Modeling the directed evolution of broad host range phages

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Abstract

Background. The host ranges of individual phages tend to be narrow, yet many applications of phages would benefit from expanded host ranges. Empirical methods have been developed to direct the evolution of phages to attack new strains, but the methods have not been evaluated or compared for their consequences. In particular, how do different methods favor generalist (broad host range) phages over specialist phages? All methods involve exposing phages to two or more novel bacterial strains, but the methods differ in the order in which those hosts are presented through time: Parallel presentation, Sequential presentation, and Mixed presentation.

Methods. We use a combination of simple analytical methods and numerical analyses to study the effect of these different protocols on the selection of generalist versus specialist phages.

Results. The three presentation protocols have profoundly different consequences for the evolution of generalist versus specialist phages. Sequential presentation favors generalist almost to the exclusion of specialists, whereas Parallel presentation does the least so. However, other protocol attributes (the nature of dilution between transfers of phages to new cultures) also have effects on selection and phage maintenance. It is also noted that protocols can be designed to enhance recombination to augment evolution and to reduce stochastic loss of newly-arisen mutants.

Key words: phage therapy, natural selection, recombination, theory, computation, ordinary differential equation

1. Introduction

Collectively, phages are the main global predators of bacteria. However, individual phages tend to have narrow host ranges, often not even encompassing entire bacterial species; even when phages are found to infect taxonomically diverse bacteria, they typically do not infect broadly within those bacterial groups (Bielke et al. 2007; Hyman and Abedon 2010; de Jonge et al. 2019; Cazares et al. 2021; Fong et al. 2021), and the diversity of bacterial anti-phage defense mechanisms virtually ensures an absence of systematically broad host ranges (Labrie, Samson, and Moineau 2010).

When phages are employed to kill bacteria, as in phage therapy, broad host ranges are desirable. Most simply, a broad host range facilitates treatment of many patients using the same formulation. The goal may be phages with individually broad host ranges or instead a

cocktail of several phages with a collective, broad host range. As few phages isolated directly from nature ever have the desired host range for any application, protocols have been developed to artificially evolve phages to attack the bacteria of interest (Adams 1959; Ross, Ward, and Hyman 2016; Hyman 2019). There are likewise cases in which it has been difficult to find phages for treating a patient's infection, for which rapidly evolving phages to attack a bacterial strain would be useful (Schooley et al. 2017; Dedrick et al. 2019; 2021). Furthermore, the facile evolution of new host ranges for a phage allows one a wide selection of phage characteristics that may be desired, in essence choosing the ideal phage first (e.g., for fast growth, low immunogenicity, use of specific bacterial receptors) and then evolving its host range to suit the application.

Protocols for host range expansion have been developed empirically, with little attention to the quantitative consequences that may affect a method's success. Part of the reason for this inattention to detail is pragmatism: if a method yields an acceptable outcome, then there is no practical need to understand why. Yet, as phage therapy looms ever larger as an alternative to antibiotics, cases are arising in which phages are limiting – there may be no phages to initiate treatment, or phages may be lacking for treatment of bacteria that evolved to resist the first round of phage therapy. This increased applicability of phage therapy warrants understanding the various methods that are available to expand its utility.

Our focus is on the 'directed' evolution of host range in phages – the use a laboratory environment to evolve one or more phages to infect several host strains. We apply mathematical/computational models to compare some of the different protocols that have been developed. This approach provides a relatively straightforward comparison of the host range characteristics likely to be evolved in the different protocols. The problem is intrinsically simple when the goal is to obtain phages growing a single (new) host. But once the goal expands to phages that grow on two or more new hosts, the possible outcomes range from 'specialist' phages that each grow on single hosts to 'generalist' phages that grow on multiple hosts, and the user may prefer generalist phages to specialists. The protocol influences these outcomes.

