Genetic Robustness and Adaptability of Viruses

Phage can develop differing sensitivities to mutations, strongly affecting their adaptive potential in varied environments

Robert C. McBride and Paul E. Turner

Biodiversity reflects billions of years of adaptation through natural selection, whereby environments help to determine which genetic variants in a population persist. This process leads some individuals in a population to possess traits that prove beneficial in a particular niche and can help to distinguish them from other organisms residing there or elsewhere. Although evolution is defined as the change in genetic makeup of a population through time, natural selection acts on phenotypes. That is, natural selection leads indirectly to changes in gene frequencies by acting on the phenotypes that genes produce.

Thus, the translation of phenotype from genotype is crucial to evolution. Robustness and brittleness are terms used for measuring and describing the relative accuracy of this genotype-to-phenotype translation. In particular, genetic robustness is the constancy of a phenotype in the face of changes in the underlying genotype. If a mutation changes a phenotype, we consider the gene or, more broadly, the genome, to be relatively nonrobust, or brittle, against mutational input. However, if a genome changes but phenotype remains unaffected, the genome is considered genetically robust. Because other types of robustness are of interest, especially environmental robustness that describes whether a phenotype persists when an environment changes, it is crucial when examining robustness to define the specific phenotype and perturbation being measured and discussed.

Many phenotypic traits could be used to study robustness. From an evolutionary perspective, however, traits that constitute fitness, the relative ability to survive and reproduce, are of greatest interest.

Comprehensive measurement of fitness is prohibitively difficult for many organisms. Fortunately, because so many microbes grow asexually, microbiologists can readily measure population-level traits for a particular microbial genotype. Thus, two proxies for genotype fitness are the reproductive growth rate and the numerical size of a population grown from that genotype. However, typically it is more desirable to measure fitness of a microbial genotype using an experimental assay where two strains are placed in the same environment and their fitness is gauged relative to one another or to some baseline, such as a single

Summary

- Robustness and brittleness describe the relative accuracy of genotype-to-phenotype translation, which is crucial to evolution.

- Examining the balance between robustness and evolvability, the capacity to adapt, can help in determining whether natural selection shapes evolution itself.

- Despite some perceived obstacles, microbial studies, particularly those involving phage, are helping to fill gaps in our knowledge of robustness.

- Because pathogenic viruses may adapt to treatments by developing greater resistance and greater potential to withstand future therapies, caution is warranted when considering mutagenic therapies.

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common-competitor genotype. In this way, researchers can efficiently and accurately measure relative fitness of microbial genotypes, and quantify genotype robustness in the face of change.

Robustness Provides Insights into the Relationship between Phenotype, Genotype

Improved understanding of the fundamental relationship between phenotype and genotype provides a clear reason for studying the evolution of robustness. On the one hand, constancy in the face of environmental and mutational changes provides obvious benefits to an organism during replication. Robustness buffers organisms against such perturbations, affording constancy in terms of cellular function, development, and offspring production. That is, robustness provides reliability in the very currencies by which natural selection judges phenotypes.

However, rigidity in the face of change may pose problems. For example, if organisms are steadfast under environmental change, how can they possibly adapt to new conditions? Because natural selection acts on phenotypic variation, robustness that buffers this variation could impede evolution.

These conflicting necessities force organisms to strike a balance between withstanding some changes and maintaining an ability to adapt to new circumstances. This compromise is the balance between robustness and evolvability, the capacity to adapt. By examining this balancing act, we may learn whether evolvability can itself evolve. Thus, we can explore the intriguing—and contentious—idea that natural selection shapes evolution itself.

How Is Robustness Studied?

Despite a longstanding interest in robustness and extensive mathematical modeling, biologists have generated few data on this subject because studying robustness presents many challenges. One hurdle is to identify organisms that vary in robustness. Traits favoring robustness are not expected to be strongly selected until a biological population reaches evolutionary equilibrium—mutation-selection balance—in a constant environment.

This prerequisite should be especially true for genetic robustness, i.e., phenotypic constancy despite mutational input. Were a population to become optimally adapted to its habitat, any mutation would be either neutral or deleterious. This scenario should then lead to strong selection for genetic robustness to evolve, protecting the phenotype against mutations. In a related sense, because spontaneous mutations are believed typically to be deleterious, selection favoring evolution of robustness should be especially strong if mutation rates are elevated, even when populations are away from equilibrium.

