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Evolvability is an important trait in the selection of bacteriophages for therapeutic use

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Elijah Horwitz

Committee in charge:

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Katie Petrie

2022

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ABSTRACT OF THE THESIS

Evolvability is an important trait in the selection of bacteriophages for therapeutic use

by

Elijah Horwitz

Master of Science in Biology

University of California San Diego, 2022

Professor Justin Meyer, Chair

The number of multidrug resistant strains of bacteria is increasing rapidly, while the discovery of new antibiotics has stagnated. Subsequently, interest in bacteriophages as anti-bacterial therapeutics has surged, in part, because there is near limitless diversity of phages to harness. While this diversity provides opportunity, it also creates the dilemma of having to decide which criteria to use to select phages. Two traits previously proposed are thermostability and reproductive rate. Here we show that focusing on phage's current abilities may be shortsighted if maximizing traits like stability and reproduction limits future evolution. We studied the ability of three phages to suppress bacteria. Each phage differed by only one amino acid at site 987 in their host-recognition protein (J), yet they varied significantly in their stability,

growth rate, and evolvability. We uncovered a three-way tradeoff such that each phage maximized only two of the three traits. The most suppressive phage was an evolvable, fast-reproducing variant, supporting the importance of evolvability and reproduction rate. We tested whether these traits were interdependent but found that each had individual effects on suppression. Seeking to test the environmental contingency of our results, we altered the experiment to reflect more challenging conditions inside a patient's body. The stable, fast-reproducing phage was most suppressive in the short term, but the fast-reproducing, evolvable phage was more suppressive in the long term. Our results highlight the importance of evolvability, an often-overlooked trait in phage therapy, and underscore the need to consider long-term dynamics when testing phage for therapeutic use.

CHAPTER 1

1.1 Introduction

The growing threat of antibiotic resistant bacteria has generated interest in the use of alternative therapeutics, such as bacteriophage (Kutter et al. 2010; Gordillo and Barr 2019). Bacteriophages are natural predators of bacteria and have been successfully used to treat multidrug resistant bacterial infections (Broncano-Lavado et al. 2021). Phages are ubiquitous in nearly every environment and are more genetically diverse than any other taxonomic group. This means that there are nearly endless varieties to harness (Zablocki et al. 2015; Jurczak-Kurek et al. 2016; Batinovic et al. 2019), whereas antibiotic drugs are far fewer and new discoveries are rare (Donadio et al. 2010; Brown and Wright 2016). To understand phage's enormous potential, there are currently more than 20,000 bacteriophages isolated and stored in the Actinobacteriophage Database, and those are the phages that infect just a single phylum of bacteria (https://phagesdb.org/). This incredible diversity is beneficial for providing practitioners with many options for treatment, but it also necessitates the development of criteria for selecting the best phage from among numerous options.

Currently, researchers select phages by first evaluating infectivity on the pathogen of interest. Infectivity is measured by testing whether phage lyse bacteria in liquid culture or by plating phage on a lawn of the pathogenic bacteria and measuring plaquing ability (Hyman 2019). If multiple candidate phages all plaque, then additional phage traits might be considered. Current literature recommends stability as one such trait (Hejnowicz et al. 2014; Casey et al. 2018), and mathematical models predict that stable phages will be more suppressive than unstable phages (Bull et al. 2019). There is also interest in deliberately enhancing stability in

therapeutic phages via directed evolution (Favor et al. 2020). Another trait that is thought to be desirable in therapeutic phages is a high reproductive rate (Casey et al. 2018). The reasoning is intuitive: phages able to rapidly generate a large population should be more effective at arresting bacterial growth (Bull et al. 2019). Despite the intuitive appeal of both stability and reproductive rate, the importance of these traits in determining bacterial suppression has not been demonstrated empirically.

A third trait that has been relatively overlooked as a criterion for therapeutic phages is evolvability (Bono et al. 2021), defined as the capacity for adaptive evolution. The reason antibiotics and even phage treatments become obsolete is that bacteria evolve resistance (Labrie et al. 2010; Blair et al. 2015). However, unlike antibiotics, phage have an endogenous algorithm – evolution by natural selection – to overcome resistance by gaining counter defenses (Bull et al. 2019). Ideally, phage therapeutics would be able to evolve counter measures to resistance during treatment without any human intervention. Phages have been shown to diverge in their ability to evolve counter-defenses, and so favoring evolvable phages during therapeutic selection might yield a more powerful treatment (Casey et al. 2018). One mechanism by which bacteria evolve resistance to phage is by mutating, deleting, or downregulating the expression of cell surface molecules that phage use as receptors during the first stage of infection (Charbit et al. 1984; Rakhuba et al. 2010; Laanto et al. 2012; Høyland-Kroghsbo et al. 2013). Under certain conditions, phage can overcome this perturbation by evolving to use a new receptor and retain suppression (Meyer et al. 2012; Borin et al. 2021). Therefore, phages with enhanced ability to evolve to use new receptors and expand their host-range should be more effective at suppressing bacteria, especially over the long term when bacteria have the opportunity to evolve resistance.

Ideally, of course, researchers should select phages that are stable, fast-reproducing, and evolvable. However, tradeoffs constrain the simultaneous optimization of multiple traits (Goldhill and Turner 2014; Edwards et al. 2021). Indeed, there is a well-known tradeoff between stability and reproduction that has been demonstrated in RNA viruses (Dessau et al. 2012; Singhal et al. 2017). Additionally, there may be a tradeoff between high levels of stability and evolvability, as demonstrated in bacteriophage λ (Strobel et al. 2022). If tradeoffs are common in nature, then it may be difficult to identify naturally occurring phages with multiple favorable traits, and it will be helpful to understand which traits to prioritize when selecting phage. Furthermore, with the development of more sophisticated genetic engineering technologies, researchers are increasingly attempting to design phage with desirable characteristics (Favor et al. 2020). Such attempts could easily be derailed without a solid understanding of which traits are most important and how tradeoffs constrain trait optimization.

We capitalized on the wealth of knowledge on bacteriophage λ evolution and molecular biology (Meyer et al. 2012; Casjens and Hendrix 2015) to generate an effective system in which to test the importance of the three phage traits for bacterial suppression. We studied three nearly identical λ genotypes from a previously created library of variants differing only by a single amino acid in the receptor binding protein J (Strobel et al. 2022). The initial library contained nine variants, and none exhibited optimal values for all three traits. Perhaps surprisingly, given their high genotypic similarity, they exhibited striking phenotypic differences across our three traits of interest, and we chose three genotypes that had each optimized a different pair of traits (Fig. 1).