Host-range expansion protocols can differ in many ways. The methods so far developed for growth on several host strains are similar in that they expose phages to the different host strains to specifically amplify the phages that grow. But three methods have been used that differ in how those multiple hosts are presented to phages:

(i) Parallel presentation. Phages are exposed to each the different hosts in separate cultures, with the phages (culture supernatant) from all cultures pooled free of cells and again distributed to the different hosts (e.g., the Appelmans protocol and variations of it, Burrowes, Molineux, and Fralick 2019; Mapes et al. 2016).

(ii) Sequential presentation. Phages are grown on one host, then the pool of phages from that host is transferred to another host, and so on (Yu et al. 2016). The process continues by rotating through each host and is then repeated. In one variation of this protocol, actual plaques are chosen for propagation from one host to the next (Yu et al. 2016).

(iii) Mixed presentation. Phages are grown in a mix of all hosts in a single culture. The bacterial mix is periodically or continually refreshed to ensure the continued presence of all hosts while maintaining the supernatant containing phage. This method

originates with d'Herelle (1926) using one non-permissive and one permissive host (as attributed by Adams 1959, p. 129).

Any of these presentation protocols may be used with few or many hosts, and the degree of bacterial strain diversity may be varied as well. Typically, all methods include exposure to a permissive host to ensure the continued maintenance of the phage and include one or more initially non-permissive hosts on which phage growth is desired. Protocols may likewise differ in the number and diversity of phages used at the start.

Perhaps the most common approach to the directed evolution of host range has been to screen for plaques on the non-permissive host (Adams 1959; Hashemolhosseini, Holmes, et al. 1994; Hashemolhosseini, Montag, et al. 1994; Yu et al. 2016). The host presentation protocols instead propagate phages agnostically, before it is known whether any phages exist that grow on the non-permissive hosts. One problem with relying on plaque isolation as the sole basis of selection is that poorly growing phages may not form plaques initially, but they may quickly evolve to do so. The blind transfer of a supernatant containing poorly growing phages facilitates the evolution of better phage growth, so that phages can eventually be obtained that do form plaques.

The focus of this study is to evaluate host presentation as it affects the directed evolution of host range. That is, how does the host-presentation protocol affect selection of generalists over specialists – phages that grow on one host versus many? The empirical goal may be to generate a single, broad host range phage, or instead to generate phages that grow best on any of several hosts, and different protocols will have different outcomes toward these goals. However, evolution also depends on the introduction of (favorable) variation and the maintenance of that variation long enough for selection to elevate its frequency. Following the treatment of selection, the paper will address some features of protocols that affect these other contributions to evolution.

2. Methods

2.1 Growth dynamics

Each of the three presentation protocols alternates a growth phase with a sampling/dilution/transfer phase. This cycle is repeated until either the desired outcome is achieved or some total duration of effort has been reached. We modeled the growth phase as a system of ordinary differential equations assuming one or two hosts and three phages. We assumed lytic phage growth with constant rate parameters and exponential growth of bacteria. Following (Weitz 2016), the general framework for our differential equations (which can be expanded to an arbitrary number of phages and hosts) is

$$\frac{dP_{i|j}}{dt} = b_{ij}\lambda_{ij}I_{ij} - k_{ij}P_{i|j}C_j \qquad (1a)$$

$$\frac{dI_{ij}}{dt} = k_{ij}P_{i|j}C_j - \lambda_{ij}I_{ij} \qquad (1b)$$

$$\frac{dC_j}{dt} = r_jC_j - \sum_i k_{ij}P_{i|j}C_j \qquad (1c)$$

Here, $i \in \{A, B, G\}$ is the index for the phage strains and $j \in \{A, B, (A, B)\}$ is the index for the bacterial environments into which the phage are placed, with (A, B) denoting the mixed environment with both hosts. $P_{i|j}$ is the density of free phage *i* growing in bacterial environment *j*; I_{ij} is the density of strain *j* cells infected by strain *i* phage; C_j is the density of bacterial strain *j*. The lysis rate for the I_{ij} infected cells is λ_{ij}, and b_{ij} is the corresponding burst size; the adsorption rate for phage strain *i* onto bacterial strain *j* is k_{ij} and r_j is the intrinsic growth rate of bacterial strain *j*. Aside from burst size, which we model as depending on the phage strain, we took the parameters to be constant even though their dependence on *i* and *j* in practice is understood. The sum in (1c) is over all phage strains *i* that are placed in a single culture of bacterial strain *j*.

