Effects of single mutations from experimental evolution of microbial proteins: Thermostability in Φ6 Cystovirus and toxin diversification in *Escherichia coli*

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Abstract

Effects of single mutations from experimental evolution of microbial proteins: Thermostability in Φ6 Cystovirus and toxin diversification in *Escherichia coli*

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Experimental and directed evolution using microbes offer powerful methods for uncovering processes of evolution across the tree of life. The goal of such experiments is to generate mutational diversity, either through propagation of microbes in stressful conditions (experimental evolution) or through artificial introduction of mutations into their genomes (directed evolution). In the case of multiple resulting mutations, each is then reverse engineered into the ancestral genotype individually to determine how it changes the phenotype of interest.

This thesis presents the results of one experimental evolution project (evolution of viral thermostability under increasing temperatures) and one directed evolution project (diversification of toxin-antitoxin protein pairs in bacteria), including both evolutionary and single-mutation analyses. In both cases, I found that mutations may persist in a population due to their pleiotropic effects on traits other than the focal one of the study. My thesis emphasizes the usefulness of laboratory evolution of microbes to guide new hypotheses about evolutionary processes.

Chapter 1. Adaptations of an RNA virus to increasing thermal stress. In an incrementally changing environment, a shift from one environmental state to another occurs over multiple organismal generations. The *rate* at which the environment changes is expected to influence both

how and how well populations adapt to the ultimate environment. To investigate this question, I evolved the lytic RNA bacteriophage Φ6 for greater thermostability by exposing viral populations to heat shocks that increased to a maximum temperature at different rates. I observed increases in the ability of many heat-shocked populations to survive high temperature heat shocks, although the survival of populations at the highest temperature and the number of mutations per population did not vary significantly according to the rate of thermal change. I then engineered specific mutations from the endpoint populations into the ancestral genotype and evaluated the effects of these mutations on viral thermostability and growth. As expected, some mutations increased viral thermostability. However, other mutations *decreased* thermostability but increased growth rate, suggesting that benefits of an increased replication rate may have sometimes outweighed the benefits of enhanced thermostability. This work highlights the importance of considering the effects of multiple selective pressures, even in environments where a single factor is changing.

***Chapter 2. Colicin mutation confers resistance to colicins in* Escherichia coli**. Colicins are toxic proteins produced by *Escherichia coli* that target and kill other *E. coli* cells. To prevent death by clone-mates, colicinogenic cells also express an immunity protein that neutralizes their own colicin by binding to it tightly and specifically. Although disruption of this binding interaction can be lethal, the colicin-immunity complex has diversified multiple times. Diversification is typically thought to occur in an immunity-led manner, through a promiscuous immunity protein that can bind multiple colicins. I aimed to test colicin-immunity diversification though directed mutagenesis of the immunity and colicin genes and screening for novel colicin-immunity pairs. I isolated a novel colicin that killed cells that expressed the ancestral immunity protein. Unexpectedly, when this novel colicin was combined with the ancestral immunity protein in the same cell, not only did the cells survive, but they also demonstrated resistance to a wide range of

other colicins. Through deeper investigation of the novel colicin, I demonstrate a mechanism of colicin resistance that depends only on the colicin genotype. That a colicin can itself protect cells from its toxic effects furthermore suggests that colicin-immunity diversification might be able to proceed in a colicin-led manner.

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Chapter 1

Adaptations of an RNA virus to increasing thermal stress

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Abstract

Environments can change in incremental fashions, where a shift from one state to another occurs over multiple organismal generations. The *rate* at which the environment changes is expected to influence how and how well populations adapt to the ultimate environment. We used a model system, the lytic RNA bacteriophage Φ6, to investigate this question empirically. We evolved viruses for thermostability by exposing them to heat shocks that increased to a maximum temperature at different rates. We observed increases in the ability of many heat-shocked populations to survive high temperature heat shocks, and on their first exposure to the highest temperature, populations that experienced a gradual increase in temperature had higher average survival than populations that experienced a rapid temperature increase. However, at the end of the experiment, neither the survival of populations at the highest temperature nor the number of

mutations per population varied significantly according to the rate of thermal change. We also evaluated mutations from the endpoint populations for their effects on viral thermostability and growth. As expected, some mutations did increase viral thermostability. However, other mutations *decreased* thermostability but increased growth rate, suggesting that benefits of an increased replication rate may have sometimes outweighed the benefits of enhanced thermostability. Our study highlights the importance of considering the effects of multiple selective pressures, even in environments where a single factor is changing.

Introduction

The process of *de novo* adaptation is typically studied in the context of the simplest form of environmental change, an abrupt shift from an old environmental state to a new one (e.g., [1]). Immediately following the environmental change, the mean fitness of the population shifts to a relatively low value (that is, it is poorly adapted to that environment). The population's mean fitness then increases as the population gains and fixes beneficial mutations. For instance, under Fisher's geometric model [2], the population is expected first to fix mutations that confer large gains in fitness, followed by mutations of increasingly smaller beneficial effect as the population approaches the optimal phenotype in the new environment [1].

However, natural environments rarely change in the simple, abrupt fashion assumed by such models. Rather, environmental changes can occur more gradually, on scales that encompass multiple organismal generations. For example, shifts between glacial and interglacial periods occurred over thousands of years (e.g., [3]). Even changes that are rapid on geological scales, such as anthropogenic climate change (e.g., [4]) or changes in pollution levels (e.g., [5]), occur over

multiple decades. Adaptation may proceed very differently in such cases of incremental environmental change [6-8].

Evolution in an incrementally changing environment is often modeled as a single quantitative trait evolving under Gaussian stabilizing selection in conditions where the optimal phenotype is constantly shifting [6, 8-13]. In contrast to adaptation under rapid environmental change, adaptation under gradual change is more likely to proceed via fixation of mutations that provide small shifts in phenotype and thus small increases in fitness [6-8]. These shifts allow an evolving population to track the optimal phenotype, but with a lag. The larger the phenotypic lag, the lower the mean fitness of the evolving population. The rate of environmental change can influence the adaptive process by setting the rate at which the population must track changes in the optimal phenotype. More rapid changes in the optimal phenotype typically result in a larger lag of the quantitative trait [6, 8, 11, 12, 14-16]. If a larger distance between the population's mean phenotype and the optimal phenotype also results in the death of a larger number of individuals, then sufficiently rapid environmental change can lead to population collapse, as small populations lose genetic variation necessary for adaptation [11-13, 17, 18].

The assumptions made by theoretical models may not always be met in biological systems. For this reason, empirical studies using microorganisms have been important in refining our understanding of evolution in incrementally changing environments. In some studies, and in line with model predictions, the rate of population extinction is lower under more gradual environmental change [19-21]. While models predict a higher mean fitness under gradual than rapid environmental change (due to a smaller lag between the population's mean phenotype and the optimal phenotype), these models tend to consider unlimited change. In contrast, many experiments set limits on the maximum amount of change in the environment. In this framework,

the level of environmental stress increases at different rates up to a maximal level, such that treatments involving more rapid change reach the maximum sooner and remain at the maximum longer. Such studies reveal heterogeneity in results of adaptation at the environmental limit. Exposure to low levels of stress can sometimes increase the probability that a population will survive at the environmental limit [20-23]. In some studies, adaptive phenotypes obtained under gradual environmental change have higher fitness in the most stressful environment than phenotypes obtained under rapid environmental change [7, 24, 25]. In other studies, adaptive phenotypes obtained under rapid environmental change are fitter [19, 20, 23]. One study found that the rate of environmental change did not affect fitness in the ultimate environment [26].

Empirical studies also reveal complexities in how the rate of environmental change affects the amount of genetic variation present during the adaptive process. Higher population sizes and less extreme selection coefficients under more gradual environmental change may permit greater genetic diversity [21, 24]. In asexual microbial populations, clonal interference, where distinct beneficial mutations arise in different genetic backgrounds and cannot recombine [27], may also be more prominent under gradual environmental change if multiple mutations of small effect are available simultaneously [24, 25]. On the other hand, when environmental change cannot exceed a maximal value, populations under gradual change must survive a greater range of environments, while populations experiencing the most rapid change must only survive the most extreme environment. If the exposure to a greater diversity of selective environments constrains mutations beneficial in all environments, then a greater diversity of mutations would be predicted under rapid change [7]. Consistent with this hypothesis, some studies find greater variability in phenotypes [7, 19] or fixed mutations [25] under rapid than attenuated environmental change.

Given the heterogeneous results from prior experiments, further experimental studies with different organisms and environmental factors are warranted. In this study, we exposed populations of the lytic RNA bacteriophage Φ6 to heat shocks that increased to a high temperature maximum at varying rates (suddenly, moderately, or gradually; Figures 1 and 2). Subjecting the viruses (but not the host) to heat stress promotes the evolution of thermotolerance via greater stability of viral proteins: Only viruses that survive heat stress with little enough damage that they can subsequently infect a host cell are able to replicate. To track adaptation over time, we measured the percent of the viral population that was able to survive heat shock at each transfer.

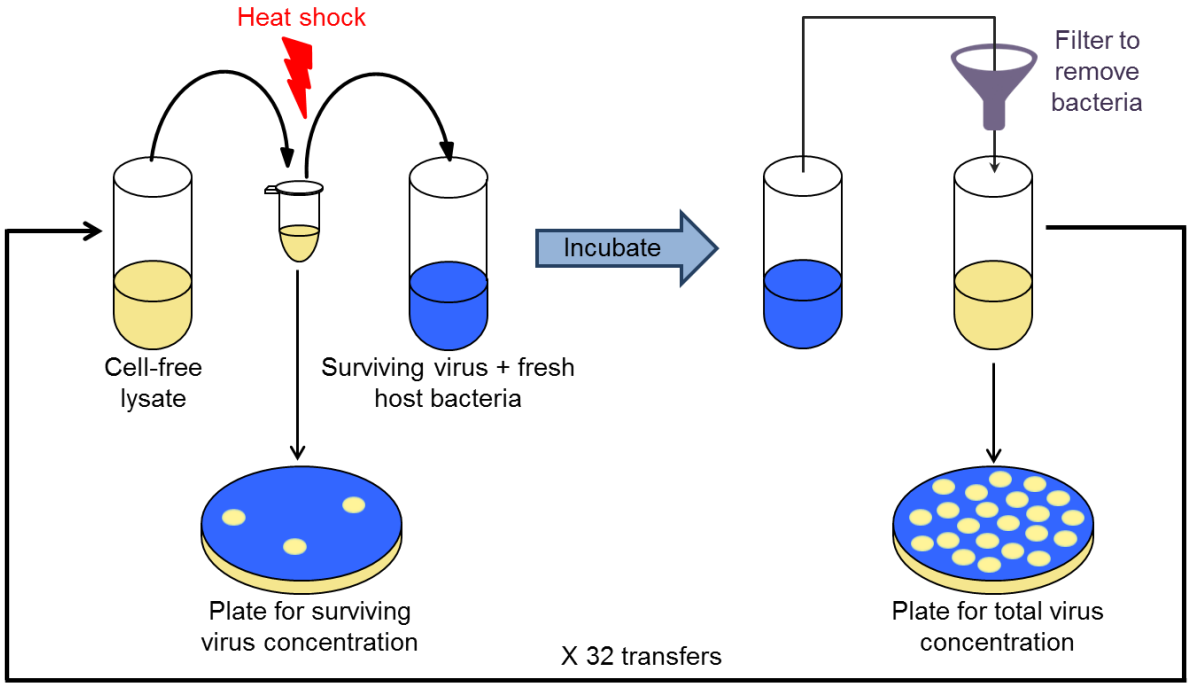


Figure 1. Schematic of the evolution experiment. Bacteria-free lysates of virus were heat shocked for 5 minutes at a pre-specified temperature (Figure 2), then added to culture with naïve host bacteria and grown overnight at 25°C. After the growth period, the viruses were separated from the bacteria by filtration, and the new cell-free lysate was again heat shocked. In order to track changes in survival to thermal stress, viral lysates were plated for concentration before and after heat shock by mixing a dilution of the lysate with abundant host bacteria in soft agar and spreading it onto a Petri dish. After overnight incubation, we counted

plaques in the bacterial lawn, each of which originated from a single viral particle. The experiment ran for 32 transfers (approximately 100 viral generations).

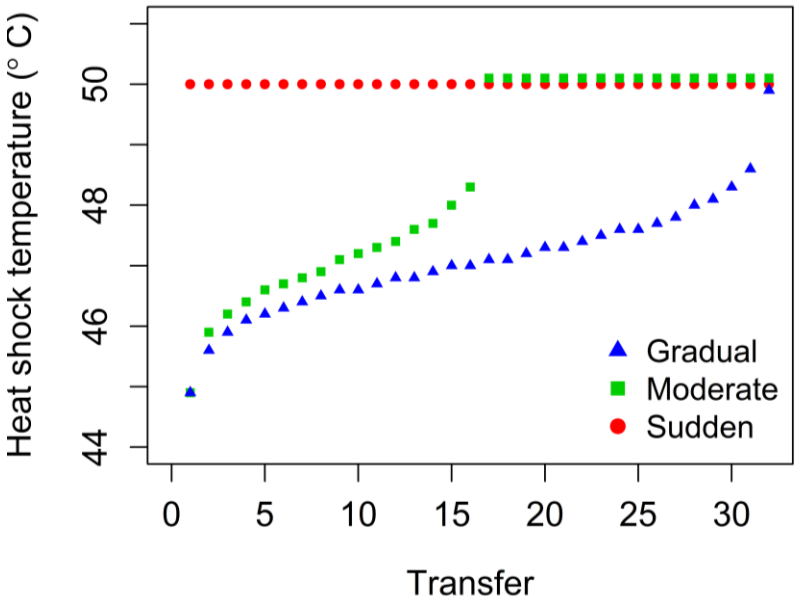


Figure 2. Temperature regimes for experimental evolution with varying rates of thermal change. Points are offset vertically at 50°C for purposes of visualization. Temperature increments were chosen such that the ancestral virus would experience constant (i.e., linear) decreases in its probability of survival. A control regime of heat shocks at a constant 25°C (not shown) accounted for evolutionary change under transfer conditions. See also S2 Table.

We aimed to address how varying rates of thermal change would affect viral evolution in:

1) *Survival on the first exposure to the most extreme environment.* Assuming that mutations that enable survival at intermediate temperatures also contribute to survival at the highest temperature, we predicted that populations under gradual thermal change would have a greater survival when they first reached the highest temperature because they had more time to gain and fix thermostabilizing mutations.

2) *Survival in the most extreme environment at the end of the experiment.* On the one hand, populations that experienced the most rapid change in temperature would also have more time to adapt under the highest temperature, favoring higher final fitness in this treatment. On the other hand, if mutations selected under intermediate temperatures serve as genetic backgrounds for additional mutations that confer high fitness under high temperatures, then populations that experienced a more gradual change in temperature might have higher survival in the ultimate environment (see [21] for a discussion of such epistatic effects in the context of adaptation in changing environments).

3) *The quantity and effect sizes of mutations that permit survival at high temperatures.* We predicted that, in environments that changed rapidly, thermostability would increase more often through single mutations of large effect, while in environments that changed more gradually, thermostability would increase more often through multiple mutations of small effect.

While we did find that evolution at intermediate temperatures enhanced the ability of populations to survive their first exposure to the highest temperature, similar adaptive endpoints were accessible under all rates of environmental change, and the ultimate survival at the highest temperature did not differ across treatments. We also found no strong relationship between the rate of environmental change and the number or effect size of mutations. In addition, we found that selective pressures orthogonal to those of the changing environment can still play a major role in shaping adaptive solutions in stressful environments.

Methods

Strains and culture conditions

A list of all viral and bacterial strains used or engineered in this study appears in S1 Table.

ϕ6 Cystovirus has a tripartite genome made of double-stranded RNA. The particular strain used in this study originated from three plasmids containing cDNAs of each of the wild type Φ6 segments [29, 30]. These plasmids were co-transformed into bacterial host cells (*Pseudomonas syringae* pathovar *phaseolicola*) to make phage particles [29, 30] (see Reverse engineering, below, for details). L. Mindich (Rutgers University, Newark, New Jersey) kindly provided the following strains: LM4286 (contains pLM687 with the ϕ6 L segment [31]), LM4284 (contains pLM656 with the ϕ6 M segment [32]), LM4285 (contains pLM659 with the ϕ6 S segment [33, 34]), and LM987 (contains pLM857 with the ϕ6 M segment and a lacH marker, which creates phages that make blue plaques [35]).

The laboratory bacterial host for Φ6 growth, *P. phaseolicola* HB10Y, derives from ATCC #21781. Transformation of the phage plasmids was performed into LM2691, a variant of *P. phaseolicola* HB10Y containing a plasmid with a T7 reverse transcriptase [36, 37] (see also Reverse engineering). Both of these hosts were kindly supplied to our laboratory by C. Burch (University of North Carolina, Chapel Hill). During competition assays, counts of clear and blue Φ6 plaques (made from plasmid pLM857) were distinguished on bacterial lawns of a second HB10Y variant, LM1034 (kindly provided by L. Mindich), which contained a plasmid with a lac omega gene [35].

Host cultures were initialized from individual colonies and grown overnight at 25°C in LC medium (Luria-Bertani broth at pH 7.5). Antibiotics (15 μg/mL tetracycline or 200 μg/mL ampicillin) were added to cultures of LM2961 or LM1034, respectively, to maintain their plasmids.

Each viral lysate was prepared from a plaque that had been isolated and stocked in 500 µL of 4:6 (v/v) glycerol:LC. A diluted sample of the virus stock was mixed with 200 µL of stationary-phase of *P. phaseolicola* in LC 0.7% top agar. The mixture was overlaid on an LC 1.5% agar base, and the agar plate was incubated overnight at 25°C. Plaques were collected and filtered in 3 mL of LC medium through cellulose acetate filters (0.2 µm pore, Thermo Scientific) to remove bacterial cells.

Evolution experiment

The evolution experiment was initialized with a lysate made from a single plaque that had resulted from the transformation from plasmids pLM687, pLM656, and pLM659 (see also Reverse engineering), prepared as described under Strains and culture conditions. This lysate was divided among 20 populations across four treatments with five replicates each (5 Gradual populations, 5 Moderate populations, 5 Sudden populations, 5 Control populations). Cell-free lysates of each population were heat-shocked at a pre-determined temperature (Figure 2, S2 Table), then added to culture with naïve *P. phaseolicola* for overnight growth at 25°C. We performed heat shocks on lysates (i.e., without the bacterial host) so that viral evolution was not affected by host heat shock responses. (We had additionally determined that the bacterial host does not survive temperatures above 45°C.)

Our thermal regimes paralleled the design used in other studies [7, 21, 25]:

1) *Sudden:* First and all subsequent heat shocks were performed at 50°C.

2) *Moderate:* Heat shock temperatures increased from 45°C over the course of evolution, reached 50°C halfway through the experiment, and remained at that temperature thereafter.

3) *Gradual:* Heat shock temperatures increased from 45°C and only reached 50°C on the final transfer.

4) *Control:* Viruses only received a mock "heat shock" at their normal growth temperature (25°C).

The exact rates of increase were determined empirically, with reference to survival of the ancestral genotype across a range of 45-50°C. Each increase in temperature in the Gradual and Moderate lineages represented equally-spaced drops in percent survival for the ancestor. (Further details on calculation of treatment heat shock temperatures are available in the Data Repository.)

Preparation for heat shock

Lysates were created from overnight liquid cultures by centrifuging 800 µL of culture at 10,000 rcf through a cellulose acetate spin filter with a 0.2 µm pore (Costar). The lysate's titer was taken as the mean of duplicate titers on *P. phaseolicola* in agar plates. To control for any density-dependent effects of heat shock on viral survival, we adjusted all lysates by dilution to match the lysate with the lowest titer for that transfer (between 8×10^9 and 2×10^{10} plaque-forming units [pfu] /mL). We note that, across treatments, lysate titers fell within less than an order of magnitude of each other, and the treatment to which lowest-titer lysate belonged varied across transfers.

The titer-adjusted lysates were diluted and plated on *P. phaseolicola* for their pre-heat shock concentrations.

Heat shock

Heat shocks were then performed on the lysates that had been diluted to the same titer. 50 µL of lysate were aliquoted into PCR strip tubes (one tube per replicate population), placed for 5 minutes on a thermocycler (BioRad, C1000 Thermal Cycler) pre-heated to the appropriate

temperature (S2 Table), and then chilled on ice. The heat-shocked lysates were diluted and plated for a count of post-heat shock concentrations.

Culturing of surviving phages

Viruses that had survived heat shock were then introduced to bacterial host cells in liquid culture for amplification. Cultures used 4 mL of LC broth and were initialized with a 1/100 dilution of naïve, stationary-phase *P. phaseolicola,* and heat-shocked lysate to a final concentration of approximately 2.5 x 10^3 viral particles/mL. (These concentrations were approximated based on survivals from the previous transfer, because the exact lysate titers were not known until following day.) We used the same initialization concentration of viruses across treatments to ensure equal mutational opportunities. Cultures were incubated for 24 hours at 25°C with orbital shaking to allow the phages to amplify. The cultures were then prepared for the next round of heat shock as described above.

Storage

After each transfer, at least 500 μL of each post-amplification population were mixed with glycerol to a final concentration of 40% and stored long-term at -20°C.

Reverse engineering

All mutations discussed in this paper were constructed on pLM659, the plasmid containing a cDNA copy of the S segment of ϕ6. Mutations were engineered into this plasmid using the QuikChange II Mutagenesis kit (Agilent) following the manufacturers' instructions. Primers for each mutation are included in S4 Table. Mutagenized plasmids were stored in *Escherichia coli*

XL1-Blue bacteria (included in the QuikChange II Mutagenesis kit), and the mutations of interest were confirmed by Sanger sequencing.

Mutation V109I was engineered using mutagenic PCR and T4 ligation. The plasmid pLM659 was PCR amplified from adjacent, overlapping primers with 5' phosphorylated ends, one of which contained the mutation of interest, using Phusion polymerase (Thermo Scientific) according to the manufacturer's instructions. To remove (unmutagenized) template plasmid, the PCR product was digested with DpnI (New England Biolabs) according to the manufacturer's instructions. Approximately 14 ng of DpnI-digested PCR product were used in a T4 ligation (New England Biolabs) according to the manufacturer's instructions, and the ligation product was transformed into electrocompetent *E. coli* DH5α, prepared as described for LM2691 below, for storage.

Creating phage particles involved transforming plasmids with each of the Φ6 genomic segments into the bacterium LM2691. We made this strain electrocompetent with the following protocol: A culture of LM2691 was grown to stationary phase, then diluted 1/10 into 50 mL of fresh media and grown to exponential phase. The cells were chilled on ice, then pelleted by centrifugation (6 minutes at 2850 rcf) and washed multiple times with the following resuspensions:

1. 50 mL of ice-cold, sterile water.
2. 15 mL of ice-cold, sterile water.
3. 2 mL of ice-cold 10% glycerol.
4. < 1 mL of ice-cold 20% glycerol (exact volume depended on the number of transformations being performed at the time).

The final suspension of cells was aliquoted into 40-μL volumes for working use.

At least 5 ng of a plasmid containing each Φ6 segment (S, M, and L) were combined with the competent cells (in some cases as much as 100 ng of each plasmid were necessary), incubated on ice for 1 minute, and electroporated on an Eppendorf Eporator in an ice-cold cuvette with a 1-mm gap. The cells were resuspended in 700 μL of SOC medium [38], added to 3 mL of LC 0.7% top agar, plated on LC 1.5% agar plates, and incubated overnight at 25°C. Successful transformations were indicated by viral plaques in the bacterial lawn. At least 6 plaques per genotype were stored for sequence confirmation (see Sequencing viral genotypes).

The ancestral genotype for the evolution experiment resulted from transformation of the original plasmids, pLM687, pLM656, and pLM659. For engineered phage, an engineered version of pLM659 was combined with the original versions of pLM687 and pLM656. A version of Φ6 marked with LacH (used for assaying viral fitness) resulted from transformation of plasmids pLM687, pLM659, and pLM857.

Sequencing viral genotypes

Sequencing was performed from viral lysates originating from either the stored populations from the evolution experiment or the stored reverse engineered plaques, and made as described in Strains and culture conditions. RNA was extracted from the lysates using the QIAamp Viral RNA Mini Kit (Qiagen) and reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen), following the manufacturers' protocols. cDNA samples were PCR amplified with phage-specific primers using touchdown cycling (annealing temperature 65-55°C for 10 cycles, reducing the temperature by 1°C each cycle, followed by 25 cycles with a 55°C annealing temperature). The resulting products were given an ExoSap-IT cleanup (Affymetrix) and Sanger

sequenced through GeneWiz. Primers were designed to permit 2x coverage of at least 90% of the genome (excluded sections at the segment ends).

Sequence alignments were performed in Geneious v. 10.0.6 and inspected by eye. The ancestral sequence (GenBank Accession # MF352213-MF352215) was built through alignment against sequence files of the plasmids containing the wild type Φ6 segments (provided by L. Mindich). All other sequences were aligned against the ancestral sequence.

Assaying viral thermostability

We assessed viral thermostability by exposing lysates to heat shocks across a range of temperatures and measuring the change in viral density under each temperature.

Cell-free lysates of each evaluated genotype were prepared as described under Strains and culture conditions and titered on *P. phaseolicola.* Each block of heat shocks included 3-5 unique genotypes (plaques) and the ancestral genotype. To control for any density-dependent effects of heat shock on viral survival, all lysates were diluted to a titer of 2.17×10^8 viral particles/mL (concentration of the lowest-titer lysate across assay blocks). The diluted lysates were plated on *P. phaseolicola* for pre-heat shock titers and were used for heat shocks.