To test which traits enhance suppression, we performed a ten-day experiment in which each phage genotype was incubated with permissive *E. coli* bacteria in 24-hour cycles, with daily

transfers to fresh media. As a metric of bacterial suppression, we monitored bacterial population size across all ten days. The study was designed such that each trait was optimized in two of the three phages, so if a single trait was most important for suppression, the bacteria would be significantly lower in two of the three populations. However, only one treatment was found to be significantly depressed, suggesting two traits worked together to make the difference. Upon finding this, we tested whether the two traits had independent effects on suppression. To achieve this, we used genotypes from the previous study, in which a key OmpF-conferring mutation was edited into the fast-evolvable and stable-evolvable backgrounds, effectively collapsing their variation to the traits: reproductive rate and stability (Strobel et al. 2022). These new genotypes were named “fast-evolved” and “stable-evolved”. Finally, we tested the environmental contingency of our first result, aiming to adjust the experimental conditions to reflect the challenging environment of the human body.

1.2 Materials and Methods:

1.2.1 Media

We prepared media exactly as described in the previous study (Strobel et al. 2022).

1.2.2 Phage Strains

The fast-evolvable, fast-stable, and stable-evolvable λ genotypes used in this study were derived from lysogenic λ phage strains of cI857 that were part of a library of phage variants created for a previous study (Strobel et al. 2022). For ease of engineering, the initial library was created by editing a lysogenic λ prophage integrated into the E. coli chromosome. This, however, presented an obstacle to our suppression experiments because the phages still contained a functional cI repressor gene, enabling them to re-integrate into the *E. coli* host chromosome and

confer high levels of resistance. To ameliorate this, we first designed a method to switch each genotype to from lysogenic to obligate lytic. We generated an oligo that would introduce two stop codons into the early part of the cI gene to render it nonfunctional and used one cycle of MAGE (Wang et al. 2009; Wang and Church 2011) to introduce it into each desired prophage genome. We used a slightly modified MAGE protocol than previously described: first, we grew up each lysogen overnight at 30 ºC with carbenicillin; then, we inoculated three replicate tubes of 3 mL LB with 100 μL of overnight culture and 4 μL of carbenicillin and incubated for 1 hour at 30 ºC; we added 20 μL of 1M arabinose to each tube and incubated for another hour at 30 ºC. Aliquots of 1 mL were then pelleted and washed three times with ice cold, sterile, nanopure water to remove media and salt residue. We added oligos at a final concentration of 5 μM and electroporated cells. Finally, after electroporation, we recovered cells in fresh media and antibiotic overnight. We inserted the stop codons with an oligo containing two different stop codons at separate locations:
C*G*C*A*CGGTGTTAGATATTTATCCCTTGCGGTGATAGATTTAACGTATGTGAACA AA AAAGTAACCATTAACACAAGAGCAGCTTGAGGAC. Then, cells were recovered overnight, and in cells that successfully integrated the cI knockout oligo, subsequent cell divisions diluted out the cI protein to the point that λ initiated its lytic cycle. Since the cI gene was knocked out, the resulting phage could not re-integrate into the E. coli chromosome.

To generate the fast-evolved and stable-evolved genotypes, we began with the lysogenic strains that had been previously edited to contain the key N1107K mutation that confers OmpF-use (Strobel et al. 2022). To make these genotypes amenable to our suppression experiments, we generated obligately lytic versions using our cI knockout method described above.

1.2.3 Host *E. coli* Strains

For the suppression experiments, we used the *E. coli* strain REL606 (Jeong et al. 2009) as the host bacterium. For detection of phages that evolved to use the OmpF receptor during the suppression experiments, we used the *LamB*⁻ strain (JW3996) from the Keio collection (Baba et al. 2006). The wild type parent of the Keio collection (BW25113, referred to as "WT" throughout this manuscript) was used for estimating phage titer during the suppression, growth rate, and stability assays.

1.2.4 Sanger sequencing

We sequenced the reactive region of the *J* gene (approximately position 2650 to 3399 of 3399 total bp) to identify mutations in genotypes that evolved to be OmpF+ in the coevolution experiment. We also sequenced the cI gene to verify successful knockouts.

For sequencing the *J* gene we used primers: Forward 5' CCT GCG GGC GGT TTT GTC ATT TA; Reverse 5' CGC ATC GTT CAC CTC TCA CT and sent unpurified PCR products to Genewiz La Jolla, CA, for sequencing with the reverse primer.

For sequencing the CI gene we used primers: Forward 5' CGA CCA GAA CACCTT GCC 3'; Reverse 5' CCC TTG CGG TGA TAG ATT TAA CG 3' and sent unpurified PCR products to Genewiz La Jolla, CA, for sequencing with both forward and reverse primer. All sequences were aligned to the appropriate reference using Unipro UGENE v1.31.1 (Okonechnikov et al. 2012).

1.2.5 Stability, reproductive rate, and evolvability measurements

Stability and net reproductive rates for the fast-stable, fast-evolvable, and stable-evolvable genotypes were measured in a previous study (Strobel et al. 2022). In the previous study, stability was measured as the rate at which phage lost infectivity in media alone (i.e. no host cells), and net reproductive rate (called simply "growth rate" in Strobel et al. 2022) was measured as the rate of increase in phage titer when incubated with permissive host cells. The latter is a combined rate of reproduction and decay, so for the current study we obtained just the rate at which phage reproduce (i.e. factoring out decay) by subtracting the decay rate from the combined rate (i.e. reproductive rate = net reproductive rate – decay). Evolvability was measured as the ability of the phage to evolve to use the non-native OmpF receptor. Several measures of evolvability were previously reported: the number of replicate populations that evolved to use OmpF, the average number of days required to evolve OmpF, or the number of mutations required to use OmpF. All metrics were correlated and so here we only report the evolutionary path length to gaining OmpF use. Fewer mutations, or a shorter evolutionary path, corresponds to higher evolvability (Kirschner and Gerhart 1998) (Figure 1.1).

For the genotypes that we edited to receive the N1107K mutation (fast-evolved and stable-evolved), we measured net reproductive rates of the fast-evolved and stable-evolved immediately after generating the obligate lytic versions. Following the protocol in Strobel *et al.* 2022, we picked a single plaque of each genotype into 100 μL of M9 Glucose and divided the volume of 100 μL into the three replicate 50ml flasks with M9 Glucose and 0.01M MgSO4. We then measured initial phage titers by diluting the flask contents and plating with WT cells infused in soft agar and added ~10^8 REL606 cells to each flask and incubated at 37 C shaking for 4 hours. After incubation, samples were taken from each flask, filtered through 0.22 μM filters to

remove bacteria, and phage titers were re-measured by diluting in M9 glucose + MgSO4 and plating with WT cells infused in soft agar. We measured growth over 4 hours rather than 24 hours (the length of time between transfers in the evolution experiment) to reduce the possibility that genotypes would evolve mutations during the growth experiment. We did not measure decay rates of the fast-evolved and stable-evolved genotypes, so we report net reproductive rates for all four genotypes in Figure 1.A.3.