For parallel host presentation, there are two separate bacterial cultures $j = A, B$ into which we place all three phage strains, resulting in phage densities $P_{i|A}$, $P_{i|B}$, which we then combine to get density $P_{i|A} + P_{i|B}$. For sequential host presentation, we have one bacterial strain at a time, *j=A* or *j=B* depending on which phase of the alternating cycle we are in. For mixed host presentation, $j = (A, B)$.

Trials followed these equations for 20 time-step 'cycles,' at which point the phages from cultures were recovered, combined (for Parallel presentation), diluted, and mixed with cells at a renewed cell density of 10^7. These new cultures were again assumed to proceed for another 20 time-step cycle, whence phages were recovered, and so on. The process ended after 1,000 time steps (50 cycles). Differences in host presentation protocols were reflected in the equations. Thus, for Parallel presentation, a separate set of equations (accommodating one cell type and three phages) was used for each host. For Mixed presentation, the same, single set of equations was used for each cycle (two cell types, three phages in a single culture), whereas Sequential presentation used a single set of equations for three phages with one cell type, but the cell type changed between cycles.

Except for burst sizes and bacterial growth rates, the same set of parameter values was used for all trails and for all (i,j). Omitting subscripts, $r = \{0.1, 0.3\}$, $b = \{10, 15, 20\}$, $\lambda = 1.0$, and $k = 10^{-9}$. If a time step is equated to an hour, these values approximate what might be used in the lab for phages from nature, but they don't span the spectrum of what is known (Levin, Stewart, and Chao 1977). Thus, a cycle would correspond to just under a day, a trial of 1000 time steps would correspond to just under two months. Bacterial growth rates would be slower than is typical for *E. coli* in liquid culture but within the realm of other bacteria under less ideal conditions. An average lysis time of 1 hr would be long for some phages, but the exponential decay stemming from ordinary differential equations results in a highly asymmetric distribution of lysis intervals, and it is known that early lysis has a disproportionate effect on phage growth when the phage population is increasing (Bull, Heineman, and Wilke 2011). And although these equations characterize phages with 3 parameters (burst size, lysis time, adsorption rate), trials varied only burst size. We consider that use of this single variable is adequate to capture other causes of differences in phage growth rate, and in turn that phage growth rate is the determinant of competition outcomes in these models.

We acknowledge that any implementation will violate some of our assumptions in ways likely to affect the outcomes quantitatively: phages will have discrete lysis times, bacterial suitability for phage growth will change over the course of the culture duration, bacteria will diversify into different structural states (planktonic and wall growth) that are differentially susceptible to

phages, and resistant bacterial will begin to evolve in the cultures. The models are thus intended to capture broad features of protocol effects on phage competition, but individual cases will certainly be affected by details. Furthermore, the major goal of this study is to compare presentation protocols for their impact on the evolution of generalists over specialists, and that comparison should be robust to parameterizations.

Numerical analyses of these equations were carried out both using C code written by us and, in many cases also with Mathematica® 13.1 using NDSolve. Individual trials were run for 1000 time steps. The C code used the Euler method with a step size of 0.001. The two approaches yielded indistinguishable patterns of evolution; all figures except Fig. 4 were derived from the C code with graphics in ggplot2 (R Core Team 2022).

2.2 Dilution protocols

Any host-presentation protocol will involve dilution of the phage. Dilutions were simulated to occur at the end of every 'cycle' (defined as 20 time steps). The specific order of events was that phages were recovered, pooled (for Parallel presentation), then diluted when being mixed with hosts at a renewed cell density of 10^7 to start a new cycle. With Parallel presentation, the different host cultures incubated at the same time were assumed to have equal volumes, so that a phage's density on one host was combined with its density on the other host weighted equally. One key assumption was that infections and free phages had different fates at the end of a cycle. To mimic how actual protocols might operate to recover phages free of bacteria (e.g., chloroform treatment or filtering), we assumed that all infected states were lost; this assumption often had little consequence, as cells had been exhausted and infected states minimized by cycle's end. Trials were also run in which the infected states were carried over to the new cultures (at the same dilutions as free phages), with no fundamental change in patterns.