Some convincing data on evolution of robustness stem from studies looking at virtual organisms—namely, self-replicating computer programs that change randomly and thus "evolve." According to one such study, elevated mutation rates can cause robust genotypes to be selectively favored over their brittle counterparts, even though robustness against mutations went hand-in-hand with lower reproductive fitness. Thus, the fittest gave way to the "flattest," with selection favoring those variants having the greatest phenotypic constancy and residing on flat regions of the fitness landscape (Fig. 1). Other studies successfully examine robustness by following changes in proteins in vitro.

Microbial populations provide another tractable choice for examining robustness because of their rapid generation times and large population sizes. However, microorganisms still require extensive time before selection begins to favor variants that evolve increased robustness. The limiting factor is time needed to achieve an adaptive optimum. For example, fitness in populations of *Escherichia coli* continues to change, even after 40,000 generations (20 years), according to Richard Lenski of Michigan State University in East Lansing and his colleagues. This result suggests a difficulty for researchers in relying on laboratory populations to examine selective pressures favoring evolution of robustness.

Although some theory suggests that evolutionary changes in robustness might be difficult to observe in laboratory populations of microbes, these systems remain the most attractive means for conducting experiments to examine evolution. Despite some perceived obstacles, microbial studies are helping to fill gaps in our knowledge of robustness. After all, microbes are immensely successful from an evolutionary standpoint; they thrive in all habitats that sup-

port life, and they are the most plentiful denizens of Earth. Over billions of years, microbes have experienced extensive selection to shape robustness.

RNA viruses seem particularly appropriate for examining genetic robustness, a view that mathematical theory supports. According to digital organism experiments, elevated mutation rates are a key prerequisite for populations to adapt by altering their genetic (mutational) robustness. Therefore, success may come from studying genetic robustness in biological systems with elevated mutation rates, making RNA viruses even better candidates because their mutation rates generally exceed those of other organisms, including DNA viruses, by at least one order of magnitude. By focusing on RNA systems, it is possible to study the evolution of robustness even when populations are not at an evolutionary equilibrium or are not subject to artificially elevated mutation rates through mutagenesis.
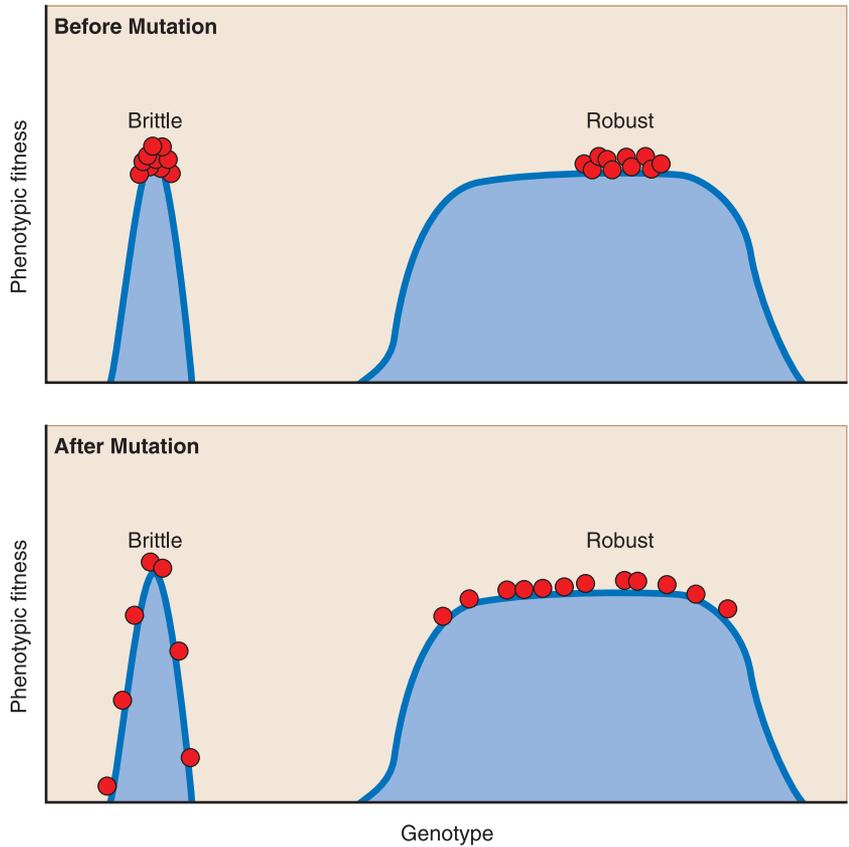
Can Robustness Evolve?

