# An evolutionary perspective of suicide as a way to protect bacteria from being killed by phage

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## Abstract

Bacteria have evolved several mechanisms to protect themselves from the ravages of infection with phages. Accounting for the evolution of almost all of these mechanisms, like envelope resistance, restriction-modification, and CRISPR-Cas - mediated immunity is straightforward. In the presence of phage, resistance or immunity are to the advantage of the individual organisms that utilize these defense mechanisms. There is, however, one form of immunity that cannot be accounted for by phage-mediated selection favoring individuals expressing the defense mechanism: abortive infection (Abi). When a bacterium with an Abi system is infected with a virulent phage it dies and the infecting phage is lost, and the infection is aborted. Recently, evidence was presented that retrons, DNA sequences that code for a reverse transcriptase and a single-stranded DNA/RNA hybrid named multi-copy single-stranded DNA (msDNA), can mediate the pathway for an abortive infection response in *Escherichia coli*. Using a general mathematical model of the population dynamics of bacteria and phage in mass culture and experiments with a retron-mediated Abi system in liquid and structured culture, we explore the conditions under which abortive infection can protect populations of bacteria from invasion by lytic phages and the conditions where an abortive infection mechanism can become established in populations without this defense. The results of our theoretical and experimental analysis question the hypothesis that Abi systems evolved and are maintained because of the protection they provide bacteria from infections with lytic phage.

## Significance Statement

Due to the increasing interest in the use of phage for therapy and the factors for determining the bacterial composition of microbiomes, in recent years there has been a resurrection of interest in bacteriophage and the mechanisms by which bacteria protect themselves against infections with these viruses. The defense system considered in this report, abortive phage infection (Abi), raises an intriguing evolutionary question. When infected with phage, a bacterium with an Abi system, dies (suicide), the infecting phage is lost. With mathematical models and experiments with a retron-mediated Abi system in *E. coli,* we explore the conditions under which Abi can protect populations of bacteria from lytic phages, and how this not-to-the advantage of individuals mechanism becomes established in populations.

## Introduction

Bacteria have evolved many mechanisms to protect themselves from being killed by infections with phages (1). From an evolutionary perspective, the most intriguing of these mechanisms is abortive infection (Abi). When a bacterium with an Abi system is infected with a virulent phage, it dies, and the infecting phage is lost and does not replicate (2). How did this suicide-based system evolve and how can it be maintained in bacterial populations when abortive infection is not to the advantage of the individual bacteria expressing this character?

The phenomenon of phage-mediated abortive infection has been known for more than 40 years (3). Abi has been most extensively studied in the bacteria employed for the production of dairy products, *Lactococcus lactis,* where phage contamination is a costly occurrence *(4-6)*. Abortive infection has been observed in a number of species of bacteria including *Escherichia coli*, *Bacillus subtilis*, *Shigella dysenteriae* and *Vibrio cholerae (7)*. Although these bacteria share the abortive phage infection phenotype, the mechanisms by which they prevent the phage from completing their lytic cycle vary (2, 4, 7).

Recently, Millman and colleagues (8) presented evidence that retrons, DNA sequences that code for a reverse transcriptase and a unique single-stranded DNA/RNA hybrid called multicopy single-stranded DNA (msDNA), can be responsible for abortive infection in *E. coli*. Until the publication of Millman et al, the function of retrons, which had been first described more than thirty years ago (9) and are present in many species of bacteria (10-12), was largely a matter of speculation. Although Millman and colleagues explore the conditions under which retrons can protect populations of bacteria from infections with phages, they did not address the underlying evolutionary question: What are the selective forces responsible for the evolution and maintenance of retrons and the contribution of abortive infection to that evolution?

Using a general mathematical model of the population dynamics of bacteria and phage in mass culture, we explore the conditions under which (i) abortive infection can protect populations of bacteria from invasion by lytic phages and (ii) an abortive infection mechanism can become established in a population without this mechanism. Using the strains of *E. coli* employed by Millman and colleagues (8) and a virulent mutant of the phage λ, $\lambda^{VIR}$, in liquid and semi-structured culture we test the hypotheses generated from our theoretical analysis. The results of our tests support the predictions of our models for Abi for protecting populations of bacteria from phage in liquid culture, but also provide evidence that the retron-mediated Abi, is only one element of this protection process. Within short-order $\lambda^{VIR}$ resistant retron-expressing cells emerge and become the dominant bacterial population. Our models predicted and our experiments confirm that in liquid culture, phage-mediated selection will not lead to the establishment of an Abi system when it is initially rare. To begin to determine whether this negative result is an artifact of liquid culture, using soft agar we tested the hypothesis that bacteria with an Abi system, retrons in this case, can become established by phage-mediated selection. Although retrons provided protection against phage in this structured habitat, when initially rare the retron-expressing population could not invade retron-lacking populations.