Two dilution protocols were evaluated: fixed volume and fixed count. The fixed-volume dilution was a simple transfer of 5% of the (free) phage density into the new cultures; with Parallel presentation, 5% of phage densities from each culture went into each of the new cultures. In contrast, fixed-count dilution reduced the phage density to 1000 in the new cultures (with Parallel presentation, 1000 phage were introduced into each of the new cultures). As phage densities at the end of a cycle often reached 10^8 or more, the two dilution protocols often resulted in orders of magnitude more phages being transferred in the fixed-volume dilution method than in the fixed-count method. Thus the two methods differ both in density of phages transferred and in the fraction of phage transferred from the previous cycle.

2.3 A historical note

An expansive Parallel presentation method referenced below is called the Appelmans protocol. We consider the Appelmans protocol to be the method described by Burrowes, Molineux, and Fralick (2019) and fundamentally similar to that of Mapes et al. (2016), who credit their method to the dissertation of Burrowes. The paper usually cited for the Appelmans protocol (Applemans 1921) merely describes serial dilution as a way of determining phage concentration; there is no mention of multiple phages or non-permissive hosts. Burrowes et al. (2019) attribute their method to the Eliava Phage Institute in Tblisi, Georgia.

3. Results

Our primary focus is the effect of protocol on selection among phages with different growth properties. The basic protocol is that phages are mixed with host cells, grown 20 time steps (a 'cycle'), diluted and redistributed to new hosts. Several simplifications are used to render the

analysis both interpretable and general: (1) host-range phage mutants are present and common enough to have escaped random loss: deterministic selection is operating; (2) all trials start with one generalist phage and two specialist phages; (3) two hosts are used, either together in one culture (Mixed), separately but concurrently (Parallel) or temporally separated (Sequential). The two hosts are designated A and B; the three phages are ϕ_A , ϕ_B , ϕ_{AB} , the subscript indicating which hosts are permissive to each phage. Thus ϕ_A is a specialist on A, ϕ_{AB} is the generalist that grows on both hosts.

Despite these simplifications, the results should generalize. For example, since it is apparent that the best-growing specialist using a host will prevail over other, 'lesser' specialists using the same host, the restriction to a single specialist per host is reasonable. The use of two hosts seems the only practical way of developing intuition; there can be little doubt that results will generalize in many ways beyond two hosts, but the combinatorial possibilities arising from more than two hosts is quickly cumbersome. By assuming deterministic dynamics, the models become agnostic to the origins of the different phages. It thus makes no difference whether the 'mutants' arose via mutation and recombination in the course of phage growth in the laboratory, were isolated from the wild and combined into a pool that is being grown, or are the products of an engineered library (Yehl et al. 2019; Latka et al. 2021).

We use two approaches in addressing questions about selection. First, we develop a simple heuristic approach that requires almost no analysis. These methods strip the selective process to the bare minimum, reducing phage growth to a single number per culture. Despite its simplicity, this approach is informative. Beyond that we use formal dynamic calculations of each host presentation protocol, with some variations.

3.1 Heuristic models

3.1.1 Parallel presentation

We begin with an approach that omits many details of phage growth but may help deconvolute the complexity of comparisons between a generalist and specialist phages. The first example assumes Parallel presentation: the phage pool is distributed to separate cultures of host A and B, grown, then pooled again before the next exposure to hosts. Phage growth in a culture is assigned a single number that reflects the number of descendants per phage at the end of the culture period, immediately before dilution (Table 1).

