**RUNNING TITLE: Small changes in host diversity alter the mode of coevolution in a bacteria-phage system**

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# Abstract

# Introduction

Genetic diversity plays a key role in the spread and evolution of infectious diseases. Host genetic diversity can limit the spread of pathogens ([1-6]) whereas parasite genetic diversity can increase the ability of parasites to adapt to local hosts [7]. Repeated and usually drastic reductions in population size are common in host-pathogen populations and can severely reduce their genetic diversity [8, 9]. Antibiotic treatment [10], the normal infection cycle of a pathogen (e.g. Lymes disease [11], HIV-1 [12], Hepatitis C virus [13], reviewed in [14]), and rapid changes in the abiotic environment such as soil structure [15] can all cause sharp reductions in diversity. Diversity also influences host-pathogen coevolution. Theoretical work suggests that reduction of host diversity can create a “monoculture effect” that increases the likelihood that pathogens can adapt to overcome host defenses [16, 17]. Others have suggested that hosts may benefit from bottlenecking by enhancing stochastic effects that resistance alleles and limit pathogen spread [18, 19], an effect which has been observed in a bacteria-phage system [20].

CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR-associated) immunity offers a tractable empirical system to examine the role of diversity in host-pathogen interactions. CRISPR-Cas is an adaptive immune system found in ~50% of bacteria and ~90% archaea [21], and confers immunity to phage infection by incorporating phage-derived sequences into CRISPR loci on the host genome. These loci consist of repeating sequences interspaced with sequences (“spacers”) derived from phage and other mobile genetic elements, typically around 30nt in length. RNA transcripts of CRISPR loci are processed and form a ribonucleoprotein complex with Cas proteins that can recognise and cleave complementary nucleic acid sequences, preventing future infections by the same phage genotype [22-24]. CRISPR loci are often diverse [25], and high CRISPR diversity has previously been shown to provide synergistic host benefits against phage in both *Pseudomonas aeruginosa* PA14 [26] and *Streptococcus thermophilus* DGCC7710 [27].

Because phage can overcome CRISPR immunity by evolving point mutations in the sequence targeted by the spacer (the protospacer) or in the protospacer-adjacent motif (PAM) [28], coevolution may occur in free-running systems, where bacteria acquire spacers over time and phage escape via point mutations in the corresponding protospacers or PAMs [29-31]. Evidence of coevolution has been observed in coexisting populations of CRISPR bacteria and phage [32-34]. The matching allele (MA) genetic architecture of CRISPR interactions suggests that CRISPR-phage coevolution may take the form of a fluctuating selection dynamic (FSD) [35], where hosts and pathogen are temporally co-adapted. This prediction is complicated however by factors such as the relative fitness of CRISPR compared to other immune mechanisms [36-38], demographic interactions between bacteria and phage [29], mutation fixation rate in phage [31], biased dilution effects [39], and reversion to sensitive phenotypes [40-42]. Further complexity is added by the observation that a coevolutionary arms race dynamic (ARD) - where hosts and pathogens escalate resistance and infectivity over time - was found to explain long-term coexistence of *S. thermophilus* and its lytic phage 2972 [43]. Clearly then, there is much still to be worked out regarding the coevolutionary processes that occur during bacteria-phage coexistence.

We previously showed that, under certain degrees of bottlenecking, *P. aeruginosa* PA14 can coexist with its lytic phage DMS3vir up to 5 days post-infection [39]. Given that the coexistence of *S. thermophilus* and phage is likely to be driven by arms race coevolution, and that reducing CRISPR diversity has been shown to promote phage persistence in *P. aeruginosa* PA14 [26], we were interested to see if coexistence in this system was driven by coevolution, and if so, what dynamic takes place. We conducted a 20-day highly-replicated co-culture experiment with *P. aeruginosa* PA14 and phage DMS3vir, repeatedly and randomly restricting host diversity (1 and 5 genotypes) at each transfer while maintaining an approximately constant phage population size. Using representative samples of this experiment, we conducted a time-shift assay to assess if coevolution had occurred, what dynamic it followed, and how both phage infectivity and host resistance evolved over time. We predicted that such severe restriction of host CRISPR diversity would lead to prolonged bacteria-phage coexistence, and that an asymmetrical coevolutionary arms race [43-45] would eventually drive phage extinct. We predicted that this dynamic would be driven by the emergence of phage-resistant surface mutants, which evolve readily in *P. aeruginosa* PA14 under high phage pressure [36].