Lysates were heat shocked over a range of temperatures from 25-54°C. For each temperature tested, three replicate samples containing 50 μL of lysate were heat-shocked for 5 minutes in a pre-heated thermocycler (BioRad, C1000 Thermal Cycler), then chilled on ice.

After heat shock, the lysates were diluted and plated for their survival. Survival of the lysates was calculated as the ratio of post- to pre-heat shock titer multiplied by 100. We estimated viral thermostability across the temperature range using an inverse Hill equation:

$$S = 100 \times \frac{{T_{50}}^n}{{T_{50}}^n + T^n}$$

(Equation 1)

where S is the percent survival, T is the heat shock temperature, T_{50} is the temperature with 50% viability, and n is the Hill coefficient. A program written in R (version 3.1.2; code available in the Data Repository) estimated parameters T_{50} and n using maximum log likelihood. Genotypes with a greater T_{50} were considered to be more thermostable.

Assaying viral competitive fitness

Viral growth rates were evaluated through growth competitions against a marked common competitor, the lacH-marked Φ6, under conditions that replicated those of growth during the evolution experiment. Plaques formed by the lacH-marked Φ6 turn blue when plated with X-Gal on LM1034 (a bacterial host containing a plasmid with the complementary lac omega gene), allowing us to distinguish the common competitor from the genotypes engineered for this study.

The common competitor was transformed from plasmids (see Reverse engineering). To pre-adapt this strain to the competition conditions, the plaque isolated from the transformation was passaged for five days in liquid LC medium. However, its growth rate remained low compared to the ancestor of the evolution experiment, so competitions were initialized at a 1:10 ratio of focal strain : common competitor. We confirmed that, for the ancestor, changing its initial ratio in the competition did not affect the measured competitive fitness (S1 Figure).

Lysates of the ancestral virus, each mutant virus, and the lacH-marked common competitor were made up from frozen stocks containing plaques, as described in Strains and culture conditions. Competition mixtures were created by combining the lysate of the focal strain, diluted

to 2.89 x 10^8 pfu/mL, with the lysate of the common competitor, diluted to 2.89 x 10^9 pfu/mL, in a 50:50 ratio. To obtain initial concentrations of each strain in the competition, the competition mixtures were plated on LM1034 with 100 µL of 40 mg/mL X-Gal (dissolved in DMSO).

Cultures were then initialized from the competition mixtures on the normal *P. phaseolicola* host. Competitions occurred in 4 mL of LC broth with a 1/100 dilution of naïve, stationary-phase *P. phaseolicola.* The competition mixture was added into this culture to a final concentration of approximately 2.5 x 10^3 viral particles/mL (the initializing concentration used in the evolution experiment; see Culturing of surviving phages, under Experimental evolution). Cultures were incubated for 24 hours at 25°C with orbital shaking.

To obtain final concentrations of each strain, aliquots of the cultures were diluted and plated on LM1034 with 100 µL of 40 mg/mL X-Gal. The competitive fitness of each focal strain was calculated as its change in relative density in the competition over time:

$$W_{1,2} = \frac{C_{f,1}/C_{f,2}}{C_{i,1}/C_{i,2}}$$

(Equation 2)

where $W_{1,2}$ denotes the calculated competitive fitness of the focal strain, C_i is the initial concentration, C_f is the final concentration, a subscript 1 denotes the focal strain, and a subscript 2 denotes the common competitor. Relative competitive fitness with respect to the ancestral genotype was then calculated by dividing the competitive fitness of each focal genotype by the mean competitive fitness of the ancestor.

Results

Changes in survival to heat shock over time

Viral survival is expected to decrease as the heat shock temperature increases. To account for this effect, we compared the percent survival of populations in the Gradual, Moderate, Sudden, and Control treatments at each transfer to the percent survival of the ancestral genotype at the heat shock temperature experienced in that transfer (Figure 3). If heat-shocked populations did not evolve greater thermostability than the ancestor, then on average there would be no difference between the survival of the population and the ancestor at each temperature (i.e., the point would fall at 0).

We did not find an improvement over time in the survival of the Control population in response to mock heat shocks at 25°C. In contrast, the percent survival of Φ6 from Gradual, Moderate, and Sudden populations was greater than ancestral values for every transfer in the second half of the experiment (Figure 3). Treatments differed in survival to their respective first exposures to 50°C (analysis of variance, $F(2,12) = 4.83$, $p = 0.03$; Figure 4); specifically, populations from the Gradual treatment had a higher survival than populations from the Sudden treatment on their first exposure to 50°C (Tukey's post-hoc test, $p = 0.03$; other comparisons were not significant). The survival data thus suggest that heat shocked populations evolved greater thermostability, even during exposure to intermediate temperatures. However, at the end of the experiment, the average survival of Gradual and Moderate populations at 50°C did not differ significantly from survival of populations from the Sudden treatment (analysis of variance, $F(2, 12) = 0.0872$, $p = 0.92$; Figure 5).

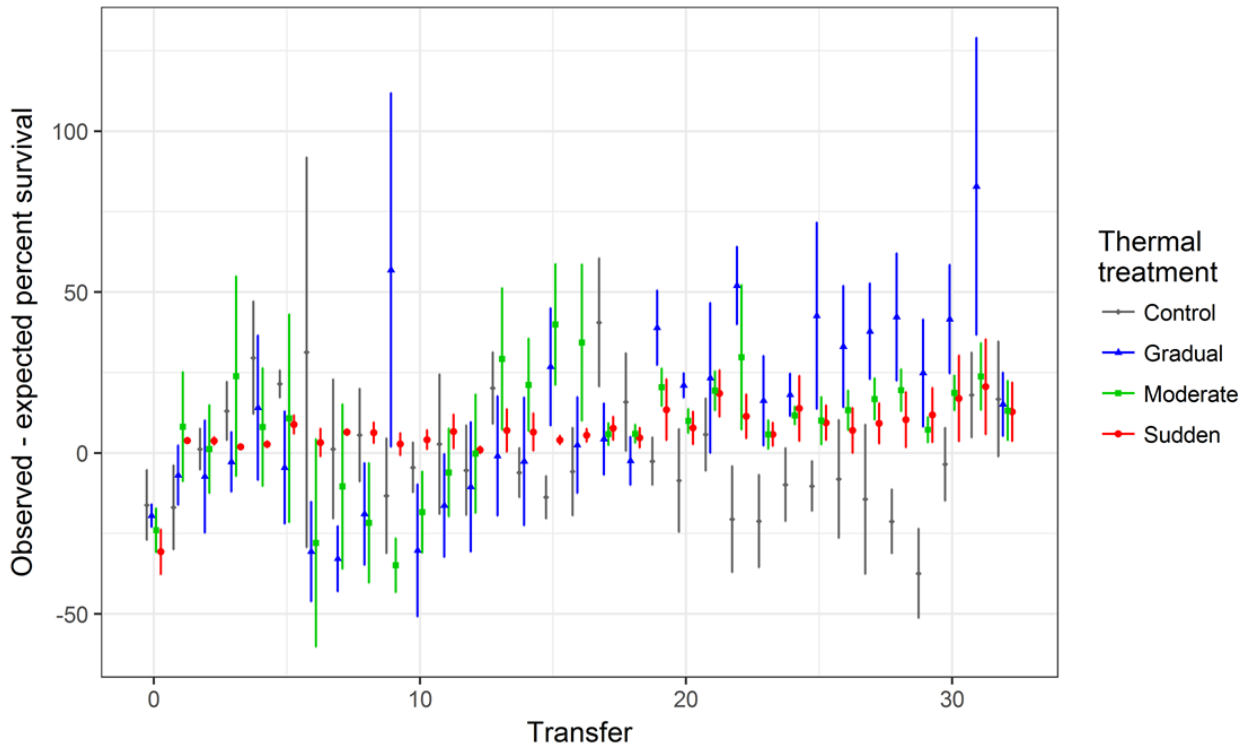


Figure 3. Changes in percent survival of viral lysates to heat shock over time. The survival of each population (Observed percent survival) at each transfer is compared to the percent survival of the ancestor (Expected percent survival) at the temperature used for heat shock (see Figure 2, S2 Table). Points represent the average difference between the population and ancestral survival; error bars represent the standard deviation of this difference. Treatments in which populations evolved better thermostability than the ancestor have a difference greater than 0. Note that, because of stochasticity in determining phage titers, differences occasionally exceed 100% (if more plaques were counted post heat shock than pre heat shock). Points are jittered horizontally for better visualization.

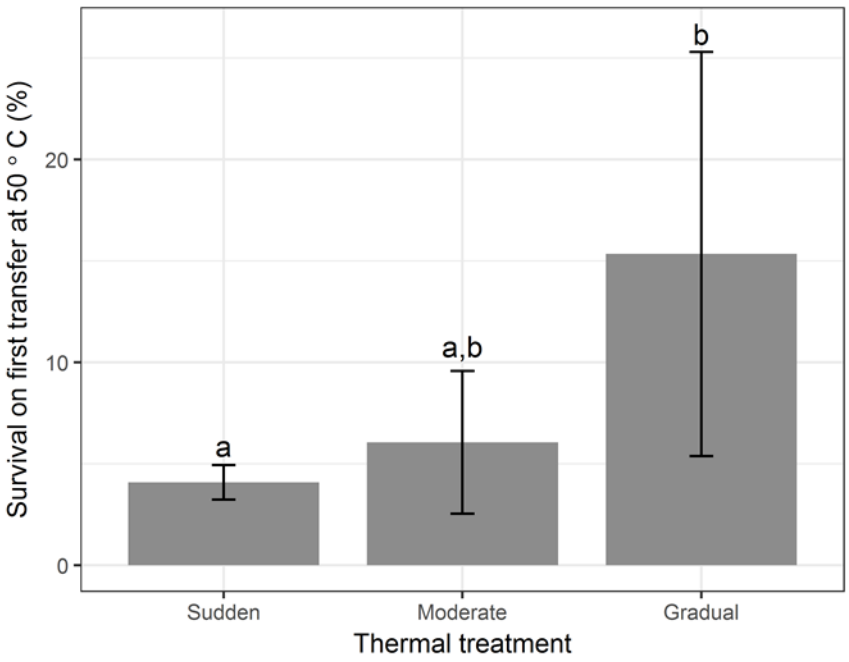


Figure 4. Average percent survival of populations on first transfer at 50°C. Sudden populations first experienced 50°C on Transfer 1, Moderate populations on Transfer 17, and Gradual populations on Transfer 32. Error bars represent the standard deviation of percent survival. Treatments with significantly different percent survivals are denoted with letters.

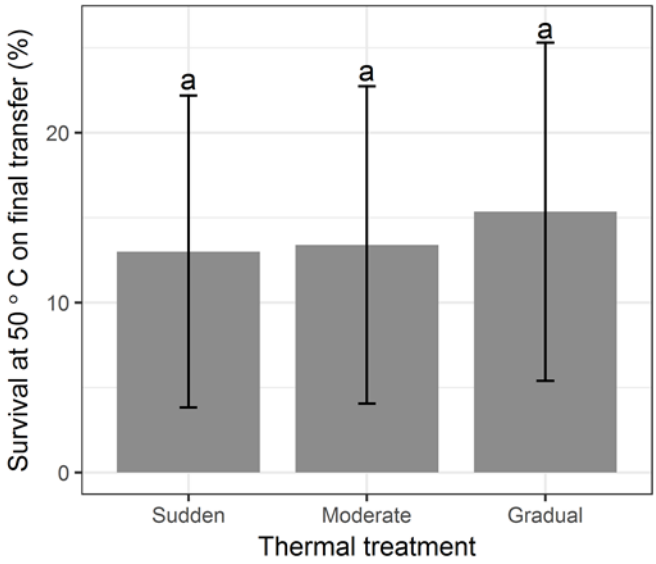


Figure 5. Average percent survival at 50°C on the final transfer (Transfer 32) of the experiment. Error bars represent the standard deviation of percent survival. Percent survival was not significantly different across treatments.

Genetic basis of thermostability

To identify mutations that may have contributed to increases in thermostability, we sequenced the endpoint populations in gene 5 (encodes the P5 lysis protein) and gene 8 (encodes the P8 outer shell protein). As proteins on the exterior of the virus that are necessary for viral infection [39, 40], both P5 and P8 are expected to experience strong selection for thermostability at high temperatures to maintain their functions.

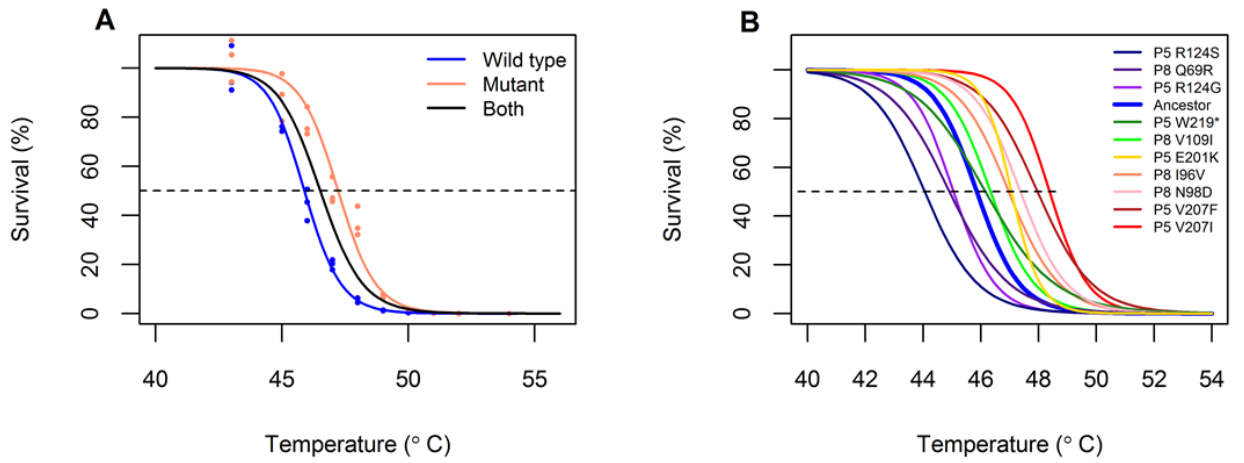
We found a total of 16 unique mutations across all populations, 11 of which were unique to populations that had experienced high-temperature heat shocks. Although populations from Gradual and Moderate treatments appeared to have accumulated more unique mutations and more mutations per lineage on average than populations from Control and Sudden treatments (Table 1),

the number of mutations per population did not vary significantly with treatment (analysis of variance, $F(3, 16) = 2.667$, $p = 0.08$).

Table 1. Number of mutations in genes encoding for P5 and P8 in each treatment.

Treatment	Number of different mutations in the treatment	Average number of mutations per population
Control	5	1.8
Gradual	7	2.4
Moderate	6	2.2
Sudden	3	1.6

We reverse engineered 10 of these mutations singly into the ancestral genetic background to evaluate their effect on viral thermostability. Nearly all of the chosen mutations appeared in more than one replicate or had been previously observed in pilot experiments (S3 Table). We evaluated the effects of the single mutations on viral thermostability by exposing bacteria-free lysates of the mutant viruses to heat shocks ranging from 25-55°C, measuring the lysate concentrations before and after heat shock. These data were used to build thermal kill curves, where the percent survivals at each temperature were fit to an inverse Hill equation (Equation 1) using maximum likelihood (Figure 6A).



394

395 **Figure 6**. **Thermal kill curves of engineered single mutants. A)** Calculations of mutant thermostability,
396 using the mutant P8 I96V as an example. Cell-free lysates were exposed to a 5-minute heat shock at each
397 temperature and plated before and afterward to calculate percent survival (circles). (Note that, due to
398 stochasticity in gauging phage titer, phage counts after heat shock can be above phage counts before heat
399 shock, accounting for survivals greater than 100%.) Equation 1 was fitted to the data in R, where the
400 parameters T_{50} (intersection of curve with dotted line) and n were estimated by maximum likelihood. A first
401 model was fit to the combined data (ancestor + mutant; black curve). A second model then estimated a
402 separate T_{50} and n for each lysate (blue, ancestor; red, mutant). The latter model was a better fit to the data
403 (log likelihood ratio test, $p < 0.0001$). **B)** Empirical thermal kill curves of the ancestor (blue) and ten
404 engineered single mutants, representing the maximum likelihood fit of all measurements taken for each
405 mutant. Data points are omitted for simplicity. In all cases, the model that used a separate T_{50} and n for the
406 ancestor and the mutants was a better fit to the data (log likelihood ratio test, $p < 0.001$). Pairwise
407 comparisons with the ancestor, as in part A, can be found in the Data Repository.

408
409 The engineered single mutants revealed that different mutations resulted in different gains
410 in thermostability in the ancestral background. As measured by an increase in the T_{50} parameter,

six of the engineered mutations increased viral thermostability by 0.3-2.1°C while three mutations decreased thermostability by 0.8-1.8°C (Figure 6B). All populations from the evolution experiment for which all mutations were evaluated and that increased in survival had at least one thermostabilizing mutation. (This pattern was most evident in the Sudden treatment; see S2 Figure.)

Four of the six thermostabilizing mutations were conservative mutations for which substitution retained non-polarity of the amino acid, while all mutations that reduced thermostability involved substitutions of polar amino acids to ionically charged amino acids or vice versa. The effect size of the mutations – that is, the amount by which the mutation increased or decreased thermostability with respect to the ancestor – did not differ significantly across heat shock treatments (analysis of variance, $F(2, 25) = 0.511$, $p = 0.61$). We note, however, that the number of mutations per population was low and that not all mutations that appeared in each population were evaluated for their effects on thermostability.

Growth effects of thermostabilizing mutations

The presence of mutations that *decreased* viral thermostability suggested that these mutations may have fixed because of non-thermal selective pressures. The mutation R124G in P5, for example, appeared in 18 out of 20 different populations, including in the Control treatment. This suggested that the mutation might improve another attribute of fitness, such as viral replication. To test whether destabilizing mutations instead improved replication, we competed all engineered mutants and the ancestor against a common competitor (see Assaying viral competitive fitness in Methods). Many of the mutations appeared to give a competitive growth advantage in comparison to the ancestor, although some mutations decreased viral growth rates (Figure 7). All

mutations that reduced thermostability enhanced relative competitive fitness, and many thermostabilizing mutations decreased relative competitive fitness.

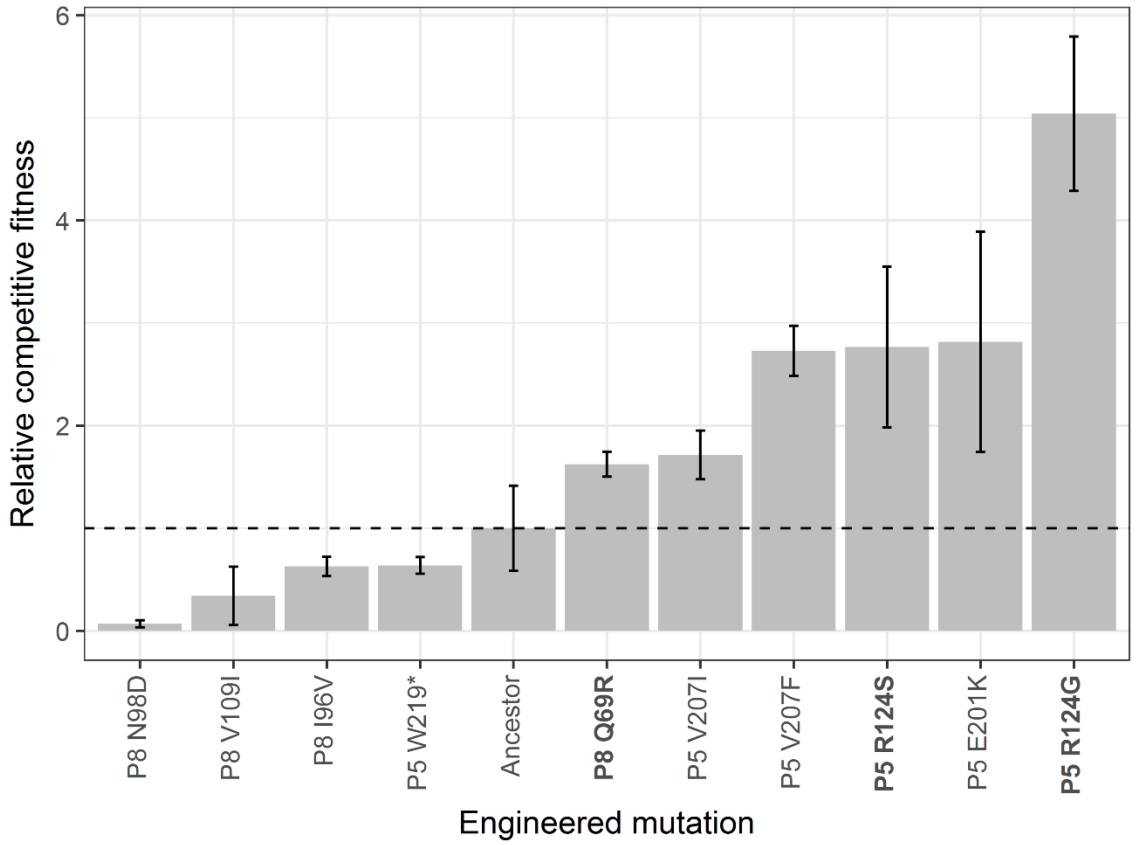


Figure 7. Competitive fitness of engineered single mutants relative to the ancestral genotype. Bar heights indicate the mean of three replicate competitions; error bars denote standard deviation. Mutations in bold font decrease viral thermostability with respect to the ancestor.

A prior study in Φ6 recorded a trade-off between thermostability and growth for one mutation in P5 [41]. In our data set, several individual mutations follow the expected pattern of low T_{50} and high growth rate, or high T_{50} and low growth rate. To test for a generalized trade-off, we regressed the relative competitive fitness of the mutants against the T_{50} values estimated from the thermal kill curves (Figure 8). Although the slope of the regression line was negative, it was not statistically different from a slope of 0 (F-statistic = 0.897, df = 9, p = 0.368).

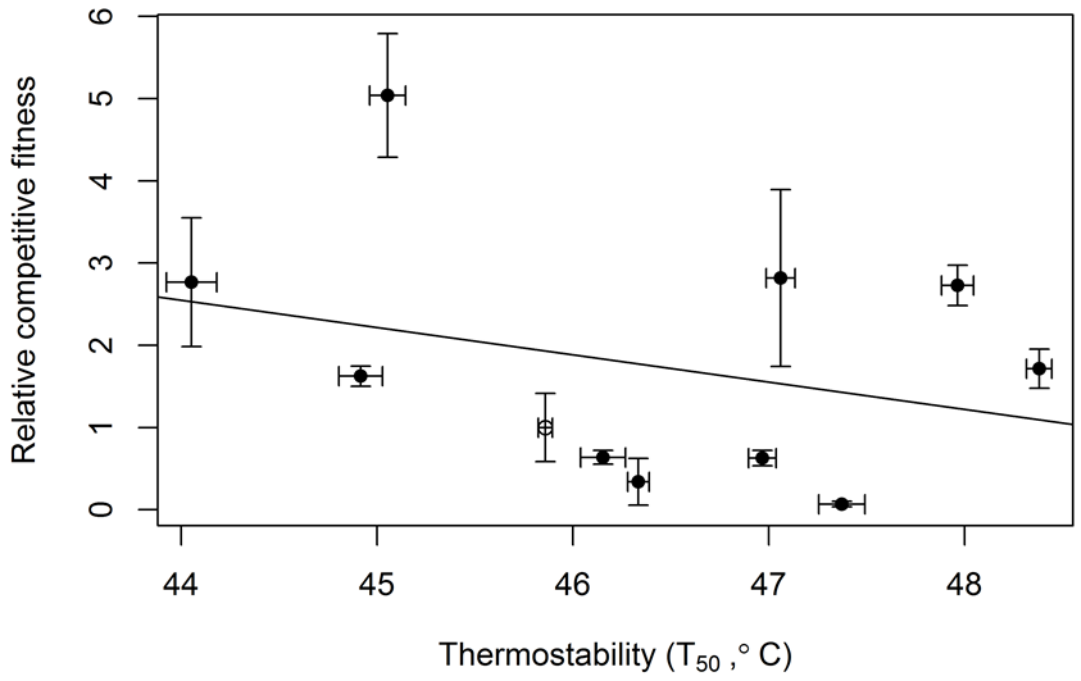


Figure 8. Relationship between relative competitive fitness and T_{50} for the ancestor and the engineered single mutants. The ancestor is marked with an open circle. X-error bars represent the standard error of the T_{50} estimate, while y-error bars represent the standard deviation of three replicate competitions. The line represents the best fit from a linear model.

Thermostabilizing and growth effects of combinations of mutations

Several populations from heat-shocked treatments did not appear to have increased their thermostability substantially over time; we expected that mutations from these populations would instead have increased viral growth rates. We confirmed this hypothesis through evaluation of the thermostability and growth rates of genotypes from one of these populations (G1, replicate 1 from the Gradual treatment). Survival of the G1 population had not increased substantially over time;

furthermore, it did not carry the common mutation, R124G in P5, that increased viral growth rate and was also found in Control populations (Figure 7, S3 Table). Two genotypes dominated G1 at the end of the experiment. Both genotypes shared the P5 mutation R124S (mutation B in Figure 9), but one genotype also had mutation E201K in P5 (mutation C), while the other had mutation Q69R in P8 (mutation A). As single mutations, all three increased viral growth rates, but only one (E201K) increased thermostability.

