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Adithi Govindan April 7, 2020

# A theoretical and experimental study of the population dynamics of bacteria and phage: implications for therapy

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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**Background:**

With the rise of infections caused by multidrug resistant organisms, alternative therapies such as phage therapy, have become of interest. Although phage therapy has successfully treated bacterial infections, in most cases bacteria evolve resistance to the phage treatment. In order to prevent or reduce the problem of resistance, it is important to address the conditions of phage-bacteria population dynamics conducive to bacterial resistance evolution.

**Results:**

Using mathematical modeling and *in-vitro* experiments, we explore three scenarios to determine if phage therapy can prevent the rise of resistance: I) phage cannot generate host range mutants so resistance to the phage can be acquired by single point mutations; II) phage are capable of evolving host range mutants that enable them to replicate on bacteria resistant to its ancestral phage; and III) a general resistance mechanism by which bacteria gain resistance to multiple phage, or mucoidy. Our theoretical and experimental results suggest that generally phage therapy, including phage cocktails, or a mixture of phages, is not effective in consistently preventing the rise of bacterial resistance. However, we observe that if phage treatment contains multiple phage, one of which as the capability of generating a host range mutation in response to resistant bacteria, the phage can prevent or delay the rise of resistant bacteria. We also observe a reduction in resistant bacteria in a population that is treated with a phage cocktail containing phage that can readily, without mutation, replicate on bacteria resistant to another phage in the population.

**Conclusions:**

Although phage therapy is an alternative treatment for multi-drug resistant infections, we suggest the phage-bacterial dynamics should be studied to elucidate the conditions that give rise to bacteria resistant to treatment. Understanding these conditions can inform the future design of phage cocktails that to prevent the rise of resistance to treatment.

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I would like to thank my friends for their constant encouragement and support throughout the project.

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## Introduction

Driven by the continuous rise in the frequency of multidrug resistant (MDR) bacterial pathogens, there has been a resurrection in an alternative therapy: bacteriophage or phage therapy. Phage are viruses that exclusively infect, replicate on and kill bacteria. The first inference of the existence of phage was made by M.E. Hankin in 1896 when he noted the bactericidal effect of the Ganges river; its waters teeming with bacteriophage could kill the bacteria *vibrio cholera* (1, 2). Phage was then re-discovered in 1915 by British scientist William Twort, and in 1917 by French scientist Felix d'Herelle, who pioneered the idea of using phage as a treatment for bacterial infections (3–5).

Despite the early discovery of phage, the West lost interest in phage therapy after World War II due to the success of antibiotics and increasing doubts surrounding the efficacy of phage therapy (6). However, in other areas of the world such as the former Soviet Union, research on phage therapy was well under way. The first Institute for phage therapy, the Eliava Institute, was established in Tblisi, Georgia and continues to conduct phage therapy research today (7). Soon, antibiotics were unable to treat MDR infections and led the West to revisit phage therapy. H.W. Smith and M.B. Huggins "resurrected" phage therapy in the West when they published a paper the 1980s that dispelled concerns and doubts of the reliability of phage therapy (7, 8). Through their mouse thigh infection model, they proved that phage can I) replicate in *E. coli* infected mice (dispelling a claim in a paper in 1943 that claimed phage are unable to replicate on bacteria in animals); II) can be more or as effective as antibiotics when used to treat patients; III) the immune system did not remove the phage as initially assumed; and IV) bacteria that evolved resistance to the single phage became avirulent (9, 10). Proceeding Smith and Huggins' paper,

several others explore the history, promises and pitfalls, and behavior of phage therapy to understand its potential for therapeutic use.

Within the past two decades, a recent resurrection of interest in phage therapy stems from the successful use of these viruses for compassionate therapy. In these cases, phage therapy was used as an alternative experimental therapy primarily due to resistance to the antibiotics administered to treat the infection. Amongst the most well-known of these successes relevant to the present study was the use of phage to treat the pancreatic pseudocyst infection of *Acinetobacter baumannii* in a patient at the University of California at San Diego (11). Despite the success of phage therapy treatment throughout history, the inevitability of the evolution of bacteria resistant to phage therapy has been and remains a significant concern (12, 13). One solution to the rise of resistance to phage therapy is phage cocktails, a collection of phages (11, 14). Consider a case in which a population of bacteria is confronted with multiple phage - bacteria can become resistant by mutation to an individual phage but may remain sensitive to the other phage in the cocktail. Additionally, bacteria may incur a fitness cost when modifying multiple genes for different receptors to combat a multi-phage attack or utilizing costly general resistance mechanism for protection against multiple phage, such as mucoidy (14,15). Thus, phage cocktails are an appealing solution to resistance, but are not the final solution because resistance can evolve even when treating bacteria with a phage cocktail (11). To properly address the resistance problem, the conditions that promote resistance in bacteria, on a broadly population level, must be understood.

The literature corroborates the promise of phage therapy as a solution to the evolution of resistance in bacteria, however only a few papers discuss the conditions that increase the likelihood of phage therapy and phage cocktail success. Exploring the phage-bacterial dynamics

at a population level following cocktail administration is one way to determine the conditions most conducive to preventing resistance (16). A few studies approach these phage-bacteria dynamics theoretically and/or experimentally (17–19). No literature address how these dynamics affect the emergence of resistance, or how changes in the phage cocktail or bacterial population conditions could affect the evolution of resistance as well. By studying the population dynamics that give rise to the evolution of resistance, a protocol for effective phage treatment can be designed to prevent or minimize bacterial resistance.

In this study, we utilize mathematical models and *in-vitro* experiments of *Escherichia coli (E. coli)* B/6 and mucoid *E. coli* K12 with their lytic phages to elucidate the conditions in which bacteria will not evolve resistance when confronted with one, two or three phages. We explore three scenarios that could be encountered when phage are used to treat bacterial infections: I) the phage cannot generate a host range mutation so bacteria acquire resistance to phage by a single point mutation; II) the treating phage can generate host range mutations to allow for their replication on resistant bacteria, so bacteria become resistant through two point mutations; and III) mucoidy, a generalized resistance mechanism to combat multiple phage. We postulate that in the case of no host range phage mutations, such that resistance is acquired through a one-point mutation, resistance will ascend in the population. We do not expect resistance to arise when resistance requires a two-point mutation. Finally, we hypothesize that mucoidy could be a generalized resistance mechanism that maintains the phage at high titers.

## Materials and Methods

### Bacteria and Phage:

All *E. coli* strains used in our experiments were derivatives of the parent strain K12 MG1655. The parent strain *E. coli* MG1655 was obtained from Ole Skovgaard at Roskilde University in Denmark. The *E. coli* B/6 strain was obtained from Seymore Lederberg in 1968. The mucoid mutant with a gain-of-function point mutation in the Rcs, "Regulator of capsule synthesis," pathway for the mucoid phenotype was constructed by the method described in (20). Bacterial cultures were grown at 37˚C in LB broth [MgSO4 2.5g/L, tryptone (Fisher Bioreagent 10g/L, yeast extract (Bacto) 5g/L, sodium chloride (Fisher Chemical) 10g/L].

Phage lysates were prepared from single plaques at 37˚C in LB medium alongside wild type *E.coli* B/6. Chloroform was added to the lysates and the lysates were centrifuged to remove any remaining bacterial cells. The T3, T4, and T5 phages were wild type phages obtained from the Bull Lab. We incorporated the lytic wild phage, EM2φB6+, into the experiments in scenario one (I) to conduct a one bacteria and three phage experiments with three lytic phages. The wild phage was a phage isolated in the Levin lab, from sewage. The wild phage is an enterobacteria phage related to Escherichia phage teqhad and Yersinia phage phiD1.

### Sampling methods:

Bacteria and phage densities were estimated by serial dilutions in 0.85% NaCl solution followed by plating. The total density of bacteria was estimated on LB (1.6%) agar plates. To estimate the densities of free phage, chloroform was added to suspensions before serial dilutions. These suspensions were mixed with 0.1 mL of overnight LB grown cultures of wild-type *E. coli* B/6

(about $5\times10^8$ cells per mL) in 3 mL of LB soft (0.65%) agar and poured onto semi-hard (1%) LB agar plates.

**Serial Transfer:**

All serial transfer experiments were carried out in 10-mL cultures grown at 37˚C with vigorous shaking. The cultures were initiated by 1:100 dilution from 10-mL overnight cultures grown from single colonies. Phage were added to these cultures to reach the initial density of approximately $10^5$ PFU/mL. At the end of each transfer, 100 $\mu$L of each culture was transferred into flasks with fresh medium (1:100 dilution). Simultaneously, 100 $\mu$L samples were taken for estimating the densities of colony forming units (CFU) and plaque forming units (PFU), by serial dilution and plating on solid agar, with selection as needed as described above.

**Testing for resistance and host range phage:**

*Liquid culture*: Colonies from the bacteria plates were streaked thrice to ensure removal of phage. Colonies from the streaked plates were used to establish overnight cultures in 2mL LB broth incubated at 37°C in a shaker. The overnight cultures were diluted 1/100 and ~1e5 phage were added to the cultures. After 24 hours of incubation, free phage in these cultures were measured. Those unable to support the phage replication were considered as resistant (19). This procedure was done for the mucoid experiments.

*Phage agar*: Phage plates were made as previously described, using 0.1 mL of overnight LB grown cultures of the isolates being tested. Dense phage stocks (108 -109 PFU/mL) were spotted onto the agar surface; susceptibility was scored according to the presence of visible plaques. This procedure was used for the non-mucoid experiments.

*Host range phage:*

When checking for host range phage for T3, the isolate being tested (from the initial serial transfers) is subjected to a spot test of the original lysate. Overnight cultures of the resistant colonies in the area where the phage was spotted, were then used as a lawn (also mixed with 3 mL of soft agar) for a subsequent spot test of T3. Upon isolating host range phage from plaques and spotting them on the *E. coli* B/6 and T3-resistant cells, we found that the host-range phage could infect both cell types, confirming the presence of a host-range phage.

**Parameter Estimates**

NOTE: Due to the lab shutting down because of COVID-19, we attempted to preliminarily measure parameters but could not include them in the study. As a result, most parameters were estimated from previous experiments, other studies, or by hand (18, 21).

The parameters critical for the interaction of T3, T4, T5 and wild phages and *E. coli* B/6 used in this study were planned to be estimated in independent experiments in LB medium. The maximum growth rate of *E. coli* B/6 was to be measured by BioScreen© as described in (22). Phage burst sizes (β) were planned to be estimated with one-step growth experiments (23). Adsorption of T3, T4, T5 and wild phage to *E. coli* was planned to be estimated as described in (23).

**The Mathematical Models and Numerical Solutions:**

All of the mathematical models in this study were restricted to continuous culture, such as in a chemostat. The bacteria and phage are maintained in a vessel of volume, vol (ml), into which a limiting resource from a reservoir where it is maintained at a concentration C ($\mu$g/ml) enters at a rate w (per ml per hour), which is the same rate at which bacteria, phage and excess resources are removed. The net rate of growth of susceptible bacteria– resistance state I, is equal to the

product of its maximum growth rate, vi, and a hyperbolic function of the concentration of the limiting resource, $\psi(r) = r/(r+k)$ where k is the concentration of the resource where the growth rate is half its maximum value, v/2 (24). The bacteria consume the resource at a rate proportional to their growth rate and a conversion efficiency parameter, e ($\mu$g)( 24).

