Mucoidy, a general mechanism for maintaining lytic phage in populations of bacteria

Waqas Chaudhry[1], Esther Lee[1], Andrew Worthy[1], Zoe Weiss[1], Marcin Grabowicz[2,3,4], Nicole Vega[1*], Bruce Levin[1,2**]

[1] Department of Biology, Emory University, Atlanta, GA 30322, USA.

[2] Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA 30322

[3] Department of Microbiology & Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA.

[4] Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA.

*nvega@emory.edu

**blevin@emory.edu

Abstract

We present evidence that mucoidy provides a general answer to the longstanding question of how lytic phage are maintained in bacterial populations. In serial transfer culture, populations of mucoid *E. coli* MG1655 that appear to be broadly resistant to lytic phage with different receptors are capable of maintaining these phage with little effect on the total density of bacteria. Based on the results of our analysis of a mathematical model, we postulate that the maintenance of phage in populations dominated by mucoid cells can be attributed to high rates of transition from the effectively resistant mucoid states to susceptible non-mucoid states. Our test of this "leaky resistance" hypothesis with both population dynamic and single cell experiments are consistent with it. We discuss reasons for the generalized resistance of these mucoid *E. coli,* and the genetic and molecular mechanisms responsible for the high rate of transition from mucoid to sensitive states.

Introduction

Bacteriophages are natural predators (parasitoids) of bacteria and are present in natural microbial communities across environments, from marine and aquatic systems [1] to the intestine of the mammalian host [2]. By mutation, bacteria can become resistant or, in the case of CRISPR-Cas, immune to these viruses [3]. Most commonly these resistant or immune cells ascend to dominate the bacterial population, but the phage continue to be maintained [4–6]. How virulent phage are maintained in populations of bacteria dominated by cells upon which they cannot replicate is a longstanding question that has been addressed both theoretically and experimentally [4–8]. Thanks to concerns about the factors responsible for maintaining and stabilizing the structure of bacterial communities, microbiomes [9–13] if you prefer, and the resurrection of interest in phage therapy [14–16], this question - and more broadly, studies of the population and evolutionary dynamics of the interactions between bacteria and phage - have become an increasingly important and relevant avenue of research.

In this study of the population and evolutionary dynamics of the interactions between bacteria and phage, we present a compelling and general answer to this question of the conditions for the existence of lytic phage [14], mucoidy. Those who work with *E. coli* K12 and other Gram negative bacteria occasionally see shiny, "snotty" looking, colonies on agar which can attributed to contamination with phage [17,18]. The overproduction of exopolysaccharide responsible for the mucoid phenotype is thought to confer resistance by providing a physical barrier between the cell surface and the would-be infecting phage [19–22].

Despite the long-standing knowledge of the relationship between mucoidy and exposure to bacteriophage, the ecological role and consequences of this association have been given little consideration. Although it is known that mucoidy confers some degree of resistance to phage [23], reversion to a non-mucoid state is generally rapid once phage have been removed [18], suggesting that mucoidy is costly. However, this costly mechanism can provide broad-range resistance to multiple lytic phage [18], suggesting a plausible advantage to mucoidy over more specific resistance mechanisms in environments with multiple phage. Not so clear is why, when *E. coli* are exposed to some but not all lytic phage, mucoidy is more likely to arise than envelope resistance.

These observations raise several questions about the population dynamics and ecology of mucoidy-based resistance. Under what conditions will mucoidy be selected for over surface or other resistance or immune mechanisms [19]? How might the ecology of phage-bacterial system differ when resistance is conferred by mucoidy rather than other resistance and immune mechanisms? Why and how, in the absence of phage, do mucoid-colony producing bacteria revert to states that produce non-mucoid colonies? What role does this reversion play in the ecology and evolution of phage-bacteria systems?

To address and answer these questions, we studied the population dynamics of mucoid colony producing in *E.coli* MG1655 following exposure of different virulent (lytic) bacteriophage, T3, T4, T5, T7 and λ^{VIR}. We present evidence that when *E. coli* K12 are exposed to T3, T4 and T7 phage, mucoid colony - producing cells are likely to arise and become the dominant population of bacteria. Moreover, whether they select for mucoidy or not, when introduced into populations of mucoid *E. coli* in serial transfer culture, all five of these lytic phage maintain high density populations whilst the density of bacteria remains at levels similar to that of phage-free cultures.

Based on the results of our analysis of the properties of mathematical/computer simulation models, we postulate that a diverse array of phage can persist on populations of mucoid *E. coli* by replicating on minority populations of sensitive bacteria that are maintained by a high rate of transition from "resistant" mucoid to sensitive non-mucoid colony types. Using a combination of computer simulations and batch culture experiments, and single-cell microscopy, we present evidence in support of this "leaky resistance" [4,24] hypothesis for the maintenance of lytic phage in populations of mucoid bacteria.

Results

<u>I- Selection for, and population dynamics of, mucoid *E. coli* in phage-infected cultures</u>

First, we sought to determine whether the lytic phages varied in their ability to select for mucoidy in *E. coli* MG1655, and these phage-selected mucoids differed from one another

in their interactions with phage. For this, wild type cultures of *E. coli* MG1655 were exposed to T3, T4, T5, or T7 phage and serially transferred over ten passages (Fig. 1). For cultures initiated with T3 or T4, the phage were maintained over all ten transfers and the dominant population of bacteria isolated at the end of the final transfer were mucoid (Fig. 1A and 1B). For T7, the phage were maintained in one the three populations, where mucoid cells were the dominant population of bacteria; in the remaining two T7 cultures, the bacteria were rapidly driven to extinction, followed by extinction of the phage (Fig. 1D). All replicates of the experiments using T5 resulted in extinction of the phage following the emergence of envelope resistance. This resistance was attributed through linkage mapping to mutations in the *fhuA* locus which encodes the T5 receptor, also see [25].

Second, we explored the capacity of these phage to be maintained in population of mucoid bacteria. For the first of these serial transfer experiments, we used the mucoids generated by selection with the phage T7. As seen in Figure 2, all four phage were maintained, and the densities of viable bacteria were high, similar to that of the phage-free controls. Stated another way, resources rather than phage limited the densities of these bacterial populations. Moreover, as can be seen in Figure S1, this result obtains in minimal medium as well as the LB used for the Figure 1 experiment. In the minimal medium experiments, however, mutants with envelope resistance arose in mucoid cultures treated with phage T4 and T5.

These results support the initial assumption that phage-selected mucoidy in *E. coli* provides broad resistance to virulent phage and indicate that co-existence of large populations of virulent phage and mucoid *E. coli* is a general phenomenon that does not depend on the phage initially used for selection. They also suggest that the capacity to select for mucoidy and/or the rate of mutation to mucoidy varied among the phages. These results also suggest that mucoidy can prevail over envelope resistance, but may not do so in all cases, dependent on the environment and on the lytic phage used.

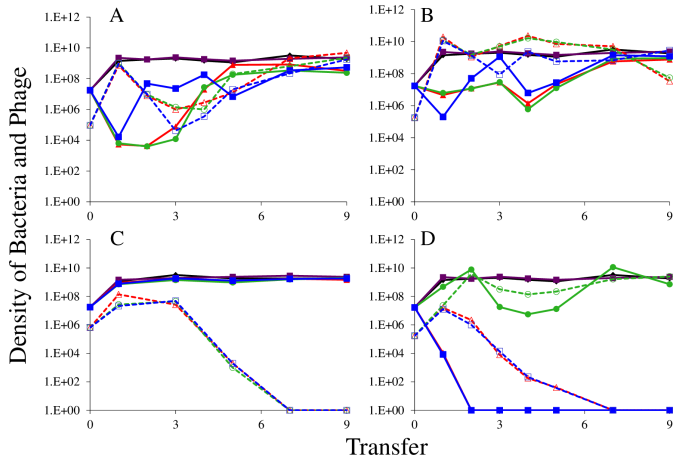


Figure 1: The population dynamics of T3 (A), T4 (B), T5 (C) and T7 (D) phage with wild-type *E.coli* MG1655. Cultures were diluted 1:100 into fresh LB medium every 24 hours; three replicates were performed per condition. Solid lines show bacterial densities in each replicate (red/green/blue); dotted lines show the phage densities in the corresponding cultures. Phage-free cultures (black and purple lines) are shown as controls, to indicate resource-limited population size for the bacteria.

