitness benefits associated with CRISPR-Cas immunity, *P. aeruginosa* will develop resistance by means of surface modification in the face of phage diversity.

Acknowledgements

I whole heartedly thank my brilliant supervisors for their advice, guidance and support throughout this Masters. I am extremely grateful to Professor Angus Buckling for getting me interested in evolutionary ecology in the first place and to Dr Edze Westra for the endless support and encouragement. Thank you both for giving me the freedom to try out new and exciting things but always being there to steer me in the right direction. It's been a fantastic experience and I hope I can continue to work with you both in the future.

A special thanks to Dr. Stineke van Houte whose cheery disposition made long days less tedious and whose help was always at hand and enthusiastically given.

I have been so lucky to be surrounded by the ever-growing, changing and brilliant Buckling lab group of Elze Hesse, Siobhan O'Brien, Adela Lujan, Florian Bayer, Andrea Dowling, Suzanne Kay, Pawel Sierocinski, Peter Stillwell, Sean Meaden, Nicole Parr, Lewis Campbell, Jenny Broniewski, Dan Morley, Helene Chabas, Iris van der Zande, Loris Capria, Mhairi Inglis and Daniella Farina.

And I could not have finished this thesis without the help of Olivia Lee whose endless supply of tea helped keep me going and remain somewhat sane. Also to "The A Team": Abigail Colclough, Aimee Murray and Amy McLeman for the unwavering support, encouragement and laughs along the way.

And finally I thank the University of Exeter for putting up with me for all these years.

Table of Contents

List of tables and figures

Introduction

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Appendix: Extended data of Chapter 5

Extended Data Table 1: Tukey HSD all pairwise comparisons of the data in Figure 3

Extended Data Figure 1: Infection with virus DMS3vir leads to rapid evolution of CRISPR-mediated immunity in WT bacteria, while CRISPR KO bacteria primarily evolve virus immunity by surface mutation

Extended Data Figure 2: No benefit of increasing spacer diversity in the absence of virus

Extended Data Figure 3: Deep sequencing analysis of the frequency of mutations in the target sequence of virus isolated at t=1 from the experiment shown in Figure 4

Extended Data Figure 4: Escape virus titres decline upon infection of diverse CRISPR populations despite increased fitness over ancestral virus

Extended Data Figure 5: Diverse populations do not acquire additional spacers during the experiments shown in Figures 2-4

Extended Data Figure 6: Persistence of phage that encodes an anti-CRISPR gene is independent of spacer diversity

Extended Data Figure 7: Virus persistence inversely correlates with the level of CRISPR spacer diversity in CRISPR immune populations of *Streptococcus thermophilus*

Extended Data Figure 8: Sensitive bacteria are unable to invade bacterial populations with CRISPR-mediated immunity in the presence of virus

Extended Data Figure 9: Virus persistence inversely correlates with the level of CRISPR spacer diversity during competition between CRISPR immune populations and the sensitive CRISPR KO strain

Extended Data Figure 10: Emergence of virus mutants that overcome CRISPR-mediated immunity during the experiment shown in Extended Data Figure 9

Extended Data Figure 11: Sensitive bacteria are unable to invade bacterial populations with CRISPR-mediated immunity in the absence of virus, independent of the level of spacer diversity

Abbreviations

CRISPR-Cas	Clustered Regular Interspaced Short Palindromic Repeats – CRISPR associated
sm	surface mutant
dpi	days post infection
LPS	Lipid polysaccharide
WT	Wildtype bacterial strain
pfu	plaque forming unit
cfu	colony forming unit
rtp	room temperature and pressure
rpm	rounds per minute
BIM	Bacteriophage-Insensitive Mutant
CRISPR KO	CRISPR Knock-out strain (also referred to as *csy3::LacZ*)
hpi	hours post infection

Foreword

Chapter 5 was the result of a collaborative effort and is currently in press. I helped to perform almost all laboratory experiments and aided in the editing of the manuscript. The title and joint authors are as follows;

van Houte, S., Ekroth, A.K.E., Broniewski, J.M., Chabas, H., Ashby, B., Bondy-Denomy, J., Gandon, S., Boots, M., Paterson, S., Buckling, A.J. and Westra, E.R. (Nature, *In Press*) The diversity-generating benefits of an adaptive immune system.

Reviews of the literature are provided and each topic discussed in each chapter. A general overview of the CRISPR-Cas system is given in the thesis introduction.

Introduction

Antagonistic coevolution is the result of host-parasite interactions where parasite infection selects for host resistance which then selects for increased parasite virulence (Thompson 1994). This endless arms race of host adaptations and parasite counter-adaptations play an important role in shaping host-parasite population dynamics. It is therefore vital to understand how different selective pressures, such as different environmental conditions or different within and between species interactions, drive the evolution of defence and counter-defence mechanisms. The defensive mechanisms of evolved immunity elicited, in the host, as a response to these interactions will be the foundation of the experiments described in this thesis.

Bacteriophages (parasites) are phages that infect bacteria (host) by injecting their genetic material into the hosts' cells. To do so, the phage must target and bind to receptors on cell surfaces. Once inside the host, phages will replicate by exploiting the bacterial cell's cellular functions, and then burst out by means of cell lysis (Figure 1) (Buckling & Rainey 2002). In order to cope with this continuous threat of infection, bacteria have developed various defence mechanisms (Figure 2), all of which can be categorised into extracellular and intracellular defences. Extracellular defences enable the bacterium to inhibit phage adsorption and block their uptake via cell surface modifications by losing, mutating or masking phage receptors. Intracellular defences, on the other hand, will cleave the DNA of these invaders by the means of either the restriction-modification (R-M) system or the CRISPR-Cas (Clustered regularly interspaced short palindromic repeats – CRISPR associated) system once phages manage to inject their genome into a bacterial cell. Alternatively, bacterial cells can act altruistically, through abortive infection, where cell death results in infection failure, and thus prevents phage proliferation and subsequent spread through the host population.

The evolution of these bacterial defence mechanisms can be a slow process. For example, the R-M system, containing the enzymes methyltransferase and restriction endonuclease, will target and modify foreign invader DNA within the bacterial cell. As

modification of foreign DNA requires specific point mutations in the targeted sequence, this process is often slow. The release and subsequent re-infection of the same invader will then be subdued as the restriction endonuclease will cleave the targeted sequence (Westra *et al.* 2012).

There are, however, two defence mechanisms that adapt rapidly to novel phages and can be studied in real-time laboratory evolution experiments. Surface modification (*sm*) is quick and provides bacterial hosts with phage immunity by altering the cell surface receptors. The other is the CRISPR-Cas system which defends the host by integrating invader DNA into its own genome, allowing the host to recognise an invader upon infection.

This thesis endeavours to understand the ecological factors that drive the evolution of *sm* and CRISPR-Cas mediated resistance of the pathogenic bacteria *Pseudomonas aeruginosa* strain PA14 to its bacteriophage parasites. The studied bacterium has a type IF CRISPR-Cas system that consists of six cas genes that are flanked by two CRISPR arrays (Figure 3). Resource limited conditions have previously shown the evolution of *P.aeruginosa* PA14 CRISPR-Cas immunity against phage DMS3vir (Westra *et al.* 2015), a mu-like phage that infects by binding to the bacterial pilus (Cady & O'Toole 2011). I use the experimental system of *P.aeruginosa* and its bacteriophage parasites to understand whether the CRISPR-Cas evolution observed for DMS3vir by the Westra *et al.* (2015) study is general across bacteriophage infections (Chapter 1). Following this, I look at the effects of multiple bacteriophage exposure on the evolution of defence mechanisms (Chapter 2). I then look at the pre-requisites of the adaptive function of CRISPR-Cas system in a novel system (Chapter 3) and a multiple bacteriophage system (Chapter 4). Finally, the co-evolutionary dynamics associated with CRISPR-Cas are explored in Chapter 5.

The history, molecular mechanism and applications of the CRISPR-Cas system

History

As an adaptive immune system, CRISPR-Cas provides acquired resistance to phages and plasmids. The CRISPR-Cas system itself was first discovered in 1987 by Ishino and colleagues, when localised repeat sequences were found within the genome of *Escherichia coli* (Ishino *et al.* 1987). These repeat sequences were shown to be adjacent to variable sequences, known today as spacers. However, it was not until 2005 that these spacer sequences were shown to be derived from foreign genetic elements (Bolotin *et al.,* Mojica *et al.* & Pourcel *et al.* 2005). Later, in 2007, Barangou and colleagues demonstrated, in *Streptococcus thermophilus*, that spaces are acquired in response to phage infection thereby providing host immunity (Barrangou *et al.* 2007). Their study demonstrated that, by removing resistance-conferring acquired spacers, once resistant bacteria became phage-sensitive. Then, by inserting the phage resistant spacer into the CRISPR array of sensitive bacteria, these sensitive bacteria in turn became resistant. This was the first demonstration of CRISPR-Cas working as an immune defence system. Furthermore, as past phage infections are recorded in the form of spacers, these sequences provide a molecular memory of past bacteriophage infections, where unique spacer-phage recognitions allow the host to defend itself upon phage re-infections (Datsenko *et al.* 2012, Vale 2010).

Mechanism

Our understanding of the CRISPR-Cas system improves as research is pinning down the processes involved in spacer incorporation. CRISPR arrays (repeat-spacer units), along with Cas proteins, form the adaptive immune system, known as CRISPR-Cas, and works as follows: 1) Upon viral infection, the bacterial Cas complex identifies alien DNA. 2) It will then incorporate, at the leader end, a unique repeat-spacer unit to the CRISPR locus (Figure 4). The leader is a sequence located on the 5' end of most CRISPR loci and can be considered as a recognition sequence because new repeat-spacer units are often added on adjacently (Sorek *et al.* 2008). 3) The novel repeat-spacer unit will then be transcribed into pre-crRNA and processed into mature crRNAs (Brouns *et al.* 2008) by Cas proteins, which use

these mature crRNAs as guides to detect (Jore *et al.* 2011) and cleave complementary invading nucleic acid (Garneau *et al.* 2010), which results in the failed infection and subsequent clearance of the parasite.

Applications

The possible benefits arising from this adaptive immune system have generated research into its application in 1) the development of antimicrobials, where the use of Cas nucleases can target and reprogram antibiotic resistance genes in bacterial populations to prevent the spread of antibiotic resistant individuals (Bikard *et al.* 2014), 2) uses in industry, whereby genetically engineered bacteriophage resistant mutants are able to provide a starter bacterial population with increased immunity against known phage (Sturino & Klaenhammer 2006) and 3) uses in genome editing, where a modified CRISPR-Cas system can be adapted to adjust or repair an organism's genome (Hwang *et al.* 2013).
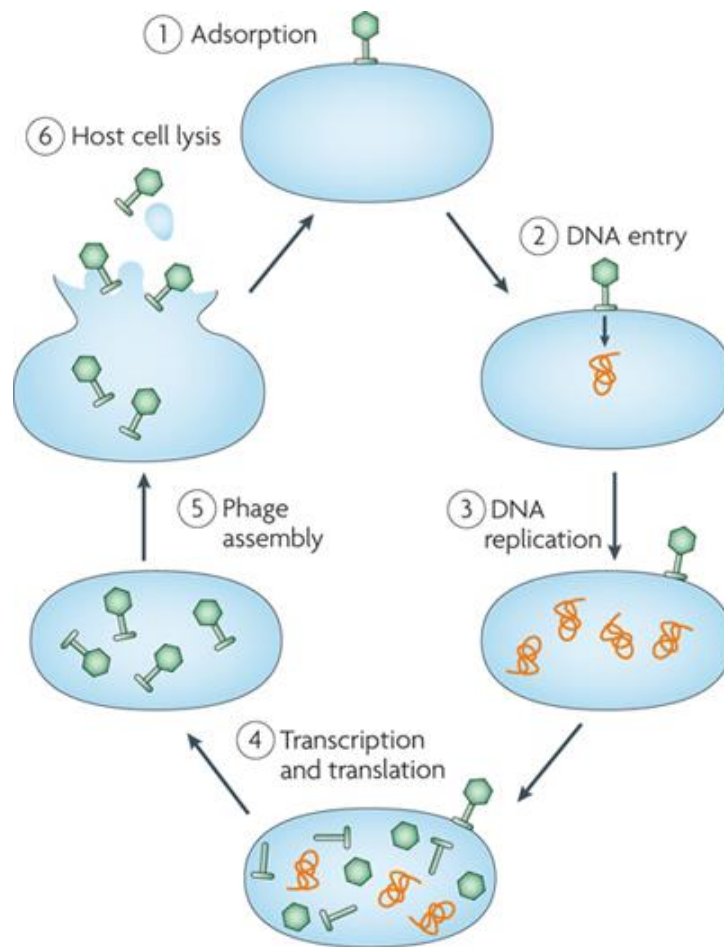
Nature Reviews | Microbiology

Figure 1: An overview of the bacteriophage lytic cycle. Image taken from Labrie *et al.* (2010) **1)** Infection is initiated by phage adsorption through a known receptor on the host cell wall. **2)** Once successful attachment has occurred, phage DNA enters the cell. **3)** Phage DNA then replicates inside the cell. **4)** Phage then exploits bacterial cell mechanisms in order for its DNA to be transcribed and translated into new progeny. **5)** After the successful transcription and translation of phage DNA, new phage particles assemble. **6)** Phages burst from the bacterial cell, by means of lysis, where the infection process of a new bacterial cell can take place.
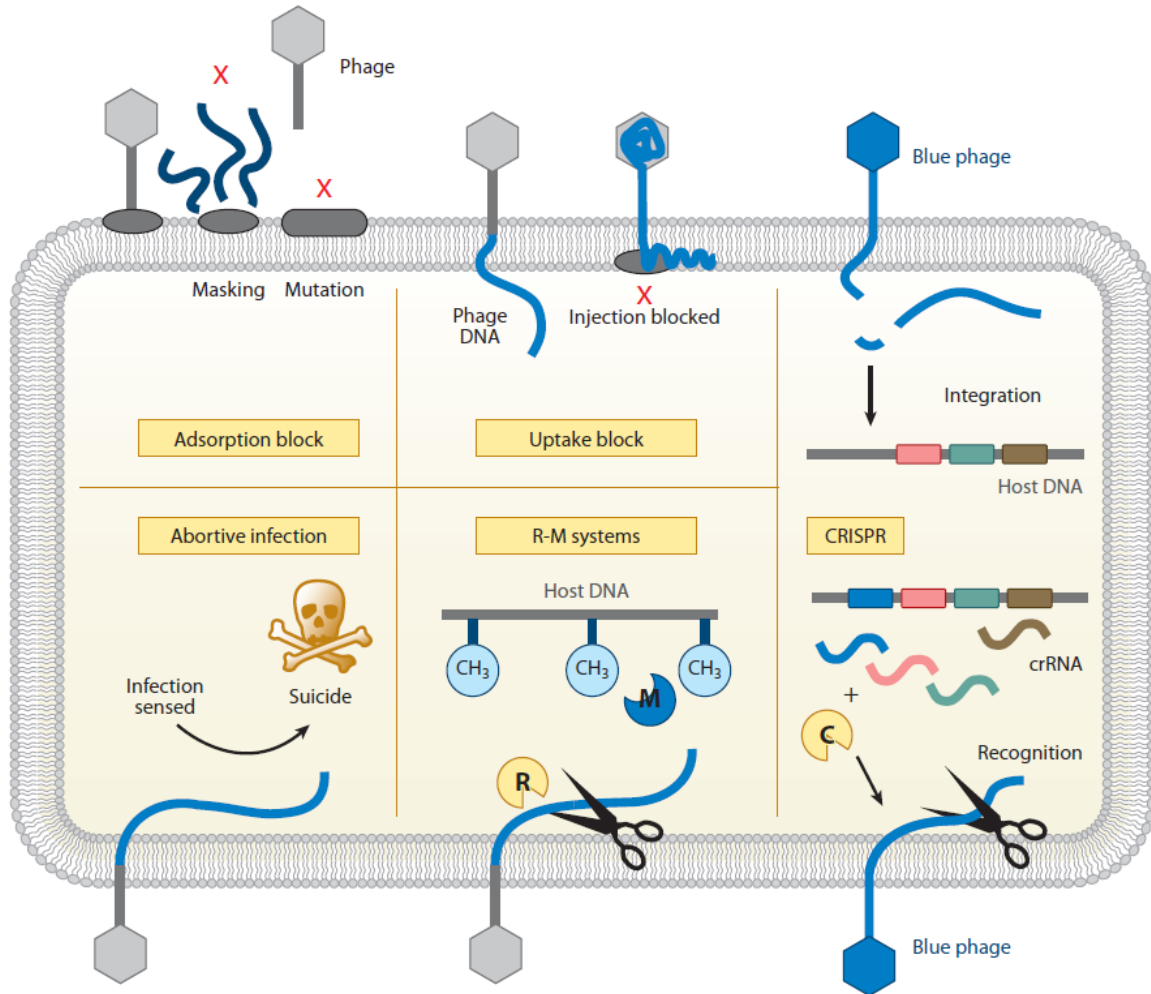
Figure 2: Diagram of bacterial defence systems. Image taken from Westra *et al.* (2012). From left to right; Phage adsorption or DNA injection may be blocked by masking or mutation of surface receptors. The self-sacrifice altruistic behaviour of abortive infections involves cell death upon infection. Phage injection may also be blocked by surface receptors (Uptake block). Restriction modification (R-M) systems will modify foreign DNA with the enzyme methyltransferase (M) and restriction endonuclease (R) will cleave the foreign DNA sequence. The CRISPR-Cas (Clustered Regularly interspaced palindromic repeats – CRISPR associated) system integrates foreign DNA (Blue phage) into the host genome and as a consequence, is recognised and cleared from the host.

Figure 3: Diagram of *Pseudomonas aeruginosa* PA14 CRISPR-Cas locus. The defence system contains two CRISPR loci, where CRISPR 1 contains 14 spacers and CRISPR 2 contains 21 spacers. There are six distinct Cas genes, which are located between loci, and are termed cas1, cas3, csy1, csy2, csy3, and cas6f. Image taken from Westra *et al.* (2015).
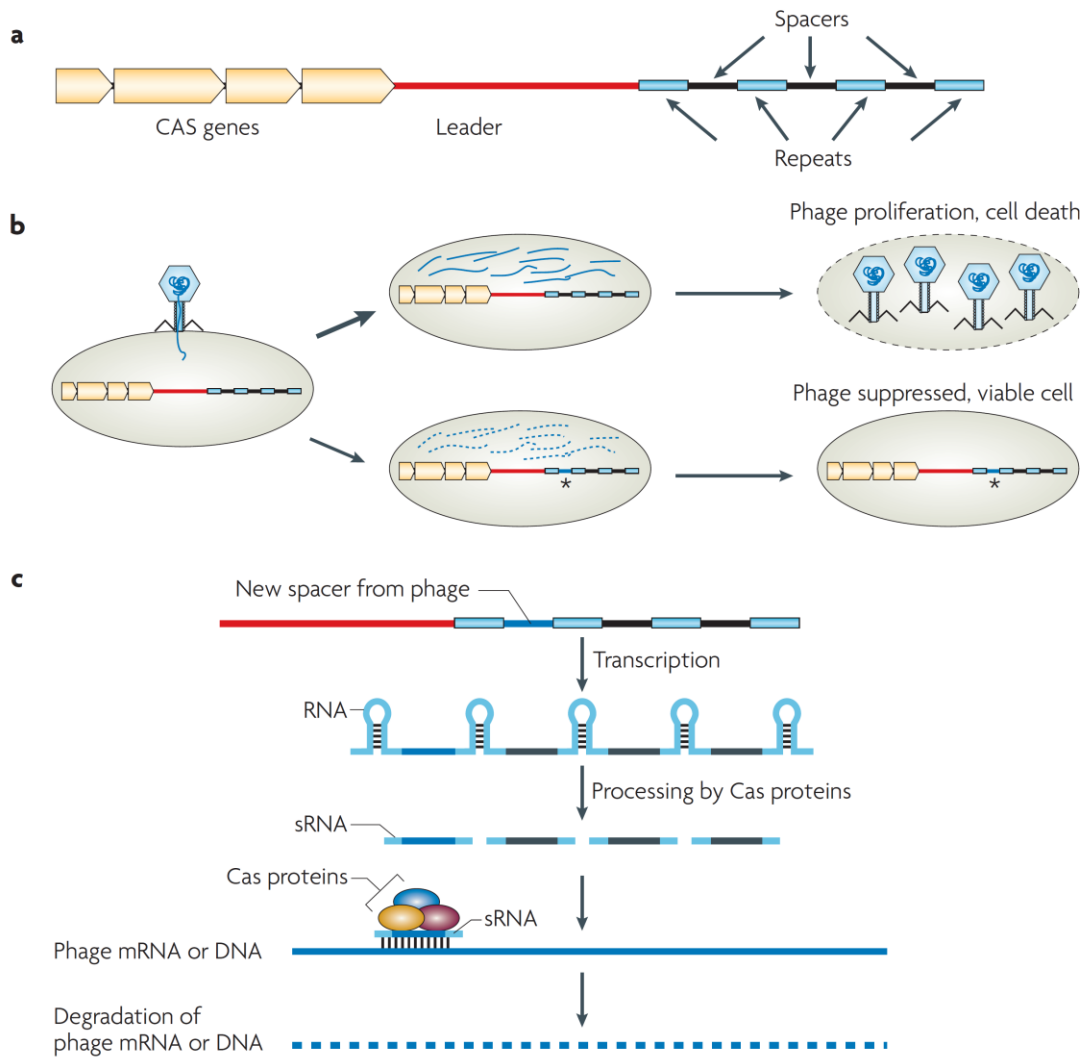
Figure 4: Mechanistic overview of the process of the CRISPR-Cas system. Modified from Sorek *et al.* (2008). **a)** Simplified structure of a CRISPR locus. **b)** Diagram of the outcomes of phage attack. Once successful attachment has occurred, the phage is able to inject itself into the cell. Phage particles then proliferate. In one instance, cell death occurs, in the other, bacteria integrate phage-derived spacers (marked by an asterisk) into its genome and survive. **c)** CRISPR mechanism overview. Once phage genetic information has been incorporated, in the form of a spacer, into the bacterial genome, the repeat-spacer unit is transcribed into RNA. Cas proteins, recognising the repeat sequences, process the RNA into small RNAs (sRNAs). Phage degradation and removal can then occur as sRNAs and Cas proteins base pair to phage mRNA.

Chapter 1

How general is the evolution of CRISPR-Cas resistance across single bacteriophage infections of the bacteria Pseudomonas aeruginosa?

Abstract

The pathogenic bacteria *Pseudomonas aeruginosa* strain PA14 can evolve resistance to bacteriophage (phage) by surface modification (*sm*) or by using the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated) adaptive immune system. In a study with *P. aeruginosa* and its phage DMS3vir, it was reported that CRISPR-Cas immunity evolves in nutrient limited conditions and *sm* in nutrient rich conditions, due to associated inducible and constitutive fitness costs. Here I investigate the generality of the evolution of the CRISPR-Cas defensive response in nutrient limited conditions by using a diverse phage collection. In contrast to results with phage DMS3vir, I found that almost all phages trigger the evolution of *sm* immunity. Interestingly, the relative benefits of CRISPR-Cas differed between phages that target different receptors. However, CRISPR-Cas failed to evolve even when it provided a fitness benefit to *sm*. This suggests that factors other than fitness costs and benefits determine the evolution of adaptive immunity in bacteria.

1. Introduction

The impact of parasites on host evolution can be affected by whether or not there is single or multiple parasite species interacting with the host. Indeed, in a snail - trematode system where the snail hosts can select to reproduce sexually or asexually, different parasitic trematode infections were shown to cause variation in the frequency of sexual individuals of host populations (King & Lively 2009). In three-spined sticklebacks (*Gasterosteus aculeatus*), the diversity of the major histocompatibility complex (involved in resistance) varied between host populations of different habitats as the result of simultaneous infections made by different combinations of parasite species (Wegner *et al.* 2003). In another system, ants

infected by multiple parasites have developed a hierarchical host defence strategy where each individual parasite was dealt with separately depending on cost to host fitness (von Beeren *et al.* 2011). Thus, the evolution of host resistance varies in different host-parasite systems and can depend on single or multiple parasite infections.

Phage infections are ubiquitous in nature, and consequently, cause bacterial host defence strategies to evolve. The most common strategies are; surface modification (*sm*), which involves the loss, masking, or mutation of cell surface receptors; and CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated) which provides host resistance by integrating phage derived sequences, termed spacers, into the host genome (van der Oost *et al.* 2014, Levin *et al.* 2013, Swarts *et al* 2012). The homologous nature of these spacer sequences to phage DNA enables the bacteria to quickly recognise and clear phage infection. In response, phages can escape CRISPR-Cas by point mutation in the cognate sequence (Datsenko *et al.* 2012). The adaptive process of the CRISPR-Cas system allows immunity to be continuously renewed and can therefore be used as a model system to study the evolution of resistance in host-parasite dynamics.

In a recent study by Westra *et al.* (2015), it was shown that resource availability greatly influenced the defence mechanism evolved upon parasite exposure. It was demonstrated that, in resource abundant environments, bacterial immunity was favoured by means of *sm*. By contrast, in resource scarce environments, bacterial immunity was achieved through the CRISPR-Cas system. It was hypothesized that the different environmental conditions caused different infection risks. Reduced bacterial growth, caused by low resources, meant that phage infection risk was lower as host-phage encounters were fewer. The pressure of varying infection risk was thus believed to affect the relative investment in either *sm*-mediated resistance or CRISPR-Cas mediated resistance. The results of the study conducted by Westra *et al.* (2015) showed clear host evolution by means of CRISPR-Cas resistance in the experimental system of *Pseudomonas aeruginosa* and its phage DMS3vir. However, this study was performed using the limited range of one phage species.

Here I examine the generality of this observation by challenging *P. aeruginosa* with other phage species and examine the evolution of *sm* and CRISPR-Cas defences in the bacterial host.