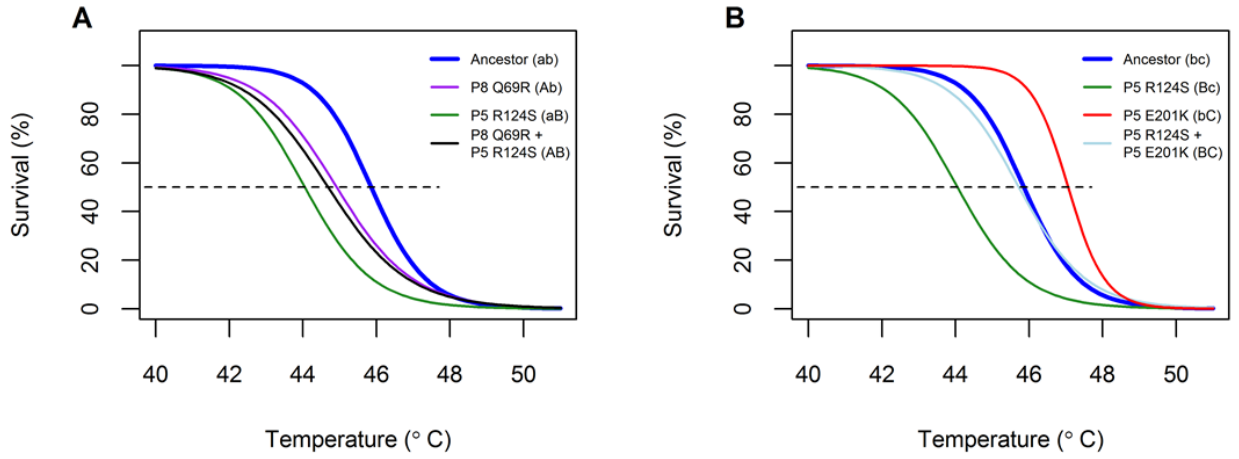


Figure 9. Evaluated thermal kill curves for two combinatorial genotypes found in one Gradual population. Mutations are given secondary labels to denote their allelic state, where a lower case letter (a, b, c) indicates the ancestral residue and an upper case letter (A, B, C) indicates the residue found in the endpoint population. **A)** Thermal kill curves for double mutant P8 Q69R + P5 R124S and its corresponding single mutants. **B)** Thermal kill curves for double mutant P5 R124S + P5 E201K and its corresponding single mutants.

We reverse engineered these mutations in their respective double combinations (AB and BC) and evaluated their effects on thermostability and viral growth. Neither double mutant improved thermostability with respect to the ancestor (Figure 9), but both double mutants improved in

relative competitive fitness with respect to the ancestor (Figure 10). Interestingly, one of the combinations exhibited sign epistasis for thermostability (mutation P8 Q69R was destabilizing in the ancestral background, but stabilizing in the P5 R124S background).

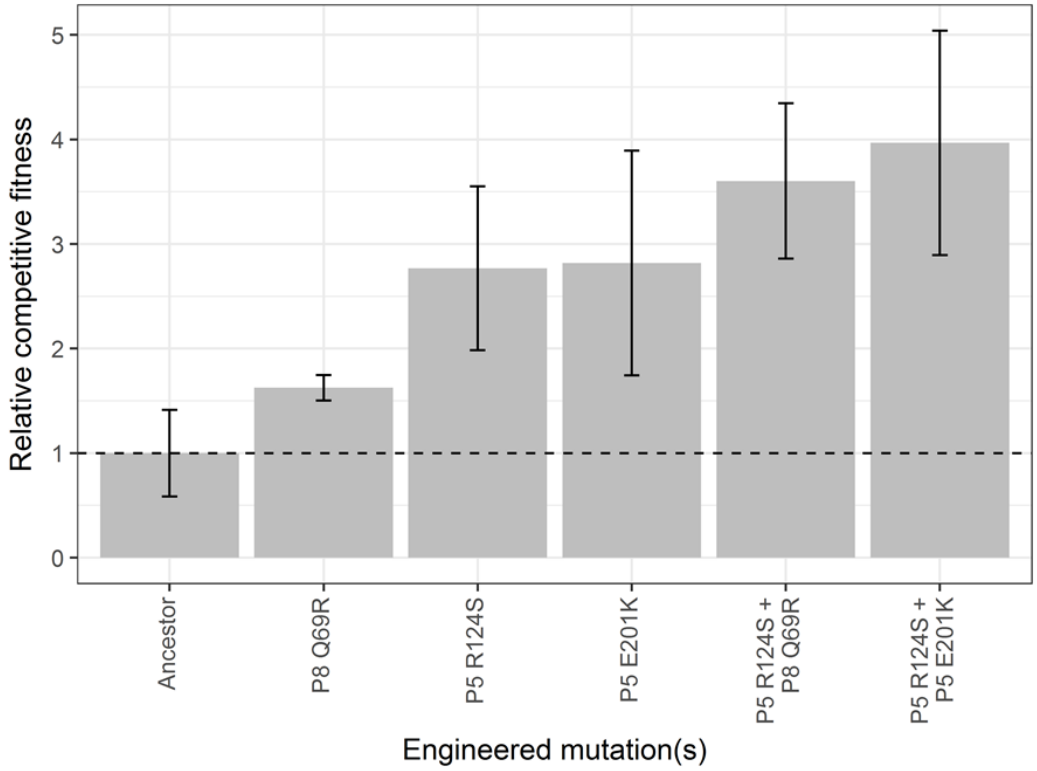


Figure 10. Relative competitive fitness of two combinatorial genotypes found in one Gradual population. Bar heights represent the mean of three replicates; error bars denote standard deviation. The relative competitive fitness of the double mutants is compared to the ancestor and the constituent single mutants. We note that addition of the double mutations did not change the overall relationship between relative competitive fitness and T_{50} portrayed in Figure 8.

Based on historical sequencing of the G1 lineage (see S2 Text), the first mutation detected in this population (P5 R124S) decreased thermostability but enhanced viral growth. Subsequent mutations (P8 Q69R and P5 E201K) increased both thermostability and competitive fitness in the

presence of P5 R124S. This sequence of mutations is consistent with stronger selection for growth rate early in a gradually changing environment and stronger selection for thermostability later on.

Discussion

Consistent with prior work in Φ6 [42, 43], we found that virus populations exposed to high-temperature heat shocks evolved greater survival to heat shock, and we identified six causative mutations that increased viral thermostability with respect to the ancestral genotype. We did not find significant differences between Gradual and Sudden treatments in endpoint survival at 50°C, or in the number or effect size of mutations, possibly due to the low number of replicates and mutations in each treatment. Instead, we found that other selective pressures may have been important during the experiment. Specifically, our experimental design permitted two places where selection had a chance to act: on survival, under the high temperature heat shocks; and on replication, when viruses were grown with their bacterial host (Figure 1). Even in heat-shocked populations, we identified mutations that reduced viral thermostability but increased growth rate, suggesting a relatively high selective pressure on viral replication.

Because replication occurred at 25°C, the typical laboratory temperature for Φ6, our experiment is reminiscent of that of Hao *et al.* [44], in which a lytic bacteriophage of *P. fluorescens* was exposed to increasing temperatures punctuated by periods of lower temperature. The authors term the fluctuations to reduced temperatures as periods of "amelioration" because they reduced selective pressures associated with heat stress. If amelioration allowed populations to recover in abundance and *de novo* mutations in the wake of an environmental stress, it could promote adaptation under stressful conditions [22, 45]. On the other hand, because amelioration relaxes the selective pressures present in a stressful environment, it may reduce the likelihood that stress-

beneficial mutations will fix [46-49]. Hao *et al.* found that fewer phage populations persisted in treatments that included temperature amelioration than in treatments where the temperature increased monotonically, indicating that periods of amelioration hindered adaptation at high temperatures. Although we did observe increases in thermostability over the course of our evolution experiment, we cannot rule out the possibility that thermostability evolution was hindered due to periods of growth at 25°C.

Amelioration is especially likely to impede adaptation to stressful environments if stress-beneficial mutations impose fitness costs under more benign conditions [44]. A prior study in Φ6 found that a highly thermostabilizing mutation decreased the ability of viruses to replicate at 25°C [41]. Although we did not find support for general trade-off between thermostability and replication in our data (Figure 8), we can identify mutations in P5 and P8 for which we measured high thermostability but low growth rates, or low thermostability but high growth rates. Interestingly, some mutations appeared to increase both thermostability and growth rate. (We note that this last class includes the particular mutation reported in [41], V207F in P5. We speculate that this is because the genetic background of our phage differed from the genotype used in [41].) It is possible that our sample of 11 genotypes is too limited to permit detection of a general trade-off. Alternatively, mutations that contribute to thermostability may not always be constrained by trade-offs. For example, due to their high mutation rates, viruses can find "cost free" adaptations [50-52], which allow them to maintain existing functions while gaining new ones. Such mutations may be particularly important during evolution in changing environments. It is also possible that we did not sample mutations that demonstrate a trade-off, since we examined mutations found at the end of the experiment. Mutations that may have exhibited a trade-off between thermostability

and relative fitness and were present at earlier time points might have been outcompeted by mutations that performed well in both dimensions.

That we find growth-enhancing but thermo-destabilizing mutations, however, highlights that organisms experience selective pressures along multiple phenotypic axes. This has potential implications for evolution in environments that change incrementally. For example, a gradually changing thermal environment imposes small differences in selective pressure on the population from generation to generation. In this case, the population may experience stronger relative selective pressure along a non-thermal axis, such as for growth. The population may then not evolve in response to the thermal environment until sufficient thermal change has occurred and relative selective pressures are high enough. For example, Gorter *et al.* [26] report that, in a yeast system, adaptation to general culture conditions preceded adaptation to high metal concentrations under conditions where the metal concentration increased slowly. Similarly, we note that while the first detectable mutation in one Gradual lineage reduced viral thermostability but enhanced growth, both mutations that rose to prominence later in the evolution of this lineage were thermostabilizing in the background of the first mutation (see S2 Text for historical sequencing of this lineage).

In extreme cases, evolution in response to a non-focal selective pressure may impose trade-offs or constraints in the changing stressful environment. Suppose, for example, that evolution for higher growth rates always reduced thermostability. Populations that experienced a gradual increase in temperature may have first fixed growth-enhancing mutations (because of stronger relative selective pressures for growth than for thermostability). However, this would have lowered their thermostability, even as thermal stress became a more prominent selective pressure over time. The population would then be in a *worse* place, in terms of thermostability, than when it started, and mutations of larger thermostabilizing effect would be required to increase its survival at high

554 temperatures. Although we are unaware of any empirical studies that look explicitly at the role of
555 such trade-offs in incrementally changing environments, this conclusion is in the spirit of studies
556 that predict greater phenotypic and genotypic constraint under slow environmental change (e.g.,
557 [7, 15, 18, 53]).

558 Other results from this study are consistent with prior work on adaptation under varying rates
559 of environmental change.

560 Evidence from prior experiments (e.g., [20-23]) suggests that evolution under mildly
561 stressful environments (such as an intermediate temperature) can enhance a population's ability to
562 withstand more stressful environments (such as a high temperature). Consistent with this
563 expectation, populations from the Gradual treatment had a higher average survival on their first
564 exposure to 50°C than did populations from the Sudden treatment on their first exposure. In other
565 words, exposure to intermediate temperatures can promote survival of Φ6 at high temperatures.

566 Several prior experiments find a greater diversity of mutations under rapid than gradual
567 environmental change [7, 19, 25]. In contrast, we find an (insignificant) pattern of more mutations
568 in endpoint Gradual and Moderate populations compared to Sudden populations. This could
569 represent a greater amount of clonal interference in Gradual and Moderate than Sudden lineages
570 (e.g., [24, 28]). (Consider, for example, that lineage G1 had two dominant genotypes at the
571 endpoint of the experiment. Sequencing the lineage at prior time points [see S2 Text] furthermore
572 suggested that both these genotypes were increasing in frequency when the experiment ended.)

573 Another possibility is that thermostability comprises a set of mutations that vary relatively
574 little regardless of thermal treatment. A study that examined the thermal adaptation of the
575 bacteriophage Qβ found that populations evolved under a constant high temperature did not
576 significantly differ in evolutionary outcomes from populations evolved under fluctuating

577 temperatures [54]. In this study, when we include data from pilot experiments, most of the
578 engineered mutations appeared in populations that had experienced diverse heat shock treatments
579 (S3 Table). Proteins tend to be marginally stable and can be destabilized by a single amino acid
580 substitution [55-57], including by mutations that are adaptive for functions besides stability (e.g.,
581 ligand binding [58, 59] or growth [60]). In contrast, computational and empirical data sets suggest
582 that relatively few substitutions will increase a protein's thermostability [56, 57, 61]. In the case
583 of an enzyme, such as the P5 lysis protein in Φ6, any mutations that increase stability must
584 simultaneously maintain the flexibility or activity necessary for the protein's function [55]. The
585 number of mutations that increase thermostability may thus be small and/or biochemically
586 constrained for any given protein, resulting in relatively few mutational pathways for
587 improvement.

588 Overall, our study emphasizes that it is important to take *all* selective pressures into account
589 during an evolution experiment. We found that populations that did not increase in thermostability
590 appeared to have increased instead in replicative ability. We speculate that this may offer an
591 alternative way for populations to persist under heat shock, rather than improving their
592 thermostability: They may be able to make up reductions in population size due to heat shock by
593 increasing their replication rate in its absence. This highlights the conclusion that multiple features
594 of organisms can evolve, even in environments that change in only a single focal factor.

595

Acknowledgments

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Supporting information

S1 Table (4 pages). Strains used and engineered in this study. Laboratory collection numbers (BK numbers) are included for request purposes. All other data presented in this study (including this table, other Supporting Information, and the Data Repository) use the project-specific (PRESS) numbers. Mutations are labeled in order ancestral base/amino acid - position - mutated base/amino acid, where the position is measured from the first nucleotide of the NCBI Reference Sequence for the S segment of Φ6 Cystovirus (Accession# NC_003714) or the first amino acid of the protein. We also note the presence of any additional (i.e., non-engineered) mutations present in the mutant viral clones. We account for the effects of these mutations with the "matched" viral clones; see S1 Text.

BK #	Project #	Organism	Species	Plasmid (bacterial strains)	Description	Source	Reference	Additional mutations (engineered strains)
225	PRESS 18	Bacteria	*Pseudomonas syringae* pv *phaseolicola* HB10Y	none	Laboratory host for phi-6	C. Burch		
258	PRESS 23	Bacteria	*P. phaseolicola* HB10Y	pLM1086 (T7 RNA polymerase)	Transformation host for phi-6 plasmids (Original name LM2691)	C. Burch	[36, 37]	
230	PRESS 28	Bacteria	*Escherichia coli* JM109	pLM687 (phi-6 L segment in pT7T319U)	Storage strain for L segment plasmid (Original name LM4286)	L. Mindich	[31]	
226	PRESS 29	Bacteria	*E. coli* JM109	pLM659 (phi-6 S segment in pT7T319U)	Storage strain for S segment plasmid (Original name LM4285)	L. Mindich	[33, 34]	

BK #	Project #	Organism	Species	Plasmid (bacterial strains)	Description	Source	Reference	Additional mutations (engineered strains)
227	PRESS 30	Bacteria	*E. coli* JM109	pLM656 (phi-6 M segment in pT7T319U)	Storage strain for M segment plasmid (Original name LM4284)	L. Mindich	[40]	
228	PRESS 31	Bacteria	*E. coli* JM109	pLM857 (phi-6 M segment + lacH in pT7T319U)	Storage strain for blue/white marked M segment plasmid (Original name LM987)	L. Mindich	[32]	
231	PRESS 32	Bacteria	*P. phaseolicola* HB10Y	pLM746B (lac omega)	Host for visualizing blue/white plaques (Original name 1034)	L. Mindich	[35]	
107	PRESS 53	Bacteria	*E. coli* DH5alpha	none	Storage strain for engineered plasmids			
368	PRESS 1	Virus	phi-6 Cystovirus		Single plaque used to initialize experimental evolution	Plasmids from PRESS 28, 29, 30	NCBI accession #MF352213, MF352214, MF352215	
370	PRESS 4	Virus	phi-6 Cystovirus		LacH marked virus for blue/white screening	Plasmids from PRESS 28, 29, 31		
352	PRESS 67	Bacteria	*E. coli* XL1-Blue	S plasmid with *a*590*g* (P8 I96V)		This study		
353	PRESS 69	Bacteria	*E. coli* DH5alpha	S plasmid with *g*629*a* (P8 V109I).	Plasmid made by T4 ligation of a PCR product	This study		
354	PRESS 71	Bacteria	*E. coli* XL1-Blue	S plasmid with *a*1989*g* (P5 R124G)		This study		
355	PRESS 73	Bacteria	*E. coli* XL1-Blue	S plasmid with *g*2238*a* (P5 V207I)		This study		
356	PRESS 75	Bacteria	*E. coli* XL1-Blue	S plasmid with *g*2238*t* (P5 V207F)		This study		
371	PRESS 83	Virus	phi-6 Cystovirus		Mutant with P8 I96V	Plasmids from PRESS 28, 30, 67		

BK #	Project #	Organism	Species	Plasmid (bacterial strains)	Description	Source	Reference	Additional mutations (engineered strains)
372	PRESS 84	Virus	phi-6 Cystovirus		Mutant with P8 V109I	Plasmids from PRESS 28, 30, 69		
373	PRESS 85	Virus	phi-6 Cystovirus		Mutant with P8 V109I; matched sample for *tt* deletion.	Plasmids from PRESS 28, 30, 69		*tt* deletion at 2443
374	PRESS 90	Virus	phi-6 Cystovirus		Mutant with P5 R124G	Plasmids from PRESS 28, 30, 71		*tt* deletion at 2443
375	PRESS 94	Virus	phi-6 Cystovirus		Mutant with P5 V207I	Plasmids from PRESS 28, 30, 73		*t* deletion at 2443
376	PRESS 96	Virus	phi-6 Cystovirus		Mutant with P5 V207F	Plasmids from PRESS 28, 30, 75		*t* deletion at 2443
377	PRESS 102	Virus	phi-6 Cystovirus		Reverse genetics control (no mutations with respect to ancestor)	Plasmids from PRESS 28, 29, 30		
357	PRESS 105	Bacteria	*E. coli* XL1-Blue	S plasmid with *g*2276*a* (P5 W219*)		This study		
358	PRESS 106	Bacteria	*E. coli* XL1-Blue	S plasmid with *a*510*g* (P8 Q69R)		This study		
359	PRESS 107	Bacteria	*E. coli* XL1-Blue	S plasmid with *a*596*g* (P8 N98D)		This study		
379	PRESS 114	Virus	phi-6 Cystovirus		Mutant with P8 N98D	Plasmids from PRESS 28, 30, 107		*c*1838*a* (synonymous), *t* deletion at 2443
381	PRESS 122	Virus	phi-6 Cystovirus		Mutant with P5 W219*	Plasmids from PRESS 28, 30, 105		
382	PRESS 128	Virus	phi-6 Cystovirus		Mutant with P8 Q69R	Plasmids from PRESS 28, 30, 106		*c*1838*a* (synonymous)
361	PRESS 151	Bacteria	*E. coli* XL1-Blue	S plasmid with *a*1991*t* (P5 R124S)		This study		

BK #	Project #	Organism	Species	Plasmid (bacterial strains)	Description	Source	Reference	Additional mutations (engineered strains)
383	PRESS 158	Virus	phi-6 Cystovirus		Mutant with P5 R124S	Plasmids from PRESS 28, 30, 151		
363	PRESS 168	Bacteria	*E. coli* XL1-Blue	S plasmid with *g*2220*a* (P5 E201K)		This study		
365	PRESS 173	Bacteria	*E. coli* XL1-Blue	S plasmid with *g*2220*a* + *a*1991*t* (P5 E201K + P5 R124S)		This study, using plasmid from PRESS 168		
366	PRESS 174	Bacteria	*E. coli* XL1-Blue	S plasmid with *a*510*g* + *a*1991*t* (P8 Q69R + P5 R124S)		This study, using plasmid from PRESS 106		
387	PRESS 182	Virus	phi-6 Cystovirus		Mutant with P5 E201K + P5 R124S	Plasmids from PRESS 28, 30, 173		
388	PRESS 184	Virus	phi-6 Cystovirus		Mutant with P5 E201K + P5 R124S; matched sample for *t* insertion + *t* deletion.	Plasmids from PRESS 28, 30, 173		*t*insertion at 2376, t deletion at 2443
389	PRESS 192	Virus	phi-6 Cystovirus		Mutant with P8 Q69R + P5 R124S	Plasmids from PRESS 28, 30, 174		*t*insertion at 2376, t deletion at 2443
390	PRESS 203	Virus	phi-6 Cystovirus		Mutant with P5 E201K	Plasmids from PRESS 28, 30, 168		*t*insertion at 2376, t deletion at 2443

733

734

S2 Table. Heat shock temperatures used at each transfer in the experimental treatments.

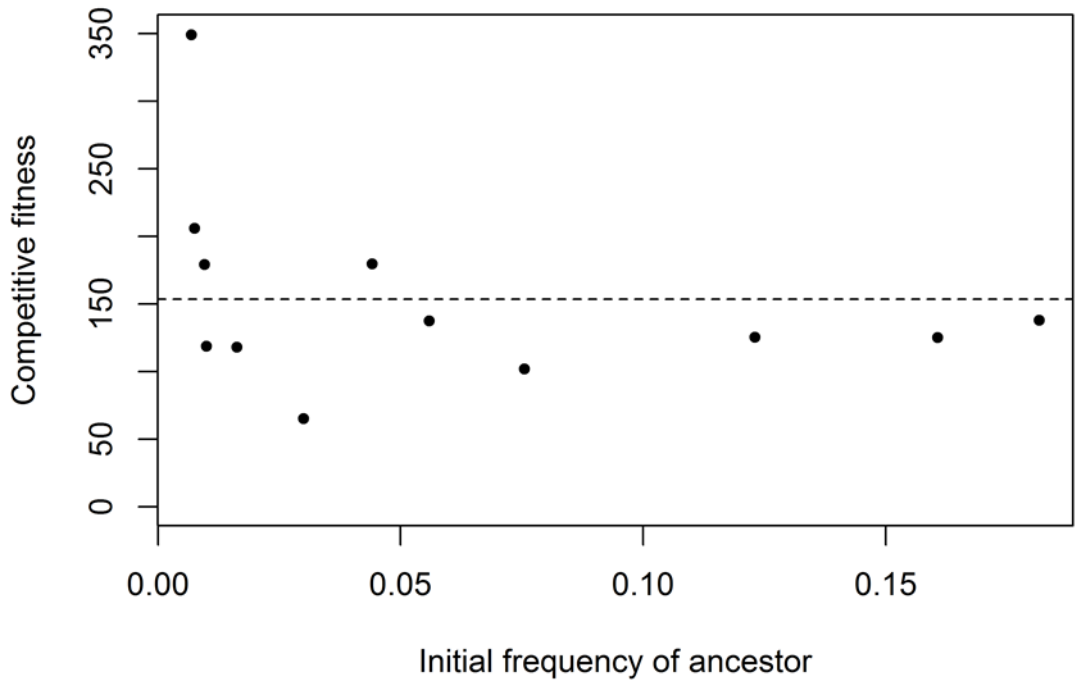
Transfer	Gradual	Moderate	Sudden	Control
0	25	25	25	25
1	44.9	44.9	50	25
2	45.6	45.9	50	25
3	45.9	46.2	50	25
4	46.1	46.4	50	25
5	46.2	46.6	50	25
6	46.3	46.7	50	25
7	46.4	46.8	50	25
8	46.5	46.9	50	25
9	46.6	47.1	50	25
10	46.6	47.2	50	25
11	46.7	47.3	50	25
12	46.8	47.4	50	25
13	46.8	47.6	50	25
14	46.9	47.7	50	25
15	47	48	50	25
16	47	48.3	50	25
17	47.1	50	50	25
18	47.1	50	50	25
19	47.2	50	50	25
20	47.3	50	50	25
21	47.3	50	50	25
22	47.4	50	50	25
23	47.5	50	50	25
24	47.6	50	50	25
25	47.6	50	50	25
26	47.7	50	50	25
27	47.8	50	50	25
28	48	50	50	25
29	48.1	50	50	25
30	48.3	50	50	25
31	48.6	50	50	25
32	50	50	50	25

S3 Table. Mutations present in genes 5 and 8 at the end of the evolution experiment. Gene 5 codes for the P5 lysis protein, while gene 8 codes for the P8 outer shell protein. All Gradual (G), Moderate (M), Sudden (S), and Control (C) populations were evaluated for mutations after 32 transfers in their specified heat shock regime. Nucleotide changes are numbered from the start of the NCBI Reference Sequence for the S segment of Φ6 Cystovirus (Accession# NC_003714). Specific mutations engineered, evaluated, and presented in this study are marked with a "Y" in the column "Evaluated for thermostability and fitness?" The column "Pilot lineages" indicates that the mutation was also found in a pilot experiment after 32 days of evolution under constant-temperature heat shock. (The exact heat shock temperature used for these pilot lineages is indicated by the number after "T" in the lineage name, while the number following "R" indicates the replicate number assigned to the population. Mutations found in multiple populations, e.g. populations 1, 2, and 3, are denoted with dashes, e.g., R1-3.)