Because we measured the net reproductive rates of the fast-evolvable and fast-stable in the previous study (lysogenic versions) and here (obligate lytic versions), we were able to assess whether knocking out the cI gene to make the obligate lytic versions altered net reproductive rate. We did not find a significant effect (2-sample t-test, n = 3 per genotype; Fast-Evolvable: t-stat = -0.110, df = 4, p = 0.918; Stable-Evolvable: t-stat = 0.566, df = 4, p = 0.602).

1.2.6 Bacterial Suppression Experiments

To determine which phage genotypes would best suppress REL606, we inoculated six 50-mL flasks per phage genotype with 10 mL modified M9-Glucose and 0.01M MgSO4 and 10^6 bacterial cells and approximately $10^5 – 10^6$ phage particles (exact values reported in Table 1.A.2). Flasks were incubated at 37 °C, shaking at 120 rpm. After 24 hours, 100 μL of each community was transferred into new flasks with 10 mL of fresh media. Flasks were passaged for 10 days for the first two suppression experiments (Figures 1.2 and 1.3) and for 6 days in the third suppression experiment (Figure 1.4). Each day, 1 mL aliquots were removed to estimate bacterial and phage densities, as well as to freeze communities with glycerol for later analysis (40 μL 80% glycerol per 200 μL sample). To assess bacterial titers, aliquots were diluted in M9-Glucose and spot plated on LB agar. For phages, 1-mL aliquots were centrifuged (1 min at 15,000 × g) to

pellet cells. Then, supernatants were serially diluted in M9-Glucose, and 2-µL aliquots were spotted on a lawn of REL606 infused in soft agar to obtain phage titers.

1.2.7 Bioinformatic prediction of J structure

We used the publicly available version (Mirdita et al. 2022) of Alphafold (Jumper et al. 2021) to predict the structure of the reactive region of the wild type J domain containing the 173 most C-terminal amino acids. We used Chimera to create Figure 1.1A (Pettersen et al. 2004).

1.2.8 Statistical Tests and Plotting

We used RStudio to create Figures 1.2, 1.3, 1.4, 1.A.1, 1.A.2, and 1.A.4 and carry out statistical tests in this manuscript. We used a two-sided Wilcoxon Rank Sum test and α value of 0.05 for statistical significance between replicates of pairs of genotypes on a single day. We used MATLAB to create Figure 1.1B and 1.A.3. For statistical comparisons of trait values in Figure 1.A.3, we conducted two-sample T-tests after verifying equal variances in MATLAB.

1.3 Results

Of the three phage genotypes, the fast-evolvable proved to be most suppressive (Figure 1.2A, 1.A.1). There are statistical differences in the bacterial densities between the fast-evolvable and fast-stable genotype on days 2, 3, 4, 5, and 6 and the fast-evolvable and fast-stable genotype on days 2 4, 5, and 6 (Table 1.A.3; Figure 1.2B-C). The other two genotypes, fast-stable and stable-evolvable, appear to be equally poor at suppressing and not statistically different from each other on any day (Table 1.A.3; Figure 1.2D). Although there are timepoints where there are not significant differences in suppression, at least one replicate of this genotype was the most

suppressive on all ten days of the experiment (Figure 1.2B, 1.2C). Because two of the three genotypes suppressed so poorly, it is difficult to draw conclusions about all three traits, but it is at least clear that stability is not of paramount importance for suppression. It appears that the reproductive rate of the phage and its evolvability may both be important phenotypic traits to allow for bacterial suppression.

This result led to a new question: does a fast reproductive rate have its own, independent effect on suppression by increasing the number of phage particles relative to the number of bacteria, or is increased reproduction just a second way of enhancing evolvability by increasing the number of generations and opportunities to evolve counter defenses? If the reproductive rate is just a function of how many opportunities the phage has to overcome resistance mutations, then perhaps the low reproductive rate of the stable-evolvable genotype prevented it from achieving sufficient replications to generate the mutations necessary to adapt to the resistant bacteria by acquiring activity on OmpF. To parse out the independent effect of reproductive rate, we edited in a mutation (N1107K) that conferred the ability to use OmpF in both the fast-evolvable and stable-evolvable genotypes (Strobel et al. 2022). By doing so, we hoped to remove evolvability from the equation by artificially making both genotypes 'evolved' with respect to OmpF use. We then repeated the suppression experiment with the two evolved genotypes, and the fast-evolved genotype was substantially more suppressive than the stable-evolved genotype (Table 1.A.3) (Figure 1.3.) In fact, not only did the stable-evolved genotype not suppress the bacteria, it did not grow fast enough to keep up with the dilution from the daily transfer, and every replicate of this genotype lost phage entirely after day two (Figure 1.A.2). Because the stable-evolved genotype was even worse at suppressing than the stable-evolvable (i.e., before receiving the OmpF^{+} granting mutation), we measured the growth rates of the evolved versions

of each genotype to verify that N1107K did not introduce an unexpected fitness cost in the stable-evolvable background. It did not, although it did cause a significant gain in growth rate (combined reproductive rate + decay rate) in the fast-evolvable background (Figure 1.A.3). Together, these results indicate that reproductive rate has an independent effect on suppression, and evolvability on its own does not confer high suppression.

Thus far, these results suggest that evolvability and reproductive rate are important criteria for suppression, but stability did not appear to predict suppression. One possible explanation for this result is that the laboratory environment of our experiments is artificially permissive of instability, compared to the more challenging environment where therapeutic phage would need to be deployed, such as inside a patient's body. In our experiment, phage had access to a homogeneous population of rapidly growing hosts. Inside a patient's body, by contrast, phage would need to survive in a spatially complex environment in which their optimal host would be intermixed with numerous other microbes from the host's microbiome. In that environment, stability might be a better predictor of suppression. To evaluate this hypothesis, we repeated the suppression experiment exactly as was done in Figure 1.2, except we decreased the amount of glucose, a limiting resource of the bacteria, by ten-fold. This lowered the carrying capacity of the bacteria in the flask, thereby decreasing the density of host cells and increasing the amount of time that phages spent in the external environment between hosts, undergoing decay. We hypothesized that under these more challenging conditions, the fast-evolvable phage might not be more suppressive than the fast-stable phage. Consistent with expectations, the fast-stable phage is the most suppressive after one day (Figure 1.4, Figure 1.A.4, Table 1.A.3). However, by day three two thirds of the replicates of the fast-evolvable phage were the most suppressive, and by the end of the experiment it was all six replicates (Figure 1.4A-C, Table

1.A.3). This finding suggests that evolvability is more important than stability for suppressing bacteria over the long term, even in the more challenging condition where stability should be favored.