2. Materials and Methods

2.1 Bacterial strains, phages and experimental conditions

Bacteria

Pseudomonas aeruginosa PA14 wild type (WT) and PA14 *csy3::LacZ* (*LacZ*) were supplied by the O'Toole lab (Cady & O'Toole 2011) at the Geisel School of Medicine (Hanover, New Hampshire, USA). Strains were grown at 37°C throughout experimental procedures. Two PA14 *LacZ* derived surface modification (*sm*) mutants, one with no pilus (*sm1*) and the other with modified LPS (Lipid polysaccharide) sites (*sm2*), were used in competition assays. These *sm* mutants were procured in evolution experiments with *LacZ* cells and phage DMS3vir (*sm1*) or phage LPB1 (*sm2*). The bacteriophage-insensitive mutant (BIM) BIM-4 was obtained through an evolution experiment with WT PA14 and phage DMS3vir, and contains one novel spacer targeting DMS3vir.

Bacterial growth media

Bacterial growth media consisted of M9 salts (60g of $Na_2HPO_4.7H_2O$, 30g of KH_2PO_4, 10g of NH_4Cl and 5g of NaCl) which were supplemented with 1mM of $MgSO_4$, 0.1 mM of $CaCl_2$ and 0.2% glucose. CRISPR mediated resistance has previously been shown to occur under these nutrient limiting conditions in response to phage DMS3vir and was thus chosen for this study (Westra et al. 2015). In order to isolate single colonies, bacterial cultures were plated onto LB (Luria-Bertani) 1.5 % agar (Miller, Molecular Genetics Powder, Fisher Scientific). The formula per Liter contained: 10g Tryptone, 5g Yeast Extract, 10g Sodium Chloride and 15g Agar.

Phages

Selections of lytic phages, that are either pilus or LPS specific, are listed in (Table 1). Pilus mediated phage infection was verified by pipetting selected phage onto ΔpilA (a pilus knockout bacterial strain) bacterial lawns.

Preparing phage stocks

Phage stock solutions were prepared by adding 10µL of 10^6 pfu (plaque forming units) /mL of desired phage to 300µL of overnight grown *LacZ* cells into 0.5% LB agar. Once mixed, the 0.5% LB agar supplemented with phage and bacteria, was poured onto prepared plates of 1.5% LB agar. Near confluent lysis (NCL) was determined from plates incubated overnight. Whereupon, plates were flooded with 10mL M9 salts and left at r.t.p (room temperature and pressure) for two hours to allow phage and bacteria to dissolve into the salt solution. The liquid mixture was then pipetted off of the plates into Falcon tubes. Falcon tubes were centrifuged at 35000 rpm (rotations per minute) for 10 minutes. Supernatant was transferred into new Falcon tubes, where a chloroform extraction, followed by further centrifugation, was conducted to kill any remaining bacterial cells. Phage titrations were performed as described below and stock solutions were stored at 4°C.

Phage titration

Chloroform extractions were performed on phage solutions, whereby 100µL chloroform (10%) was added to 900µL of each sample replicate. Phage titres were determined by serially diluting phage solutions in M9 salts. 5µL of virus at 10-fold dilutions were spotted onto 1.5% LB agar containing a bacterial lawn top layer. Bacterial lawns were made by mixing 300µL of *LacZ* cells (phage sensitive bacteria) with 9mL 0.5% LB agar layer. After overnight incubation at 37°C, plaques were counted to determine phage titre.

2.2 Streak assays

Upon experimental conclusions, replicate populations were diluted and plated onto 1.5% LB agar. A total of 16 single isolated colonies per replicate were selected and grown overnight in 0.2% glucose M9 media. Overnight cultures were streaked across a horizontal line of 25µL of a given phage at 1×10^9 pfu/mL. Resistance was determined when bacteria showed no growth inhibition, while sensitivity was determined as inhibition of growth across the line of phage.

2.3 Spacer acquisition

Once stocks of single colonies had been obtained, they were diluted 10-fold into sterile H_2O. As *P. aeruginosa* PA14 has two CRISPR loci, both sites were amplified by PCR in order to detect spacer acquisition. The master mix contained 5µL of DreamTaq Green (2x) (Thermo Scientific), 0.1µL of 100pmol/µL forward primer, 0.1µL of 100pmol/µL reverse primer, 3.8µL of H_2O and 1µL of 10-fold diluted bacterial single colony per reaction. The CRISPR1 locus primers were as follows; forward CTAAGCCTTGTACGAAGTCTC, reverse CGCCGAAGGCCAGCGCGCCGGTG. CRISPR2 locus contained a forward primer of GCCGTCCAGAAGTCACCACCCG and a reverse primer of TCAGCAAGTTACGAGACCTCG. The product amplification process followed a PCR program of a single cycle of 10 minutes at 95°C, 35 cycles with 30 seconds at 95°C, 30 seconds at 50°C and 45 seconds at 72°C followed by a final cycle of 7 minutes at 72°C. Products were then run through a 2% Agarose gel, comprised of TAE (40mM Tris-Acetate and 1mM EDTA) solution (Fisher Scientific) and containing RedSafe™ Nucleic Acid Staining solution (20 000x) (iNtRon biotechnology). Spacers were counted by observing the amplicon size in comparison to 1kb Hyperladder (Bioline) and the original WT strain.

2.4 Evolution experiment

In order to investigate the evolution of CRISPR immunity to the collection of phages in Table 1, approximately 10^6 cfu/mL *P. aeruginosa* PA14 (WT) were infected,

separately, with 10^4 pfu/mL of phages DMS3vir, 1214, 109, 68, M4, LPB1, LMA2 and LUZ24 at time point 0. In addition, daily infections of 10^4 pfu/mL, were set up in parallel to test which host defence mechanism would evolve in continuous parasite exposure. Each experiment was performed in 6 replicates and bacteria were grown at 37°C in shaking conditions of 180rpm. Cultures were transferred on a daily basis, into fresh medium at a proportion of 1:100 for a total of 19 days. The experiment was conducted over this set amount of time with the hypothesis that resistance may develop slowly with novel phages. Single colonies were isolated at 19 days post infection (dpi) to establish phage resistance and to determine spacer incorporation. Colonies were picked at random. In addition, morphology of individual colonies were analysed since surface modification mediated resistance against pilus-specific phage is associated with a round and smooth colony morphology, whereas sensitive cells or CRISPR-Cas mediated resistance show spreading morphologies. In addition, chloroform extractions were conducted to deduce final phage concentrations in all replicates.

2.5 Competition assays

Competition assays were conducted to measure the fitness associated with CRISPR immunity relative to surface modification mutation. In order to test this, bacteriophage-insensitive mutants (BIMs; bacteria that have acquired a spacer that provides CRISPR immunity) and the surface mutants (*sm1* and *sm2*) were independently grown overnight in 10mL 0.2% glucose M9 media. Overnight cultures were then combined at a 1:1 ratio by mixing 500µl of each BIM and *sm* mutant culture, where BIM-4 and BIM-1214 were mixed, separately, with *sm1* and BIM-LPB1 with *sm2*. Mixed cultures were inoculated, in replicates of 6, into microcosms containing 6mL of 0.2% glucose M9 media and allowed to incubate for 24h, whereupon cultures were transferred into fresh media. In addition, competitions between BIM-LPB1 and *sm2* were performed in glucose concentrations of 0.02% and 0.002% to test the effect of lower resource abundance on the relative fitness of CRISPR immunity. Competitions were then tested in the presence of phage, where 10^6 pfu/mL phage was added at the start of the competition to all 6 replicates of mixed cultures. Samples were taken every 24h, diluted and plated onto 1.5% LB

agar supplemented with 50µg/mL of X-gal. Plates were incubated for 48h to determine the relative frequencies of the competing strains at each time point. The blue colour of *sm* colonies, resulting from the *LacZ* insertion into the ancestral strain, allowed them to be distinguished from the BIM colonies (white). Colony frequencies were counted and used to calculate the relative fitness using the equation:

$$Rf = \frac{(fs\ X\ at\ t = x) * (1 - (fs\ X\ at\ t = 0))}{(fs\ X\ at\ t = 0) * (1 - (fs\ X\ at\ t = x))}$$

Equation describing the Relative fitness (Rf) (Ross-Gillespie *et al.* 2007), where fs is the fraction of strain X, X is the fraction of CRISPR colonies to *sm* colonies, and t is the time point. When the relative fitness =1, fitness of CRISPR = fitness of *sm*. CRISPR provides a fitness benefit when the relative fitness > 1, and a cost when the relative fitness < 1.

2.6 Statistical analysis

Data were analysed using SPSS software (IBM Corp. Released 2012. IBM SPSS statistics for Windows, Version 21.0. Armonk, NY, USA) and Sigma-plot (SigmaPlot version 12.5, Systat Software, Inc., San Jose, California, USA) was used for the creation of graphs.

As the data did not meet the assumptions of a parametric test, non-parametric tests were used to test for significance. To assess whether the proportion of resistant and sensitive mutants between single phage treatments, at 19dpi, were significantly different, Kruskal-Wallis tests were used. A Wilcoxon signed-rank test was then used to assess whether the proportions of CRISPR and *sm* mediated resistance differed between phage treatments.

The relative fitness results were analysed to determine whether the relative fitness of different BIM mutants were significantly different and whether the relative fitness of CRISPR mutants differed between mutants with LPS or pilus binding derived phage DNA. I used parametric t-tests or one-way ANOVA where model assumptions could be met, otherwise non-parametric equivalents were used. To test for significance, all analyses applied α = 0.05.

Table 1: Phages used in experimental procedures

| Name	Source	Reference	Morphology	Morphotype species	Receptor
DMS3vir	Patient at Dartmouth Hitchcock Medical Center, Vermont, USA, 2004	Cady & O'Toole (2011)	Siphoviridae	D3112	Pilus
1214	Sewage, Lindberg collection, Texas, USA, 1964	Lindberg & Latta (1974)	Myoviridae A1	PB-1	Pilus
109	Sewage, Lindberg collection, Texas, USA, 1964	Lindberg & Latta (1974)	Myoviridae A1	PB-1	Pilus
68	Sewage, Lindberg collection, Texas, USA, 1964	Lindberg & Latta (1974)	Myoviridae A1	φkz	Pilus
M4	Sewage, Lindberg collection, Texas, USA, 1964	Lindberg & Latta (1974)	Myoviridae A1	Li-24	Pilus
LPB1	Hospital sewage, Belgium, 2006	Ceyssens *et al.* (2009)	Siphoviridae	D3112	LPS
LMA2	River, Maastricht, Holland, 2007	Ceyssens *et al.* (2009)	Myoviridae	PB-1	LPS
LUZ24	Hospital sewage, Leiden, Holland 2004	Ceyssens *et al.* (2009)	Podoviridae	LUZ24	LPS

3. Results

3.1 Evolution experiments

When exposing PA14 to phages described in Table 1, both with single infections at time point 0 and with daily infections, I found that resistance evolved in all phage treatments (Figure 1 and Figure 2). However, CRISPR-mediated immunity was limited in response to any of the phages tested. Instead, bacteria evolved almost exclusively surface modification immunity. Statistical analysis showed that there was no significant difference in CRISPR-mediated resistance between phage treatments with single infections (Kruskal-Wallis: $X^2 = 9.182$, df = 7, P = 0.240, Figure 1) or daily infections (Kruskal-Wallis: $X^2 = 13.3$, df = 7, P = 0.065, Figure 2). Thus, the previously observed high level of CRISPR immunity against DMS3vir (Westra et al. 2015, Figure 1 Chapter 3) is not a general observation across phage infecting *P. aeruginosa* strain PA14.

A Wilcoxon signed-rank test then assessed the proportion of resistance, irrespective of defence mechanism, to different phage treatments. Results showed that the total proportion of resistance (i.e. CRISPR and *sm*-mediated combined) did differ between different novel phages (Wilcoxon signed-rank test: T = 3, n = 48, N = 48, P<0.001), showing that, under some phage conditions, bacterial populations contain more sensitive individuals than others.

With a limited number of CRISPR clones across all single phage treatments, phage extractions were conducted at 19dpi to determine whether phage had persisted or ceased. Phage presence was seen in treatments with phages 1214 (3×10^8 pfu/mL), 109 (6×10^8 pfu/mL), 68 (9×10^6 pfu/mL), M4 (1×10^8 pfu/mL), LPB1 (1×10^9 pfu/mL) and LUZ24 (4×10^9), confirming that with populations containing high levels of *sm* mutants, phage is able to persist. However, phage DMS3vir and LMA2 had been driven extinct at 19dpi.
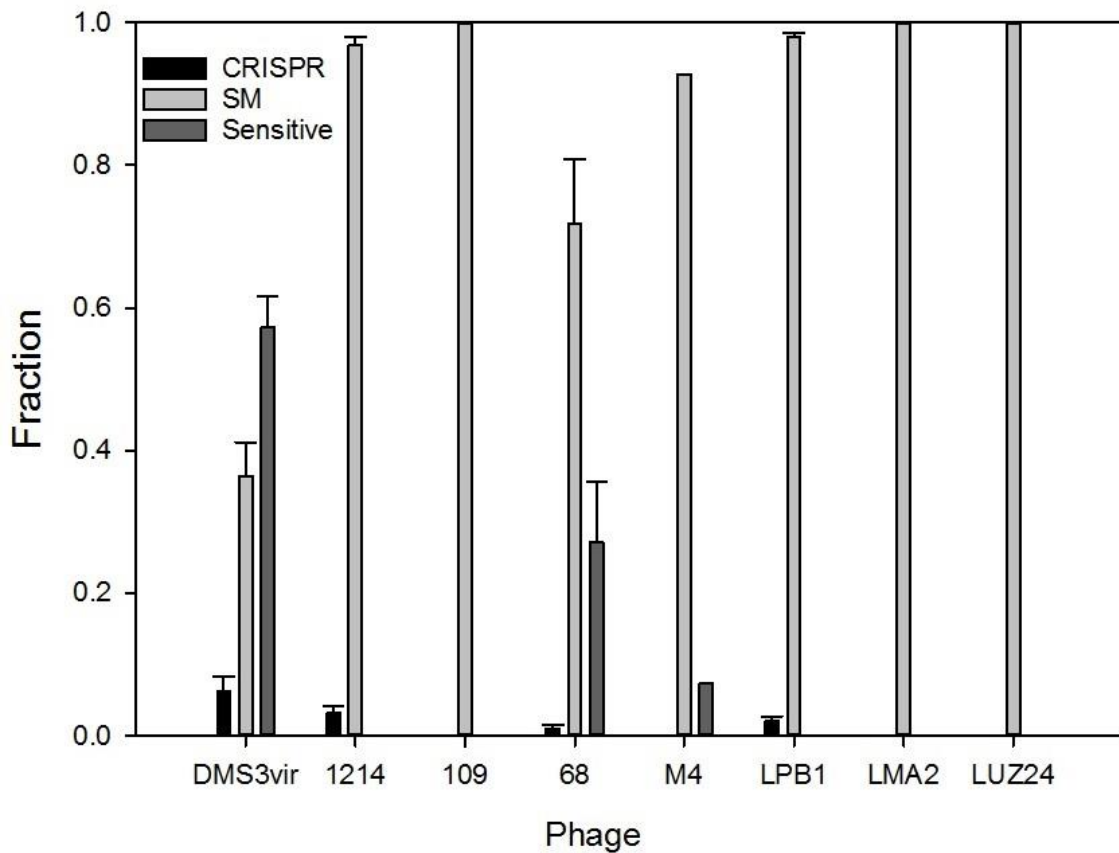
Figure 1: Average fraction of CRISPR-Cas resistance, *sm*-mediated resistance and sensitive cells in different phage treatments at 19 dpi (days post infection). Initial WT infection consisted of 10^4 pfu/mL phage. Error bars are measured as 95% confidence intervals.
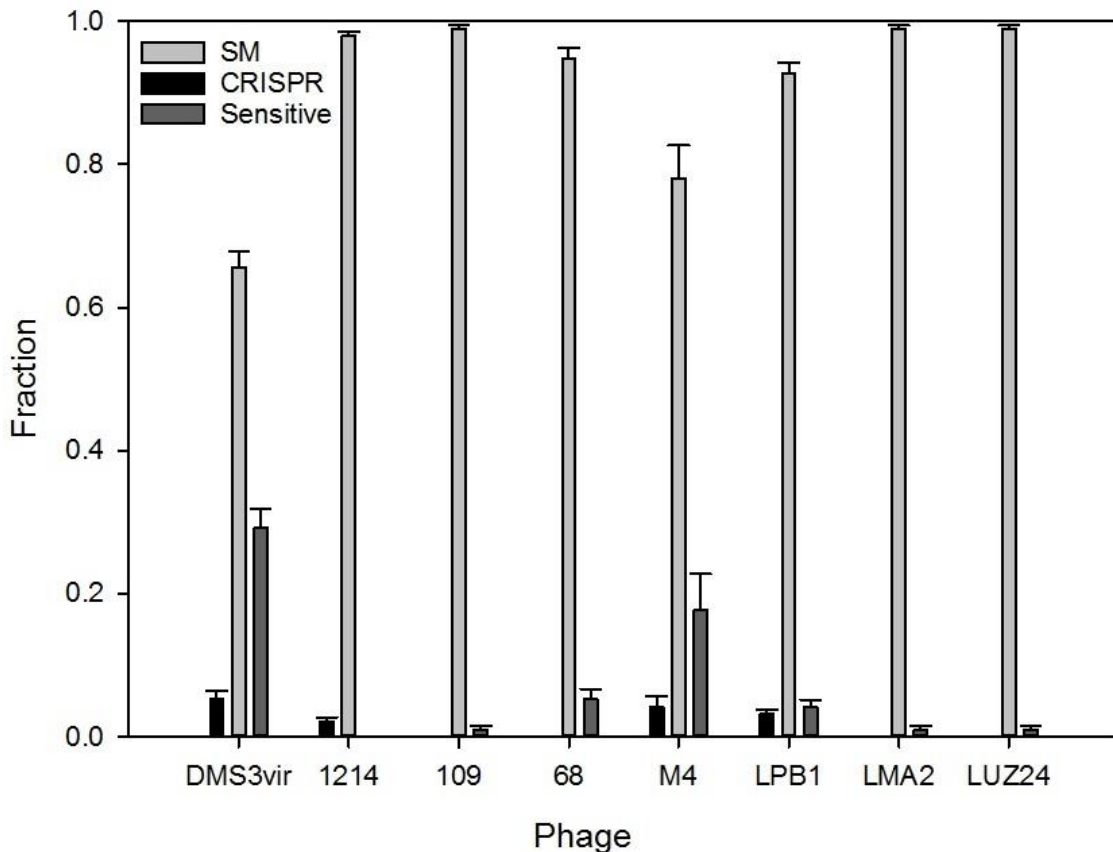
Figure 2: Average fraction of CRISPR-Cas mediated resistance, *sm*-mediated resistance and sensitive cells in different phage treatments under daily phage infections at 19 dpi (days post infection). Phage was added on a daily basis, where each infection consisted of 10^4 pfu/mL phage. Error bars are measured as 95% confidence intervals.

3.3 Competition assays

It was previously reported that CRISPR immunity provides a clear fitness benefit over *sm* in the presence of low titres of phage DMS3vir (Westra *et al.* 2015). To understand why CRISPR evolution was limited in response to other phages, I examined the fitness associated with CRISPR relative to *sm* in the context of CRISPR clones acquired from the above mentioned evolution experiments with other phage species. To this end, competitions between CRISPR and *sm* mutants were carried out. A total of three CRISPR mutants were selected (BIM-4, BIM-1214 and BIM-LPB1) to compete with *sm1* (lacking a pilus) or *sm2* (modified LPS site)

mutants. To test the constitutive fitness costs associated with *sm*-mediated resistance, competitions were performed in the absence of phage (Figure 3A). Inducible fitness costs associated with CRISPR-mediated resistance were tested for in competitions in the presence of phage (Figure 3B).

In the absence of phage (Figure 3A), the relative fitness average was >1 for BIM-4 (2.29 ± 95% CI [1.2]) and BIM-1214 (3.43 ± 95% CI [1.0]). This indicates that CRISPR has a higher relative fitness to *sm1* mutant in the absence of phage, which is in agreement with a constitutive cost associated with *sm* (Buckling & Brockhurst 2012, Bohannan & Lenski 2000, Westra *et al.* 2015). By contrast, the competition assay between the CRISPR mutant with a spacer obtained from a LPS binding phage (BIM-LPB1) and the *sm2* mutant, showed a relative fitness average < 1 (0.85 ± 95% CI [0.13]) in the absence of phage (Figure 3A). The LPS modified *sm* mutant (*sm2*), thus provided a higher fitness benefit compared to the CRISPR mutant (BIM-LPB1). Statistical analyses confirmed that the relative fitnesses of CRISPR mutants, in the absence of phage, were significantly different to *sm* mutants in all competition assays (One-sample t-test: BIM-4: $t_5 = 9.998$, P < 0.001, BIM-1214: $t_5 = 16.445$, P < 0.001, BIM-LPB1: $t_5 = 26.376$, P < 0.001).

In contrast to Westra *et al.* (2015), there was no significant difference between the relative fitness of CRISPR to *sm*1 in the presence of phage DMS3vir (relative fitness average = 1 for BIM-4: 1.01 ± 95% CI [0.38], Figure 3B). Interestingly, CRISPR mutants carrying spacers targeting the pilus-specific phage 1214 were fitter than the selected surface mutant (relative fitness average > 1 for BIM-1214: 1.9404 ± 95% CI [0.12], Figure 3B). This demonstrates that the cost of CRISPR-mediated resistance, in the presence of phage, is less costly than *sm*-mediated resistance. Again, the CRISPR mutant with a spacer targeting the LPS-specific phage LPB1, had a lower fitness than *sm2* in the presence of phage (Figure 3B), with a relative fitness average of < 1 (BIM-LPB1 0.756 ± 95% CI [0.14]). In this study, surface modification to the LPS binding site used by phage LPB1 is therefore less costly than initiating an adaptive immune response in the presence of the same phage. These results are supported by statistical analysis, where the difference in relative fitnesses of

CRISPR to *sm* were significantly different (One-sample t-test: BIM-4: $t_5 = 4.316$, P = 0.008, BIM-1214: $t_5 = 31.716$, P < 0.001, BIM-LPB1: $t_5 = 10.282$, P < 0.001). This explains why CRISPR immunity fails to evolve in response to the LPS binding phage LPB1.

Given that different phage derived BIM mutants were used to measure relative fitnesses, the differences between BIM mutants were compared to see whether the relative advantage of CRISPR over *sm* differed between phages. An ANOVA showed that there was no difference in CRISPR fitness between treatments in the absence (one-way ANOVA: $F_{2,15} = 3.369$, P = 0.062) of phage but there was a difference in presence (one-way ANOVA: $F_{2,15} = 27.597$, P < 0.001) of phage. Thus there was limited constitutive costs in the absence of phage and inducible fitness costs in the precence of phage.