Several independent studies show that robustness can increase or decrease, depending on the particulars of the selective environment. For example, we used a single genotype of the lytic RNA bacteriophage φ6 to found three populations that were allowed to adapt to growth on *Pseudomonas syringae* pathovar *phaseolicola*. These lineages experienced low multiplicity of infection, where individual phage particles infected host cells to produce progeny. In parallel, three additional lineages evolved at a higher multiplicity, in which two to three phage particles on average coinfected a single cell. This experiment continued for 300 phage replication cycles.

The key fundamental difference between the two experimental treatments is that high-multiplicity viruses could experience complementation, a mechanism that can confer robustness against mutations. In theory, adaptive robustness assumes that phenotypic expression results solely from the underlying genotype. However, this assumption should not hold true for viruses that can experience complementation, a mecha-

FIGURE 1

Before Mutation

Brittle   Robust

Phenotypic fitness

After Mutation

Brittle   Robust

Phenotypic fitness

Genotype

Schematic where brittle and robust organisms are defined by their fitness response to mutational change, using the metaphor of fitness landscapes. Fitness is vertical height on the landscape. Mutation causes genotypes to move away from their original position on the horizontal axis. After mutation, brittle individuals experience large changes in fitness as they are "pushed off" the narrow fitness peak. In contrast, robust individuals reside on flatter portions of the landscape, and are therefore phenotypically buffered against mutational change. (Figure modified from C. O. Wilke and C. Adami, Mutational Res. Frontiers **522**:3–11, 2002.)

nism whereby a lower fitness viral genotype can phenotypically profit from intracellular proteins made by a coinfecting virus of higher fitness. Therefore, complementation during coinfection should automatically buffer viral phenotypes against mutations. Viral complementation plays the same role as gene duplication and diploidy that could provide robustness to cellular or multicellular organisms.

Because complementation buffers mutational effects, it offers a built-in mechanism for robustness in coinfecting lineages of phage φ6. By this logic, conditions fostering complementation (and hence, coinfection) may weaken selection for phage φ6 genomes to maintain their individ-

ual-level robustness because coinfection provides mutational buffering. We therefore predicted that the degree of coinfection—high multiplicity versus low multiplicity—should influence evolution of robustness in phage φ6 populations. To be more specific, we hypothesized that selection for robustness should be greatly relaxed in the φ6 lineages that arose under high levels of coinfection. If true, this would mean that the high-multiplicity populations should be dominated by φ6 genotypes that are relatively less robust to mutations.

