

The Population and Evolutionary Dynamics of *Vibrio cholerae* and Its Bacteriophage: Conditions for Maintaining Phage-Limited Communities

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ABSTRACT: Although bacteriophage have been reported to be the most abundant organisms on earth, little is known about their contribution to the ecology of natural communities of their host bacteria. Most importantly, what role do these viral parasitoids play in regulating the densities of bacterial populations? To address this question, we use experimental communities of *Vibrio cholerae* and its phage in continuous culture, and we use mathematical models to explore the population dynamic and evolutionary conditions under which phage, rather than resources, will limit the densities of these bacteria. The results of our experiments indicate that single species of bacterial viruses cannot maintain the density of *V. cholerae* populations at levels much lower than that anticipated on the basis of resources alone. On the other hand, as few as two species of phage can maintain these bacteria at densities more than two orders of magnitude lower than the densities of the corresponding phage-free controls for extensive periods. Using mathematical models and short-term experiments, we explore the population dynamic processes responsible for these results. We discuss the implications of this experimental and theoretical study for the population and evolutionary dynamics of natural populations of bacteria and phage.

Keywords: population dynamics, coevolution, bacteria, bacteriophage, continuous cultures, mathematical models.

Introduction

The third chapter of Darwin's *Origin of the Species* is devoted to a question that continues to dominate ecology: what limits the densities of natural populations of organisms? Almost all of the research addressing this question has involved eukaryotes. Are the densities of natural populations of animals and plants limited by the nutrients and resources that they consume or by the organisms that consume them (herbivores, predators, parasitoids, and para-

sites) or are their densities unregulated, such that they simply wax and wane with season and climate (Hairston et al. 1960)? In this article, we address this question for prokaryotic populations, bacteria, and their viral parasitoids, bacteriophage, which are the most abundant organisms on earth (Weinbauer 2004; Suttle 2005). Under what conditions will bacteriophage, rather than resources, limit the population densities of their host bacteria?

In theory, there are broad conditions where lytic bacteriophage can limit the population densities of their host bacteria (Levin et al. 1977). Experimentally, when low numbers of *Escherichia coli* strain B and the lytic phage T7 were mixed in continuous culture, resources remained abundant, and the phage limited the densities of the bacteria for extensive periods (Chao et al. 1977). In these continuous cultures, the densities of the bacterial population remained two or three orders of magnitude lower than in phage-free control cultures. However, in this study, as well as in all other studies that we know of that have addressed this question (Levin et al. 1977; Middelboe 2000; Mizoguchi et al. 2003; Wei et al. 2010), mutant bacteria resistant to the phage eventually emerged and ascended to dominance. This ascent of resistant bacteria is particularly rapid when the initial number of bacteria is great and phage-resistant mutants are, as a result, already present. Once resistant bacteria increased, resources, rather than the viral parasitoids, limited the bacterial population.

The ascent of phage-resistant mutant bacteria need not be the sole evolutionary process. There can be and often is a coevolutionary arms race with resistant mutants among the bacteria countered by host-range mutant phage that are capable of replicating on the resistant bacteria. Even with single bacteria and phage species, this arms race can promote considerable diversity in both populations (Weitz et al. 2005; Forde et al. 2008). However, in most if not all cases, these arms races between viruses and bac-

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teria terminate with the ascendancy of resistant bacteria for which the phage are unable to generate host-range mutants (Levin et al. 1977; Lenski and Levin 1985; Wei et al. 2010). In theory, the adaptive immune system known as clustered regularly interspaced short palindromic repeats, or CRISPR (Jansen et al. 2002; Barrangou et al. 2007), could promote an almost indefinite arms race between bacterial immunity and host-range mutations in the phage (Levin 2010; Vale and Little 2010). In practice, however, we are aware of only two studies that report an extended genetic arms race between bacteria and phage (Buckling and Rainey 2002; Gomez and Buckling 2011), and it is unclear whether the phage, rather than resources, limited the bacterial population in those investigations.

On first consideration, it may seem that, with multiple phages, each with different receptors and therefore requiring independent resistance mutations, it would be possible for the viruses to continue to limit the densities of the bacteria. That is the strategy employed to prevent multiple resistance to chemotherapeutic agents in long-term infections, such as HIV infection (Hammer et al. 1997) and tuberculosis (Onyebujoh et al. 2005). However, the results of the only experimental test of this multiple-phage hypothesis of which we are aware did not support it (Korona and Levin 1993). In that investigation, three phages that use different receptors were introduced into cultures of susceptible bacteria bearing a restriction-modification system to which all three phages were susceptible. Within 48 h, bacterial mutants with envelope resistance to all three phage ascended to dominance, and the bacterial population went from a phage-limited to a resource-limited state.

Here we present the results of an experimental study of the population and evolutionary dynamics of *V. cholerae* N16961 and two of its phages and computer simulations of these processes. The results of these experiments and associated modeling support the hypothesis that, when two or more phages are present, there are conditions in which viruses can limit densities of bacterial populations for extended periods of time. We discuss the implications of these experimental and theoretical results for natural populations of phage and how the predictions made from in vitro investigation can be tested in natural populations of bacteria and phage.

Material and Methods

Bacteria and Phage

The *Vibrio cholerae* used in this study were derived from El Tor O1 N16961, a spontaneous streptomycin-resistant mutant of a clinical isolate from Bangladesh. John J. Mekalanos and Shah Faruque generously provided these bacteria. A spontaneous rifampicin-resistant mutant of

N16961 was used as the marked strain for fitness studies. John Mekalanos also provided lysates of a cholera bacteriophage, designated Peru-2, isolated from a natural population.

Media, Culture, and Sampling Procedures

Liquid cultures of *V. cholerae* were maintained in either full-concentration Luria-Bertani broth (LB) or a medium composed of autoclaved 5% LB and 95% tap water at 30°C. Long-term population dynamics of N16961 and phage were performed in a continuous culture system of the homemade design first presented in the appendix of Levin et al. (1977). For a modern version, see <http://www.ecfl.net>. The 5% LB tap water medium was used for these continuous culture populations and batch culture experiments estimating fitness.

The density of *V. cholerae* N16961 was estimated from colony count data, expressed as colony-forming units (CFU) on LB agar. The density of phage was estimated from plaque counts, expressed as plaque-forming units (PFU) on LB agar overlaid with 3-mL soft-agar lawns of N16961. Unless otherwise noted, estimates of phage titers from these continuous cultures were made from samples taken directly from the culture vessel. In a few cases, a drop of chloroform was added to samples to distinguish free phage from infected cells. No difference was found between the estimated phage titers with and without chloroform (data not shown). We interpret this to mean that the majority of phage present in the culture were free virus particles, rather than particles adsorbed to or within cells.