As the competition assays between *sm2* and BIM-LPB1, both in the absence and presence of phage, showed a CRISPR relative fitness average <1, competitions were also performed in media of lower glucose content (0.02% and 0.002%). This was to test whether the relative fitness of CRISPR depends on resource concentrations, i.e. whether the cost of *sm* is increased under further nutrient limiting conditions. This hypothesis is based on previously observed resource-dependent costs of resistance in other organisms (Boots 2011) as well as bacteria (Lopez-Pascua & Buckling 2008). However, the relative fitness average remained <1 (Figure 4), again in 0.2% glucose (0.954 ± 95% CI [0.04]), and also in both 0.02% glucose (0.911 ± 95% CI [0.022]) and 0.002% (0.772 ± 95% CI [0.05]) glucose media. This shows that, even in reduced resource environments, a CRISPR with a spacer targeting a LPS-specific phage does not confer a higher relative fitness to a LPS *sm* mutant. Relative fitness's were confirmed to be significantly different between nutrient limiting conditions (One-sample t-test: 0.2% glucose: $t_5 = 11.1$, P < 0.001, 0.02% glucose: $t_5 = 19.81$, P < 0.001, 0.002% glucose: $t_5 = 7.53$, P = 0.001), where the relative fitness of CRISPR is seen to decrease with decreasing glucose levels. However, there was no significant difference in CRISPR fitness between treatments (one-way ANOVA: $F_{2,15} = 1.36$, P = 0.287). Thus, this shows that the relative fitness

of CRISPR immunity relative to a LPS *sm* mutant is not only consistently lower in different nutrient limiting environments, but also decreasing with decreasing nutrient availability.
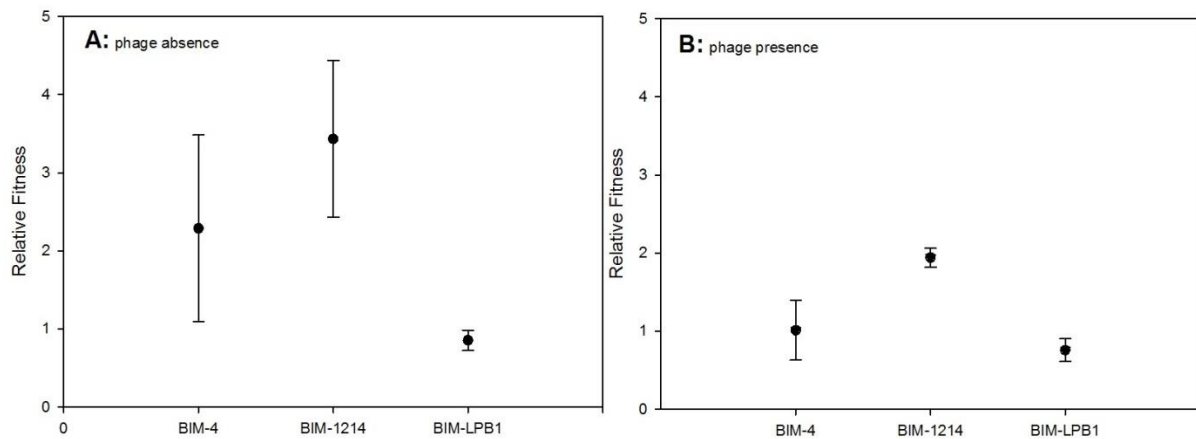
Figure 3: Relative fitness of CRISPR versus *sm*-mediated resistance. Competitions were held in the absence (**A**) and presence (**B**) of phage. The competitions were; DMS3vir resistant CRISPR mutant (BIM-4) with *sm1* mutant (lacking a pilus), phage 1214 resistant CRISPR mutant (BIM-1214) with *sm1* mutant and LPB1 resistant CRISPR mutant (BIM-LPB1) with *sm2* mutant (with modified LPS binding sites). Final phage titrations in competitions experiments in the presence of phage were; 1.6×10^{10} pfu/mL (BIM4), 6×10^{10} pfu/mL (BIM-1214) and 1×10^{10} pfu/mL (BIM-LPB1). Error bars show 95% confidence intervals.
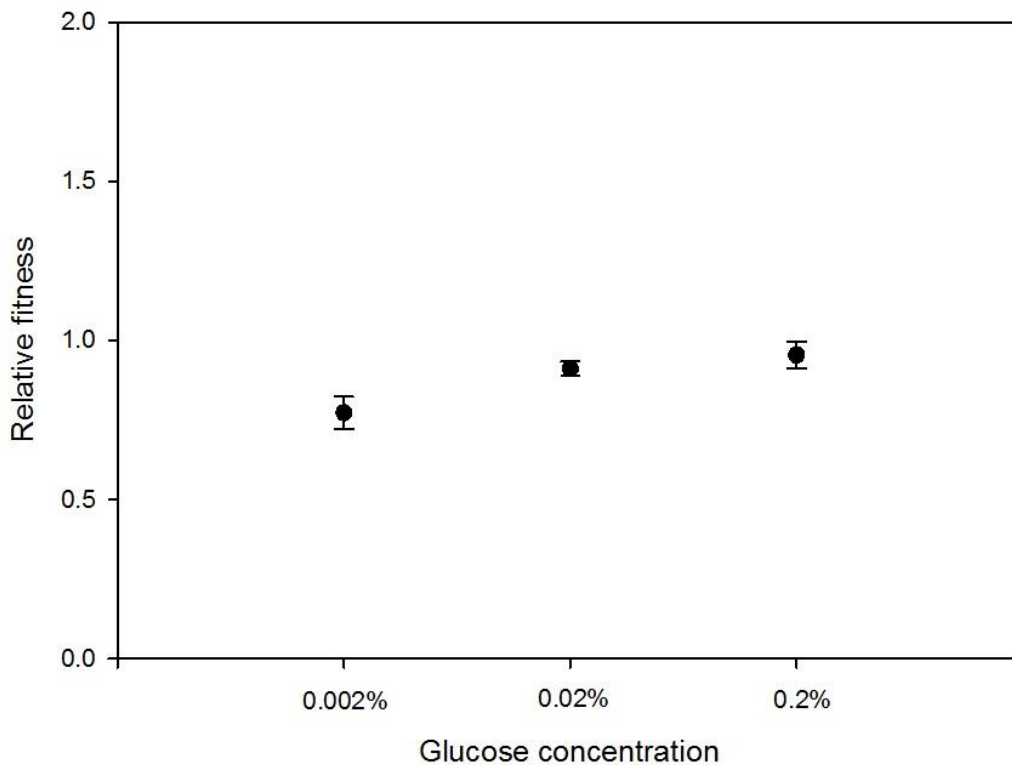
Figure 4: Competition assay of BIM-LPB1 in different nutrient limiting conditions. The graph shows the relative fitness of a LPS phage derived CRISPR mutant when competed with a LPS mutant. Relative fitness's of BIM-LPB1 were all <1. Error bars are shown as 95% confidence intervals.

4. Discussion

Here I show how novel phages do not necessarily induce CRISPR-Cas mediated resistance in their sensitive hosts, even when CRISPR generally provides higher fitness than the alternative immune strategy of *sm*. Single phages were used to infect the pathogenic bacteria *Pseudomonas aeruginosa* to test which defence mechanism, CRISPR-Cas or *sm*, would be elicited upon infection. In both cases of single (Figure 1) and continuous (Figure 2) infections, resistance was shown to be mainly expressed through cell surface modification.

It was curious that at 19dpi (days post infection) of phage DMS3vir, little CRISPR mediated resistance was observed as a previous study showed that DMS3vir infections of *P. aeruginosa* WT PA14 conveyed an almost 100% CRISPR mediated resistance at 3dpi (Westra *et al.* 2015, Figure 1 Chapter 3). In addition, phage removal from the environment had led to renewed DMS3vir sensitivity. If the presence of a phage derived spacer sequence in the host genome does not infer a fitness cost in the absence of phage, then CRISPR mutants should have been able to persist in the environment. As it is, they did not. Even though competition assays, with pilus mutants, demonstrated that CRISPR mutants inferred a greater relative fitness benefit than *sm*, in the absence of phage, the numbers of CRISPR mutants in these populations were low. It may be that, phage had already overcome host resistance. If a bacterial population evolved to contain only one or two CRISPR clones, counter-resistance by phage could easily be achieved by point mutations in the phage genome (Deveau *et al.* 2008). This would allow the phage to escape the response of the adaptive immune system and instead remove the limited number of CRISPR clones from the population. Another explanation is that, as phage were in some cases cleared from the environment the spacer sequences that recognised these phages were lost over time due to limited selection for resistance. This suggests that there is still a cost of CRISPR-Cas resistance in the absence of phages.

Phage managed to persist in six out of eight single phage infections, where complete phage extinction was observed for DMS3vir and LMA2. Phage extinctions are not uncommon and have been documented in experimental evolution experiments with *E.coli* and virulent T-phages (Lenski & Levin 1985, Bohannan & Lenski 2000). Phage extinction with concern to phage DMS3vir was not surprising as a new study has shown its early extinction is due to the generation of high levels of CRISPR diversity (Chapter 5). The rate of evolution between bacteria and phage can be seen as different as spacer acquisition by the CRISPR-Cas system is fast and point mutations in specific proto-spacer sequences in the phage are slow. This explains why bacterial populations can rapidly drive phage to extinction. On the other hand, as resistance was seen to be 100% *sm* mediated and no CRISPR-Cas mediated resistance was detected in the case of a LMA2 infection, it was surprising to see

phage clearance. As extinction has occurred, phage disappearance can be explained by the gradual dilution over time, as a result of experimental transfers.

It could also be explained by the degradation of the phages over time, which has been observed to occur in a different bacteriophage-host environment (Cairns *et al. 2009*). Another explanation, arising from deeper DNA sequence analysis, is that the CRISPR locus of *P. aeruginosa* carried a perfect match to phage LMA2. This means that the recognition, and possible resistance, was already in place in the bacterial genome prior to experiments carried out in this study. Therefore, as new spacers were not generated, and the WT strain is sensitive to this phage, it is likely that LMA2 carries a CRISPR inhibitor. It has recently been documented that some phage may have anti-CRISPR genes (or CRISPR inhibitors) (Bondy-Denomy *et al.* 2013), where these genes allow the phage to evade the CRISPR-Cas system. Thus, the inability of the bacteria's CRISPR-Cas system to incorporate new phage LMA2 spacers is why resistance was conveyed via surface modification.

Curiously, a CRISPR clone demonstrated a lower relative fitness to its competitor; the LPS surface mutant (*sm2*) in both the presence and absence of phage. This shows that the alterations to LPS sites, in these experiments, did not induce the same level of costs as seen with a pilus *sm*. The functions of the altered bacterial LPS sites are not presently known, which means that the costs associated with their alterations could not be fully understood. Also, this study was limited to testing the competition of only one CRISPR clone containing a LPS binding phage derived spacer, making it difficult to conclude that the modification of LPS binding sites are generally less costly than an adaptive immune response. However, the disadvantage of having CRISPR resistance could not have been due to differences in resource levels (Figure 4). This suggests that there are still other factors that are important in driving the evolution of a CRISPR-Cas adaptive immune response.

It is unclear as to why a CRISPR-Cas-mediated resistance was not prevalent across different phage species. With resistance through spacer incorporation being high in

DMS3vir infections of *P. aeruginosa* (Westra *et al.* 2015), it was surprising that different phage species did not reflect this observation. The concept of priming may be key to understanding these results. Priming relies on partial spacer complementarity to phage genomic sequences in order for increased spacer incorporation to occur (Datsenko *et al.* 2012). As priming is important with *P. aeruginosa* to phage DMS3 (Cady *et al.* 2012), the lack of complementarity of spacers to the novel phages used in this study may explain why limited spacer incorporation, and therefore lack of priming possibilities, was observed across phage infections.

Chapter 2

The effects of multiple bacteriophages on the evolution of resistance in the bacteria Pseudomonas aeruginosa.

Abstract

Two key mechanisms employed by prokaryotes for parasite defence are surface modification (*sm*) and the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated) system. CRISPR-Cas provides highly specific immunity, whereas *sm* immunity can either be very specific, with a subtle mutation to a receptor providing immunity to a single phage, or broad range where a drastic mutation (such as receptor loss) can provide immunity to a group of phages. As described in Chapter 1, *Pseudomonas aeruginosa* evolves CRISPR-Cas immunity primarily in response to phage DMS3vir, while other phage species in our collection mainly trigger *sm*-mediated immunity. Although it is important to study the effects of single phage species infections on the evolution of defence mechanisms, single infections may be rare in a natural setting. Here, I investigate how phage diversity might affect the evolution of bacterial defence mechanisms. Firstly, I mixed pilus-specific phages, including DMS3vir, where I found that mixed infections consistently trigger the evolution of *sm* immunity. Secondly, the exposure of bacteria to LPS-specific phage mixes, that all trigger highly specific *sm* (i.e. no initial cross resistance), resulted in broad range *sm* resistance against all phages. Collectively, these data show that phage diversity in the environment drives *sm* immunity over CRISPR-Cas.

1. Introduction

Hosts are under constant threat by parasite infection where an infection can drive the evolution of different resistance mechanisms (Brockhurst *et al.* 2005, Frank 1993). As such, the force of infection or different ecological factors will determine the benefit of one defence mechanisms over another. Thus, the optimum defence mechanism will depend on the type of infection(s), where parasite(s) cause either a general or

specific resistance response. Parasite diversity may also be instrumental in the evolution of different resistance mechanisms, however, little is known on how parasite diversity will affect the choice in elicited immune response.

Host resistance often carries a fitness cost (Koskella *et al.* 2011, Perron *et al.* 2007, Rigby *et al.* 2002). For example, bacterial cell surface modifications (*sm*) may be costly if unable to provide effective resistance to highly specific parasites (Koskella *et al.* 2011), novel parasites or if multiple modifications to surface structures are required (Bohannan *et al.* 1999, Levin & Bull 2004). If multiple structural alterations occur, the number of necessary specific resistances may result in an accumulative fitness cost as each specific resistance carries a cost (Koskella *et al.* 2011). Additionally, the alterations made in generating multiple structural modifications may be costly to the host continued in the absence of parasites. On the other hand, *sm*-mediated resistance may provide cross-resistance against phages targeting similar receptors, reducing the cost of acquiring the modification (Hall *et al.* 2012), and proving beneficial in parasite diverse environments where this defence provides general host immunity.

Adaptive immunity, such as the parasite-specific resistance provided by the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated) system, is another form of host defence and is cost free in the absence of parasites (Hamilton *et al.* 2008, Westra *et al.* 2015). Through the incorporation of unique parasite sequences, known as spacers, the adaptive function of the CRISPR-Cas system allows an organism to adapt to specific and multiple different parasites as resistance is continuously renewed (Barrangou & Marraffini 2014). Even though an adaptive immune system imposes a cost when activated (Vale *et al.* 2015, Westra *et al.* 2015), the host is saved from continued fitness costs inflicted from a parasitic attack upon clearance of the very same infection.

It has been proposed that, in the face of parasite diversity, the probability of rapid spacer acquisition to several bacteriophages (phages) at once by the CRISPR-Cas

system is rare (Weinberger *et al.* 2012). Where studies have shown eventual phage extinction in bacterial evolution experiments containing single phage species infections, the presence of a second phage species showed extended host-phage co-evolution (Paez-Espino *et al.* 2015). Thus, with the addition of another phage species, the host's ability to eliminate phage presence decreased with increasing phage diversity. The population-genetic mathematical model of virus-CRISPR dynamics described by Weinberger *et al.* (2012) speculates that if a CRISPR clone has simultaneously acquired a spacer against two phages, it will sweep through the bacterial population in the presence of both phages. Hence, this CRISPR clone is able to go to fixation if it acquires immunity to both viral-subpopulations in a population. This suggests that spacer acquisition to multiple phage species is possible, however, it is unclear which phage conditions (i.e. phage species targeting different host receptors) are likely to select for CRISPR-Cas immunity.

In the current study, I exposed the opportunistic pathogen *Pseudomonas aeruginosa* strain PA14 to different multiple bacteriophages to determine which host defence mechanism (CRISPR-Cas system or *sm*) evolves. By infecting *P. aeruginosa* with a gradual increase of pilus-binding bacteriophages, I could test whether the previously observed high levels of CRISPR-Cas resistance to DMS3vir (Westra *et al.* 2015) is maintained in combination with other pilus-specific phages. In this context, *sm* provides cross-resistance and was hypothesized to be favoured over CRISPR-Cas. Next, *P. aeruginosa* was exposed to different LPS-specific phage mixtures. LPS specific phages were used as bacterial surface modifications associated with resistance to these phages are specific and thus offer no cross-resistance. Hence, it was hypothesized that mixed infections would favour CRISPR-Cas mediated resistance.

2. Materials and Methods

The same bacterial strains and phages as described in Chapter 1 were used throughout the following experiments. Bacterial growth media also remained the

same. Spacer acquisition was monitored and streak assays were performed to measure resistance, both of which were carried out as in Chapter 1.

2.1 Multiple phage experiments

Evolution experiments with combinations of multiple phages were set up to test which immune response would be elicited in multiple parasite conditions. Mixed phage populations were created by combining 1 x 10^6 pfu/mL solutions of each appropriate phage. A full description of all phages used can be found in Table 1 of Chapter 1. The pilus binding phages used were DMS3vir, 1214, 109, and M4. As resistance to DMS3vir at 3dpi (days post infection) is almost 100% CRISPR-Cas mediated (Westra *et al.* 2015), all pilus binding mixtures contained DMS3vir. This was to test whether DMS3vir in combination would elicit the same resistance response as with single phage species infection. Thus, the treatments consisted of two phages (DMS3vir and 1214), three phages (DMS3vir, 1214, and 109) and four phages (DMS3vir, 1214, 109, and M4). This is not a comprehensive list of combinations, however, these combinations will provide an insight to whether the gradual increase in phage diversity affects the resistance evolution between *P. aeruginosa* and phage DMS3.

Mixtures of LPS binding phages were also used to test whether the pressure of modifying different LPS sites would affect which defence mechanism would be elicited upon infection. The combinations composed of two (LMA2 and LPB1, LMA2 and LUZ24, LPB1 and LUZ24) and all three phages (LMA2, LPB1 and LUZ24).

Multiple phage experiments were performed in 6 replicates of 6mL 0.2% glucose M9 media, which were inoculated from an overnight culture with ~ 10^6 WT cells. Approximately 10^4 pfu/mL of combined phage particles were then added to their respective treatment. After three days of daily transfers, treatments were diluted and plated onto 1.5% LB agar. Single colonies were randomly selected for PCR amplification and subsequent spacer acquisition determination. Colonies were also tested for resistance to all phages involved in each treatment. Furthermore, for

experiments containing two LPS binding phages, cross-resistance was tested for by performing a streak assay across a LPS binding phage not used in the given mixed treatment.

2.2 Statistical analysis

A description of the statistical packages used can be found in Chapter 1.

Kruskal-Wallis tests were used to determine whether the difference between the fractions of CRISPR versus *sm* mutants, in the multiple phage experiments, were likely to have occurred due to a particular phage treatment. Pilus and LPS experiments were grouped separately where differences in the fraction of CRISPR mediated resistance were tested across treatments. To test for significance, all analyses applied α = 0.05.

3. Results

3.1 Multiple phage experiments

It is unclear whether CRISPR-Cas mediated resistance is advantageous in the face of multiple parasite infections. *P. aeruginosa* evolves almost exclusively CRISPR-Cas mediated immunity against DMS3vir at 3dpi (Westra *et al.* 2015), but evolves *sm*-mediated resistance against other pilus specific phage species (Chapter 1). The same qualitative results as in Westra *et al.* (2015) at 3dpi of DMS3vir are shown in figure 1 of Chapter 3. This surface modification results in cross-resistance between the different phages (Figure 1). So, in all multiple pilus binding experiments results showed, mixes of; two (DMS3vir and 1214), three (DMS3vir, 1214 and 109), or four phages (DMS3vir, 1214, 109 and 68) almost solely evolved *sm*-mediated resistance, with low levels of CRISPR-mediated resistance occurring in treatments with two or four phages (Figure 1).

P. aeruginosa was exposed to mixes of LPS-specific phages. Again, *sm*-mediated resistance was the most prevalent means of resistance when different LPS-specific phages were combined (Figure 2). Each of these phages trigger specific surface modification based resistance on their own. Crucially, bacterial clones with *sm* resistance against one LPS-specific phage remained sensitive to other LPS-specific phages (Figure 3). Hence, no cross-resistance was observed.

Statistical analysis showed no significant difference between mixed phage treatments on the level of CRISPR mediated resistance immunity for the multiple pilus binding phage experiments (Kruskal-Wallis: X^2 = 2.267, df = 2, P = 3.22) or multiple LPS binding phage experiments (Kruskal-Wallis: X^2 = 4.016, df = 3, P = 0.260). This shows that the differences in phage species used in multiple phage treatments did not cause differences in expressed resistance. These data show that the presence of multiple phages consistently triggers the evolution of *sm* based immunity.
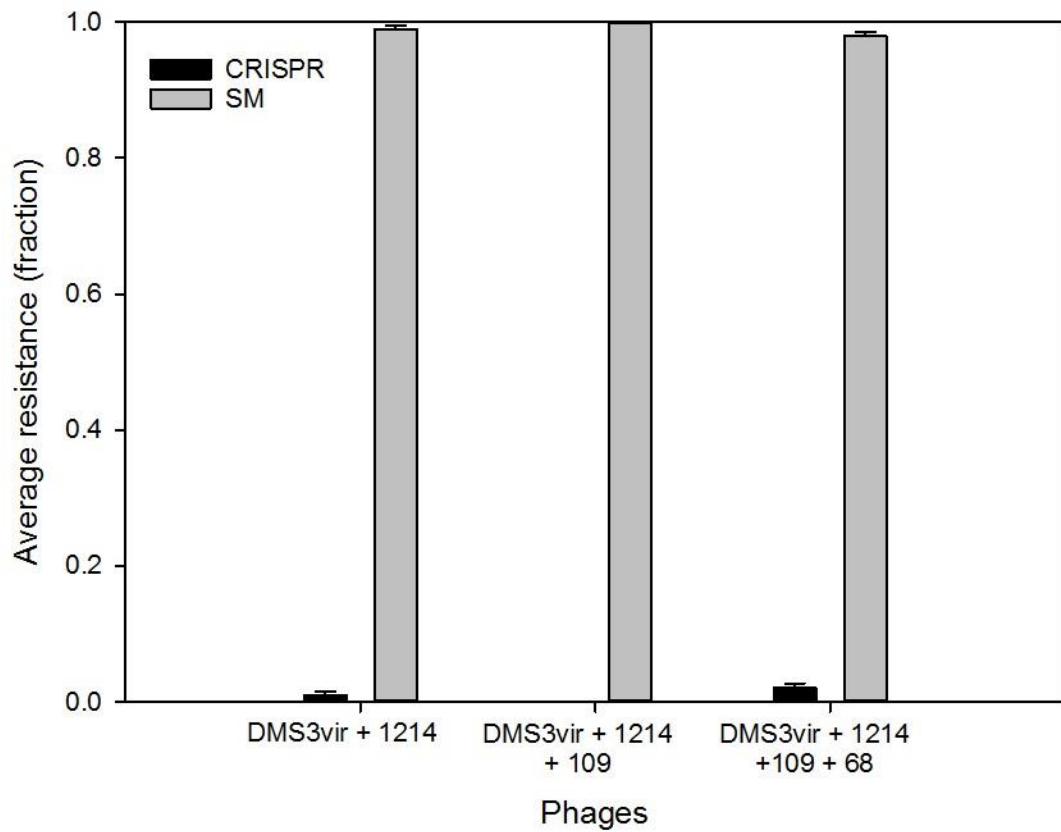
Figure 1: Average fraction of CRISPR-Cas mediated resistance versus *sm*-mediated resistance in treatments containing multiple pilus binding phages at 3 dpi (days post infection). The treatments are as follows; two phages (DMS3vir and 1214), three phages (DMS3vir, 1214, and 109) and four phages (DMS3vir, 1214, 109, and 68). Error bars indicate 95% confidence intervals.
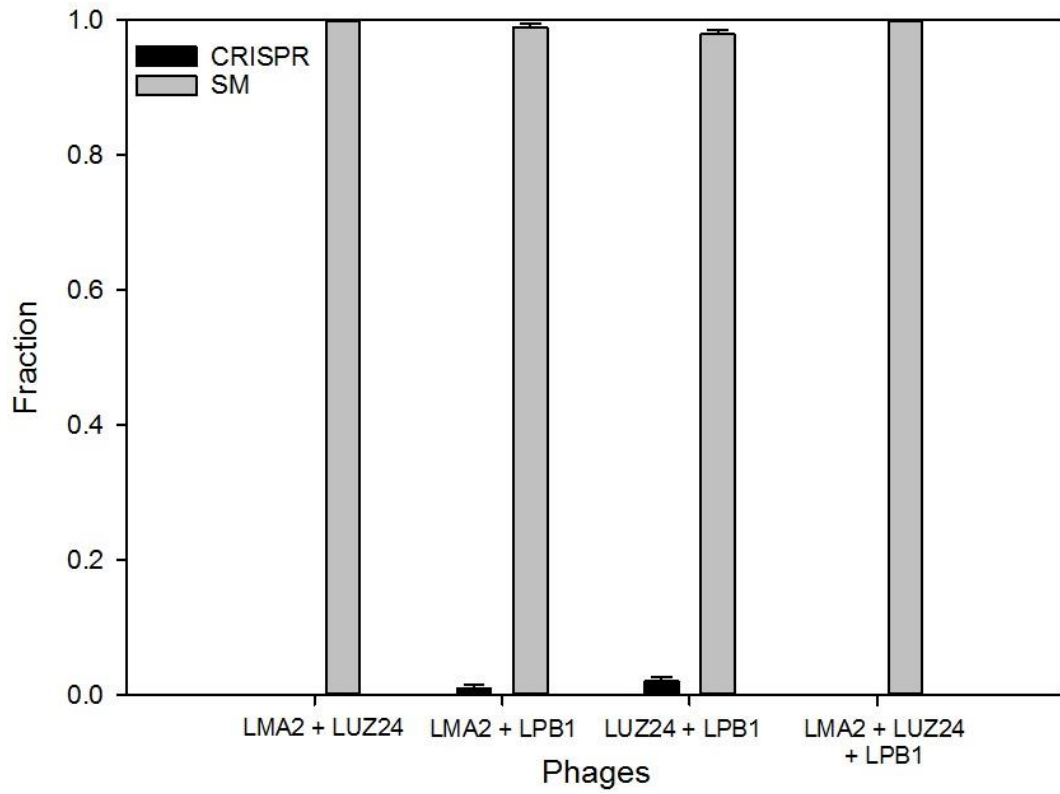
Figure 2: Average fraction of CRISPR-Cas mediated resistance versus *sm*-mediated resistance in multiple LPS binding phage treatments at 3 dpi. WT was infected with treatments of two or three phages. The phage treatments are represented on the x-axis. Error bars indicate 95% confidence intervals.
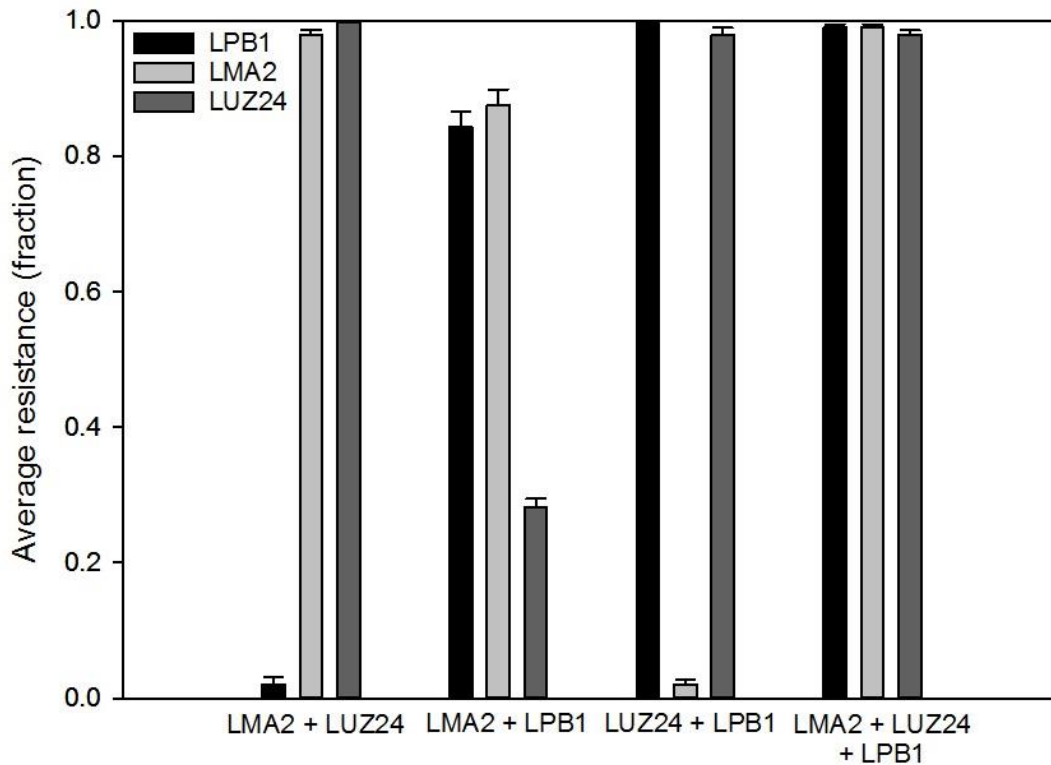
Figure 3: Cross-resistance in multiple LPS binding phage experiments. The graph shows the average fraction of phage resistance across multiple LPS phage treatments at 3dpi. Cross-resistance was tested for by exposing treatments to a different LPS phage. Each bar represents resistance to phage LPB1, LMA2 or LUZ24. Error bars indicate 95% confidence intervals.