To test the idea, we isolated 10 phage clones at random from each of three low- and high-multiplicity lineages. Each of these 60 clones was then subjected to mutation accumulation under extreme daily bottlenecks of a single virus particle (Fig. 2). Specifically, we plated a dilution of each phage φ6 population on a host lawn and the next day chose a plaque at random for the next round of population growth. Thus, the population is propagated via daily plaque-to-plaque transfers imposing an extremely small population size, making genetic drift overwhelm selection, and enabling the phage to accumulate nonlethal mutations at random. Because random mutations are expected to be deleterious, fitness of a lineage is expected to decline as mutations accumulate.
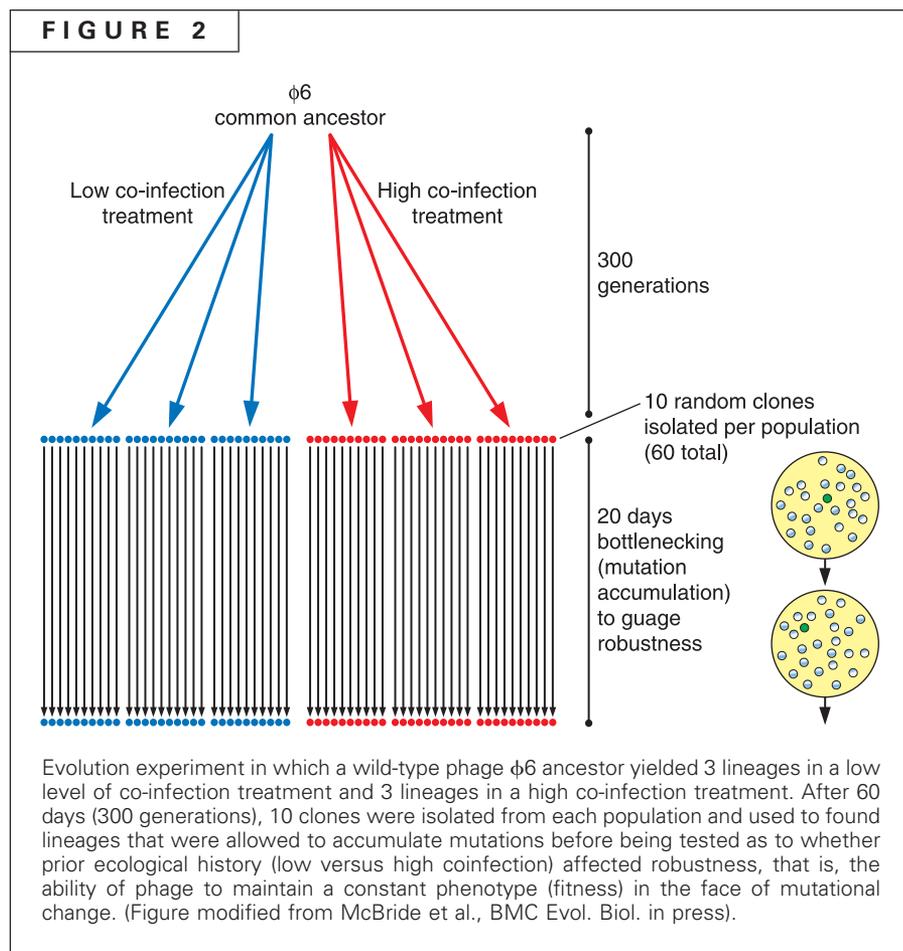
The 60 phage lineages were subjected to 20 consecutive days of extreme bottlenecking, providing opportunity for about 1.3 mutations to fix in each population, based on an estimated rate of 0.067 mutations per generation in phage φ6. To examine how the amassed mutations affected phenotypic fitness (W, relative growth rate on the host bacteria), we measured $\log_{10} W$ of each pre- and post-bottleneck lineage. The difference between these two values, $\Delta\log_{10} W$, reveals the sensitivity of the lineage to phenotypic effects of the accumulated mutation(s). Support for the hypothesis would be that the 30 lineages initiated by clones historically evolved under high multiplicity (frequent coinfection) would show greater variance in $\Delta\log_{10} W$ values, owing to weakened selection for them to maintain robustness as an individual trait. Additional support would come from a greater mean magnitude of $\Delta\log_{10} W$ values for the high-multiplicity lineages, indicating that they suffered a greater drop in fitness on average, because they are less able to withstand the deleterious effects of mutations. The data support the general predictions (Fig. 3), confirming that selection to maintain mutational robustness is weaker with viral coinfections. So far, the exact molecular mechanism responsible for robustness in phage φ6 has not been determined. But some clues regarding the mechanism stem from an additional set of experiments examining the relationship between robustness and evolvability.

Does Robustness Promote or Hinder Evolvability?