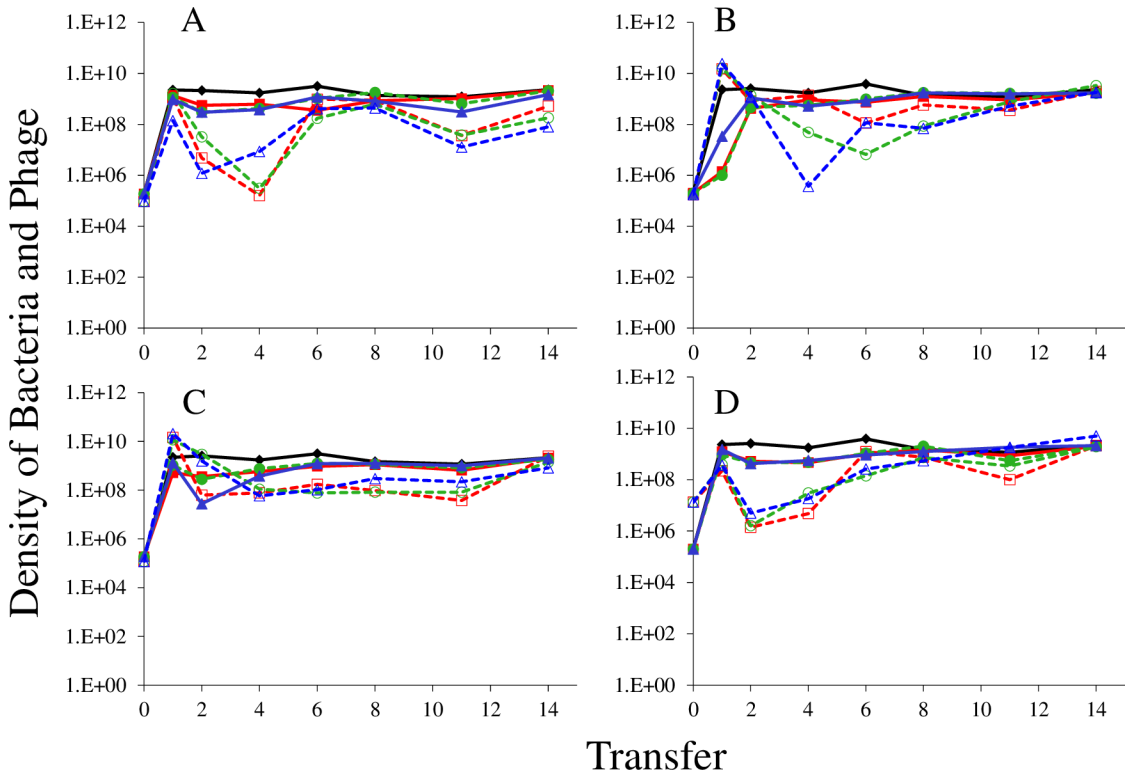


Figure 2: The population dynamics of phage T3 (A), T4 (B), T5(C) and T7 (D) phage with T7 generated mucoid *E.coli* MG1655 as the host strain in LB. Triplicate cultures were diluted 1:100 into fresh LB media every 24 hours, and CFU/PFU measurements were taken via dilution plating. Solid lines show bacterial densities in each replicate (red/green/blue); dotted lines show the phage densities in the corresponding cultures. Phage-free cultures (black and purple lines) are shown as controls, to indicate resource-limited population size for the bacteria. Phage-free controls lost the mucoid phenotype entirely after the 4th serial transfer. At the end of the 15th serial transfer, three colonies from each phage-infected culture were streaked at least three times to get rid of phage, yielding non-mucoid revertants that were again sensitive to the relevant phage.

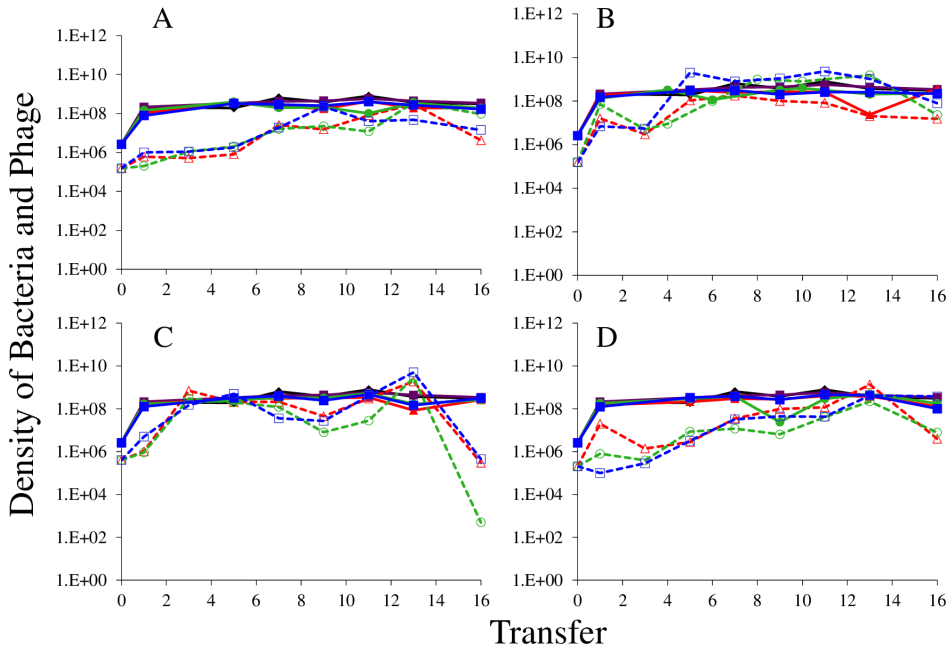


Figure S1: The population dynamics of T3 (A), T4 (B), T5 (C) and T7 (D) phage with T7 generated mucoid *E.coli* MG1655 as the host strain. Cultures were diluted 1:100 into fresh M9 + 0.4% glucose every 24 hours, and CFU/PFU measurements were taken via dilution plating. Solid lines show bacterial densities in each replicate (red/green/blue); dotted lines show the phage densities in the corresponding cultures. Phage-free cultures (black and purple lines) are shown as controls, to indicate resource-limited population size for the bacteria. Note that phage-free controls lost the mucoid phenotype. At the end of the last serial transfer, three colonies from each phage-infected culture were streaked at least three times to get rid of co-evolving phage. These isolated clones were tested against the phage used in the experiment; envelope resistance arose in T4 and T5-infected cultures, accompanied by a loss of mucoidy.

2- Mechanism of phage-induced mucoidy

Next, we investigated the ability of defined mutations in the *E coli* colanic acid synthesis pathway to recapitulate the observed ecological interactions between bacteria and phage, specifically the high-level maintenance of phage on apparently resistant hosts. The "regulator of capsule synthesis" (Rcs) phosphorelay system regulates colanic acid capsule production in *Escherichia coli* [26]. The Rcs phosphorelay is complex, but at its core: RcsF is a sensory lipoprotein that causes activation of the RcsC sensor kinase in an environment-dependent manner; RcsC causes phosphorylation of the RcsB transcription factor; phosphor-RcsB is a positive regulator of the colanic acid biosynthetic operon at the *cpsB* promoter. Overproduction of colanic acid via this pathway results in the distinctive "mucoid" colony phenotype, and mucoid strains featuring mutations in this pathway are selected for by exposure to virulent phage in *E. coli* MG1655 [18].

To understand the dynamics of mucoid selection and the role of mucoidy in phage maintenance, we sought to determine whether EPS-impaired strains, which do not produce mucoid colonies, would be able to maintain phage populations during serial passage. To this end, we tested two gene knockout strains in the Rcs signaling pathway

(Δ*rcsB* and Δ*rcsF*) as well as one gene knockout strain in the EPS operon (Δ*cpsB*), all of which are impaired in capsule synthesis. We also tested a strain (*rcsC137 ompC::Tn5*) that has an activating A904V substitution in RcsC and is therefore constitutively mucoid [27], to determine if the simple induction of mucoidy via a known mechanism was sufficient to allow maintenance of phage. We found that the *rcsC*137 constitutive-mucoid mutant was able to maintain large populations of phage and bacteria for all phage tested, while phage exposure in loss-of-function mutants impaired in mucoidy (Δ*rcsB*, Δ*rcsF*, Δ*cpsB*) resulted in extinction of both phage and bacterial populations when T3 phage were used (Fig. 3). Exposure to T4 or T7 phage resulted in bacterial population collapse in some conditions but not others, largely dependent on whether evolution of envelope resistance occurred; in the Δ*rcsF* background, mucoid colonies were observed, presumably downstream of a mutation compensating for the mucoidy defect in the knockout. Notably, the inactivating mutation in *ompC* in the *rcsC137* constitutive mutant strain, which can confer envelope resistance to some phage [28], had no noticeable effect in these assays. For reasons that are not yet clear, T5 phage were maintained on all host backgrounds regardless of mucoidy, despite the fact that evolution of envelope resistance generally leads to extinction of this phage (Fig. 1).

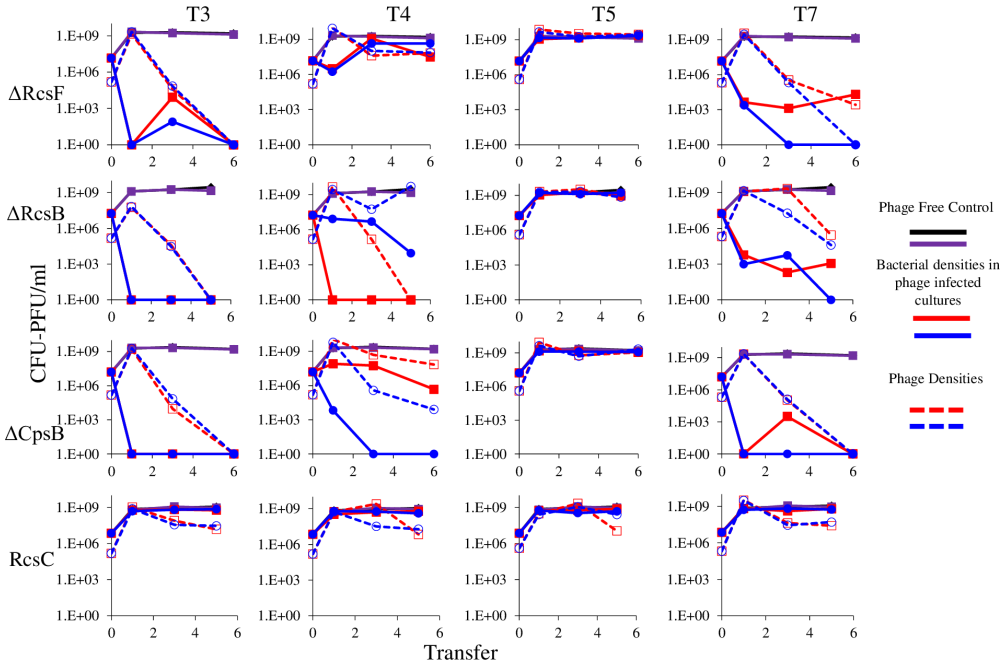


Figure 3. Constitutive presence or absence of mucoidy affects the dynamics of *E. coli*-phage interactions. T3, T4, T5, or T7 phage were co-cultured with genetically modified strains of *E.coli* MG1655 with known gain or loss of function in the Rcs pathway. Strains used were: Δ*rcsF*::kan (pathway loss-of-function knockout of the outer membrane Rcs input), Δ*rcsB*::kan (pathway loss-of-function knockout of the RcsB transcription factor), Δ*cpsB*::kan (pathway loss-of-function knockout of biosynthesis of exopolysaccharide colonic acid), and *rcsC*137 *ompC*::Tn5 (gain-of-function point mutant that is a defined activating mutation of mucoidy). Cultures were diluted 1:100 into fresh LB medium every 24 hours; two replicates were performed per condition. Solid red and blue lines show bacterial densities, while dotted lines show the phage densities in the

corresponding cultures. Phage-free cultures are shown as controls (black and purple lines), to indicate resource-limited population size for the bacteria.