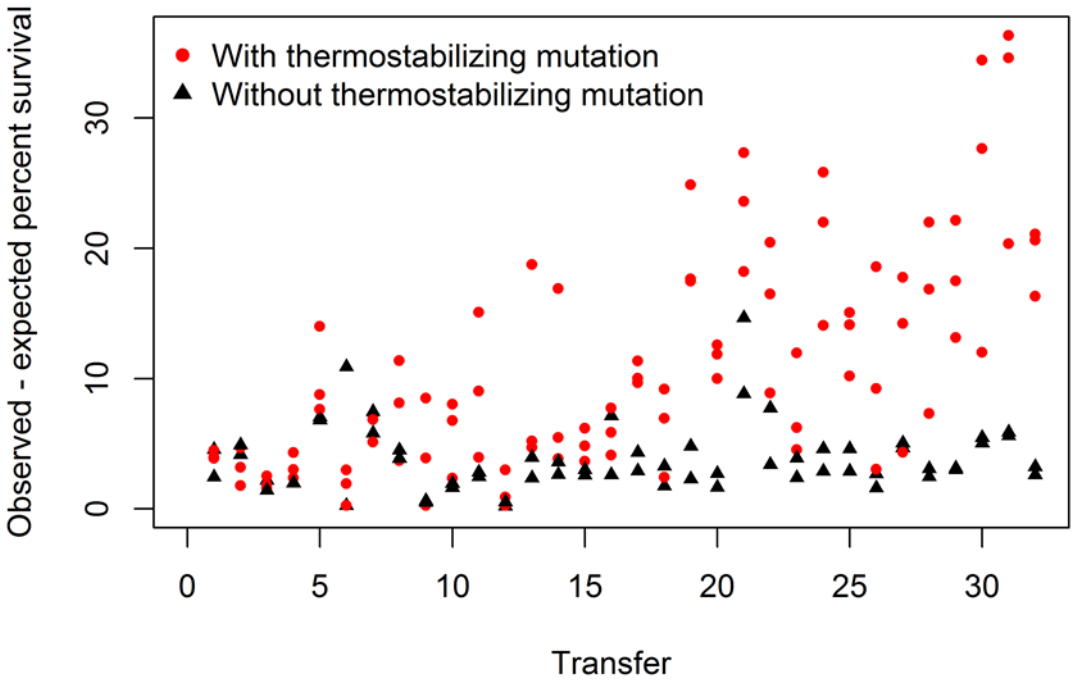
Gene	Nucleotide change	Amino acid change	Lineages	Pilot lineages	Evaluated for thermostability and fitness?
gene 8	*a*510*g*	gln 69 arg	G4, G1	T50 R3	Y
gene 8	*a*590*g*	ile 96 val	S2, S3	T47 R2, T50 R4, 6, & 7, T52 R1	Y
gene 8	*a*596*g*	asn 98 asp	G5, G2	T50 R4	Y
gene 8	*g*629*a*	val 109 ile	M1, M4, M5	T47 R1	Y
gene 5	*c*1625*t*	synonymous	C4	none	N
gene 5	*a*1675*g*	lys 19 arg	M2	none	N
gene 5	*a*1989*g*	arg 124 gly	C1-5, G2-5, M1 & 3-5, S1-5	T47 R1-2, T48 R1-2, T50 R1-7, T52 R1-2	Y
gene 5	*a*1991*t*	arg 124 ser	G1	none	Y
gene 5	*g*2220*a*	glu 201 lys	M2, G1, S5	none	Y
gene 5	*g*2229*c*	val 204 leu	M3	none	N
gene 5	*g*2238*a*	val 207 ile	M2	T50 R3	Y
gene 5	*g*2238*t*	val 207 phe	G3	T50 R5	Y
gene 5	*g*2241*a*	ala 208 thr	G5	none	N
gene 5	*g*2275*a*	trp 219 STOP	C5	none	N
gene 5	*g*2276*a*	trp 219 STOP	C3	T52 R1-2	Y
gene 5	*g*2282*c*	STOP 221 tyr	C2	none	N

S4 Table. Primers used for reverse engineering of Φ6 mutants. The engineered mutation is indicated with upper case in the nucleotide sequence. Where not otherwise stated in Reverse engineering (Methods), primers were prepared according to instructions in the QuikChange II mutagenesis kit. Primers for mutant V109I in P8 were given a standard desalting and used in a T4 ligation reaction; melting temperatures for these primers were calculated using the OligoAnalyzer (Integrated DNA Technologies, https://www.idtdna.com/calc/analyzer).

Mutation	Primer sequence	Orientation	Tm (deg. C)
P8 V109I	5'Phos gct gga act gAt cga aga tgc	Forward	56.1
P8 V109I	5'Phos tct tcg cgg tac tga ccg	Reverse	57
P8 I96V	cag ccg gat ttc Gtc ggt aac ctc ggt c	Forward	77.9
P8 I96V	gac cga ggt tac Cga cga aat ccg gct g	Reverse	77.9
P5 R124G	gca gac gtg gga tGg act ccg tcg ttt ag	Forward	77.8
P5 R125G	cta aac gac gga gtc Cat ccc acg tct gc	Reverse	77.8
P5 V207F	ctg tcg cgg cgT ttg cgg ctg cg	Forward	78.5
P5 V207F	cgc agc cgc aaA cgc cgc gac ag	Reverse	78.5
P5 V207I	ctg tcg cgg cgA ttg cgg ctg cg	Forward	78.5
P5 V207I	cgc agc cgc aaT cgc cgc gac ag	Reverse	78.5
P5 W219*	cat gtc aaa gag agt tgA gct tag ccc tga act g	Forward	77.9
P5 W219*	cag ttc agg gct aag cTc aac tct ctt tga cat g	Reverse	77.9
P8 Q69R	gac gcc tat cag cGg ctt ctg gag aat cat c	Forward	78.9
P8 Q69R	gat gat tct cca gaa gcC gct gat agg cgt c	Reverse	78.9
P8 N98D	cgg att tca tcg gtG acc tcg gtc agt acc	Forward	79
P8 N98D	ggt act gac cga ggt Cac cga tga aat ccg	Reverse	79
P5 E201K	ccg aag caa agc Aag gcc gct gtc gc	Forward	78.4
P5 E201K	gcg aca gcg gcc tTg ctt tgc ttc gg	Reverse	78.4
P5 R124S	cag acg tgg gat agT ctc cgt cgt tta ggc	Forward	79.2
P5 R124S	gcc taa acg acg gag Act atc cca cgt ctg	Reverse	79.2



S1 Figure. Competitive fitness of the ancestral genotype initialized at different frequencies. Competitive fitness was calculated using Equation 2. The mean competitive fitness is indicated with a dashed line. The competitive fitness values of the ancestral genotype are not significantly correlated with its initial frequency (Pearson's correlation test, ρ = -0.32, p = 0.30).



764

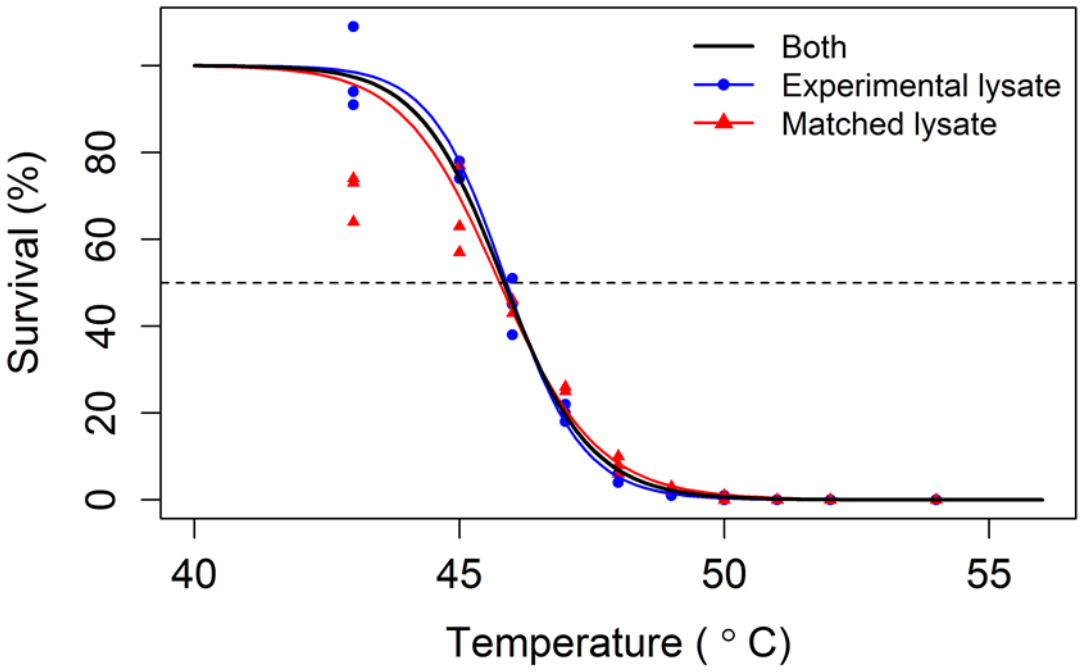
765 **S2 Figure. Changes in percent survival of Sudden populations with and without**
766 **thermostabilizing mutations**. At each transfer, the survival of each population (Observed percent
767 survival) was compared to the percent survival of the ancestor (Expected percent survival) at 50°C.
768 Lineages are distinguished according to whether at least one mutation evaluated to be
769 thermostabilizing was present in the endpoint population. (Note that, although the entire lineage
770 has been colored for the purpose of visualization, the exact point in time at which the
771 thermostabilizing mutation arose was not evaluated in this study.)

772

S1 Text. Effects of additional mutations in the engineered viral mutants on thermostability and relative competitive fitness.

Because Φ6 mutates rapidly, several engineered genotypes used in this study contained additional mutations in regions other than the locus of interest. Often, these additional mutations were in extended *t*-runs in the 3' untranslated region (UTR) of the S segment and were most likely due to slippage during viral replication. (The specific mutations are recorded in S1 Table.) Because these slippage mutations occurred commonly, we were sometimes able to isolate pairs of mutants containing the same focal mutation, one of which had specific 3' UTR mutations and the other of which did not.

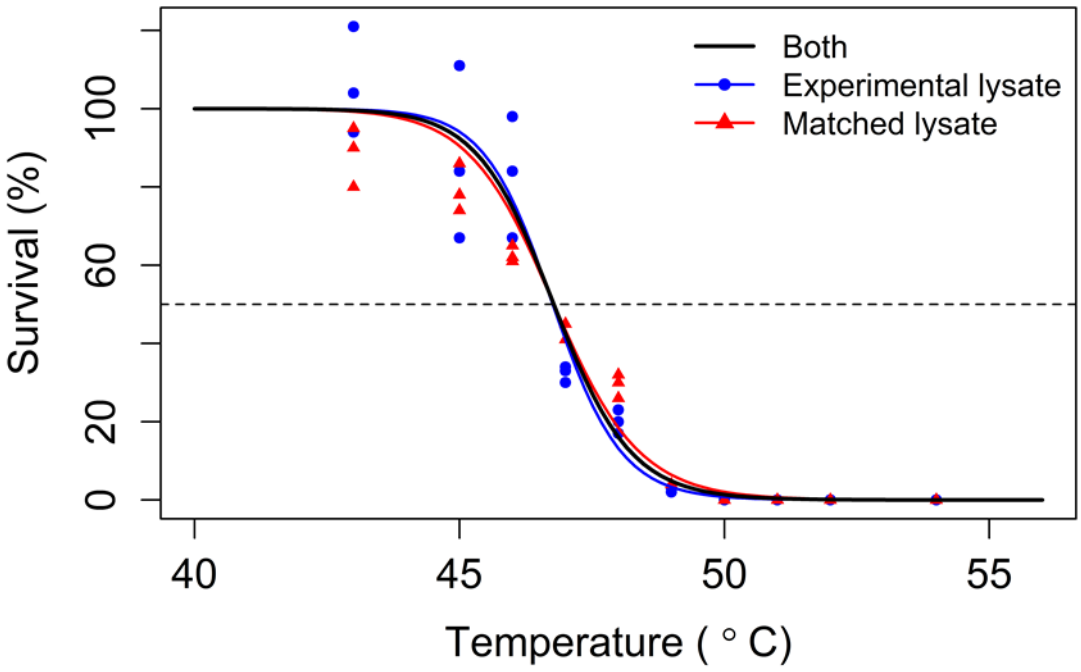
To account conservatively for the effects of the 3' UTR mutations on thermostability (Figure A-C), we exposed cell-free lysates to a 5-minute heat shock at temperatures ranging from 25°C-55°C and plated before and afterward to calculate percent survival. We then fit Equation 1 to the data, estimating the parameters T_{50} (intersection of curve with dotted line) and n by maximum likelihood. For each pair, a first model was fit to the combined data (mutant without 3' UTR mutations + mutant with 3' UTR mutations; black). A second model then estimated a separate T_{50} and n for each lysate (blue, mutant without 3' UTR mutations; red, mutant with 3' UTR mutations).



790

791 **Figure A.** Thermal kill curve comparisons between the isolated ancestral genotype and an independently

792 engineered plaque with identical sequence, to account for plaque-specific effects.



793

794 **Figure B.** Thermal kill curve comparison of two isolates with P8 V109I, one of which also contained a

795 double-*t* deletion at position 2443 in the 3' UTR.

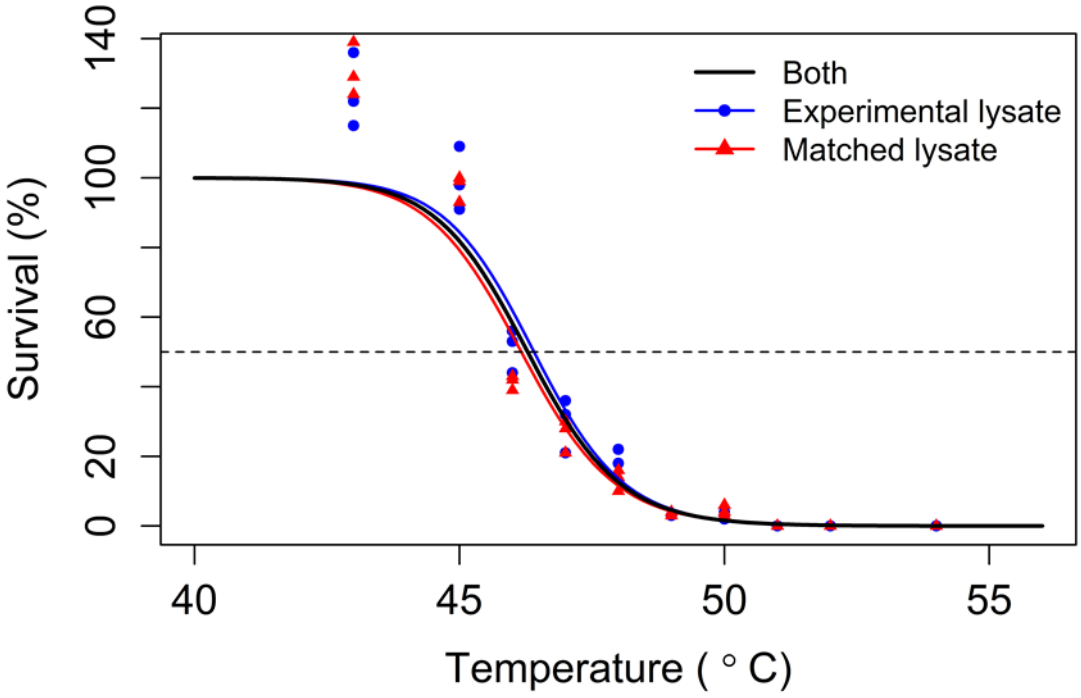


Figure C. Thermal kill curve comparison of two isolates with P5 R124S + P5 E201K, one of which also contained a *t* insertion at position 2376 and a *t* deletion at position 2443 in the 3' UTR.

In two cases (Figures A-B), the latter model was a better fit to the data after Bonferroni correction, suggesting that the additional mutation affected viral thermostability. However, subsequent tests that allowed either T_{50} or n to vary while holding the other parameter constant indicated that the T_{50} estimate was not significantly different in lysates with the 3' UTR mutations (see the Figshare Data Repository). (We note that the T_{50} values of the experimental mutants are highly correlated regardless of whether our model allows only T_{50} to vary or both T_{50} and n, so this choice of model does not alter any of the conclusions of our study. See also Data Repository.)

We also competed the mutants with the 3' UTR mutations against a common competitor to evaluate their relative competitive fitness in comparison to the ancestral genotype (Figure D). In

these cases, relative competitive fitness was compared to the genotype with the same focal mutation but lacking the 3' UTR mutations. We found no significant effect of the 3' UTR mutations on the relative competitive fitness of the viruses.

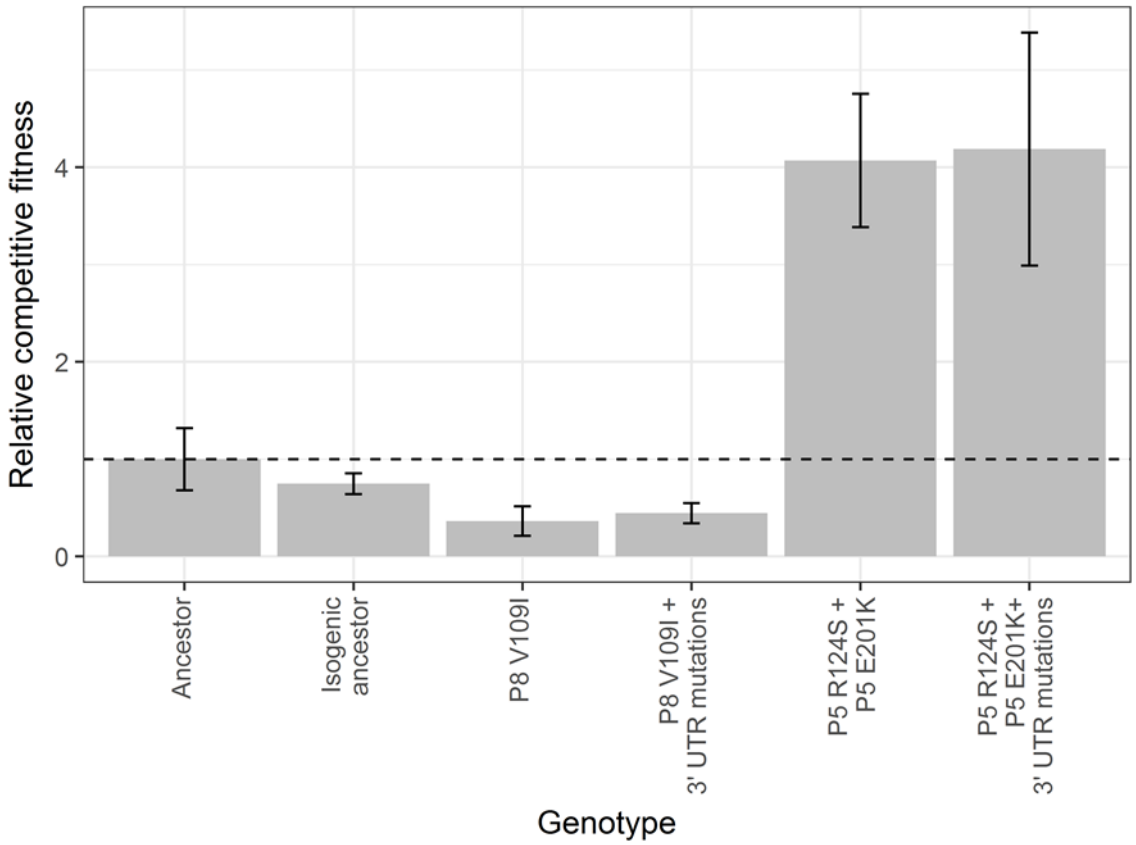


Figure D. Competitive fitness of all sets of matched mutants, relative to the ancestral genotype. Bar heights indicate the mean of three replicate competitions; error bars denote standard deviation. Pairs that share focal amino acid mutation(s) do not differ significantly in fitness (2-sample t-test, $p > 0.3$ for all pairs).

S2 Text. Historical sequencing of population Gradual 1.

We sequenced lineage G1 (Replicate population 1 from the Gradual treatment) back in time to understand when its mutations arose (Table A).

Table A. Sanger sequencing results of mutations in P5 and P8 in the G1 lineage over time. The ancestral genotype is denoted with lower case (a, b, c), while mutations are denoted with upper case (mutation A = P8 Q69R, B = P5 R124S, C1 = P5 E201Q, C2= P5 E201K). Polymorphisms (a double peak in the chromatogram) are denoted by listing both alleles. A slash separating the alleles indicates that the peak for the first allele was higher than the peak for the second allele, while an equals sign indicates that the peak heights were roughly equivalent.

Transfer	Mutations detectable
8	None
16	b / B c / C1
24	C1
28	b / B C1 / C2
30	a / A b = B C1 / C2
31	a / A b = B c = C1 = C2
32	a = A b = B c = C2

From these data, we concluded that the R124S mutation in P5 arose relatively early in evolution (prior to the halfway point, Transfer 16). An additional mutation (not evaluated in this

study), E201Q in P5, arose separately between Transfers 8 and 16. The genotype with this mutation increased in frequency through Transfer 24. Its frequency then declined as the mutations P8 Q69R and P5 E201K, presumably on a genetic background with P5 R124S, rose to appreciable frequencies in the final 4-6 days of evolution.

Chapter 2

A poison that is its own antidote: Colicin mutation confers resistance to colicins in *Escherichia coli*

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Abstract

Colicins are toxic proteins produced by *Escherichia coli* that target and kill other *E. coli* cells. To prevent death by clone-mates, colicinogenic cells also express an immunity protein that neutralizes their own colicin by binding it with high affinity and specificity. Diverse colicins, each with a unique cognate immunity protein, exist in nature, and many of them appear to have common ancestry. Because disruption of colicin-immunity binding can be lethal, diversification of the colicin-immunity complex diversifies is typically thought to occur in an immunity-led manner, through a promiscuous intermediate immunity protein that can bind multiple colicins. We aimed to test colicin-immunity diversification by mutagenizing the immunity and colicin genes and screening mutants for their ability to survive non-cognate colicins or kill cells with ancestral immunity, respectively. We isolated a novel colicin that killed ancestral immunity-producing cells. Unexpectedly, when this novel colicin was combined with the ancestral immunity gene in the same cell, not only did the cells survive, but they also demonstrated resistance to a wide range of other colicins. A deeper investigation into the properties of cells with the novel colicin revealed that the presence of the novel colicin protein altered expression or presentation of the BtuB outer membrane protein, which many colicins use to enter the cell. Our study reveals a colicin-dependent

mechanism of colicin resistance. Furthermore, that a colicin can itself protect cells from its toxic effects suggests that colicin-immunity diversification may be able to proceed in a colicin-led manner.

Introduction

Modifications of the interactions between protein partners can result in the evolution of novel organismal functions. Sometimes, this occurs when mutations alter the affinity of a protein for typical or atypical partners (e.g., James & Tawfik 2003, Bloom *et al.* 2006, Bloom *et al.* 2007, Harms & Thornton 2014, Anderson *et al.* 2016) and can expand the repertoire of intracellular protein interactions. In other cases, such as two-component signaling (Skerker *et al.* 2008, Urano *et al.* 2008, Podgornaia & Laub 2015) or toxin-antitoxin systems in bacteria (Riley 1993a, Riley 1993b, Aakre *et al.* 2015, Nuckolls *et al.* 2017), interaction between particular protein partners is necessary for function. Diversification of such tightly-associated systems requires corresponding mutations between both partners that preserve their binding interaction.

We focus here on the nuclease E colicins, a family of closely related, proteinaceous toxins produced by *Escherichia coli* that target and kill susceptible *E. coli* cells through degradation of nucleic acid polymers (Cascales *et al.* 2007, Riley 1993a, Riley 1993b). Colicins have been interpreted as an anti-competitor mechanism, where release of the toxin eliminates susceptible competitors of clone mates (Riley 1993a). The colicin is encoded on a plasmid that also carries an immunity gene, or the cognate antitoxin, and a lysis gene (Figure 1a). When induced by an SOS promoter, colicin, immunity, and lysis genes are expressed. (The immunity gene is also expressed from a constitutive promoter found in the coding region of the colicin gene [Chak & James 1985].) The immunity protein binds to the colicin protein, neutralizing its toxic effect. The lysis protein

then bursts the colicinogenic cell and releases the colicin-immunity complex. The colicin binds to outer membrane proteins on neighboring bacteria, dislodging the immunity protein in the process (Zakharov *et al.* 2006), and is imported across the periplasm by the cell's translocation machinery. Clone mates of the colicinogenic cell carry the same plasmid and encode for the cognate immunity protein, which binds to and neutralizes the colicin. Non-clones that do not carry the cognate immunity protein, on the other hand, will be killed by the colicin.

Colicins and their cognate immunity proteins bind each other with both high affinity (K_d up to 10^{-17}, Wallis *et al.* 1995, Kleanthous & Walker 2001, Li *et al.* 2004) and high specificity (for example, cells expressing immunity to colicin E2 remain susceptible to colicins E3-E9), akin to a lock and key. Their converse functions of poison and antidote, respectively, make both the colicin and the immunity protein essential to the survival of a colicinogenic cell. At the same time, sequence comparisons indicate that the colicin-immunity complex has undergone multiple diversifications (Riley 1993a, Tan & Riley 1997). Patterns of sequence divergence among closely related colicin operons reveal an elevated rate of base substitution in the immunity gene and immunity-binding portion of the colicin gene (Riley 1998). This suggests diversification through compensatory mutation (Juan *et al.* 2008, Aakre *et al.* 2015), where mutations to one protein are matched (compensated) by mutations in its partner. However, disruption of colicin-immunity binding can be lethal (Masaki *et al.* 1991), and the likelihood of gaining matching mutations simultaneously is low.

