**TITLE: Pathogens benefit from diversity when infecting equally diverse hosts**

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# Abstract

# Introduction

Host diversity often associates with reduced pathogen success. This idea has gained ample support from numerous observational and experimental studies spanning a wide range of host-pathogen systems (1-3). The effects of host diversity on pathogen success may be due to limitation of evolutionary emergence of novel pathogen genotypes (4-9), negative density-dependence whereby diversity “dilutes” focal hosts (10-17) or feedback between these evolutionary and ecological effects (8, 18-20).

Less well-understood is how pathogen diversity may interact with host diversity. Like their hosts, pathogens are often diverse (21). Within-species pathogen diversity may be related to traits under constant selection by the host immune system such as antigens and surface proteins (ref); traits relating to epidemiology such as virulence, latency, or transmission (ref); or due to spatial and/or temporal population structure (22). Indeed, pathogens may be among the most diverse organisms on Earth, as their lifecycle is often characterised by short generation times, high mutation rates, large population sizes, and rapid selection (refs).

Given the recognition that pathogens tend to have high within-species diversity, it makes sense that the effects of host diversity on pathogen success are likely to depend on the relative levels of host-pathogen diversity. Maybe a discussion of how it might matter is warranted here? A small number of studies have investigated how host and pathogen diversity might interact and influence epidemiology and host success. Experiments with the water flea *Daphnia magna* and its microsporidium parasite *Octosporea bayeri* found that parasite diversity did not have a notable effect on parasite prevalence when interacting with mono- or polyclonal hosts (23). However, a later study in a similar system found that diverse parasites were more successful in both mono- and polyclonal hosts (24). To our knowledge, these are the only two published studies where both host and pathogen diversity were explicitly experimentally manipulated. A recent meta-analysis suggested that there is not a significant interaction between host and pathogen diversity (1), however all but one study analysed to derive this result did not make pathogen diversity explicit nor included it as a controlled variable. The effect of relative host-pathogen diversity, if any, on pathogen success is therefore poorly understood and ambiguous.

The interaction between lytic bacteriophage (phage) and the bacterial CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR-associated) immune system is a tractable model system to study the role of relative host-pathogen diversity (9, 25, 26). CRISPR-Cas immune systems can incorporate short DNA fragments (spacers) of about 30 base pairs derived from the phage genome into CRISPR loci on the host genome (27). Processed CRISPR transcripts guide Cas immune complexes to identify and cleave the invading phage genome, preventing successful re-infections (28-31). In turn, phage can evolve to overcome CRISPR immunity by point mutation (32-35). Phage evolution to overcome CRISPR immunity can lead to CRISPR-phage coexistence and coevolution (36-39). CRISPR diversity can provide increased resistance by limiting the ability of phage to acquire the mutations needed to overcome CRISPR resistance, which in turn can drive rapid phage extinction (9, 18, 25, 40).

# Materials & Methods

## Bacterial strains and phage

Evolution experiments were carried out using *Pseudomonas aeruginosa* UCBPP-PA14 (which has two CRISPR loci, CRISPR1 and CRISPR2), UCBPP-PA14 *∆pilA* (this strain lacks the pilus, which is the phage DMS3 receptor, and therefore displays surface-based resistance) and phage DMS3vir (41). We used *P. aeruginosa* UCBPP-PA14 *csy3::lacZ* (42), which carries an inactive CRISPR-Cas system, for phage amplification, and for top lawns in phage spot and plaque assays.

## BIM-phage library

To control the levels of host and phage diversity in our experiments, we used a library of 24 *P. aeruginosa* PA14 clones each carrying a single spacer in CRISPR2 (bacteriophage-insensitive mutants; BIMs) and 24 DMS3vir clones that could infect each BIM (escape phage). The pattern of host-pathogen specificity in this library corresponds with a matching allele model. To monitor the population dynamics and relative fitness of individual bacterial clones within the mixed populations during the co-culture experiments, we transformed 8 BIMs to carry a *lacZ* reporter gene. The BIMs chosen for transformation were such that a single clone could be monitored in each of the 3-clone mixtures (that is, BIMs 1, 4, 7, 10, 13, 19, and 22). This enabled us to measure the selection rate of a labelled BIM through time by performing a blue:white screen when plating on LB agar supplemented with 40g/ml X-gal. The design of the library and cloning procedures to generate *lacZ*-labelled BIMs have been previously described in Common, Walker-Sünderhauf (18).