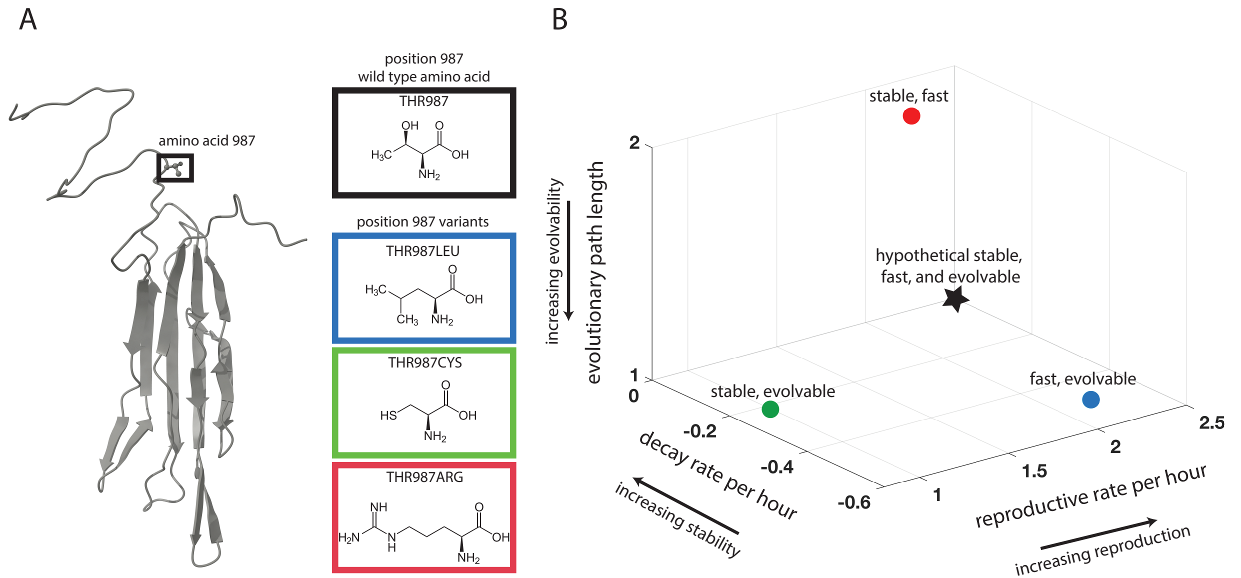

Figure 1.1 - A trio of closely related phage genotypes demonstrate a three-way trade-off between stability, reproduction, and evolvability. Panel A: AlphaFold prediction of the domain of the λ receptor binding protein that determines host range. The three λ genotypes in this study were identical except for a single amino acid difference in this domain (black box). Insets show the wild type amino acid and three variant amino acids. Panel B: Three-dimensional plot of phage trait values. Stability is measured by decay rate, the rate at which phage lose infectivity in an environment lacking hosts. Reproduction is measured by reproductive rate, the rate at which phage replicate on their host bacteria, adjusted to account for the phage lost to decay. Evolutionary path length is the number of mutations required to infect through the non-native receptor. It is the inverse of evolvability because a genotype requiring fewer mutations to achieve a new function is more evolvable. The position on the graph corresponding to optimality of all three traits is indicated by the star in the lower back corner. No phage was able to optimize all three traits.

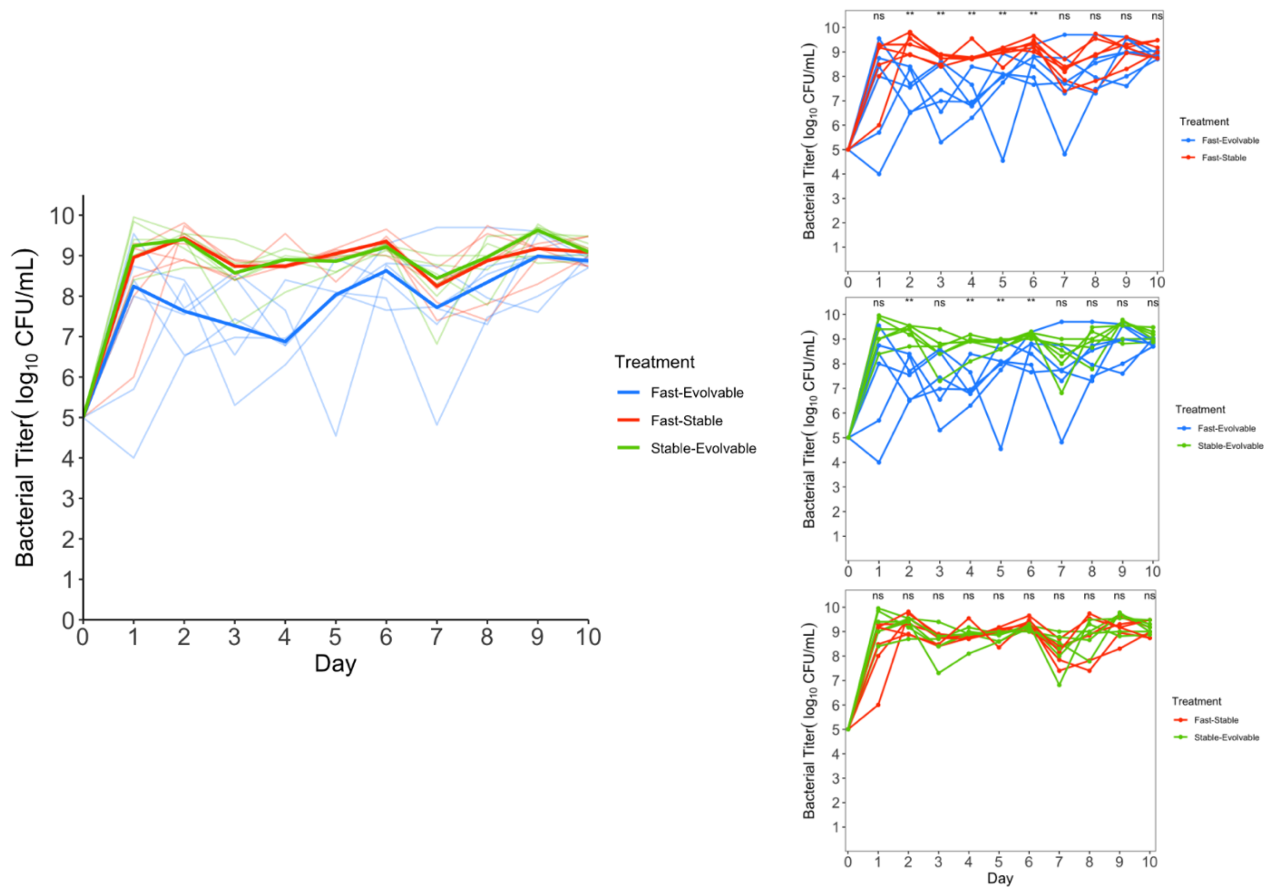

Figure 1.2 - λ suppression of bacteria monitored daily for 10 days. Each line corresponds to the bacterial titer in a single replicate flask population. Six replicate flasks were initiated for each phage genotype. Across all genotypes, the same bacterial strain was used to initiate the flask and approximately the same ratio of phage and bacteria were added. Panel A: All three phage genotypes are shown together. Median lines for bacterial population replicates A-F suppressed by a given genotype are shown in bold, and individual populations are shown by translucent lines. Panel B-D: pairs of genotypes are shown for ease of visualizing differences. Statistical differences are present on days 2-6 between the Fast-Evolvable replicates and Fast-Stable replicates, days 2, 4, 5, and 6 between the Fast-Evolvable replicates and Stable-Evolvable replicates, and statistical differences were not detected between the Fast-Stable replicates and Stable-Evolvable treatments at any time (Table 1.A.2). A Wilcoxon Ranked Sum Test was used to test statistical significance between levels of suppression between two phage genotypes.