4. Discussion

I investigated the effect of multiple phage species on elicited immune defence in the bacterium *Pseudomonas aeruginosa*. Specifically, I tested if pilus-binding phage DMS3vir would still evoke CRISPR-Cas mediated immunity when mixed with other pilus-specific phages. I also investigated whether phages that do not inflict *sm* cross-resistance in their hosts during single infections, would trigger CRISPR-Cas immunity when mixed due to the otherwise multiple surface alterations needed to provide general resistance. It has been suggested, that an adaptive immune system

is beneficial in the face of phage diversity (Barrangou & Marraffini 2015), however, my results contradict this hypothesis. Neither multiple pilus nor LPS binding phage combinations, produced resistance by spacer incorporation through the CRISPR-Cas system. Instead, these results overwhelmingly show cell surface modification as a means of resistance.

It is surprising that surface modification was the most prevalent mode of resistance as, for example, the consequences of losing pili are reduced biofilm formation (O'Toole & Kolter 1998) and limited motility (Burrows 2012), which are vital mechanisms in a movement dependent environment. Reversing such modifications is possible but rare (Taylor *et al.* 2015). Thus, the cost of losing this structure can only be accounted for if another factor, such as cell death, imposes an even higher cost, or if resistance to multiple phages can be gained when all phages bind to the same receptor. This explains the high level of *sm* mediated resistance seen in the multiple pilus phage experiments.

On the other hand, competition between phages may explain the lack of adaptive evolution in multiple pilus phage treatments containing DMS3. There is evidence that different phage species may compete for the same receptor site (Lenski 1988). Thus, co-infection of multiple pilus binding phages may have resulted in phage-phage competition, reducing the chances of phage DMS3 triggering resistance evolution by adaptive immunity. Phage-phage competition may also affect lysis time as cell lysis will be determined by the phage with the fastest lysis time (Refardt 2011). Therefore, if phage DMS3 has a slow lysis time, reduced numbers of DMS3 phage may explain why limited adaptive evolution has occurred.

It was surprising to see modifications to several LPS sites in the multiple LPS phage evolution experiment as alterations to many different surface structures have been predicted to be costly (Koskella *et al.* 2011). A future direction of this study would be to combine pilus binding and LPS binding phages. Even though phage species are genetically dissimilar, the *sm* required to confer cross-resistance to all phages may be too costly if phages bind to different host receptors. In a study by Avrani *et al.*

(2011), it was shown that host mutations that offered resistance to some phages, caused rapid infection by others. If parasites challenge different receptors on the cell surface, it is likely that the cost of modifying several of these sites will be too great, thus prompting another form of defence, such as adaptive immunity, to cope with the threat of infection.

The fact that adaptive immunity failed to evolve against most individual phages (Chapter 1) may explain why it is not observed in multiple phage treatments. A reason for this may be that *P. aeruginosa* needs to be primed before the adaptive process of the CRISPR-Cas system can actively incorporate phage derived spacer sequences. However, in order for *P. aeruginosa* to be primed, a spacer needs to already offer a partial match to a phage sequence. This mismatch will trigger the generation of new spacers as it offers some recognition to a phage sequence (Richter *et al.* 2014, Swarts *et al.* 2012). Host immunity can then be actively renewed by the positive-feedback process of priming as phages simultaneously co-evolve to mutate mismatches of the targeted spacer sequences in its own proto-spacer (Fineran *et al.* 2014). Several studies have shown that the first spacer incorporation event highly influences the positive-feedback process of the CRISPR-Cas adaptive immune system (Datsenko *et al.* 2012, Swarts *et al.* 2012). Thus, the lack of a primed system in this chapter and Chapter 1 may explain why adaptive immunity has been limited. Therefore, establishing a primed system between *P. aeruginosa* PA14 WT strain and a novel phage may enlighten the results observed in this chapter. My next chapter will therefore examine the importance of the priming phenomenon in the evolution of CRISPR-Cas mediated immunity in this experimental system.

Chapter 3

The importance of priming in CRISPR-Cas mediated resistance

Abstract

Bacteria can evolve immunity by surface modification or through the CRISPR-Cas
(Clustered regularly interspaced short palindromic repeats - CRISPR-associated)
system. However it has been noted that, even though CRISPR-Cas immunity is often
associated with higher fitness levels, it frequently fails to evolve against single
(Chapter 1) and multiple (Chapter 2) phages. Here I investigate the importance of a
process known as "priming" for the evolution of CRISPR immunity. Priming occurs
when a pre-existing spacer has partial complementarity to the infecting phage. It is
well-established that priming increases the rate of incorporation of new spacers in
many different environmental systems. I found that removing a spacer from the
CRISPR locus that is predicted to mediate priming against DMS3vir reduces the rate
of CRISPR-Cas evolution. Additionally, I found that bacterial clones carrying a
spacer against phage 68 evolved increased levels of CRISPR-Cas immunity in
comparison to the ancestral bacterial host. However, the levels of evolved immunity
against phage 68 by primed bacteria were much lower than those observed for
DMS3vir, suggesting that other phage life history traits may be important.

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPRs), along with
CRISPR-associated (Cas) proteins provide adaptive immunity to foreign genetic
material in prokaryotes (Sorek *et al.* 2008). Bacteria will incorporate bacteriophage
(phage) nucleic acid sequences, termed spacers, into its CRISPR loci when this
novel foreign invader is encountered (Yosef *et al.* 2012). Host immunity is gained as
spacer sequences are highly specific and provide homologous sequence recognition
to the corresponding phage proto-spacer sequence. However, immunity can be lost
if phage acquire point mutations to either the target proto-spacer sequence, the PAM

(proto-spacer adjacent motif) or the seed region of the phage proto-spacer (Datsenko 2012, Semenova *et al.* 2011, Sun *et al.* 2013). These nucleotide mismatches allow the phage to bypass the recognition and prospective clearance by the host CRISPR-Cas system, resulting in phage sensitivity (Barrangou *et al.* 2007). Although these mismatches allow phage to overcome host immunity, they also trigger a greatly increased rate of spacer acquisition, known as "primed" spacer acquisition. The positive-feedback process of priming leads to the incorporation of new spacers to restore immunity, and presumably relies on partial recognition to escape viruses (Fineran *et al.* 2014). Thus, by acquiring multiple spacers for the same invader, the host is strengthening its recognition to it and tightening the bacteria-phage interaction (Swarts *et al.* 2012).

The molecular memory of the CRISPR locus provides an overview of host-parasite evolutionary past. Studies have shown how vital matching or mismatching in a pre-existing spacer to a previously encountered parasite is for efficient priming to occur. In fact, priming is considered a prerequisite for the evolution of CRISPR-Cas immunity and has been shown in *Escherichia coli,* (Datsenko *et al.* 2012), *Pectobacterium atrosepticum* (Richter *et al.* 2014) and *Haloarcula hispanica* (Li *et al.* 2013). Another study showed that priming can occur when a novel parasite resembles a previously encountered parasite. Therefore, host resistance can be rapidly generated to a previously unknown parasite, simply by having mismatches in spacer sequences to a known one (Fineran *et al.* 2014). Thus, different mismatches can trigger different strengths of priming.

Here I investigate whether the prerequisite for priming explains why *Pseudomonas aeruginosa* strain PA14 evolves CRISPR-Cas immunity against some, but not all, phage species (Chapter 1). I investigate the importance of priming using two complementary approaches. First, I generate bacteriophage insensitive mutants (BIMs) with CRISPR-Cas immunity against phage 68, followed by the generation of phages that bypass this CRISPR-Cas immunity. Next, I test the frequency of spacer acquisition in response to these escape phages using the ancestral strain PA14 and BIMs as hosts. Additionally, I examine the effect of removing a spacer that is

predicted to mediate priming of strain PA14 against DMS3vir, in order to test the evolution of CRISPR-Cas immunity against DMS3vir in an unprimed system.

2. Materials and Methods

2.1 Bacterial strains, phages, and media

Bacteria and bacterial growth media

Pseudomonas aeruginosa wild type (WT) PA14, *P. aeruginosa* CRISPR-Cas knock-out strain *csy3::LacZ* (*LacZ*), and *P. aeruginosa* WT PA14 derived knock-out strains CRISPR2 Δ spacer 1-2 (SMC4707) and CRISPR2 Δ spacer 2-21 (SMC4577) were used throughout experiments and were all supplied by the O'Toole lab (Cady & O'Toole 2011) at the Geisel School of Medicine (Hanover, New Hampshire, USA). The bacterial growth media, described in Chapter 1, was used throughout the following experiments.

Phage

Phage 68 is a part of the Lindberg phage collection, supplied by the Davidson lab (University of Toronto, Ontario, Canada), and was chosen to test the efficiency of priming to a novel phage. A stock solution of phage 68 was generated by combining 10µL of 10^6 pfu (plaque forming units) /mL and 300µL of overnight grown *LacZ* cells to 9mL of 0.5% LB agar and poured onto 1.5% LB agar where overnight growth in 37°C allowed near confluent lysis to be reached. Plates were then flooded and left for 2 hours with 10mL M9 salts to retrieve phage and bacteria from the LB agar. To clear bacterial cells from phage lysate, the liquid was chloroformed and centrifuged at 35000 rpm (rotations per minute) for 10 minutes. Whereupon, final phage stock titrations, in pfu/mL, were determined by diluting and pipetting 5µL of 10-fold supernatant dilutions onto *LacZ* 1.5% LB agar bacterial lawns. The stock was stored at 4°C.

Partial matches of the phage DMS3vir to *P. aeruginosa* WT PA14 genome exist and were thus used in the CRISPR knock-out experiments, described below, as a model for a primed system. DMS3vir was supplied by the O'Toole lab (Cady & O'Toole 2011) (Geisel School of Medicine, Hanover, New Hampshire, USA).

2.2 Streak assays and phage titration

Streak assays and phage titrations were performed as described in Chapter 1.

2.3 Spacer acquisition

Acquisition of spacers into both, CRISPR1 and CRISPR2, CRISPR loci were detected through colony PCR. The primers for CRISPR1 locus were: forward CTAAGCCTTGTACGAAGTCTC, reverse CACCGGCGCGCTGGCCTTCGGCG. CRISPR2 locus had a forward primer of GCCGTCCAGAAGTCACCACCCG and a reverse primer of CGAGGTCTCGTAACTTGCTGA. Separate primers had to be designed for the CRISPR2 Δ experiments. To detect spacer acquisition for CRISPR2 Δ spacer 1-2 a forward primer of AGCCACTGTGTCGGCCAAAACC was used. In treatments using the strain CRISPR2 Δ spacer 2-21, a forward primer of TGTGTGAGGAGCGTGAGCTTCC was designed. The reverse primer for CRISPR2 remained the same, CGAGGTCTCGTAACTTGCTGA, in both cases.

PCR reactions were prepared in the same manner as in Chapter 1, followed by the identical PCR program for product amplification. All materials and procedure for gel electrophoresis can also be found in Chapter 1.

2.5 CRISPR knockout experiment

To test the importance of pre-existing spacer sequences in the process of priming, CRISPR knock-out strains were challenged with phage DMS3vir. Approximately 10^6 cfu/mL bacterial cells from overnight grown cultures of CRISPR knock-out strains; CRISPR2 Δ spacer 1-2 and CRISPR2 Δ spacer 2-21, along with WT, were

inoculated into microcosms containing 6mL of 0.2% glucose M9 media. Each treatment contained 6 replicate populations, all infected with 10^4 pfu/mL of DMS3vir. A 1:100 transfer into fresh media was performed at daily intervals until 3 days post infection (dpi) was reached. Cultures were diluted to 10^{-5} and plated onto 1.5% LB agar plates, where single colonies were isolated and inoculated into fresh 0.2% glucose media. Streak assays tested colony resistance against DMS3vir. Single colony PCRs determined whether spacers had been incorporated. Phage extractions were also performed to establish presence or absence of phage.

2.6 Bacteriophage insensitive mutant (BIM) selection and CRISPR escape phage (CEP) generation

In order to generate a BIM, *P. aeruginosa* WT was infected with 10^4 pfu/mL of phage 68 and inoculated into 0.2% glucose M9 media and allowed to incubate in temperatures of 20°C, 28°C and 37°C. Cultures were transferred daily (1:100) into fresh medium for a total of three days. Two BIMs containing one additional phage derived spacer were obtained as a result of this experiment.

Next, escape phages to both BIMs were generated. A 1:1 mix of BIM and *LacZ* cells were inoculated into LB media containing 10^6 pfu/mL of phage 68. The culture was allowed to grow overnight. Next, a chloroform extraction to remove bacterial cells was performed. Remaining phage were serially diluted and spotted onto bacterial lawns comprised of BIMs to confirm the presence of escape phage. Escape phage was only generated for one of these BIMs. As this BIM contained one spacer and was obtained through evolution with phage 68, it was given the name BIM68-1. Plaque assays where then prepared to isolate a single CRISPR escape phage (CEP) plaque (CEP-68-1). To isolate a single colony, 10µL of 10^4 pfu/mL of escape phage stock mixture was added to 300µL BIM68-1 culture and 9mL 0.5% LB agar and poured onto 1.5% LB agar and allowed to incubate at 37°C overnight. This single CRISPR escape phage plaque (CEP-68-1) was then amplified to be used in evolution experiments.

BIM68-1 was infected with 10^4 pfu/mL CEP-68-1, in 0.2% glucose M9 media, and transferred daily into fresh media for three days. Nineteen BIMs containing a second additional spacer were obtained from this experiment. Thus, these BIMs contained a total of two spacers of genetically derived material from phage 68. This time a 1:1 mix of all nineteen BIMs and *LacZ* were inoculated with CEP-68-1. Again, escape phage was only generated for one BIM clone, resulting in the name BIM68-2. A single CRISPR escape phage plaque (CEP-68-2) was isolated by plaque assay, this time on bacterial lawns containing BIM68-2, and amplified into a stock solution.

A summary of the BIMs and CEPs obtained can be found in Table 1 below.

Table 1: BIMs and escape phages used in experimental procedures

Bacteriophage insensitive mutant (BIM)	Number of spacers	CRISPR Escape phage	Resistant to
BIM68 – 1	1	CEP-68-1	Phage 68 and DMS3vir
BIM68 – 2	2	CEP-68-2	Phage 68 and DMS3vir

2.7 Priming experiments

P. aeruginosa clones, exhibiting different levels of primed recognition to phage 68 (BIM68-1 and BIM68-2), were evolved separately with CRISPR escape phage 68 (CEP-68-1 and CEP-68-2). In replicates of 6, ~10^6 cfu/mL of overnight grown WT, BIM68-1 and BIM68-2 were inoculated into 0.2% glucose media and infected with phage (10^4 pfu/mL). Primed strains, along with WT, were compared by infecting cells with variants of phage 68 as follows; WT with phage 68, BIM68-1 with escape phage CEP-68-1 and BIM68-2 with escape phage CEP-68-2. Experiments were conducted over a three day period, where transfers of 1:100 of overnight treatments into fresh media were done at daily intervals. Upon 3dpi, replicates were diluted and plated onto 1.5% LB agar and incubated overnight to allow single colony formations.

Bacterial colonies were isolated, picked and suspended in 0.2% glucose M9 media overnight. Viral titrations along with phage resistance and preferred resistance mechanism were determined at the conclusion of the experiment.

2.8 Statistical analysis

Statistical software used was the same as in Chapter 1.

A Kruskal-Wallis test was used to assess whether there was a significant difference in CRISPR-mediated defence between both CRISPR knockout strains (CRISPR2 Δ spacer 1-2 and CRISPR2 Δ spacer 2-21) and WT, and between BIMs (BIM68-1 and BIM68-2) and WT. The frequency of CRISPR-Cas across replicates was also tested across bacterial strains with a Fisher's exact test. To test for significance, all analyses applied α = 0.05

3. Results

3.1 CRISPR knockout experiment

As spacers on the CRISPR locus reflect prior phage encounters, the removal of these should limit the fluidity in which additional spacers are added, thus slow the priming process. It has been predicted that spacer 1 on the CRISPR2 locus mediates priming against DMS3vir (Cady *et al.* 2012) due to the partial matches with the phage proto-spacer (Figure 3). As such, the CRISPR knockout strain containing the removed leader-end first two spacers (CRISPR2 Δ spacer 1-2) is believed to have a stronger effect on priming in comparison to the other knockout strain (CRISPR2 Δ spacer 2-21).

Interestingly, CRISPR2 Δ spacer 1-2 showed 100% *sm*-mediated immunity in the presence of DMS3vir (Figure 1). This demonstrates that, spacers 1-2 are very important in new CRISPR resistance acquisition events and that the mismatches observed between the CRISPR2 locus to DMS3vir play a role in priming. By contrast, CRISPR2 Δ spacer 2-21 exhibited ~80% *sm*-mediated immunity, with some

CRISPR mediated resistance and sensitivity in the population (Figure 1). However, the number of spacer acquisition events in CRISPR2 Δ spacer 2-21 experiments did not exhibit the same high numbers observed in experiments using WT (Figure 1). As a result, there was a significant difference in CRISPR-mediated resistance across strains (Kruskal-Wallis: X^2 = 14.14, P = 0.001). This highlights the importance of spacer 1, because even though the majority of the locus was removed, the presence of this spacer resulted in renewed spacer acquisition.
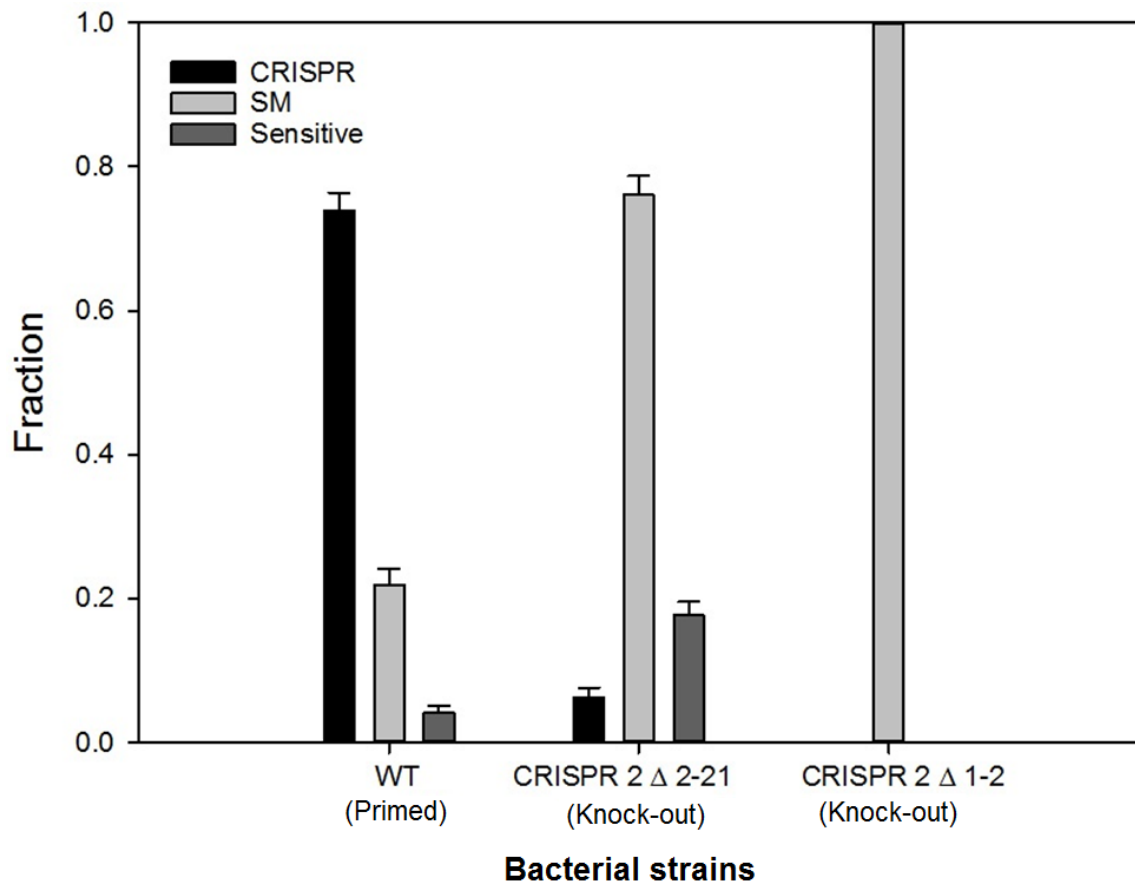
Figure 1: Average fraction of resistance (CRISPR or *sm*-mediated) or sensitivity in evolution experiments with WT or CRISPR knock-out strains in the presence of phage DMS3vir at 3dpi (days post infection). WT bacteria are primed to phage DMS3vir, whereas knock-out strains CRISPR 2 Δ 2-21 and CRISPR 2 Δ 1-2 are no longer primed to DMS3vir. Final phage DMS3vir titrations were as follows; 6×10^6 pfu/mL (WT), 8×10^{10} pfu/mL (CRISPR 2Δ 2-21) and 4×10^9 pfu/mL (CRISPR 2Δ 1-2). Error bars are measured in 95% confidence intervals.

3.2 Priming experiments

Based on previous studies on *E. coli* showing that point mutations in the phage PAM (proto-spacer adjacent motif) stimulate renewed spacer acquisition in the host (Datsenko *et al.* 2012, Swarts *et al.* 2012), it was predicted that similar observations would occur in *P. aeruginosa*. To determine whether primed strains of *P. aeruginosa* to the novel phage 68 results in increased levels of spacer acquisition, BIMs (BIM68-1 and BIM68-2) were evolved in the presence of escape phage 68 (CEP-68-1 and CEP-68-2).

The majority of mediated resistance in both bacterial populations (BIM68-1 and BIM68-2) occurred by *sm* when infected with phage 68 escape phages (Figure 2). Spacer acquisition by BIM68-1 and BIM68-2 was limited, and there was no significant difference between bacterial strains on observed CRISPR-mediated resistance (Kruskal-Wallis: $X^2 = 5.57$, df = 2, P =0.062). Resistance in a non-primed system (WT infected with phage 68, Figure 2) occurred by *sm*, showing that the acquisition of spacers by means of CRISPR-Cas is a rare occurrence in this particular phage-host interaction.