## A General Abortive infection model

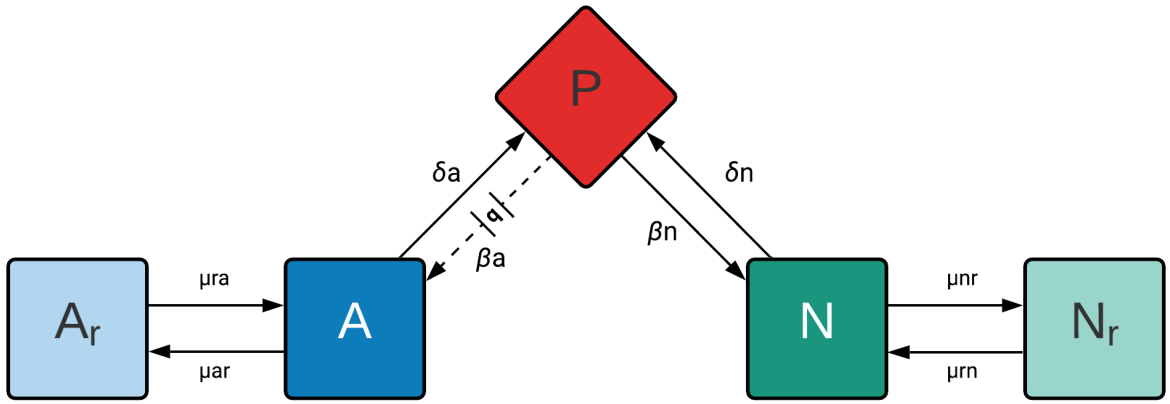


**Figure 1.** A mass-action diagram representing five populations: a lytic phage (P), a phage-sensitive $Abi^+$ bacteria population (A), a phage-resistant $Abi^+$ bacteria population ($A_r$), a phage-sensitive $Abi^-$ bacteria population (N), and a phage-resistant $Abi^-$ bacteria population. The phage can adsorb and replicate on populations A and N, and the bacteria can transition between their phage-resistant and phage-sensitive states.

The model developed here (Figure 1) is a general model of the population dynamics of bacteria and phage with a generalized mechanism of abortive infection. There is a single population of phage, with a density of phage particles express as plaque forming units per ml and designation, P. The bacteria are of four states (populations): A, $A_r$, N, and $N_r$ are the densities of these populations, cells expressed as colony forming units per ml, as well as their designation, where A have a functional abortive infection system (are $Abi^+$), $A_r$ are $Abi^+$ and resistant to P, whereby P cannot reproduce on this population. N are $Abi^-$ and sensitive to phage infection, while $N_r$ are $Abi^-$ and resistant to infections with P. By mutation or other processes, the bacteria change states, $A \rightarrow A_r$ and $A_r \rightarrow A$ at rates $\mu_{mra}$ and $\mu_{mar}$, and $N \rightarrow N_r$ and $N_r \rightarrow N$ at rates $\mu m_{nr}$ and $\mu_{mrn}$, per cell per hour, respectively.

The bacteria grow at maximum rates, $v_a$, $v_{ar}$, $v_n$, and $v_{nr}$, per cell per hour, for A, Ar, N and $N_r$, respectively with the net rate of growth being proportional to the concentration of a limiting resource, R as in Monod 1949 (13): where K is the concentration of the resource where the net growth rate is half its maximum value, the Monod constant. The limiting resource is consumed at a rate proportional to the function $\Psi(R)$ and a conversion efficiency parameter, e μg/ml, which is the amount of the resource needed to produce a new cell (14). To account for the fact that as the population approach stationary phase, R=0, phage infection and mutation rates decline as we assume that all of these transition rates are proportional to ψ(R) and thus hyperbolic. As reported

before, we assume phage infection is a mass action process whose rate is equal to the product of the density of bacteria and phage and a rate constants of phage infection, $\delta_A$ and $\delta_N$ (ml·cells/hour) for infections of A and N, respectively (15). Infections of A by P are aborted with a probability, q per infection ($0 \leq q \leq 1$), and the phage do not replicate. All infections of N by P produce $\beta_n$ phage particles, and the (1-q) infections of P with A that do not abort, produce $\beta_a$ phage particles. All infected A and N bacteria are killed. The latent period of phage infection is not considered in this model or the numerical solution employed to analyze its properties. Thereby the short-term population dynamics of the phage-bacterial interactions is not well-mimicked by this model. However, as suggested in (15), this simplifying assumption has little impact on our numerical solutions at 24 hours as presented.