III. A hypothesis for maintenance of phage in mucoid populations of bacteria.

We now consider the population dynamics of lytic phage and mucoid hosts to address two questions. One, how are the phage maintained in mucoid populations of bacteria? Two, under what conditions will envelope resistant mutant bacteria emerge and replace the mucoid cells?

We postulate that the maintenance of high densities of phage in populations of mucoid bacteria can be attributed to a substantial density of sensitive cells generated by a high rate of transition from the mucoid to non-mucoid states, a mechanism that has been called "leaky" resistance [4]. To illustrate how this mechanism can account for the observed maintenance of high densities of phage with little effect on the density of bacteria, and for the common failure to see the ascent of mutants with surface resistance, we use a simple mass-action model of the population dynamics of bacteria and phage (Fig. 5, Equations 1-5). In this model, there are three populations of bacteria – sensitive (S), mucoid (M), and resistant (NR), with densities in cells per ml, and a single population of lytic phage (P), density in particles per ml in liquid culture.

The bacteria grow at maximum rates $v_s > v_m > v_r$ (cell^{-1} hr^{-1}) respectively (S2 Fig), with the realized growth rates dependent on the concentration of the shared limiting resource (R, μg/mL) via a hyperbolic function $\psi(R)=R/(R+k)$ [29], where the Monod constant k (μg/mL) is the concentration of limiting resource where the growth rates are half their maximum value. The phage adsorb to the sensitive and mucoid cells with rate constants $\delta_S >> \delta_M$ (hr^{-1}mL^{-1}) but do not adsorb to the envelope-resistant bacteria. Upon adsorption, both mucoid and sensitive cells produce β phage particles (neglecting the latent period, for simplicity). Transition from sensitive to mucoid states S→M occurs at a rate x, and from mucoid to sensitive M→S at a rate y, both per cell per hour with all rates proportional to $\psi(R)$. The model also allows for transitions from S → NR and from NR → S at rates xs and xr, respectively. As in [4] we assume that the limiting resource is taken up at a rate proportional to the net growth rate of each bacterial population, modified by the biomass conversion parameter e (μg), which is the amount of resource needed to produce a new cell [30]. Serial transfer is simulated by diluting the population of bacteria and phage by a factor $d = 0.01$ every 24 hours, removing the existing resource, and adding C μg/ ml of the limiting resource.

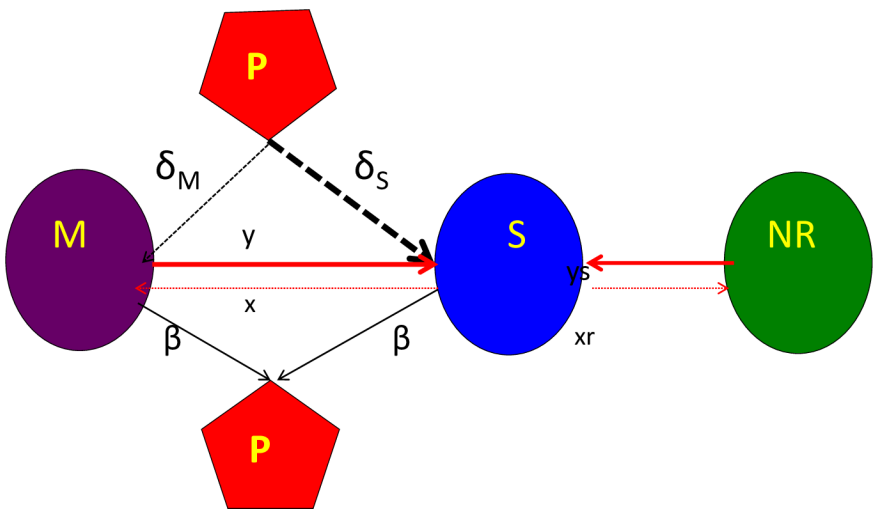


Figure 4 - Diagram of the mass-action model, showing transitions between states. Bacteria may be mucoid (M), sensitive (S) or envelope-resistant (NR). Mucoid and sensitive cells adsorb phage (P), but resistant cells do not. See the text and Methods for full definitions of the parameters, variables, and assumptions behind the construction of this model.

$$\frac{dR}{dt} = -\psi(r) \bullet e \bullet (vs \bullet S + vm \bullet M \bullet vr \bullet N_R) \tag{1}$$

$$\frac{dS}{dt} = vs \bullet \psi(R) \bullet S \ - \delta_S \bullet P \bullet S \bullet \psi(r) \ + \psi(R) \bullet (-x \bullet S + y \bullet M - xs \bullet S + yr \bullet NR) \tag{2}$$

$$\frac{dM}{dt} = vm \bullet \psi(R) \bullet M - \delta_M \bullet M \bullet P \bullet \psi(R) \ + \psi(r) \bullet (x \bullet S - y \bullet M) \tag{3}$$

$$\frac{dN_R}{dt} = vr \bullet \psi(R) \bullet N_R \ + \psi(r) \bullet (yr \bullet S - xr \bullet NR) \tag{4}$$

$$\frac{dP}{dt} = \delta_S \bullet P \bullet S \bullet \beta \bullet \psi(R) \ + \delta_M \bullet P \bullet M \bullet \beta \bullet \psi(R) \tag{5}$$

$$where \ \ \psi(R) = \frac{R}{(R+k)}$$

These equations were solved numerically and the dynamics were simulated with Berkeley Madonna™. The values of the phage infection parameters δ_S and β for the sensitive cells used in these simulations are in the range estimated in [4,31] and in growth rate experiments. Copies of the program used are available from www.eclf.net.

In these simulations, two qualitative observations have to be accounted for: (1) the stable maintenance of a high density of lytic phage with a high (resource-limited level) density of mucoid bacteria in serial transfer culture (Figs. 1 and 2), and (2) the failure of envelope resistant mutants to ascend and replace the mucoid bacteria as the dominant bacterial population. With realistic parameters, the above model can account for these observations.

In populations initially dominated by mucoid cells, within short order the density of phage increases to levels only slightly less than that of the mucoid population, the density of which is near the maximum for the concentration of resource, $\sim 1000/5\text{x}10^{-7} = 2\text{x}10^{9}$. The sensitive cell population rapidly increases in density and then levels off to an apparent equilibrium (Fig. 5A). The equilibrium density of sensitive cells decreases with a greater adsorption rate constant, $\delta_S = 10^{-7}$, rather than 10^{-8} (Fig. 5B). The equilibrium densities of phage, mucoid, and sensitive bacteria in populations initially dominated by sensitive cells are the same as those in populations initially dominated by mucoid bacteria (Fig. 5C). While cells with envelope resistance can initially increase when rare, once the dominant population of mucoids, sensitive cells, and phage reach their equilibrium densities, the density of resistant cells declines because of the higher fitness cost imposed for resistance (Fig. 5D).

The population dynamic mechanisms responsible for establishment and maintenance of the phage in populations dominated by mucoid cells and the failure of bacteria with envelope resistance to become established can be seen from the short-term changes in densities of bacteria, phage and the concentration of resources (Fig. 5E). As the density of sensitive cells increases, so too does the phage population, until growth is halted by depletion of resources. Because of their greater fitness, in the absence of phage the density of sensitive cells increases at a rate greater than the resistant mucoid and these revertants become the dominant population of bacteria (Fig. 5F).

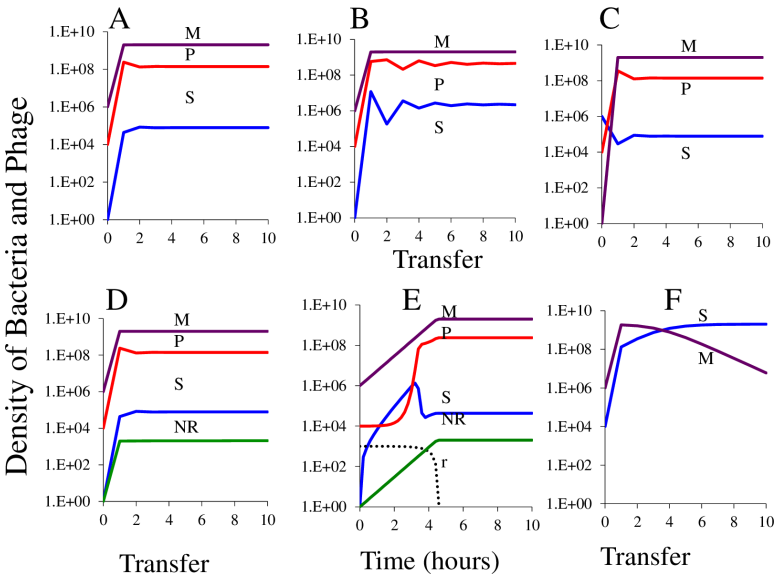


Figure 5. Simulation results, changes in the densities of mucoid, M, sensitive, S, and envelope-resistant, NR, bacteria and virulent phage, P. Standard parameters (C=1000, $e=5\text{x}10^{7}$, $v_s=1.4$, $v_m=1.3$, $v_r=1.2$, k=1, $\beta=100$, $x=10^{-3}$, $y=10^{-5}$, xs=0, yr=0. $\delta_M = 10^{-11}$, $\delta_S = 10^{-8}$), unless otherwise noted. A- A serial transfer population initiated with a high density of mucoid bacteria, free phage and one sensitive cell per ml. B- Populations initiated as in A, but with $\delta_S = 10^{-7}$. C- Populations initiated with a high density of sensitive cells and phage and one mucoid cell per ml. D- Populations initiated as in A, but with one envelope-resistant cell (NR). E- Short term dynamics of the changes in density of populations with the standard parameter values and the starting conditions in A. F- Changes in the densities of mucoid and sensitive bacteria in the absence of phage.