One possibility for preserving colicin-immunity interactions during diversification is through a "promiscuous" intermediate, specifically a promiscuous immunity protein that can bind multiple colicins (including its native cognate colicin; Figure 1b, d). Promiscuous intermediates have been found in other protein diversifications (Aharoni *et al.* 2005, Khersonsky *et al.* 2006,

Khersonsky & Tawfik 2010, Harms & Thornton 2014). In the case of colicins, a single amino acid changes in E6 or E9 immunity proteins can extend their protection to colicins E3 or E7, respectively (Masaki *et al.* 1991, Levin *et al.* 2009). Furthermore, cells with expanded immunity phenotypes have a competitive advantage *in vitro*: A numerically rare bacterial strain with an immunity protein that protects against both its own colicin and the colicin of the majority strain will rapidly invade the community (Tan & Riley 1996).

The promiscuous immunity protein acts like a master key that can open multiple locks. In the background of this immunity protein, the colicin protein could then gain a corresponding mutation. This novel colicin would be neutralized by the promiscuous immunity protein, but not the ancestral immunity protein, resulting in a "superkiller" cell that could poison its ancestor. Multiple rounds of this two-step process (broadening of the immunity protein's protection, followed by corresponding changes in the colicin) would result in a new colicin-immunity pair (Riley 1993b).

Alternatively, diversification may occur through duplication of the immunity gene (e.g., Hittinger & Carroll 2007; Figure 1c, e). This would be akin to having an extra key for the same lock. One copy of the immunity protein would retain binding to the native colicin, while the other copy would be able to mutate freely. The steps would be similar to the case of a promiscuous immunity intermediate, but because binding is no longer constraining, duplication may increase the range of acceptable mutations. Many bacterial strains do in fact carry multiple immunity genes in their colicin operons (Tan & Riley 1996).

Under both "master key" and "extra key" hypotheses, diversification of the colicin-immunity complex is immunity-led: The immunity gene takes the first mutational step. In contrast, a colicin-led diversification, where the colicin gene mutates first, is not expected to be selectively favorable.

A colicin mutation that does not alter binding of colicin to the immunity protein (i.e., a neutral mutation) would not have a selective advantage over the ancestral colicin. A mutation that affected colicin-immunity binding could make the colicin toxic to cells with the ancestral immunity protein (cells with a non-ancestral or non-cognate immunity protein can be killed by the original colicin). However, because the mutant cell would also possess the ancestral immunity protein, it would be poisoned by its own colicin, making this an evolutionary non-starter.

We originally aimed to test the immunity-led hypotheses for colicin-immunity diversification by mutagenizing first the immunity gene, then the colicin gene. Unexpectedly, we isolated a novel colicin gene whose properties were independent of the cell's immunity background. Further exploration of this colicin gene revealed that a single base mutation conferred both toxicity to other cells and protection to the producing cell, a phenomenon that has not previously been described in colicin systems.

Methods

Strains and culture conditions

A list of bacterial, plasmid, and viral strains used or engineered in this study appears in Table S1.

Liquid bacterial cultures were initialized from freeze-down stocks and grown overnight in Lysogeny broth (LB; Difco) at 37°C with orbital rotation at 220 rpm. Colonies were grown on LB plates with 1.5% agar. Kanamycin (final concentration of 50 μg/mL) or ampicillin (final concentration of 100 μg/mL) were added to cultures of strains that contained the respective antibiotic resistant marker.

Isolating colicin supernatant

Colicin protein was isolated as supernatant from a bacterial strain with the corresponding plasmid. A culture of the specific strain was grown overnight to saturation, then diluted 1/50 into fresh media and grown until the culture reached an OD595 of 0.2 (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices). Mitomycin C was added to the culture to a final concentration of 10 μg/mL to induce the colicin operon, and the culture was allowed to grow for an additional 2 hours. The cells were then lysed with 1 μL of chloroform for every 1 mL of culture, and the tube was centrifuged to pellet bacterial debris. The supernatant containing the colicin protein was transferred to a new tube and stored long-term at 4°C.

Colicin spot tests

A culture of the strain on which various colicin supernatants would be tested was grown overnight. 300 μL of this culture were added into 4mL of LB soft (1%) agar and poured over a Petri dish containing LB 1.5% agar and kanamycin at 50 μg/mL. The soft agar layer was allowed to solidify for 20 minutes. 2.5 μL spots of colicin supernatant, undiluted and diluted by 10-fold, 100-fold, and 1000-fold, were pipetted on top of the soft agar layer in a grid-like pattern. After overnight incubation, cells would have grown over spots of colicins to which they were resistant (e.g., through possession of a cognate immunity protein or other means), while spots of colicins to which the cells were sensitive would leave clearings in the bacterial lawn.

Mutagenesis and screening for novel immunity proteins.

We present here a summary of our mutagenesis and engineering methods. Complete details are available in the Supplemental Information.

The colicin operon used in this study for mutagenesis originated from the native E3 colicin operon. We first mutagenized the E3 immunity gene via error-prone PCR (GeneMorph II Random Mutagenesis kit) according to the manufacturer's instructions. However, we note that our mutagenesis technique is not saturating. The mutagenized immunity alleles were ligated into a pCR2.1 vector using TA cloning (Invitrogen) and transformed into chemically competent Top-10 cells, according to the manufacturer's instructions. Transformed cells were plated on Petri dishes containing LB 1.5% agar, kanamycin (50 µg/mL), X-Gal (for blue/white screening), and pre-spread with 200 µL of supernatant containing colicin E6 (as the selective condition: cells that grew in the presence of colicin E6 would be candidates for immunity proteins with broadened function). Candidate colonies were Sanger sequenced and tested for growth against both colicin E3 (the native cognate colicin, to which cells should maintain immunity) and colicin E6 (a non-cognate colicin to which cells should gain immunity, with respect to cells containing the ancestral E3 immunity protein). Two novel immunity alleles were isolated from this process. A second round of mutagenesis on one of these novel immunity alleles and screening in the presence of colicin E6 resulted in two additional novel immunity alleles (Table S3).

Mutagenesis and screening for novel colicin proteins

Creation of plasmid backbones with differing immunity genes

Prior to mutagenesis of the colicin gene, we created plasmid backbones containing the novel immunity genes. We moved the E3 operon to a pGA3K3 backbone with a kanamycin resistance marker for ease of manipulation, and we engineered plasmids containing either one or two immunity genes. The native E3 colicin operon has genes that encode cognate immunity proteins to both E3 and E8 plasmids. For plasmids with two immunity genes, we used Gibson cloning

(Gibson *et al.* 2009) to replace the E8 immunity gene with either one of the novel immunity alleles or a second E3 immunity gene. To make operons with only one copy of the novel immunity genes, the common copy of the ancestral E3 immunity gene was removed from the double-copy plasmids, and the plasmids re-ligated using T4 ligation. All resulting plasmids were pooled to make a library of immunity backgrounds.

Isolation of novel colicin genes

The gene for colicin E3 was mutated using error-prone PCR (GeneMorph II Random Mutagenesis kit) and re-ligated back into the immunity library using Gibson assembly. (Again, we note that this mutagenesis technique is not saturating.) The resulting library, containing mutated colicin alleles in operons with either one or two immunity genes, at least one of which was a novel immunity gene, was cloned into chemically competent C2984 cells (NEB). Transformant colonies were screened by their ability to leave a clearing in a lawn of bacteria that produced only the ancestral E3 immunity protein (Levin *et al.* 2009; colicin is not produced). An overnight culture of these E3 immunity-producing cells were added to LB soft agar and spread over a base of LB 1.5% agar, and the top layer of agar was allowed to dry. Transformant colonies were then stabbed in a grid-like pattern into the top agar. After overnight incubation, any transformants with a novel colicin would kill the E3 immunity-producing cells, leaving a clearing in the lawn. As controls, we also stabbed colonies that produced the ancestral colicin E3 (do not leave a clearing in an E3 immunity-producing lawn) or colicin E6 (leave a clearing, since the lawn lacks the cognate immunity protein).

Identifying the causative colicin mutation and necessary immunity background

We created a backbone for testing non-synonymous changes in the novel colicin allele by separately amplifying the isolated colicin plasmid, excluding the colicin gene, and the E3 colicin gene from the native E3 plasmid. The fragments were ligated through Gibson cloning. We then added the specific colicin mutations individually into this background using overlapping mutagenic primers (27J25_col1_F / 27J25_col1_R or 27J25_col2_F / J27J25_col2_R; see Table S2). The same mutagenic primers were used to add the causative colicin mutation into previously-made backbones containing a single copy of each immunity gene (imm21, imm50, or the ancestral E3 immunity gene).

Construction of strains to test immunity production

We constructed additional strains to test whether the causative colicin mutation affected immunity production. The first construct included only the immunity-promoter region of the colicin gene and the immunity gene. We PCR-amplified pGA3K3 with primers that complemented the Vf2 and Vr sequences for the backbone (primers Vf2_revcomp and Vr_revcomp; see Table S2), and the final 568 bases of the colicin gene and the complete immunity gene for the insert (primers E3_promtag_F and E3_promtag_R; these primers included adapters for Vf2 and Vr). The segments were ligated in a Gibson reaction.

The second construct changed the causative serine mutation from a TCA serine to an AGT serine, resulting in a base change at every position. The mutation was encoded on two overlapping mutagenic primers (col_F_addSer and col_R_Sertag), one of which also contained an adapter. The engineered plasmid that contained the single causative mutation in the colicin gene and the ancestral E3 immunity gene was amplified with these primers and re-ligated in a Gibson reaction.

As controls, the same processes were performed on plasmids containing the ancestral E3 colicin and one copy of the ancestral E3 immunity gene.

qPCR to analyze immunity protein production

Cultures of cells containing an ancestral immunity gene and either the ancestral E3 colicin or the novel colicin with the causative mutation were grown in LB with kanamycin to 50 µg/mL to log phase. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad, no. 732-6820), quantified using the Epoch Microplate Spectrophotometer, and 1 µg was used for cDNA synthesis through the iScript cDNA synthesis kit (Bio-Rad, no. 1708890). Quantitative real time-PCR was carried out using a KAPA SYBR fast qPCR kit according to manufacturer's instructions. Separate reactions amplified the E3 immunity gene (experimental sample) and a 16s rRNA gene (control). Normalized-fold expression was calculated using the Pfaffl (2001) method.

Construction of a strain to test protective effects of colicin production

We knocked out colicin production but maintained the context of the colicin operon by changing the start codon of the colicin gene from an ATG to a TTG. Using the plasmid containing the single, causative colicin mutation and the ancestral E3 immunity gene, we amplified the pGA3K3 backbone and the colicin operon separately, then re-ligated them in a Gibson reaction. The forward and reverse primers of each reaction were the reverse complement of the other's (that is, the insert's forward primer matched the backbone's reverse primer and vice versa). The start codon mutation was encoded on the forward primer of the insert and the reverse primer of the backbone. (Primers used were colATG_F / bglbrk_Suffix_R and bglbrk_Prefix_F / colATG_R; see Table S2.) As a control, the same process was performed on a plasmid containing the ancestral E3 colicin and one copy of the ancestral E3 immunity gene.

BF23 phage assay

A culture of the bacterial strain that we wanted to test for BF23 infection was grown overnight. 400 µL of this culture were mixed with a dilution of a BF23 phage stock in LB 1% soft agar and overlaid on an LB 1.5% agar plate. After overnight incubation, plaques would form on sensitive lawns with low concentrations of phage, while no plaque would form on resistant lawns, even when plated with high concentrations of phage.

Fluorescence assay

Total internal reflection fluorescence microscopy was used to evaluate binding of fluorescently-marked colicin to outer membrane BtuB and was performed in laboratory of Dr. Colin Kleanthous according to protocols in Rassam *et al.* 2015. Briefly, cells containing the ancestral E3 immunity protein and either the ancestral E3 colicin or the novel colicin with the causative mutation were inoculated from frozen stocks in LB broth and grown to exponential phase at 37°C, then transferred into M9-glucose minimal media (0.1 mM $CaCl_2$, 0.1 mM $FeSO_4$, 2mM $MgSO_4$, 1 g NH_4Cl, 0.05% [w/v] casamino acids, 0.4% [w/v] D-glucose) supplemented with 50 µg/mL of kanamycin and incubated overnight at 37°C. 200 µL of culture were pelleted by centrifugation, washed with 200 µL of Phosphate Buffer Solution, and resuspended in 200 µL of M9 minimal media with a fluorophore (colicin E9 labeled with Alexa Fluor 488-maleimide according to the protocol in Rassam et al. 2015). Tubes with the resuspension were incubated for 12 minutes with regular inversion to allow the fluorescently-labeled colicin E9 to bind to outer membrane BtuB. The cells were again pelleted by centrifugation and washed three times in 400 µL of M9-glucose minimal media, followed by a single wash in Phosphate Buffer Solution. Cells

were loaded onto an agar pad (made with 200 µL of M9 containing 1% UltraPure^{TM} agarose [w/v]) and a coverslip (a 1.5 cm × 1.6 cm Gene Frame matrix [Thermo Fischer Scientific]).

Cells were visualized at room temperature using a custom-made total internal reflection fluorescence (TIRF) microscope built around the Rapid Automated Modular Microscope (RAMM) System (ASI Imaging). GFP and TMR excitation were controlled by a 488 nm and a 561 laser respectively, provided by a multi-laser engine (iChrome MLE, Toptica). At the fiber output, the laser beams were collimated and focused (100x oil immersion objective, NA 1.4, Olympus) onto the sample under an angle allowing for highly inclined thin illumination. Fluorescence emission was filtered by a dichroic mirror and notch filter (ZT405/488/561rpc & ZET405/488/561NF, Chroma). Images were collected by projecting the AF488 emission onto an EMCCD camera (iXon Ultra, 512x512 pixels, Andor). The pixel size was 96 nm. Transmission illumination was provided by an LED source and condenser (ASI Imaging). Sample position and focus were controlled with a motorized piezo stage, a z-motor objective mount, and autofocus system (MS-2000, PZ-2000FT, CRISP, ASI Imaging) with a 100 ms frame rate.

Results

Isolation of novel colicin and immunity genes

We used the native E3 colicin operon as our ancestral genotype. Using error-prone PCR, we first mutagenized the E3 immunity gene and screened for “promiscuous” immunity proteins that could protect cells against colicin E6, a distinct but closely related colicin to E3 (Figure S1a). We isolated four immunity alleles with broadened function (Table S3).

Using the mutated immunity alleles as a genetic background, we then looked for novel colicin proteins that were toxic to their E3 ancestor. We mutagenized the E3 colicin gene through error-prone PCR, cloned it into plasmids containing our isolated immunity genes, and screened transformed cells for their ability to produce clearings on a lawn of ancestral E3 immunity-producing bacteria (indicating that the ancestral immunity protein no longer bound the mutagenized colicin well; Figure S1b). One problem we anticipated was that any novel colicin that could not be neutralized by either the ancestral or a mutated immunity protein would also kill its producing cell. However, imperfect binding of an immunity protein might be compensated by a higher abundance of immunity proteins (Li *et al.* 2004). Therefore, to optimize our chances of finding a novel colicin, we cloned the mutagenized colicin alleles into a library of plasmids that contained either one or two immunity genes (one of the mutated immunity alleles on its own, or one copy of a mutated immunity allele and one copy of the ancestral E3 immunity gene).

Among 6,000 colonies screened from the library, we isolated one colony that formed a clearing in a lawn of cells producing ancestral E3 immunity protein (Figure S1c). However, we note that our mutagenesis process was not exhaustive (error-prone PCR does not necessarily cover every possible mutation; it may introduce multiple mutations that are lethal in combination; and some screened members from the library were most likely wild type).

Genetics of the evolved colicin allele and associated immunity background

We isolated and sequenced the plasmid of the novel colicinogenic colony to characterize its genotype. The plasmid contained two different mutated immunity alleles, which we called imm21 and imm50 (see Table S3). We had not cloned the mutagenized colicin genes into any backgrounds

containing two mutagenized copies of the immunity gene, so this was most likely a chimeric backbone that arose during PCR amplification of the plasmid library, which contained multiple nearly identical immunity alleles (e.g., Wang & Wang 1996). The mutagenized colicin allele carried two nonsynonymous mutations (*g*1150*a*, corresponding to an amino acid change of A384T; and *c*1450*t*, corresponding to P484S) and one synonymous mutation (*t*273*c*).

We first determined the cytotoxic phenotypes of the nonsynonymous mutations from the novel colicin allele. We engineered each mutation individually into the ancestral E3 colicin gene, using the double immunity background of the isolated colony. A colony containing only the *c*1450*t* mutation left a clearing in a lawn of cells that produced the ancestral E3 immunity protein, while a colony containing only the *g*1150*a* mutation did not. Consistent with hypotheses of immunity-led colicin diversification, the causative mutation appeared in the immunity-binding region of the colicin protein (Figure 2). For the remainder of the paper, we consider only the colicin gene with the *c*1450*t* mutation. We refer to this allele as `c*` and its protein product as `C*`. (The ancestral E3 colicin allele and protein are denoted as `c` and `C`, respectively.)

Our next aim was to determine which of the two mutated immunity proteins neutralized our novel colicin. We cloned `c*` into genetic backgrounds with one copy of either imm21 or imm50. Cells with `c*` were viable in both single-copy immunity backgrounds, and they showed the same toxicity phenotype as `c*` in the double-immunity background (i.e., a clearing in a lawn of cells expressing ancestral E3 immunity).

As a negative control, we also cloned `c*` into a plasmid with one copy of the ancestral E3 immunity gene (allele denoted as `i`, protein product as `I`). Because the cells with the `c*` allele killed cells with the ancestral immunity gene, `c*i` cells were not expected to survive. To our

surprise, we isolated abundant colonies from this transformation. This perplexing result changed the direction of our research and led us to investigate the *c*i* genotype more deeply.

Was the cell with the *c*i* plasmid chromosomally resistant to colicin?

We first considered that the *c*i* cell may have had chromosomal resistance to colicins, where mutations in genes that encode receptors or translocation machinery common to multiple colicin import pathways allow cells to survive in the presence of various colicins (Feldgarden & Riley 1999). To test this hypothesis, we spotted lysates of colicins E3 and E6 onto lawns containing either *c*i* cells or ancestral *ci* cells. Because both cells produced the ancestral E3 immunity protein, we expected that *c*i* cells would show the same immunity profile as the *ci* ancestor: They would be immune to colicin E3 but sensitive to colicin E6 (Figure 3a). Instead, we found that *c*i* cells were resistant to both colicins E3 and E6 (Figure 3b), consistent with chromosomal resistance.

In a cell with chromosomal resistance to colicins, the effects of the *c*1450*t* mutation would be masked. We isolated the *c*i* plasmid and moved it to a naive batch of competent cells (retransforming the *ci* plasmid into the same batch as a control). However, we obtained the same results. Cells with *c*i* were resistant to both colicins E3 and E6, while the *ci* control exhibited typical resistance to E3 and sensitivity to E6. In other words, cells with the *c*i* plasmid were not protected from colicins E3 and E6 (as well as C*) because of mutations on the chromosome, but because of the *c*1450*t* mutation in the colicin gene.

Was enhanced resistance of cells with the *c*i* plasmid due to regulatory changes?

The *c*1450*t* mutation in *c** falls inside the putative region of the promoter for the immunity gene (Chak & James 1985). This suggested to us that the mutation, in addition to conferring toxicity to ancestral cells, might simultaneously change regulation of the immunity gene. Specifically, if the ancestral immunity protein bound the C* colicin poorly but the *c*1450*t* mutation *increased* immunity expression, then the increase in the abundance of immunity proteins might have compensated for their lower binding affinity (Li *et al.* 2004), allowing cells with the *c*i* plasmid to survive.

We ran three experiments to test this hypothesis. We first asked whether a cell that only produced the ancestral immunity protein (i.e., no colicin production) would be protected by the *c*1450*t* mutation in the immunity promoter region. To test this, we built an "immunity-only" plasmid containing only the 3' end of the colicin gene, including the *c*1450*t* mutation, and the full immunity gene (the immunity protein should be produced and regulated according to the *c*1450*t* mutation, but the colicin protein itself is not produced). However, cells with this construct recovered sensitivity not only to colicin E6 (Figure 3c), but also, unexpectedly, to colicin E3 – even though they possessed the cognate E3 immunity protein! A mutated immunity promoter alone was insufficient to confer enhanced colicin protection.

Because the immunity-only construct encoded multiple changes to the colicin operon (it knocked out the SOS promoter, the 5' end of the colicin gene, and the lysis gene) that may have influenced the effect of the *c*1450*t* mutation, we came up with an alternative strategy to test immunity-regulating effects. We maintained the entire *c*i* operon, but manipulated the S484

codon to an alternate serine. If the *c*1450*t* mutation were necessary for enhanced colicin resistance through upregulation of the immunity gene, then we would expect that changing the base sequence – but not the encoded amino acid – would negatively affect immunity production and result in a loss of colicin protection. However, cells with the alternate serine continued to show resistance to colicin E6 (Figure 3d). Because the alternate nucleotide sequence also enhanced the cells' colicin protection, the *c*1450*t* mutation specifically was *not* necessary for upregulation of the immunity protein.

Finally, we decided to test immunity expression directly using qPCR. Expression levels of the immunity gene in cells with the `ci` plasmid versus the `c*i` plasmid did not differ significantly (Figure S2). In other words, the *c*1450*t* mutation did *not* appear to change regulation of the immunity gene.

Was enhanced resistance of cells with the `c*i` plasmid linked to colicin production?

One of the main differences between the immunity-only construct and the alternate serine construct was the production of the mutant colicin protein: Colicin protein is not produced in the immunity-only construct, but *is* produced in the alternate serine construct. Because cells with the immunity-only construct were sensitive to colicins but cells with the alternate serine construct were resistant, this suggested that the `C*` protein *itself* might contribute to colicin resistance. To test this hypothesis, we knocked out colicin production by altering only the start codon of the colicin gene. This construct preserved all other elements of the colicin operon, including the SOS promoter and the remainder of the `c*` allelic sequence. Colonies with the start codon mutation failed to produce a clearing in a lawn of cells expressing the ancestral E3 immunity protein (Figure

S3), suggesting that production of the colicin protein was indeed negatively affected. The cells recovered sensitivity to colicins E3 and E6 (Figure 3e), supporting the hypothesis that the C* protein itself plays a role in enhanced colicin resistance.

How does a mutant toxin confer immunity its producing cell?

To explore the mechanism by which C* protects cells that produce it, we took advantage of the natural diversity of colicins. We spotted lawns of cells containing either *c*i* or *ci* plasmids with colicins that spanned a range of receptor binding sites, translocation pathways, and killing mechanisms. The colicins to which *c*i* cells demonstrated enhanced resistance relative to *ci* cells used the BtuB outer membrane protein as their binding receptor, while colicins to which *c*i* did not differ in relative resistance from *ci* did not use the BtuB receptor (Figure 4).

These results suggested to us that the C* colicin may compromise the ability of BtuB to bind to its target colicins. Under this hypothesis, other processes that require a functional version of BtuB, such as bacteriophage binding, may also be affected. We infected cells with the *c*i* and *ci* plasmids with the bacteriophage BF23, which uses the BtuB receptor for infection (Hong *et al.* 2008). Cells with the *c*i* construct were fully resistant to BF23, while *ci* cells could still be infected (Figure 5a). We also used total internal reflection fluorescence microscopy with a tagged colicin E9 protein (Rassam *et al.* 2015) to determine whether BtuB was present in a bindable form on the cell surface. Cells with the *ci* plasmid showed a fluorescent signal for BtuB on the membrane, while *c*i* cells had a weak or absent signal (Figure 5b). These results suggested that BtuB was either not being presented on the cell surface at all, or it was presented in a form that did not permit these binding interactions. In other words, C* appears to have pleiotropic effects that

compromise normal functioning of BtuB. This means that BtuB-binding colicins, including C* itself, cannot bind to the cell, rendering it colicin resistant.

Discussion

We produced four new immunity alleles through random mutagenesis of the E3 immunity gene and screening of mutants on colicin E6, and one mutagenized E3 colicin allele that was able to kill cells producing the ancestral E3 immunity protein. The causative mutation that conferred toxicity resulted in a change from a proline to a serine at residue 484 in the colicin protein. This amino acid lies in a hydrophobic pocket at the colicin-immunity binding interface (Figure 2), and the ancestral proline residue engages in van der Waals interactions with a phenylalanine from the E3 immunity protein (Carr *et al.* 2000). Furthermore, the ancestral P484 is conserved between colicins E3 and E6, but varies in colicin E4 (Carr *et al.* 2000), suggesting that the residue may affect the nature of the interaction between particular colicin and immunity proteins. Taken together, these lines of evidence suggest that the P484S mutation may alter binding of the C* colicin to the native E3 immunity protein, conferring toxicity to cells that only possess the E3 immunity gene.

Unexpectedly, we found that cells with the C* colicin were themselves resistant to both colicins E3 and E6. This phenotype was independent of the strain's immunity background. In fact, when the *c** colicin allele was combined with E3 immunity in the same cell, the strain not only survived (despite the fact that C* kills cells with E3 immunity), but it also continued to show resistance to E6 (which ancestral *ci* cells cannot survive)! As additional evidence supporting the independence of *c** from the effects of a particular immunity protein, the immunity residues that

were altered in the mutated alleles (residues 35, 48, and 52; see Table S2) may not play essential roles in colicin binding or specificity (Carr *et al.* 2000).

Instead, the C* colicin appears to have pleiotropic effects that confer both toxicity to other cells, and protection to the producer. Specifically, expression or presentation of the outer membrane protein BtuB appears to be compromised in *c** cells, rendering the cells resistant to BtuB-binding colicins, including C* itself. In this way, the poison effectively creates its own antidote.