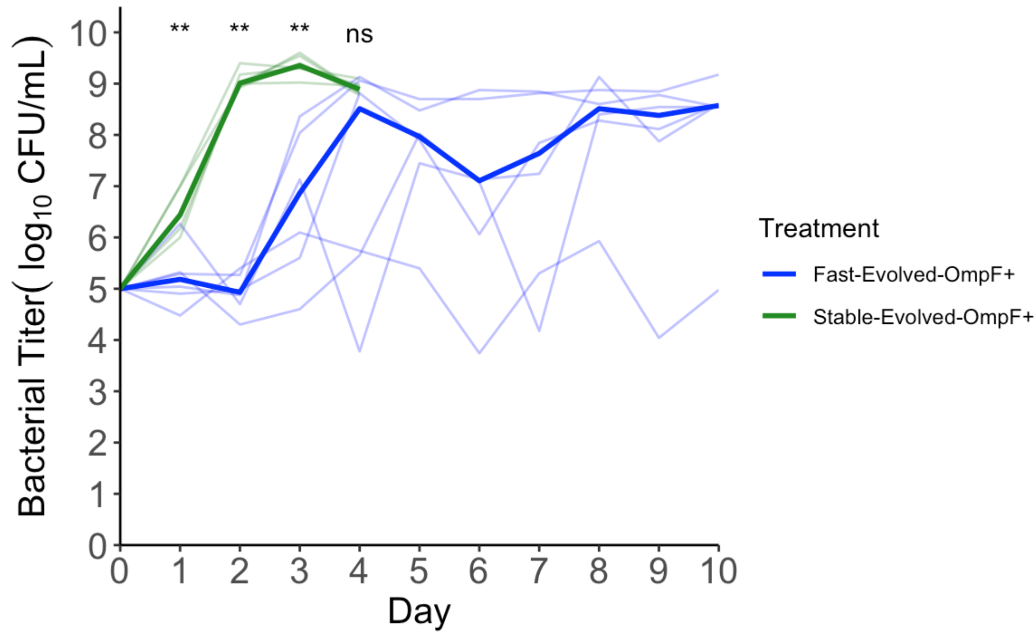

Figure 1.3 - Bacterial population dynamics exposed to two λ genotypes differing only in their reproductive rates. Both genotypes were engineered to contain a key mutation that conferred activity on the OmpF receptor, allowing infection of bacteria that are resistant to $OmpF^{-}$ phage. Six replicate flask populations were initiated for each phage genotype, with the same bacterial strain. Panel A: Median lines for bacterial population replicates A-F of a given genotype are shown in bold, and individual populations are shown by translucent lines. Each translucent line corresponds to the bacterial titer in a single replicate flask population. The stable, slow reproducing phage (green) poorly suppressed the bacteria and the phages passed below our limit of detection after the first day. The stable-evolved replicates were discontinued after three days of no phage detection. Statistical differences between replicate populations are present on days 1, 2, and 3 (Table 1.A.2). A Wilcoxon Ranked Sum Test was used to test statistical significance between levels of suppression between the two phage genotypes.

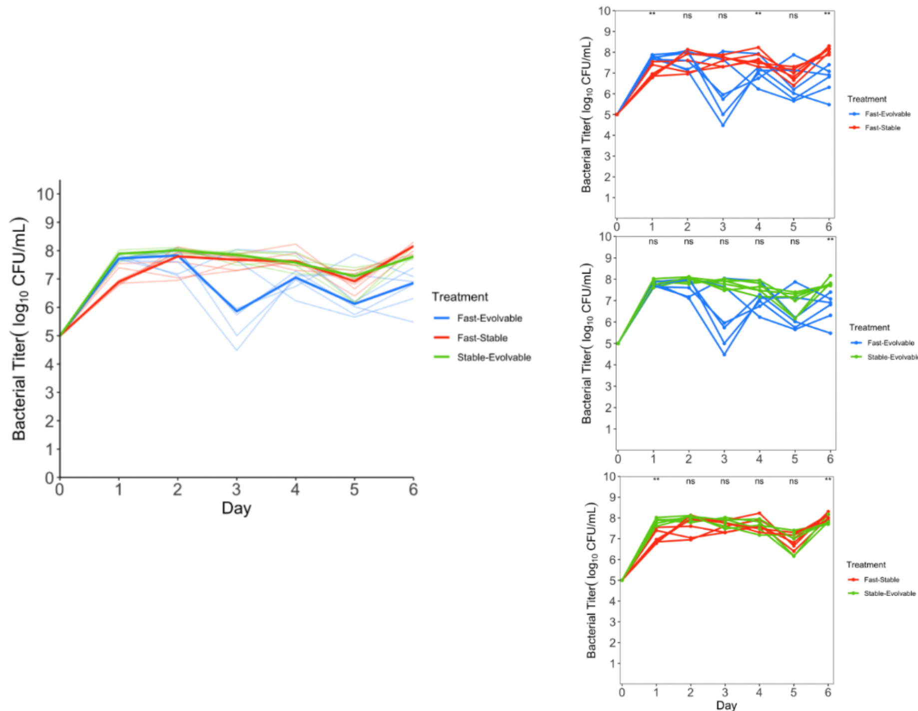

Figure 1.4 - Environmental contingency of bacterial suppression dynamics. The conditions of this experiment were identical to those of Figure 1.2, except that the available glucose was reduced by ten-fold, limiting the maximum potential bacterial carrying capacity throughout the duration of the experiment. Panel A: All three phage genotypes are shown together. Median lines for bacterial population replicates A-F suppressed by a given genotype are shown in bold, and individual populations are shown by translucent lines. Panel B-D: pairs of genotypes are shown for ease of visualizing differences. Statistical differences are present on days 1, 4, and 6 between the Fast-Evolvable replicates and Fast-Stable replicates, day 6 between the Fast-Evolvable replicates and Stable-Evolvable replicates, and days 1 and 6 between the Fast-Stable replicates and Stable-Evolvable replicates (Table 1.A.2). A Wilcoxon Ranked Sum Test was used to test statistical significance between levels of suppression between two phage genotypes.