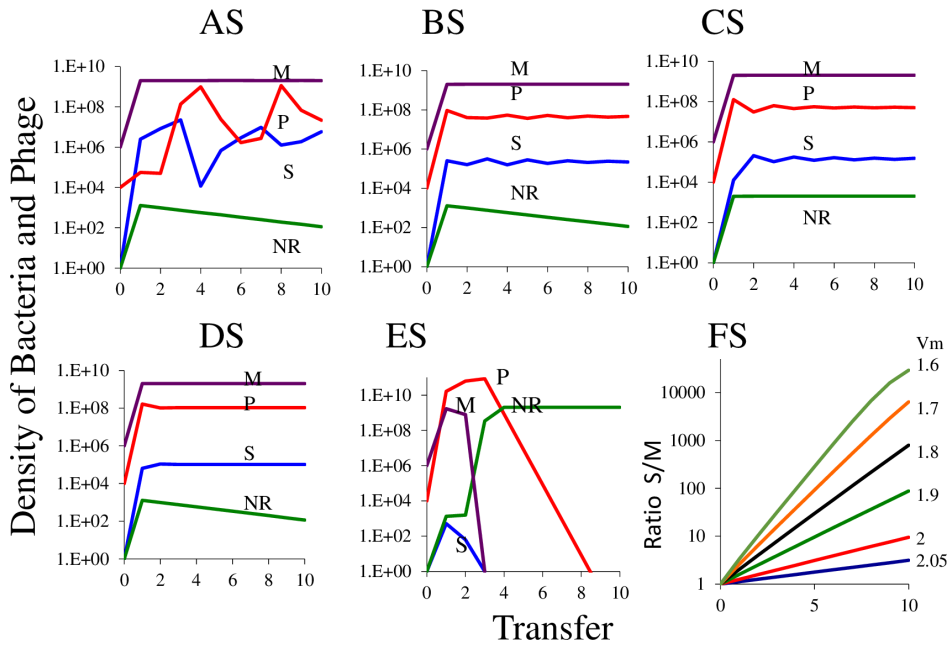


Figure S2: Simulation results, sensitivity to parameter values. Populations shown are mucoid, M, sensitive, S, and envelope-resistant, NR, bacteria and virulent phage, P. Standard parameter values, C=250, β=100, vs=2.1, x=10^{-5}, k=1.0, e=5x10^{-7}. The values of the parameters for above figures are noted in the below table.

	δs	**δm**	**vm**	**y**
AS	2.00E-08	1.00E-11	1.7	1.00E-04
BS	2.00E-07	1.00E-11	1.7	1.00E-04
CS	2.00E-07	1.00E-11	1.6	1.00E-03
DS	2.00E-07	1.00E-12	1.7	1.00E-03
ES	2.00E-07	1.00E-10	1.7	1.00E-03

FS – Changes in the ratio of S/M for different values of the growth rates of sensitive cells.

IV. Test of the leaky resistance hypothesis

In accord with the model, for the sustained density of sensitive cells to be sufficiently great to maintain the phage at the high densities observed, the rate of transition or reversion, from mucoid to sensitive non-mucoid bacteria has to be high, on the order of 10^{-4} to 10^{-3} per cell, and the mucoids have to be substantially less fit than sensitive non-mucoids. The phage adsorption rate and fitness cost parameters also have to be in the range modeled, for resistant cells not to ascend to dominate the culture and lead to the loss of the phage. More information about the range of parameter values needed to account for the population dynamics observed can be seen in Figure S2. In the following, we present two lines of evidence in support of the hypothesis that there is a high rate of transition from mucoid to sensitive, on the order of 10^{-3} per cell per hour.

(i) Population dynamic evidence for a high rate of reversion from mucoids to non-mucoids: One line of evidence for a high rate of transition from mucoid to sensitive non-mucoid states comes from population dynamics experiments using a method similar to that employed in [4] with genetically marked λ phage, λ^{CAM}. This method is based on the

premise that lysogens will be only be formed in populations of effectively resistant mucoid bacteria by infecting sensitive cells in the minority population of non-mucoid bacteria. We first performed experiments demonstrating that like the T-phages, λ^{VIR} maintains high densities in mucoid populations of different types (Fig. S3); notably, as for T5 phage (Fig. 1), mucoidy does not arise in an initially sensitive wild-type population in this medium. We then introduced λ^{CAM} to cultures of T3- and T7-selected mucoids and the *rcsC*137 constitutive-mucoid mutant. We followed the changes in the densities of free phage, the total densities of cells and, by selective plating (see the Materials and Methods), the lysogens and non-mucoid (sensitive) cells generated during the course of the experiment (Figure 6, A, B, and C). While the rates of accumulation of lysogens varied, these data indicate that reversion from mucoid to non-mucoid occurred frequently during growth for all strains.

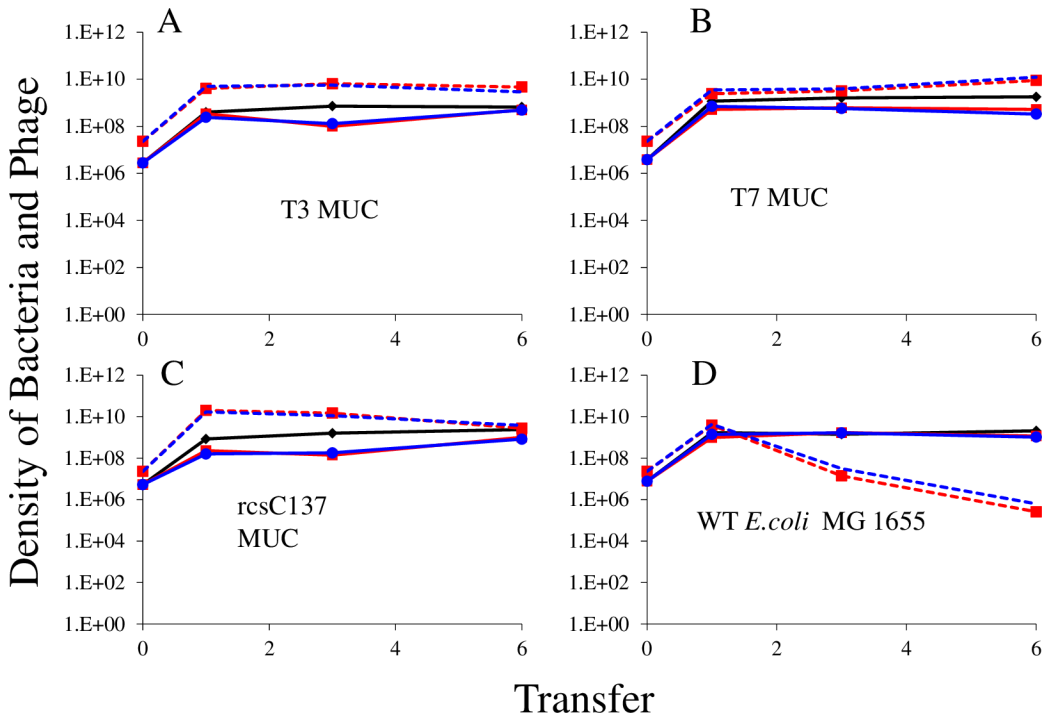
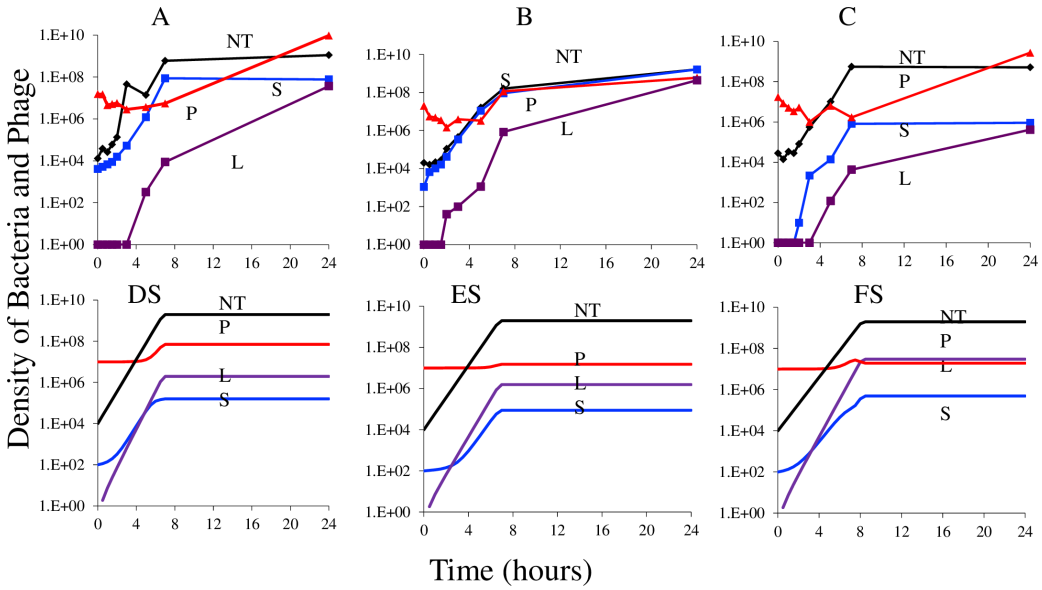


Figure S3: Population dynamics of λ^{VIR} phage with T3-generated mucoid, T7-generated mucoid, *rcsC*137 *ompC*::Tn5 (constitutive mucoid) and WT *E.coli* MG1655. Cultures were inoculated into LB medium with λ_{vir} phage (1:1) and diluted 1:100 into fresh LB medium every 24 hours; two replicates were performed per condition. The decline of phage in wild-type cultures corresponds to the emergence of envelope resistance against λ^{VIR}. Solid red and blue lines show bacterial densities while dotted line show the phage densities in the corresponding cultures. Phage-free cultures (black lines) are shown as controls, to indicate resource-limited population size for the bacteria.