With these definitions and assumptions, the rates of change in the densities of bacteria and phage and the concentration of the limiting resource are given by the system of time-dependent coupled differential equations listed in Equations (1-7).

$$\frac{dR}{dt} = -\Psi(R) \cdot e \cdot (v_n \cdot N + v_{nr} \cdot NR + v_{ab} \cdot A + v_{ar} \cdot AR) \tag{1}$$

$$\frac{dN}{dt} = \Psi(R) \cdot \left(v_n \cdot N - \delta_n \cdot N \cdot P + (\mu_{mrn} \cdot NR - \mu_{mnr} \cdot N)\right) \tag{2}$$

$$\frac{dNR}{dt} = \Psi(R) \cdot \left(v_{nr} \cdot NR - (\mu_{mrn} \cdot NR - \mu_{mnr} \cdot N)\right) \tag{3}$$

$$\frac{dA}{dt} = \Psi(R) \cdot \left(v_{ab} \cdot A - (\mu_{mra} \cdot AR - \mu_{mar} \cdot A)\right) \tag{4}$$

$$\frac{dAR}{dt} = \Psi(R) \cdot \left(v_{ar} \cdot AR - (\mu_{mra} \cdot AR - \mu_{mar} \cdot A)\right) \tag{5}$$

$$\frac{dP}{dt} = \Psi(R) \cdot (\delta_n \cdot \beta_n \cdot P \cdot N \cdot -\delta_a \cdot q \cdot P \cdot A + (1-q) \cdot \delta_a \cdot P \cdot A \cdot \beta_a - \delta_a \cdot P \cdot AR) \tag{6}$$

$$\text{Where: } \Psi(R) = \frac{R}{R+K} \tag{7}$$

## Results

We open this consideration of the population and evolutionary dynamics of abortive phage infection with an analysis of the protection a perfect Abi system provides a population against lytic phage predation. For this (Figure 2) and the other considerations of an Abi system below, we present the predicted densities of bacteria and phage at time 0 and 24 hours, the initial and final densities, respectively.