Assay for Phage Resistance

Resistance of N16961 to phage was determined in two ways. First, by spotting $10\text{--}20 \times 10^9$ PFU/mL of the phage lysates on 500- μ L soft-agar lawns on 100-mm-diameter petri dishes containing LB agar (four lawns per plate). Isolates were determined to be resistant if no clearing was evident. Resistant isolates were confirmed by a liquid phage growth assay. Approximately 10^5 PFU was added to exponentially growing cultures of bacteria in 5% LB. After 8–10 h, the densities of phage were estimated by serial dilution and plating on N16961 lawns. If there was no increase in phage density, the bacteria were deemed to be resistant. For bacterial samples taken from the continuous cultures, colonies were passaged on LB agar before testing to minimize the likelihood of contamination with coexisting phage.

Assay for Lysogeny

Samples were taken from the middle of the plaques, where phage densities were high, and were streaked for single colonies on LB agar. After three colony-to-colony passages, single colonies were grown in 5% LB in 12-well plates at 30°C. When their optical density was approximately half of the maximum, the cultures were exposed to UV (long-wave UV-366 nm) at a distance of 5–10 cm for 45 s to induce any prophage and then allowed to grow overnight. A drop of chloroform was added, the cell debris was removed by centrifugation, and the cleared supernatant was spotted onto lawns of susceptible cells. In this assay, we assume that, if the cells from the center of the plaques were lysogens for the phage, there would be free phage in the UV-induced culture and plaques would be seen on susceptible cells (Lwoff 1953, 1966).

Relative Malthusian Fitness of Phage-Resistant *V. cholerae* N16961

Pairwise competition experiments were performed to estimate the Malthusian fitness of phage-resistant bacteria relative to susceptible bacteria. As a marker for the susceptible cell line, we used a spontaneous rifampicin-resistant mutant of the wild-type N16961, designated Rif.R. Approximately equal frequencies of the phage-resistant strains and the Rif.R common competitor from overnight cultures were mixed 1 : 100 in 5% LB, were cultured at 30°C, and their densities were estimated from colony count data at two time points. The Malthusian selection coefficient s was calculated using the formula

$$s = \frac{[\ln(W_t) - \ln(W_0)]}{\ln\left(\frac{N_t}{N_0}\right)},$$

where W_t and W_0 are the ratios of the densities of the phage-resistant isolates to Rif.R at time t and the initial time 0, respectively, and N_t and N_0 are the total densities of bacteria at those two times (Travisano and Lenski 1996). The relative fitness of each individual phage-resistant mutant to Rif.R was calculated as $1/(1 - s)$. As a control for the fitness cost of the rifampicin marker, pairwise competition experiments were performed with Rif.R and wild-type N16961 from phage-free continuous cultures isolated at the same time as the phage-resistant mutants.

Fitness of the Phage

To estimate the growth of specific phage on N16961, we used a method similar to that employed by Wichman et al. (2005). The bacteria were grown in 12-well microtiter plates in 2 mL 5% LB at 30°C to a density of approximately

10^7 CFU/mL. Between 10^3 and 10^4 PFU/mL of phage was added to these agitated, exponentially growing bacterial cultures. The phage densities were estimated immediately (P_0) and after one hour of incubation (P_1). As our estimate of the fitness of the phage, we use the doubling time during that period, $T_2 = \log(P_1) - \log(P_0)$.

Results

The Original Observations

The motivation for this study and the questions we address in the following sections were somewhat serendipitous. While performing continuous-culture experiments with mixtures of *Vibrio cholerae* N16961 and a phage designated Peru-2, we observed the long-term (750 h, ~1,200 cell generations) persistence of a phage-limited bacterial population. Compared with the phage-free controls, the bacterial densities in the presence of phage were nearly two orders of magnitude lower ($\sim 2 \times 10^6$ rather than 2×10^8 CFU/mL), and the cultures remained clear for the period of the experiment, which indicated an abundance of unconsumed resources. The viral population was also maintained throughout the experiment, with average densities exceeding that of the bacteria (see app. A, available online).

Although there was no evidence for changes in the morphology of the bacterial colonies, there were two distinct types of phage plaques, big (designated B) and turbid (designated T). Based on restriction-fragment analysis, the B and T phages identified in our original experiments are genetically different from each other and therefore likely to be different species (see app. A). Qualitatively and quantitatively similar results were obtained in a replicate experiment in which B and T phages were tracked. Both phages were maintained during the course of the experiment (see app. A).

We interpret the results of these first experiments as evidence for the existence of conditions in which densities of bacterial populations can be limited for extensive amounts of time by bacteriophage, rather than by resources. The remainder of this report is a jointly experimental and theoretical quest to elucidate and understand the population dynamic and evolutionary conditions required for phage to limit bacterial populations.

Single-Phage Dynamics

Are two species of phage necessary to maintain the bacteria in a phage-limited state? To address this question, we introduced pure single-plaque lysates of B and T phages into continuous cultures with established populations of N16961.

The results of these experiments with the B phage are presented in figure 1. Shortly after the introduction of these viruses, the density of bacteria rapidly decreased while that of the phage increased. By the second day, however, the bacterial population recovered. After this recovery, the bacterial density of the cultures with phage was the same as that of the phage-free control cultures. Based on the average harmonic means (fig. 1E), the estimated viable cell densities were somewhat lower than those for the control cultures but were substantially greater than those for the phage-bearing cultures observed in our preliminary experiments. In all of these cultures, the phage continued to maintain their population throughout the course of the experiment.

High cell densities in the presence of high phage densities suggested that phage resistance had evolved in the cells, and this was confirmed with a spot assay. The majority of single clones of bacteria isolated after, but not before, the initial population recovery appeared to be resistant to the coexisting phage. The resistance was confirmed in a liquid culture assay; the B phage do not replicate on the bacteria deemed resistant according to the spot assay. These B phage-resistant cells continued to dominate the bacterial population for the duration of the experiments. There was no evidence of morphological variation in the plaques produced by the dominant population of phage sampled. We assume that these phage are maintained by replicating on a minority population of susceptible cells (Chao et al. 1977; Levin et al. 1977), but we were unable to isolate this subpopulation because of the absence of selectable markers in these experiments. Although we cannot exclude the possibility that there are minority populations of host-range phage mutants capable of growing on the resistant bacteria, we were not able to isolate such mutants on lawns of bacteria that were resistant to the B phage. On the other hand, it was clear that the B phage populations evolved during the course of these experiments. The rate of replication of phage from later samples on wild-type, susceptible N16961 exceeded that of the ancestral B lysates (fig. 1F). This evolution of higher fitness for the B phage was not restricted to continuous-culture experiments and was observed within 30 h in batch culture.

In figure 2, we present the results of parallel experiments with the T phage introduced into established populations of susceptible N16961. The initial decrease in the density of bacteria after the introduction of the T phage was less than that noted for B viruses. In four of the five independent replicas, the density of the bacteria after the initial recovery approached the density of the bacteria of the corresponding phage-free control cultures, which is consistent with a resource-limited state. However, in figure 2C, the bacterial density in the culture with phage re-

mained substantially less than that in the control culture for the duration of the experiment. Overall, the average harmonic mean of the estimated density of bacteria in the five cultures with phage was only slightly less than that in the control cultures (fig. 2D) and was some two orders of magnitude greater than that in the experiments with the original mixed Peru-2 lysate (see fig. A1A,B). In all four cultures, the phage continued to maintain their population for the duration of the experiments.