# Materials & Methods

## Bacterial strains & phage

The bacterial strains and phages used in this study have all been described previously. Evolution experiments were carried out using *Pseudomonas aeruginosa* UCBPP-PA14 (WT) and phage DMS3vir [46]. WT *P. aeruginosa* PA14 does not carry any CRISPR spacers targeting DMS3vir. *P. aeuruginosa* UCBPP-PA14 *csy3::lacZ* [47], which has a non-functional CRISPR system, was used for overnight amplification of glycerol stocks of phage and plaque assays. An anti-CRISPR phage, DMS3vir-AcrF1[26], was used in streak assays.

## Co-culture experiment

Evolution co-culture experiments were established in glass vials by inoculating 6ml of M9 minimal media (supplemented with 0.2% glucose) with ∼1x106 cfu (colony- forming units) from an overnight culture of WT *P. aeruginosa* PA14. Approximately 1x104 pfu (plaque-forming units) of DMS3vir was added to each glass vial. 180μl of culture was taken from each vial and phage was extracted using chloroform. Phage titres were determined by serially diluting extracted phage eight times in 1x M9 salts, and then spotting 5μl of each dilution on a top lawn of *P. aeruginosa* PA14 *csy3::lacZ*. The detection limit of phage spot assays is 102 pfu ml–1. Samples of culture were serially diluted in M9 minimal media, plated on M9 agar (1.5% w/v), and incubated overnight at 37˚C.

After approximately 24hrs of growth, cfus were counted to determine host densities. Either one or five individual colonies were picked from each replicate and re-suspended in 100 or 500μl M9 media, respectively. 60μl of each cell suspension was then used to inoculate 6ml of fresh M9 media. These treatments correspond to the 1- and 5-clone treatments referred to in the Results. To ensure that phage titres remained approximately constant through each transfer, 60μl of chloroform-extracted phage from the corresponding replicate was added to the fresh M9 media. The vials were then incubated at 37˚C while shaking at 180rpm. The process of sampling, overnight incubation and transfer was repeated until a replicate had no detectable phage for 2 days. The experiment was terminated after 20 days post-infection (dpi). Each treatment was performed in twelve independent replicates (N = 12).

## Determining host immune phenotype and CRISPR spacer acquisition

Bacterial immunity against ancestral phage was determined at 1, 3, 5 and 7 dpi, using three independent assays as described in Westra, van Houte [36]. First, bacteria were plated on LB agar, and 24 randomly-selected individual clones per replicate per timepoint were streaked through either DMS3vir or DMS3vir-AcrF1. Clones sensitive to both phage genotypes were scored as ‘sensitive’; those resistant to the DMS3vir but sensitive to DMS3vir-AcrF1 were scored as ‘CRISPR’; and those resistant to both were scored as ‘surface mutant (SM)’. Second, each clone was also grown for 24hrs in M9 media in the presence or absence of DMS3vir, and the OD600 was measured. Cultures that had a reduced growth rate were scored as sensitive. Third, spacer acquisition in the CRISPR loci was determined by PCR with primers CTAAGCCTTGTACGAAGTCTC and CGCC- GAAGGCCAGCGCGCCGGTG (for CRISPR 1) and GCCGTCCAGAAGTCAC- CACCCG and TCAGCAAGTTACGAGACCTCG (for CRISPR 2). Results from streak assays, OD600 measurements and PCR were cross-referenced to confirm the phenotype of each clone.