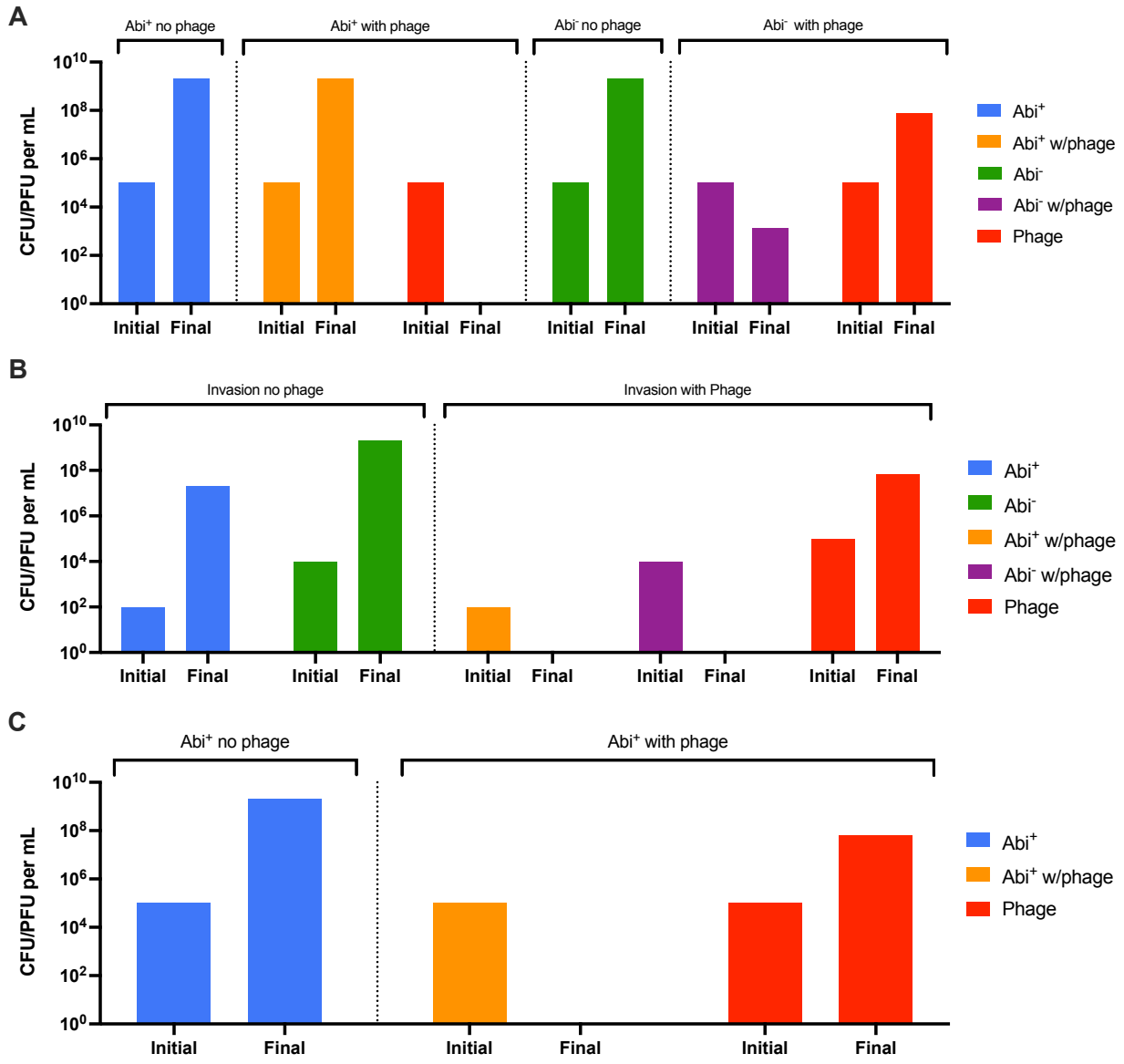


**Figure 2.** Simulation results. Changes in the densities of bacteria and phage over a 24 hour period. A detailed consideration of the dynamics of these simulations are presented in Supplemental Figure S1. **A-** An Abi$^+$ population without and with phage, and an Abi$^-$ population without and with phage in a perfect Abi system (q=1.00). **B-** A high density of Abi$^-$ bacteria and a low density of Abi$^+$ with and without phage with a perfect Abi system (q=1.00). **C-** A replica of the Abi$^+$ simulations in Figure 2A with a less than perfect Abi system (q=0.98).

As can be seen in Figure 2A, a completely effective Abi system (q=1.00) is able to protect a population of bacteria from invasion by phage. By 24 hours the phage population is gone and the $Abi^+$ population is at its maximum density. As noted in Figure S1, this protection occurs even when the initial density of phage substantially exceeds that of the bacteria. When the $Abi^-$ bacteria are confronted with phage, by 24 hours the bacteria are gone and there is a substantial density of free phage. When the $Abi^+$ bacteria are rare relative to the $Abi^-$, even in the presence of phage, they are lost. The density of phage increases whilst that of the $Abi^-$ declines (Figure 2B). The ability of the $Abi^+$ population to prevent the ascent of the phage and protect the bacterial population is critically dependent on the efficacy of abortive infection. If the probability of an Abi system aborting a lytic phage infection is less than 98%, the bacteria are killed off and the phage replicate and reach high densities (Figure 2C).

In Figure 3, we present the results of our experimental tests of the Abi protection hypotheses presented in Figure 2. We explore these hypotheses with a retron-expressing cell line that is capable of aborting phage infections (16). For this we follow the changes in the densities of bacteria and phage with the retron-expressing strain and $\lambda^{VIR}$. As a control, we consider a phage sensitive strain that does not express the retron. As anticipated from the model (Figure 2A), by 24 hours the phage population is gone or nearly so, and the bacterial density is at the level of a phage-free control (Figure 3A). The results of the control experiment with the sensitive strain are inconsistent with the prediction of the model (Figure 2B). As anticipated from the model, the phage density increased over the 24 hours, but contrary to what is expected, the bacteria are not lost, but rather increased to the density anticipated in the absence of phage (Figure 3A).