*Co-culture experiments*

We designed 4 polyclonal treatments which varied in absolute diversity but had the same degree of relative host-pathogen diversity. These 4 treatments were based on the BIM library: 3-clone x 3-phage, 6-clone x 6-phage, 12-clone x 12-phage, 24-clone x 24-phage. Each BIM was paired with its corresponding escape phage. In each replicate, a single *lacZ*-labelled BIM was included.

From fresh overnight cultures of each BIM, we made mixes of equal proportion of each clone according to the treatments described. To monitor the population dynamics and competitive performance of the CRISPR-resistant population as a whole, we also added PA14 *∆pilA* (surface mutant; SM, which is fully resistant to phage DMS3vir and has a distinct “smooth” colony morphology) to each mix in equal proportion to the CRISPR population. We then inoculated 6ml of M9m 1:100 from each mix. We made mixes of equal proportion of each escape phage according to the treatments, and added approximately 1x106 pfu ml-1 of the escape phage mixture to each vial. Treatments consisted of 8 biological replicates (*N*=8) to ensure that both BIMs and phage were equally represented across treatments. Glass vials were incubated at 37˚C while shaking at 180rpm. At 1, 2, and 3 days-post infection (dpi), the sampling of the phage and bacterial culture was repeated as described. Cultures were transferred 1:100 to fresh media after sampling had been carried out. The experiment was terminated at 3 dpi.

Each day 180μl of culture was taken from each vial and phage was extracted using chloroform. Extracted phage were serially diluted in 1x M9 salts. Total phage titres were determined by either spotting 5μl of each dilution on a top lawn of *P. aeruginosa* PA14 *csy3::lacZ* (spot assay), or by mixing 100µl of extracted phage with 200µl of PA14 *csy3::lacZ* to create a top lawn (plaque assay), which were then incubated at 37˚C for 24hrs. Titres of each phage genotype were determined similarly using the phage’s corresponding BIM instead of PA14 *csy3::lacZ*. The detection limits of spot assays and plaque assays are 102 pfu ml-1 and 100 pfu ml-1 respectively. To monitor bacterial densities, culture samples were serially diluted in 1x M9 salts, and then plated on LB agar + 40µg/ml X-gal, and incubated for ~48hrs at 37˚C. The density of SM, CRISPR and the labelled BIM was then calculated. SM were differentiated from CRISPR clones by their “smooth” colony morphology, and the labelled BIM was identified by the blue:white screen.

We assessed the competitive performance of the CRISPR relative to SM clones and the labelled BIM relative to non-labelled BIMs by calculating selection rate (*rn* = (ln [density strain A at tn/density strain A at tn-1] – ln[density strain B at tn/density strain B at tn-1])/day) (43, 44), which expresses competitive performance as the natural log of the relative change in density of one competitor against another.

## Phage evolution

We examined phage evolution during the experiment by sampling 12 individual plaques from each replicate that had detectable levels of phage from 1 to 3 dpi, which were amplified on PA14 *csy3::lacZ* overnight in LB broth, at 37˚C and shaking at 180rpm. Phage were extracted using chloroform, and then diluted 1000-fold. Samples of each phage were then applied on lawns of each of the 24 BIMs and WT PA14 *csy3::lacZ*. A successful infection was indicated by a clear lysis zone on the top lawn. Phage were classified according to whether they had expanded their infectivity range (could infect the original susceptible clone and a new clone in the BIM library).