A Fisher's exact test was used to assess whether number of replicates producing CRISPR-Cas resistance was significant between bacterial strains. As a result, there was a significant difference (one-tailed Fisher's exact test, P < 0.001). This shows that, while the difference in the fraction of spacer acquisition was insignificant, the detection of CRISPR-Cas resistance is greater in strains already containing recognition to phage 68.
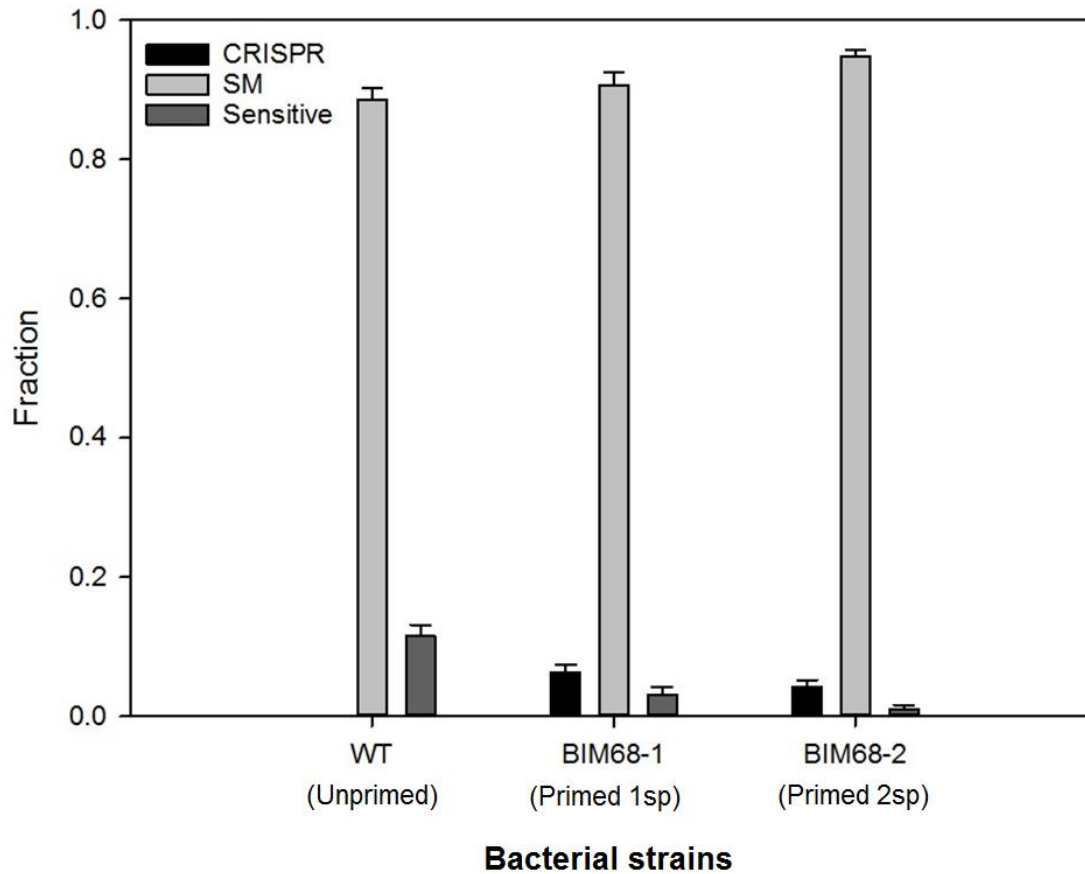
Figure 2: *P. aeruginosa* PA14 (WT) and primed strains; BIM68-1 and BIM68-2 at 3dpi of phage 68. Phage 68 infections differed and were as follows; WT with ancestral phage 68, BIM68-1 with escape phage 68 (CEP-68-1) and BIM68-2 with escape phage 68 (CEP-68-2). Bacterial strains are unprimed (WT) or primed with one (BIM68-1) or two (BIM68-2) spacers matching phage 68. Each bar shows the average fraction of phage sensitivity or immunity through CRISPR or *sm.* Final phage 68 concentrations were as follows; 8×10^9 pfu/mL (WT), 2×10^9 pfu/mL (BIM68-1) and 1×10^{11} pfu/mL (BIM68-2). Error bars are measured as 95% confidence intervals.
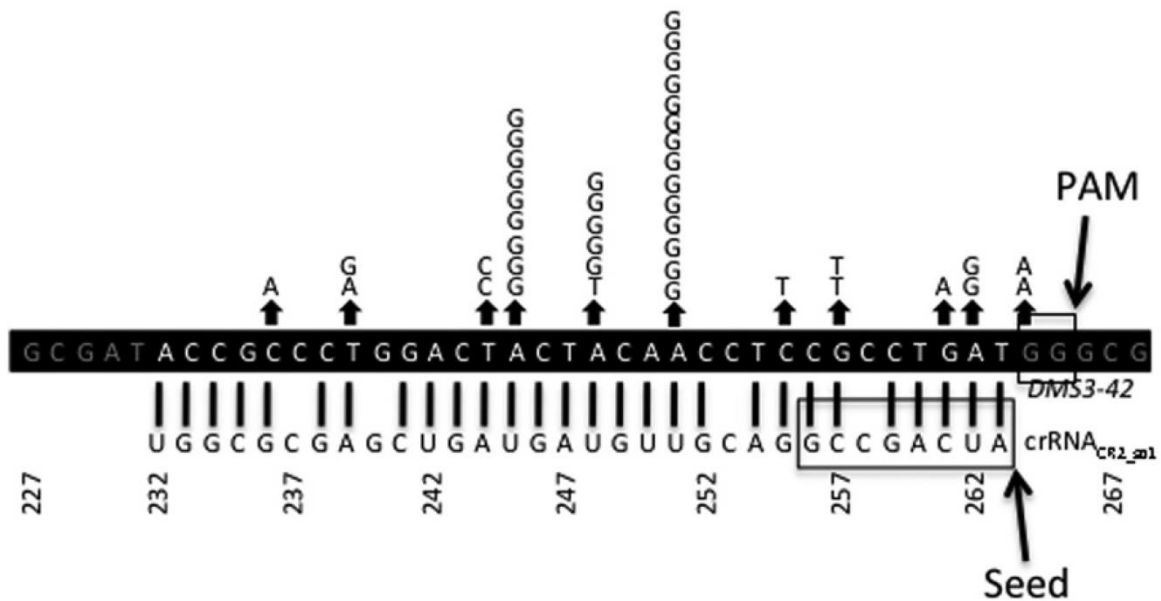
Figure 3: Diagram of partial match of spacer 1 on the CRISPR2 locus to DMS3vir. The spacer is represented on the bottom as crRNA$_{CR2\_sp1}$, and phage DMS3vir on the top as DMS3-42 T255. The orientation of the locus is simplified by boxing the crRNA seed and the phage PAM sequence. Arrows above the DMS3vir sequence indicate point mutations enabling evasion of CRISPR-Cas. Image taken from Cady *et al.* 2012.

4. Discussion

Here I demonstrate that the presence of spacers targeting specific phage sequences can enhance the priming process of the CRISPR-Cas system. By first evolving engineered *Pseudomonas aeruginosa* spacer knockout strains, with phage DMS3vir, I was able to test the importance of prior spacer sequences on the priming efficiency of the CRISPR-Cas system. Spacer removal, or their reduced numbers, resulted in host defence system change to *sm*-mediated resistance. Following this, *P. aeruginosa* bacteriophage insensitive mutants (BIMs) were generated by evolving with a novel phage species, phage 68, to test whether increased phage recognition would increase priming. In spite of limited spacer acquisition, detection of CRISPR-

Cas increased. However, the most prevalent means of bacterial resistance was mainly evolved through *sm*.

Studies have shown that the positive feedback loop of priming is greatly influenced by the first spacer acquisition event (Datsenko *et al.* 2012, Swarts *et al.* 2012) and that in comparison to naive adaptation, priming is rapid (Savitskaya *et al.* 2013). It is therefore not surprising that, with spacer removal, *P. aeruginosa* knockout strains showed a distinctive lack of renewed spacer acquisition with DMS3vir infection. The, albeit limited, priming observed for CRISPR 2 Δ 2-21 (Figure 1) can be explained by the partial match of spacer 1 to DMS3vir (Figure 3). Previous infection of WT cells with DMS3vir has been shown to promote high levels of resistance by CRISPR-Cas spacer incorporation (Westra *et al.* 2015). Figure 1 is also qualitatively consistent to the evolution experiment conducted in the Westra et al. (2015) study. So, once spacer 1 is deleted (CRISPR 2 Δ 1-2, Figure 1), spacer acquisition is removed. This is consistent with the theory that spacers play an important role in the process of priming (Datsenko *et al.* 2012, Li *et al.* 2014, Richter *et al.* 2014) and when removed, will remove the adaptive ability of the CRISPR-Cas system and instead result in host defence system change, which in this case was *sm*-mediated resistance.

It has also been hypothesized that early phage infection history has an important effect on the generation of new resistance. However, the data obtained using phage 68 does not support this. It was believed that once a host had acquired a phage derived spacer, the incorporation of additional spacers would occur at a higher frequency. Even though renewed CRISPR-Cas resistance is detectable, *sm* remains largely the dominant resistance mechanism. Perhaps the lack of CRISPR-Cas resistance is the result of high phage virulence. The lytic lifecycle of phage 68 may be too fast for active spacer incorporation to occur, especially with high infection titres. Therefore, if virulence is too strong and priming too weak, the host may evolve a more costly form of resistance by modifying its surface.

The main conclusion is that priming is important in this system. Indeed, immune priming is also found to be beneficial in other systems. The bumble bee *Bombus terrestris*, for example, when infected by similar parasites where shown to have improved immune system "memory" upon successive parasite challenges (Sadd & Schmid-Hempel 2006). In an experiment using the beetle *Tenebrio molitor*, Moret and Siva-Jothy (2003) were able to demonstrate that the survival of pathogen infection is greatly increased by previous encounters with pathogens. However, increased phage recognition did not increase priming equally between two different phage species in this study. It is still puzzling why we observe high levels of CRISPR-Cas mediated resistance in *P. aeruginosa*-DMS3vir interactions and not in other *P. aeruginosa*-phage interactions. Deep sequencing of bacterial populations will help to elucidate the result of reduced priming towards phage 68 by showing total CRISPR resistance in a population rather than estimating the frequency of total evolved adaptive immunity in a subset of individuals, and will thus be conducted in future investigation.

Chapter 4

The effects of multiple bacteriophage infection on the evolution of CRISPR resistance in primed bacterial strains

Abstract

In Chapter 2, I demonstrated how CRISPR-Cas immunity typically fails to evolve when *Pseudomonas aeruginosa* was exposed to mixtures of phages. However, bacteria were either naive (unprimed) against all phage species, or primed against only a single phage species. Here I investigate whether CRISPR-Cas immunity evolves when bacteria are primed prior to infection against all phages involved. To this end I use the *P. aeruginosa* PA14-derived strains described in Chapter 3, primed to both phage 68 and DMS3vir. Thereby, exposure to either phage is able to trigger host spacer incorporation. Although detectable levels of CRISPR-Cas immunity evolve upon exposure to single phages, CRISPR-Cas resistance fails to evolve upon mixed infection. This further supports my earlier conclusion that CRISPR-Cas consistently fails to evolve when exposed to multiple phages, where the host will instead favour the evolution of more broad range resistance mechanisms. Perhaps the pressure of multiple parasites on the CRISPR-Cas system is too great for efficient resistance to occur by means of spacer incorporation; alternatively, the benefit of surface modification could be greater as this confers resistance to both phages.

1. Introduction

An adaptive immune system is predicted to be beneficial when faced with parasite diversity as adaptive immunity is associated with low fitness costs (Horvath & Barrangou 2010). However, as described in Chapter 2, adaptive immunity against multiple phages consistently fails to evolve. This may be driven by a lack of priming against all phages involved in these studies, as priming has been shown to be key to

the evolution of CRISPR-Cas immunity in Chapter 3 and other experimental systems (Datsenko *et al.* 2012, Swarts *et al.* 2012, Finneran *et al.* 2012, Li *et al.* 2013, Richter *et al.* 2014).

Priming in the CRISPR-Cas system relies on sequence mismatches between a pre-existing spacer and a phage proto-spacer, and triggers increased rates of spacer acquisition (Datsenko *et al.* 2012, Fineran & Charpentier 2012, Heler *et al.* 2014). As the CRISPR locus can be considered a library of past infections, spacer sequences are not limited to single phage species and presumably offer recognition to a diverse set of parasites. Indeed, metagenomic and bioinformatic analyses show that this system is adaptive to diverse pathogens (Andersson & Banfield 2008, Horvath *et al.* 2008). However, despite priming having been demonstrated to renew resistance to an invader, it is not yet firmly established how efficient this system is when multiple spacer acquisition events are required, or if it is always the most ideal defence system. If a parasite diverse environment contains phage species that target different receptors on the host cell surface, then each specific surface modification required may be too costly for the host (Koskella *et al.* 2011), making CRISPR-Cas resistance beneficial as it is not dependent on the phages mode of infection. On the other hand, if all phages target the same receptor, it may be more beneficial to alter the receptor as resistance will be applied against multiple phages.

I have previously shown how parasite diversity induces *sm*-mediated defence when the host is unprimed and infected with mixed phages (Chapter 2). Therefore, this chapter investigates whether the CRISPR-Cas system is able to rapidly evolve when the host is primed against all phages present in an environment. However, even though the host is primed, the phages all target the same host receptor (pili). Thus, the prediction is that *sm* will be the most prevalent resistance mechanism.

2. Materials and Methods

2.1 Bacterial strains, phages and media

Bacteria and bacterial growth media

Pseudomonas aeruginosa wild type (WT) PA14, *P. aeruginosa* CRISPR-Cas knock-out strain *csy3::LacZ* (*LacZ*), along with bacteriophage insensitive mutants (BIMs) described in Table 1 (BIM68-1 and BIM68-2) were used in the following experimental procedures. Bacterial growth media is as described in Chapter 1.

Phage

The rationale and preparation of phage 68 is described in Chapter 3. Sequencing analysis showed 100% homogeneity of both BIM-1 and BIM68-2 to phage DMS3vir, meaning that the spacer targeting phage 68 also targets phage DMS3vir. Thus, DMS3vir escape phages were obtained for both BIMs. A 1:1 mixture of 500µL BIM (either BIM68-1 or BIM68-2) and 500µL *LacZ* cells were inoculated into LB media and infected with 10^4 pfu (plaque forming units)/mL DMS3vir. Chloroform extractions were performed on overnight cultures, and plaque assays onto each respective BIM bacterial lawn enabled a single CRISPR escape phage plaque (CEP-DMS3vir-1 and CEP-DMS3vir-2) for each BIM to be isolated. Plaque assays contained 10µL of 10^6 pfu/mL and 300µL of either BIM68-1, or BIM68-2 overnight grown cells which were added to 9mL of 0.5% LB agar and poured over 1.5% LB agar. Stock solutions of CEP-DMS3vir-1 and CEP-DMS3vir-2 were generated and stored at 4°C.

2.2 Streak assays, phage titrations and spacer acquisition

The method for streak assays can be found in Chapter 1. In experiments containing phage combinations, single colonies were streaked against both phages involved in the procedure.

Phage titrations were performed as described in Chapter 1. Overall titrations for experiments containing phage combinations were noted where phage type was not separated.

Spacer acquisition was measured in the same manner as in Chapter 1.

2.5 Priming experiments

To test the effect of multiple phages on spacer acquisition, all bacterial strains were infected with combinations of phage 68 and DMS3vir. Bacterial strains and phages used are described in Table 1. WT strain was infected with ancestral phage 68 and DMS3vir, BIM68-1 with CEP-68-1 and CEP-DMS3vir-1, and BIM68-2 with CEP-68-2 and CEP-DMS3vir-2. Phage 1:1 mixtures were created by combining 500µL of 10^6 pfu/mL of each phage, where 10^4 pfu/mL of the combination was added to ~10^6 cfu/mL overnight grown bacterial cultures in 6 replicates of 0.2% glucose M9 media and allowed to evolve over a total of three daily transfers in 37°C.

In parallel, and under the same conditions, WT, BIM68-1, and BIM68-2 were infected with 10^4 pfu/mL of ancestral phage 68, CEP-68-1, and CEP-68-2 respectively. Also, bacterial strains were infected with ancestral DMS3vir, CEP-DMS3vir-1, and CEP-DMS3vir-2. Again, these cultures were inoculated, in replicates of 6, into 0.2% glucose M9 and transferred into fresh media over three days.

All three evolution experiments were, at 3 days post infection (dpi), diluted into M9 salts and plated onto 1.5% LB agar. Overnight incubation in 37°C produced single colonies, 16 of which for each replicate were randomly picked and re-suspended into 0.2% glucose M9 for overnight growth. Resistance and mode of resistance, along with phage titrations were determined at 3dpi. PCRs were also performed to account for any spacer acquisition.

Table 1: BIMs and escape phages used in experimental procedures

Bacteriophage insensitive mutant (BIM)	Number of spacers	CRISPR Escape phage	Resistant to
BIM68 – 1	1	CEP-DMS3vir-1	DMS3vir
		CEP-68-1	Phage 68 and DMS3vir
BIM68 – 2	2	CEP-DMS3vir-2	DMS3vir
		CEP-68-2	Phage 68 and DMS3vir

2.6 Statistical analysis

Description of statistical packages used can be found in Chapter 1.

In the interest of determining any significant differences of CRISPR-Cas mediated immunity across bacterial strains, Kruskal-Wallis tests were used in statistical analysis. To test for significance, all analyses applied α = 0.05.

3. Results

To compare the evolution of defence mechanisms between a primed bacterial strain and an unprimed bacterial strain, WT *Pseudomonas aeruginosa* PA14 was infected with ancestral phage 68 and DMS3vir, alone and in combination. In agreement with previous findings (Chapters 2 and 3), bacteria evolved CRISPR-Cas immunity against DMS3vir, but not against phage 68, nor against the combination of both (Figure 1). As such, there was a clear significant difference between all phage treatments on CRISPR-Cas mediated resistance using WT cells (Kruskal-Wallis: $X^2 = 16.25$, df = 2, P < 0.001, Figure 1).

In order to test the effect of phage diversity on the evolution of defensive mechanisms in bacterial strains primed to all phages, BIMs; BIM-68-1 and BIM-68-2 were infected, singularly, with escape phages 68 and DMS3vir and in combination of both. CRISPR-Cas resistance was observed for DMS3vir and phage 68, but not in mixtures of both (Figure 2 and Figure 3). As such, CRISPR-Cas resistance was significantly different between contrasting phage infections with BIM68-1 (Kruskal-Wallis: $X^2 = 13.15$, df = 2, P = 0.001, Figure 2) and BIM68-2 (Kruskal-Wallis: $X^2 = 12.35$, df = 2, P = 0.002, Figure 3). Thus, phage infection(s) had a crucial effect on mediated resistance response in *P. aeruginosa*. This shows that, when bacterial strains are primed against both phage species, the mixtures of both phages result in fewer spacer acquisitions than when with single infections.

It should be noted that in all mixed phage experiments, CRISPR-Cas mediated phage resistance was measured against both phages. Upon mixed infection, some spacer acquisition was observed for both BIM68-1 and BIM68-2, but these spacers only provided resistance against DMS3vir. Importantly, compared to DMS3vir single infection, spacer acquisition in combination was greatly reduced. This shows that CRISPR-Cas immunity towards DMS3vir is significantly different when combined with phage 68 as opposed to single phage infection (Kruskal-Wallis: $X^2 = 9.4$, df = 2, P = 0.009).
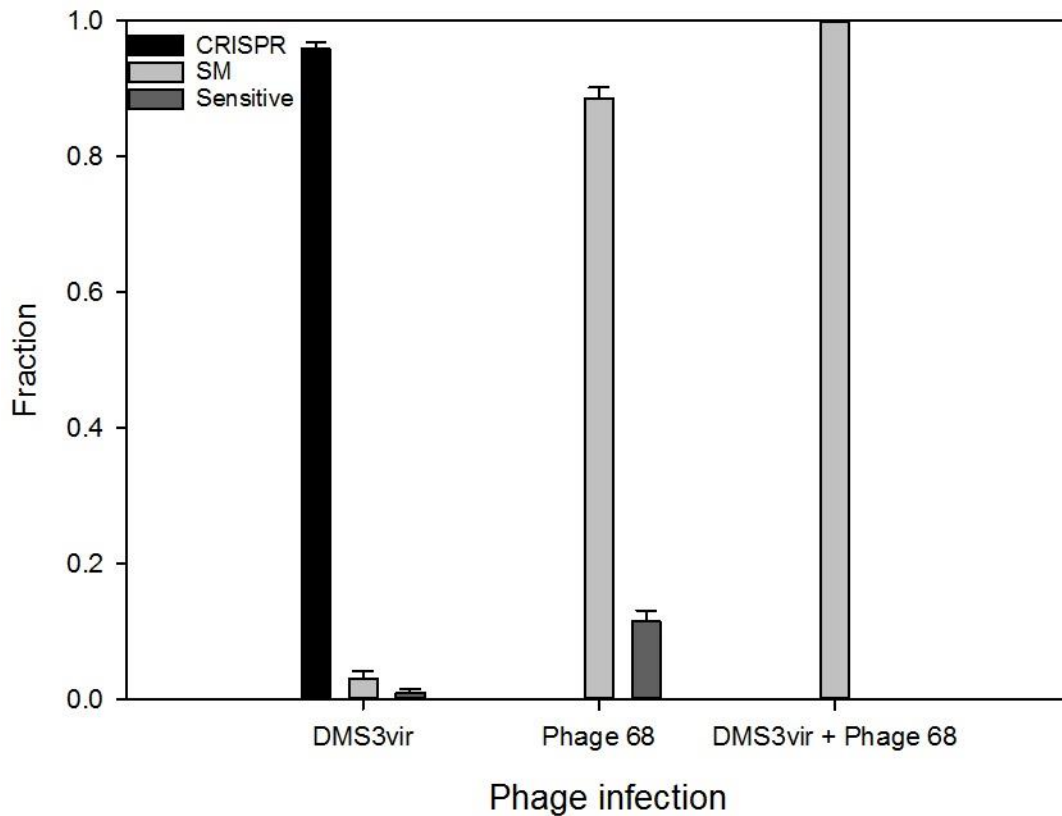
Figure 1: Average fraction of *P. aeruginosa* strain PA14 (WT) population immunity and sensitivity at 3dpi. The infections were; phage DMS3vir, phage 68, and the combination of phage (DMS3vir and phage 68). Immunity is divided into CRISPR-Cas mediated or *sm*-mediated. Final phage concentrations averaged to 8×10^{7} pfu/mL (DMS3vir), 8×10^{9} pfu/mL (phage 68) and 6×10^{10} pfu/mL (DMS3vir + phage 68). Error bars are measured as 95% confidence intervals.
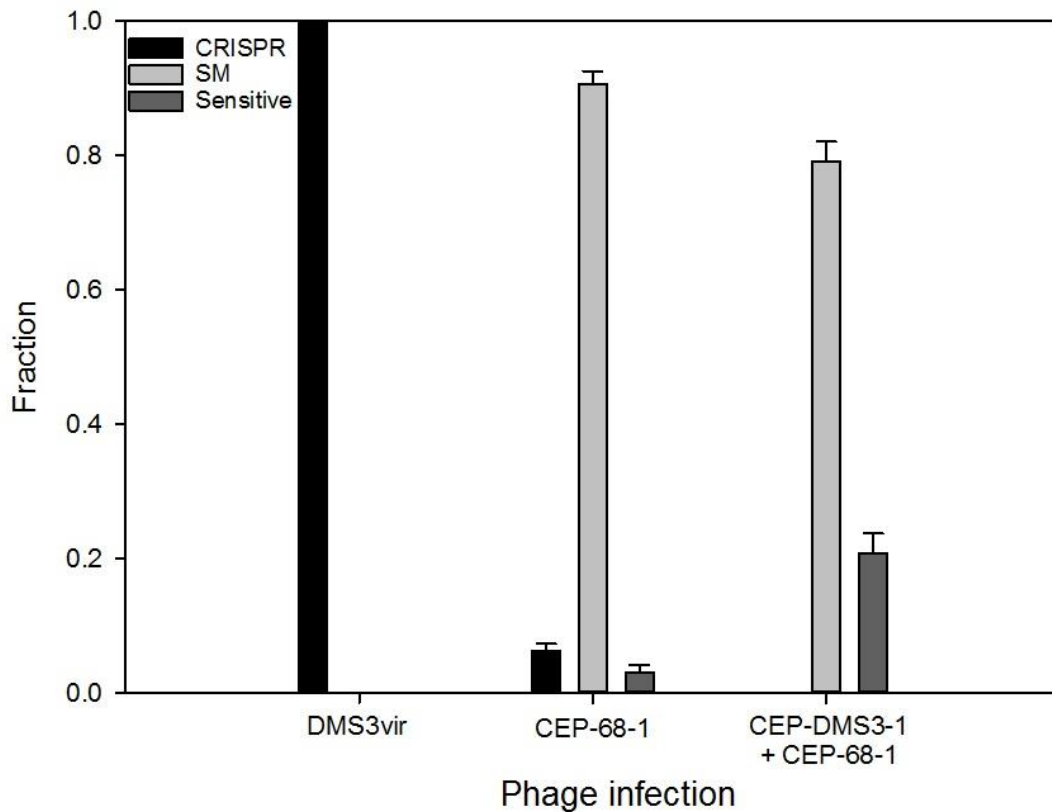
Figure 2: Average fraction of bacterial strain BIM68-1 population immunity and sensitivity at 3dpi. The infections were; escape phage DMS3vir (CEP-DMS3vir-1), escape phage 68 (CEP-68-1) and the combination of escape phage (CEP-DMS3vir-1 and CEP-68-1). Immunity is divided into CRISPR-Cas mediated or *sm*-mediated. Final phage concentrations averaged to 1 x 10^7 pfu/mL (CEP-DMS3vir-1), 2 x 10^9 pfu/mL (CEP-68-1) and 2 x 10^{10} pfu/mL (CEP-DMS3vir-1 + CEP-68-1). Error bars are measured as 95% confidence intervals.
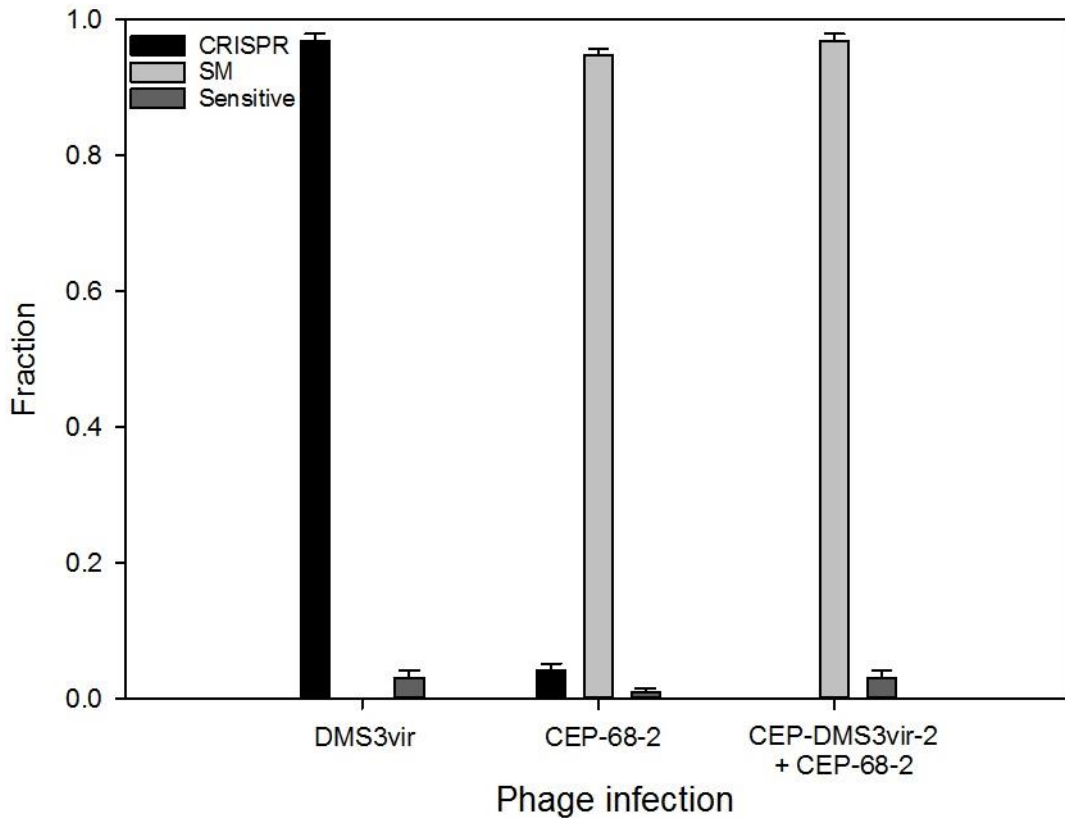
Figure 3: Average fraction of bacterial strain BIM68-2 population immunity and sensitivity at 3dpi. The infections were; escape phage DMS3vir (CEP-DMS3vir-2), escape phage 68 (CEP-68-2), and the combination of escape phage (CEP-DMS3vir-2 and CEP-68-2). Immunity is divided into CRISPR-Cas mediated or *sm*-mediated. Final phage concentrations averaged to 1 x 10^{11} pfu/mL (CEP-DMS3vir-2), 5 x 10^4 pfu/mL (CEP-68-2) and 2 x 10^{11} pfu/mL (CEP-DMS3vir-2 + CEP-68-2). Error bars are measured as 95% confidence intervals.