The phage adsorb to the bacteria at a rate equal to the product of the density of bacteria and phage of that state, Pi and Si, an adsorption rate parameter, $\delta$, per hour per ml (26). To allow for the fact that the rate of phage adsorption and replication depends on the physiological state of the bacteria, we assume that the rate of adsorption is proportional to $\psi(r)$. We neglect latent periods and assume that upon adsorption the phage produce $\beta$ phage particles. Mutation to phage resistance, reversion from resistance to sensitivity, and host range mutations for the phage occur respectively at rates, $\mu$b, $\mu$m and $\mu$p per cell or per phage per hour. Here too we assume that the rate of mutation depends on the physiological state of the bacteria and thereby is proportional to the concentration of the resource via the $\psi(r)$ function.

All equations were solved numerically and the dynamics were simulated with Berkeley Madonna™ with integration by the Euler method and values of the parameters in the range estimated experimentally. To allow for the stochastic nature of mutation for resistance for the bacteria and host range mutations for the phage, we use Monte Carlo simulations. At each time interval, dt (dt=0.0001 hours) a rectangularly distributed random number, x ($0 \leq x \leq 1$) is generated. If $x < \mu$*N*dt*vol*$\psi(r)$ then 1/(Vol*dt) resistant bacteria (or host range) mutants are added to the resistant or host range population and the same number removed from the population from whence the mutation was generated. N in this case is the density of the bacteria or phage. We assume that the reversion from resistant to sensitive states are deterministic. Copies of the Berkeley Madonna™ programs use are presented in the Appendix to this Thesis.

**Research plan:**

We consider three separate scenarios that are likely to be encountered when phage are used to treat bacterial infections. In the first, (I), by single point mutations sensitive bacteria can become resistant to the phage. Using mathematical and computer simulation models we explore *a priori* conditions under which bacterial populations will evolve resistance and thereby no longer be limited, controlled, when confronted with one, two and three phages that require independent mutations for resistance. With population dynamic and other experiments with *E. coli* and its lytic phages, we explore the validity of the predictions of these models. In the second, (II) the phage are able to generate host range mutants that enable them to replicate on first order resistant mutants, but not the resistant mutants that evolve in response to these host range phage. Using a mathematical and computer simulation models, we determine the *a priori* conditions under which populations of bacteria will escape limitations by phage with these properties. We test the validity of these predictions with population dynamic experiments with phage and E. coli that require two mutation for full resistance to all the bacteria. In the third, (III) the bacteria generate mutations that provide resistance to phage with different receptors. Using experimental populations of *E. coli* with this generalized resistance we explore the conditions under phages can limit their densities.

## Results

### Scenario I. One-point mutations required for resistance

#### *Theoretical Considerations*

**1** - Populations of bacteria confronted with one phage

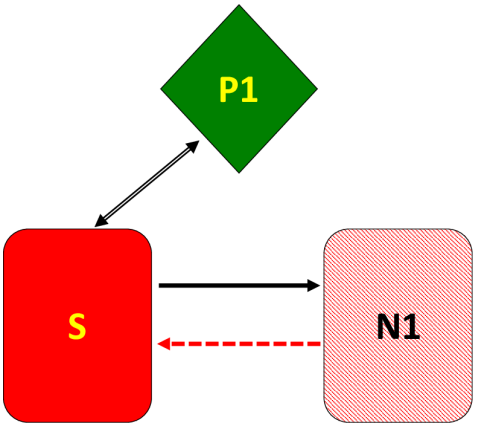
In this first model (Figure 1), there is a single population of phage, P1, bacteria that are sensitive to those phage, S and by mutation resistant cells are generated, N1. The parameters and variables of this model and the models that follow are presented in Table 1.

**Table 1**

| Parameter (symbol) | Description | unit |
|---|---|---|
| **R** | Resource concentration in culture | µg $ml^{-1}$ |
| **c** | Resource concentration in reservoir | µg $ml^{-1}$ |
| **w** | Flow rate | $hour^{-1}$ |
| **k** | Monod constant, resource concentration at which bacterial growth is v/2 | µg $ml^{-1}$ |
| **vs** | Maximum growth rate for S | $hour^{-1}$ |
| **vr** | Maximum growth rate for N1, N2, N3 | $hour^{-1}$ |
| **vr2** | Maximum growth rate for N12, N13, N21, N23, N31, N32 | $hour^{-1}$ |
| **vr3** | Maximum growth rate for N123 | $hour^{-1}$ |
| **e** | Efficiency parameter, efficiency of bacterial conversion of resource into daughter cells | µg |
| **δ** | Adsorption rate constant | cell phage $hr^{-1}$ |
| **β** | Burst size | particles infected $cell^{-1}$ |
| **µm** | Reversion rate | $hour^{-1}$ |
| **µb** | Mutation rate for resistance | $hour^{-1}$ |
| **µp** | Mutation rate for host range phage | $hour^{-1}$ |

| Variables | Description |
|---|---|
| **S** | Sensitive cells |
| **N1, N2, N3** | Resistance to P1, P2, P3 respectively |
| **N12, N13, N23** | Resistant to P1 and P2, resistant to P1 and P3, resistant to P2 and P3; P3, P2, and P1, respectively can replicate on each bacterial mutant |
| **N123** | Resistant to P1, P2, and P3 |
| **P1, P2, P3** | Phage 1, phage 2, phage 3 |
| **N0, N1, N2** | Two-step mutation; original bacteria, mutant 1, mutant 2 |
| **P0, P1** | Host-range phage 0 and 1 |

**Table 1.** Parameters and variable for the numerical solutions of the simulations all models are shown. Parameters and variables used for numerical solutions of the simulations are shown. Initial densities of *E. coli* B/6 were estimated by plating before each experiment. Certain parameters and variables were approximated by hand in attempt to capture the dynamics observed *in-vitro*. Resource concentration, conversion efficiency, and Monod constants are approximated because the exact concentration of limiting resource in LB is unknown. Growth rates were also estimated.




$$\frac{dr}{dt} = \underbrace{w \bullet (C - r)}_{\text{resource input}} \quad \underbrace{-\psi(r) \bullet e \bullet (vs \bullet S + vr \bullet N_R)}_{\text{resource uptake}}$$

$$\frac{dS}{dt} = \underbrace{vs \bullet \psi(r) \bullet S}_{\text{growth } S} \quad \underbrace{-\delta \bullet S \bullet P1 \bullet \psi(r)}_{\text{phage adsorption}} \quad \underbrace{+ v \bullet N_R \bullet \psi(r)}_{\text{mut } N_R \rightarrow S} \quad \underbrace{- \mu \bullet S \bullet \psi(r)}_{\text{mut } S \rightarrow N_R} \quad \underbrace{- w \bullet S}_{\text{flow out}}$$

$$\frac{dP1}{dt} = \underbrace{\delta \bullet S \bullet P1 \bullet \beta \bullet \psi(r)}_{\text{phage replication}} \quad \underbrace{- w \bullet P1}_{\text{phage flow out}}$$

$$\frac{dN_R}{dt} = \underbrace{vr \bullet \psi(r) \bullet N_R}_{\text{growth } N_R} \quad \underbrace{+ \mu \bullet S \bullet \psi(r)}_{\text{mut } S \rightarrow N_R} \quad \underbrace{- v \bullet N_R \bullet \psi(r)}_{\text{mut } N_R \rightarrow S} \quad \underbrace{- w \bullet N_R}_{\text{flow out}}$$

Where $\psi(r) = r/(r+k)$

**Figure 1.** Diagram and equations for a model of the population and evolutionary dynamics of one bacteria and one phage with mutation to resistance. There is one phage P1 and two populations of bacteria, sensitive, S, and resistant, N1. The phage infect and replicate on the sensitive bacteria, line with two arrows, but not the resistant. Resistance is generated by mutation, S→N1, solid black line, and resistance bacteria can revert back to sensitive N1→S, broken red line.

At the start of the simulations, S = $1x10^6$ cells/ml, and P1 = $10^5$ phage particles/ml. In Figure 2A we present a run where resistance does not evolve and in 2B, where it does. Since we allow for a high rate of reversion of the resistant cells to sensitive cells, N1→S, although P1 is unable to replicate on N1, it is maintained in the population through replication on the sensitive cells and revertant cells (27). The bacteria are maintained in a resource limited state. To provide an idea of the likelihood for resistance to the phage evolving and the time before that evolution occurs, we ran 100 independent simulations with the same parameters as in Figure 2A and B, see Figure C. By the word fixed we mean populations where the density of resistant cells is at or near the maximum. By 400 hours, 53% of the runs were fixed, the bacteria were no longer limited by the phage and by 1000 hours, 80% of the runs were fixed. In these simulations the rate of mutation to resistance was $10^{-9}$ per cell per hour. If the rate of mutation is higher, $10^{-8}$ fixation occurs earlier, Figure 2D.

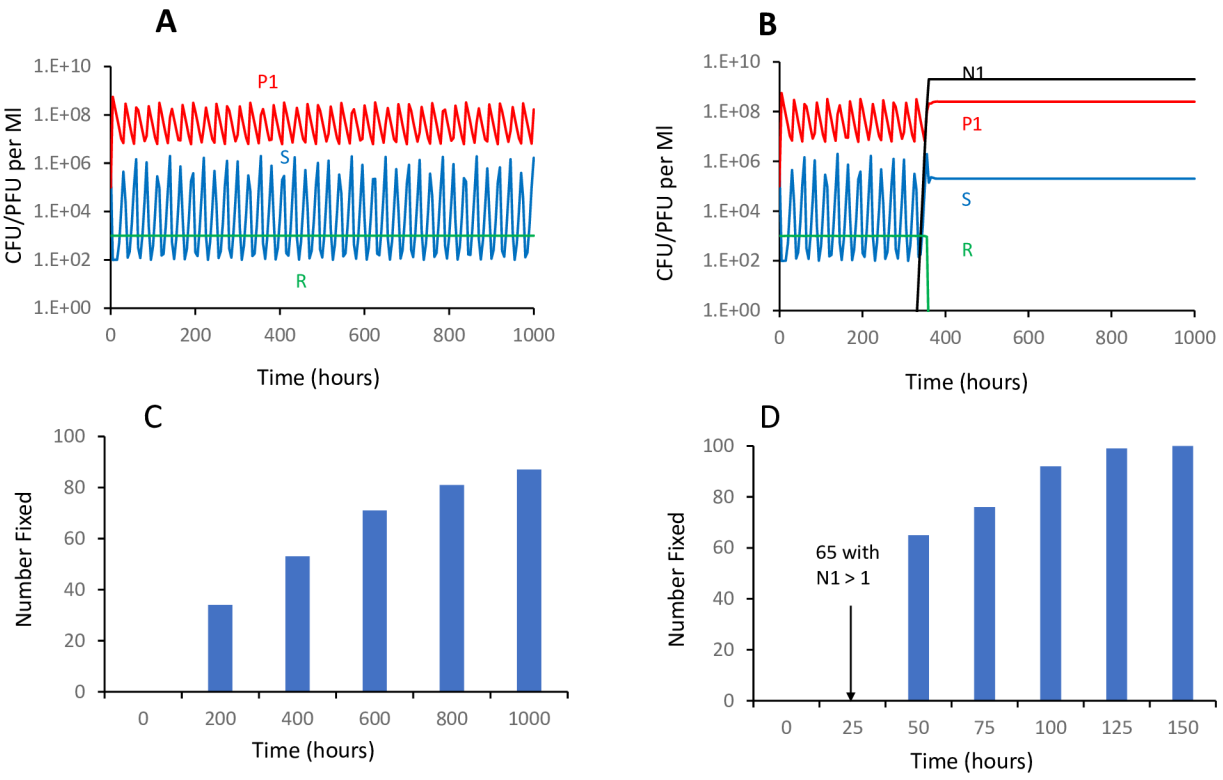



**Figure 2.** Simulation of the one bacteria and one phage dynamics. Initial densities of the bacteria and phage, respectively $S=10^5$ and $P=10^6$ cells and particles/ml. Parameter values vs=1, $e= 5x10^{-7}$, k=0.25, $\delta=1x10^{-8}$, $\beta=100$, w=0.2, $\mu m= 5x10^{-5}$, vol = 10 ml. (A) Changes in the densities of bacteria and phage in simulation where resistance failed to evolved, $\mu p=10^{-9}$. (B) Changes in the densities of bacteria and phage in a simulation where resistance evolved. (C) Number of 100 simulations dominated, fixed, for N1 at different time $\mu p=10^{-9}$. (D) Number of 100 simulations dominated, fixed, for N1 at different times $\mu p=10^{-8}$.