## Infectivity and resistance evolution

To measure whether host resistance and phage infectivity evolved during the evolution experiment, we isolated phage clones and bacterial clones from replicates where phage persisted for at least 7 days. Due to a trade-off between sample size and the number of timepoints chosen for analysis, we conducted a power analysis using the pwr package [48] in R. We derived reference Cohen’s *D* values for a small effect from Common *et al.* 2019 [43] (0.57), and a large effect from Hall *et al*. 2011[49] (1.28). This showed that sampling at least four time points was suitable to detect both effect sizes in the 1-clone treatment (small: D1,6 = 0.43; large: D1,6 = 0.76) and 5-clone treatment (small: D1,4 = 0.30; large: D1,4 = 0.56). Phage extracted from 1, 3, 5, and 7 dpi were subjected to plaque assays. For each replicate and time point, twelve plaques were randomly picked and amplified in 96 well plates containing LB inoculated with *P. aeruginosa* PA14 *csy3::lacZ* . Twelve colonies from each replicate and timepoint were picked at random from the 24 clones isolated as part of the immune phenotype assays. To examine the evolution of phage infectivity for each of the eight replicates, the 48 phage clones that were isolated were spotted onto 48 bacterial lawns corresponding to the bacterial clones isolated from the same replicate. Phage were classified as being infective against a bacterial clone if a clear lysis zone was visible on the lawn after overnight incubation at 37˚C. If no lysis zone was visible, the host was classified as resistant.

Using this data, we measured the evolution of phage infectivity range as the proportion of bacterial clones that phage from each time point from the same replicate experiment could infect. In a similar way, we measured the evolution of host resistance range as the proportion of all phage genotypes from the same replicate experiment that could be resisted by bacteria from each time point. Infectivity or resistance was analysed in a Generalised Linear Model (GLM) with genotype as a fixed effect and a binomial family with a logit link function. Mean infectivity or resistance was then analysed for each time point in a Generalised Linear Mixed Model (GLMM) using the lme4 package [50], with time point as a fixed effect and replicate as a random effect. Model coefficients and confidence intervals were transformed from logits to probabilities prior to presentation.

## Time-shift experiment

Because the susceptibility and resistance of bacterial clones to phage from past, present or future time points was determined, our infectivity assay also served as a time-shift experiment [51]. Our phage infectivity and host resistance data was analysed as a time-shift experiment by first scoring each pairwise challenge as *Past*, *Contemporary*, or *Future*, with reference to the phage’s background compared to the host. Infectivity was then analysed in a GLMM with phage background as a fixed effect and replicate, phage timepoint and host timepoint as random effects. Models had a binomial family with a logit link function.

# Results

We tested how long *P. aeruginosa* PA14 and phage DMS3vir (hereafter “phage”) could coexist under conditions of very low and repeatedly randomized host diversity. We set up a co-culture experiment by infecting *P. aeruginosa* PA14 with 106 plaque-forming units (pfus) of phage in 24 replicate populations. After 24hrs of growth and infection, phage was extracted from samples of each replicate, and samples of each replicate were plated on M9 agar. 1 colony-forming unit (cfu) was picked from 12 of the plates and 5 cfus were picked from the remaining 12, which were resuspended in fresh media. These suspensions were then used to inoculate a larger volume of fresh media. These two treatments are hereafter referred to as 1-clone and 5-clone. Finally, a sample of the extracted phage was added to the media in order to maintain an approximately constant phage population size during the transfer. This procedure was repeated for 20 days, during which time we monitored the bacterial and phage population densities daily.

**Figures 1A** and **1B** show the population dynamics during the 20-day experiment. In the absence of reducing host diversity as described, phage is driven extinct by 4 days post-infection (dpi) (**Figure S1**). However, with the exception of 4 replicates in the 1-clone treatment, all had detectable phage until 5 days post-infection (dpi), after which phage extinction occurred more often. Average time to phage extinction was 7.42±1.03 and 6.58±0.43 days for 1- and 5-clone treatments, respectively (means ± SEs). A Cox proportional hazards model further showed that there was no significant difference between the treatments in terms of phage survival (HR = 2.07 [0.83, 5.20], *z* =1.55, *p* = 0.12) (**Figure S2)**. We did not observe any extinctions of the bacterial host. Interestingly, lower phage titres were correlated with high host densities in the 1-clone treatment (*c* = -1.16 [-1.61, -0.70], *z*=-4.97, *p*<0.0001), but no statistically significant relationship between phage titres and host densities was observed in the 5-clone treatment (*c*= 0.13 [-0.55, 0.81], *z*= 0.37, *p*=0.26).