Experiments with phage φ6 are helping to address the question of whether robustness promotes or hinders evolvability. Because in nature phage φ6 attack *Pseudomonas* spp., plant pathogens that colonize leaf surfaces, we typically

FIGURE 2

Evolution experiment in which a wild-type phage φ6 ancestor yielded 3 lineages in a low level of co-infection treatment and 3 lineages in a high co-infection treatment. After 60 days (300 generations), 10 clones were isolated from each population and used to found lineages that were allowed to accumulate mutations before being tested as to whether prior ecological history (low versus high coinfection) affected robustness, that is, the ability of phage to maintain a constant phenotype (fitness) in the face of mutational change. (Figure modified from McBride et al., BMC Evol. Biol. in press).

culture φ6 at 25°C. When exposed to 45°C heat shock for as little as 5 minutes, roughly 80% of bacteria-free lysates of wild-type φ6 lose the capacity to infect *P. syringae* pv. *phaseolicola*. Presumably, the 45°C heat shock damages viral proteins that are needed for infectivity.

When we subjected 12 robust and 12 brittle genotypes of φ6 to the same heat shock, all showed similar initial sensitivity in terms of average percent survival (%S), with mean survival for both robust and brittle genotypes of only 14%. Because both groups of φ6 were earlier subject to incubations at a benign 25°C, we did not expect the robust and brittle clones to differ in their sensitivity to high temperature. However, this information led us to conduct an experiment that examined whether robustness enhanced or suppressed evolvability under heat-shock conditions.

We next subjected 24 lineages of half-robust and half-brittle clones to a 50-generation (10-day) experiment where each lineage experienced growth on *P. phaseolicola* at 25°C, with periodic (every fifth generation) exposure to 45°C heat shock (Fig. 4A). Then we measured mean %S at 45°C for each founding clone and its derived endpoint population to estimate Δ%S, the change in percent survival after 50 generations of selection to resist damaging heat shock. The results show that the lineages founded by robust genotypes are more evolvable, indicating that robustness promotes evolvability in phage φ6, at least as it adapts to high temperatures (Fig. 4B).
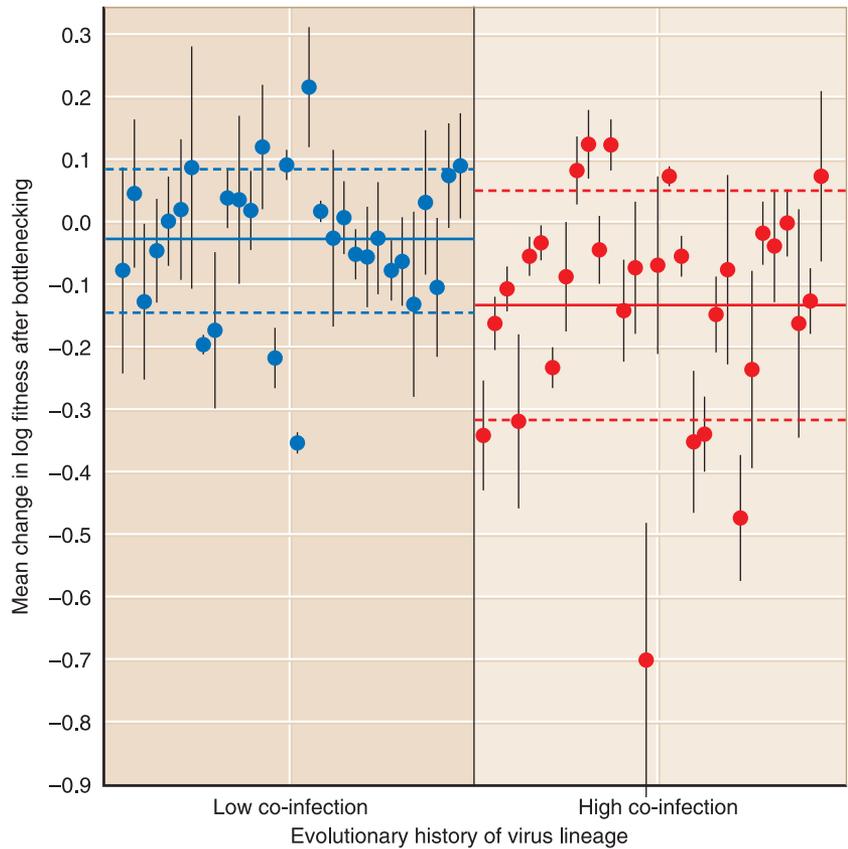
Robust genotypes of phage φ6 may feature proteins that tolerate mutations while maintaining proper folding. Thus, despite equivalent sensitivity to heat shock between the robust and brittle founding strains, a greater tolerance to mutational change by the robust lineages may account for their evolvability advantage. Thus, we surmise that one or more proteins of the robust viruses maintain proper folding even while accumulating spontaneous mutations that led to thermostable genotypes. This combination of traits could explain the relative advantage in evolvability for robust viruses when