4. Discussion

Here I show that, despite spacer incorporation occurring with single phage infections of primed strains of *Pseudomonas aeruginosa* PA14, the same strains fail to evolve CRISPR-Cas based immunity upon mixed infections. Instead, surface modification (*sm*) was observed to be the most prevalent means of host resistance. Thus

complementing the results of Chapter 2 where phage diversity triggered *sm*-mediated resistance and not CRISPR-Cas mediated resistance.

Although bacteria consistently failed to evolve CRISPR-Cas immunity against both phages during mixed infections, some spacer incorporation was observed. However, this was directed exclusively against DMS3vir. This result is interesting as spacer acquisition is highly prevalent in single infection of DMS3vir (Westra *et al.* 2015). Yet, when in combination with another phage species, the level of spacer acquisition is greatly reduced. This may not be surprising as, according to the mathematical model by Weinberger *et al.* (2012), the chance of rapid spacer acquisition in a parasite diverse environment is rare. Reduced CRISPR immunity in experimental conditions containing two phages may therefore be accounted for due to the limited chance in the simultaneous acquisition of multiple spacers. The pressure imposed by two simultaneous phage infections may be too great for the adaptive function of the CRISPR-Cas system, and would explain why the evolution of more general resistance by surface modification is observed.

As phages are ubiquitous in nature and can outnumber their hosts by tenfold (Suttle 2005), the probability of encountering multiple different phages is high. Thus, it is important to understand how different infections impact the evolution of different host defences. These results suggest that CRISPR-Cas mediated resistance is limited in the presence of two pilus binding phage. If phage target different receptors, it would be reasonable to predict that the multiple alterations of the cell surface binding sites necessary for resistance would be too great a cost (Frank 2000) in comparison to spacer incorporation by the CRISPR-Cas system. As such, it would be interesting to test the effect of bacterial strains primed to both LPS and pilus binding phages, on mediated resistance and should thus be the subject for future investigation.

As *sm*-mediated resistance was observed to be the most prevalent means of resistance for *P. aeruginosa* it would be interesting to understand how the evolution of this defence mechanism would affect bacterial virulence in a natural setting. For

example, in cystic fibrosis patients, *P. aeruginosa* relies on biofilm formation to spread within its host (Woods *et al.* 1980). Thus, the loss of pili may reduce disease prevalence and limit infection, meaning that phage therapy may benefit from a treatment strategy involving phages that target bacterial receptors used for motility. In a different system, *Pseudomonas syringae* relies on pili to infect their host by means of adhering to plant leaves. When infected with a pilus-specific bacteriophage, *P. syringae* were found to have a reduced ability to adsorb to leaf surfaces due to the loss of their pili (Romantschuk *et al.* 1993). The importance of this structure explains why the adaptive immune system of CRISPR-Cas exists. However, it is still unclear as to which selective forces favour one defence mechanism over the other, when the CRISPR-Cas system is likely to be activated, and whether indeed its sole function lies in host defence.

Chapter 5

The diversity-generating benefits of an adaptive immune system

Abstract

Prokaryotic CRISPR-Cas adaptive immune systems insert spacers derived from viruses and other parasitic DNA elements into CRISPR loci to provide sequence-specific immunity (van der Oost et al. 2014, Barrangou et al. 2007). This frequently results in high within-population spacer diversity (Andersson & Banfield 2008, Paez-Espino et al. 2013, Paez-Espino et al. 2015, Westra et al. 2015), but it is unclear if and why this is important. Here, we show that as a result of this spacer diversity, viruses can no longer evolve to overcome CRISPR-Cas by point mutation, which results in rapid virus extinction. This effect arises from synergy between spacer diversity and the high specificity of infection, which greatly increases overall population resistance. We propose that the resulting short-lived nature of CRISPR-dependent bacteria-virus coevolution has provided strong selection for the evolution of sophisticated virus-encoded anti-CRISPR mechanisms (Bondy-Denomy et al. 2013).

1. Introduction, Results and Discussion

We previously reported that *Pseudomonas aeruginosa* strain UCBPP-PA14 evolves high levels of CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR-associated) adaptive immunity against virus DMS3vir under laboratory conditions (Westra *et al.* 2015). However, viruses can readily evolve to overcome sequence specific CRISPR immunity (Deveau *et al.* 2008, Semenova *et al.* 2011). To study how CRISPR-Cas impacts virus persistence, we measured titres of virus DMS3vir over time upon infection of either wild type (WT) *P. aeruginosa* or a functional CRISPR-Cas knock-out (CRISPR KO) strain. Virus that infected the WT strain went extinct at 5 days post-infection (dpi) (Figure 1A), whereas virus infecting

the CRISPR KO strain persisted in all replicates until the experiment was terminated at 30 dpi (Figure 1B). WT bacteria exclusively evolved CRISPR-mediated immunity, while the CRISPR KO strain evolved immunity by mutation, loss or masking of the receptor (i.e. surface mutation) (Extended Data Figure 1). The observation that CRISPR-Cas drives virus extinct so rapidly was unexpected since viruses can escape CRISPR immunity by a single point mutation (Deveau *et al.* 2008, Semenova *et al.* 2011).

Virus extinction might result from the high level of spacer diversity that naturally evolves upon virus exposure in this and other CRISPR-Cas systems (Andersson & Banfield 2008, Paez-Espino *et al.* 2013, Paez-Espino *et al.* 2015, Westra *et al.* 2015). Both theory and data suggest that host genetic diversity can synergistically reduce the spread of parasites if the infection process is specific (i.e. a parasite genotype can infect a restricted number of host genotypes) and a failed infection results in parasite death (Childs *et al.* 2014, Lively *et al.* 2010, King & Lively 2012, Van Baalen & Beekman 2006, Altermatt & Ebert 2008, Schmid-Hempel & Crozier 1999, Levin *et al.* 2013, Iranzo *et al.* 2013, Keesing *et al.* 2010); assumptions that hold for CRISPR-Cas-virus interactions. While the protective effect of host diversity may be lost following the evolution of single viruses that escape from multiple spacers (Childs *et al.* 2014, Iranzo *et al.* 2013), host diversity has the additional benefit of limiting such viral adaptation. Specifically, lower virus population sizes resulting from host diversity (Lively 2010a, King & Lively 2012) reduce the probability of escape mutations, and the greater the diversity the more escape mutations needed.

To examine these hypotheses, we generated bacterial populations in which we manipulated the level of spacer diversity; we used 48 individual clones with CRISPR-based immunity against virus DMS3vir to generate bacterial populations with five distinct diversity levels: 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 surface mutant (Westra *et al.* 2015) in the presence or absence of virus DMS3vir and virus levels were monitored over time.

This experiment revealed a strong inverse relationship between virus persistence and the level of spacer diversity in the bacterial population (Figure 2). Virus titers remained high in 44 out of 48 replicates when the CRISPR population consisted of a monoculture (Figure 2A). However, as diversity increased, virus persistence decreased (Figures 2B-E) and virus was driven extinct rapidly and reproducibly when the CRISPR population consisted of a 48-clone mixture (Figure 2E).

Next, we examined the fitness consequences of generating spacer diversity. In the absence of virus there was no significant effect of diversity on the relative fitness associated with CRISPR-Cas compared to a resistant surface mutant (Extended Data Figure 2; $F_{1, 52}=3.20$, p=0.08). However, in the presence of virus CRISPR-associated fitness increased with increasing spacer diversity (Figure 3; $F_{4,71}=40.30$ p<0.0001 and Extended Data Table 1), with mean fitness increasing 11-fold from monoculture to the highest diversity population. In monoculture, the CRISPR population was outcompeted by the surface mutant (rel. fitness < 1; T=-11.68, p<0.0001). However, as diversity increased, the CRISPR population consistently outcompeted the surface mutant (rel. fitness > 1; 6-clones: T=3.05, p=0.0093; 12-clones: T=3.95, p=0.0028; 24-clones: T=3.48, p=0.0088; 48-clones: T=3.06, p=0.014; all significant after sequential Bonferroni correction), showing that the generation of spacer diversity is an important fitness determinant of CRISPR-Cas (Figure 3).

Given that all bacterial clones used in the experiment were initially resistant, we hypothesized that the benefit of spacer diversity emerges from an inability of virus to evolve escape mutants. To examine this, 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. As expected, we could not detect escape virus in the ancestral virus (Figure 4A; left column, indicated in green). However, in 43 of the 48 CRISPR

monocultures, virus evolved within 2 days to overcome CRISPR immunity (Figure 4A; indicated in red). For 5 clones no escape virus could be detected, and virus went extinct in 4 of these instances (Figure 4A, asterisks). Three of these 5 clones carried multiple spacers targeting the virus, which limits the emergence of escape virus (Levin *et al.* 2013). The emergence of escape virus decreased as diversity increased to 6, 12, 24 and 48 CRISPR alleles (Figure 4); in the latter, 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 (Extended Data Figure 3). This is because virus needs to overcome multiple spacers in the diverse host population if it is to increase in frequency (Extended Data Figure 4). Consistent with a lack of escape virus emerging against all host genotypes, the spacer content of mixed populations of 6, 12, 24 and 48 clones did not increase between t=0 and t=3 (Wilcoxon Signed Rank p>0.2 for all treatments), whereas monocultures acquired novel spacers in response to emerging escape virus (Wilcoxon Signed Rank W=333, DF=47, p<0.0001; Extended Data Figure 5). These data show that while escape viruses can clearly evolve against most of the clones, escape viruses do not emerge when these clones are mixed.

We hypothesized that the benefit of within-population spacer diversity is because of synergy between the different clones. However, diversity will also increase the chance that a single clone with one or more spacers that the virus is unable to overcome will be present in the population. Indeed, we observed 5 clones against which escape mutants were never detected, and presence of these clones in many of the diverse populations could explain the fitness advantage of diversity. To investigate if synergy plays an important role in the benefit of diversity beyond this "jackpot" effect, we compared the fitness of diverse populations with the fitness of the fittest constituent clone, as measured in monoculture. This analysis revealed that synergism contributed an approximately 50% growth rate advantage when in competition with surface mutants (Mean ± SEM difference in fitness between mixtures and fittest constituent monoculture = 0.47 ± 0.18; P < 0.01).

The short-lived nature of coevolution between CRISPR-resistant bacteria and virus escape mutants beyond a host diversity threshold may explain the evolution of sophisticated anti-CRISPR mechanisms to overcome CRISPR-Cas (Bondy-Denomy *et al.* 2013). Indeed, a virus carrying an anti-CRISPR gene (Bondy-Denomy *et al.* 2013) was found to persist independent of CRISPR diversity levels (Extended Data Figures 6A-B) and caused similar extinction of CRISPR-resistant monocultures and 48-clone populations that competed against a surface mutant (Fisher's exact test, p=1.0 at t=1, p=0.33 at t=3 dpi; Extended Data Figure 6C).
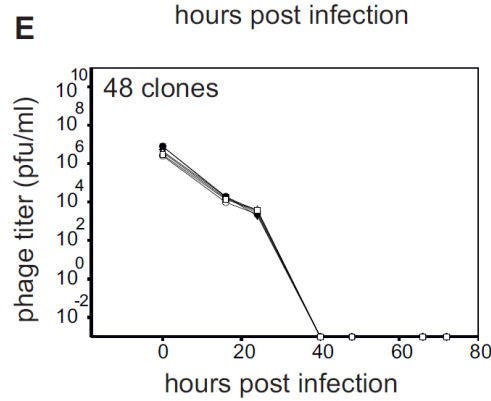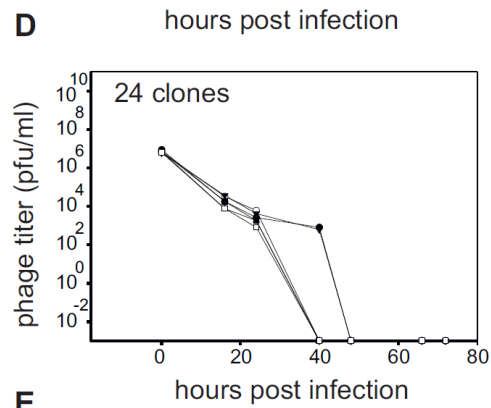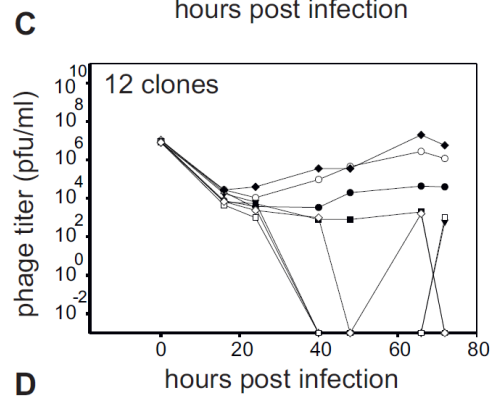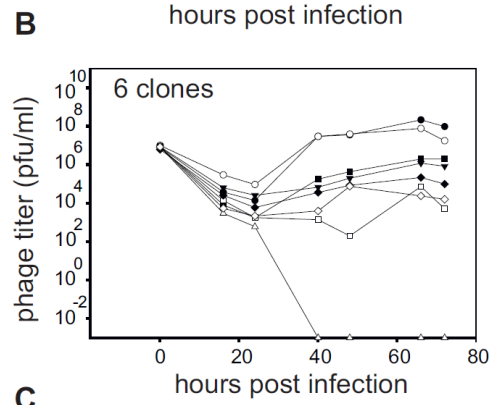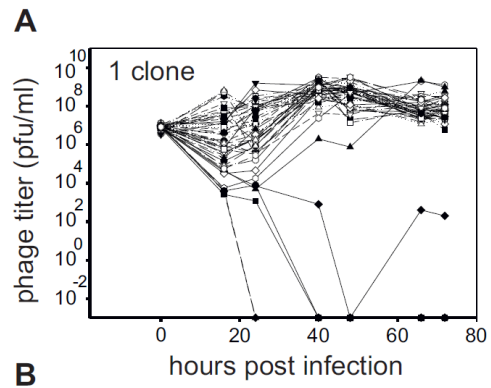
Finally, to test that our results were not limited to the *P. aeruginosa* PA14 Type I-F CRISPR-Cas system, we performed a similar experiment with *Streptococcus thermophilus* DGCC7710 clones that evolved resistance against virus 2972 using a Type II-A CRISPR-Cas system. As shown in Extended Data Figure 7, we found a similar effect of CRISPR resistance allele diversity on virus persistence and escape virus emergence. However, during coevolution experiments the levels of evolved diversity are lower in *S. thermophilus* (data not shown and Paez-Espino *et al.* 2013, and Paez-Espino *et al.* 2015), which, consistent with theory (Childs *et al.* 2014, Iranzo *et al.* 2013), allows for more persistent coevolution (Paez-Espino *et al.* 2013, Paez-Espino *et al.* 2015). Lower levels of evolved spacer diversity might be due to a more weakly primed CRISPR-Cas system (Datsenko *et al.* 2012, Swarts *et al.* 2012, Fineran *et al.* 2014).

Collectively, our data demonstrate that the propensity to generate host genetic diversity is a key fitness determinant of CRISPR-Cas adaptive immune systems because it limits the emergence of escape virus. Consistent with the idea that it is harder for a parasite to adapt to a heterogeneous host population (Hamilton *et al.* 1990), virus rapidly evolved high levels of infectivity on monocultures, but not on a diverse mix of the same host genotypes. Parasites are often invoked as the selective force driving the evolution of diversity generating mechanisms (Hamilton *et al.* 1990, Pal *et al.* 2007, Morran *et al.* 2011, Howard & Lively 1994, Ashby & King 2015). In most cases, individual-level selection is assumed to be the driver of these traits, because individual benefits are high, and group selective benefits would be opposed

by the invasion of individuals who do not pay the fitness costs associated with these mechanisms (e.g. sex and increased mutation rates) (Ashby & King 2015, Lively 2010b, Peters & Lively 1999). In the case of CRISPR-Cas, we speculate that population-level selection may have contributed to its evolution. First, there were large benefits associated with synergy between diverse genotypes. Second, costs of CRISPR-Cas are conditional on virus exposure (Westra *et al.* 2015, Vale *et al.* 2015) and clones lacking CRISPR immunity cannot invade populations (Extended Data Figures 8-11). Third, the highly structured nature of bacterial populations, and the resulting high relatedness, promotes between-population selection (Gardner & Grafen 2009). Future tests of this hypothesis are needed to reconcile the selective forces that have shaped the evolution of CRISPR-Cas systems.
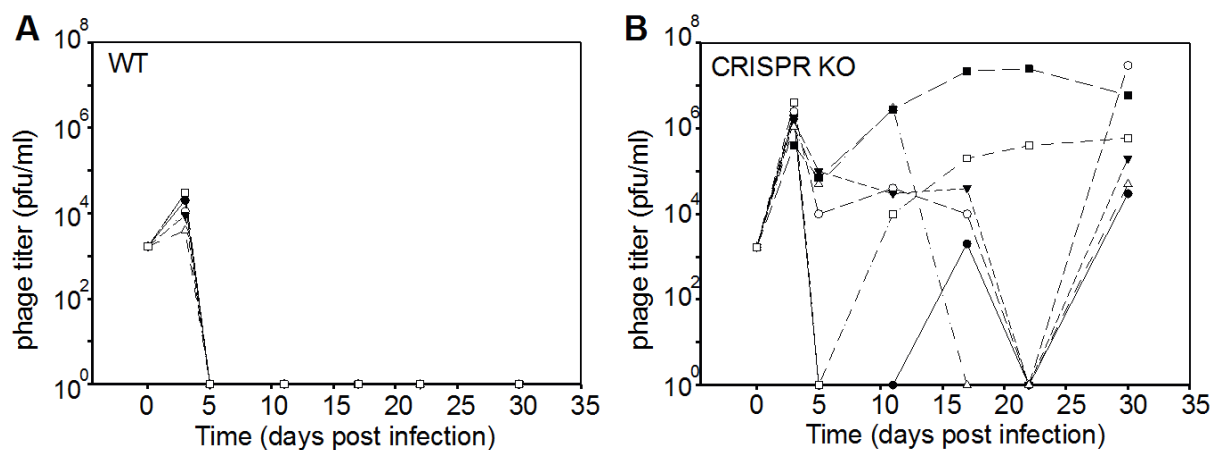
Figure 1: Evolution of CRISPR-mediated immunity leads to rapid extinction of virus. Titre (pfu/ml) of virus DMS3vir over time upon infection of **A)** WT *P. aeruginosa* and **B)** *P. aeruginosa* strain *csy3::LacZ* (CRISPR KO strain). Each line indicates an individual replicate experiment (n=6). The limit of detection is 200 pfu/mL.

A

1 clone

phage titer (pfu/ml)

hours post infection

B

6 clones

phage titer (pfu/ml)

hours post infection

C

12 clones

phage titer (pfu/ml)

hours post infection

D

24 clones

phage titer (pfu/ml)

hours post infection

E

48 clones

phage titer (pfu/ml)

hours post infection

Figure 2: Virus persistence inversely correlates with the level of spacer diversity. Virus titres (pfu/ml) over time upon infection of a bacterial population consisting of an equal mixture of a surface mutant and **A)** a monoculture with CRISPR-mediated immunity (n=48), or polycultures with CRISPR-mediated immunity consisting of **B)** 6 clones (n=8), **C)** 12 clones (n=8), **D)** 24 clones (n=6), **E)** 48 clones (n=6). The number of replicates is chosen such that all clones are equally represented in each treatment. Each line indicates an individual replicate experiment. The limit of detection is 200 pfu/ml.
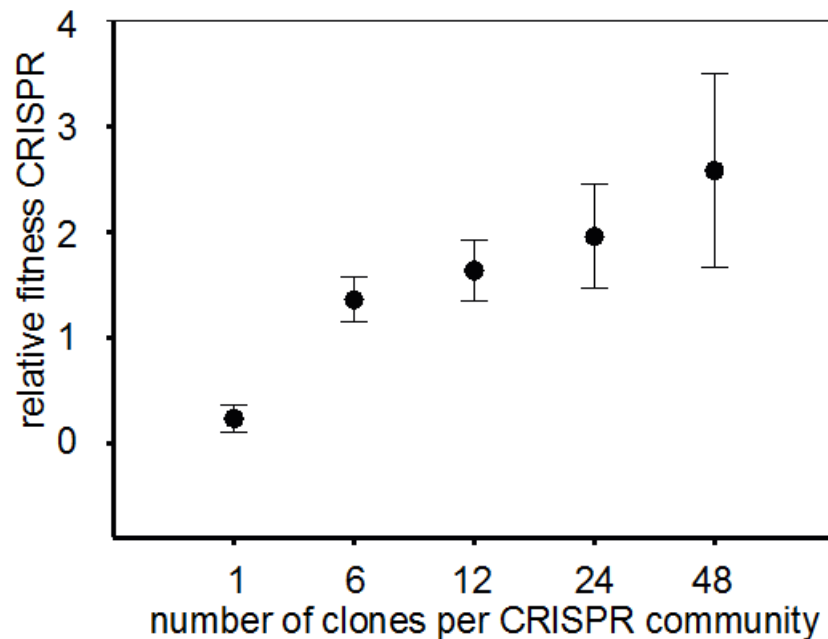
Figure 3: Relative fitness of bacterial populations with CRISPR-mediated immunity positively correlates with increasing spacer diversity. Relative fitness of bacterial populations with CRISPR-mediated immunity, with spacer diversity as indicated, at 3 days post infection when competing with a surface mutant. Error bars indicate 95% confidence intervals.
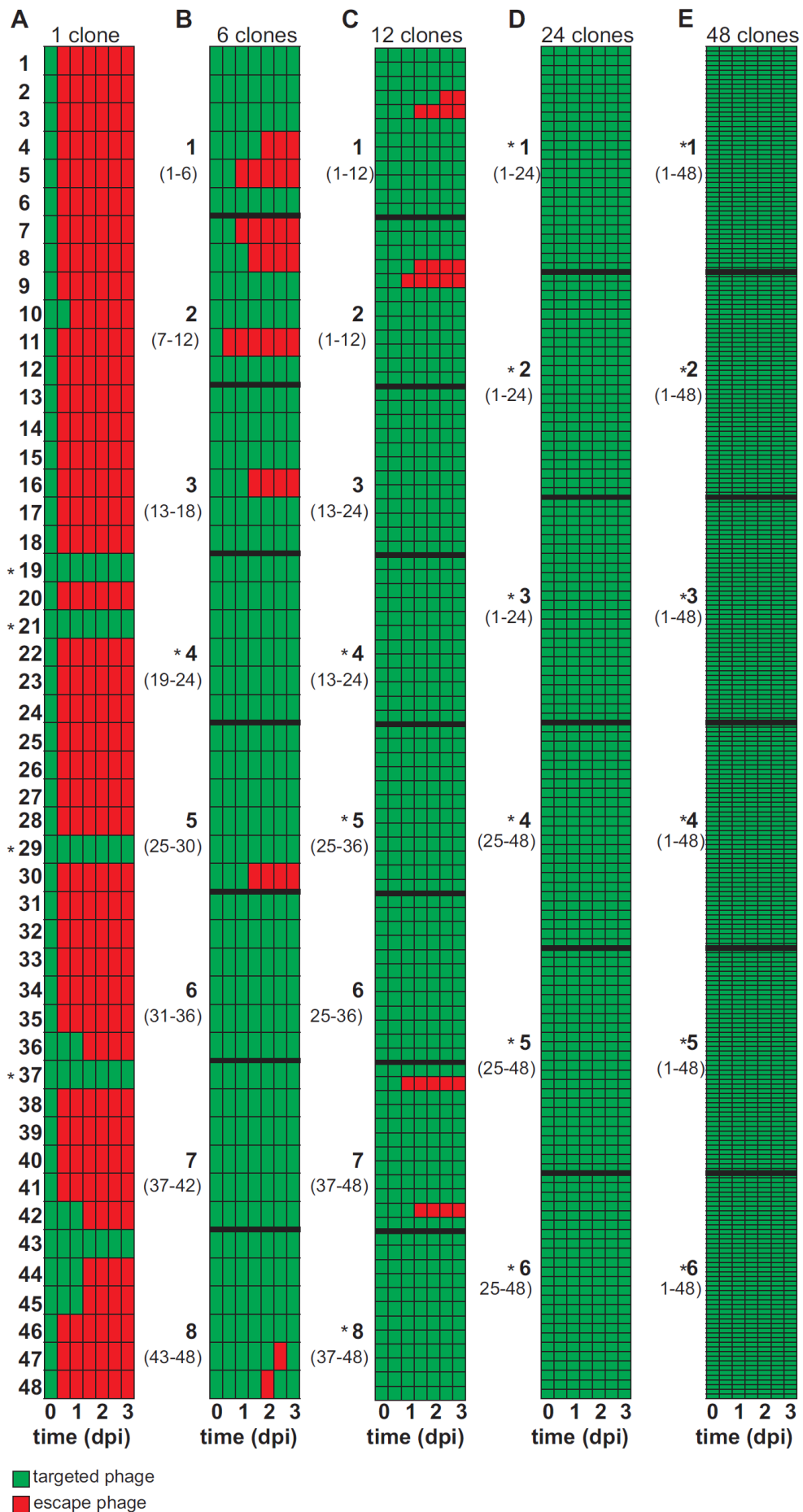
targeted phage
escape phage

Figure 4: Emergence of virus that overcomes host CRISPR immunity (escape virus) during the experiment shown in Figures 2 and 3. Each column in a table represents a time point where virus was isolated (0. 16, 24, 40, 48, 64 and 72 hours post infection, as indicated below the table (in days post infection)). Green: no escape virus. Red: escape virus. Panels A-E correspond to each of the experiments shown in Figure 2 A-E. Bold numbers indicate replicate experiments. Numbers between parentheses indicate the identity of the clones that are present in the CRISPR population. Asterisks indicate that virus went extinct during the experiment.