Natural and clinical *E. coli* isolates typically exhibit a high proportion of cells that are resistant to one or multiple colicins (Riley & Gordon 1996, Feldgarden & Riley 1998). Mutations to *btuB* are a common mechanism of resistance, particularly after laboratory exposure of *E. coli* to E-series colicins (Hufton *et al.* 1995, Feldgarden & Riley 1998, Feldgarden & Riley 1999, Calcuttawala *et al.* 2015). In contrast, the form of resistance found here is due not to genetic mutation of the *btuB* gene, but to the presence of a single-base mutation in the colicin gene. The precise mechanisms by which C* affects BtuB are unknown: whether expression of the *btuB* gene is reduced, its transcript not translated, the peptide trapped inside the cell or periplasm, or the protein presented on the cell surface in a dysfunctional conformation. One hypothesis might be that intracellular protein-protein interactions between C* and BtuB result in BtuB being sequestered (and possibly degraded) away from the membrane, or in BtuB assuming a non-bindable conformation within the membrane. However, BtuB is transcribed as an amino acid precursor that is immediately chaperoned to the membrane, and only assumes its tertiary form on insertion into the outer membrane (Tamm *et al.* 2004, Walther *et al.* 2009). Alternatively, the C* colicin may influence transcription or translation of BtuB. For example, because E3 colicins are RNAses, the C* colicin may target and digest *btuB* transcripts. However, E3 colicins specifically

target ribosomal RNA (Boon 1971, Bowman *et al.* 1971, Senior *et al.* 1971), and it seems unusual that a mutation in the colicin's immunity-binding region, which involves residues that are distinct from the residues of the cytotoxic active site (Kleanthous *et al.* 1999, Kleanthous & Walker 2001), would alter its specificity to include *btuB* transcripts. Possibly, the colicin protein interferes with BtuB expression or presentation indirectly, for example by negatively affecting expression of a chaperone, or by inducing stress responses in the cell that result in downregulation of the *btuB* gene (e.g., Lee *et al.* 2006). Any of these options would confer resistance to any BtuB-binding colicin, and addressing them will require a finer investigation into the interactions between colicins, immunity proteins, and BtuB.

Because BtuB is the binding site for vitamin B_{12} (cobalamin; Chimento *et al.* 2003), its reduction or loss may entail fitness costs. On the one hand, knockout mutants of *btuB* are still able to survive in both rich and minimal media (Baba *et al.* 2006), indicating that loss of BtuB is not lethal. On the other hand, BtuB loss may be accompanied by lower growth rates (Baba *et al.* 2006). Fitness costs due to BtuB loss are likely to be particularly relevant in a nutrient-limited environment. The prevalence of corrinoids such as cobalamin may also shape microbe-microbe interactions in environments such as the human gut (Degnan *et al.* 2014), which would imply a role for BtuB in microbial competition.

In the case that the benefits of novel allelopathy do outweigh the costs of downregulation or removal of the BtuB receptor, then we would expect the c^* genotype to displace its c ancestor. Once c^* fixes, and assuming that there are no other colicins in the environment, the cells would no longer benefit from allelopathy (all cells are resistant to BtuB-binding colicins), but they would still pay the cost of BtuB loss. At this point, there are three potential routes for a subsequent mutational step. One possibility would be to restore BtuB function through back-mutation of c^*

to C. However, a back-mutant would not gain an allelopathic benefit in the population of colicin-resistant C^* cells, and restoration of BtuB would make it susceptible to the C^* colicin. A second option would be to restore BtuB function through a compensatory mutation in the *btuB* gene. However, as before, restoration of BtuB would leave a *btuB* mutant susceptible being killed by the C^* colicin. We speculate that a third option might involve mutation of the immunity gene. If both the killing and protective effects of the C^* colicin are due to a lower binding affinity with the ancestral immunity protein, then perhaps a mutation in the immunity gene that restored typical colicin-immunity binding interactions would also restore normal BtuB function. Although this cell would be susceptible to BtuB-binding colicins, it would maintain protection against the C^* colicin through its matching immunity protein.

Finally, although we have highlighted a new mechanism of resistance for one mutant, similar interactions between colicins and BtuB may also occur in cells with the ancestral E3 colicin operon. Specifically, when we eliminated production of colicin E3 from ancestral cells (either by truncating the colicin gene or altering its start codon), they became more susceptible to the E3 colicin (Figure S4) – even though they still produced the cognate E3 immunity protein. This result is consistent with a scenario in which the absence of the colicin protein leads to greater abundance of surface-exposed BtuB, potentially allowing more colicin from the environment to enter the cell than can be neutralized by the produced immunity proteins.

Although we started our experiment exploring immunity-led hypotheses of colicin diversification, we discovered that a mutation to the colicin can ensure protection from the colicin itself. The potential for a new poison to serve as its own antidote highlights that there are aspects of colicin-immunity systems that are incompletely understood. Further exploration of this

phenomenon may increase our understanding not only of colicin evolution, but also of the molecular mechanisms of colicin action.

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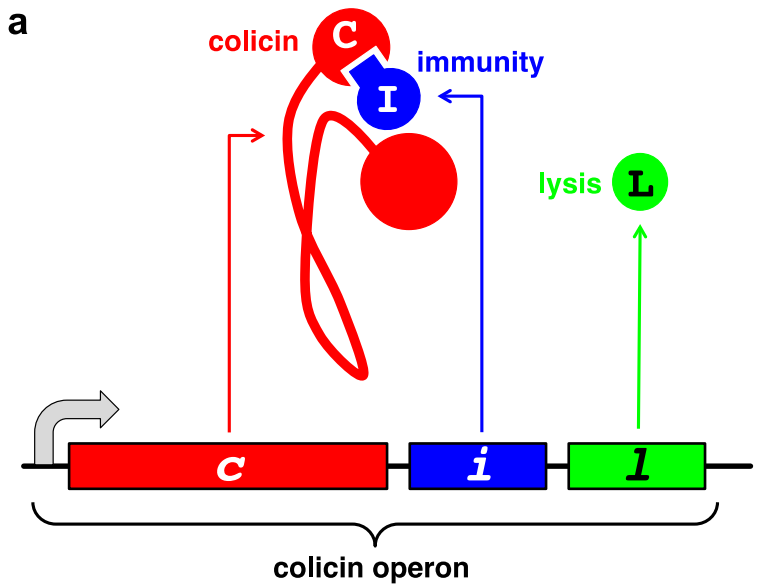
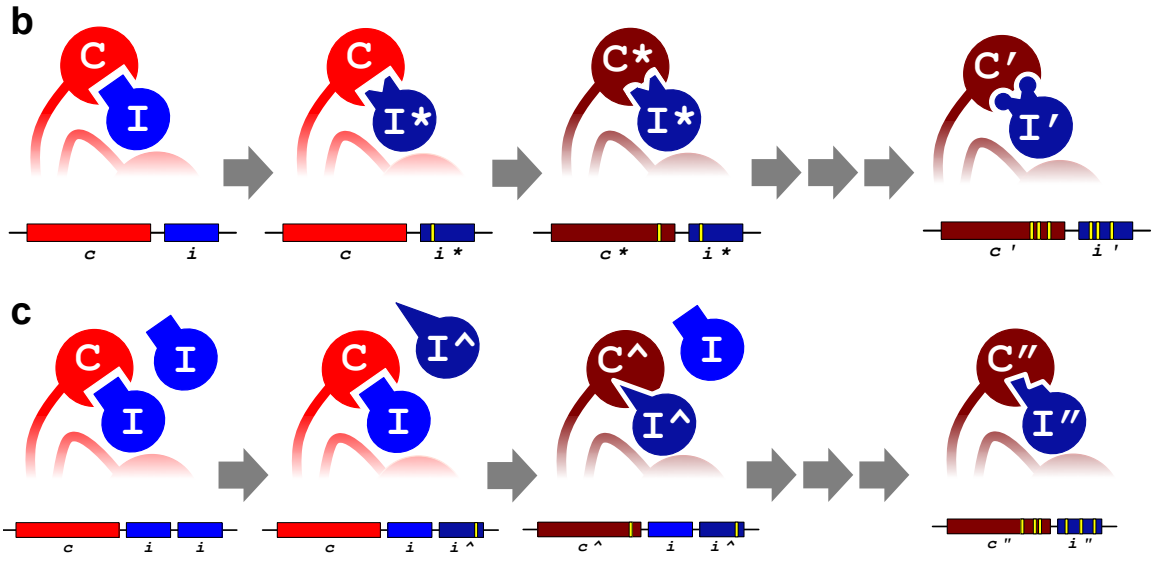
Figures





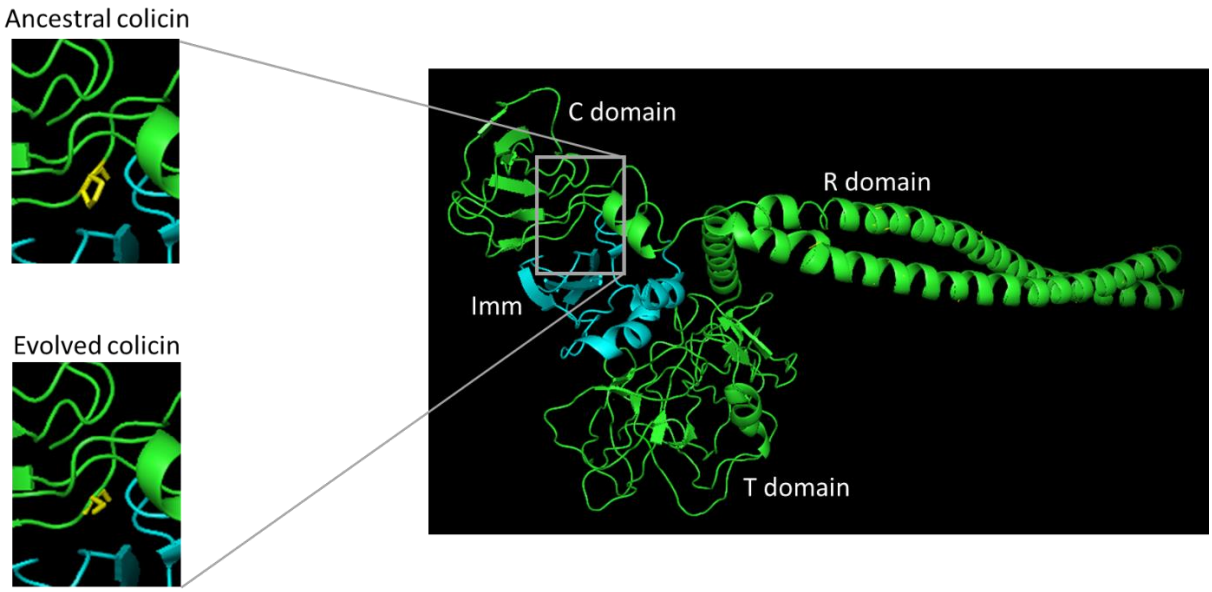
d

	C	C*	C′
I	binds well	binds poorly	binds poorly
I*	binds well	binds well	binds poorly
I′	binds poorly	binds poorly	binds well

e

	C	C^	C″
I	binds well	binds poorly	binds poorly
I^	binds poorly	binds well	binds poorly
I″	binds poorly	binds poorly	binds well

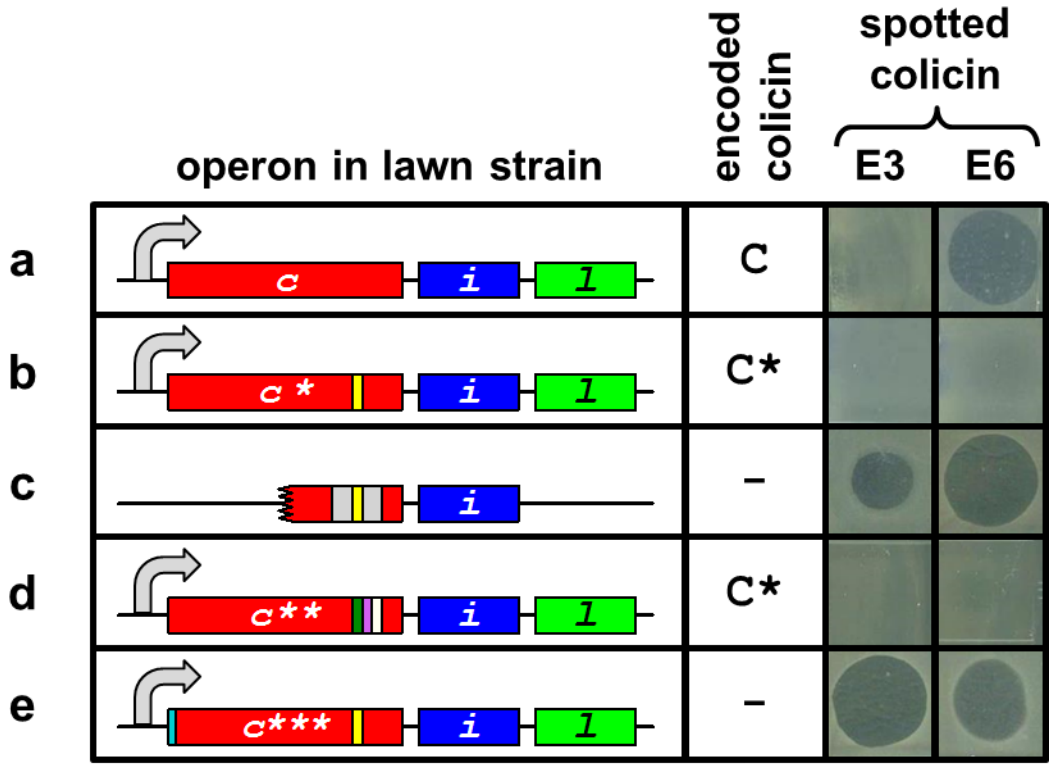
Figure 1. **a)** Colicins are expressed off an operon that is regulated by an SOS promoter. The colicin (red) is able to kill sensitive cells. A constitutively produced cognate immunity protein (blue) binds to and neutralizes the colicin so the colicinogenic cell is not killed by clone-mates. A lysis protein (green) kills the colicinogenic cell, releasing colicins in the process. **b)** Colicin diversification via promiscuous immunity intermediate. The immunity gene gains a mutation (*i**) that gives the cell broadened protection against colicin. The colicin gene then gains a mutation (*c**) that allows better binding to the I* immunity protein. Multiple rounds of this process could result in a novel colicin-immunity pair (C′I′). **c)** Duplication hypothesis for colicin diversification. Duplication of the immunity gene allows one copy to handle binding to the ancestral colicin, while the other copy is able to diversify (*i^*). The colicin protein then gains a corresponding mutation (*c^*) that permits better binding to the I^ immunity protein. **d)** Under the hypothesis of a promiscuous intermediate immunity protein, the first mutated protein, I*, binds both the ancestral colicin (C) and the mutated colicin (C*), whereas the ancestral immunity protein (I) only binds the ancestral colicin. This allows *c***i** cells from part **b** to asymmetrically kill their *ci* ancestor. Multiple rounds of this process produce a specific colicin-immunity pair (C′I′) that is distinct from the ancestor (e.g., I′ binds C′ but not C, and I binds C but not C′). **e)** Under the duplication process, every colicin-immunity pair can be completely specific. Here, the *c^i^i* cells are able to asymmetrically kill their *ci* ancestor because I binds C, but not C^. Further rounds of duplication and mutation can continue to generate new specific colicin-immunity pairs.



634

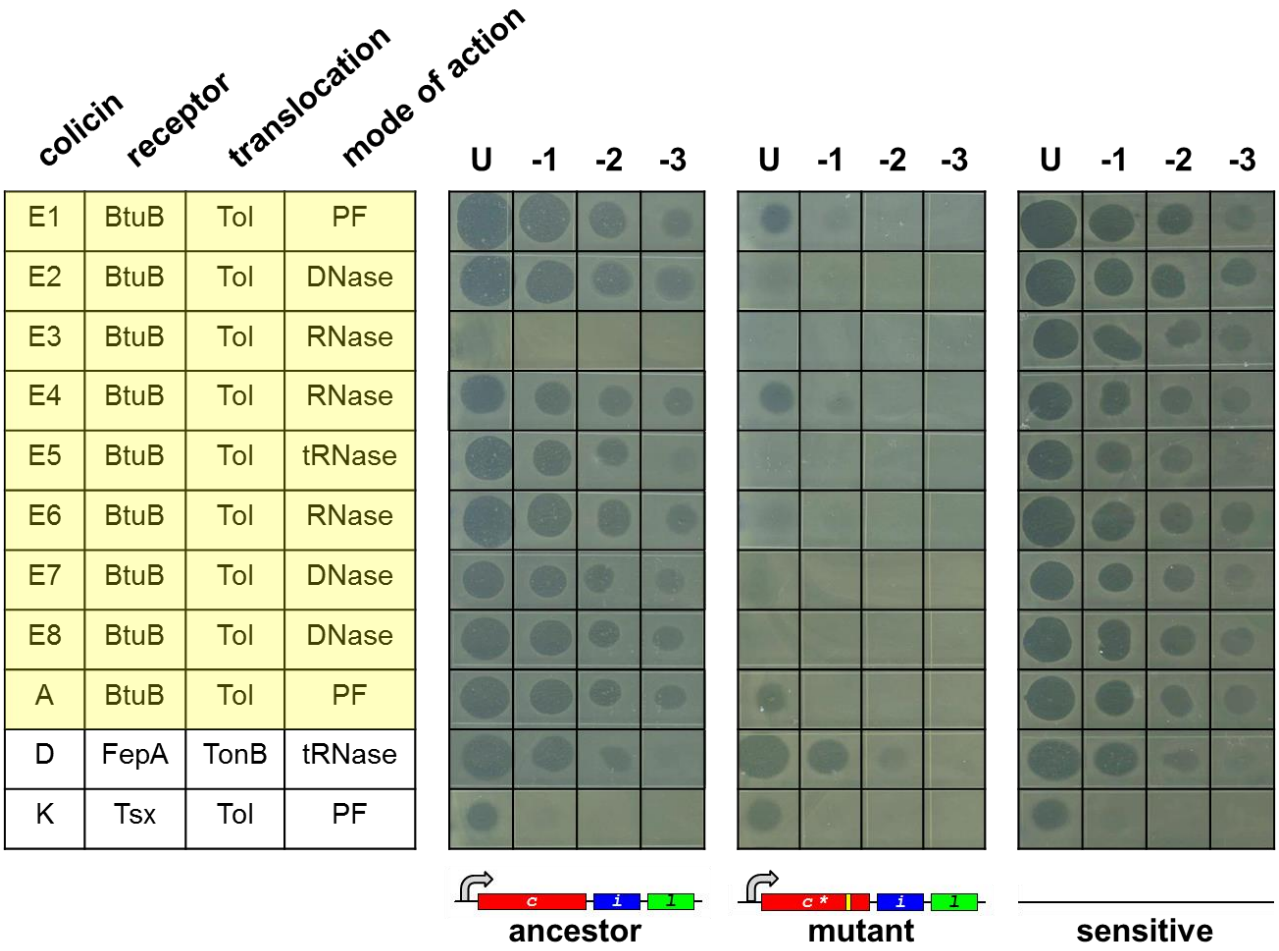
635 **Figure 2.** Overlay of the causative P484S mutation (bottom left), which allows the novel colicin to kill cells
636 producing the ancestral E3 immunity protein (top left), on Protein Data Bank structure 1jch (Soelaiman *et*
637 *al.* 2001). The mutation appears in the immunity-binding region of the colicin protein.

638



639

640 **Figure 3.** Resistance phenotype of cells with the particular engineered operon to colicins E3 and E6. The
641 isolated *c*1450*t* mutation is marked in the colicin gene as a yellow bar. Column "Encoded colicin" indicates
642 what colicin, if any, is produced by the operon. Cells with a clearing in the "Spotted colicin E3" or "Spotted
643 colicin E6" columns are sensitive to colicin E3 or E6, respectively, while cells with no clearing are resistant
644 to colicin E3 or E6. **a)** The ancestral E3 colicin operon produces the ancestral colicin and is killed by colicin
645 E6. **b)** The evolved colicin operon produces a novel colicin (C*) and is not killed by colicin E3 or E6. **c)**
646 When the region containing the mutation and the putative promoter (grey area) is isolated, cells recover
647 sensitivity to E3 and E6. **d)** When the P484S mutation is changed to an alternate serine (indicated by a blue,
648 pink, and white bar in the colicin gene), cells retain resistance to E3 and E6. **e)** Knocking out the start codon
649 of the colicin gene (indicated by a blue bar) returns E3 and E6 sensitivity to the cells.



650

651 **Figure 4.** Results of spot tests when lawns containing the ancestral E3 colicin operon (center-left panel),
652 an operon with *c*1450*t* in the colicin gene (center-right panel), or sensitive cells (right panel) are spotted
653 with a range of colicins with different modes of action, binding receptors, and translocation pathways (left
654 panel). Cells were spotted with undiluted (U), 10^{-1}, 10^{-2}, and 10^{-3} dilutions of the colicin. The presence of a
655 clearing indicates that the cells were sensitive to the spotted colicin. In comparison to the cells with the
656 ancestral E3 operon or lacking a colicin operon, *c** cells showed less sensitivity to BtuB-binding colicins.

657

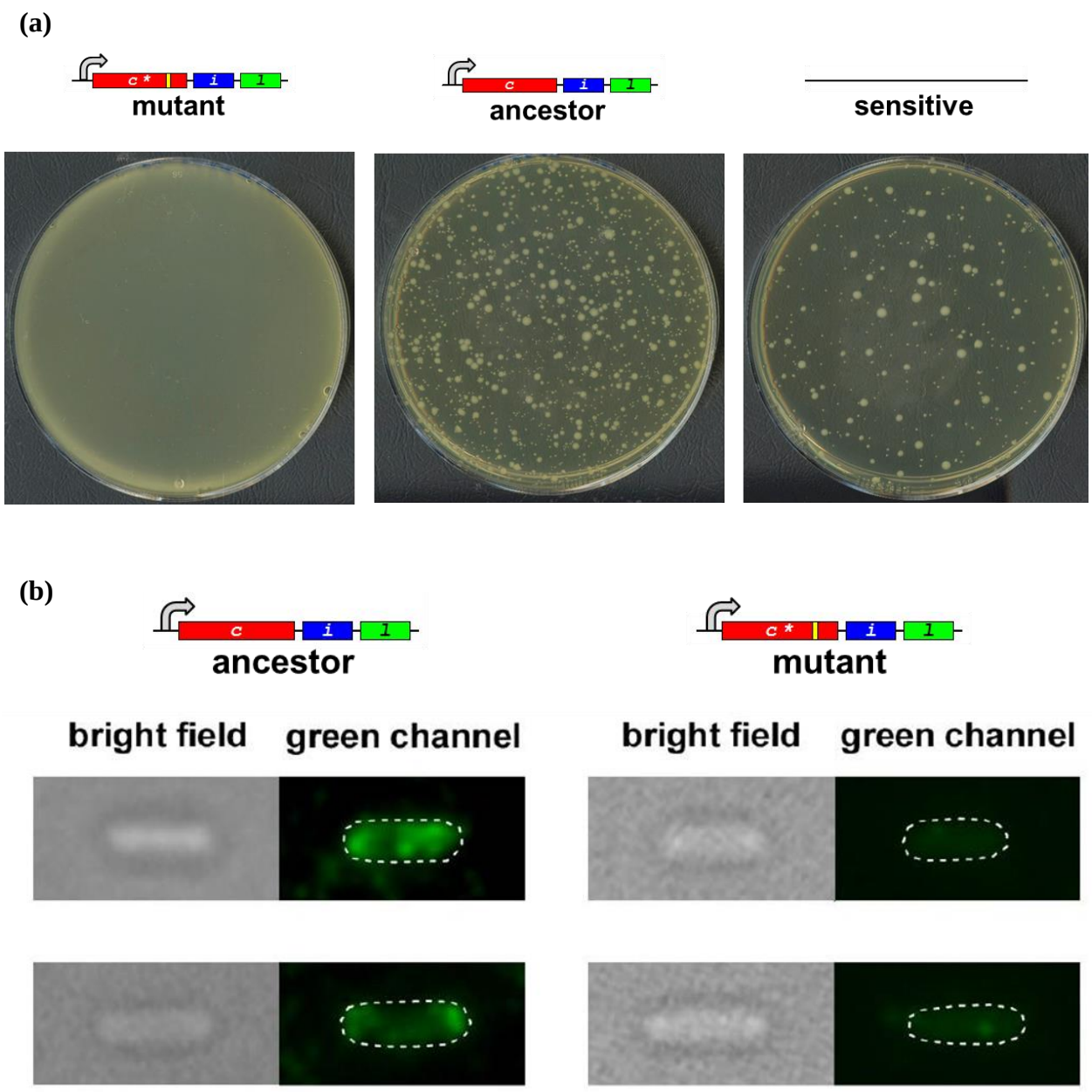


Figure 5. a) Cells with the ancestral E3 operon, the c^* colicin operon, and lacking a colicin operon were plated with 10^9 BF23 phage particles. Phage susceptibility is indicated by reduced density of the bacterial lawn. **b)** Total internal reflection fluorescence microscopy of fluorescently-marked colicin E9 to the BtuB outer membrane protein shows more fluorescence in cells with the ancestral E3 operon than in cells with the c^* colicin operon.

Supplemental information

Supplemental tables

Table S1 (4 pages). Bacterial, plasmid, and viral strains used or engineered in this study.