In all of the T phage cultures, bacteria resistant to the ancestral and coexisting phage were identified by spot assay. In contrast with the findings of the B phage experiments depicted in figure 1, the resistant cell population was not always the dominant population. For example, at 417 h, the fraction of isolates resistant to ancestral and coexisting phage in figure 2B and 2D was 9/10 and 5/10, respectively. At 525 h, the resistant fractions were 5/10, 8/10, and 4/10 for figure 2A, 2B, and 2D, respectively. We failed to detect cells resistant to the ancestral or coexisting T phage in 10 isolates obtained at 417 h from the culture in figure 2C. We were also unable to isolate host-range T phage mutants on lawns of resistant bacteria.

Independent Resistance Mechanisms

At this juncture, the B and T *V. cholerae* phages are uncharacterized; we do not know the sites to which they adsorb, nor do we have information about the physiology or molecular biology of the infection process. What is clear from the present results and is most important to this ecological/evolutionary story is that resistance to these phages by N16961 is through separate mechanisms. B phage replicate on cells that are resistant to T phages, and T phages replicate on *V. cholerae* that are resistant to B phages (data not shown).

Two-Phage Dynamics

We interpret the results of these experiments with T and B phages as support for the hypothesis that a single species of phage cannot limit the densities of bacterial populations for extensive periods of time. Bacteria resistant to the phage emerge and increase to densities similar to that found in phage-free cultures; resources, rather than viruses, limit the bacteria in these cultures. Can two species of phage for which resistance is through separate mechanisms (i.e., for which there is no cross resistance) limit the bacterial densities for extensive periods of time?

To answer this question, we introduced mixtures of pure lysates of B and T phages into established continuous cultures of N16961. The results of three independent two-phage experiments are presented in figure 3. Soon after the introduction of the two-phage cocktail, the cultures

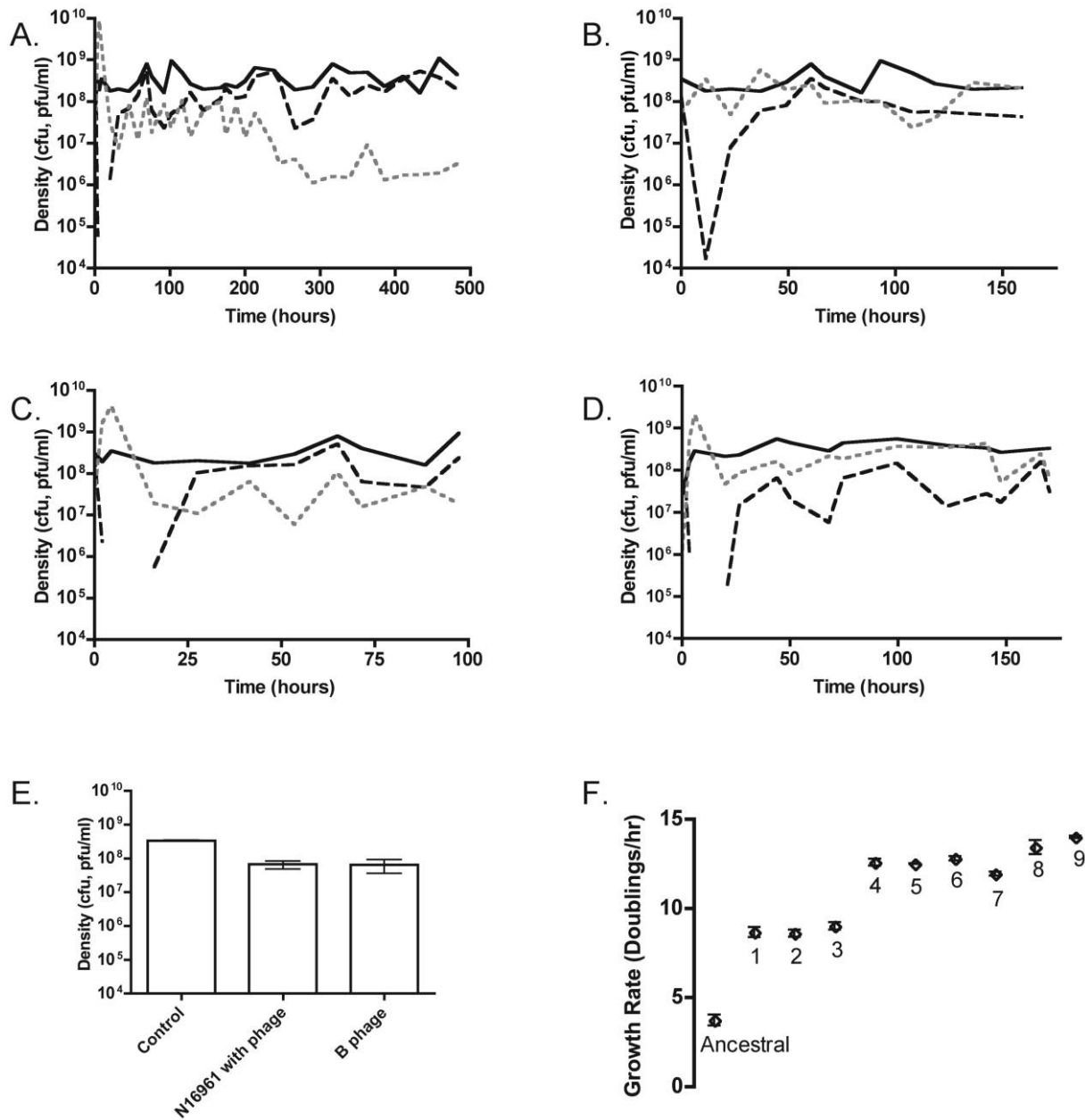


Figure 1: Population dynamics and evolution of big (B) phage and *Vibrio cholerae* N16961 in continuous culture. *A–D*, Changes in the densities of bacteria and phage, showing the estimated density of bacteria in phage-free control cultures (solid black line), cultures with phage (dashed black line), and phage (dotted gray line). Dilution rates (w) in *A*, *B*, *C*, and *D* are $0.3\text{--}0.4\text{ h}^{-1}$, $0.5\text{--}0.6\text{ h}^{-1}$, $0.3\text{--}0.4\text{ h}^{-1}$, and $0.5\text{--}0.6\text{ h}^{-1}$, respectively. *E*, Average harmonic mean of cell or phage densities of *A–D*, calculated from 50 h to termination of the experiments, with standard errors. *F*, Mean doublings per hour of B phage derivatives grown in N16961. Points 1, 2, and 3 show lysates from 3 separate continuous cultures at approximately 30 h; 4, 5, 6, and 7 show single-plaque isolates from 2 separate batch cultures at approximately 50 h; 8 and 9 show single-plaque isolates from 2 separate continuous cultures at approximately 24 h. Bars represent the standard error. CFU = colony-forming units; PFU = plaque-forming units.