## **2** - Populations of bacteria confronted with two phage Model 1

In this model (Figure 3) the bacteria can generate mutations to prevent infection by host range phage of two types, those that attack through receptor 1 and those that attack though receptor 2. The sensitive population of bacteria, S, is confronted with a phage, P (that can only replicate on the sensitive population), which by single point mutations can generate resistance by modifying receptor site 1 or receptor site 2, respectively N1 and N2. The phage can generate host range mutants of two types, P1 which can replicate on N2, and P2 which can replicate on N1. By mutation, the bacteria can generate cells that are resistant to both P1 and P2, N12. Until N12 is

generated and ascends, the phage can regulate the densities of the bacterial population. Because of a high rate of transition from the resistant to the sensitive states, N1→S, N2→S, N12→N1 and N2 the bacteria and phage can co-exist (27).

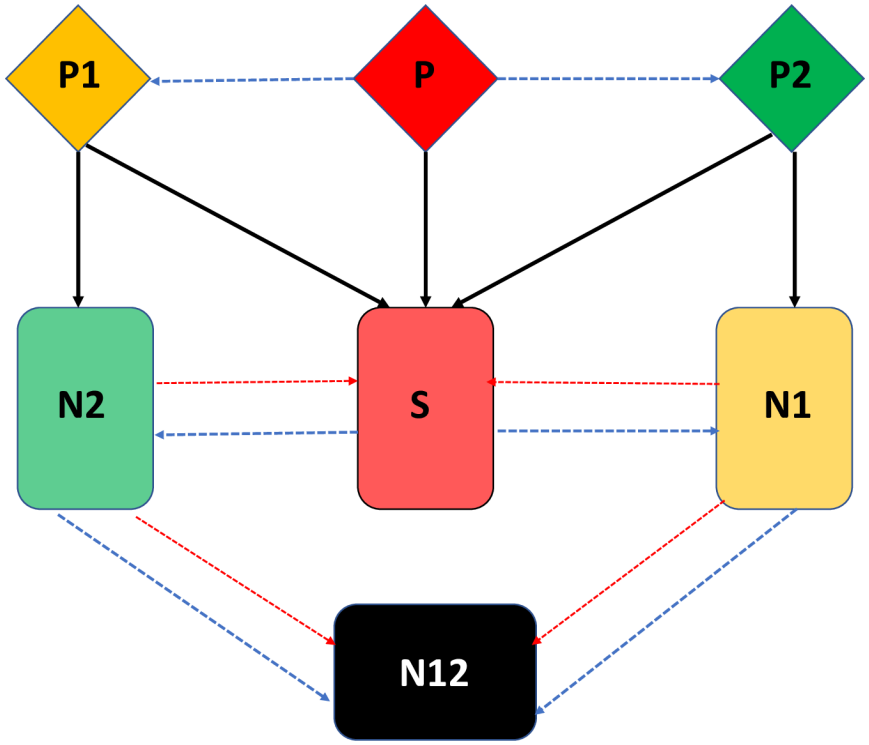



**Figure 3.** Diagram for a model for four states of the bacteria, sensitive to both phage, S, N1 resistant to phage P1, N2, resistant to phage P2 and N12 resistant to both phage. Solid lines indicate infection and phage replication, broken blue lines denote transitions between states by random mutation, broken red lines denote deterministic changes between states. Model information is given in the appendix.

In Figure 4 we present the results of simulation of the model depicted in Figure 3. Although two mutations are required for the bacteria to be resistant to phage of both states, these double resistant mutants evolve and ascend to become the dominant population (Figure 4A) and the bacterial population is no longer limited by the phage as it is in Figure 4B. With the seemingly low mutation rate to phage resistance and host range phage, $\mu=10^{-9}$ per cell or phage per hour, by

500 hours 50% of the simulations were dominated by, fixed for, the double resistant mutants, and by 1000 hours, 80% are fixed (Figure 4D, blue bars – model 1). If the mutation rate is higher, within a 125 hours after first exposure to the naïve phage, 99% of the simulationed populations are fixed for bacteria with resistance to the two phages (Figure 4E, blue bars – model 1). However, when compared to model 2 (figure 5), orange bars, the time for fixation of the double mutant is delayed when compared to model 1. When the seemingly low mutation rate, $\mu=10^{-9}$ per cell or phage per hour, by 250 hours 13% of the simulations are fixed and by 100 hours 78% are fixed. At a higher mutation rate, $\mu=10^{-8}$ per cell or phage per hour, by 200 hours 97% of the simulated populations are fixed.

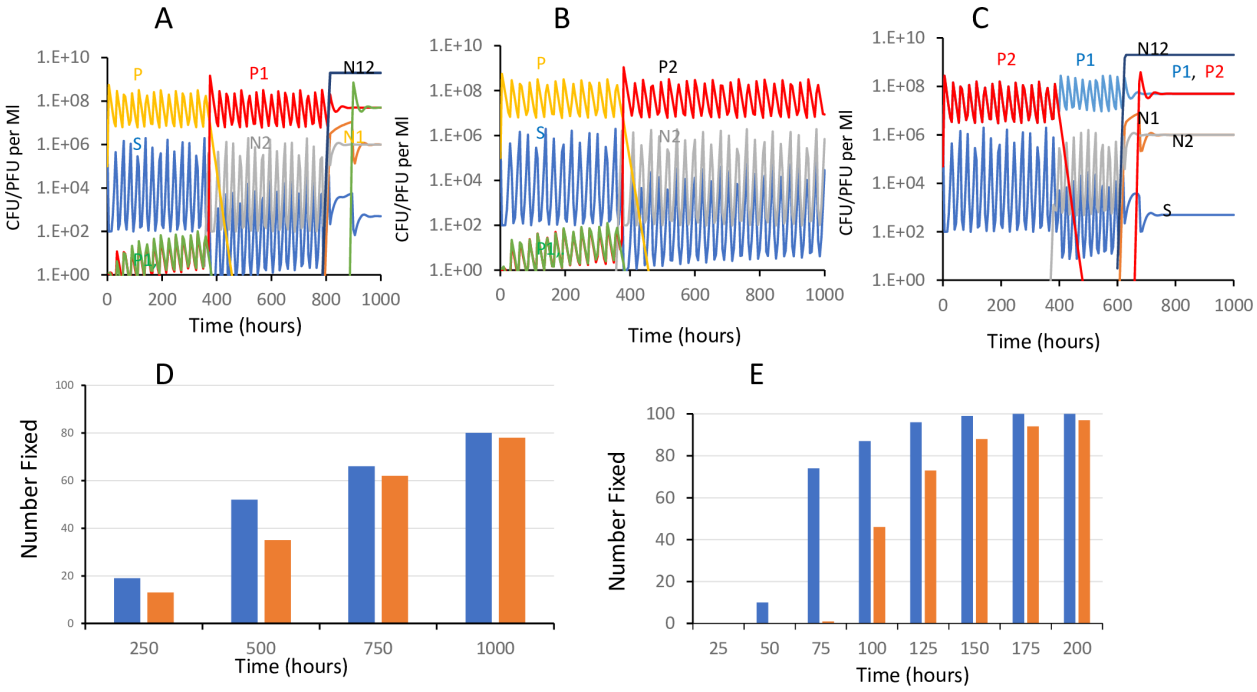



**Figure 4.** Simulation results for one bacteria and two state phage model 1 and 2 depicted in Figures 3 and 5, respectively. Initial densities of bacteria and phage in the Figure 3 and 5 model, $S=10^6$ and $P=10^5$, and $S=10^6$, cells and particles/ml. $P1=P2=5x10^4$ particles/ml in the Figure 5 model. Parameter values vs=1, $e= 5x10^{-7}$, k=0.25, $\delta=1x10^{-8}$, $\beta=100$, w=0.2, $\mu m= 5x10^{-5}$, Vol = 10 ml. (A) and (B) simulate the model in Figure 3 model, and (C) simulates the model in Figure 5. A and B show the changes in the densities of bacteria in single simulation runs for

the Figure 3 model and (C) for the Figure 5 model $\mu b=\mu p=10^{-9}$. (D) Number of 100 simulations dominated by bacteria resistant to phage of both host range states, N12, at different times; blue the Figure 3 model, orange the Figure 5 model, $\mu b=\mu p=10^{-9}$. (E) Number of 100 simulations dominated by bacteria resistant to phage of both host range states, N12, at different times. Blue - Figure 3 model, orange - the Figure 5 model, $\mu b=\mu p=10^{-8}$

In Figure 5, we present model of bacteria confronted with two phage P1 and P2, where P1 is capable of replicating on bacteria resistant to P2, and P2 is capable of replicating on bacteria resistant to P1. This model should be more ameanable to having the phage control the infection than that presented in Figure 3 since at the start of their encounter with the bacteria the phage are already capable of replicating on cells resistant to a single phage. The results of the simulations in Figure 4 support this interpretation. This can be seen from the results of the 100 simulations, (Figure 4D and Figure 4E). When the phage first confronting the bacteria are capable of replicating on the bacteria resistant to one phage, the populations are phage-limited earlier than when the initial phage can only replicate on sensitive cells. In the second situation, host range mutants are necessary for those phage to replicate on the bacteria resistant to the phage. The population is dominated by resistant bacteria, or are phage limited later than in the populations simulated in Figure 4 A and B.

**3-** Populations of bacteria confronted with two phage Model 2

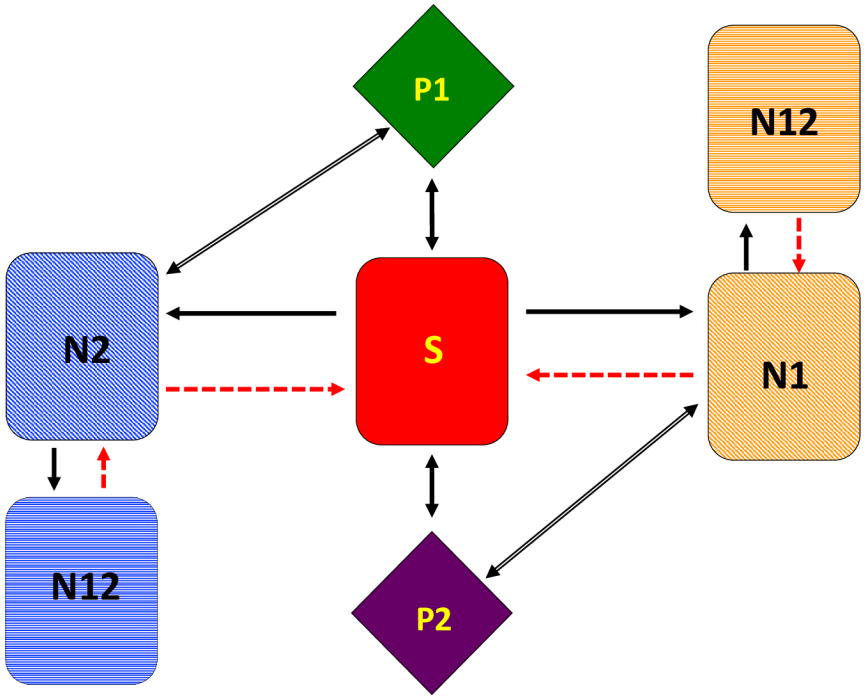



**Figure 5.** Diagram for a model with two phage, P1 and P2, and bacteria of three states, S, sensitive to both phage, N1, resistant to P1, N2, resistant to P2, and N12 resistant to P1 and P2, Solid lines with arrows at each end indicate infection and phage replication, solid lines denote transitions between states by random mutation, S→N1 and S→N2, N1→N12, N2→N12. Broken red lines indicate deterministic transitions between states N1→S, N2→S. Model information is given in the appendix.