Strain #	Plasmid(s) or phage	Bacterial host	Description	Reference
BK1	pColE1	BZB1011	native colicin E1 plasmid	Riley 1993a
BK2	pColE2	BZB1011	native colicin E2 plasmid	Riley 1993a
BK3	pColE3	BZB1011	native colicin E3 plasmid	Riley 1993a
BK4	pColE4	BZB1011	native colicin E4 plasmid	Riley 1993a
BK5	pColE5	BZB1011	native colicin E5 plasmid	Riley 1993a
BK6	pColE6	BZB1011	native colicin E6 plasmid	Riley 1993a
BK8	pColE8	BZB1011	native colicin E8 plasmid	Riley 1993a
BK83	pcolE7	BZB1011	native colicin E7 plasmid	Gordon & O'Brien 2006
BK86	pcolA	BZB1011	native colicin A plasmid	Gordon & O'Brien 2006
BK89	pcolD	BZB1011	native colicin D plasmid	Gordon & O'Brien 2006
BK93	pcolK	BZB1011	native colicin K plasmid	Gordon & O'Brien 2006
BK95	pcolM	BZB1011	native colicin M plasmid	Gordon & O'Brien 2006
BK294	pNC3K3	REL606	3K3 plasmid, sensitive control (Kanamycin resistant)	This study
BK280	pNC3K3E3.im2	DH5-alpha	Colicin E3 operon with extra copy of ancestral im gene in pGA3K3 (Kanamycin resistant)	This study
BK288	pNC3K3E3.im1	C2984	Colicin E3 operon in pGA3K3 (Kanamycin resistant)	This study
NICE21	pNCCRe3i.21	Top10	pCR2.1 + mutagenized E3 im (im21), demonstrates enhanced immunity to colE6	This study
NICE25	pNCCRe3i.25	Top10	pCR2.1 + mutagenized E3 im (im25), demonstrates enhanced immunity to colE6	This study

Strain #	Plasmid(s) or phage	Bacterial host	Description	Reference
NICE45	pNCCRe3i.45	Top10	pCR2.1 + mutagenized E3 im (im45), demonstrates enhanced immunity to colE6	This study
NICE50	pNCCRe3i.50	Top10	pCR2.1 + mutagenized E3 im (im50), demonstrates enhanced immunity to colE6	This study
BK276	pNC3K3E3.im2_21	DH5-alpha	Colicin E3 operon with extra copy of im21 gene in pGA3K3 (Kanamycin resistant)	This study
BK277	pNC3K3E3.im2_25	DH5-alpha	Colicin E3 operon with extra copy of im25 gene in pGA3K3 (Kanamycin resistant)	This study
BK278	pNC3K3E3.im2_45	DH5-alpha	Colicin E3 operon with extra copy of im45 gene in pGA3K3 (Kanamycin resistant)	This study
B279	pNC3K3E3.im2_50	DH5-alpha	Colicin E3 operon with extra copy of im50 gene in pGA3K3 (Kanamycin resistant)	This study
BK272	pNC3K3E3.im21	DH5-alpha	Colicin E3 operon with im21 gene in pGA3K3 (Kanamycin resistant)	This study
BK273	pNC3K3E3.im25	DH5-alpha	Colicin E3 operon with im25 gene in pGA3K3 (Kanamycin resistant)	This study
BK274	pNC3K3E3.im45	DH5-alpha	Colicin E3 operon with im45 gene in pGA3K3 (Kanamycin resistant)	This study
BK275	pNC3K3E3.im50	DH5-alpha	Colicin E3 operon with im50 gene in pGA3K3 (Kanamycin resistant)	This study
BK289	pNC3K3E6.im1	C2984	Colicin E6 operon in pGA3K3 (Kanamycin resistant)	This study
BK259	pNC3K3E3.im1	REL606	Colicin E3 operon in pGA3K3 (Kanamycin resistant)	This study
BK295	pNC3K3E3_ColEvol.im50,21	C2984	Original evolved colicin plasmid isolated from colicin evolution experiment	This study

Strain #	Plasmid(s) or phage	Bacterial host	Description	Reference
BK307	pNC3K3E3_c1450t.im50_im21 in REL606	REL606	Colicin E3 operon in pGA3K3 (Kanamycin resistant) containing im50,im50,im21 and c1450t causative mutation	This study
BK308	pNC3K3E3_g1150a.im50_im21 in REL606	REL606	Colicin E3 operon in pGA3K3 (Kanamycin resistant) containing im50,im50,im21 and g1150a non-causative mutation	This study
BK260	pNC3K3E3_c1450t.im1	REL606	c1450t Colicin E3 operon in pGA3K3 (Kanamycin resistant)	This study
BK309	pNC3K3E3_c1450t.im50 in REL606	REL606	Colicin E3 operon in pGA3K3 (Kanamycin resistant) containing im50 and c1450t causative mutation	This study
BK310	pNC3K3E3_c1450t.im21 in REL606	REL606	Colicin E3 operon in pGA3K3 (Kanamycin resistant) containing im21 and c1450t causative mutation	This study
BK287	pNC3K3E3_cca1450agt.im1	REL606	Replaced Pro484 with alternate Ser484 (AGT) in pNC3K3E3.im1 (Kanamycin resistant)	This study
BK296	pNC3K3E3_ΔPsosΔ5'col.im1	REL606	Psos and 5' end of colicin gene deleted from pNC3K3E3.im1 (Kanamycin resistant, no col production)	This study
BK297	pNC3K3E3_c1450t_ΔPsosΔ5'col.im1	REL606	Psos and 5' end of colicin gene deleted from pNC3K3E3_c1450t.im1 (Kanamycin resistant, no col production)	This study
BK285	pNC3K3E3_a1t.im1	REL606	Colicin gene start codon changed to TTG in pNC3K3E3.im1 (Kanamycin resistant, no col production)	This study
BK286	pNC3K3E3_c1450t_a1t.im1	REL606	Colicin gene start codon changed to TTG in pNC3K3E3_c1450t.im1 (Kanamycin resistant, no col production)	This study

Strain #	Plasmid(s) or phage	Bacterial host	Description	Reference
BK282	pTrc99a_KanR	JM83	pTrc99a_Kan (Kanamycin resistant)	Li *et al.* 1997, this study added Kan resistance
BK281	pTrc99a_E3im_KanR	JM83	E3 immunity gene in pTrc99a_Kan (Kanamycin resistant, inducable expression of immunity)	Li *et al.* 1997, this study added Kan resistance
BK283	pTrc99a_colicinE3 + pNC3K3E3_a1t.im1	JM83	Colicin E3 gene in pTrc99a (Ampicillin resistant, inducable expression of colicin) and colicin E3 operon in pGA3K3 (Kanamycin resistant) with no colicin production (start codon ATG changed to TTG)	This study
BK284	pTrc99a_ColicinE3_c1450t + pNC3K3E3_a1t.im1	JM83	Evolved colicin E3 gene in pTrc99a (Ampicillin resistant, inducable expression of colicin) and colicin E3 operon in pGA3K3 (Kanamycin resistant) with no colicin production (start codon ATG changed to TTG)	This study
BK290	BF23 phage	n/a	BF23 phage	Hong *et al.* 2008
BK291	no plasmid	ZK126	E. coli strain used for farming BF23 phage	Hong *et al.* 2008

Table S2 (2 pages). Primers used during construction and testing of novel colicin and immunity genes.

Bases in upper case indicate adapter tags.

Primer name	Primer sequence
E8imm_XbaI tag_F	[Phos]CGTTCTAGAgggctgaaatatgaatgccgg
E8imm_NarItag_R	[Phos]CTAGGCGCCcgttcatccatgaacactagaagcc
E3_NdeI_tag_F	[Phos]AACATatgggacttaaattggatttaacttgg
E3_NdeI_tag_R	[Phos]cctctcaaagatatttcttgatatttcg
E3imm_NarItag_F	CGCGGCGCCctttgagaggaagttatgggacttaaattgg
E3imm_XbaItag_R	CCGTCTAGAccctgataatattcgatcaccaatcacc
operon_3K3tag_F	TCTGGAATTCCTGCTGCGGAGATCTggatcccatgagtggaagcggg
operon_3K3tag_R	CGGACTCGAGAACCCTGTTGGATCCCTGCAGcgcatcagaatcagcc
bglbrk_Suffix_F	ggatccaacagggttctcgag
bglbrk_Prefix_R	agatctccgcagcaggaattc

Primer name	Primer sequence
SpliceExtraImm_F	[Phos]gggctgaaatatgaatgccgg
SpliceExtraImm_R	[Phos]cgttcatccatgaacactagaagcc
E3im_1_F	caagaaatatctttgagaggaacatatggga
E3im_1_R	caactcatccctgataatatttgatcaccaatc
E3im_1_F_revcomp	aagtcccataTGttcctctcaaagatatttcttg
E3im_1_R_revcomp	gattggtgatcaaatattatcagggatgagttg
E3_imm_F	caactcatccctgataatatttgatca
E3_imm_R	caagaaatatctttgagaggaacatatg
E3_Imm_F_revcomp	catatgttcctctcaaagatatttcttg
E3_imm_R_revcomp	tgatcaaatattatcagggatgagttg
E6operon_3K3tags_F	GAATTCCTGCTGCGGAGATCTaactcggttttaatcagacctgg
E6operon_3K3tags_R	CTCGAGAACCCTGTTGGATCCcgatcggtttcgtgcgt
Vf2_biobrick	tgccacctgacgtctaagaa
Vr_biobrick	attaccgcctttgagtgagc
Vr_revcomp	gctcactcaaaggcggtaat
Vf2_revcomp	ttcttagacgtcaggtggca
E3colmutF	caactcatccctgataatatttgatca
E3colmutR	caagaaatatctttgagaggaacatatg
E3col_mut_F_revcomp	cattgggtcatgggcaaat
E3col_mut_R_revcomp	gagaggaacatatgggacttaaattg
E3col_F	ttatgagcggtggcgatggac
E3col_R	acaggcaatcagttgaaaggtccag
E3col_F_revcomp	gtccatcgccaccgctcataa
E3col_R_revcomp	ctggacctttcaactgattgcctgt
27J25col1_F	[Phos] tcacagaatgtggcaaatggc
27J25col1_R	[Phos] ccgccagTcattgggtcat
27J25col2_F	[Phos] ttaagcctgggataTcaaaaacac
27J25col2_R	[Phos] gatcaccaagccctttaatattctc
Col_R_Sertag	CCACCATTCTGCTTTGGTGTTTTACTtatcccaggcttaagatcaccaagc
Col_F_addSer	cgattcaggcctggtatgagtcag
Col_F_Ser	AGTaaaacaccaaagcagaatggtgg
Col_R_addSer	ctgactcataccaggcctgaatcg
E3imm_promtag_F	TGCCACCTGACGTCTAAGAActcttgctgatgcaatagctg
E3imm_promtag_R	CTCGAGAACCCTGTTGGATCCcatccctgataatatttgatcacc
ColATG_F	gggaatttttTtgagcggtgg
ColATG_R	ccaccgctcaAaaaaattccc
E3imm_qPCR_F	ggacttaaattggatttaacttgg
E3imm_qPCR_R	caatcaccatcacgataatcaaac
16s_357_F	ctcctacgggaggcagcag
16s_357_R	gwattaccgcggckgctg

674

675

676 **Table S3.** Genetic details of immunity proteins isolated from mutagenesis of the E3 immunity gene and

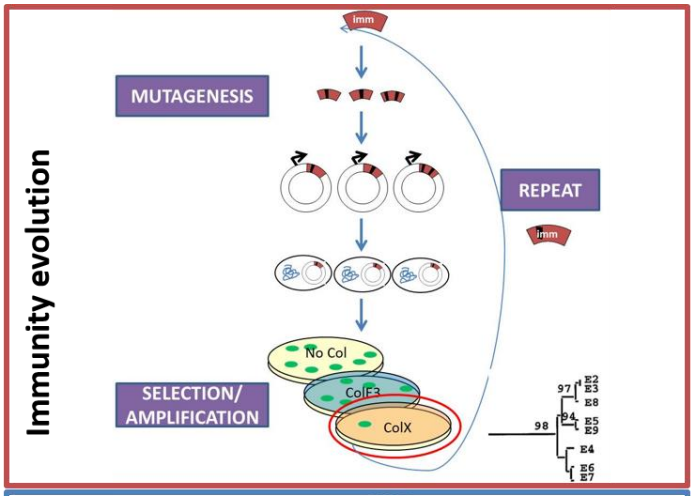
677 screening on colicin E6.

Name	Nucleic acid changes	Amino acid changes
Imm21	a103t, a154t, tt171-2ac	S35C, I52L, synonymous
Imm25	a35t	K12I
Imm45	g38t, a103t, a154t, tt171-2ac	S13I, S35C, I52L, synonymous
Imm50	a103t, g143a, a154t, tt171-2ac	S35C, C48Y, I52L, synonymous

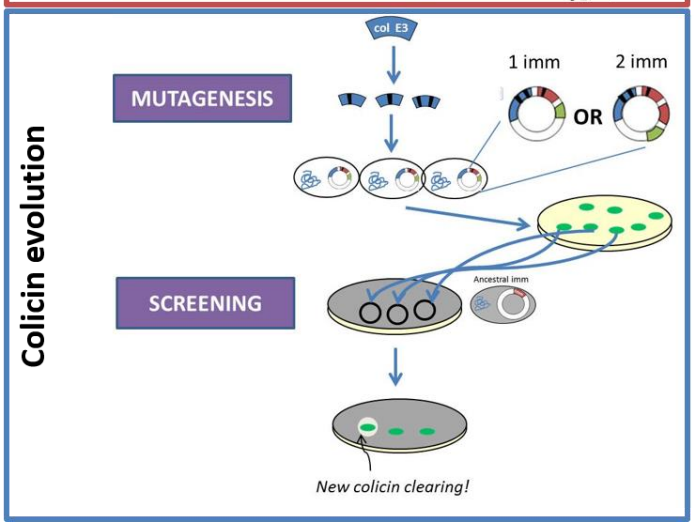
678

Supplemental figures

(a)



(b)



(c)

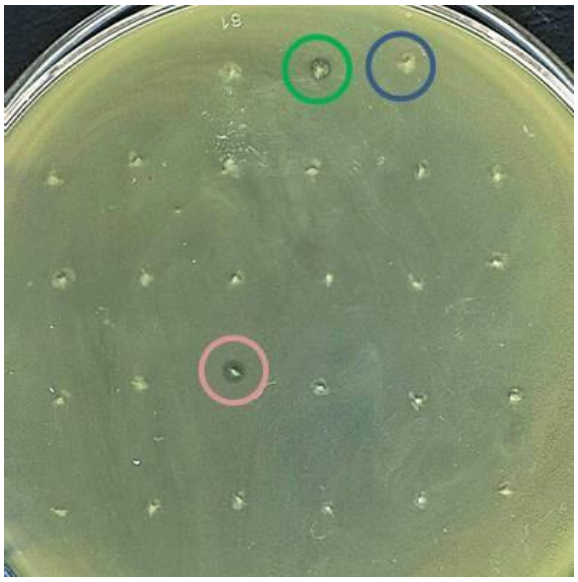
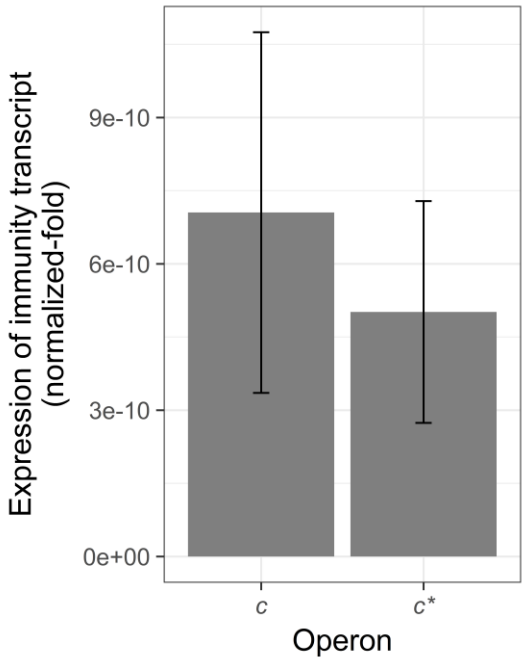


Figure S1. Schematic of experimental scheme used to isolate novel immunity and colicin genes.

a) *Immunity evolution.* The E3 immunity gene underwent random mutagenesis so that each mutagenized gene contained approximately 3 mutations. The mutagenized genes were cloned into a plasmid backbone, and colonies were screened for growth in the presence of colicin E6 (indicates that the mutagenized immunity protein is able to protect the cell against a non-cognate colicin). The immunity gene from one colony that was able to grow in the presence of colicin E6 underwent a second round of mutagenesis and screening. **b)** *Colicin evolution.* The E3 colicin gene underwent random mutagenesis so that each mutagenized gene contained approximately 1-6 mutations. The mutagenized colicin genes were cloned into plasmids that contained either a single copy of a novel immunity gene (isolated from part A), or one copy of a novel immunity gene and one copy of the ancestral E3 immunity gene. Colonies were screened for activity on a lawn of cells that produced the ancestral E3 immunity gene. Clearings in the lawn indicated that the cells were producing a colicin that could not be neutralized by the ancestral E3 immunity protein, and the cells that produced the clearing were candidates for a novel colicin gene. **c)** *Isolated colony* containing the novel colicin (pink circle) leaves a clearing in a lawn of cells that produce the ancestral E3 immunity protein. A positive control (green circle; colony producing colicin E6) and negative control (blue circle; unmutagenized colony producing colicin E3) are shown for comparison.



699

700 **Figure S2.** There is no difference in expression of the immunity gene between cells that produce the
701 ancestral E3 colicin (left) or that produce the novel colicin (right). Bar heights are the median normalized
702 expression of 2 biological replicates; error bars indicate standard error.

703

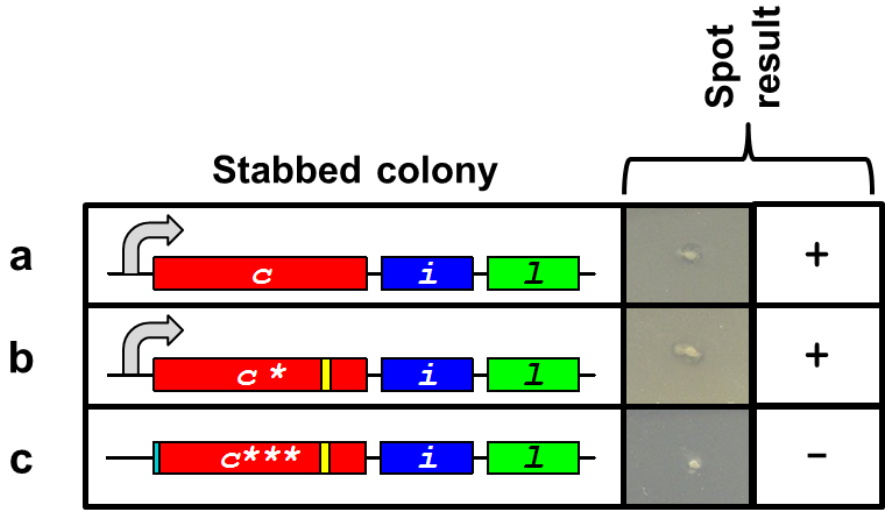


Figure S3. Clearing tests of engineered constructs stabbed into a lawn of sensitive cells. a) Cells with the ancestral E3 operon leave a clearing in a sensitive lawn. b) Cells with the c* colicin gene leave a clearing in a sensitive lawn. c) Cells with an altered start codon in the colicin gene do not leave a clearing in a sensitive lawn.

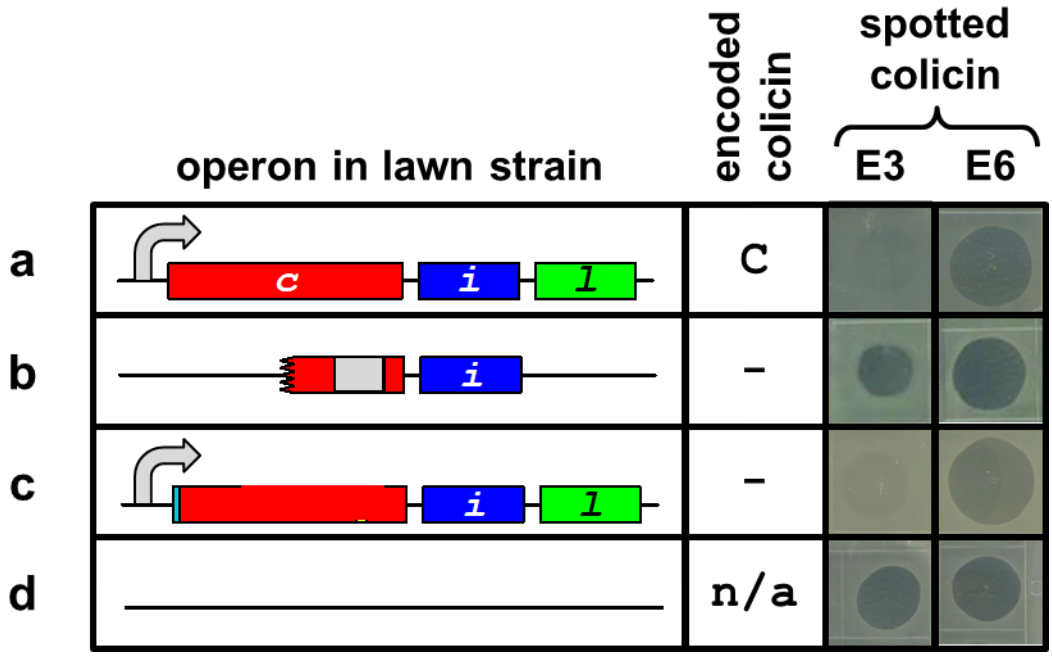


Figure S4. Resistance phenotype of cells with the particular engineered operon to colicins E3 and E6. Column "Encoded colicin" indicates what colicin, if any, is produced by the operon. Cells with a clearing in the "Spotted colicin E3" or "Spotted colicin E6" columns are sensitive to colicin E3 or E6, respectively, while cells with no clearing are resistant to colicin E3 or E6. **a)** The ancestral E3 colicin operon produces the ancestral colicin and is killed by colicin E6. **b)** When the region containing the putative immunity promoter (grey area) is isolated, cells recover sensitivity to E3. **c)** Knocking out the start codon of the colicin gene (indicated by a blue bar) increases E3 sensitivity. **d)** Resistance phenotypes of sensitive cells that do not contain a colicin operon for comparison.

Supplemental methods

Isolating colicin supernatant from inducible colicin strains

To ensure strong expression of the ancestral and evolved colicin proteins, each colicin operon was moved to an IPTG-inducible pTrc99a plasmid (Li *et al.* 1997). To prevent cell death

due to leaky expression of the IPTG-induced promoter, we also transformed plasmids with the colicin start codon knocked out (see Analysis of colicin tolerance, below) into the same cell for constitutive expression of the immunity protein.

A 1/50 dilution of overnight culture was transferred into fresh LB+Kan broth in a 50 mL flask. Cultures were incubated for approximately 6 hours until the culture reached OD595~0.3. IPTG was added to a final concentration of 1 mM to induce colicin expression, and cultures were incubated for an additional 90 minutes. Cultures were then transferred to a centrifuge tube, and 1 uL of chlorform was added for every 1 mL of culture. Tubes were vortexed for 30 seconds, then centrifuged at 4000 g for 5 minutes. The supernatant was immediately pulled off and placed in new sterile tube, being careful not to disturb pellet. Supernatant was stored long-term at 4°C.

Colicin streak test

Streak tests can be performed using either overnight culture or a colony. 15 uL of colicin supernatant were pipetted in puddle on the agar at the top of a petri dish, then the plate was lifted vertically to run the supernatant puddle down to the opposite end of the plate. The plate was allowed to dry for 20 minutes. If assaying a colony, the colony was scraped up using a pipette tip, and the tip was dragged across the surface of the plate, perpendicular to the supernatant. If assaying culture, 5 uL of culture were pipetted and puddled on one side of plate, then a pipette tip was run through the culture and across the center of the plate, perpendicular to the supernatant. Plates were incubated agar-side down overnight at 37°C.

Molecular techniques

PCR and Cloning, DNA quantification, and clean-up techniques

Unless otherwise noted, all molecular techniques were performed as follows.

Plasmid miniprep: The strain containing plasmid of interest was grown from a scraping of a freezer stock or a single colony into an 18 mm test tube containing 4 mL of LB broth (with antibiotics as necessary) and incubated overnight at 37 C with rotation at 220 rpm. Using a Qiagen Spin Miniprep Kit, plasmid was isolated from 1 mL of culture; product was eluted in 50C milli-Q water.

PCR: DNA was amplified using Thermo-Fisher Scientific Phusion® High-Fidelity DNA Polymerase with HF buffer as per manual instructions for 35 cycles (anneal temperatures, primers, and extension times are specified in the following sections). Expected fragment sizes were confirmed using gel electrophoresis.

PCR purification: All PCR products were purified using the QIAquick PCR Purification Kit as per manual instructions; product was eluted in 30 uL of milli-Q water heated to 50C.

DNA quantification: dsDNA was quantified from 2 uL of PCR product or plasmid miniprep product (ng/uL) using an Epoch Microplate Spectrophotometer.

Electrocompetent cell transformation: 1 ul (1-50 ng) of DNA product was added to a thawed vial of electrocompent cells. Cells were incubated on ice for 1 minute, then transferred to a clean cuvette. Cells were electroporated at 1250V on an Eporator (Eppendorf), then recovered in 1 mL of LB broth for 1 hour at 37°C. 100 uL of recovery culture were plated on and LB+antibiotics (as needed) agar petri dish. Plates were incubated overnight at 37°C.