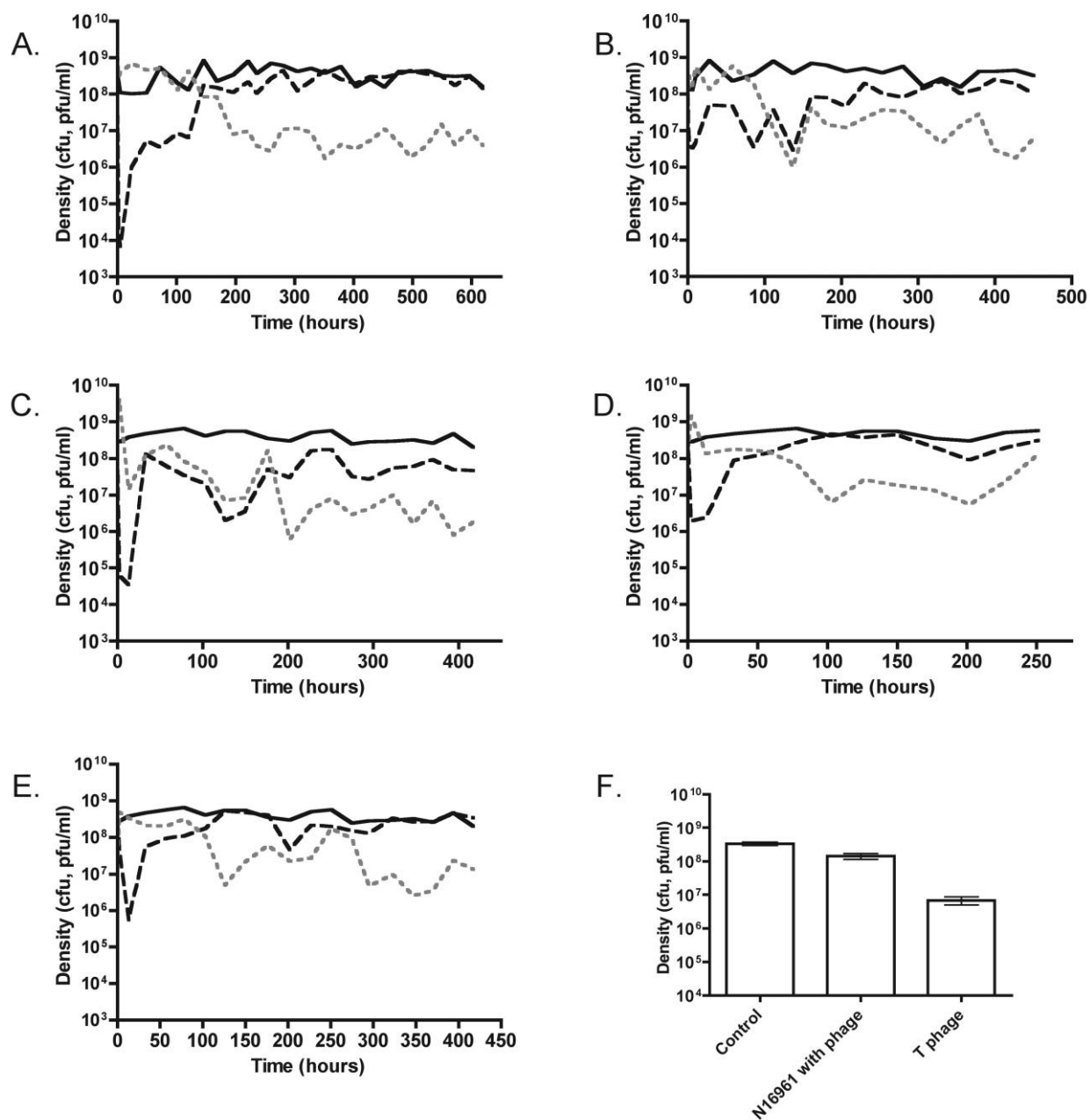


Figure 2: Population dynamics and evolution of turbid (T) phage and *Vibrio cholerae* N16961 in continuous culture. A–E, Changes in the densities of bacteria and phage, showing the estimated density of bacteria in phage-free control cultures (solid black line), cultures with phage (dashed black line), and phage (dotted gray line). Dilution rates (w) in A, B, C, D, and E are $0.36\text{--}0.44\text{ h}^{-1}$, $0.2\text{--}0.3\text{ h}^{-1}$, $0.2\text{--}0.3\text{ h}^{-1}$, $0.2\text{--}0.3\text{ h}^{-1}$, and $0.5\text{--}0.6\text{ h}^{-1}$, respectively. F, Average harmonic mean of cell or phage densities for the five (A–E) independent experiments, calculated from 100 h to the termination of the experiment, with standard errors. CFU = colony-forming units; PFU = plaque-forming units.