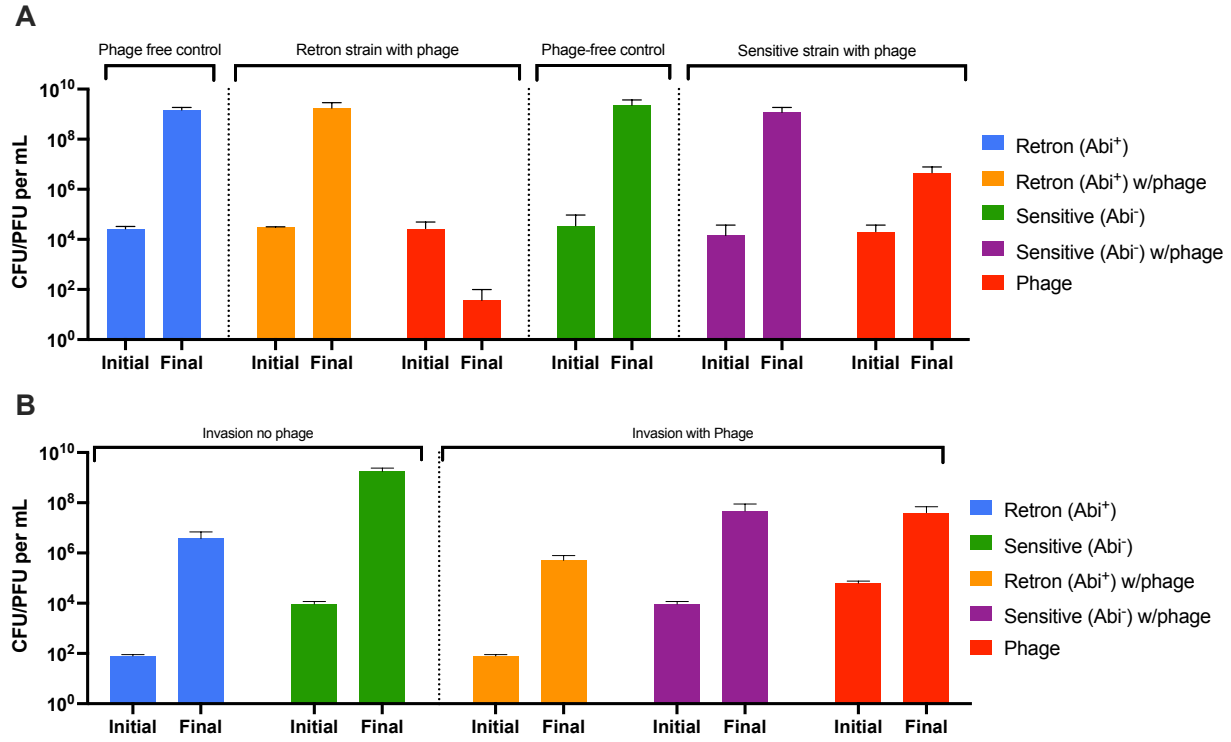


**Figure 3.** Competition experiments measuring the changes in the densities of bacteria and phage over 24 hours for bacteria expression the retron in the absence of phage (blue) and in the presence of phage (orange) and a sensitive population of bacteria in the absence of phage (green) and in the presence of phage (purple), when challenged with $\lambda^{VIR}$ (red). The data are the means and standard

errors of the phage and bacterial densities of three independent replicas. **A-** The protective ability of the Abi system as simulated in Figure 2A and 2C. **B-** The ability of an $Abi^+$ population to invade an $Abi^-$ population as simulated in Figure 2B.

To understand reasons for this deviation from the theory, we tested for resistance by the cross-streak method (Supplement Table S3). By this criterion the vast majority of initially sensitive bacteria recovered at 24 hours are resistant to $\lambda^{VIR}$. Curiously, this is also the case for the retron-expressing bacteria recovered at 24 hours. Does this mean that retron-mediated Abi is not sufficient to prevent invasion of the phage?

To elucidate the reasons for the deviation from the predictions of the initial simulations, we return to the model, but now allow for generation of $\lambda^{VIR}$ resistant $Abi^+$ (AR) and $Abi^-$ (NR) bacteria. As noted in Chaudhry et al (17), there is a high rate of generation of $\lambda^{VIR}$ resistant bacteria for *E. coli* K12 (MG1665), with transition rates, $\mu_{mar}$ and $\mu_{mnr}$ in the model of $10^{-5}$ per cell per hour or greater. If we allow for that high transition rate for sensitive $Abi^-$ cells, both the phage and resistant bacteria ascend (Figure 4). This parallels our observation in the previous experiments with $\lambda^{VIR}$ and a sensitive *E. coli* (Figure 3B). For the $Abi^+$ population, the situation is a bit more complex. If the efficacy of the Abi is too great, q=1.00, the phage are quickly lost and, although resistant cells are produced they do not ascend to the resource- limiting level. If, however, Abi is less than perfectly effective, q=0.98, the phage density initially increases but then the phage are lost and abi resistant cells do ascend to dominate the population (Supplemental Figure S2). This consistent with what we observed. If we allow for phage resistant mutants, the $abi^+$ population can increase in initial frequency as $abi^+$ resistant bacteria, but the dominant population will be phage-resistant $abi^-$ cells.