FIGURE 3

A mutation accumulation study reveals that phage φ6 strains that evolved under low coinfection are more robust than those evolved under high coinfection. Each point is the mean change in $\log_{10}$ fitness resulting from mutation accumulation, for an independent lineage founded by a virus clone previously evolved under low level of coinfection (blue circles) or high level of coinfection (red squares). Horizontal lines are grand means among lineages within a treatment, and the dashed lines indicate one standard deviation away from the mean. (Figure modified from Montville et al., PLoS Biology, **3**:1939–1945, 2005.)

adapting to heat shock. In contrast, the brittle viruses were constrained in their ability to adapt because their proteins accumulated mutations that increased thermostability but compromised viral reproduction.

Future Work

Our studies with phage φ6 suggest several intriguing possibilities for further research. Despite evident biodiversity, species also die out, as is readily seen in fossil records. Robustness could enable organisms to innovate and become more easily adaptable, as shown in our studies. But can robustness also protect a lineage from
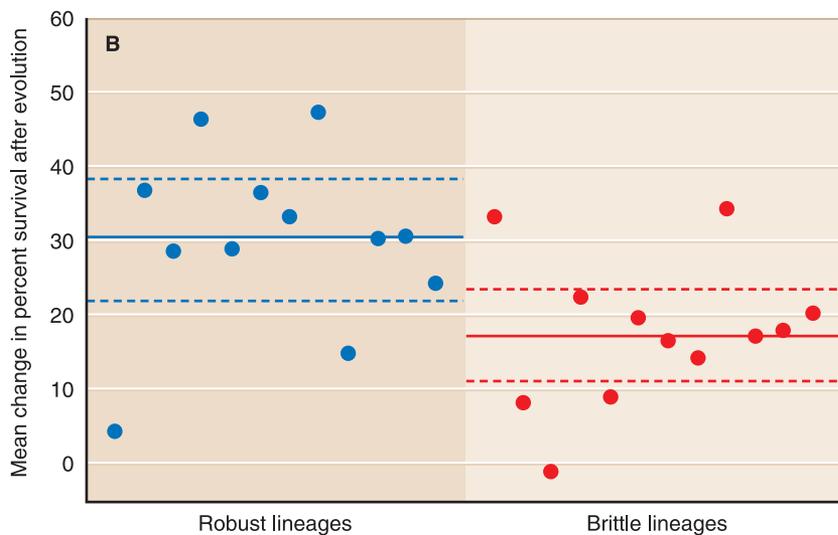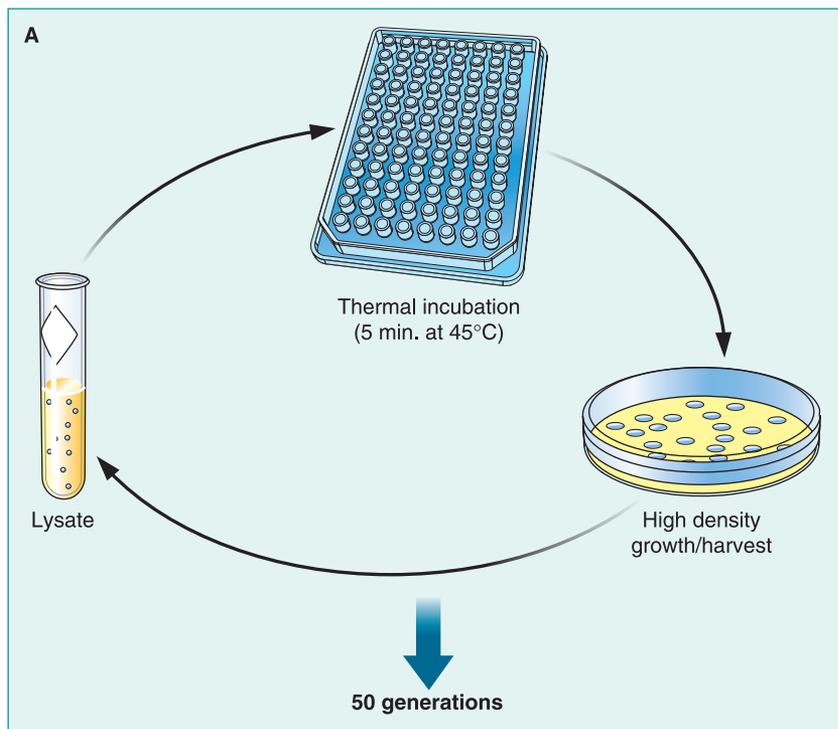
FIGURE 4