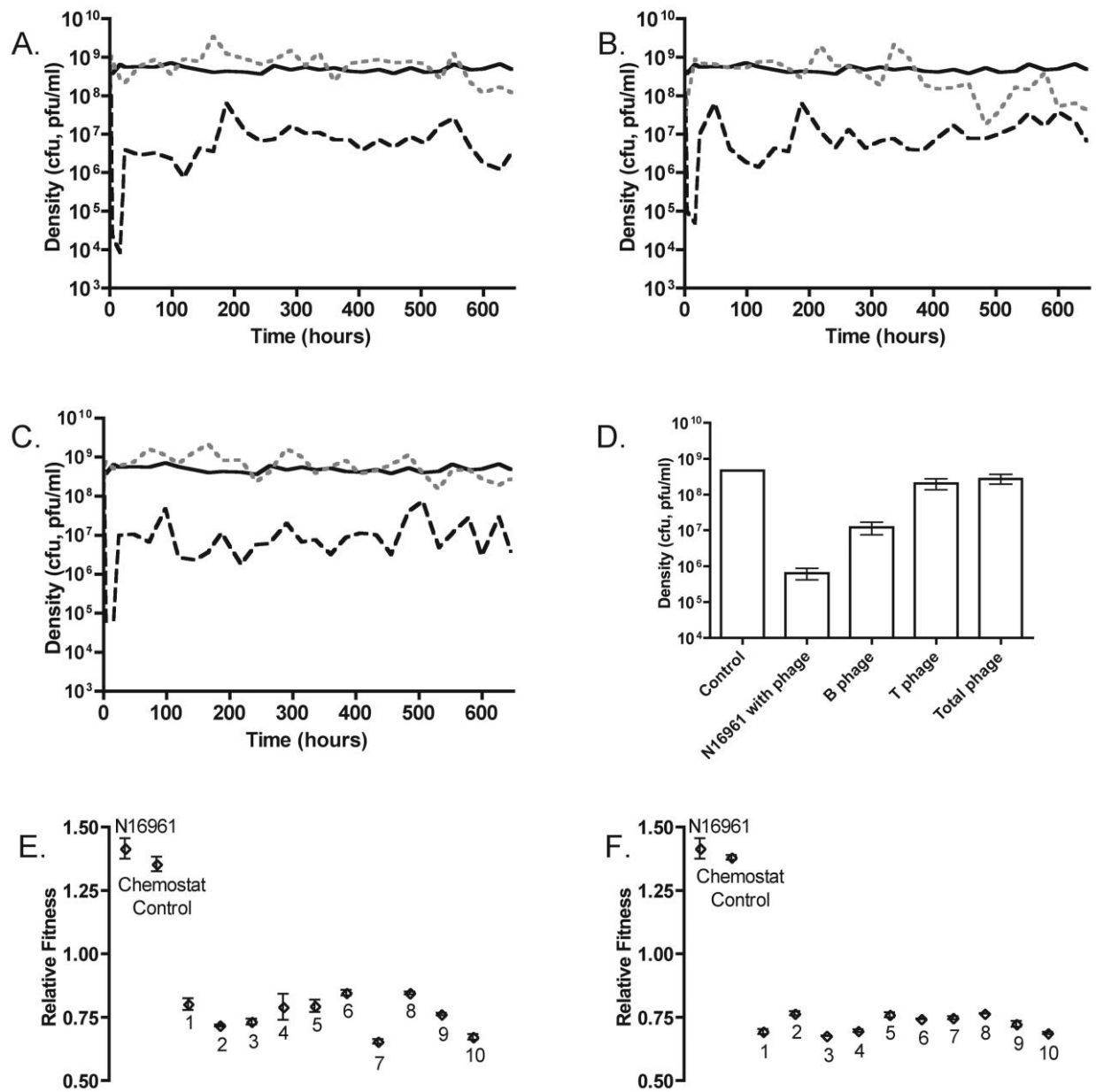


Figure 3: Population dynamics of N16961 with a mixture of turbid (T) and big (B) phage in continuous culture. A–C, Changes in the density of bacteria and phage, showing the estimated density of bacteria in phage-free control cultures (solid black line), cultures with phage (dashed black line), and phage (dotted gray line). Dilution rates in A, B, and C are 0.29–0.35 h⁻¹, 0.44–0.52 h⁻¹, 0.36–0.44 h⁻¹, respectively. D, Average harmonic mean of cell or phage densities from A–C, calculated from 100 h to the termination of the experiment with standard errors. E and F, Means and standard errors of the relative fitness of the doubly (B and T) resistant bacteria, compared with a susceptible rifampicin-resistant control strain. E, Isolates from approximately 500 h. F, Isolates from approximately 650 h. CFU = colony-forming units; PFU = plaque-forming units.

became clear, and they remained that way for the duration of the experiments, which is consistent with an abundance of available resources. The bacterial densities leveled off and remained 2–3 logs lower than those of the phage-free controls. In all three two-phage experiments, both the B and T phages were present at substantial densities throughout the course of the experiment. As the experiments proceeded, however, the relative density of T phage increased. This change corresponded with the increase in the density of cells resistant to B phage, which soon dominated the bacterial population. Bacteria resistant to the T phage were detected in only one of the three experiments, shown in figure 3C. In this experiment, T phage resistance was detected simultaneously with resistance to the B phage at approximately 500 h. Although we were able to isolate cells that were resistant to both B and T phage in these cultures, these remained a minority population of bacteria.

Why did those bacteria resistant to both the T and B phages not ascend to dominance? One possibility is that they had a very large fitness cost relative to the single phage-resistant cells and the susceptible cells. To test this hypothesis, we performed pairwise competition experiments with 20 mutants that were resistant to both the B and T phages randomly chosen from the third continuous culture (fig. 3C); 10 from ~500 h, and 10 from the end of the experiment. As can be seen from figure 3E and 3F, all of the doubly resistant isolates were substantially less fit than the reference strain, Rif.R, which itself is approximately 20% less fit than the wild-type N16961. The average relative fitness of these doubly resistant isolates is approximately 55% of the wild-type N16961.

Accounting for the Dynamics and Stability

We interpret the results of the experiments presented in figure 3 as support for the hypothesis that, collectively, multiple species of phage can limit the densities of their host bacterial population. This, however, accounts for only part of what we need to understand about the role of phage in limiting the densities of bacteria. These single-phage and two-phage experiments raise a number of issues about the dynamics of the phage-bacteria interaction and their apparent stability, which we now address experimentally and with mathematical models.

Lysogeny

How did the susceptible bacteria in the figure 2 experiments maintain their population in the presence of the T phage without a dominant, phage-resistant population? One possible explanation is that the bacteria exposed to the phage acquired phenotypic resistance to killing by these viruses but still produced phage. This is what would be

anticipated for a temperate, rather than lytic, life cycle, and the phenomenon of lysogeny (Lwoff 1953; Stewart and Levin 1984). In the case of a classical temperate phage, the bacteria would acquire the phage genome as a prophage and would appear resistant when confronted with modest densities of that phage (Barksdale and Arden 1974). This could also account for the turbid nature of the T phage plaques; immune lysogens would form small colonies within the plaques.

At one level, the results of our experiments are consistent with the lysogeny hypothesis for the T phage. We estimated the density of viable bacteria within the plaques and from phage-free areas of the lawn. There were substantial densities of viable cells in both sites: $4.02 \pm 0.15 \times 10^7$ CFU/mL within plaques and $7.47 \pm 0.34 \times 10^8$ CFU/mL in the lawn. The density of the phage within the plaques was $2.35 \pm 0.17 \times 10^9$ PFU/mL. However, when tested with the spot assay, the bacteria recovered from these plaques appeared to be susceptible to the T phage. Additionally, the classical lysogeny assay described in “Materials and Methods” was negative for the bacteria recovered from these plaques, with no evidence for induction of free phage. Based on these results, we conclude that the T phage is not temperate, at least not by the classical definition.

The Stability

As noted in Levin et al. (1977), with bacterial growth and phage infection parameters (adsorption rates, burst sizes, and latent periods) in realistic ranges, the densities of susceptible bacteria and phage in continuous cultures would be anticipated to oscillate with increasing amplitude. Within short order, either the susceptible bacteria or the phage would be lost. This was not observed in these experiments. These communities of bacteria and lytic phage are substantially more stable than was anticipated by simple mass-action models. The results of our modeling and experiments suggest two mechanisms of phenotypic resistance that contribute to the stability of these communities: persistence and wall populations.

Persistence in Batch Culture

Although not sufficient to explain the recovery of a high-density population of phage-susceptible bacteria, the presence of a subpopulation of nongrowing, planktonic cells that are refractory to phage killing could play a role in these dynamics. Such subpopulations have long been known for antibiotic-treated cultures (Bigger 1944; Balaban et al. 2004; Wiuff et al. 2005), and there is evidence that bacteria of this persist state are refractory to phage (Pearl et al. 2008). Were this the case, when an exponen-

tially growing population of N16961 is exposed to lytic phage, killing would not be complete, the densities of viable cells would level off, and a substantial fraction of the bacteria would survive. To illustrate this hypothesis and provide a framework to test it, we use a mathematical model of the population dynamics of the bacteria and phage with a persistent subpopulation.