1.4 Discussion

The aim of this study was to evaluate which phage trait is most predictive of bacterial suppression. We hypothesized that evolvability would be most predictive, and therefore the fast-evolvable and stable-evolvable genotypes would be most suppressive. The first suppression experiment with all three genotypes revealed that a single phage, the fast-evolvable genotype, was suppressive, while the fast-stable and stable-evolvable genotypes were not. This somewhat unexpected result validated the importance of evolvability over stability but also suggested that a fast reproductive rate, in addition to evolvability, is critical for suppression. In a follow-up experiment, growth rate had independent effect after controlling for evolvability. Finally, having shown that stability was the least predictive of suppression, we asked whether our experimental design favored that outcome by using conditions that are artificially permissive to unstable genotypes. To alter our experiment to better reflect the more challenging environment with fewer permissive hosts, we repeated the first suppression experiment using only 10% of the glucose, limiting the bacterial carrying capacity of the flask. Initially, the fast-stable phage was most suppressive, validating the hypothesis that decreasing host availability penalized unstable phage. However, by the end of the experiment the fast-stable phage had become non-suppressive, while the fast-evolvable phage had become suppressive. We confirmed that the gain in suppression was due to the fast-growing evolvable phage achieving robust growth on OmpF much earlier than the other genotypes. These findings emphasize the importance of considering not only the current traits of therapeutic phages, but also their capacity for adaptive evolution. The reversal of suppression ability across a multi-day timescale suggests the importance of measuring suppression over an entire course of treatment since resistance and counter-resistance traits only take days to evolve.

Although the need to consider the future evolutionary potential (i.e. evolvability) has not yet permeated *in vivo* phage therapy practice, numerous studies have demonstrated that providing an evolutionary advantage to phage generates more suppression. In a technique called 'phage training', an evolutionary advantage is given by coevolving a candidate phage with the target bacteria, allowing an evolutionary arms race to play out, and then isolating an evolved phage strain for use against the naïve bacteria (Laanto et al. 2020; Borin et al. 2021). Another strategy aims to enhance the evolution of more suppressive phage by increasing opportunities for recombination among different strains, allowing beneficial mutations to be shuffled into a single, highly suppressive genotype (Burrowes et al. 2019). Our study raises the possibility that there is substantial genetic variation in evolvability, even among closely related genotypes. Relatively simple laboratory experiments on existing collections of phages might identify genotypes with unusually high evolutionary potential.

With the rise of genetic engineering technology, there is increasing interest in designing phage with the traits deemed desirable for suppression. Our study sheds light on some possible pitfalls that might frustrate such attempts. In the phage variant library codon 987 was targeted because it had mutated during selection for increased stability (Strobel et al. 2022). Seeking additional variants with a range of stabilities, different amino acids were edited in at codon 987. The assumption was that altering the amino acid at codon 987 affected only stability and evolvability but might not have obvious pleiotropic effects on other traits. This appeared so for most variants; however, the 987CYS (the stable-evolvable genotype used in this study) had a markedly reduced reproductive rate. This result demonstrates that a genotype that seems to "break" one tradeoff (stability vs. evolvability) might pay a cost in another trait (reproductive rate). Had multiple axes of variation not been considered, this seemingly tradeoff-breaking phage

might have appeared to be an ideal therapeutic phage, but the current study demonstrated the opposite result. Better outcomes might be achieved from 'bioprospecting' naturally occurring phages or using directed evolution to evolve enhanced genotypes, rather than attempting to design optimal genotypes, because selection should penalize genotypes with low fitness.

There are several limitations to the current study. First, we examined only three phage genotypes, and their genomes were identical except for a single codon. The evolutionary history and idiosyncrasies of this experimental system could prevent our results from being applied generally across the vast diversity of phages. In λ, there is a known tradeoff between stability and evolvability (Strobel et al. 2022) that might not exist in other phages or might be less pronounced. In other phages, different combinations of traits might produce tradeoffs, and our study provides a general framework for understanding how tradeoffs limit the optimization of therapeutic phages. Another limitation is that we studied suppression dynamics in flasks under controlled laboratory conditions and used a single phage and a single bacterial host. These conditions are unlike the environment in which therapeutic phages would be deployed. The human gut, for example, where λ-like phages might be used, is replete with myriad host-associated microbes in addition to the target, many of which would likely be unavailable as prey for the therapeutic phage (Lozupone et al. 2012). We began exploring this dimension with our limited glucose environment, and our results were robust to the perturbation, but we did not test the effect of non-host microbes. Phage may also encounter extremes of temperature, pH, and chemicals *in vivo* that would penalize unstable genotypes (Blazanin et al. 2022). Despite these differences between λ's natural environment and laboratory conditions, comparisons between laboratory and natural populations of λ revealed that the sites that receive mutations that drive host-range expansion in the laboratory are present in natural populations, suggesting that

laboratory studies are informative for understanding natural dynamics (Maddamsetti et al. 2018). Prior work specific to phage therapy suggests that despite differences between *in vitro* and *in vivo* conditions, similar evolutionary dynamics can play out *in vitro* and *in vivo*, validating the use of in vitro experiments to inform clinical practices (Castledine et al. 2022).

This work demonstrates the important role of understanding evolutionary biology in phage therapy. Although phage have properties that are similar to chemical therapeutics, it is critical to remember that they are biological entities and have their own ability to propagate and evolve. When using directed evolution to create phage therapeutics, phage must be selected to evolve the properties that enable them to suppress bacteria while considering that evolving or engineering one desirable phenotypic characteristic may come at the cost of sacrificing another characteristic. Tradeoffs are difficult to avoid in biological systems, so evaluating traits in the context of tradeoffs is necessary. Considering evolutionary potential alongside conventional attributes, like stability and reproductive rate, allowed us to predict the genotype that best suppressed bacterial population across a multi-day timeframe.

1.5 Acknowledgements

Chapter 1 in full, is a reprint of the material currently being prepared for publication. Horwitz EK, Strobel HM, Meyer JR (2022). Evolvability is a key trait in the selection of bacteriophages for therapeutic use. The thesis author was the primary investigator and author of this paper.