The population dynamic data show that bacteria and phage can coexist much longer than would be expected under control conditions. Although phage in all replicates across both treatments were eventually driven extinct, fluctuations in the densities of both phage and host in several replicates suggested that selective sweeps were likely to have occurred, indicative of coevolution. To test if coevolution had indeed taken place, and if so, what kind of dynamic it followed, we conducted a time-shift experiment. We first identified replicates where bacteria and phage coexisted for at least 7 days, giving 7 replicates from the 1-clone treatment and 5 replicates from the 5-clone treatment, and isolated 12 bacterial clones and 12 phage clones from each replicate at 1, 3, 5 and 7 dpi. We then performed a phenotypic time-shift assay by exposing bacteria to phage from their past, present or future [51, 52]. This gives a measure of changes in infectivity patterns over time. Generalized linear mixed models (GLMMs) with replicate, host timepoint, and phage timepoint as random effects and phage background as a fixed effect were used to analyse the time-shift data. This assay design also allowed us to examine whether the phage and bacteria evolved increased infectivity and resistance over time. This was done by measuring the resistance of each individual bacterial clone against all phage clones derived from the same replicate, and measuring the infectivity of each individual phage clone against all bacterial clones from the same replicate in a GLMM.

Analysis of time-shift data showed that, independent of treatment, the original time-point of the phage with respect to the host had a significant overall effect on infectivity (χ2(2,10) = 7.82, *p* < 0.05, *R2* = 0.70), with no overall effect of treatment on infectivity patterns (χ2(2,10) = 4.95, *p* = 0.08, *R2* = 0.70). Infectivity was generally low in both treatments, compared to *S. thermophilus* [43] which has a less-active CRISPR system.

In the 1-clone treatment (**Figure 2A**), hosts were clearly more susceptible to contemporary phage than those from their past, and slightly more so compared to phage from the future. The difference in susceptibility between contemporary and future phage was marginally statistically insignificant (*p* = 0.56). Analysis of host susceptibility to contemporary phage at each timepoint in the assay showed that, apart from at 1 dpi, there were consistent and significant decreases in susceptibility from 3 dpi onwards (**Figure 2B**). Analysis of the evolution of infectivity and resistance showed that phage evolved increased infectivity from 1 to 3 dpi, but then remained statistically similar (**Figure 2C**), while hosts evolved decreased resistance at 3 dpi, followed by an increase until 7 pi, where all bacterial clones had developed resistance to all phage challenges (**Figure 2D**). Together, these data suggest that bacteria and phage coevolved following a weak arms race dynamic (ARD) in the 1-clone treatment, where hosts and pathogens escalated resistance or infectivity over time, but one in which host resistance rapidly outpaced pathogen infectivity.

Turning to the 5-clone treatment, hosts were significantly more susceptible to infection from contemporaneous phage compared to those from either the past or future (**Figure 3A**). Patterns of susceptibility to contemporary phage from this treatment show a general increase, with a drop at 5 dpi, across the four time points included in the time-shift assay (**Figure 3B**). The evolution of infectivity and resistance is broadly similar in this treatment compared to the 1-clone treatment, with important differences. As in the 1-clone treatment, phage evolved increased resistance from 1 to 3 dpi, after which it remains statistically similar (**Figure 3C**). Evolved host resistance again showed a similar trend, however, while resistance was always high, mean resistance did not reach totality (**Figure 3D**). Together, data from the 5-clone treatment is therefore strongly suggestive of a fluctuating selection dynamic (FSD) in which phage adapt to be most infective to bacterial hosts from the same moment in time, so that infectivity evolves to remain approximately constant, and resistance evolution is never complete.

Given that different coevolutionary dynamics appear to have driven the coexistence of bacteria and phage in the co-culture experiment in the 1- and 5-clone treatment, we next wanted to know if the different treatments had influenced the evolution of immune phenotype. High phage pressure is known to favour the evolution of surface modification (SM) based resistance [36], which is a general, innate phage defence mechanism that could feasibly contribute to an ARD. We also examined the spacer content of CRISPR clones using PCR of the CRISPR1 and CRISPR2 loci, as increasing number of spacers per clone is known to limit the evolution of escape phage (reference).