In this model, there are two bacterial populations, susceptible cells (N) and persister cells (X), and one phage population (P), where N , X , and P are densities (cells or particles per mL) as well as the designations of these populations. The persister subpopulation (Bigger 1944; Balaban et al. 2004; Pearl et al. 2008) is derived from N and returns to the N state but does not divide. The switch between N and X states occurs at rates proportional to the resource concentration and coefficients nx and xn , respectively. The susceptible bacteria, N , grow at a rate proportional to the concentration of a limiting resource, R (mg/mL) via a Monod function (Monod 1949)

$$\psi(R) = \frac{\nu R}{(R - k)},$$

where ν (h^{-1}) is the maximum rate of replication and k (mg/mL) is the concentration of the resource where the growth rate is half of its maximum. As in Levin (2010), we assume that the resource concentration, which determines rate of cell division, is a measure of the physiological state of the bacteria.

Phage infection is assumed to be dependent on the physiological state of the bacteria. Thus, as the concentration of the resources decreases, the rate of adsorption δ and burst size β of the phage also decreases.

$$\delta(R) = (1 - x)\delta_{\max} + x\delta_{\max} \frac{R}{R + k},$$

$$\beta(R) = (1 - x)\beta_{\max} + x\beta_{\max} \frac{R}{R + k},$$

where x ($0 \leq x \leq 1$) is a coefficient to denote the magnitude of the resource effect on these phage infection parameters and δ_{\max} and β_{\max} are the maximum values of the adsorption rate parameter and burst size, respectively.

There is a latent period L (s) between the time of infection and the burst. At any given time t , there are M phage-infected cells per milliliter that burst and produce $\beta(R)$ phage when time is equal to $t + L$. With these definitions and assumptions, the rates of change in the density of bacteria and phage and the concentration of the resource at time t are given by

$$\frac{dR}{dt} = -\psi(R)Ne,$$

$$\frac{dN}{dt} = \psi(R)N(t) - \delta(R)NP - \frac{R}{R + k}[nxN - xnX],$$

$$\frac{dM}{dt} = \delta(R)NP - M(t - L),$$

$$\frac{dX}{dt} = \frac{R}{R + k}[-nxN + xnX],$$

$$\frac{dP}{dt} = M\beta(R) - \delta(R)NP,$$

where $M(t - L)$ is the density of phage-infected cells L time units earlier and e is the conversion efficiency parameter (Levin et al. 1977), which is the amount of resource needed to produce a new bacterium.

To analyze the properties of this and the following models, we use numerical solutions to these differential equations with parameters in a range similar to those estimated for *Escherichia coli* and its lytic phages, T2 and T7, and *V. cholerae* and its phage, JSF4 (Levin et al. 1977; Wei et al. 2010). These simulations were programmed in Berkeley Madonna and are available at <http://www.eclf.net/programs>.

In figure 4A and 4B, we present the results of these simulations with different initial ratios of phage and bacteria (multiplicities of infection; MOIs). In the absence of phage, the bacteria grow to and maintain a density that is limited by the amount of resource available. In the presence of phage, but without persisters, the bacterial population initially increases but then dies off as the phage population increases (no persisters, MOI $1\text{E-}4$). However, with persisters present at an initial frequency of 1×10^{-4} , the bacteria do not die off completely. Instead, the bacterial density levels off, and the bacterial population is maintained despite high densities of phage ($1\text{E}4$). The density at which the bacterial population levels off is inversely proportional to the MOI, which is a testable hypothesis. The density of surviving bacteria and the rate of killing should be proportional to the initial ratio of phage to bacteria.

To test this persister hypothesis, we followed the changes in phage and bacterial density in batch culture with different initial MOIs (fig. 4C, 4D). All of the phage-treated cultures showed a significant decrease in viable cell density followed by a leveling off in the density of phage recovered. Even with >100 phage particles to each cell, at least 10^4 bacteria survived. We interpret the results of this experiment to be consistent with the presence of persisters, a subpopulation of bacteria that are phenotypically refractory to the T phage.