1.A Appendix

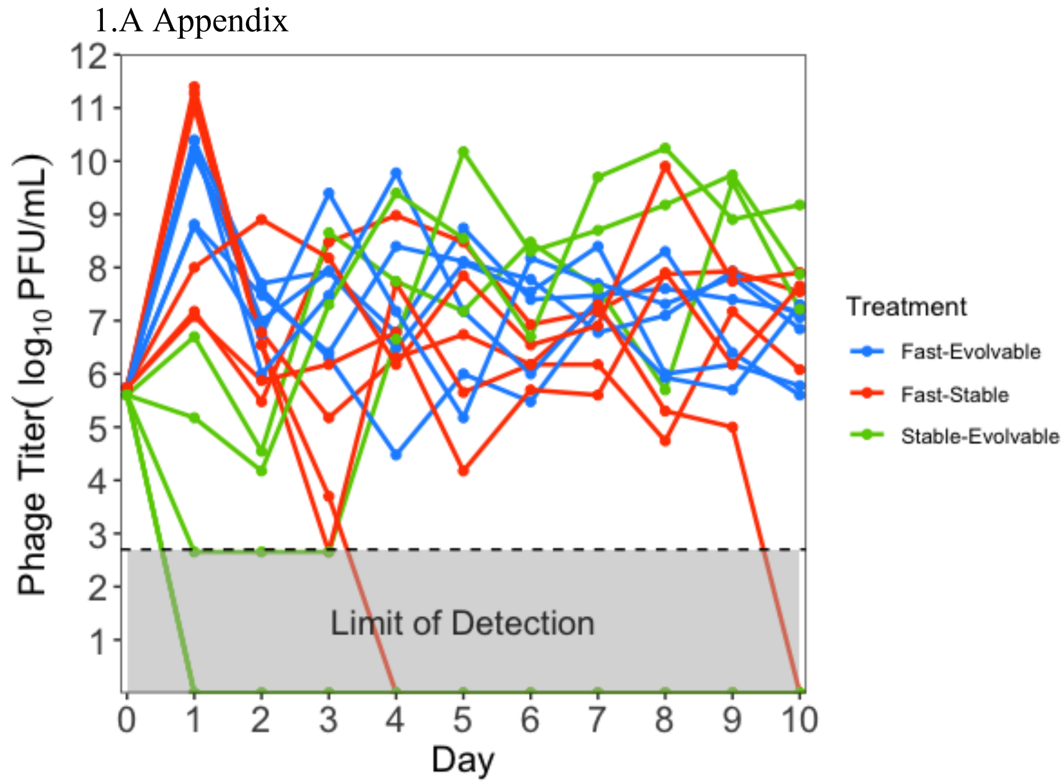

Figure 1.A.1 - Phage Titer from Figure 1.2
Phage densities were measured each day for all replicates of all 3 genotypes.

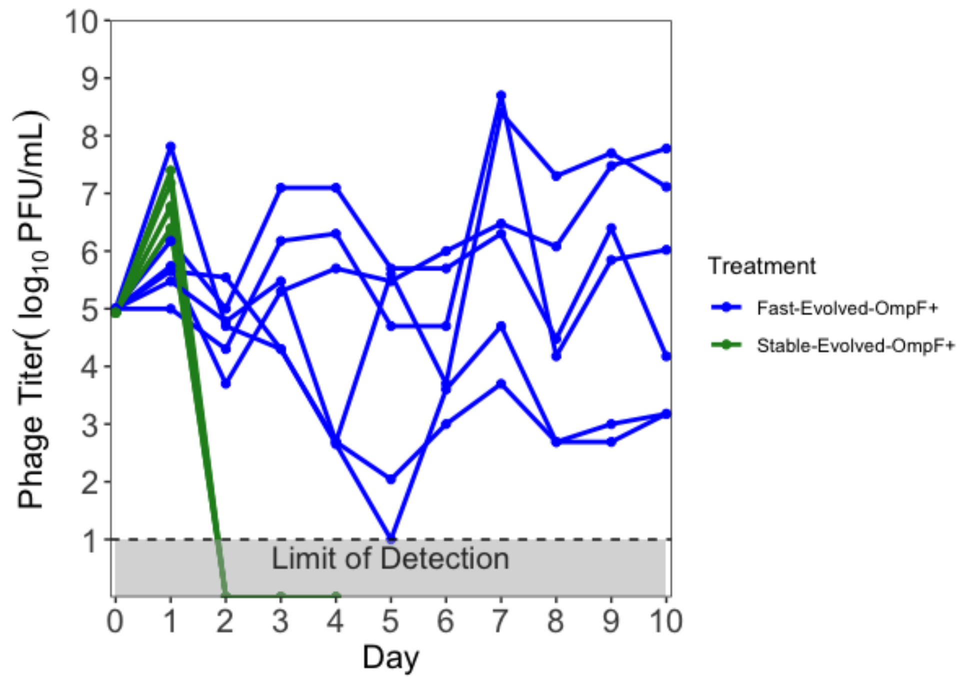

Figure 1.A.2 - Phage Titer from Figure 1.3
Phage densities were measured each day for all replicates of both genotypes.