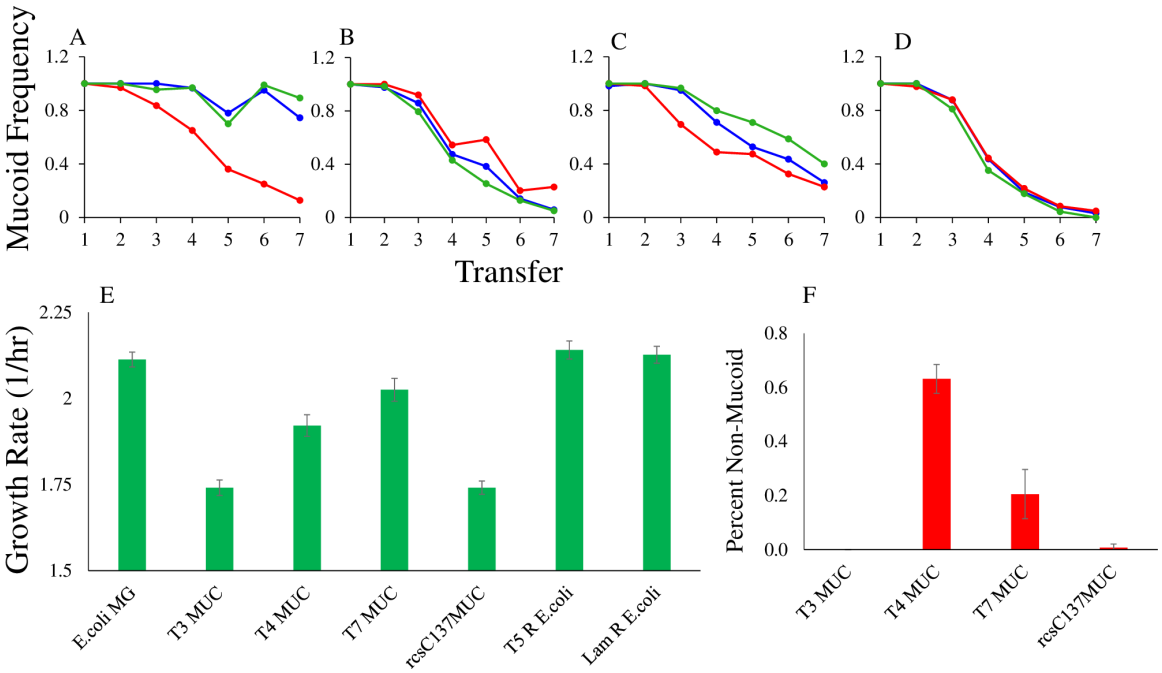
.

Figure 6: Short-term population dynamics of lysogen formation. A, B and C: experimental results, changes in the densities of total cells NT, free λ^{CAM} phage P, lysogens L, and sensitive non-lysogens (revertants) S. A- T3 selected mucoids, B- T7 selected mucoids, C- genetically constructed mucoid *E.coli* MG1655 (*rcsC*137 *ompC*::Tn5). DS, ED, and FS: simulations, changes in population density, standard parameters, Vs=2.1, Vl=2.1, d = $2E10^{-7}$, dm=10^{-11}, x=10^{-5}, C=1000, k= 1, e=$5x10^{-7}$, probability of lysogeny, λ= 0.01, ind = 10^{-4}. DS- Standard parameters with y=10^{-3}; Vm=1.8, ES - standard parameters with y=10^{-4}, Vm=1.8, FS- Standard parameters with y=10^{-3} and Vm=1.5.

To obtain a rough estimate of the rates of formation of lysogens, we used a simulation similar to that employed in [4] (Fig. 6 DS, ES, FS). It is clear that this simple model is not an strictly accurate analogue of the short-term population dynamics of lysogen formation for several reasons. First, in the simulations, λ^{CAM} phage does not increase in density at a rate even close to that observed nor do they reach as high a density. Next, save for the *rcs*137 MUC strain, the predicted density of revertants (S) in simulation is lower than observed in experiments. Most importantly, the predicted rate of increase in the density of lysogens for the T3 and T7 selected mucoids is, with the M->S transition rate of 10^{-3} per hour, less than that observed experimentally and, for the *rcs*137 mucoids, similar to that observed. Nevertheless, these simulations demonstrate that a high rate of transition from mucoidy is necessary to even approximate the observed trends in lysogen formation in an initially mucoid population of cells.

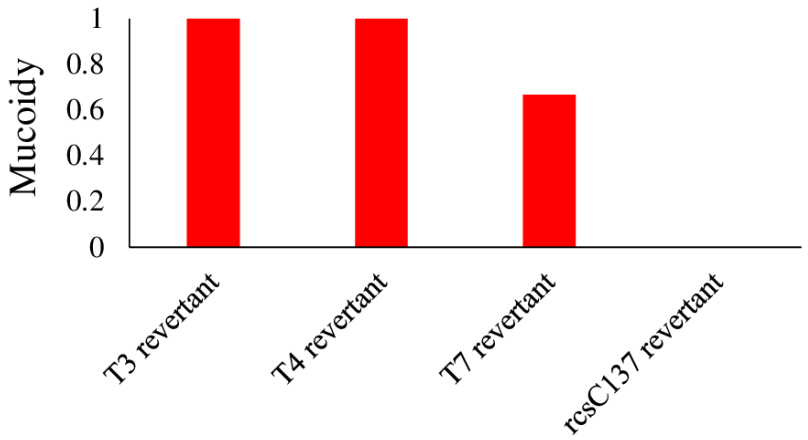
Additional evidence for a high rate of transition from mucoid to non-mucoid and presumably sensitive cells is presented in Figure S4A-D. In this experiment, we followed the numbers of mucoid and non-mucoid colonies in serial transfer culture. It is clear that for the T7 and T4-generated mucoids, there is a high rate of transition to non-mucoid. This is not the case for the T3-generated mucoids or the genetically constructed mucoids (*rcsC*137). In Figure S4E we present the estimates of the maximum growth rates of wild-type MG1655, various mucoid lineages, and mutants with envelope resistance against T5

and λ^{VIR}. These data indicate that mucoidy does incur a substantial growth rate cost, as indicated by the model analysis. Furthermore, envelope resistance to T5 and λ^{VIR} incurs no such defect under these culture conditions, and may well be the reason why mucoidy is not observed when *E. coli* K12 is challenged with these phage (Fig. 1, S4 Fig. E-F).



S4 Figure. **Reversion from mucoid occurs at different rates in lineages derived from exposure to different T phage, and reversion restores wild-type growth rates.** (A-D) To analyze the reversion rate of mucoid to non-mucoid phenotype, single mucoid colonies were picked from the 9th serial transfer of T3, T4 and T7 phage infected cultures of *E.coli* MG1655 (Fig 1) and grown in liquid culture in the absence of phage, with dilution 1:100 into fresh LB broth every 24 hours. At the end of every transfer, the culture was serially diluted and plated on LB agar for CFU/ml counts and colony morphology determination. (E) Mucoid (MUC) cultures suffer a substantial growth rate defect as compared with wild-type *E. coli* MG1655, while envelope resistance (R) to T5 and λ phage incurs no growth rate penalty under these conditions. Note that the "T4 Muc" and "T7 Muc" cultures immediately produce a mixed culture of mucoid and revertant colonies, as seen during CFU plating of 24-hour growth curve cultures (F), and the estimated growth rate for the mixed culture is likely to reflect the more rapid growth rate of the substantial revertant fraction. Data were obtained from OD_{600} growth curves in LB at 37°C. Error bars represent mean ± SD of ten (E) or three (F) replicates.

Notably, we observed that these revertants to a non-mucoid phenotype were in some cases able to return to mucoidy upon re-exposure to phage (S5 Fig.).



S5 Figure. The proportion of mucoid colonies is shown for populations of the ancestral revertant. Mucoidy was scored after exposure to phages on the phage plate. Three individual revertant non-mucoid colonies were picked from each ancestor mucoid background (bacteria from transfer 8 derived from mucoid ancestors, Figure S4 A, B, C, D). 100 ul of overnight cultures of these clones was mixed with 100 µl of a high titer of each respective phage along with 3ml soft agar and poured on the plate; rcsC137 revertants were exposed to T7 phage.

(ii) Single-cell observations of mucoidy, reversion-induced heterogeneity, and phage infection. The second line of evidence for a high rate of transition from mucoid to normal colony sensitive cells comes from single-cell observation of phage infection in mucoid and non-mucoid cultures. Prior work has shown that reversion from phage-induced mucoidy occurs through secondary mutations within the Rcs system rather than through direct reversion of the initial mutation [18], and that these mutations restore expression of Rcs to roughly wild-type levels. If this is the case, it should be possible to identify individual mucoid cells based on expression levels in the Rcs pathway. To this end, we used a plasmid encoding a fluorescent reporter for Rcs expression (pBAD18Cm::P_{rprA}-mCherry) to visualize heterogeneity within initially mucoid populations (Fig. 7, S4 Fig). Mucoid and non-mucoid cells do not appear morphologically distinct on a single-cell level, and mucoids do not clump or agglomerate in well-mixed liquid culture, which might have protected these cells against phage [32]. However, fluorescence expression from the Rcs promoter is clearly different between wild type and mucoid lineages.

If reversion to non-mucoid eliminates over-expression of Rcs and consequently increases sorption by virulent phage, this should be visible as differences in single-cell sorption to phage which anti-correlate with expression of the Rcs pathway. Using cells expressing the mCherry Rcs fluorescent reporter as described above, we infected cultures with GFP-fluorescent T7 phage [32]; the T7-derived mucoid lineage was chosen due to the rapid reversion to non-mucoidy and high expected levels of heterogeneity in these cultures, and wild-type non-mucoid MG1655 was used for comparison. We observed that, in wild-type cultures of MG1655 where expression of Rcs is uniformly low, most to all individual cells sorb phage and become infected (Fig. 7A,B). By contrast, in T7-induced mucoid *E. coli*, a substantial minority population of low-Rcs cells, apparently corresponding to the

revertants expected in this culture, become infected with T7 phage, while cells strongly expressing Rcs do not become infected (Fig. 7 C, D).