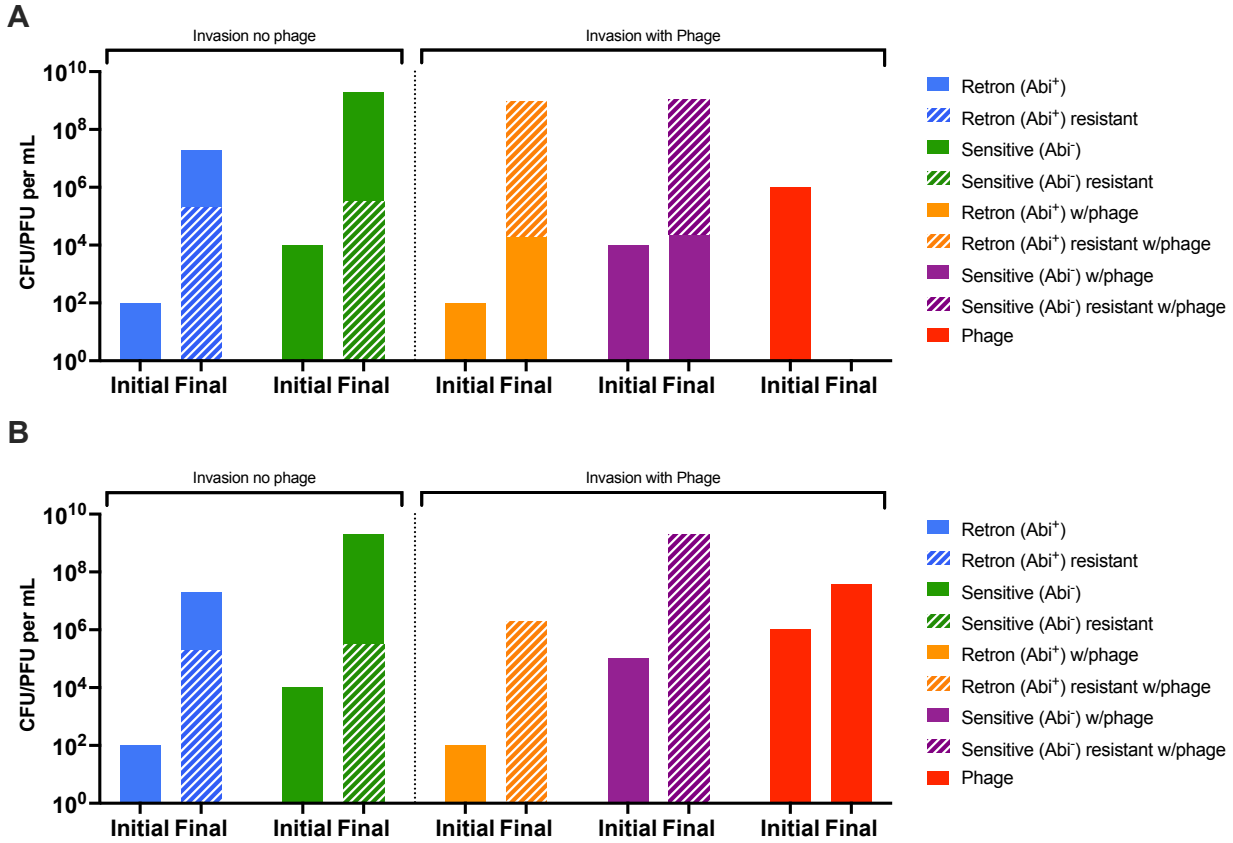

**Figure 4**. The simulation results in Figure 2B repeated but allowing for resistance to be selected for within populations (AR and NR in Equations 1-7). Changes in the densities of bacteria and phage after 24 hours in two invasion situations where a minority retron population is competed against a sensitive population. We present in the first invasion a competition of retron expressing ($Abi^+$) bacteria (blue) while allowing for the generation of phage-resistant retron expressing mutants (overlay of white dashes on blue bar). For this invasion, sensitive bacteria ($Abi^-$) are presented in green with a white dashed overlay representing the phage-resistant $Abi^-$ population. The second invasion simulation was computed in the presence of a significant phage population (show in red). In this case, retron expressing ($Abi^+$) bacteria are shown in orange with an overlayed resistant population presented with white dashes. Phage-sensitive $Abi^-$ bacteria are depicted in purple with their corresponding resistant population presented with dashed white lines overlayed on a purple bar. **A-** Simulations with complete Abi effectiveness (q=1.00). **B-** Simulations with a less-than-perfect Abi system (q=0.98).

Abortive Infection in a Physically Structured Populations

In the real world there may be situations where, as assumed by our models and mimicked by our liquid culture experiments, bacteria exist as planktonic cells all of which have equal access to resources, wastes, each other, and phage. On the other hand, in natural habitats bacteria are likely to be physically structured where they are present as colonies and microcolonies. With phage present, some of these colonies will be infected while others will not and there will be variation in the time during the growth cycle that colonies are infected with phage and the number of phages infecting that colony. Although not explicitly described in this way, in the cartoon presented in Figure 1 of the review of abortive infection by Lopatina and colleagues(2), if the bacteria are growing as colonies, an infection of a cell in an $Abi^+$ colony would not spread to the rest of the colony as the infected cell will die, but no phage would be produced. If the infected colony is $Abi^-$, phage would be produced and the infection would spread to the rest of the colony. Stated another way, in physically structured populations in the presence of phage $Abi^+$ cells would have an advantage over $Abi^-$, and populations of $Abi^+$ cells could increase when initially rare in a community of $Abi^-$ bacteria. Evidence for this being the case is presented in (18). In their experiments with *E. coli* growing as colonies in structed environments, depending on the number and size of the colonies, bacteria with their Lit $Abi^+$ system were substantially more fit than the competing population of $Abi^-$.