2. Material and Methods

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 Westra *et al.* (2015) and references therein. Phage DMS3vir+*acrF1*, which carries the anti-CRISPR gene *acrF1* (formerly 30-35), was made by inserting *acrF1* into the DMS3vir genome using methods described in Bondy-Denomy *et al.* (2013). *Streptococcus thermophilus* strain DGCC7710 and its virus 2972 has been described in Barrangou *et al.* (2007).

Coevolution experiments

The coevolution experiments shown in Figure 1 were performed in glass microcosms by inoculating 6 ml M9 supplemented with 0.2% glucose with approximately 10^6 colony forming units (cfu) bacteria from fresh overnight cultures of the WT *P. aeruginosa* UCBPP-PA14 or CRISPR KO strain and adding 10^4 plaque forming units (pfu) of virus DMS3vir, followed by incubation at 37 ºC while shaking at 180 rpm (6 replicates). Cultures were transferred daily 1:100 to fresh broth. Virus titers were determined at 0, 3, 5, 11, 17, 22 and 30 days after the start of the coevolution experiment by spotting virus samples isolated by chloroform extraction on a lawn of CRISPR KO bacteria. The analysis of virus immunity was performed by cross-streak

assay and PCR as described previously (Westra *et al.* 2015).

Generation of populations with different levels of CRISPR diversity

For the competition experiments, shown in Figures 2-4 and Extended Data Figures 2-6 and 8-11, we generated *P. aeruginosa* populations with varying levels of CRISPR spacer (allele) diversity. To this end, we isolated from the 6 replicates of the coevolution experiment (Figure 1) a total of 48 individual clones that had acquired CRISPR immunity against virus DMS3vir. We have previously shown that individual clones tend to have unique spacers (Westra *et al.* 2015). 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 (details of the composition of each population can be found below, under "number of replicate experiments").

Competition experiments

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 CRISPR population and either the surface mutant (Figures 2-4 and Extended Data Figures 2, 4-6, 8) or the CRISPR KO strain (Extended Data Figures 7-11). At the start of each experiment 10^9 pfu of virus 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 µg·ml^{-1} X-gal (to allow discrimination between WT-derived CRISPR clones (white) and CRISPR KO or surface mutant (blue)). The relative frequencies of the WT strain were used to calculate the relative fitness (rel. fitness = [(fraction strain A at t=x) * (1 − (fraction strain A at t=0))] / [(fraction strain A at t=0) * (1 − (fraction strain A at t=x)]). At 0, 16, 24, 40, 48, 66 and

72 hpi, samples were taken and chloroform extractions were performed to isolate total virus, which was spotted on a lawn of CRISPR KO bacteria for quantification. All subsequent statistical analyses were carried out using JMP (v12) software.

Determination of escape virus emergence

To determine the emergence of escape virus during the competition experiments, every isolated virus sample was spotted onto 48 different bacterial lawns, corresponding to each of the different CRISPR clones. 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 Figures 2-4 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 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.

Determining the acquisition of new spacers

To examine spacer acquisition during the competition experiments shown in Figures 2-4, we examined by PCR for each diversity treatment the spacer content of 384 randomly isolated clones at both t=0 and t=3 (192 clones per time point). For each replicate experiment, the difference in the total number of spacers between t=0 and t=3 was divided by the number of clones that were examined to calculate the average change in the number of spacers per clone.


Number of replicate experiments

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 6 for sufficient statistical power. Hence, competition experiments with the 1-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 4 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 2 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).

Escape phage degradation and fitness

In the experiment shown in Extended Data Figure 3, approximately 10^8 pfus of either ancestral virus or escape virus, which was isolated from the competitions between monocultures 1-6 and the surface mutant, was used to infect a monoculture of the corresponding CRISPR clone or the 48-clone polyculture. Phage samples were taken at 0, 9, 20 and 28 hpi by chloroform extraction and titrated on a lawn of the CRISPR KO strain. Fitness of each of the escape phages was determined by a competition experiment between ancestral and escape virus; a 50:50 ratio of escape and ancestral phage (10^9 pfus total) was used to infect either a monoculture of the corresponding CRISPR clone or the 48-clone polyculture. Virus samples were taken at t=0 and t=20 hpi by chloroform extraction and used in a plaque assay on CRISPR KO. Next, individual plaques (48 plaques per replicate) were isolated and amplified on the CRISPR KO strain. To determine the ratio of escape and ancestral virus, virus from each individual plaque was spotted on a lawn of *1*) CRISPR KO (both ancestral and escape virus form plaques) and *2*) the corresponding CRISPR immune clone (only escape virus can form a plaque).

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 Barrangou *et al.* (2007). A total of 44 individual clones 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.
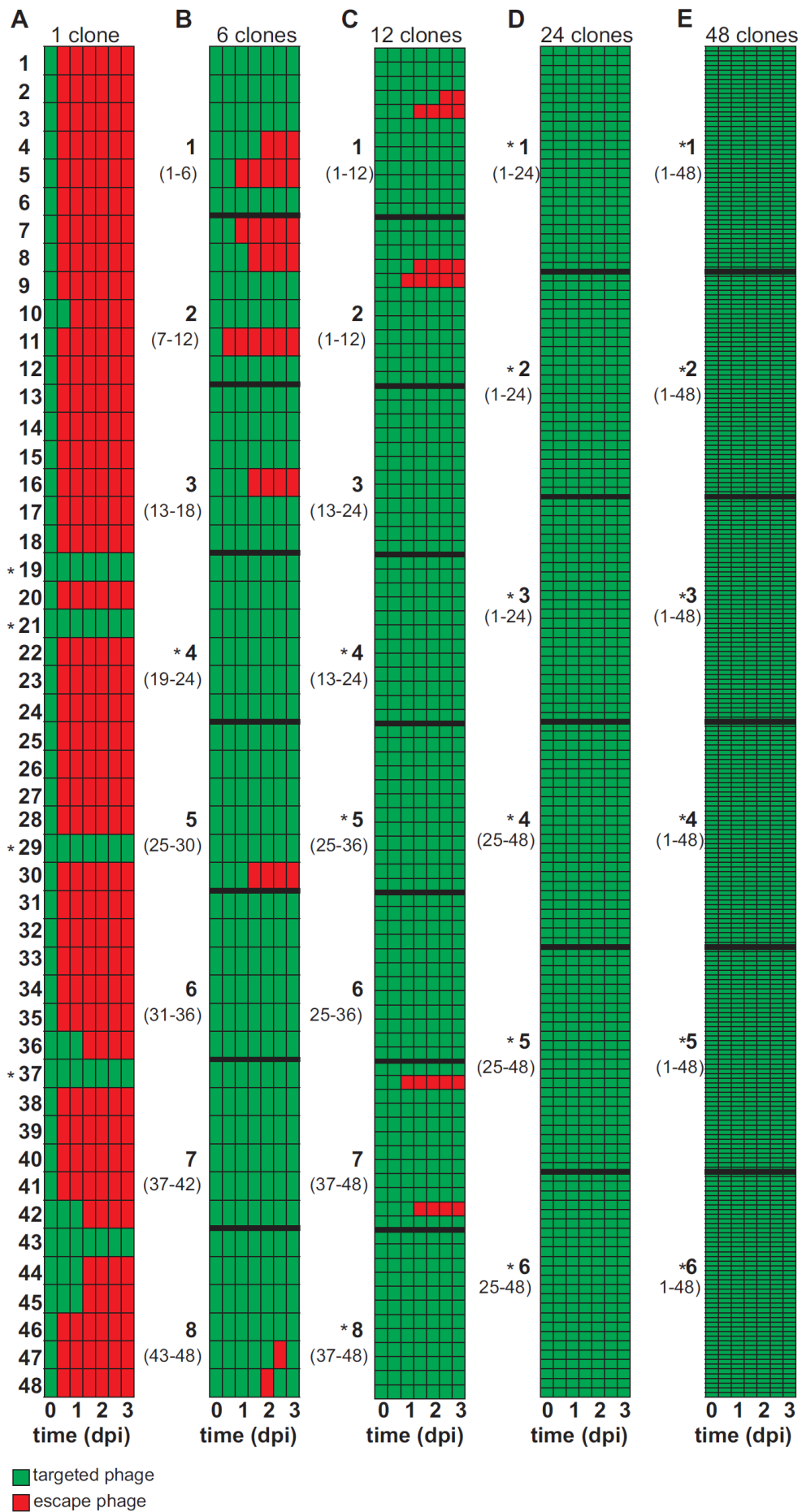
Figure 4: Emergence of virus that overcomes host CRISPR immunity (escape virus) during the experiment shown in Figures 2 and 3. Each column in a table represents a time point where virus was isolated (0, 16, 24, 40, 48, 64 and 72 hours post-infection, as indicated below the table (in days post-infection)). Green: no escape virus. Red: escape virus. Panels A-E correspond to each of the experiments shown in Figure 2 A-E. Bold numbers indicate replicate experiments. Numbers between parentheses indicate the identity of the clones that are present in the CRISPR population. Asterisks indicate that virus went extinct during the experiment.

General discussion

In the face of pathogen infection, hosts have evolved a wide array of defence mechanisms. In particular, bacteria can adapt to threats using at least five different mechanisms within the ranges of intracellular and extracellular defences, from surface modification, the restriction modification system, abortive infection, and invader uptake block to the CRISPR-Cas system. While the molecular mechanisms of these immune strategies have been explored in great detail, there is limited knowledge about which selective forces favour one mechanism over the other. A recent study revealed how the risk of infection can drive the evolution of constitutive (surface modification) versus inducible (CRISPR-Cas) defences (Westra *et al.* 2015). While co-evolution experiments between a single bacterial species and single lytic phages are crucial for examining elicited immune responses, they tell us little about host defence evolution in a natural setting where phage diversity may exist.

This thesis has endeavoured to understand the effects of different phage conditions on the evolution of the CRISPR-Cas system in the pathogenic bacterial host *Pseudomonas aeruginosa.* Although it is not conclusive, I provide novel evidence that bacteria primed to multiple phages, will not necessarily continue to prime in parasite diverse conditions (Chapter 4). Indeed, by exploring naive (Chapter 2) as well as primed bacteria (Chapter 4), I found that increased phage diversity consistently favoured the evolution of surface modification based resistance. Understanding the evolution of this defensive mechanism is important as the implications that result from the modifications to cell surfaces affect the bacteria's ability to colonize and infect its host. *P. aeruginosa* adherence to the human upper respiratory epithelium of cystic fibrosis patients is mediated through pili and allows the formation of biofilms which are fundamental in the spread of infection (Woods *et al.* 1980, O'Toole & Kolter 1998). Thus, the loss or modification of this structure will affect disease spread. Therefore, the application of phages that trigger pili loss could, potentially, be useful in developing new methods of phage therapy in biofilm forming bacterial pathogens.

Host resistance by surface modification or CRISPR-Cas have different implications for both ecological and evolutionary interactions between bacteria and phage. Specifically, we have shown how phage persists in conditions where host populations evolve resistance by surface modification, whilst being rapidly driven extinct when bacteria evolve CRISPR-Cas immunity (Chapter 5). This effect emerges from the high levels of CRISPR allele diversity that naturally evolves in initially clonal populations, making it harder for phage to overcome host resistance due to the number of diverse host genotypes. We have been able to demonstrate that, where bacterial monocultures suffer high parasite infectivity, with increasing CRISPR polycultures, parasite evolution is limited and eventually driven extinct. This highlights the importance of the production of genetic diversity in increasing herd immunity and decreasing the spread of disease (King & Lively 2012); a concept which has been demonstrated in crop management where genetically diverse rice crops limit the severity of rice blast disease outbreak in comparison to genetically homogenous crops (Zhu *et al.* 2000).

Little is known about what conditions favour CRISPR-Cas systems in nature. However, we know that priming is important in the *P. aeruginosa* - bacteriophage system (Chapter 3) as well as in other systems (Datsenko *et al.* 2012, Li *et al.* 2013, Richter *et al.* 2014). As priming is important, relatedness between phages is advantageous to the bacterial host. Spacers targeting one phage species may, in some cases, offer partial matches to related phage species. As a result, rapid spacer acquisition will occur to the related phage species (Andersson & Banfield 2008). This leads to the speculation that CRISPR-Cas systems may be favoured in spatially structured environments as it produces high levels of spacer diversity (Chapter 5, Haerter & Sneppen 2012). High relatedness between locally adapted phages means that diversity generated by the CRISPR-Cas system will be beneficial to the local host. This is shown in Chapter 5 with CRISPR driving the local phage population to extinction. Harsh environmental conditions, such as acid mine drainage sites, could also favour the evolution of CRISPR-Cas immunity due to low population densities and limited species richness of both hosts and phages (Baker & Banfield 2003). Indeed, these environments allow for more intense host-parasite interactions. As

such, metagenomic analyses of biofilms forming these communities reveal active CRISPR-Cas evolution (Denef *et al.* 2010).

In conclusion, my thesis has aimed to draw attention to the different effects that familiar, novel, and diverse phage populations have on shaping the evolution of resistance in their bacterial host. While research into the molecular biology of the CRISPR-Cas system is booming, there is little understanding of what environmental factors affect the evolution of this defence strategy. The knowledge of how multiple pathogens can affect the evolution of host defence strategies is still limited, and continued research is vital in light of the development of therapeutic application of phages to reduce the spread of bacterial disease (Debarbieux *et al.* 2009). Finally, the importance of priming as well as naive resistance will help to establish how often, and under what conditions, CRISPR-Cas is mediated in natural environments. Further investigation into the ecological pressures responsible for the evolution of CRISPR-Cas immunity could ultimately lead to a greater understanding of host-parasite arms races.

Appendix

Extended data Chapter 5

Extended Data Table 1: Tukey HSD all pairwise comparisons of the data in Figure 3. 1 = monoculture, 6 = 6-clone polyculture, 12 = 12-clone polyculture, 24 = 24-clone polyculture, 48 = 48-clone polyculture

| Comparison		Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
1	6	-1.12680	0.2141986	-5.26	<.0001*	-1.72637	-0.52724
1	12	-1.40303	0.2141986	-6.55	<.0001*	-2.00259	-0.80346
1	24	-1.72790	0.2428783	-7.11	<.0001*	-2.40775	-1.04806
1	48	-2.35252	0.2428783	-9.69	<.0001*	-3.03236	-1.67267
6	12	-0.27622	0.2804518	-0.98	0.8612	-1.06124	0.50879
6	24	-0.60110	0.3029225	-1.98	0.2842	-1.44901	0.24682
6	48	-1.22571	0.3029225	-4.05	0.0012*	-2.07363	-0.37780
12	24	-0.32488	0.3029225	-1.07	0.8200	-1.17279	0.52304
12	48	-0.94949	0.3029225	-3.13	0.0205*	-1.79741	-0.10158
24	48	-0.62462	0.3238378	-1.93	0.3119	-1.53108	0.28184
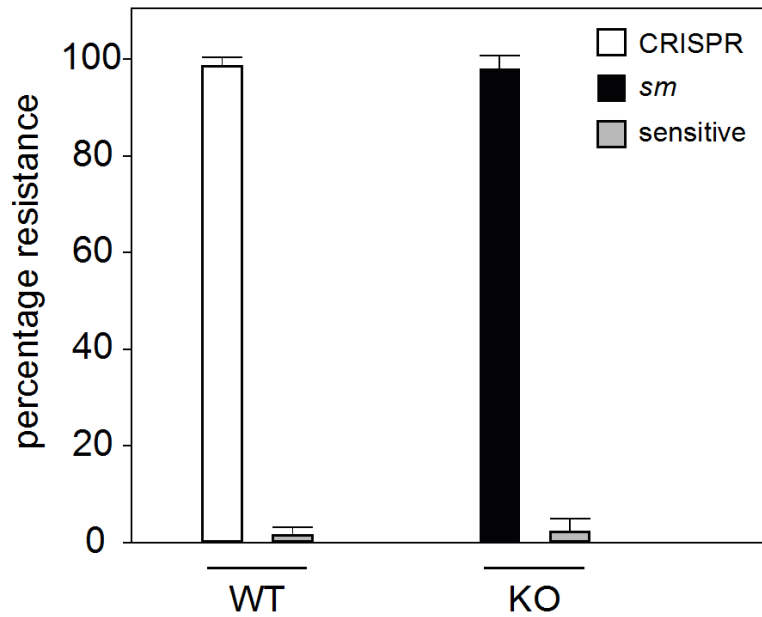
Extended data Figure 1: Infection with virus DMS3vir leads to rapid evolution of CRISPR-mediated immunity in WT bacteria, while CRISPR KO bacteria primarily evolve virus immunity by surface mutation. Percentage bacteria at 5 days post-infection that have evolved immunity by CRISPR-Cas (white bar), surface mutation (black bar) or that have not evolved immunity (sensitive; grey bars). Error bars indicate 95% confidence intervals (CI).
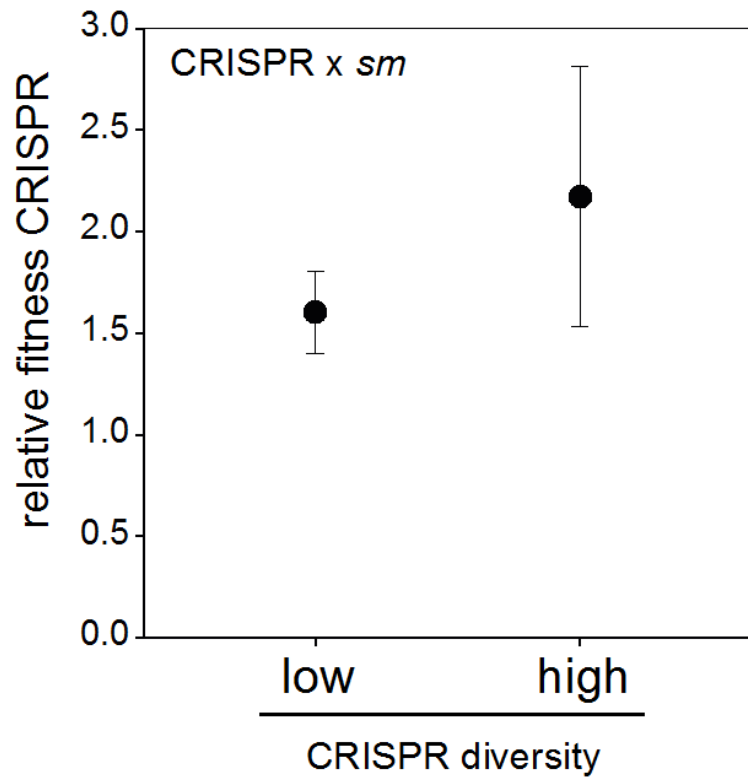
Extended data Figure 2: No benefit of increasing spacer diversity in the absence of virus. Relative fitness of CRISPR immune monocultures (single spacer; low diversity) and polycultures (48 spacers; high diversity) at 3 days post-infection when competing with a surface mutant (*sm*) in the absence of virus. Error bars indicate 95% CI.
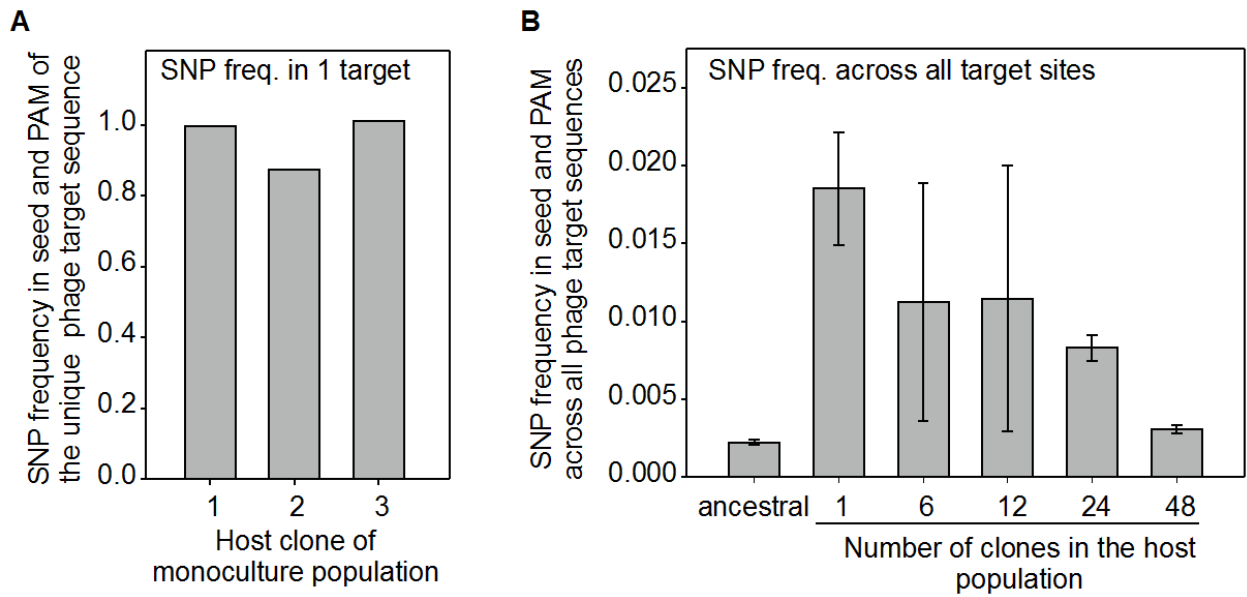
Extended data Figure 3: Deep sequencing analysis of the frequency of mutations in the target sequence (seed sequence and the adjoining PAM) of virus isolated at t=1 from the experiment shown in Figure 4. **A)** Frequency of point mutation in the single target sequence of a viral population isolated from the monocultures of clones 1-3. **B)** 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 replicates from a single diversity treatment. Error bars indicate 95% (CI).
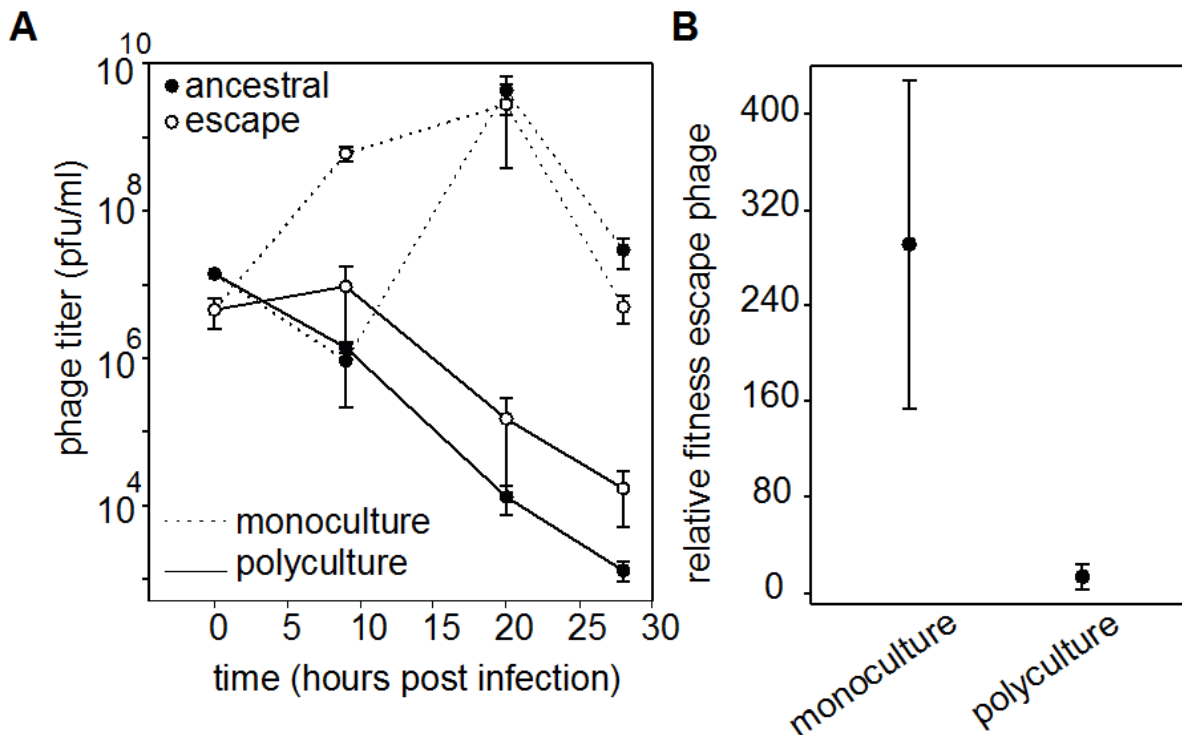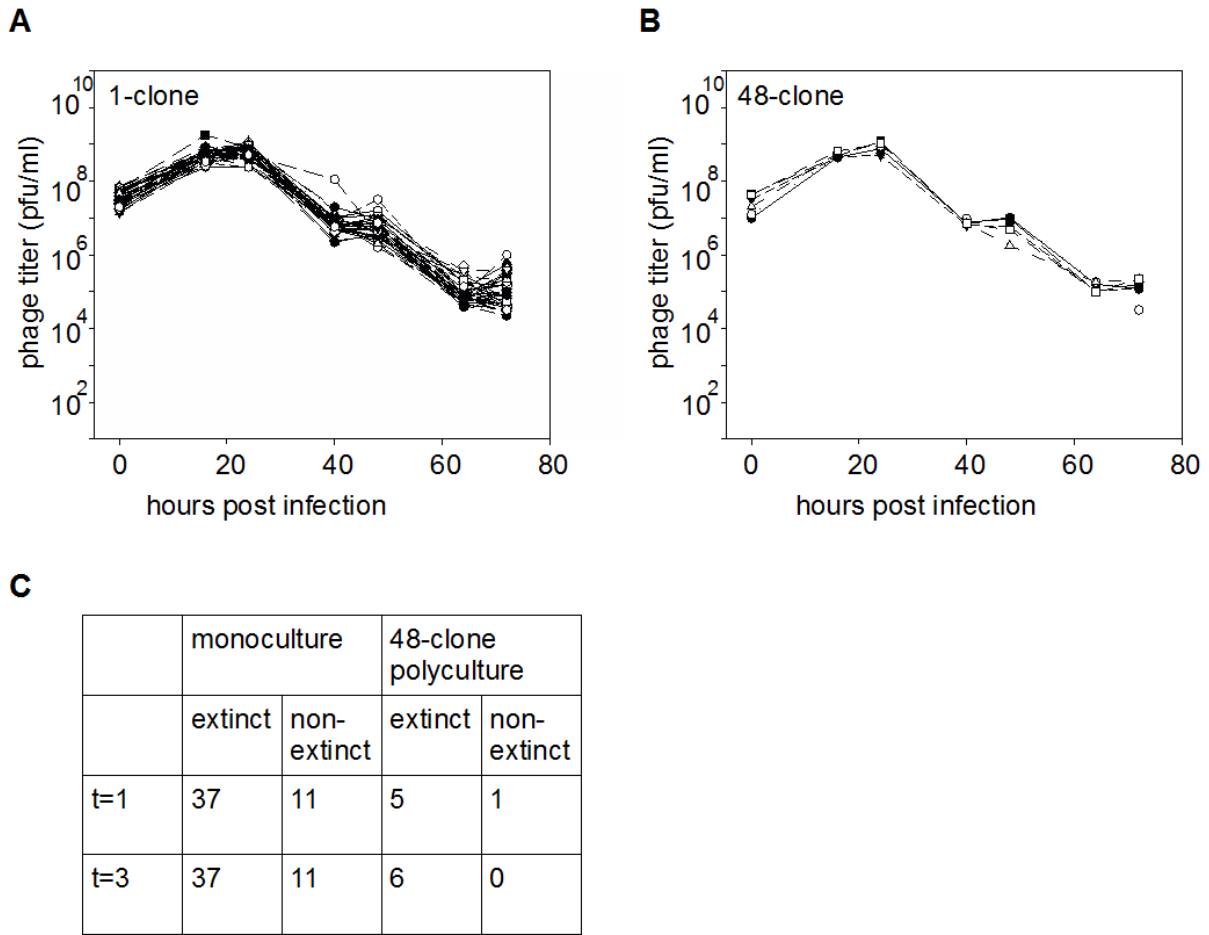
Extended data Figure 4: Escape virus titres decline upon infection of diverse CRISPR populations despite increased fitness over ancestral virus. Escape virus was isolated from monocultures of clones 1-6 competing with the surface mutant at 24 hpi (Figure 3 and Extended Data Figure 2). **A)** Virus titres (pfu/ml) over time upon infection with approximately 10^7 pfu individual escape virus or ancestral virus of a bacterial population consisting of a monoculture (dotted line) or 48-clone polyculture (solid line). **B)** Relative fitness of escape virus and ancestral virus during infection of a CRISPR resistant monoculture or polycultures consisting of 48 clones. All experiments were performed in 6 replicates. Error bars indicate 95% CI. The limit of detection is 200 pfu/ml.
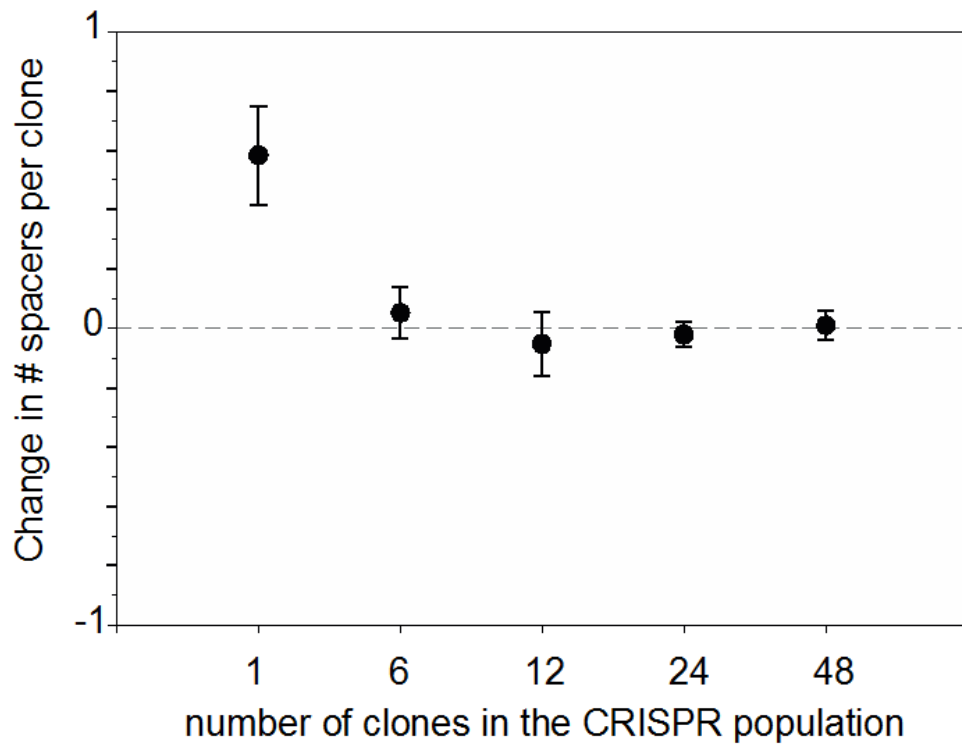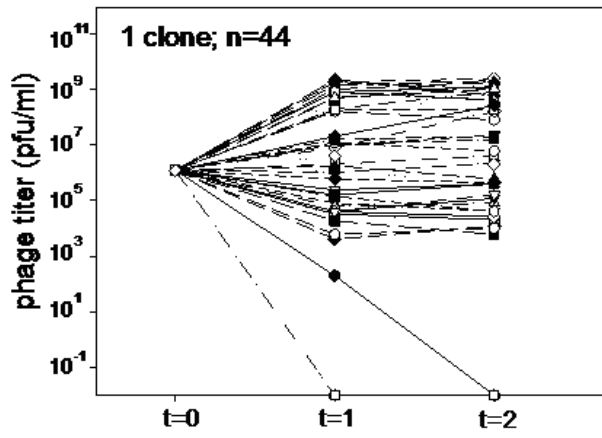
Extended data Figure 5: Diverse populations do not acquire additional spacers during the experiments shown in Figures 2-4. For each diversity treatment we examined the spacer content of 192 randomly isolated clones at both t=0 and t=3 (384 clones in total per diversity treatment). The change in the total number of spacers between t=0 and t=3 was calculated independently for each replicate experiment and divided by the number of clones that were examined. The graph indicates the average across the replicates of the change in spacer content per clone and error bars indicate 95% CI.