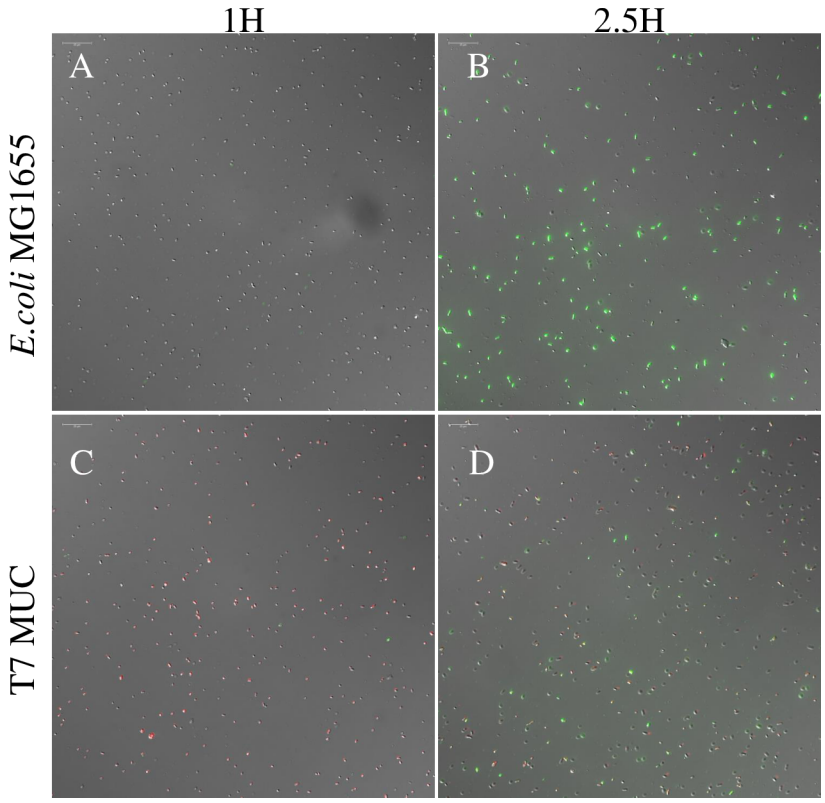


Figure 7 Rcs pathway induction is negatively correlated with T7 phage infection on the single-cell level. In MG1655 wild-type non-mucoid culture (top), expression of Rcs (red) is low, and T7 phage infection (green) is universal. By contrast, in T7-induced mucoid culture (bottom), Rcs expression is high in the bulk of the population and low in a minority of putative revertants, and only the low-Rcs sub-population is infected with T7 phage. Images represent separate aliquots from 1 mL LB cultures incubated at 37°C, sampled approximately 1 hour (left panels) and 2.5 hours (right panels) after inoculation. Images are an overlay of DIC, TRITC, and GFP channels, acquired using a 40X objective (total magnification 400X). Scale bar is 25 μM.

Discussion

A longstanding, fundamental question about the ecology, population and evolutionary biology of bacteria and phage is how these viruses are maintained following the ascent of resistant cells upon which they cannot replicate [4–7,33]. The results of this study provide support for "leaky" reversion from mucoid to sensitive states as a mechanism for the maintenance of phage in bacterial populations. Unlike those presented in other studies

addressing this question, this mechanism is not specific to any particular bacteria and phage. It also provides a possible explanation for why mucoid colony producing bacteria are commonly found in natural populations of Gram-negative bacteria [34–36].

Here we observe that a high rate of genetic or phenotypic transitions (reversion) from mucoidy to phage-sensitivity allows for the maintenance of both bacteria and phage at high densities, and thereby adding generality of "leaky resistance" [4,24] but also [8] as a mechanism by which bacteria and phage co-exist. The mucoid - leaky resistance observed here differs from that reported by [4] in that the rate of transition from mucoid to sensitive is substantially greater (10^{-3} rather than $5x10^{-5}$). Most importantly, unlike the specific receptor site or other specific forms of surface resistance [37], as suggested earlier [18,38], mucoidy provides a general mechanism for bacteria to be simultaneously resistant to multiple phages. Further, through single-cell microscopy, we demonstrate that propagation of phage in cultures of mucoid *E. coli* relies on infection and lysis of these revertant cells, which have restored wild-type levels of expression through the Rcs capsule synthesis system. Finally, our models and experimental results suggest that if mucoidy evolves first, mutants with more specific resistance mechanisms are, with the exceptions like T5 (see the following), unlikely to ascend.

At one level, the population dynamic and experimental and modeling observations and results are mechanistic, in that they explain how mucoid *E. coli* can maintain high-density populations of lytic phage. However, at the genetic, physiological and molecular levels, these results and explanations raise mechanistic questions which they may allude to, but do not answer. Included among those questions are: (1) How does mucoidy provide the broad "resistance" to phages? (2) Why (really how) is the rate of transition from mucoid to non-mucoid so high? (3) What are the genetic, physiological, and molecular basis of the observed mucoid phenotypes? (4) Why does exposure to some phage, such as T5 in our experiments, not select for mucoidy, but instead selects for envelope resistance? (5) What might the consequences of mucoidy be for microbial populations in natural environments?

This study and others have found that phage-induced mucoidy in *E. coli* produces a generalized resistance to infection by lytic phage [18]. Cross-resistance to phage is common where resistance is conferred by a broad mechanism, such as mutation of alternative sigma factors, rather than by mutation of a specific cell surface receptor recognized by the phage. It has been suggested that capsule production blocks sorption of phage to cell surface receptors by generating a physical barrier, which would likely be sufficient to provide generalized resistance [19–22]; however, we did not test this hypothesis directly. We do observe that phage resistance corresponds to Rcs expression on a single-cell level, consistent with this hypothesis.

Consistent with other studies, we found that mucoid phage-resistant mutants revert quickly to a non-mucoid, phage-sensitive type when phage are removed, probably due to additional mutations elsewhere in the Rcs pathway rather than through reversion of the original mucoidy-inducing mutation [18]. Reversion appeared to occur at different rates in *E. coli* mucoids obtained through exposure to different T phage, such that T3-derived mucoids appeared to be relatively stable and T4- and T7-derived mucoids reverted more rapidly. Observations of the growth rate defect in mucoid cells (S5 Fig.) indicated that T3-derived mucoids had a strong growth defect, suggesting that the difference in

observed reversion was not due to a lack of selective pressure for improved growth. It may therefore be the case that the specific mutation(s) that produce mucoidy in our T3-derived lineages are more difficult to reverse than the mutations that arise in other phage-derived lineages, or even that direct reversion of the original mutation is required.

The reversion rate from mucoid to non-mucoid appears to be very high in our more unstable lineages, on the order of 10^{-4}-10^{-3} revertants per cell. This rate is rather extraordinary, but is nonetheless supported by multiple observations over several different mucoid lineages. The large size of the Rcs regulon no doubt contributes to the rapid rate of reversion by providing an abundance of mutational targets [18]. However, it is not clear that this is sufficient to explain the observed rates of reversion, as *E. coli* is only expected to experience roughly one mutation per thousand cells per generation overall [39], and rapid establishment of multiple mutator lineages in these experiments is *a priori* unlikely. The mechanisms (genetic or otherwise) underlying this very rapid reversion are therefore unknown.

We observed that, in some cases, revertants from mucoidy were able to re-gain the mucoid phenotype upon re-exposure to phage. This is in contrast with a previous study which found that mutations leading to reversion of mucoidy prevented a return to the mucoid phenotype [18]. In that study, the tested non-mucoid revertants had acquired large gene deletions spanning the *rcs* operon or an IS insertion in *rcsC*. The nature of these mutations likely explains why subsequent return to the mucoid phenotype was not possible: deletions are a loss of genetic information that cannot be reverted, and excision of an IS element occurs infrequently. We therefore suggest that reversion of mucoidy occurs via other means in our study, most likely nucleotide changes that cause gene loss-of-function but that can subsequently be reverted to wild type. As mentioned above, given the high rates of observed reversion from mucoidy, it is possible that our mucoid lineages are not (all) genetic mutants; if there is a phenotypic component, both the high rate of reversion and the ready re-emergence of mucoidy are easily accounted for.

We found that bacterial populations rapidly evolved mucoidy rather than envelope resistance after infection with T phage, and that the resulting population remained mucoid until phage were removed. Specifically, we found in simulations that envelope resistance should not be able to invade and maintain itself within these mucoid-resistant populations, under the assumption that resistance incurs greater or equivalent fitness costs to mucoidy. Even without this assumption, this result is perhaps unsurprising; mucoidy can arise via mutation in a large number of genes associated with colanic acid production and regulation [18,26], and the large mutational target presented might allow mucoidy to arise more readily than resistance in *E. coli* MG1655. In an established mucoid lineage, envelope resistance should not provide an additional advantage in terms of phage susceptibility, and the additional fitness costs associated with spontaneous envelope resistance will make any resistant-mucoid mutants unlikely to persist long enough to revert from mucoidy. On the other hand, spontaneous sensitive revertants from mucoidy are unlikely to persist and reproduce in the presence of high phage titers and are therefore unlikely to be a source of new envelope resistant mutants. We therefore expect that a mucoid lineage will generally remain mucoid and fail to generate envelope resistant cells over extended periods in the presence of virulent phage, due to the low probability associated with this event.