To begin to explore the contribution of physical structure on the capacity of the retron system to protect bacteria from phage and the ability of a retron-expressing bacteria to invade in a population dominated by retron-lacking bacteria, we performed the Abi protection and invasion when rare experiments depicted in Figure 3 in 0.6% using protocol similar to that in (19). As observed in liquid culture, in soft agar retron-mediated Abi, protects the bacterial population from being killed off by the phage and prevents the replication of the phage (Figure 4). The retron-lacking population grows to maximum density and the phage are lost. And, as observed in liquid culture, the retron-expression population is not able to increase when rare. Also, as observed in liquid culture, the bacteria recovered at 24 hours were resistant to $\lambda^{VIR}$.

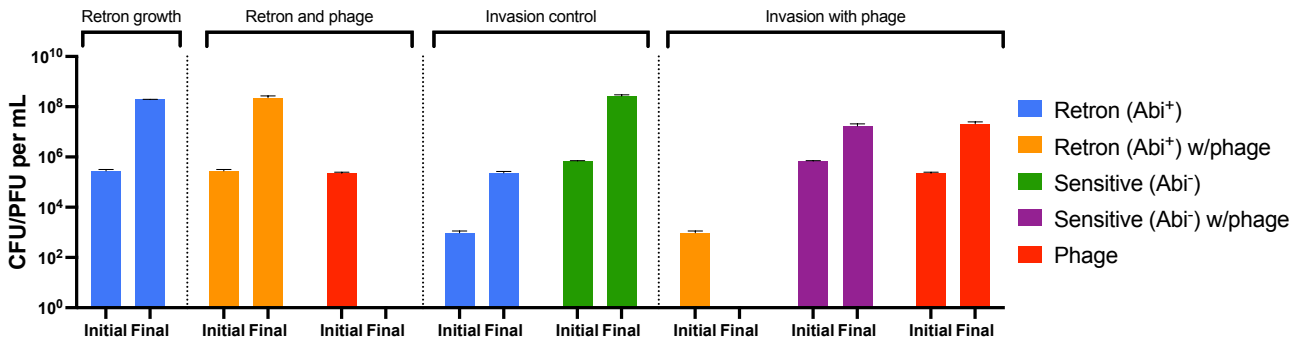


**Figure 5**. Replica of Figure 3 in a structured environment. Demonstrated here is the Abi's ability to protect bacterial populations and its ability to invade Abi$^-$ populations. Presented are means and standard errors from three independent replicas. The competitions are measuring the changes in the densities of bacteria and phage over 24 hours for retron-expressing bacteria in the absence of phage ( blue) and in the presence of phage (orange) and retron-lacking bacteria in the absence of phage (green) and in the presented of phage (purple), when challenged with $\lambda^{VIR}$ (red).

## Discussion

Over the past forty or so years there have been number of reports of abortive phage infection systems, Abi, protecting populations of bacteria from infections with lytic phage. Using genetic procedures, these studies have elucidated the different mechanisms by which the replication of an infecting phage is aborted and the cell commits suicide. However, with the exception of (18) we have not been able to find studies addressing the population dynamic, ecological and evolutionary questions considered here: Under what conditions will Abi protect populations of bacteria from infections with phage? Under what conditions will Abi evolve in populations of bacteria without this defense mechanism?

The results of our analysis of a mathematical model of the population dynamics of abortive infection identified a major constraint on the conditions under which Abi can protect bacteria from phage infection. The Abi system has to be nearly perfectly effective. If an Abi system fails to protect the bacterium from virulent phage infection with a probability of 2% or more, the phage will proliferate, and the bacterial population will be eliminated unless there is some mechanism of bacterial resistance or persistence in the presence of phage. Stated another way, our model predicts that for an Abi system to be able to defend bacteria from the ravages of phage infection, it must be more than 98% effective.

Our model predicts that even when an Abi defense system is 100% effective for preventing lytic phage replication and there are an abundance of phage, an initially rare population of $Abi^+$ bacteria will not be able to become established in an $Abi^-$ population. Our experiments testing this hypothesis are consistent with this prediction. In liquid culture, the retron-expressing populations were unable to become established in populations dominated by retron-lacking competitors (Figure 4). In these experiments, the *E. coli* population surviving this encounter with $\lambda^{VIR}$, was dominated by retron-lacking $\lambda^{VIR}$ resistant mutants.

It has been suggested that abortive infection mechanisms evolved in physically structured habitats, where the bacteria exist as colonies rather than free cells (20). Under these conditions, when phage infect retron-expressing colonies, the infected cells die but do not produce phage, and the vast majority of bacteria in the colony survive. To test this hypothesis for the ecological role and evolution of Abi, we performed our protection assay and invasion-when-rare experiments in soft agar. The results of these experiments indicate that in physically structured population the retron-mediated Abi can protect bacteria from being killed off by phage and prevent the replication of the phage. However, as in the case of our liquid culture experiments, our results indicate that abortive infection is only part of the reason retron-expressing populations are protected from invasion by phage. Within short order phage-resistant cells emerge and ascend to dominate the bacterial population. Furthermore, as observed in liquid culture retron-expressing *E. coli* cannot increase when rare in populations dominated by retron-lacking bacteria. There is a caveat to the latter conclusion in that our soft agar physically structured population may not be adequately structured to allow the retron-expressing to increase when they are initially rare.