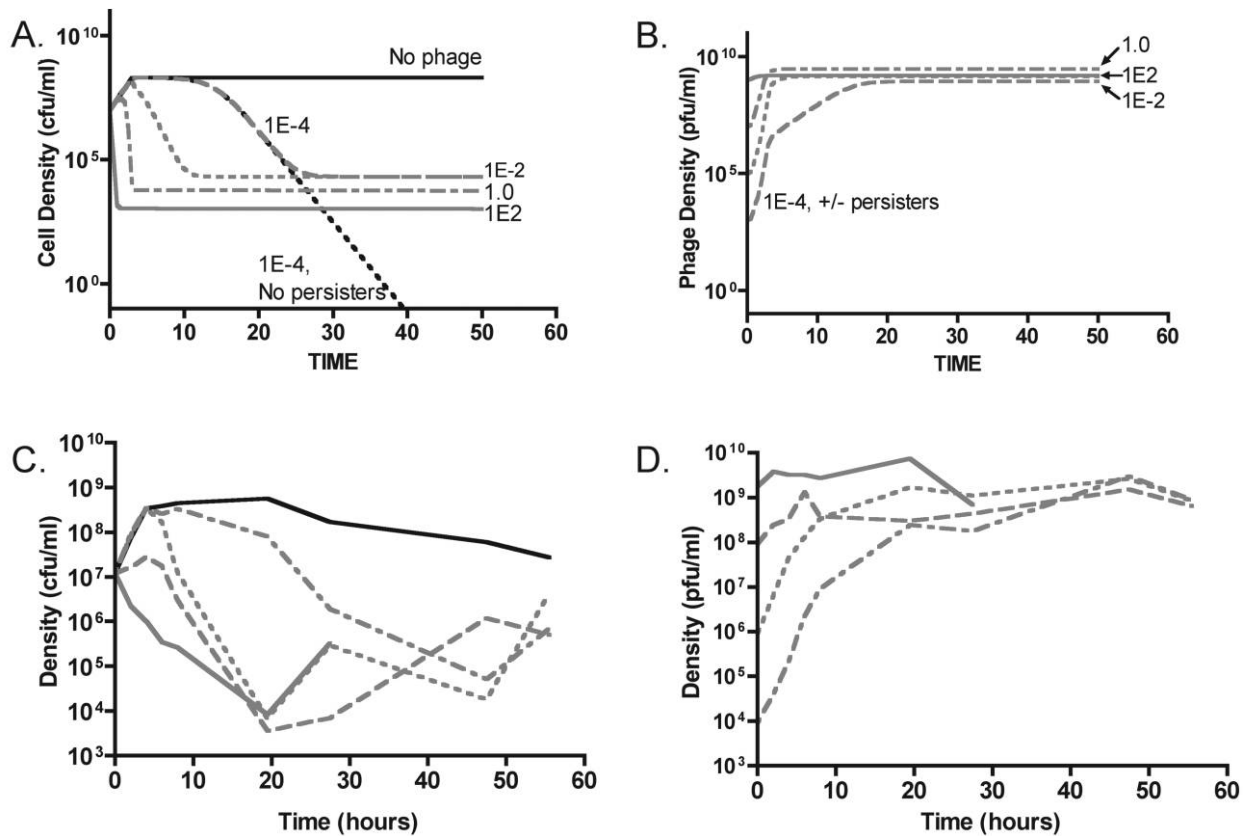


Figure 4: Role of persisters in the population dynamics of turbid (T) phage and N16961. Changes in the density of bacteria and phage in batch culture. *A* and *B*, Results of computer simulations. *A*, Bacteria. *B*, Corresponding populations of phage. Parameter values, $\nu = 1.0$, $k = 0.25$, $e = 5E-7$, $\delta_{\text{MAX}} = 1E-8$, $\beta_{\text{MAX}} = 50$, $x = 0.9$, $L = 0.2$, $nx = 1E-4$, $xn = 1E-3$, initial $R = 100$, initial $N = 1E7$, and initial $X = 1E3$ (in the simulation without persisters, initial $X = 0$ and $nx = xn = 0$). *C* and *D*, Experiments with T phage and susceptible N16961 in batch 5% Luria-Bertani broth culture. *C*, Changes in the density of bacteria. *D*, Corresponding changes in the density of phage. The solid black line represents the phage-free control cultures, the dashed and dotted gray line represents a multiplicity of infection (MOI) of $7.5E-4$, the dotted gray line represents an MOI of $7.5E-2$, the dashed gray line represents an MOI of 7.5 , and the solid gray line represents an MOI of $1.5E2$. CFU = colony-forming units; PFU = plaque-forming units.

Phage-Refractory Wall Populations

The other process contributing to the relative stability of the phage-bacteria association in these continuous cultures are the bacteria adhering to the walls of the culture vessels. Although genetically susceptible, bacteria in biofilms, such as those adhering to the walls of the culture vessels, are phenotypically refractory to phage (Cerca et al. 2007). As demonstrated both theoretically and experimentally by Schrag and Mittler (1996), the continuous migration of cells to and from these wall populations prevents the loss of susceptible bacteria and stabilizes the phage-bacterial community. In appendix A, we illustrate this effect and consider the combined effect of persistence and a wall population.

Two-Phase Dynamics

To better understand the processes contributing to the phage-limited dynamics in our two-phage experiments, we extended the above model for the two-phage community. In this incarnation of the model, there are two populations of phage with densities P_1 and P_2 and four populations of planktonic, nonpersister bacteria, defined as follows: N , susceptible to both phage; N_1 , resistant to phage 1; N_2 , resistant to phage 2; and N_{12} , resistant to both phages. For convenience, we assume there is no latent period and that the rates of conversion of the bacteria to the corresponding persistent and wall population states are the same: nx , xn , nw , and wn . Although the rate of resource uptake is the same, we allow for differences in the maximum growth rates of the bacteria, ν , νn_1 , νn_2 , and νn_{12} ,

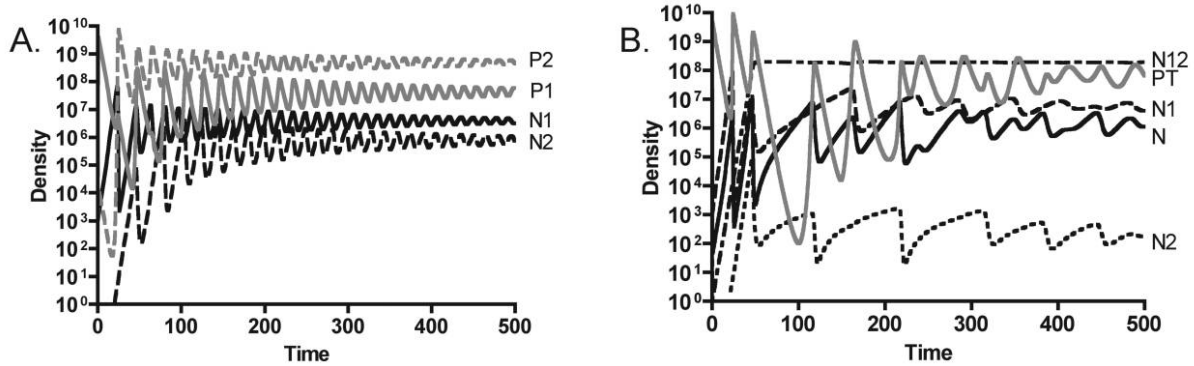


Figure 5: Model of two-phage dynamics in continuous culture. Changes in the densities of planktonic bacteria resistant to single phages and to both phages (N_1 , N_2 , and N_{12}), phage P_1 and P_2 , and total phage (PT) predicted from computer simulations. Standard parameters were as follows: $\nu = 1.0$, $k = 0.25$, $e = 5 \times 10^{-7}$, $\delta_{\max 1} = 1E-8$, $\delta_{\max 2} = 1E-9$, $\beta_{\max 1} = 50$, $\beta_{\max 2} = 100$, $x = 0.5$, $w = 0.4$, $ww = 0.01$, $C = 100$, $nx = xn = 0.0001$, $nw = 0.01$, and $wn = 0.005$. A, No doubly resistant bacteria, $\nu_1 = 0.9$, $\nu_2 = 0.85$. B, Doubly resistant bacteria present, $\nu_1 = 0.9$, $\nu_2 = 0.85$, $\nu_{12} = 0.80$, initial $N_{12} = 1$.

for the N , N_1 , N_2 , and N_{12} populations, respectively. With these definitions and assumptions and those of the preceding incarnations of this model, the rates of change in the densities of the bacterial and phage populations and concentration of the limiting resource are given by

$$\begin{aligned} \frac{dR}{dt} &= w(C - R) - \frac{R}{k + R}e(\nu N \\ &\quad + \nu_1 N_1 + \nu_2 N_2 + \nu_{12} N_{12}), \\ \frac{dN}{dt} &= \psi(R)N - \delta_1(R)NP_1 - \delta_2(R)NP_2 - \frac{R}{R + k}[nxN \\ &\quad - xnX] - nwN + wnNW - wN, \\ \frac{dN_1}{dt} &= \psi_1(R)N_1 - \delta_1(R)N_1P_1 - \delta_2(R)N_1P_2 - \frac{R}{R + k}[nxN_1 \\ &\quad - xnX_1] - nwN_1 + wnNW_1 - wN_1, \\ \frac{dN_2}{dt} &= \psi_2(R)N_2 - \delta_1(R)N_2P_1 - \delta_2(R)N_2P_2 - \frac{R}{R + k}[nxN_2 \\ &\quad - xnX_2] - nwN_2 + wnN_2 - wN_2, \\ \frac{dN_{12}}{dt} &= \psi_{12}(R)N_{12} - wN_{12}, \\ \frac{dNW}{dt} &= nwN - wnNW - wwN, \\ \frac{dNW_1}{dt} &= nwNW_1 - wnNW_1 - wwN_1, \end{aligned}$$

$$\frac{dNW_2}{dt} = nwN_2 - wnNW_2 - wwNW_2,$$

$$\frac{dX}{dt} = \frac{R}{R + k}[nxN - xnX] - wX,$$

$$\frac{dX_1}{dt} = \frac{R}{R + k}[nxN_1 - xnX_1] - wX_1,$$

$$\frac{dX_2}{dt} = \frac{R}{R + k}[nxN_2 - xnX_2] - wX_2,$$

$$\frac{dP_1}{dt} = P_1(\delta_1(R)(N + N_2) + \delta_1(R)N_2(\beta_1(R) - 1)) - wP_1,$$

$$\frac{dP_2}{dt} = P_2(\delta_2(R)(N + N_1) + \delta_2(R)N_1(\beta_2(R) - 1)) - wP_2,$$

where the growth rates of each of the bacterial populations, $\psi_x(R)$, are the product of their respective maximum growth rates, ν , ν_1 , ν_2 , and ν_{12} , and the resource function, $R/(k + R)$.