**4** - Populations of bacteria confronted with three phages

In Figure 6 we present the diagram of the model for bacteria and three phages. For simplicity, the phage are not depicted in the diagram of this model. The phage are of three states, and the bacteria of 7 states. The phage are capable of replicating on bacteria that are not resistant to that phage, for example phage P2 can replicate on sensitive bacteria, S, and bacteria resistant to P1 and P3, N1, N3, and N13 (N31). Bacteria of state N123 are resistant to phage of all three states. Phenotypically, N13 and N31 are identical, but were generated by mutation in two different orders, N1 →N13 and N3 →N31.

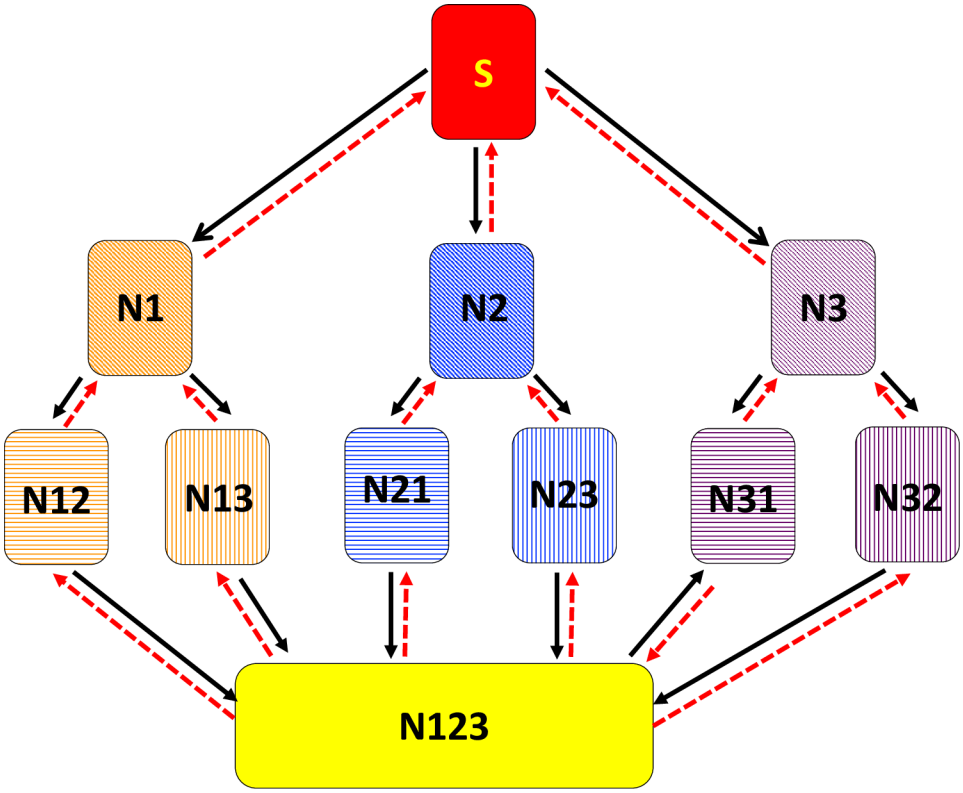



**Figure 6.** Diagram for a model of bacteria confronted with three phages**.** The bacteria can be sensitive to all three phage, S, resistant to one phage, N1, N2 and N3, resistant to two phages, N12 (N21), N13 (N31), N23 (N32), and resistant to all three phage, N123. Phage resistance is acquired by random mutation, solid black lines, and resistant mutants can revert back to the sensitive state, broken red lines.

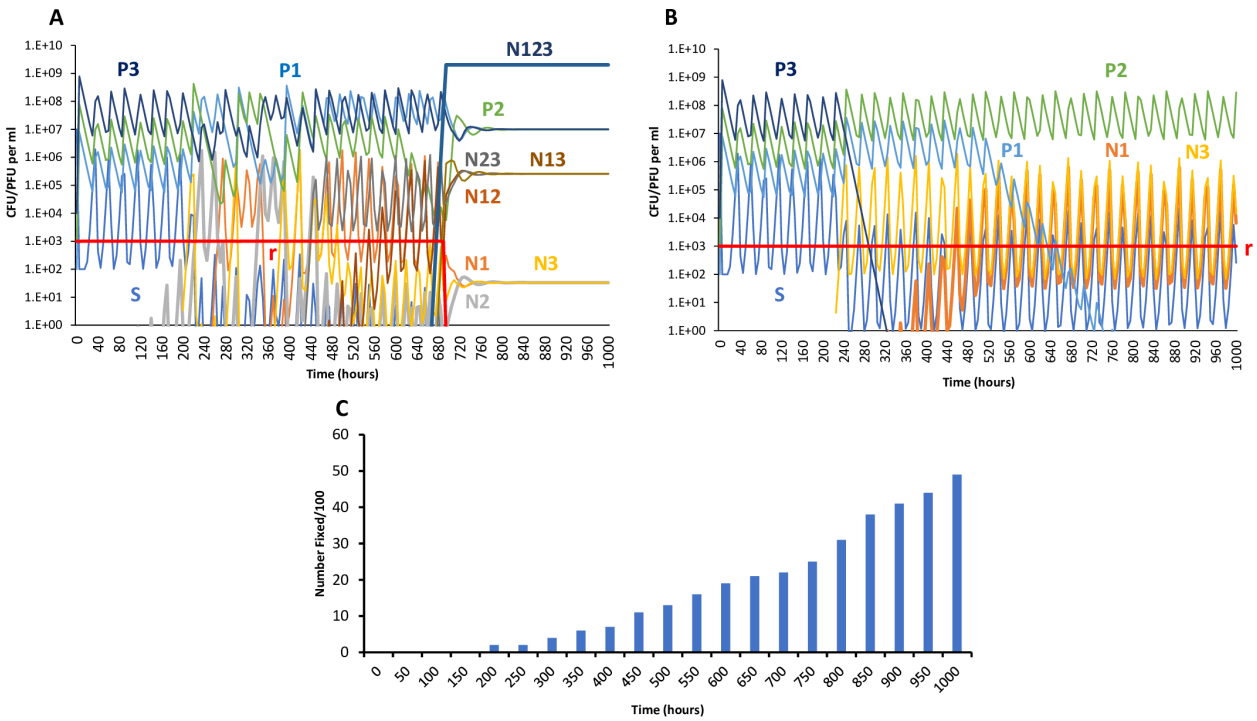



**Figure 7.** Simulation results, bacteria confronted with three phage. Parameter values vs=vr=1, e= $5x10^{-7}$, k=0.25, $\delta=1x10^{-8}$, $\beta$=100, w=0.2, $\mu$m= $5x10^{-5}$, vol = 10 ml. The simulations were started with $10^7$ S and $10^2$, $10^3$, and $10^4$ for P1, P2 and P3 phage respectively. (A) and (B) single simulations where resistance to all three phage, N123 evolved and ascended to dominance, (A) and where it did not and the bacterial population remained under control by the phage. (C) Results of 100 simulations, number of runs in which the triple resistant mutants, N123 evolved and ascended to dominance, became fixed at different times.

Although there are three phages, resistance to all three requires independent mutations. If the rate of mutation is low, $10^{-9}$ per cell per hour, after 500 hours of exposure triple resistant mutant dominated in more than 10% of the simulations and by 100 hours, 50%.

***Experimental Results***

***A Caveat:*** *Thanks to that pesky coronavirus our lab was shut down before we were able complete the planned experimental studies. Consequently, the experimental results presented albeit informative should only be considered preliminary and in need of replication and expansion.*

**1-** Populations of bacteria confronted with one phage

The theory predicts that under broad conditions, when populations of bacteria are confronted with a single phage for which they, by mutation, can generate resistance, resistant mutants will evolve and dominate the bacterial population. At one level the results of our experiments with *E. coli* B/6 and the lytic wild phage, T4, and T5 are consistent with this hypothesis (Figures 8 and Figure 9), the densities of the bacteria in these cultures ascends to levels similar to that of the phage-free controls. All of the bacteria exposed to T5 isolated at 6$^{th}$ transfer were resistant to this phage, and about 70% of the T4 exposed *E. coli* isolated at this time were resistant. We did not have a chance to check whether the *E. coli* exposed to the wild phage were sensitive or resistant to this phage. Contrary to the corresponding model where, because of the high rate of transition to sensitive, the phage were maintained, this was not the case for T5, which were lost following the ascent of resistance.

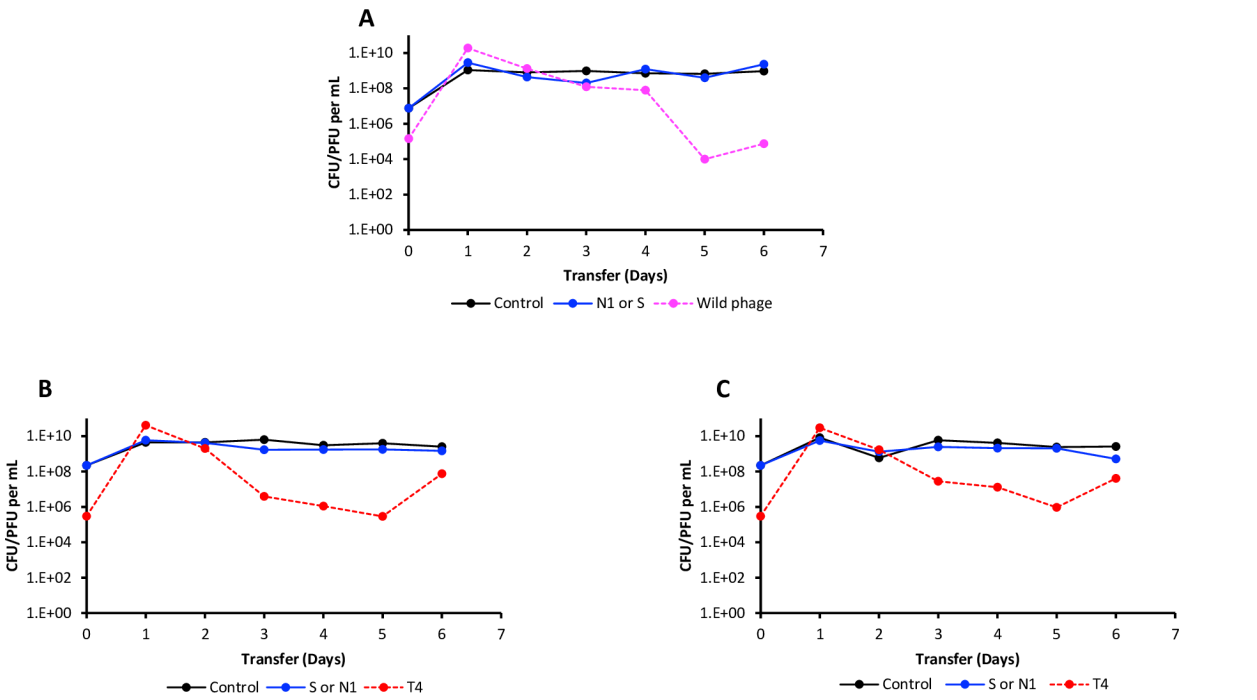



**Figure 8.** The single phage dynamics with *E. coli* B/6 (A) The six day serial transfer of *E. coli* B/6 and the wild phage. (B) and (C) The six day serial transfer of *E. coli* B/6 and T4, replica one and two respectively.