However, in cases where envelope resistance is less costly than mucoidy, any envelope resistant mutants that do enter the population will increase in frequency (S2, S4 Fig). Consistent with this, we observed envelope resistance arising within mucoid cultures under some conditions. Most notably this occurs with T5 phage, where envelope resistance is expected to have minimal or no cost [25], and other studies have found that mucoidy and other resistance mechanisms can arise together within single cultures [18]. Our results indicate that the evolution and utility of mucoidy, as compared with other phage resistance mechanisms, will depend heavily on the strains involved and the environmental context of the interaction. Under these conditions (laboratory *E. coli* exposed to single strains of phage in liquid media), exponential growth rate was a reasonably informative measure of fitness, but this is known to be untrue in other scenarios [40], and particularly in more complex environments[6,41,42]. Understanding the selective forces that are likely to dominate in different contexts will therefore be relevant for a global understanding of mucoidy as a part of phage-bacteria interactions.

Importantly, these studies were performed in well-mixed liquid media, where we did not observe the formation of cellular collectives (Fig. 7). In stationary cultures where surface adhesion and biofilm formation occur, the formation of collectives could allow exopolysaccharide production to be used as a common good [32,43]. Bacteria-phage dynamics in surface-adhered conditions might therefore differ from those observed here; for example, creation of surplus extracellular matrix by mucoid bacteria might provide some protection to phage-sensitive bacteria, either by impeding free diffusion of phage or by providing a scaffolding of phage-sorbing cellular material, allowing maintenance of a sensitive sub-population alongside mucoids and phage [44]. As the Rcs pathway in *E. coli* is a known regulator of biofilm formation [26], it is very plausible that mutations in this pathway that generate mucoidy in the interests of phage resistance will also produce changes in collective behavior.

These results may be relevant for understanding the evolution and maintenance of mucoidy in other systems. Mucoidy has been observed in chronic *Pseudomonas aeruginosa* infections of the cystic fibrosis lung and marks progression of the disease into a more debilitating stage [43,45]. While it is at present not known whether phage-imposed selection plays a role in this clinical phenomenon, selection for mucoidy has been observed in *Pseudomonas* exposed to lytic phage [34,36]. As interest in phage-based therapy for cystic fibrosis lung infection grows [14,16,46,47], clarifying the role of phage in disease-associated mucoidy will be critical in developing safe and useful treatments for these intransigent infections.

Acknowledgements

The authors would like to thank Cary Nadell, Jim Bull, and Sylvain Moineau for generously providing us with the phage used in these experiments and Ole Skovgaard for providing the *E. coli* MG1655 employed here. This research was funded by grants from the U.S. National Institutes of General Medical Science GM098175-17 to B.R. L., 1R35GM133509-01 to M.G., and Emory University start-up funds (MG, NV). We are grateful to Ingrid McCall for managing the logistics of this enterprise and for providing editorial suggestions, and to Melony Ivey for technical assistance.

Materials and Methods

Bacteria, phage and growth media

Strains used in these experiments are shown in Table 1. Bacterial cultures were grown at 37˚C either in M9 media [M9 salts (248510, Difco) supplemented with 0.4% glucose (6363-53-7, Fisher Scientific), 1 mM $MgSO_4$ (Sigma Aldrich), 0.1 mM $CaCl_2$ (JT Baker), and 0.2% Thiamine B1 (Sigma Aldrich)] or LB broth [$MgSO_4$ 2.5g/L, tryptone (Fisher Bioreagent 10g/L, yeast extract (Bacto) 5g/L, sodium chloride (Fisher Chemical) 10g/L]. All *E. coli* strains used in our experiments were derivatives of the parent strain K12 MG1655. Rcs pathway knockout strains were constructed by the method described in [48]. Gain-of-function point mutant for mucoid phenotype strain was constructed by the method described in [49].

Phage lysates were prepared from single plaques at 37˚C in LB medium alongside wild-type *E.coli* MG1655. Chloroform was added to the lysates and the lysates were centrifuged to remove any remaining bacterial cells.

The λ^{VIR} strain used in these experiments was obtained from Sylvain Moineau. The construction of temperate phage λ^{KAN} and λ^{CAM} is described in [31].

Table 1. Bacterial strains and phage used in this study.

#	Bacterial Strain	Properties	Reference
1	*E.coli* K12 (MG1655)	Wild Type	Ole Skovgaard at Roskilde University in Denmark
2	T7 Mucoid *E. coli* MG1655	T7 phage selected mucoid	This study
3	T3 Mucoid *E. coli* MG1655	T3 phage selected mucoid	This study
4	*E.coli* K12	Δ*rcsF*::kan	This study
5	*E.coli* K12	Δ*rcsB*::kan	This study
6	*E.coli* K12	Δ*cpsB*::kan	This study
7	*E.coli* K12	*rcsC*137 *ompC*::Tn5	This study
#	Phage	Properties	Reference
1	T3	Wild Type	Jim Bull Lab

2	T4	Wild Type	Jim Bull Lab
3	T5	Wild Type	Jim Bull Lab
4	T7	Wild Type	Jim Bull Lab
5	T7	GFP-labelled T7	Cary Nadell
6	λ^{vir}	Virulent mutant of λ	Sylvain Moineau Lab
7	λ^{KAN}	Wild type λ with Kanamycin antibiotic cassette	[31]
8	λ^{CAM}	Wild type λ with chloramphenicol antibiotic cassette	[31]

Sampling bacterial and phage densities

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 λ-KAN or λ-CAM lysogens, cultures were plated on LB (1.6%) agar with 25 μg/mL kanamycin (AppliChem Lot# 1P0000874) or chloramphenicol (Merck Lot# 2430B61), respectively.

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 MG1655 (about 5×10^8 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 10^6 PFU/mL. At the end of each transfer, 100 μL of each culture was transferred into flasks with fresh medium (1:100 dilution). Simultaneously, 100 μL 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.

Bacteriophage and bacteria parameter determination

The parameters critical for the interaction of λ phages and *E. coli* MG1655 used in this study were estimated in independent experiments in LB medium. The maximum growth rate of *E. coli* MG1655 was measured by Bioscreen as described in [40]. Phage burst sizes (β) were estimated with one-step growth experiments [50] in a manner similar to [51]. Adsorption of λ to *E. coli* was estimated as described in [50]. The procedure for estimating the probability of lysogeny and the rate of spontaneous lysogen induction are presented in [31].

Short term dynamics experiments

Overnight cultures of *E. coli* MG1655 were grown in LB media. The cultures were diluted in a 1:1000 ratio on 20 mL of medium in 100 mL flasks to which λ_{KAN} or λ_{CAM} phage was added to an initial density of $1x10^7$ PFU/mL. Short term dynamics were conducted culturing the mixture at 37 ˚C in shaking conditions. Total bacteria count was conducted by sampling 100 µL at regular intervals and plating dilutions on LB agar plates. Lysogen counts were obtained by sampling 100 µL and plating dilutions on LB agar with 25 µg/mL kanamycin or chloramphenicol. Phage counts were obtained by sampling 1 mL at regular intervals, adding chloroform to kill bacteria, and plating for PFU quantification as previously described.

Resistance Testing

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[52].

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 (10^8-10^9 PFU/mL) were spotted onto the agar surface; susceptibility was scored according to the presence of visible plaques.

Single Cell Microscopy

Overnight cultures of WT and mucoid *E.coli* MG1655 carrying plasmid pBAD18Cm::P_{rprA}-mCherry (reporter for induction of the Rcs pathway [53]) were grown in LB media. These cultures were diluted 1/1000 in fresh LB and incubated for 30 minutes with vigorous shaking (300 RPM) at 37°C. We then added GFP-labelled T7 phage [32] at 0.1 MOI and returned to incubation in a stationary incubator at 37°C. The phage free control and phage infected culture slides were sampled at 15-30 minute intervals and observed on an inverted fluorescent microscope (Leica DMi8 with LasX Premium software). Images are an overlay of DIC, TRITC, and GFP channels, acquired using a 40X objective (total magnification 400X).

Works Cited

1. Chibani-Chennoufi S, Bruttin A, Dillmann M-L, Brüssow H. Phage-Host Interaction: an Ecological Perspective. J Bacteriol. 2004;186: 3677–3686. doi:10.1128/JB.186.12.3677-3686.2004

2. Abeles SR, Pride DT. Molecular Bases and Role of Viruses in the Human Microbiome. J Mol Biol. 2014;426: 3892–3906. doi:10.1016/j.jmb.2014.07.002

3. Gurney James, Pleška Maroš, Levin Bruce R. Why put up with immunity when there is resistance: an excursion into the population and evolutionary dynamics of restriction–modification and CRISPR-Cas. Philos Trans R Soc B Biol Sci. 2019;374: 20180096. doi:10.1098/rstb.2018.0096

4. Chaudhry WN, Pleska M, Shah N, Weiss H, McCall I, Meyer J, et al. Leaky resistance and the conditions for the existence of lytic bacteriophage. PLOS Biol. 2018;16: e2005971. doi:https://doi.org/10.1371/journal.pbio.2005971

5. Levin BR, Stewart FM, Chao L. Resource-Limited Growth, Competition, and Predation: A Model and Experimental Studies with Bacteria and Bacteriophage. Am Nat. 1977;111: 3–24.