Table 1: Host-specific growth of different phages per culture

		Host A	Host B
Phage	ϕ_A	$N_{A\|A}$	1
	ϕ_B	1	$N_{B\|B}$
	ϕ_{AB}	$N_{AB\|A}$	$N_{AB\|B}$

The subscript of $N_{i|j}$ gives the designation of the phage (i) followed by the host on which it is grown (j). A value of 1 is assigned to each of the specialists in cultures of their respective non-permissive hosts to represent phage survival without growth.

After one round of growth, the numbers of phages in the pool combining A and B cultures will have changed in proportion to the numbers given in Table 2:

Table 2: Combined growth after pooling

ϕ_A	$N_{A\|A}$ +1
ϕ_B	1 + $N_{B\|B}$
ϕ_{AB}	$N_{AB\|A}$ + $N_{AB\|B}$

The ratios of the A-specialist to the generalist have changed by

$$\frac{N_{A|A} + 1}{N_{AB|A} + N_{AB|B}} \quad . \qquad (2a)$$

Similarly, the B-specialist has fared against the generalist according to

$$\frac{N_{B|B} + 1}{N_{AB|A} + N_{AB|B}} \quad . \qquad (2b)$$

If either ratio exceeds 1, the specialist on that host will be increasing over the generalist, and vice versa.

These results reveal that, with Parallel presentation, a generalist has an arithmetic advantage over specialists from its growth on multiple hosts. But this advantage is modest such that a relatively meagre superiority of the specialist will allow it to avoid being displaced by the generalist. There is an asymmetry favoring the generalist: whereas the superiority of the generalist on one host ensures the loss of that specialist, a superiority of the specialist on one host does not ensure loss of the generalist because the generalist may be maintained via its growth on the other host. There are three possible outcomes. (1) the generalist has a selective advantage over both specialists. (2) the two specialists each have advantages over the generalist. (3) the generalist has an advantage over one specialist but not both. If the goal is to obtain a generalist phage, the second outcome is the worst because the generalist will be lost. Even if the generalist displaces only one of the specialists, and thus is not the only phage in the evolved culture, it can be recovered from the selection by testing individual phages.

The simplicity of these numbers belies a complexity. Specifically, the $N_{A|A}$ and $N_{AB|A}$ are not independent: rapid growth of one phage on A will affect host abundance and thus affect the numbers attained by the other phage on A (likewise for $N_{B|B}$ and $N_{AB|B}$). Yet this interdependence will matter only if the culture is grown long enough that bacterial numbers are overwhelmed by phage growth. The $N_{i|j}$ are also not necessarily constant, depending on the numbers of phages added to a culture (which may differ across cycles). These simple numbers are therefore offered merely as a heuristic. Furthermore, the numbers $N_{i|j}$ cannot be analytically calculated from easily measured phage properties such as burst size, lysis time and absorption rate. Indeed, if the bacteria grow rapidly enough relative to the phage, a phage with a low growth rate on one host may actually attain a higher $N_{i|j}$ than if it had a high growth rate (Kerr et al. 2006).

The ratios in (2) suggest a possible undesirable outcome of parallel presentation, what might be called a ‘tyranny’ of generalists on good hosts. Suppose that, regardless of phage, host A is

very productive for phage infection but B is not ($N_{A|A}$ and $N_{AB|A}$ are large, $N_{B|B}$ and $N_{AB|B}$ are small). The ratios indicate that, even if the specialist on B is much better than the generalist on B, the generalist may displace that specialist because of the generalist's high output on A. If host B is an important target for phage therapy despite its unsuitability for phage growth, a parallel presentation protocol will work against the evolution of specialist phages that excel on B. Below, we suggest a modification of Parallel presentation to avoid this outcome.

A further complication is that these ratios reflect the relative fates of the different phages but not whether they grow fast enough to be maintained. Maintenance will depend on both selection and dilution. In the trivial case, the phage pool could be diluted so much that all phages vanish – the dilution across transfers exceeds the largest $N_{i|j}$. Less obvious, strong differences in phage production between hosts A and B combined with extreme dilutions can result in the phage pool purging any specialist growing on the poor host for demographic reasons, not because of its evolutionary inferiority. The specialist on the poor host could have a selective advantage over the generalist on that host, yet be lost because it is diluted too much, while the generalist is maintained because of its growth on the good host. Such a case will be illustrated below.