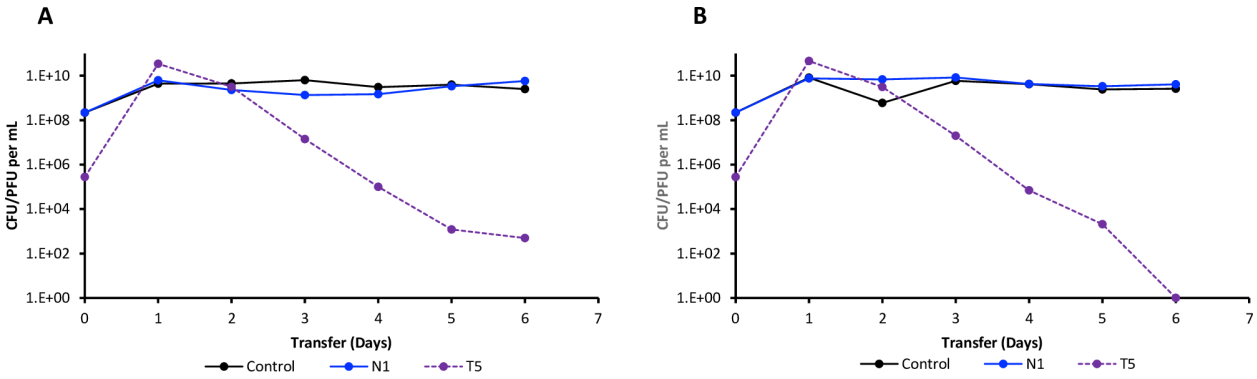



**Figure 9.** Single phage dynamics with the T5 and *E. coli* B/6. Replica one (A) and two (B) of the six-day serial transfer results.

## 2- Populations of bacteria confronted with two phage

In the theoretical results for a population of one bacteria and two phage, the evolution of a double mutant commonly, but not inevitably, will arise and ascend to dominate the population. In the experiment with mixtures of T4 and T5, the population of *E. coli* ascended to levels similar to that of phage free cultures (Figure 10A). In the experiment with a mixture of the wild

phage and T4, over the periods sampled, the bacteria were limited by the phage (Figure 10B). In one of the two experiments with the wild phage and T5, the bacterial population appeared to be limited by the phage, while in the replica after an initial decline in the density of the bacteria, they recovered to levels similar to that of the resource-limited phage-free control (Figure 11A and B). In Figures 10 and 11, the bacteria (blue line) is labeled "N1/S" because we could not confirm resistance due to the lab shutdown.

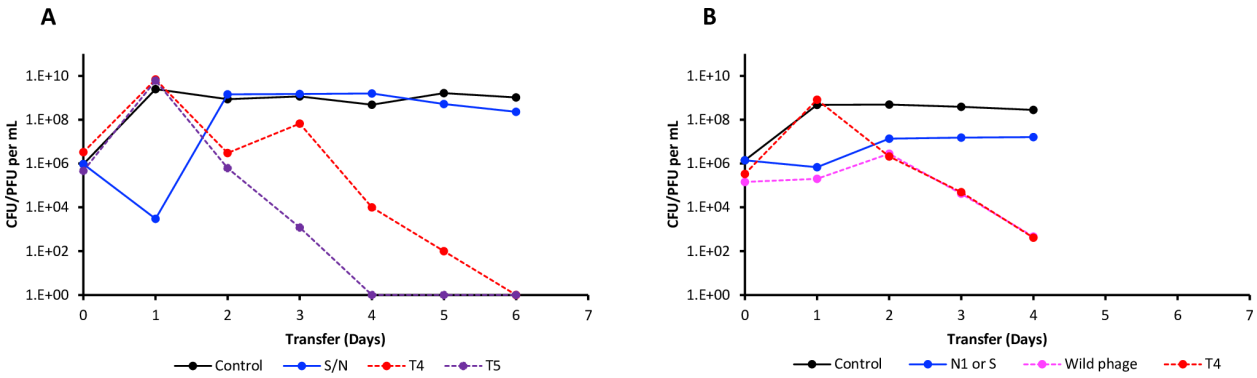



**Figure 10.** Two phage dynamics with *E. coli* B/6 (A) Six day serial transfer results of T4 and T5 with *E. coli* B/6. (B) Two phage dynamics of the wild phage and T4 with *E. coli* B/6.

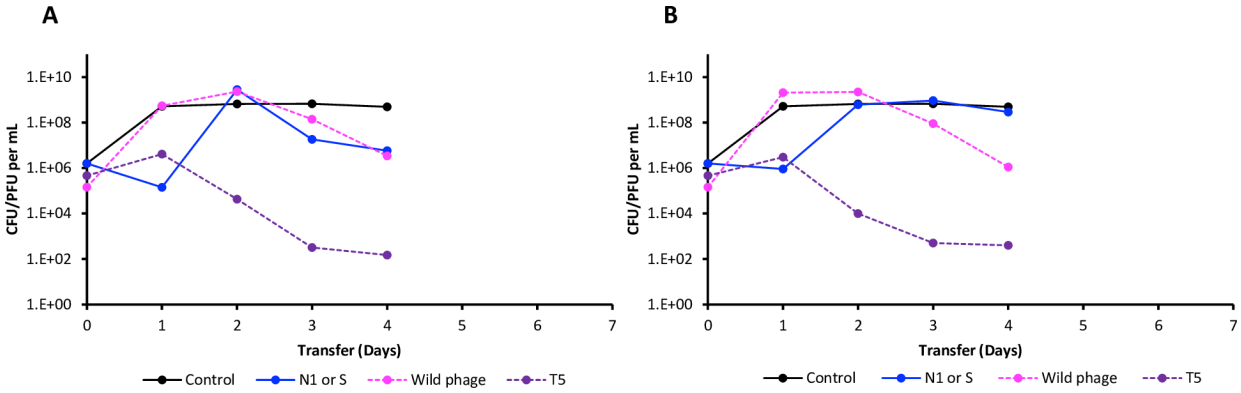



**Figure 11.** Two phage dynamics with the wild phage and T5 with *E. coli* B/6. (A) Replica one and (B) replica two of the six day serial transfer.

## 3 - Populations of bacteria confronted with three phage

Alas, these experiments were planned and all set to go but the coronavirus nixed the endeavor.

**Scenario II. <u>Two-point mutations required for resistance</u>**

***Theoretical Considerations***

**1** - Populations of bacteria confronted with one phage

In Figure 12 we present a model of the population and evolutionary dynamics of bacteria and a single phage in which resistance to the phage requires two independent mutations.

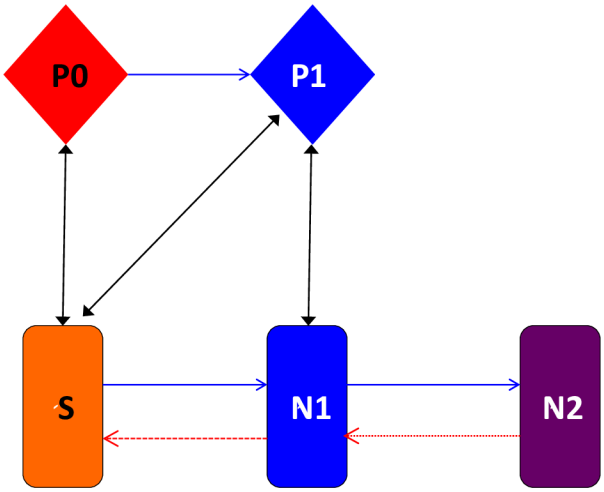



**Figure 12.** Model of the population and evolutionary dynamics of bacteria and phage where there is a host range mutation, P0→ P1 resistance to the phage requires two mutations, S→N1 and N1→N2. By random mutation, the solid blue line S→ N1 and N1→ N2 and by a deterministic transition, the broken red line N2→N1 and N1→S.

In Figure 13 we present the results of simulations of the model in Figure 12. Figure 13A represents a population in which bacteria resistant to all phages emerge, while Figure 13B represents a population where that is not the case. One host range mutation in the phage and two resistant mutations in the bacteria are required before the bacteria resistant to the phage are generated. Nevertheless, even when the mutation rate is low, $10^{-9}$ per cell or phage per hour, by 200 hours nearly 40 % of the simulated populations are dominated by bacteria resistant to the phage and by 1000 hours more than 80% are fixed for the resistant N2 cell line (Figure 13C).

When those mutation rates are $10^{-8}$ per cell per hour, by 50 hours nearly 90% of the simulated populations are dominated by mutants resistant to the phage (Figure 13D).