## Statistical analysis

All statistical analyses were carried out in R v3.5.3 (45). The packages dplyr (46), tidyr (47) and magrittr (48) were used throughout for data handling. Generalised linear mixed models (GLMMs) were used throughout, and replicate was treated as a random effect in all models. Model selection followed a nested approach, where full versus reduced models were compared using information criteria (49, 50), and the similarity between observed and predicted values. The overall statistical significance of the single and interaction effects of fixed effects (that is, treatment and dpi) was assed using likelihood ratio tests (LRTs), which give an *F*- and associated *p*-value. Simple Bayesian GLMMs from the MCMCglmm (ref) package were used to analyse phage evolution. These models used a probit transformation (the inverse standard normal distribution of the probability) and a flat prior. The overall statistical significance of the single and additive effects of fixed effects in these models was assessed using χ2 tests and their associated *p-*values using the VCVglmm package (ref). Where possible, exact *p-*values are given, but R is unable to give exact values when *p* < 2.2 x 10­­-16. When phage titre was considered as the response variable, data was log-transformed to improve model fit. Confidence (or in the case of Bayesian models, highest probability density) intervals around model coefficients and predicted means were calculated to the 95%, 89% and 67% level to give the reader a clearer indication of effect size. The package ggplot2 (51) was used to generate figures.

# Results

Phage benefited from being diverse. Although phage population size was still strongly negatively affected by host diversity when controlling for the effect of time (*F*3, 114 = 47.03, *p* < 2.2 x 10-16; **Figure X**), compared to monoclonal phage populations, polyclonal phage were generally able to reach larger overall population sizes (**Figure X**). This benefit of phage diversity is clear when the effects of diversity treatment and time were statistically controlled (difference in mean ln pfuml-1between mono- and polyclonal phage treatments: *β* [95% CI] = 4.65 [3.85, 5.39], *t*(2)178 = 11.34, *p* < 2 x 10-16­). Phage were also observed at 3 dpi in the 12- and 24-clone treatments – when monoclonal phage were mostly extinct by this time (**Figure X**). Phage therefore reached larger population sizes, and persisted longer, when phage diversity matched that of the CRISPR host population.

The CRISPR population did not benefit from being diverse (**Figure X**). CRISPR selection rate compared to SM was not positively associated with CRISPR allele diversity (*F*3, 83 = 1.01, *p* = 0.39), and it did not change over the course of the experiment (*F*2, 83 = 1.30, *p* = 0.28). When diverse CRISPR populations were challenged with a pre-evolved monoclonal phage there was also no effect of host diversity or time on the selection rate of CRISPR hosts resistant to the phage (**Figure X**)(18), and these hosts were generally as competitive as SM. Importantly, CRISPR selection rate was overall lower when host diversity was matched by phage diversity (difference in mean selection rate between mono- and polyclonal phage treatments: *β* [95% CI] = -0.57 [-0.74, -0.40], *t*(2)178 = -6.17, *p* = 4.39 x 10-9)(**Figure X**). The CRISPR population was also generally less competitive than SM across treatments and timepoints (mean selection rate [95% CI]: -0.92 [-1.10, -0.72). CRISPR diversity therefore did not seem to protect susceptible hosts from phage, and the whole CRISPR population was supressed by the phage epidemic.

Individual susceptible CRISPR clones also did not benefit from population-level host diversity. When challenged by a monoclonal pre-evolved phage, susceptible hosts become more competitive compared to the CRISPR population as a whole as CRISPR diversity increases (**Figure X**)(18). This is likely because host diversity reduces the relative density of susceptible individuals, which in turn reduces phage reproduction. We predicted that this dilution effect would still play a role when phage diversity matched that of the host, because the contact rate with a given phage genotype’s focal host might still be limited by dilution. This is observable in improved competitive ability of the susceptible clone (**Figure X**)(18). Contrary to this prediction, selection rate of the *lacZ*-labelled clone did not increase with host CRISPR diversity (*F*1,77 = 0.08, *p* = 0.98; **Figure X**). Indeed, in three replicates in the 12- and 24-clone treated with polyclonal phage, the labelled clone went extinct - we did not observe this in the same treatments with monoclonal phage (**Figure X**). Together, this indicates that the dilution effect of host diversity is insufficient to limit pathogen success when challenged by an equally diverse pathogen population.

# Discussion

# Acknowledgements

# Figure Captions

# Figures

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