3.1.2 Sequential presentation

Here the entire phage pool is cultured on one host (A), whence the resulting phage pool (cleared of bacteria) is grown on host B, and the cycle is repeated. Yu et al. (2016) proposed two versions of a sequential protocol, and they specifically suggested that sequential was a good method for evolving generalists. One method (Method A) selected plaques on one host to continue the transfer to the next host. The other method (B) involved the transfer of supernatants from host to host. Method A could not possibly retain specialists or generalists that did not form visible plaques. Our focus is therefore on Method B. Using our heuristic approach, phage growth across one round of alternation will be proportional to the following:

Table 3: Phage outputs during a cycle of sequential presentation

ϕ_A	$N_{A\|A} \cdot 1$
ϕ_B	$1 \cdot N_{B\|B}$
ϕ_{AB}	$N_{AB\|A} \cdot N_{AB\|B}$

with relative performances

$$\frac{N_{A|A}}{N_{AB|A} \cdot N_{AB|B}}, \qquad (3a)$$

$$\frac{N_{B|B}}{N_{AB|A} \cdot N_{AB|B}}. \qquad (3b)$$

It is now easily seen that, in contrast to Parallel presentation, the generalist grown under alternating hosts realizes a multiplicative/geometric advantage of its growth on each host; the specialists grow only on their permissive hosts. This should strongly favor the generalist.

3.1.3 Mixed presentation

This protocol is easy to implement but offers the greatest challenge for intuition. All three phages are growing in one culture of both hosts. In contrast to the other two protocols, the generalist quantities $N_{AB|A}$ and $N_{AB|B}$ cannot be separated from each other because the generalist is infecting both hosts in the same culture: its numbers from one host affect its numbers on the other host. To create a combined value for the generalist merely trivializes the problem to the point of uselessness. Some of these challenges have been addressed in models that assume a constant abundance of hosts (Bull 2006), but the protocols for host range evolution often violate this assumption. We thus rely entirely on numerical analyses for Mixed presentation.

3.2 Numerical analyses

3.2.1 Parallel presentation

To provide a more quantitative sense of selection, we numerically analyze ordinary differential equations similar to those in (1) that encode phage growth, bacterial growth, and bacterial death from phage attack (see Methods for the structure of equations, see Data Availability for access to the files). Each panel in Fig. 1 is a heat map illustrating the fate of one of the three phages under Parallel presentation with a fixed-count dilution (the phage pool was diluted to a density of 1000 every cycle). For a single pair of specialist burst sizes, the figure shows whether the generalist or specialists are maintained across a set of generalist burst sizes. Note that phage maintenance will generally depend both on evolution and demography, but for these trials, the outcomes are due entirely to evolution.

The results are clear (Fig. 1) and in agreement with the impressions from eqns (2). The fate of the generalist is governed by the coordinates of the pair of specialist burst sizes (15 and 15, given by the black-on-white ring): the generalist is lost if its burst sizes on both hosts are less than those of the specialists, whereas the specialists are lost if the generalist has superior bursts on both hosts. There are intermediate zones, and the generalist is not completely lost until its burst sizes fall somewhat below those of the specialists. Each specialist is lost as the respective generalist burst size exceeds 15, but there is a slight shoulder effect in which the specialist is more prone to loss as the generalist performs well on the other host. This latter effect is consistent with the ratios in (2), that the generalist is somewhat better at suppressing one specialist as it does relatively better against the other specialist. Under these conditions, Parallel presentation is a ‘fair arbiter’ of phage performance on each host: the best-growing phages are retained on each host.

Overall, the generalist is retained in approximately ¾ of the space shown. The space shown does not necessarily reflect the spectrum of biological possibilities, of course. If the generalist was constrained so that it could not outperform specialists, for example, the relevant space would be limited to the lower left quadrant, for which the generalist would be mostly absent.