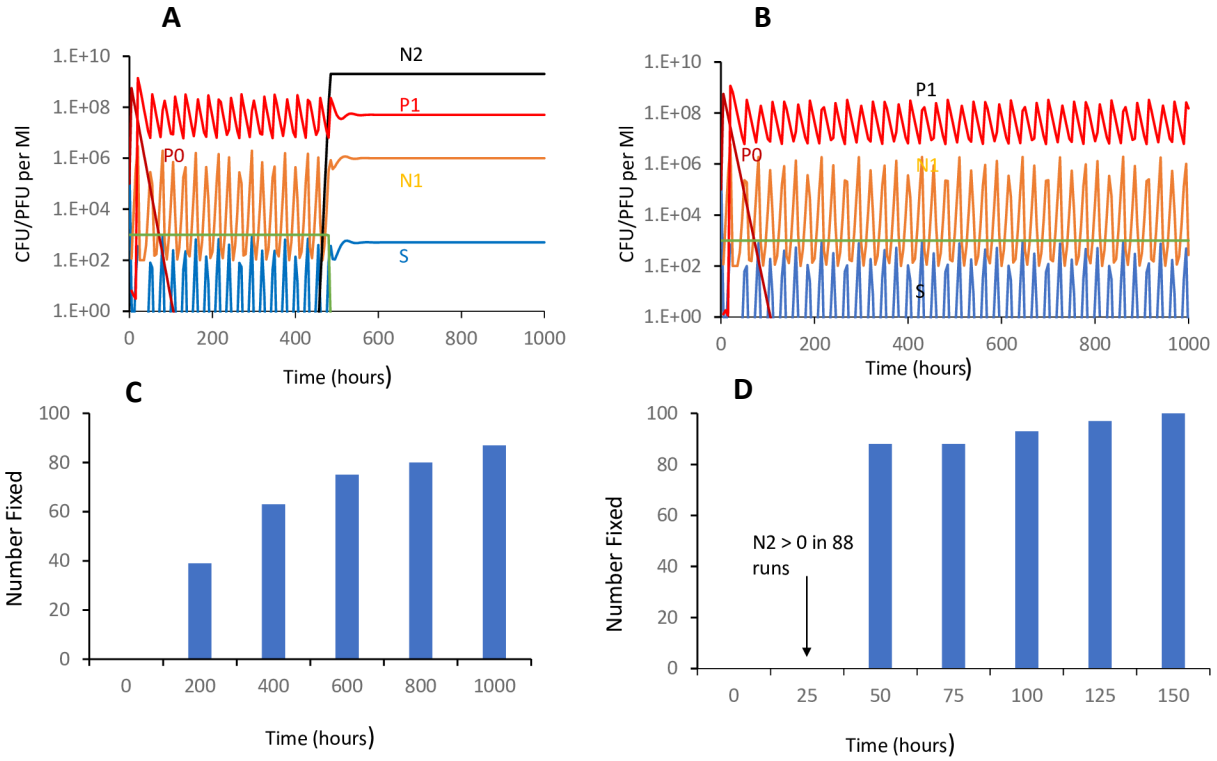



**Figure 13.** Simulation results – model of the population and evolutionary dynamics of bacteria and phage where there are host range mutations in the phage P0→P1 and two mutations are required for resistance to the phage, S→ N1 → N2. Standard parameter values v=1 for all, e= $5x10^{-7}$, k=0.25, δ=$1x10^{-8}$, β=100, w=0.2, $\mu$m= $5x10^{-5}$, vol = 10 ml. Initial densities S=$10^6$, P0=$10^5$. Changes in the densities of bacteria and phage in two simulated runs $\mu$b=$\mu$p=$10^{-9}$. (A) Simulation where resistance, N2, evolved and B where it did not. (C)Number of 100 runs fixed for N2 at different times $\mu$b=$\mu$p=$10^{-9}$. (D) Number of 100 runs fixed for N2 at different times, $\mu$b=$\mu$p=$10^{-8}$.

***Experiments Results:***

<u>**1**- Populations of bacteria confronted with one phage</u>

For these experiments we use the phage T3. With *E. coli* B/6 we generate mutants resistant to these phage and, when we expose the resistant mutants to T3 we get a host range phage that replicate on the resistant mutants. Then, by exposing the resistant B/6 to the host range phage we

get the second order resistant mutants. This is consistent with the model depicted in Figure 12. At this time we do not know whether the arms race can continue with a host range phage capable of replicating on the second order resistant mutant. In Figure 14, we present the result of two serial transfer experiments with T3 and *E. coli* B/6. In both cases, over the period of the experiment, the bacteria remained limited by the phage.

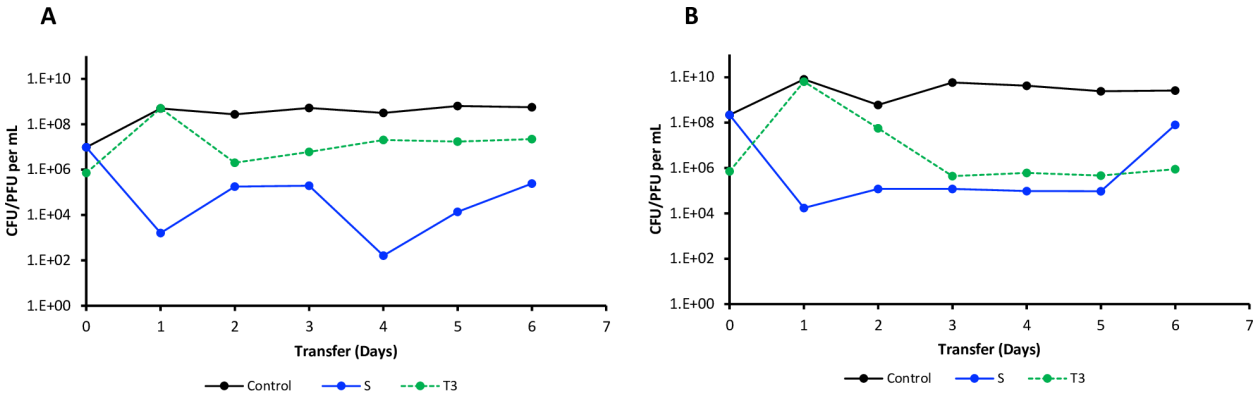



**Figure 14.** Serial transfer experiments with T3 and *E. coli* B/6. (A, B) Changes in the densities of phage and bacteria.

In Figure 15, we explore the population dynamics of T3 in combination with T4 and T5. When cultured with T3 and T4, the population remains phage-limited during the course of the experiment (Figure 15A). When *E. coli* B/6 is cultured with both T3 and T5 the population is nutient limited (Figure 15B). We also performed an experiment with T3 in combination with both T4 and T5. In both cases the *E. coli* B/6 population remained phage limited for the course of the experiment (Figure 16 A and B).

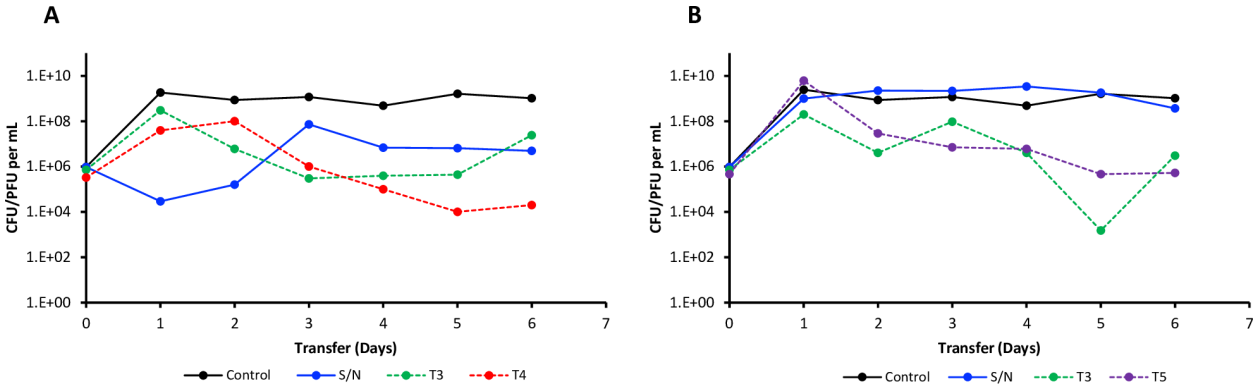



**Figure 15.** Serial transfer experiments with two phage and *E. coli* B/6. Changes in the densities of phage and bacteria with (A) T3 and T4 and (B) T3 and T5.

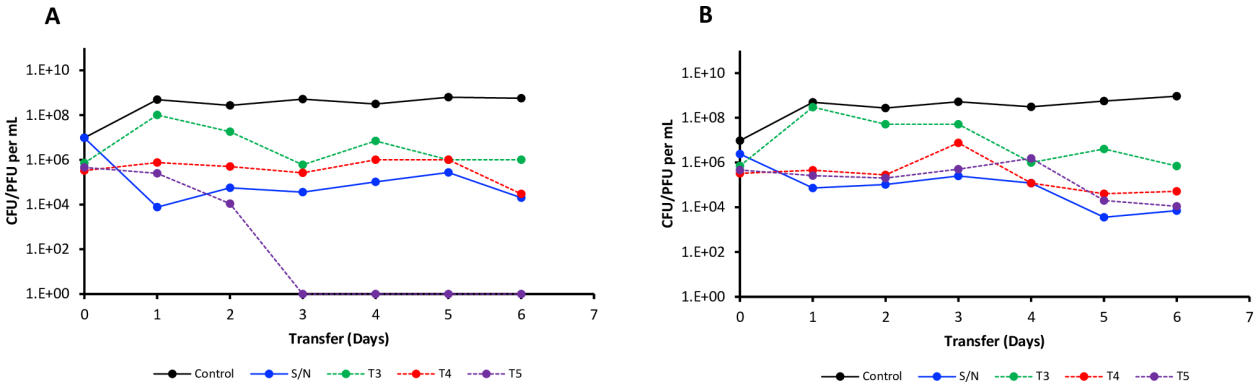



**Figure 16.** Serial transfer experiments with three phage and *E. coli* B/6. (A, B) Two replicas of changes in the densities of phage and bacteria with *E. coli* B/6 and T3, T4, and T5.

## Scenario III. Mucoidy, a generalized mechanism of resistance to multiple phage

### *Experimental Results*

Chaudhry et al. demonstrates the maintenance of a high phage titer of a single phage on mucoid *E. coli* MG1655 *(21)*. Thus, our interest in this paper is the multiple phage dynamics with mucoid *E. coli* K12.

**1 -** Population of mucoid bacteria confronted with two phage

In Figure 17 we present the six-day serial transfer experiments of mucoid *E. coli* K12 and two phages. In populations mixed with T3 and T4, T4 and T5, and T3 and T5 the bacteria ascend and remain at a nutrient limited density in Figure 17A-B, 18A-B, 19A-B, respectively. The

sensitivity tests revealed that the population was dominated by T3 sensitive and T4 resistant bacteria in Figure 17A-B. In 18A, the population of *E. coli* was resistance to T4 and sensitive to T5, while the bacterial density remained nutrient limited. In 19A-B, bacteria was found to be sensitive to T3 and T5 through a spot test, on day six of the transfer. The population was limited by the nutrients.

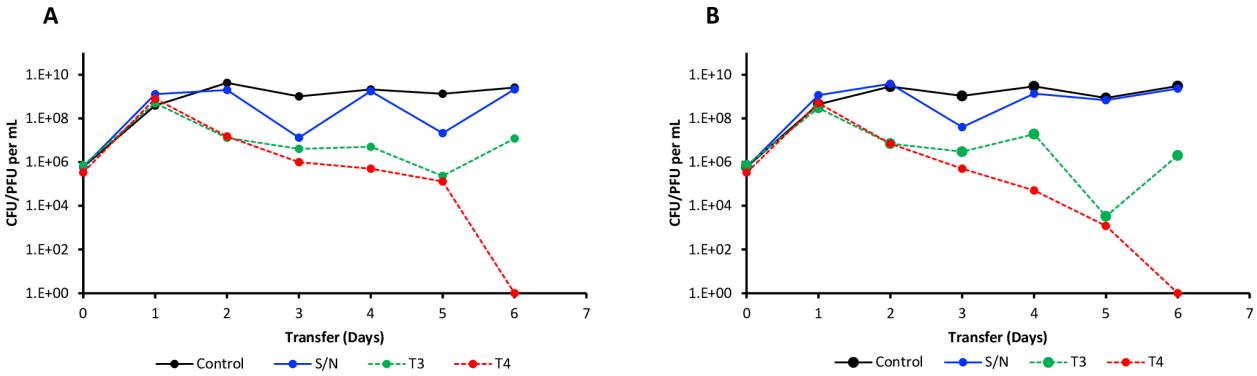



**Figure 17.** Serial transfer of one mucoid bacteria and two phage. (A,B) Changes in the densities of phage and bacteria of *E. coli* B/6 with T3 and T4 in a six day serial transfer.