(A) Design for an evolution experiment where phage φ6 lineages were selected to withstand damaging effects of heat shock. A phage lysate was exposed to 45°C incubation for 5 minutes, and a dilution of the surviving progeny was plated on a lawn of *P. phaseolicola* bacteria. Overnight plaque formation at 25°C corresponded to 5 generations of phage evolution. The plaques were then harvested, and the process repeated for 10 days, equivalent to 50 phage generations (modified from McBride et al., submitted). (B) Mean change in percent survival after selection with heat shock (45°C) is greater for virus lineages founded by 12 robust strains (blue circles), relative to lineages initiated by 12 brittle strains (red squares). All populations were subjected to 10 days (50 generations) of periodic heat shocks. Solid line is the grand mean for the group, and dashed lines are 95% confidence intervals. (Figure modified from McBride et al., BMC Evol. Biol., in press).

going extinct? Fitness is determined by the relative ability to survive *and* to reproduce. Our viral studies showed that robustness enhanced relative ability for viruses to evolve the ability to enhance their survival. However, whether enhanced survival trades off with reproduction is unknown. That is, a population may evolve to produce a high *quantity* of offspring that survive some environmental stress without producing offspring of high *quality*.

This limitation might pertain to contemporary populations of frogs and other amphibians. Despite producing very large quantities of offspring per generation, amphibians are declining worldwide, perhaps owing to adverse effects of toxins and parasites on average offspring quality. Sustainability of any population is governed by whether its birth rate matches or exceeds its death rate. If robustness enhances both the ability to survive and to reproduce, we might conclude that robustness promotes evolvability and prevents extinction. However, it could be that robustness only enhances survival, so that robust lineages are no better able to avoid extinction than their brittle counterparts. Future research on microbial systems could help to address this issue.

From a disease standpoint, RNA viruses exact deadly tolls in humans, wildlife, and agricultural systems. For example, many of the newly identified infectious diseases in humans are due to zoonotic RNA viruses that have shifted from other animal species into human populations. Antiviral drugs are becoming increasingly important for controlling viruses because vaccines are often unavailable or ineffective in treating RNA virus infections and disease.

Some of these drugs are purposefully designed to elevate the mutation rate of an RNA virus population within the host, perhaps to a level where the population goes extinct because it accumulates too many mutations for individual virions to remain viable. Such mutagens increase mutation rates, causing a decrease in average viral fitness. But these

drugs also produce strong selection for viruses to resist mutational degeneration. One possible mechanism is for viruses to develop increased genetic robustness, which reduces the deleterious effects of drug-imposed mutagenesis. Studies such as ours indicate that robustness is a viral trait that can change through natural selection. More alarmingly, our work suggests that pathogenic viruses may respond by not only evolving greater resistance to current treatments, but a greater potential to adapt to withstand future therapies. Thus, caution is warranted when considering the usefulness of mutagenic therapies.

Finally, by showing that robustness can in-deed evolve and by suggesting that robustness may positively relate to evolvability, our work sheds light on a fundamental tension that exists in explaining how organisms persist in the face of changing conditions. The ability to withstand mutational perturbation while simultaneously adapting to environmental change is an achievement that many researchers deemed implausible. By demonstrating a positive relationship between robustness and evolvability in biological populations, our phage studies show that these seemingly incompatible tasks can be achieved, and establish that evolution itself has the potential to evolve.

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