We found that CRISPR-based immunity was the most frequent phenotype at all timepoints in both treatments (**Figures 4A** & **B**). Sensitive hosts were not detected in either treatment after 5 dpi. SM phenotypes occurred at a low frequency at all timepoints in the 1-clone treatment, and significantly fewer were found in the 5-clone treatment, with no SM hosts detected at 7 dpi in this treatment. There were no statistically significant differences in the number of acquired spacers per clone between treatments, with the exception of 5 dpi, where average spacer number was higher in the 5-clone treatment (**Figures 4C** & **D**). Together, the immune phenotype and spacer sequence data show that coexistence of bacteria and phage in both treatments, as well as the coevolutionary dynamics we observed, occurred against a host background composed mostly of CRISPR hosts with multiple spacers.

# Discussion

We conducted a 20-day highly-replicated co-culture experiment with *P. aeruginosa* PA14 and its lytic phage DMS3vir. We tested how repeated and random restrictions of host diversity to 1 and 5 clones at each transfer, while maintaining an approximately constant phage population size, would affect bacteria-phage coexistence and coevolution. In line with our predictions, we found that *P. aeruginosa* and DMS3vir could coexist over multiple generations, compared to a control treatment. This effect was similar in both diversity treatments. Our time-shift and infectivity/resistance evolution data show that coexistence was likely driven by antagonistic coevolution. Further, it suggests that even slight changes in host diversity can lead to different coevolutionary scenarios. A weak arms race dynamic (ARD), with hosts and phage evolving increased resistance and infectivity over time, played out in the 1-clone treatment. By contrast, time-shift data suggests that coexistence in the 5-clone treatment was likely driven by a fluctuating selection dynamic (FSD) [53], in which hosts and phage were temporally coadapted. Surprisingly, coexistence and coevolution in both treatments took place against a host background composed mostly - and in some cases entirely - of CRISPR-mediated immunity.

The observation that bacteria-phage coexistence can be explained by a FSD experimentally demonstrates that this coevolutionary dynamic is possible in CRISPR-phage interactions. Our experimental design enabled a more realistic scenario of repeated restrictions of host diversity while reducing, to the best of our ability, the confounding effects of phage dilution [39] and host density on pathogen spread [54]. While we concede that our experimental conditions are necessarily vastly simplified compared to those found in natural environments, our observations reveal at least some of the requirements for FSD in coexisting bacteria-phage populations. More broadly we have experimentally shown that evolutionary predictions derived from mathematical modelling, namely that a matching allele (MA) genetic architecture of infectivity/resistance [35], can be replicated in a living host-pathogen system*.*

A key question arising from our data is: why were different coevolutionary dynamics observed in the two diversity treatments? It is a particularly surprising result given our prediction that coexistence would be driven by an ARD in both treatments, and that FSD in CRISPR-phage interactions can be easily destabilized given that the difference in diversity between the treatments was not large. Although we can only speculate, we suggest two alternative explanations. One possibility is that the presence of SM hosts in the 1-clone treatment may have weakened a FSD. The immune phenotype data shows that hosts with SM-mediated resistance occurred at low but persistent frequencies at all sampled timepoints in the 1-clone treatment. Maintenance of SM may have been facilitated by higher multiplicity of infection (MOI) in this treatment, a result of monoclonal diversity, lower host diversity, or both. High phage MOI has previously been shown to promote the evolution of SM in *P. aeruginosa* PA14 [36]. Such a low frequency of SM hosts may have caused the marginally insignificantly lower infectivity of future compared to contemporary phage. A second possibility is that increased stochasticity in the 1-clone treatment meant that resistant hosts were more likely to be fixed, and sensitive hosts more likely to be lost. Previous theoretical work has shown that stochastic reductions in diversity can weaken FSD by increasing the speed of allele fixation, independent of initial allele frequency [18]. Experimentally, Hesse and Buckling [20] have suggested that such stochastic gains and losses caused rapid phage extinction following strong bottlenecking events during coevolution. This effect would have weakened FSD in our experiment by making novel resistant CRISPR or SM variants more likely to reach high frequencies or fixation, while removing already rare sensitive hosts. This exciting result certainly merits further investigation to clarify the precise reasons these different dynamics occurred, and demonstrates how even slight changes in diversity can influence coevolution.