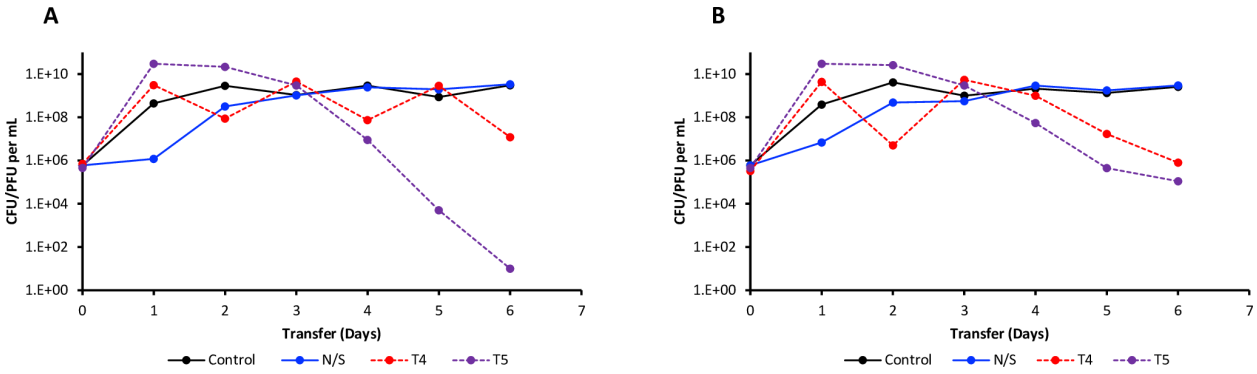



**Figure 18.** Serial transfer of one mucoid bacteria and two phage. (A,B) Changes in the densities of phage and bacteria *E. coli* B/6 with T4 and T5 in a six day serial transfer.

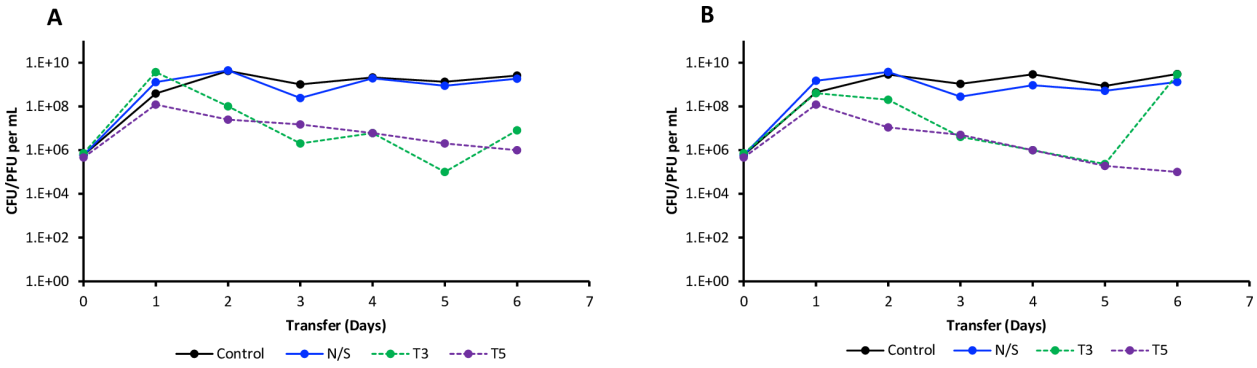



**Figure 19.** Serial transfer of one mucoid bacteria and two phage. (A,B) Changes in the densities of phage and bacteria of *E. coli* B/6 with T3 and T5 in a six day serial transfer.

## 2 - Population of mucoid bacteria confronted with three phage

Figure 20A-B show the population dynamics experiment with mucoid *E. coli* and T3, T4, and T5. The population is nutrient limited in both experiments, while the all phage apart from T5 were maintained in the transfer.

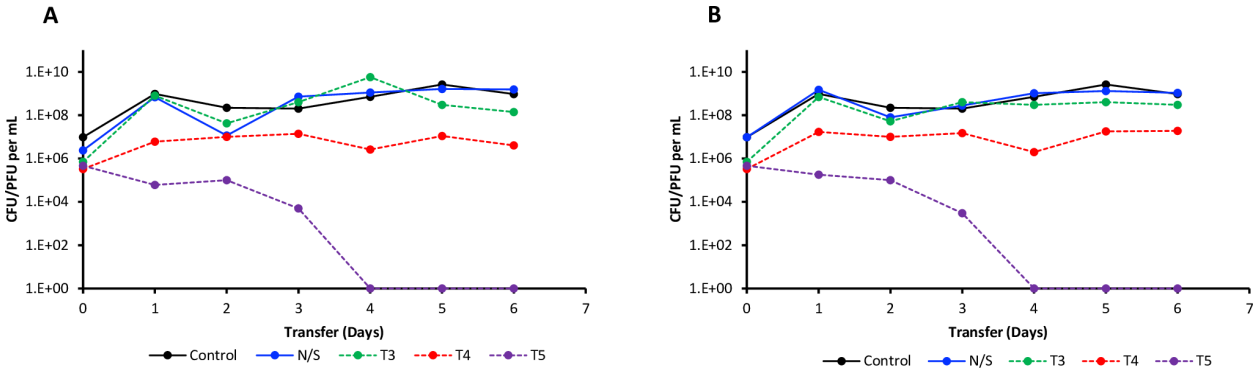



**Figure 20.** Serial transfer of one mucoid bacteria and three phage. (A,B) Changes in the densities of phage and bacteria of *E. coli* B/6 with T3, T4 and T5 in a six day serial transfer.

## Discussion

The seemingly inevitable issue of bacterial resistance to phage therapy must be addressed, so we explore the conditions in which resistance evolves in a population of bacteria confronted with phage. With a better understanding of the phage-bacterial population dynamics, phage treatment practices can be established to improve their efficacy in treating life-threatening bacterial infections. Our theoretical results predict in the condition of a single point mutation for resistance in bacteria, resistance will evolve in almost every case. In scenario I, in the case of a single phage in a population of bacteria, resistance will almost invariably evolve, so that the population is no longer limited by the phage. In the case of *E. coli* B/6 treated with two phage, we consider two situations: One, where resistance evolves rapidly (scenario I, model I) and two, where resistance is delayed (scenario I, model 2). In the first case, resistance to both phage is evolves rapidly if the mutation rate is low, $10^{-9}$, as the initial phage can only replicate on sensitive cells. In the second case where resistance to the phage is delayed, host-range phage mutants, P1 and P2, that we assume previously evolved to replicate on resistant bacteria, N2 and N1 respectively, significantly delay the rise of the double resistant mutant, N12, in the population. Stated in another way, phage in model 2 are ahead of phage in model 1 because the phage can readily replicate on resistant bacteria in the population without generating a mutation. Finally, in the case of a three-phage combination, resistance to all three phage can evolve when the rate of mutation is high, $10^{-8}$. The corresponding experimental results suggest a similar qualitative result: the populations of bacteria treated with one, two and three phage combinations of the lytic phages, wild phage, T4, and T5, become nutrient limited, suggesting the evolution of resistance to the phage in solution.

In scenario II - bacteria that generate a two-point mutation for resistance to the treating phages capable of generating host range phage mutants - the theory suggests bacteria will evolve resistance to all states of the phage and remain fixed in the population. The parallel experimental results do not corroborate the theoretical results. The population of *E. coli* B/6 treated with the host range phage we consider, T3, is consistently phage limited. In the two phage combination with T3, we observe two qualitatively different outcomes. A T3 and T4 treated population was phage limited while the T3 and T5 treated population was nutrient limited. When a population of *E. coli* B/6 is confronted with T3, T4, and T5, the bacterial density is significantly phage limited, suggesting that the bacteria did not evolve resistance to all three phage. In the third solely experimental scenario we consider, mucoid *E. coli* are confronted with a combination of two or three phage. We found that the population rapidly and consistently becomes nutrient limited in both conditions.

When exploring the conditions in which resistant bacteria evolve when confronted with phage through three different scenarios, we observe distinct phage-bacteria and phage-phage dynamics for each result. In the context of phage therapy use, the results do not indicate a clear solution to eliminate resistance but rather a step towards elucidating effective phage treatment regimens that address these phage-bacteria dynamics. Although resistance generally evolves in all theoretical and several experimental results, we determine three conditions that reduce or delay bacterial resistance in a population treated with phage. One, multiple phage treatment is superior to a single phage treatment, two, phage therapy should include at least one phage with the capability of responding to phage-bacteria dynamics with host range mutations to prevent or delay the rise of resistant bacteria, and three, a phage cocktail should begin with a phage able to

replicate on bacteria resistance to other phage in the population to improve the success of phage therapy.

To address the first treatment condition able to delay resistance, we determined using lytic phages wild phage, T4 and T5 alone cannot substantially reduce the bacterial density, confirming the theoretical results. However, when combining multiple phages to treat a population of bacteria, resistance is delayed, or the population is moderately phage limited. The support for multiple phage treatment was confirmed in scenario I and II because the coexistence of phage and degree to which the population was limited by the phage was correlated with the number of types of the treating phage. Multiple studies, *in-vitro* and *in-vivo* have confirmed this observation and noted its importance in improving phage therapy (11, 14, 16). Mechanistic reasons for the success of multiple phage, phage cocktails, include the hierarchy idea and synergistic effects. With respect the hierarchy as a mechanism, if multiple phage are used for treatment it is likely for a bacteria to become resistant to one phage but remain sensitive to the other phage (16). Phage can also act synergistically, as one phage can have an enzyme that degrades the extracellular carbohydrates on the bacterial cell for infection that another phage can exploit (16). A similar dynamic is observed in Coberley et al. suggests cells resistant to one phage could provide a "spatiotemporal refuge" for the other phage (28). Both phenomena could explain why T5, which is normally lost due to rapid evolution of bacterial resistance, is maintained when mixed with T3 (Figure 15). For example, the multiple host range phage mutants of T3 could refuge T5 and allow for their coexistence. In the T3, T4, and T5 case, the phage-bacteria and phage-phage dynamics are complex. The dynamics suggest a hierarchy or synergy mechanism for the phage coexistence and ability to limit the bacterial population. While multiple studies have addressed the success and mechanisms responsible for the success of phage

cocktail treatment, no study discusses the implication for host range mutations in the phage used for phage therapy or a phage cocktail, and their capacity to delay or prevent resistance – condition two and three.

Scenario I and II present results that support condition two and three that we propose reduce or delay the evolution of resistant bacteria. Condition two suggest that the ability of phage therapy to eliminate or delay the ascent of bacteria resistant to all treating phage is dependent on the inclusion of a phage with the capability of generating a host range mutant and condition three suggests that using a phage that can replicate on the bacteria resistant to phage that adsorb at a separate receptor decreases the rise of the resistant bacteria. Condition two indicates that phage with host range mutation capabilities should be heavily considered when designing a phage cocktail because of their capacity to limit a bacterial population and prevent the evolution of a multiply resistant bacterial mutant. All experimental cases, apart from the T3 and T5 combination, suggest any combination of phage with T3 significantly limits the population of *E. coli* B/6 or delays resistance. We were unable to test for the mechanism of T3, but we hypothesize that parameters specific to T3 such as a low mutation rate or longer latent period, could be responsible for its behavior. Others such as Fraser et al. confirm the speculation that T3 can generate host range mutants through recombination experiments that prove T3 mutants differ at more than one locus (29). In addition, Fraser et al. suggests T3 has an extended latent period, implying that delayed latency could allow for the capability of host range mutation (29). Condition three supports the idea of designing phage therapy to be inclusive of phage that can replicate on resistant bacteria. When comparing the frequency and rate at which resistance arises in scenario I, model 1 vs. model 2, both frequency and rate of resistance are substantially lowered in the case that phage can replicate on bacteria resistant to another phage in the

population, model 2. When designing a phage cocktail suggested by the condition three, one could isolate the infecting bacteria from a patient, generate phage resistant to different mutants of the bacteria, and administering those phages in the treatment regimen. Treatment regimens that involve using the patient to generate an improve phage or phage cocktail is suggested in (30). Although time consuming, this treatment type could allow for a personalized treatment that can rapidly and significantly delay the evolution of bacteria resistant to multiple phage.