Chemically competent cell transformation: 1 uL (1-50 ng) of DNA product was added to a thawed vial of chemically competent cells (NEB) on ice. Cells were incubated on ice for 30

minutes, heat shocked for 30 seconds at 42 C, incubated on ice for an additional 5 minutes, then recovered in 950 uL of SOC at 37°C for 1 hour. 100 uL of recovery culture were plated on an LB+antibiotics (as needed) agar petri dish. Plates were incubated overnight at 37°C.

DpnI digest to remove residual methylated background plasmid prior to Gibson Assembly: DpnI digests (NEB) were performed as per manual instructions: 40-80 ng of DNA was digested at 37°C for 1 hour, then heat denatured enzyme at 80°C for 20 minutes.

Preparation of electrocompetent cells

5 ml of an overnight culture in LB (+ antibiotics as needed) were added to a 1-liter Erlenmeyer flask containing 250 mL of YENB broth (0.75% Bacto yeast extract (Difco) and 0.80% Bacto Nutrient Broth (Difco) in distilled water). The culture was incubated at 37°C with shaking at 200rpm for 2-4 hours, until ideal OD was reached (OD595~0.3 for REL606). The flask was chilled on ice at 4°C for 10 minutes, then the culture was transferred to a cold, sterile 250 mL centriufge tube (Corning). The tube was centrifuged in a Beckman-Coulter centrifuge chilled to 4°C at 2850 g for 5 minutes. The supernatant was poured off, and 100 mL of sterile, cold water were added. The pellet was gently resuspended, then centrifuged in 4°C-chilled centrifuge at 2850 g for 5 minutes. The supernatant was poured off. 5 mL of cold 10% glycerol were added, the pellet was resuspended, and the tube was centrifuged in a 4°C-chilled centrifuge at 2850 g for 5 minutes. The supernatant was poured off, 1 mL of cold 20% glycerol added, and the pellet resuspended. 40 uL of the resuspension were aliquoted into a cold 1.5 mL Eppendorf tubes. Tubes were stored at -80°C for no more than 3 months.

Construction of pNC3K3E3.im2 and pNC3K3E3.im1

The E3 colicin operon was prepared with both single and duplicated copies of the E3 immunity gene and set up construct for directed evolution of immunity gene to be possible through either (Gibson *et al.* 2009) or restriction cloning. The colicin operon was also moved to a smaller plasmid backbone to increase efficiency.

Removed E8 imm and added restriction sites to clone in extra E3 immunity gene: BK3 contains two immunity genes that correspond to E8 and E3. From a plasmid miniprep of BK3, we amplified 1 ng of pcolE3 to amplify with E8imm_NarItag_R and E8imm_XbaI tag_F to remove the E8 imm gene while adding XbaI and NarI restriction sites for cloning in an additional E3 immunity gene (70°C annealing temperature, 3 minute extension time, expected fragment size ~7kb). DNA was purified and quantified. We ligated 50 ng of PCR product with T4 DNA ligase (NEB) per manual instructions (incubated 2 hours at 22°C) and transformed 1 uL of ligation product into electro-competent (ec) DH5α. The recovery culture was plated on LB + 200 uL of colicin E3 supernatant, and the plate was incubated overnight at 37C. We confirmed deletion of E8 immunity gene using colony streak tests with colE3 and colE8 and by sequence confirming restriction site addition. A successful transformant was cultured overnight in LB broth, and the plasmid extracted by a plasmid miniprep.

Added NdeI restriction site upstream of E3 immunity gene: We amplified 1 ng of plasmid (pColE3 with E8 imm deletion and addition of NarI and XbaI restriction sites) with E3_NdeI_tag_F and E3_NdeI_tag_R to add an NdeI site upstream of remaining E3 immunity gene (60°C annealing temperature, 3 minute extension time, expected fragment size ~7kb). DNA was purified and quantified. We ligated 50 ng of PCR product with T4 DNA ligase (NEB) per manual instructions (incubated 2 hours at 22°C) and transformed 1 uL of ligation product into ecDH5α. The recovery culture was plated on LB agar + 200 uL of colicin E3 supernatant, and the plate was incubated overnight at 37°C. 3 transformant colonies were selected randomly and cultured overnight in LB broth. Their plasmids were extracted via plasmid minipreps and the presence of NdeI restriction site confirmed by digestion with NdeI, confirming fragment sizes on a gel, and by sequence confirming.

Added extra E3 immunity gene in place of E8 immunity gene: From plasmid miniprep of BK3, we amplified 1 ng of pcolE3 with E3imm_NarItag_F and E3imm_XbaItag_R, isolating the E3 immunity gene and adding NarI and XbaI tags for cloning (72°C annealing temperature, 1 minute extension time, expected fragment size ~0.3kb). DNA was purified and quantified. 640 ng of E3 immunity gene were digested with NarI (NEB, 1 uL), XBaI (NEB, 1 uL), 1X buffer 4, and 1X BSA in a 20uL reaction at 37°C for 1 hour. 570 ng of previously cloned plasmid (pColE3 with E8 imm deletion and addition of NarI, XbaI, and NdeI restriction sites) were digested with NarI (NEB, 1 uL), XBaI (NEB, 1 uL), 1X buffer 4, and 1X BSA in a 20ul reaction at 37°C for 1 hour. We ligated 85 ng of digested vector and 26 ng of digested insert (E3 immunity gene) overnight at 4°C. The ligation product was heat deactivated ligation at 65°C for 20 minutes, then 1 uL was transformed into ecDH5α. The recovery culture was plated on LB agar + 200 uL of colicin E3 supernatant and incubated overnight at 37°C. 3 transformant colonies were randomly selected and cultured overnight in LB broth. Their plasmids were extracted via plasmid minipreps and the presence of an extra E3 immunity gene confirmed by sequencing.

Cloned colicin E3 operon into GA3K3 plasmid backbone: We amplified 1 ng of successfully cloned plasmid (pColE3 with E8 imm deletion, E3 imm addition, and addition of NarI, XbaI, and NdeI restriction sites) with operon_3K3tag_F and operon_3K3tag_R primers to amplify the colicin operon and add tags for Gibson cloning into pGA3K3 backbone (62°C annealing temperature, 1 minute extension time, expected fragment size ~2.6kb). We amplified 1 ng of pGA3K3 (medium copy number plasmid, http://parts.igem.org/Part:BBa_K590014) with bglBrk_prefix_R and bglBrk_suffix_F primers (70°C annealing temperature, 1 minute extension time, expected fragment size ~2.7kb). DNA from PCR products was purified and quantified. We performed a DpnI digest on the GA3K3 backbone using 80 ng of prduct (final concentration 40 ng/uL). We performed Gibson assembly using 1X NEB Gibson Assembly master mix, 40 ng of GA3K3 DpnI digested backbone, and 40 ng of colicin operon insert. The mixture was incubated at 50C for 15 minutes, then diluted 3x with Milli-Q water. 1 uL of diluted Gibson product was transformed into ecDH5α. The recovery culture was plated on LB agar + Kanamycin and incubated overnight at 37°C. We randomly selected 3 transformant colonies to culture overnight in LB+Kan broth,

then performed plasmid minipreps and sequence confirmed successful addition of the colicin operon into GA3K3 backbone. This plasmid is pNC3K3E3.im2; in REL606 host (BK271) and DH5α (BK280).

Remove additional E3 immunity gene to generate single E3 immunity gene plasmid: We amplified 1 ng of pNC3K3E3.im2 with SpliceExtraImm_F and SpliceExtraImm_R primers to remove the additional E3 immunity gene (62°C annealing temperature, 3 minute extension time, expected fragment size ~5.3kb). DNA from PCR products was purified and quantified. We ligated 50 ng product with T4 DNA ligase (NEB) per manual instrutions (incubated 2 hours at 22°C) and transformed 1 uL of ligation product into ecDH5α. The recovery culture was plated on LB agar + Kanamycin and incubated overnight at 37°C. We randomly selected 3 transformant colonies, cultured them overnight in LB+Kan broth, performed plasmid minipreps on them and confirmed their sequence. This plasmid is pNC3K3E3.im1; in REL606 host (BK259) and C2984 (BK288).

Mutagenesis and selection of immunity E3 gene

Prepared mutagenized imm insert

We established the ideal amplicon amount of 1 ng/uL for mutagenesis using calculations (Hall & Barlow 2008) and sequence confirming to achieve ~12 mutations/kb (immunity gene is ~0.25kb). To achieve 1 ng amplicon, we amplified 20 ng of pNC3K3E3.im1 (isolated using miniprep of BK288) to amplify with E3im_1_F and E3im_1_R primers using the GeneMorph II Random Mutagenesis kit as per manual instructions (56°C annealing temperature, 1 minute extension time, 29 cycles, expected fragment size ~0.25kb). DNA was purified and quantified.

TA cloning, transformation, and plating

To ensure the presence of poly-A overhangs on the mutagenized imm gene fragment, 50 ng (3 uL) of the fragment were incubated with 1 uL of dNTPs and 1 uL of Taq polymerase (NEB) for

15 minutes at 72°C. The enzyme was subsequently heat deactivated at 65°C for 10 minutes. We ligated 8 ng (1 uL) of insert with 1 uL of 10X ligation buffer and 100 ng (4 uL) of pCR2.1 vector from a TA cloning kit (Invitrogen) overnight at 14C. 1 uL of TA cloning product was transformed into 40 uL of chemically competent Top-10 cells (NEB). 100 uL of the transformed culture were plated on 9 different LB+Kan+X-gal plates spread with 200 uL of colE6 supernatant. As controls, 50 uL of the transformed culture were plated on LB+Kan+X-gal agar petri dishes containing no colicin or spread with colicin E3.

Analyze potential broadened immunity genes and repeated mutagenesis

We analyzed white or light blue colonies appearing on the colicin E6 plate (light blue/white colony, in presence of X-gal, indicates successful insertion of immunity gene) by inoculating colonies in LB+Kan broth, incubating overnight, and performing streak test across colE6 and colE3 supernatant at 0.01 and 0.001 dilutions. A second round of mutagenesis was repeated using im21 gene.

Building pNC3K3E3.imx, pNC3K3E3.imx_2, and pNC3K3E6.im1 plasmids for colicin evolution experiment

Building pNC3K3E3.imx_2 plasmids

From a plasmid miniprep of BK280, we amplified 1 ng of pNC3KE3.im2 with E3_imm_R_revcomp and E3_imm_F_revcomp primers to create the backbone containing one copy of the ancestral (E3) immunity protein (63°C annealing temperature, 3 minute extension time, expected fragment size ~5.3kb). DNA was purified and quantified. We performed a DpnI digest using 200 ng of PCR product (final concentration 100 ng/uL). From plasmid miniprep of NICE21,

25, 45, and 50, we amplified 1 ng of pNCCRe3i.21, pNCCRe3i.25, pNCCRe3i.45, and pNCCRe3i.50 with E3_imm_F and E3_imm_R to isolate the broadened immunity genes (63°C annealing temperature, 1 minute extension time, expected fragment size ~.25kb). DNA was purified and quantified. Using the 2x NEB Gibson Assembly Master Mix kit, 1 uL (20 ng) of DpnI digested backbone and 1 uL (2 ng) of insert were ligated at 50°C for 15 minutes. 1 uL of the Gibson product was transformed into 40 uL of electro-competent REL606. 100 uL of the transformed culture were plated on LB+Kan agar petri dishes. We randomly selected 3 transformant colonies, cultured them overnight in LB+Kan broth, performed plasmid minipreps and confirmed the sequences of plasmids pNC3KE3.im2_21, pNC3KE3.im2_25, pNC3KE3.im2_45, and pNC3KE3.im2_50.

Building pNC3K3E3.imx plasmids

We amplified 1 ng of each of these plasmids (pNC3K3E3.im2_21, etc.) with SpliceExtraImm_F and SpliceExtraImm_R primers to remove the additional E3 immunity gene (62°C annealing temperature, 3 minute extension time, expected fragment size ~5.3kb). DNA was purified and quantified from the PCR products. The fragment from the PCR reaction was re-ligated with T4 DNA ligase (NEB) using 50 ng of PCR product as per manual instrutions (incubated 2 hours at 22°C), and 1 uL was transformed into electro-competent REL606. 100 uL of transformed culture were plated on LB+Kan agar petri dishes. We randomly selected 3 transformant colonies, cultured them overnight in LB+Kan broth, performed plasmid minipreps and confirmed the sequences of plasmids pNC3KE3.im21, pNC3KE3.im25, pNC3KE3.im45, and pNC3KE3.im50.

Building pNC3K3E6.im1

From plasmid miniprep of BK6, we amplified 1 ng of pColE6 with E6operon_3K3tags_F and E6operon_3K3tags_R primers to extract the colicin E6 operon and add 3K3 tags (66°C annealing temperature, 2 minute extension time, expected fragment size ~3kb). From plasmid miniprep of BK288, we amplified 1 ng of pNC3K3E3.im1 with bglBrk_prefix_R and bglBrk_suffix_F primers to create the 3K3 backbone (70°C annealing temperature, 1 minute extension time, expected fragment size ~2.7kb). DNA was purified and quantified from all PCR products. We performed Gibson assembly using 1X NEB Gibson Assembly master mix, 50 ng of 3K3 backbone, and 50 ng of colicin E6 operon insert. Gibson reaction was incubated at 50°C for 15 minutes, then 1 uL was transformed into 40 uL of chemically competent C2984 cells (NEB). 100 uL of transformed culture were plated on LB+Kan agar petri dishes spread with 200uL of colicin E6 and incubated overnight at 37°C. We selected 1 transformant colony, cultured it overnight in LB+Kan broth, performed plasmid miniprep, and sequence confirmed successful addition of colicin E6 operon into the GA3K3 backbone. This plasmid is pNC3K3E6.im1 in C2984 host (BK289).

Mutagenesis and screening of colicin E3 gene

Preparation of backbone

We created a mix of plasmids isolated from BK272-BK280 by adding 1 ng of each plasmid to a PCR tube and vortexing. 1 ul of this plasmid mix was used to amplify the backbone for mutagenesis of the colicin gene with E3col_mut_F_revcomp and E3col_mut_R_revcomp primers (63°C annealing temperature, 3 minute extension time, 3.7-4 kb expected fragment size depended

on immunity gene copy number in the backbone). DNA was purified and quantified. We performed a DpnI digest using 65 ng of the PCR product (final concentration 52 ng/uL).

Preparation of mutagenized col insert

From plasmid miniprep of BK288, we amplified 1 ng of pNC3K3E3.im1 with Vf2_biobrick and Vr_biobrick primers to prepare the insert (63°C annealing temperature, 1 minute extension time, expected fragment ~2.9 kb). DNA was purified and quantified. We established the ideal amplicon amount of 1 ng for mutagenesis using calculations (Hall & Barlow 2008) and sequence confirming to achieve 2-12 mutations/kb (colicin gene fragment is 0.5kb). To achieve 1 ng of amplicon, we amplified 3' end of colicin E3 gene from 6 ng of the Vf2/Vr fragment with E3colmutF and E3colmutR primers using the GeneMorph II Random Mutagenesis kit as per manual instructions (56°C annealing temperature, 1 minute extension time, 29 cycles). Gel electrophoresis confirmed the expected fragment size of ~0.55 kb. DNA was purified and quantified.

Gibson assembly, transformation, and plating

Using the 2x NEB Gibson Assembly Master Mix kit with 1 uL (52 ng) of DpnI digested backbone and 1 uL (3 ng) of insert, we ran a ligation at 50°C for 15 minutes. 1 uL of Gibson product was transformed into 40 uL vial of chemically competent C2984 cells (NEB). 100 uL of the transformed culture were plated on 10 different LB+Kan agar petri dishes and plates were incubated overnight at 37°C.

Screening the mutagenized colicin library

All transformant colonies were screened by stabbing them into a soft agar lawn of NICE88 bacteria in petri dishes using sterile toothpicks, including controls (BK289 produces colicin E6

and should leave a clearing; BK288 produces colicin E3 and should not leave a clearing), in a grid-like format. Plates were incubated plates overnight at 37°C, then inspected for clearings. For each possible clearing, the colony of interest was harvested by stabbing clearing-making colony and streaking it out on an LB/Kan petri dish. The stab test was repeated with 5 colonies from the streak plate, including controls.

Analysis of evolved colicin gene

Cloning c1450t and g1150a into original immunity background (im50, im50, im21) to identify causative mutation

From a plasmid miniprep of BK288, we amplified 1 ng of pNC3K3E3.im1 with E3col_F and E3col_R primers to obtain the ancestral colicin gene (72°C annealing temperature, 1 minute extension time, expected fragment ~1.6kb). From a plasmid miniprep of BK295, we amplified 1 ng of pNC3K3E3_ColEvol.im50,50,21 to obtain the backbone, including the 3 immunity genes (72°C annealing temperature, 2.5 minute extension time, expected fragment ~4.6kb). DNA was purified and quantified. We performed a DpnI digest on the backbone fragment using 92 ng of PCR product (final concentration 46 ng/uL). We performed a Gibson assembly using 46 ng of DpnI digested backbone and 9 ng of colicin gene fragment using the 1X NEB Gibson Assembly master mix and incubating at 50°C for 15 minutes. The Gibson product was diluted 3x in Milli-Q water, then 1 uL of diluted product was used for transformation into ecBK26. 100 uL of the transformed culture were plated on LB+Kan agar petri dishes and incubated overnight at 37°C. We randomly selected 2 transformant colonies, cultured them overnight in LB+Kan broth, performed plasmid miniprep, and sequence confirmed successful addition of ancestral colciin gene into im50/im50/im21 immunity background.

We amplified 1 ng of this plasmid using 27J25col1_F and 27J25col1_R primers (68°C annealing temperature, 3 minute extension time, expected fragment ~6kb) to clone in g1150t, or 27J25col2_F and 27J25col2_R primers (64°C annealing temperature, 3 minute extension time, expected fragment ~6kb) to clone in c1450t separately to identify which mutation was causative. DNA was purified and quantified. The PCR product was re-ligated using 50 ng of product with the T4 DNA ligase (NEB) as per manual instrutions (incubated 2 hours at 22°C) and 1 uL of ligation product was transformed into electro-competent REL606. 100 uL of transformed culture were plated on LB+Kan agar petri dishes and plates were incubated overnight at 37°C. We randomly selected 2 transformant colonies, cultured them overnight in LB+Kan broth, performed plasmid minipreps and confirmed the sequences of plasmids pNC3K3E3_g1150a.im50_im50_im21 (BK308) and pNC3K3E3_c1450t.im50_im50_im21 (BK307) in REL606.

We performed stab tests using single colonies of BK307 and BK308 into a lawn of NICE 88 to test which genotype (BK307 or BK308) leaves a clearing in the E3 immunity producing lawn.

Cloning c1450t into im21, im50 and im1 immunity backgrounds

From plasmid minipreps of BK272, BK275, and BK259, we amplified 1 ng of pNC3K3E3.im21, pNC3K3E3.im50, and pNC3K3E3.im1 using 27J25col2_F and 27J25col2_R primers to add the c1450t mutation into each immunity background (64°C annealing temperature, 3 minute extension time, expected fragment ~6kb). DNA was purified and quantified. The fragments were re-ligated using 40 ng product with the T4 DNA ligase (NEB) as per manual instrutions (incubated 2 hours at 22°C), and 1 uL of ligation product was transformed into electro-competent REL606. 100 uL of transformed culture were plated on LB+Kan agar petri dishes and incubated overnight at 37°C. We randomly selected 5 transformant colonies from each plate and

performed a stab test into NICE 88 lawn to confirm insertion of c1450t with clearing phenotype. Candidate colonies were cultured overnight in LB+Kan broth, plasmids extracted via miniprep, and insertion of c1450t mutation sequence confirmed, generating pNC3K3E3_c1450t.im1 (BK260), pNC3K3E3_c1450t.im50 (BK309), and pNC3K3E3_c1450t.im21 (BK310).

Analysis of *c*i* genotype

Cloning in alternate Serine codon

From a plasmid miniprep of BK288, we amplified 1 ng of pNC3K3E3.im1 with Col_R_Sertag and Col_F_addSer primers (71°C annealing temperature, 1.5 minute extension time, expected fragment ~2.9 kb) and with Col_F_Ser and Col_R_addSer primers (71°C annealing temperature, 1.5 minute extension time, expected fragment ~2.4 kb) to replace Proline 484 with Serine (AGT). DNA was purified and quantified. We performed a Gibson assembly of the two fragments using the 1X NEB Gibson Assembly master mix and 40 ng of each fragment. Reaction was incubated at 50C for 15 minutes, then diluted 3x in Milli-Q water. 1 uL of diluted product was used for transformation into ecBK26. 100 uL of tranformed culture were plated on LB+Kan agar petri dishes and incubated overnight at 37C. We randomly selected 2 transformant colonies, cultured them overnight in LB+Kan broth, performed plasmid miniprep, and sequence confirmed successful addition of the alternate Serine codon. This plasmid is pNC3K3E3_cca1450agt.im1 in REL606 host (BK287).

Cloning out 5' col, lysis, and P_{SOS}

From plasmid minipreps of BK288 and BK260, we amplified 1 ng of pNC3K3E3.im1 and pNC3K3E3_c1450t.im1 with E3imm_promtag_F and E3imm_promtag_R primers to extract the

immunity promoter region in the colicin gene and the immunity gene (64°C annealing temperature, 1 minute extension time, expected fragment ~1.5 kb). We also amplified the 3K3 backbone from 1 ng of pNC3K3E3.im1 with Vr_revcomp and Vf2_revcomp primers (63°C annealing temperature, 2 minute extension time, expected fragment ~2.5kb). DNA from both reactions was purified and quantified. We performed a Gibson assembly of the 2 separate reactions using 50 ng of 3K3 backbone and 12.5 ng of each immunity promoter region/immunity gene fragment with 1X NEB Gibson Assembly master mix. Reaction was incubated at 50°C for 15 minutes, then diluted 3x in Milli-Q water. 1 uL of diluted product was used for transformation into ecBK26. 100 uL f transformed culture were plated on LB+Kan agar petri dishes and incubated overnight at 37°C. We randomly selected 2 transformant colonies from each plate, cultured them overnight in LB+Kan broth, performed plasmid miniprep, and sequence confirmed successful cloning of immunity promoter region and immunity gene into 3K3 backbone to generate pNC3K3E3_ΔPsosΔ5'col.im1 (BK296) and pNC3K3E3_c1450t_ΔPsosΔ5'col.im1 (BK297) in REL606.

Mutating start codon of colicin gene

From plasmid minipreps of BK288 and BK260, we amplified 1 ng of pNC3K3E3.im1 and pNC3K3E3_c1450t.im1 with primer set 1 (ColATG_F and bglbrk_Suffix_R, expected fragment ~2.3 kb) and primer set 2 (ColATG_R and bglbrk_Suffix_F, expected fragment ~3 kb) to create 2 fragments containing the ATG->TTG mutation (69°C annealing temperature, 2 minute extension time). DNA was purified and quantified. We performed a Gibson assembly of the 2 separate reactions using 28 ng of fragment 1 and 32 ng of fragment 2 with the 1X NEB Gibson Assembly master mix. Reaction was incubated at 50°C for 15 minutes, then diluted 3x in Milli-Q water. 1 uL of the diluted Gibson product was used for transformation into ecBK26. 100 uL of the

transformed culture were plated on LB+Kan agar petri dishes and incubated overnight at 37°C. We randomly selected 2 transformant colonies from each plate, cultured them overnight in LB+Kan broth, performed plasmid miniprep, and sequence confirmed successful site-directed mutagenesis of ATG->TTG to generate pNC3K3E3_c1450t_a1t.im1 (BK286) and pNC3K3E3_a1t.im1 (BK285) in REL606.

qPCR to analyze immunity protein production

Total RNA was extracted from cultures grown to log phase in LB with 50 ug/mL of Kanmycin (OD555~0.24) using the Aurum Total RNA Mini Kit (Bio-Rad, no. 732-6820), and the RNA yield was quantified using the Epoch Microplate Spectrophotometer. RNA (1 μg) was used for cDNA synthesis through the iScript cDNA synthesis kit (Bio-Rad, no. 1708890). Quantitative real time-PCR was performed using 2 ul of 1:10 diluted (for the experimental primers E3imm_qPCR_F and E3imm_qPCR_R) and 1:100 diluted (for the control primers 16s_357_F and 16s_357_R) cDNA. Reactions of 25 ul were carried out using a KAPA SYBR fast qPCR kit according to manufacturer's instructions and using the appropriate primers to amplify the E3 immunity gene (experimental) and a rRNA 16s gene (control). Real-time PCR was performed using a Bio-Rad CFX96 Real-time qPCR system (Hot start 95°C for 3 min, 40 cycles of 95°C for 3 sec and 60°C for 30 sec). Normalized-fold expression was calculated using the Pfaffl method (Pfaffl 2001).

Farming BF23 phage

We grew BF23 phage from a scraping of freezer stock in 18 mm test tube with 4ml LB+Kan overnight at 37°C with rotation at 220 rpm. 400 uL of bacterial culture were added to a 13mm tube containing 4 mL of SA and BF23 phage (diluted to 10^-7, -5, or -3). The tube was swirled to mix

the contents, and spread over an LB agar petri dish. The plate was incubated overnight, agar-side down, at 37°C. The following day, we looked for a plate with a "lacy" lawn (i.e., containing more than 1000 phage plaques). The soft agar layer was scraped off using a sterile spatula, added to a tube containing 4mL of LB, and vortexed for 30 seconds to break up the soft agar. The tube was centrifuged at 4000 g's for 5 minutes to pellet the soft agar. Then the supernatant was transferred to a new tube, added 50ul of chloroform to kill bacterial cells, vortexed for 10 seconds, and centrifuged again at 4000 g's for 5 minutes. The supernatant was pulled off and stored in a fresh tube at 4°C.