We end on a somewhat philosophical note. People commonly assume that the phenotype observed is the object of natural selection. For example, resistance generated by modifications of the receptor sites to which phage adsorb evolves through selection mediated by phage. That selection

can be easily demonstrated by exposing sensitive bacteria to phage. There is, however, another side to this. Clearly, the receptor sites to which the phage adsorb did not evolve to adsorb phage. Throughout this investigation we, and all of the abortive infection articles cited, implicitly or explicitly assert that Abi evolved in response to selection mediated by phage. Could it be that retrons and other Abi systems evolved and are maintained by selection for factors other than as defenses against phage infection?

## Materials and Methods:

### Growth media and strains

Bacterial cultures were grown at 37 °C in MMB broth (LB broth (244620, Difco) supplemented with 0.1 mM $MnCl_2$ and 5 mM $MgCl_2$). The *E. coli* strain containing the Ec48 retron plasmid was obtained from Rotem Sorek. The sensitive *E. coli* used for controls was MG1665 marked with streptomycin resistance and the Ec48 was marked with ampicillin resistance to differentiate in the invasion experiments. Phage lysates were prepared from single plaques at 37 °C in LB medium alongside *E. coli* C. Chloroform was added to the lysates and the lysates were centrifuged to remove any remaining bacterial cells. The $\lambda^{VIR}$ strain used in these experiments was obtained from Sylvain Moineau.

### Sampling bacterial and phage densities

Bacteria and phage densities were estimated by serial dilution in 0.85% saline followed by plating. The total density of bacteria was estimated on LB hard (1.6%) agar plates. In invasion experiments, diluted samples were placed on LB hard (1.6%) agar plates supplemented with ampicillin (2.5%) or streptomycin (4%) plates to distinguish bacteria type. To estimate the densities of free phage, chloroform was added to suspensions before serial dilution. These suspensions were plated at various dilutions on lawns made up of 0.1 mL of overnight LB-grown cultures of *E. coli* C (about $5\times10^8$ cells per mL) and 4 mL of LB soft (0.65%) agar on top of semihard (1%) LB agar plates.

### Resistance Testing with Cross Streaks

Bacteria were tested by streaking in straight lines ten colonies from 24-hour plates across 20 µL of a $\lambda^{VIR}$ lysate (>$10^8$ plaque-forming units [pfu]/mL) on LB hard (1.6%) agar plates. Susceptibility to $\lambda^{VIR}$ was noted as breaks in the lines of growth. Continuous lines were interpreted as evidence for resistance.

### Growth Rate Estimations

Growth rates were estimated in a Bioscreen C. 48-hour overnights of each strain to be tested were diluted in MMB broth to an initial density of approximately 1e5 cells per ml. 10 replicas of each strain were loaded into 100-well plates and grown at 37c with shaking for 24 hours taking OD (600nm) measurements every five minutes. See Supplemental Table S2.

### Preparing liquid culture experiments

Bacterial overnight cultures grown at 37 °C in MMB Broth were serially diluted in 0.85% saline to approximate initial density and 100 µL were added to flasks containing 10 mL MMB. $\lambda^{VIR}$ lysate (>$10^8$ [pfu]/ml) was serially diluted to an MOI of ~1 and 100 µL was added to the appropriate flask. These flasks were sampled for both phage and bacterial initial densities and were then grown at 37°C with constant shaking. The flasks were once again sampled for phage and bacterial densities.

### Preparing experiments in physically structured soft agar cultures

Bacterial cultures grown at 37°C in MMB and $\lambda^{VIR}$ lysate were serially diluted in 0.85% saline to appropriate initially densities. The final dilutions were sampled for phage and bacterial initial densities and 100 µL of diluted phage and bacteria were added to 4 mL of LB soft (0.65%) agar and poured into small petri dishes which were grown at 37°C. After 24 hours, the agar was placed

into a tube containing 6 mL of saline, vortexed and sonicated for 1 hour. These tubes were serially diluted and sampled for final phage and bacterial densities.

**Numerical solutions – computer simulations.**
To analyze the properties of this model we use Berkeley Madonna to solve the differential Equations 1-7. The growth rate and phage infections parameters used for these simulations are those estimated for *E. coli* and $\lambda^{VIR}$. See Supplemental Table S1 for the values of these parameters. Copies of this program are available at www.eclf.net.

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