In figure 5, we follow the simulated changes in the densities of the bacteria and phage populations for two situations: where there are no bacteria resistant to both phage ($N_{12} = 0$; fig. 5A) and where there are doubly resistant bacteria (fig. 5B). In the absence of double resistance, both singly resistant populations, N_1 and N_2 , are maintained in a phage-limited state, despite the difference in fitness and phage susceptibility of the bacteria. However, the population susceptible to both phage, N , is eliminated (data not shown). Both phage species coexist in an apparently

stable state as well (fig. 5A). In the presence of doubly resistant bacteria, the phage density oscillates with decreasing amplitude as time proceeds. Over the run period, both phage continue to be maintained along with the susceptible N , single-phage resistant N_1 and N_2 populations, as well as the dominant N_{12} .

Save for a major exception, we interpret these theoretical results to be consistent with those observed in figure 3. That exception is the failure to see an increase of the doubly resistant bacteria in the figure 3C experiment. At this time, we do not know why the doubly resistant (i.e., T- and B-resistant) bacteria in these cultures do not increase in density. Although they were substantially less fit than the susceptible bacteria, our independent estimates of their maximum growth rates exceeded the dilution rate of the continuous cultures (data not shown). In accordance with our models, in the presence of both phages, they should have increased to dominate the bacterial population under these conditions (fig. 5B). One possibility is that doubly resistant mutants would increase to dominate the cultures if these experiments were continued for a longer period.

Discussion

The molecular geneticist Jacques Monod once quipped, "What is true for *E. coli* is true for elephants, only more so." (Freidmann 2004). This adage, which some may see as a compelling form of inductive inference, does not seem to apply to *Vibrio cholerae* and the lytic phage studied in this investigation. There are elements of the bacteria-phage dynamics observed in this study that we have not seen in other investigations of this ilk. In this regard, the T phage experiments and the continued maintenance of a high density of susceptible cells in the resource-limited community in figure 2 is the most remarkable finding. However, relative to the broader question that motivated this study, whether phage can limit the densities of bacterial populations, the unusual features of the phage-bacterial dynamics reported here are nuances. We interpret the results of this study as support for the hypothesis that, for extensive periods of time, bacterial populations can be limited by multiple, but not single, phage species.

Qualitatively, and to a large extent quantitatively, the results of these single-virus continuous culture experiments with the B and T phage are similar to those of other experimental studies of the population dynamics of bacteria and single species of phage (Chao et al. 1977; Levin et al. 1977; Lenski and Levin 1985; Middelboe 2000; Wei et al. 2010). Mutants resistant to these phages emerged and soon became dominant strains, and the density of the bacteria in the cultures with these viruses approached that in the phage-free control cultures. Although the B phage

evolved to become better able to replicate on the ancestral, susceptible N16961, there was no evidence for either of these viruses evolving to be able to replicate on resistant strains of these bacteria. Although *V. cholerae* susceptible to the coexisting phage continued to persist at high densities in the T phage experiment (for reasons we do not fully understand), these cultures also became limited by resources rather than by phage. Following the development of resistant strains, the turbidity of these cultures was the same as that for the phage-free control cultures.

Based on both their turbidity and colony count estimates of density, the continuous cultures of N16961 with mixtures of T and B phage were clearly limited by phage. Although doubly resistant mutants could be isolated from some of these experimental populations, it is not clear why they did not become dominant and establish a resource-limited community. Although the results of our pairwise competition assay indicated that the double mutants were less fit than their susceptible ancestor, their exponential growth rate was high enough to overcome the flow rate. It may be that, if we ran these experiments for a longer period, higher-fitness doubly resistant bacteria would evolve and become dominant.

In an earlier study, Schrag and Mittler (1996) presented evidence that the stability of communities of bacteria and phage in continuous cultures can be attributed to subpopulations of phenotypically resistant but genetically susceptible bacteria on the walls of these vessels. The results of the current experiments suggest that, in addition to a wall-adhering subpopulation, persists, defined as slowly replicating or nonreplicating phage-refractory planktonic cells (Bigger 1944; Balaban et al. 2004; Wiuff et al. 2005; Pearl et al. 2008), may also play a role in stabilizing these communities.

The results of this study with two phage contrasts markedly with those obtained in the *Escherichia coli* restriction-modification phage dynamic studies of Korona and Levin (1993). In those experiments with three phage species, triple resistance evolved <48 h after the bacteria's first encounter with phage, and the bacterial population became limited by resources. The reason for this is an order hierarchy of phage replication (Weigle and Delbrück 1952). Upon infection of a cell with multiple phages, the phage first in the hierarchy suppresses the replication of the others. As a result, selection sequentially favors resistance to single phages in the order of their hierarchy. As a consequence of this hierarchy, the density of the bacterial population is effectively limited by one phage at a time, and resistance to that phage is selected for. We postulate that, in the *V. cholerae* phage system used in this study, there is little or no hierarchy of replication in joint infections of a single cell. Consequently, resistance to one phage

provides little or no advantage to a bacterium in the presence of the other phage.

We interpret the results of this investigation as support for the hypothesis that, collectively, multiple species of phage can play a substantial role in determining the structure of natural communities of bacteria by regulating the densities of their host populations of bacteria. However, we also recognize the inferential caveats of this theoretical and experimental study in this regard. The most important of these caveats is subsumed in our use of the phrase “for extended periods” to describe the amount of time that these phage-limited cultures remained in that state. In the world beyond mathematical models and microcosm experiments, 700 h is not much time. With more time, it may well be that bacteria resistant to multiple phages with sufficiently high fitness will emerge.

Conversely, this inferential caveat and the limitations of *in vitro* experiments can be addressed empirically; these experiments and models make a prediction (provide hypotheses) that can be tested in the real world. If in a natural community bacteriophages limit the densities of their bacterial hosts, these bacteria would be susceptible to the collective of coexisting phages. If these bacteria are resistant, the collective population of bacteriophages would not be regulating their densities but, rather, persisting by replicating on susceptible minority populations. We are aware of only one study that addressed this question for bacteria and phage from natural sources (Waterbury and Valois 1993). In that investigation with marine cyanobacteria, the dominant population of bacteria were resistant to the coexisting phage, and the authors concluded that the phage were not limiting the density of their host bacteria. How generalizable this finding is remains to be seen. Viruses that infect bacteria and archaea may be the most abundant organisms in the world, and they have been postulated “to play a profound role in the organization and functions of microbial communities, but their diversity and host range are seldom evaluated in community-level studies, and science knows relatively little about them” (Harwood and Buckley 2008, p. 15).

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