In contrast to the findings that mucoidy supports the high density of phage in Chaudhry et al., we see that when mucoid *E. coli* is in culture with two phages, they cannot maintain a high density of phage. This could be due to the high reversion between sensitivity and resistance in the mucoid phenotype (21). The reversion could allow the development of resistant and knockdown of the phage density as a result. In the context of phage therapy, the result suggests that phage therapy would be highly problematic when used to treat mucoid infections in the lung for example, and should be considered for combination therapies with antibiotics or nanoparticles (10, 31,32).

Although resistance to phage remains a problem for phage therapy, exploiting certain conditions of the phage-bacterial dynamics could allow for the prevention or delay of resistant bacteria from ascending and dominating the population. Prevention would be the ultimate goal of phage cocktails, however a delay in the ascent of resistance could allow time for the immune system to assemble and coordinate an attack on the resistant bacteria so that the infection can be cleared (16). With continued research on the mechanisms responsible for the success and failure of phage therapy, it can become a more robust therapy that can reduce and delay resistant bacteria and treat multi-drug resistant infections.

**Caveats and excuses**

Here we address certain discrepancies and caveats we make in the paper. Our model simulated in Berkeley Madonna™ considered chemostats, while the experiment we conduct are in a serial transfer condition. We will generate serial transfer models for these experiments.

**Future Directions**

There are several experiments we would like to complete in order to address all the questions and hypotheses in this paper. For instance, we like to estimate parameters, conduct a three-phage experiment with lytic phage unable to generate host range mutations, and to generate models for the cases of two and three phage combinations with T3, a phage capable of producing a host range mutant. In addition, we would like to conduct experiments that could elucidate the phage-phage and phage-bacterial dynamics and/or synergies we observe in certain experiments.

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# Appendix

Berkeley Madonna Programs

## I – Model for Figure 1

```
{Lytic Phage Model - Chemostat culture no time delay}
METHOD EULER
STARTTIME = 0
STOPTIME=1000
DT = 0.001
DTOUT = .5
init S=1E6  {Sensitive}
init P = 1E2 {Phage}
init NR=100 {Resistant}
init R=100 {Initial Resource}
vs = 1 {Growth rate sensitive}
vr = 1 {Growth rate resistant}
c=1000 {Reservoir resource}
w=0.2 {Flow rate}
e=5E-7 {Conversion efficiency}
d=1e-8 {adsorption rate constant}
k=0.25 {Monod constant}
bu=50 {Bursrt size}
mu =1E-7 {Rate of mutation to resistance}
nu =1E-6 {Rate of transition to sensitive|}
vol=10 {Volume}
psi=R/(R+k)
d/dt (R) = w*(c-R)  - (vs*S +vr*NR)*psi*e
d/dt (S) = vs*S*psi - d*S*P*psi*DX +nu*NR-XN/DT- w*S
d/dt (NR) = vr*NR*psi +XN/DT -nu*NR  - w*NR
d/dt (P) =  S*P*d*bu*DX- w*P

{Refuge - When S < SREF, the phage don't adsorb}
SREF=0
DX=IF S < SREF THEN 0 ELSE 1
{Mutation - Monte Carlo}
x=RANDOM(0,1)
XN=IF x < S*mu*DT*Vol*psi THEN 1/VOL ELSE 0
```

**Figure 3 Model**

```
{Two step phage resistance continuous culture}
```

```
{Two states}
METHOD EULER
STARTTIME = 0
STOPTIME = 100
DT = 0.0001
DTOUT=1

Init N0 = 1e7 {Sensitive to P0 and  P1}
init N1=0 {Resistant to P0 resistant sensitive to P1}
init N2=0 {Resistant to P0 and P1}
init P0=100 { Can only replicate on N0}
init P1=0 {Can replicate on N0 and N1}
init R=1000 {Resource concentration}

mb=1e-9 {Mutation rate N0->N1, N1-->N2}
nb =1e-7 {Transition rate N1-->N0, N2-->N1 deterministic}
mp =1e-7 {Host range P0-->P1}
w =0.1 {Flow rate}
vol = 1 {Volume}
v0 =1 {Max growth N0}
v1=1 {Max growth N1}
v2=1 {Max growth rate N2}
d0 =1e-7 {Adsorption rate P0}
d1=1E-7 {Adsorpton rate P1}
e =5E-7 {Conversion efficiency}
B=50 {Burst size all}
C=1000 {Resource in the reservoir}
k=1 {Monod constant}
psi=R/(R+k)

d/dt (R) = w*(C-R) -psi*e*(v0*N0+v1*N1+v2*N2)
d/dt (N0) =v0*N0*ZZ*psi - N0* (d0*P0+d1*P1)*psi*Z -XN1/DT + N1*nb  - w*N0
d/dt (N1) = v1*N1*psi -N1*d1*P1*psi*Z +XN1/DT -N1*nb +N2*nb  -w*N1
d/dt (N2) = v2*N2*psi +XN2/DT  -N2*nb- w*N2
d/dt (P0) = d0*N0*P0*B*psi*Z -YP1/DT -w*P0
d/dt (P1)  = P1*(d0*N0 +d1*N1)*B*psi*Z +YP1/DT -w*P1

{Mutation}
z=RANDOM(0,1)
XN1=IF z<mb*N0*DT*vol*psi THEN 1/VOL ELSE 0
z1=RANDOM (0,1)
XN2 = IF z1<mb*N1*dt*vol*psi THEN 1/VOL ELSE 0
z2= RANDOM (0,1)
YP1= IF z2<mp*P0*vol*dt*psi THEN 1/VOL ELSE 0

{Refuge}
```

```
ref =100
Z= IF (N0+N1+N2)<ref THEN 0 ELSE 1
ZZ= IF N0<0.5 THEN 0 ELSE 1
```

**Three phage model – used for the two phage simulations Figure 5 and the three phage model Figure 6**

```
METHOD EULER

STARTTIME = 0
STOPTIME=10
DT = 0.02

{Lytic Phage Model - Chemostat no time delay}
{Three Phage Model}
METHOD EULER
STARTTIME = 0
STOPTIME=1000
DT = 0.001
DTOUT = .5


init S =1E6  {Sensitive}
init N1=0 {Resistant 1}
init N2 =0 {Resistant 2}
init N3 =0 {Resistant 3}
Init P1 = 100 {Replicates on S, N2, and N3}
init P2=100 {Replicates on S N1 and N3}
init P3=100 {Replicates on S, N1, and N2}
init N12=1 {Resistant to 1 and 2}
init N23=1 {Resistant to 2 and 3}
init N13=1 {Resistant to 1 and 3}
init N123= 0 {Resistant to 1, 2, and 3}
init R=100 {Initial Resource}
vs = 1 {Growth rate sensitive}
vr=1 {Growth rate resistant N1, N2, and N3}
vr2=1 {Max growth resistant N12, N13, N23}
vr3=1 {Max growth resistant N123}
c=100 {Reservoir resource}
e=5E-7 {Conversion efficiency}
d=1e-10 {adsorption rate constant}
k=0.25 {Monod constant}
bv=100 {Burst size}
m= 1e-5 {Transition rate Resistant to sensitive}
ub= 1E-7 {Mutation rate to resistance S->N1, S->N2, N1-> N12, N2->N12, N12->N123 etc}
```

```
ref= 100 {Refuge density of cells that can support the phage, when that's too low, there's no adsorption}
w=0.2 {Flow rate}
vol=10 {Volume}
C=1000 {Reservoid resource}

d/dt (R) =  w*(C-R)  - (vs*S +vr*N1+vr*N2+vr*N3 +
vr2*N12+vr2*N23+vr2*N13+vr3*N123)* (r/(r+k))*e

d/dt (S) = (vs*R/(R+k))*S - d*S*(P1+P2+P3)*X +m*(N1+N2+N3) - ub*S - GN1/DT -GN2/DT -GN3/DT -w*S

d/dt (N1) = (vr*R/(R+k))*N1-d*N1*(P2+P3)*X-m*N1 +m*N12 + m*N13 +GN1/DT-GN121/DT -GN131/DT -w*N1

d/dt (N2) = (vr*R/(R+k))*N2-d*N2*(P1+P3)*X-m*N2 +m*N12 + m*N23 +GN2/DT-GN122/DT -GN232/DT-w*N2

d/dt (N3) = (vr*R/(R+k))*N3-d*N3*(P1+P2)*X-m*N3 +m*N13 + m*N23 +GN3/DT-GN133/DT -GN233/DT-w*N3

d/dt (N12) = (vr2*R/(R+k))*N12 -d*N12*P3*X - 2*m*N12 +m*N123
+GN121/DT+GN122/DT - GN12312/DT -w*N12

d/dt (N23) = (vr2*R/(R+k))*N23 -d*N23*P1*X - 2*m*N23 +m*N123
+GN232/DT+GN233/DT-w*N23


d/dt (N13) = (vr2*R/(R+k))*N13 -d*N13*P2*X - 2*m*N13 +m*N123
+GN131/DT+GN133/DT-w*N13

d/dt (N123) = (vr3*R/(R+k))*N123 +GN12312/DT+GN12323/DT + GN12313/DT - 3*m*N123
-w*N123

d/dt (P1) = P1*d*bv*((S+N2+N3+N23)*X)-w*P1
d/dt (P2) = P2*d*bv*((S+N1+N3+N13)*X)-w*P2
d/dt (P3) = P3*d*bv*((S+N1+N2+N12)*X)-w*P3

NT=S+N1+N2+N3+N12+N13+N23+N123
PT=P1+P2+P3
{Monte Carlo Mutation}

ran1=RANDOM(0,1)
GN1= IF ran1< (S*ub*dt*vol) AND S >0 THEN 1/vol ELSE 0

ran2=RANDOM(0,1)
```

```
GN2=IF ran2<(S*ub*dt*vol) AND S>0 THEN 1/vol ELSE 0

ran3=RANDOM(0,1)
GN3=IF ran3<(S*ub*dt*vol) AND S>0 THEN 1 ELSE 0

ran121=RANDOM(0,1)
GN121=IF ran121<(N1*ub*dt*vol) AND N1>0 THEN 1/vol ELSE 0

ran131=RANDOM(0,1)
GN131=IF ran121<(N1*ub*dt) AND N1>0 THEN 1/vol ELSE 0

ran133=RANDOM(0,1)
GN133=IF ran121<(N3*ub*dt*vol) AND N3>0 THEN 1/vol ELSE 0

ran122=RANDOM(0,1)
GN122=IF ran122<(N2*ub*dt*vol) AND N2>0 THEN 1/vol ELSE 0

ran232=RANDOM(0,1)
GN232=IF ran232<(N2*ub*dt*vol) AND N2>0 THEN 1/vol ELSE 0

ran233=RANDOM(0,1)
GN233=IF ran233<(N3*ub*dt*vol) AND N3>0 THEN 1/vol ELSE 0

ran12312=RANDOM(0,1)
GN12312=IF ran12312<(N12*ub*dt*vol) and N12>0 THEN 1/vol ELSE 0

ran12313=RANDOM(0,1)
GN12313=IF ran12312<(N13*ub*dt*vol) and N13>0 THEN 1/vol ELSE 0

ran12323=RANDOM(0,1)
GN12323=IF ran12312<(N23*ub*dt*vol) and N23>0 THEN 1/vol ELSE 0

{refuge saving}
X= IF(S+N1+N2+N3)<ref AND (P1+P2+P3)>0 THEN 0 ELSE 1
```