6. Gómez P, Buckling A. Bacteria-Phage Antagonistic Coevolution in Soil. Science. 2011;332: 106–109. doi:10.1126/science.1198767

7. Wei Y, Kirby A, Levin BR. The Population and Evolutionary Dynamics of Vibrio cholerae and Its Bacteriophage: Conditions for Maintaining Phage-Limited Communities. Am Nat. 2011;178: 715–725. doi:10.1086/662677

8. Weissman JL, Holmes R, Barrangou R, Moineau S, Fagan WF, Levin B, et al. Immune loss as a driver of coexistence during host-phage coevolution. ISME J. 2018;12: 585–597. doi:10.1038/ismej.2017.194

9. Reyes A, Wu M, McNulty NP, Rohwer FL, Gordon JI. Gnotobiotic mouse model of phage-bacterial host dynamics in the human gut. Proc Natl Acad Sci U S A. 2013;110: 20236–20241. doi:10.1073/pnas.1319470110

10. Draper LA, Ryan FJ, Dalmasso M, Casey PG, McCann A, Velayudhan V, et al. Autochthonous faecal virome transplantation (FVT) reshapes the murine microbiome after antibiotic perturbation. bioRxiv. 2019; 591099. doi:10.1101/591099

11. Hannigan GD, Duhaime MB, Koutra D, Schloss PD. Biogeography and environmental conditions shape bacteriophage-bacteria networks across the human microbiome. PLoS Comput Biol. 2018;14. doi:10.1371/journal.pcbi.1006099

12. Stern A, Mick E, Tirosh I, Sagy O, Sorek R. CRISPR targeting reveals a reservoir of common phages associated with the human gut microbiome. Genome Res. 2012;22: 1985–1994. doi:10.1101/gr.138297.112

13. Manrique P, Bolduc B, Walk ST, Oost J van der, Vos WM de, Young MJ. Healthy human gut phageome. Proc Natl Acad Sci. 2016;113: 10400–10405. doi:10.1073/pnas.1601060113

14. Cafora M, Deflorian G, Forti F, Ferrari L, Binelli G, Briani F, et al. Phage therapy against Pseudomonas aeruginosa infections in a cystic fibrosis zebrafish model. Sci Rep. 2019;9: 1527. doi:10.1038/s41598-018-37636-x

15. Lu TK, Collins JJ. Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. Proc Natl Acad Sci U A. 2009;106: 4629–34.

16. Forti F, Roach DR, Cafora M, Pasini ME, Horner DS, Fiscarelli EV, et al. Design of a Broad-Range Bacteriophage Cocktail That Reduces Pseudomonas aeruginosa Biofilms and Treats Acute Infections in Two Animal Models. Antimicrob Agents Chemother. 2018;62: e02573-17. doi:10.1128/AAC.02573-17

17. Mizoguchi K, Morita M, Fischer CR, Yoichi M, Tanji Y, Unno H. Coevolution of Bacteriophage PP01 and Escherichia coli O157:H7 in Continuous Culture. Appl Environ Microbiol. 2003;69: 170–176. doi:10.1128/AEM.69.1.170-176.2003

18. Wielgoss S, Bergmiller T, Bischofberger A, Hall A. Adaptation to Parasites and Costs of Parasite Resistance in Mutator and Nonmutator Bacteria. Mol Biol Evol. 2016;33: 770–782. doi:https://doi.org/10.1093/molbev/msv270

19. Labrie S, Samson J, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol. 2010;8: 317–327.

20. Kim MS, Kim YD, Hong SS, Park K, Ko KS, Myung H. Phage-Encoded Colanic Acid-Degrading Enzyme Permits Lytic Phage Infection of a Capsule-Forming Resistant Mutant Escherichia coli Strain. Appl Environ Microbiol. 2015;81: 900–909. doi:10.1128/AEM.02606-14

21. Qimron U, Marintcheva B, Tabor S, Richardson CC. Genomewide screens for Escherichia coli genes affecting growth of T7 bacteriophage. Proc Natl Acad Sci. 2006;103: 19039–19044. doi:10.1073/pnas.0609428103

22. Scholl D, Adhya S, Merril C. Escherichia coli K1's Capsule Is a Barrier to Bacteriophage T7. Appl Env Microbiol. 2005;71: 4872–4874. doi:10.1128/AEM.71.8.4872-4874.2005

23. Harrison E, Truman J, Wright R, Spiers A, Paterson S, Brockhurst MA. Plasmid carriage can limit bacteria–phage coevolution. Biol Lett. 2015;11. doi:https://doi.org/10.1098/rsbl.2015.0361

24. Delbruck M. Bacterial Viruses or Bacteriophages. Biol Rev. 1946;21: 30–40. doi:10.1111/j.1469-185X.1946.tb00451.x

25. Lenski RE, Levin BR. Constraints on the Coevolution of Bacteria and Virulent Phage: A Model, Some Experiments, and Predictions for Natural Communities. Am Nat. 1985;125: 585–602.

26. Clarke DJ. The Rcs phosphorelay: more than just a two-component pathway. Future Microbiol. 2010;5: 1173–1184. doi:10.2217/fmb.10.83

27. Stout V. Identification of the promoter region for the colanic acid polysaccharide biosynthetic genes in Escherichia coli K-12. J Bacteriol. 1996;178: 4273–4280. doi:10.1128/jb.178.14.4273-4280.1996

28. Morona R, Tommassen J, Henning U. Demonstration of a bacteriophage receptor site on the Escherichia coli K12 outer-membrane protein OmpC by the use of a protease. Eur J Biochem. 1985;150: 161–169. doi:10.1111/j.1432-1033.1985.tb09002.x

29. Monod J. The Growth of Bacterial Cultures. Annu Rev Microbiol. 1949;3: 371–394. doi:10.1146/annurev.mi.03.100149.002103

30. Stewart FM, Levin BR. Partitioning of Resources and the Outcome of Interspecific Competition: A Model and Some General Considerations. Am Nat. 1973;107: 171–198.

31. Pleška M, Lang M, Refardt D, Levin BR, Guet CC. Phage-host population dynamics promotes prophage acquisition in bacteria with innate immunity. Nat Ecol Evol. 2018;2: 359–366. doi:10.1038/s41559-017-0424-z

32. Vidakovic L, Singh PK, Hartmann R, Nadell CD, Drescher K. Dynamic biofilm architecture confers individual and collective mechanisms of viral protection. Nat Microbiol. 2018;3: 26. doi:10.1038/s41564-017-0050-1

33. Campbell A. Conditions for the Existence of Bacteriophage. Evolution. 1961;15: 153–165. doi:10.1111/j.1558-5646.1961.tb03139.x

34. Martin DR. Mucoid Variation in Pseudomonas Aeruginosa Induced by the Action of Phage. J Med Microbiol. 1973;6: 111–118. doi:10.1099/00222615-6-1-111

35. Klieneberger-Nobel E. Capsules and Mucoid Envelopes of Bacteria. J Hyg (Lond). 1948;46: 345–348.

36. Scanlan PD, Buckling A. Co-evolution with lytic phage selects for the mucoid phenotype of Pseudomonas fluorescens SBW25. ISME J. 2012;6: 1148–1158. doi:10.1038/ismej.2011.174

37. Wright R, Friman V-P, Smith M, Brockhurst MA. Cross-resistance is modular in bacteria–phage interactions. PLOS Biol. 2018;16: e2006057. doi:10.1371/journal.pbio.2006057

38. Tazzyman SJ, Hall AR. Lytic phages obscure the cost of antibiotic resistance in Escherichia coli. ISME J. 2015;9: 809–820. doi:10.1038/ismej.2014.176

39. Lee H, Popodi E, Tang H, Foster PL. Rate and molecular spectrum of spontaneous mutations in the bacterium Escherichia coli as determined by whole-genome sequencing. Proc Natl Acad Sci. 2012;109: E2774–E2783. doi:10.1073/pnas.1210309109

40. Concepción-Acevedo J, Weiss HN, Chaudhry WN, Levin BR. Malthusian Parameters as Estimators of the Fitness of Microbes: A Cautionary Tale about

the Low Side of High Throughput. PLOS ONE. 2015;10: e0126915. doi:10.1371/journal.pone.0126915

41. Björkman J, Nagaev I, Berg OG, Hughes D, Andersson DI. Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance. Science. 2000;287: 1479–1482. doi:10.1126/science.287.5457.1479

42. Santos-Lopez A, Marshall CW, Scribner MR, Snyder D, Cooper VS. Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. bioRxiv. 2019; 581611. doi:10.1101/581611

43. Malhotra S, Limoli DH, English AE, Parsek MR, Wozniak DJ. Mixed Communities of Mucoid and Nonmucoid Pseudomonas aeruginosa Exhibit Enhanced Resistance to Host Antimicrobials. mBio. 2018;9: e00275-18. doi:10.1128/mBio.00275-18

44. Simmons M, Bond MC, Drescher K, Bucci V, Nadell CD. Evolutionary dynamics of phage resistance in bacterial biofilms. bioRxiv. 2019; 552265. doi:10.1101/552265

45. Li Z, Kosorok MR, Farrell PM, Laxova A, West SEH, Green CG, et al. Longitudinal Development of Mucoid Pseudomonas aeruginosa Infection and Lung Disease Progression in Children With Cystic Fibrosis. JAMA. 2005;293: 581–588. doi:10.1001/jama.293.5.581

46. Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophages ϕMR299-2 and ϕNH-4 Can Eliminate Pseudomonas aeruginosa in the Murine Lung and on Cystic Fibrosis Lung Airway Cells. mBio. 2012;3: e00029-12. doi:10.1128/mBio.00029-12

47. Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, et al. Bacteriophages Can Treat and Prevent Pseudomonas aeruginosa Lung Infections. J Infect Dis. 2010;201: 1096–1104. doi:10.1086/651135

48. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. 2006;2: 2006.0008.

49. Gottesman S, Trisler P, Torres-Cabassa A. Regulation of capsular polysaccharide synthesis in Escherichia coli K-12: characterization of three regulatory genes. J Bacteriol. 1985;162: 1111–1119.

50. Ellis EL, Delbrück M. THE GROWTH OF BACTERIOPHAGE. J Gen Physiol. 1939;22: 365–384.

51. Abedon ST, Herschler TD, Stopar D. Bacteriophage Latent-Period Evolution as a Response to Resource Availability. Appl Environ Microbiol. 2001;67: 4233–4241. doi:10.1128/AEM.67.9.4233-4241.2001

52. Wei Y, Ocampo P, Levin BR. An experimental study of the population and evolutionary dynamics of Vibrio cholerae O1 and the bacteriophage JSF4. Proc R Soc B Biol Sci. 2010;277: 3247–3254. doi:10.1098/rspb.2010.0651

53. Konovalova A, Grabowicz M, Balibar CJ, Malinverni JC, Painter RE, Riley D, et al. Inhibitor of intramembrane protease RseP blocks the σE response causing lethal accumulation of unfolded outer membrane proteins. Proc Natl Acad Sci U S A. 2018;115: E6614–E6621. doi:10.1073/pnas.1806107115