A

B

C

	monoculture		48-clone polyculture	
	extinct	non-extinct	extinct	non-extinct
t=1	37	11	5	1
t=3	37	11	6	0

Extended data Figure 6: Persistence of phage that encodes an anti-CRISPR gene is independent of spacer diversity. **A)** Virus titres (pfu/ml) over time upon infection of a bacterial population consisting of an equal mixture of a surface mutant and **A)** a monoculture with CRISPR-mediated immunity (n=48) or **B)** a 48-clone polyculture with CRISPR-mediated immunity (n=6). Each clone is equally represented in each treatment. Each line indicates an individual replicate experiment. The limit of detection is 200 pfu/ml. **C)** The number of replicate experiments in which the CRISPR immune population went extinct (no detectable white colonies) at 1 and 3 dpi.

A
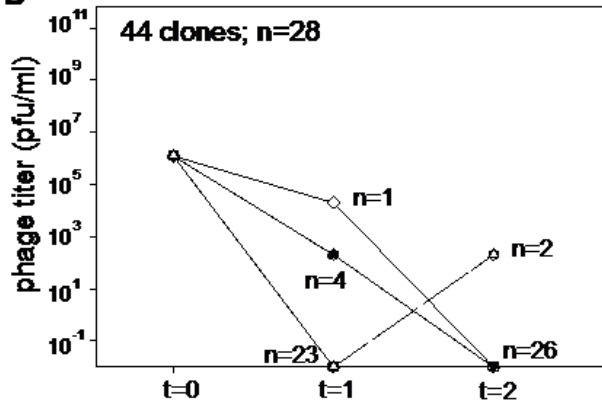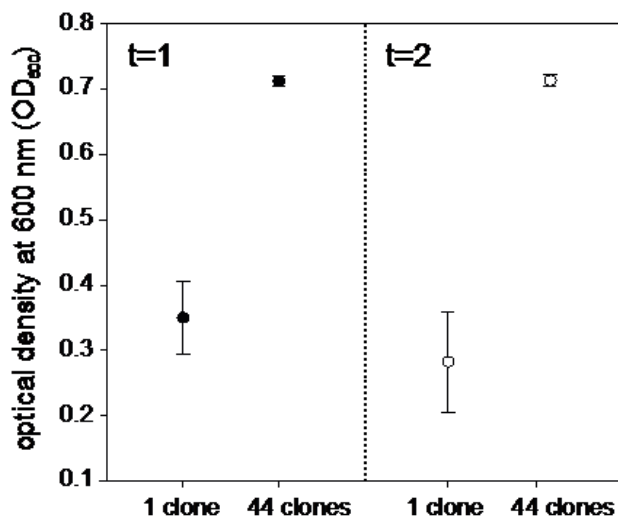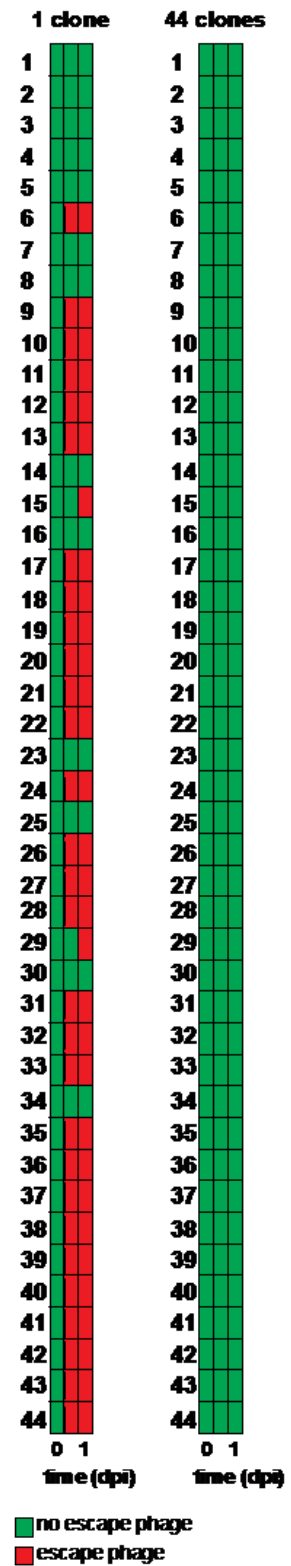
1 clone; n=44

phage titer (pfu/ml)

10^{11}
10^9
10^7
10^5
10^3
10^1
10^{-1}

t=0   t=1   t=2

B

44 clones; n=28

phage titer (pfu/ml)

10^{11}
10^9
10^7
10^5
10^3
10^1
10^{-1}

n=1
n=4
n=2
n=23
n=26

t=0   t=1   t=2

C

optical density at 600 nm (OD_{600})

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

t=1   t=2

1 clone 44 clones 1 clone 44 clones

D

1 clone   44 clones

0 1   0 1
time (dpi)   time (dpi)

▉ no escape phage
▉ escape phage

98

Extended data Figure 7: Virus persistence inversely correlates with the level of CRISPR spacer diversity in CRISPR immune populations of *Streptococcus thermophilus*. Virus titres (pfu/ml) over time upon infection of a bacterial population consisting of **A)** a monoculture with CRISPR-mediated immunity (n=44) or **B)** 44-clone polycultures with CRISPR-mediated immunity (n=28). Each clone is equally represented in each treatment. Each line indicates an individual replicate experiment. The limit of detection is 200 pfu/ml. **C)** OD600 of monocultures and polycultures at 1 and 2 days post infection. Error bars indicate 95% confidence intervals. **D)** Emergence of virus mutants that overcome CRISPR-mediated immunity after 0, 16, and 24 hours post-infection. Green indicates no escape virus. Red indicates emergence of escape virus. All polyculture experiments showed no escape virus.
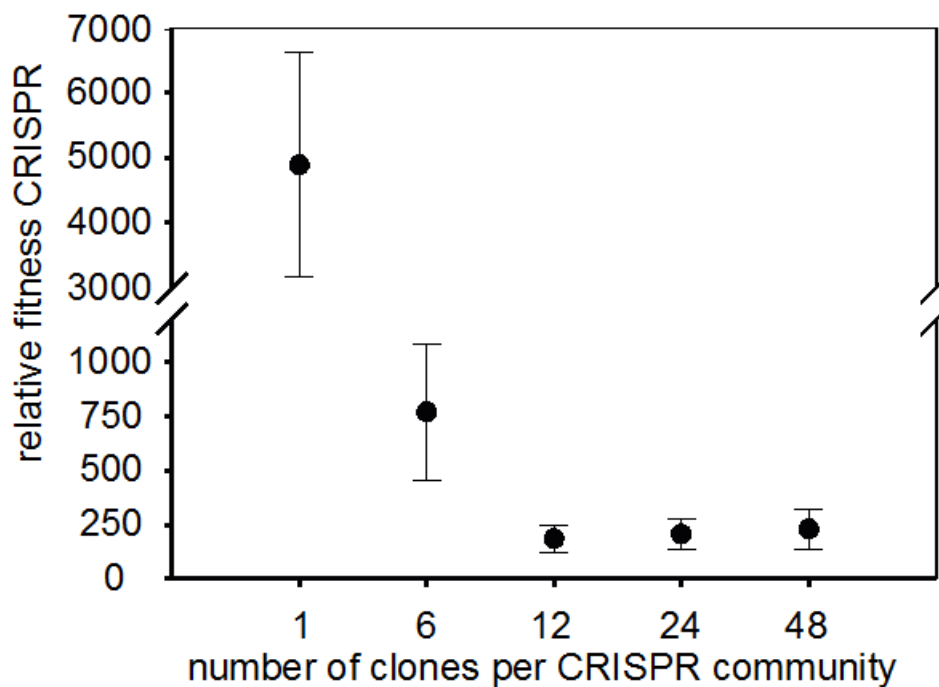
Extended data Figure 8: Sensitive bacteria are unable to invade bacterial populations with CRISPR-mediated immunity in the presence of virus. Relative fitness of CRISPR populations with indicated spacer diversity at 3 days post-infection when competing with the sensitive CRISPR KO strain. Relative fitness of CRISPR populations decreases with increasing spacer diversity due to the rapid virus extinction, which benefits sensitive bacteria, but is higher than 1 in all cases. Error bars indicate 95% CI.
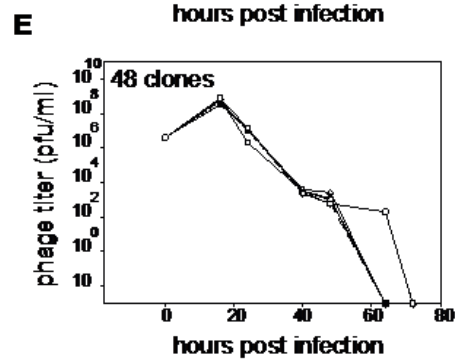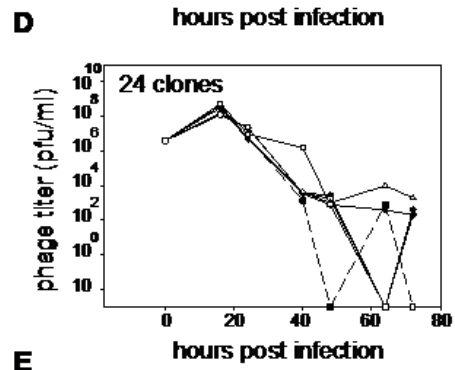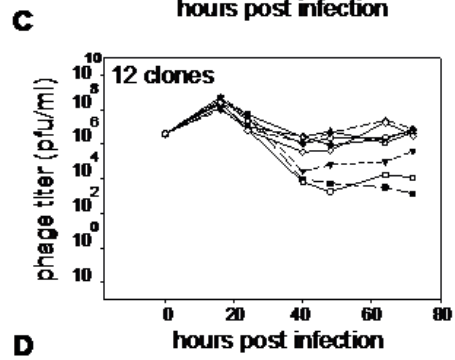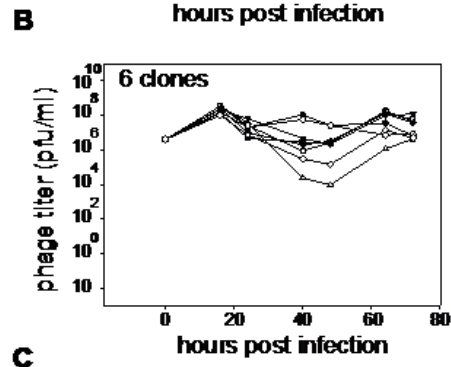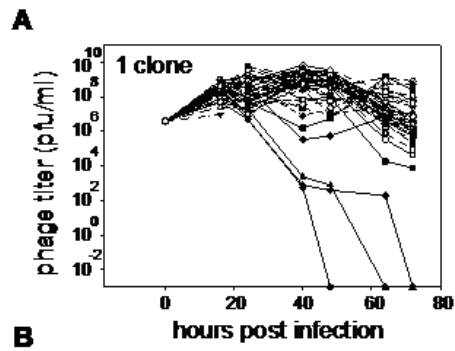
A

B

C

D

E

100

Extended data Figure 9: Virus persistence inversely correlates with the level of CRISPR spacer diversity during competition between CRISPR immune populations and the sensitive CRISPR KO strain. Virus titres (pfu/ml) over time upon infection of a bacterial population consisting of an equal mixture of a CRISPR KO clone and **A)** a monoculture with CRISPR-mediated immunity (n=48), or polycultures with CRISPR-mediated immunity consisting of **B)** 6 clones (n=8), **C)** 12 clones (n=8), **D)** 24 clones (n=6), **E)** 48 clones (n=6). The number of replicates is chosen such that all clones are equally represented in each treatment. Each line indicates an individual replicate experiment. The limit of detection is 200 pfu/ml.

A 1 clone
B 6 clones
C 12 clones
D 24 clones
E 48 clones

targeted phage
escape phage

102
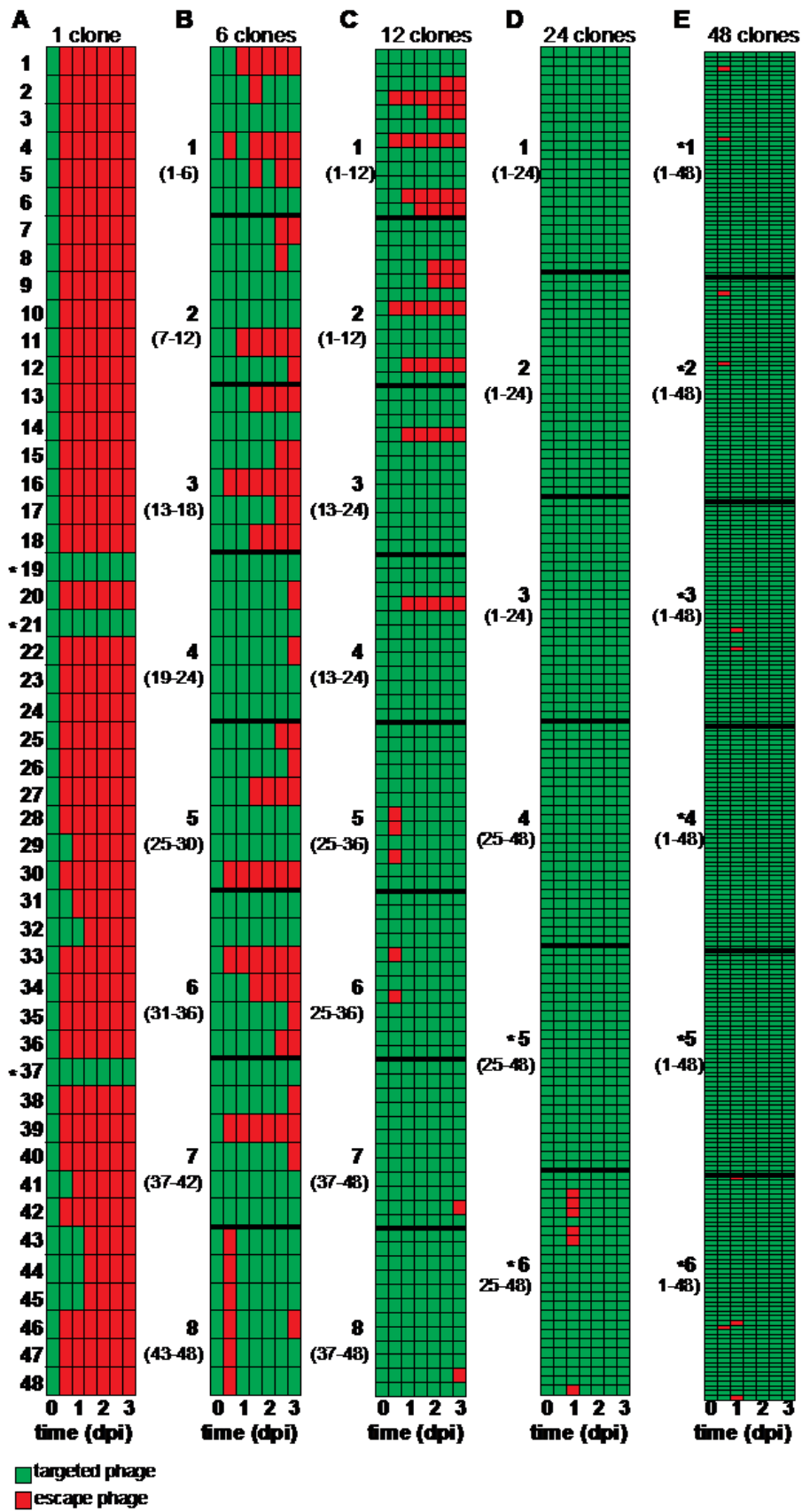
Extended data Figure 10: Emergence of virus mutants that overcome CRISPR-mediated immunity during the experiment shown in Extended Data Figure 9. Each column in a table represents a time point (0, 16, 24, 40, 48, 64 and 72 hours post-infection, as indicated below the table (in days post-infection)) where virus was isolated. Green indicates no escape virus. Red indicates emergence of escape virus. Panels A-E correspond to each of the experiments shown in Extended Data Figure 9 A-E. Bold numbers indicate replicate experiments. Numbers between parentheses indicate the identity of clones that are present in a population with CRISPR-mediated immunity. Asterisks indicate replicate experiments where virus went extinct during the experiment.
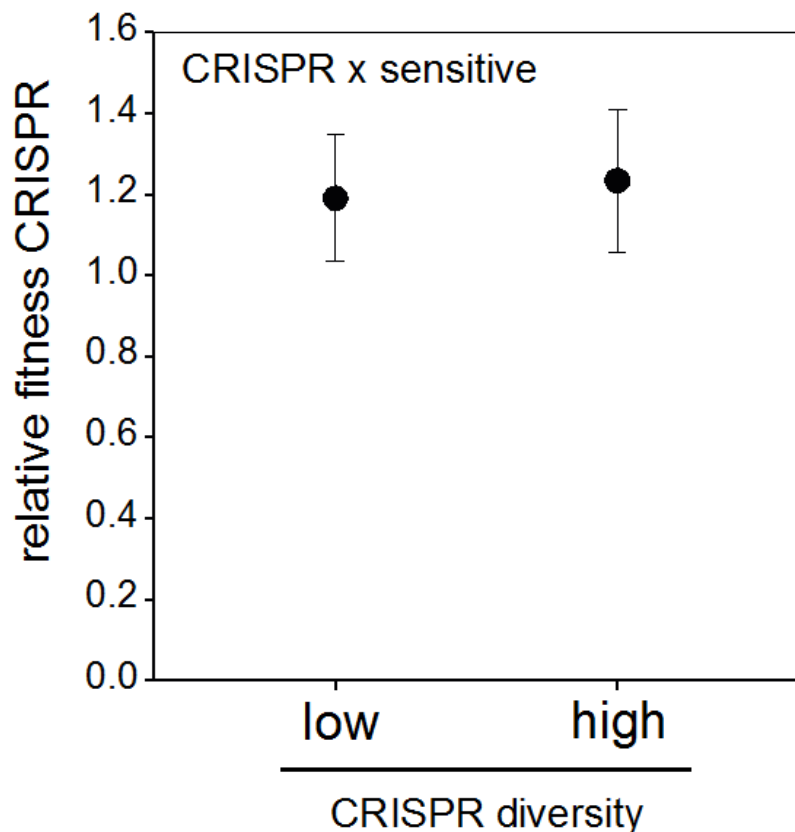

Extended data Figure 11: Sensitive bacteria are unable to invade bacterial populations with CRISPR-mediated immunity in the absence of virus, independent of the level of spacer diversity. Relative fitness of monoculture (single spacer; low diversity) and polyculture (48 spacers; high diversity) at 3 days post-infection when competing with the CRISPR KO strain (sensitive) in the absence of virus. Error bars indicate 95% CI.

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