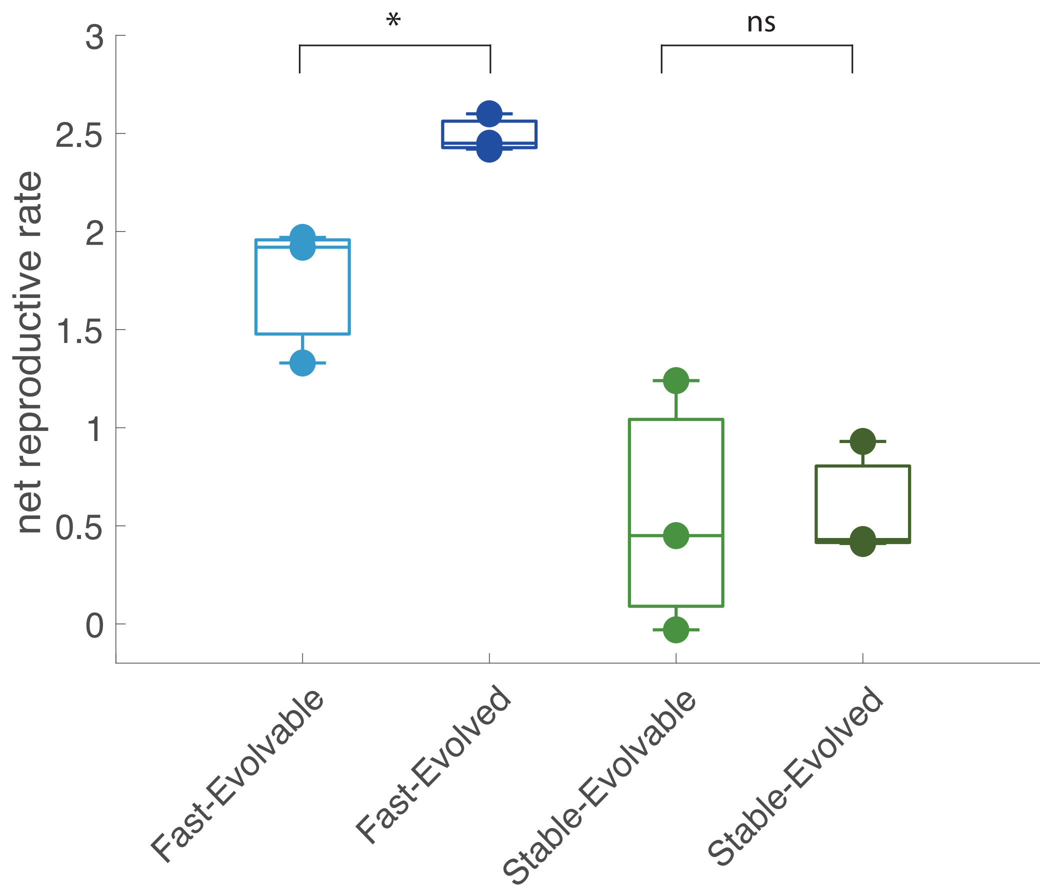

Figure 1.A.3 - Net reproductive rate (reproductive rate + decay rate) of the fast reproducing, unstable and fast reproducing, stable genotypes without the final mutation ("evolvable") and after receiving the final mutation via engineering ("evolved"). These genotypes were used in the suppression experiment from Figure 1.3. The net reproductive rate of Fast-Evolved was slightly higher than and Fast-Evolvable (2-sample T-test, n = 3 per genotype; t-stat = -3.523, df = 4, P = 0.0244), whereas the net reproductive rates of Stable-Evolvable and Stable-Evolved were indistinguishable. (2-sample T-test, n = 3 per genotype; t-stat = -0.09, df = 4, P = 0.933).

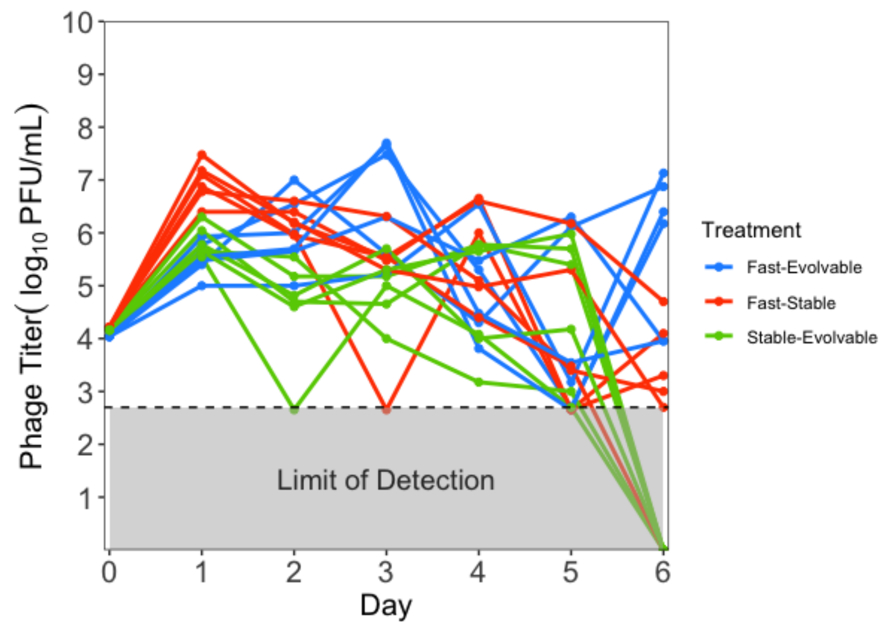

Figure 1.A.4 - Phage Titer from Figure 1.4
Phage densities were measured each day for all replicates of each 3 genotypes.

Table 1.A.1 - Phage trait values used to create Figure 1.1A. Decay rates and evolutionary path length were first reported in Strobel, Horwitz, and Meyer 2022. Reproductive rates were computed from previously reported net growth rates and decay rates from the same paper.

genotype	net reproductive rate per hour	decay rate per hour	reproductive rate per hour	distance to adaptation (# mutations)
THR 987LEU (Fast-Evolvable)	1.606601368	-0.485	2.091601368	1
	1.828305097	-0.47	2.298305097	
	1.714115496	-0.511	2.225115496	
THR987ARG (Fast-Stable)	1.631190757	-0.155	1.786190757	2
	1.873172219	-0.138	2.011172219	
	1.968209044	-0.119	2.087209044	
THR987CYS (Stable-Evolvable)	0.798291867	-0.211	1.009291867	1
	1.041989095	-0.215	1.256989095	
	0.50178022	-0.22	0.72178022	

Table 1.A.2 - Phage titers used to initiate suppression experiments.

	Suppression Exp. 1 (Figure 1.2)	Suppression Exp. 2 (Figure 1.3)	Suppression Exp. 3 (Figure 1.4)
Phage Genotype	Phage added to flask replicates	Phage added to flask replicates	Phage added to flask replicates
LEU	5.50E+05	1.00E+06	1.10E+05
ARG	5.50E+05		1.65E+05
CYS	4.00E+05	8.50E+05	1.45E+05

Table 1.A.3 Statistics from Figure 1.2, 1.3, and 1.4

Figure 1.2			
comparison	day	W	P-Value
fast-evolvable vs. fast-stable	1	12.5	0.4217
	2	0	0.002165
	3	3.5	0.02447
	4	0	0.004847
	5	1	0.004329
	6	2	0.008658
	7	14	0.5887
	8	12	0.3939
	9	14.5	0.6298
	10	10	0.2273
fast-evolvable vs. stable-evolvable	1	5.5	0.05382
	2	0	0.004922
	3	7	0.09123
	4	1	0.007687
	5	5	0.04113
	6	4	0.03035
	7	13	0.4848
	8	10	0.2403
	9	8	0.1262
	10	7	0.09155
fast-stable vs. stable-evolvable	1	10	0.2281
	2	21	0.6863
	3	23	0.4673
	4	11	0.2928
	5	28.5	0.1087
	6	26	0.2248
	7	17	0.5211
	8	13.5	0.9372
	9	17	0.2281
	10	10	0.7457
Figure 1.3			
comparison	day	W	P-Value
fast-evolved vs. stable-evolved	1	2	0.01291
	2	0	0.002165
	3	0	0.004998
	4	13	0.468
Figure 1.4			
comparison	day	W	P-Value
fast-evolvable vs. fast-stable	1	36	0.004922
	2	21.5	0.6304
	3	9	0.1727
	4	5	0.04113
	5	10	0.2403
	6	0	0.004998
fast-evolvable vs. stable-evolvable	1	9	0.1712
	2	10.5	0.2615
	3	8	0.132
	4	6	0.06508
	5	11	0.3095
	6	0	0.004998
fast-stable vs. stable-evolvable	1	0	0.004998
	2	10	0.2403
	3	9	0.1712
	4	20.5	0.7466
	5	16.5	0.8721
	6	32	0.02919

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