The divergent coevolutionary outcomes in the 1- and 5-clone treatments may explain why phage densities were negatively related to those of the host in the former, but not in the latter. Under a FSD, a proportion of the antagonists are temporally coadapted and consequently “it takes all the running you can do to keep in the same place” [55], so the densities of both remain roughly stable. Under an asymmetrical ARD, at any given time the host population can resist phage infection, such that increases in host density are associated with declines in phage density. This result highlights how coevolutionary dynamics can influence and be reflected in population demographic changes.

The predominance of CRISPR hosts probably led to the eventual extinction of phage in all replicates in both treatments. Bacterial hosts are often “ahead” during antagonistic coevolution [45], an effect observed in *Streptococcus thermophilus* where the presence of CRISPR led to an asymmetry in which the evolution of host resistance “outpaced” that of phage infectivity [43]. The limited host range and the consistent decline in infectivity of contemporary phage in the 1-clone treatment are consistent with this idea. The mechanistic basis of CRISPR immunity and phage escape mutation suggests how this asymmetry takes place. Hosts can acquire multiple novel spacers with only a marginal cost [56], but phage mutation is limited by mutation supply [44, 57, 58]. In addition, full phage infectivity requires mutations in all the protospacers targeted by the host CRISPR array, which becomes increasingly difficult when individual hosts and populations acquire a greater number and diversity of spacer sequences over time [26, 57]. Understanding of this mechanism provides an insight into why phage was consistently driven extinct in the 5-clone treatment, despite the occurrence of a FSD and increasing infectivity of contemporary phage. Phage can adapt to contemporary host’s CRISPR spacers and coexist so long as the spacer content of the host population remains below a threshold level, either of spacer diversity or number. Once this threshold is crossed, and mutation supply limits the evolution of escape phage, extinction occurs. Together this suggests that while the mode of coevolution may differ depending on host diversity levels, the ultimate outcome for bacteria-phage coexistence can depend on the immune mechanisms involved.

# Figure Captions

**Figure 1: Phage and host population dynamics**

Phage and host population titres over time per replicate in **A)** 1-clone and **B)** 5-clone treatments. Phage titres (plaque-forming units; pfu ml-1) are shown in black and host densities (colony-forming units; cfu ml-1) are shown in blue. The level of phage detection is 200 pfu ml-1 (dashed line).

**Figure 2: Results from time-shift experiment – 1-clone treatment**

**A)** Proportion of hosts infected when phage were from the host’s past, present or future. **B)** Proportion of hosts infected by phage from the same time point (days post-infection). **C)** Phage infectivity over time, represented as the proportion of host genotypes from all time points that were infected by a given phage genotype in each replicate. **D)** Host resistance over time, represented as the proportion of phage genotypes from all time points that were resisted by a host genotype in each replicate. The dotted lines are for illustrative purposes. Means are shown. 95% CIs represent the variation of the mean among replicates. (*N* = 12).

**Figure 3: Results from time-shift experiment – 5-clone treatment**

**A)** Proportion of hosts infected when phage were from the host’s past, present or future. **B)** Proportion of hosts infected by phage from the same time point (days post-infection). **C)** Phage infectivity over time, represented as the proportion of host genotypes from all time points that were infected by a given phage genotype in each replicate. **D)** Host resistance over time, represented as the proportion of phage genotypes from all time points that were resisted by a host genotype in each replicate. The dotted lines are for illustrative purposes. Means are shown. 95% CIs represent the variation of the mean among replicates. (*N* = 12).

**Figure 4: Evolution of immune phenotypes and spacer acquisition**

Relative frequencies of bacterial clones with CRISPR immunity, surface mutation (SM) resistance, or sensitive phenotypes, at 1, 3, 5 and 7 days post-infection (dpi) in **A)** 1-clone and **B)** 5-clone treatments. Total number of spacers per clone at 1, 3, 5 and 7 dpi in **C)** 1-clone and **D)** 5-clone treatments. Means are shown. 95% confidence represent variation of the mean among replicates (*N*=12).

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