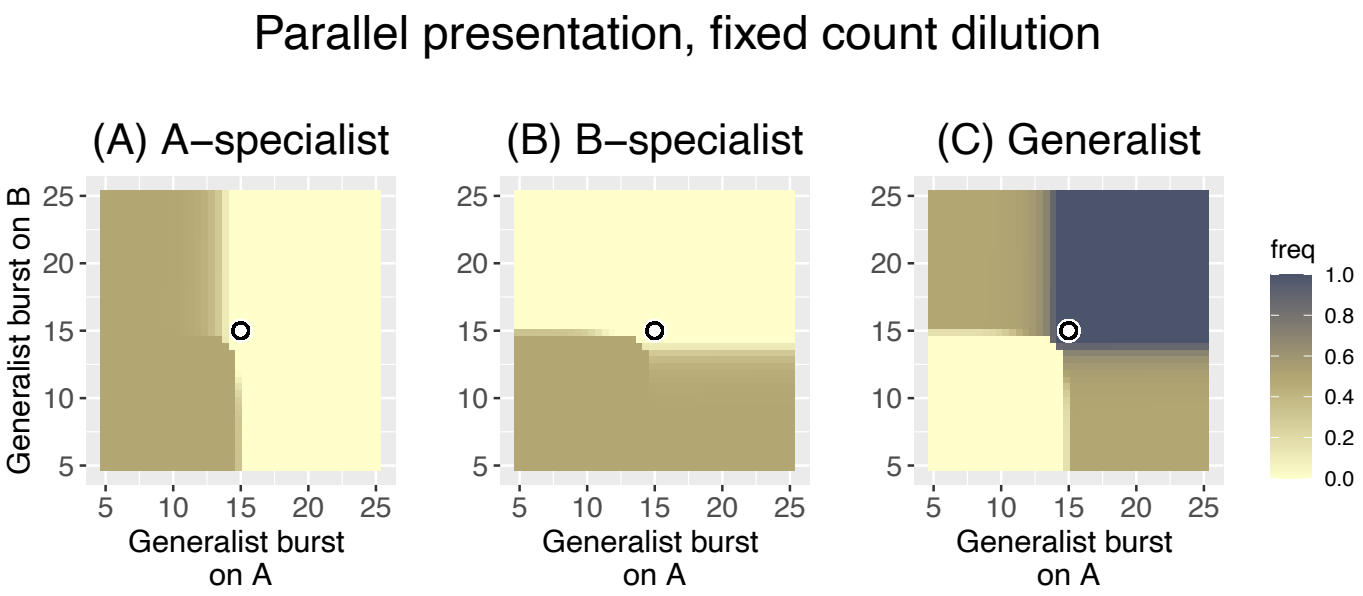

Fig. 1. Heat maps of Parallel presentation with equal burst sizes of the two specialists (15, 15, as indicated by the coordinates of the black-on-white ring). The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. The generalist is maintained on either or both hosts according to whether its burst size exceeds that of the specialist on that host (as given in the key, phage maintenance is indicated by a color darker than light yellow); there are substantial zones in which two phages are maintained, although only the generalist is maintained in the upper right quadrant. Each panel shows, for the phage indicated in the title, the spectrum of frequencies after 1,000 time steps. Each specialist is lost when the generalist burst size exceeds that of the specialist. The generalist is lost only when its burst sizes are less than those of the specialists on both hosts. Parallel presentation is thus a 'fair' arbiter of phage retention. There is a slight advantage to being a generalist in that the generalist displaces the specialist even when the generalist bursts are both slightly less than that of the respective specialist. Bacterial growth rate is $r = 0.3$ and the fixed-count dilution reduced phage density to 1000 every 20 time steps. Phage frequencies were calculated as a phage's density relative to the combined density of all phages; the key shows the colors for phage frequencies after 1000 time steps.

Now consider a case when the specialists have different burst sizes (b_A =20, b_B =10, Fig. 2). This case could arise if one host is better for phage growth than the other, or just if one specialist happens to be poor at growth. The pattern for the generalist from Fig. 1 is shifted to the new specialist coordinates but is otherwise largely the same as before.

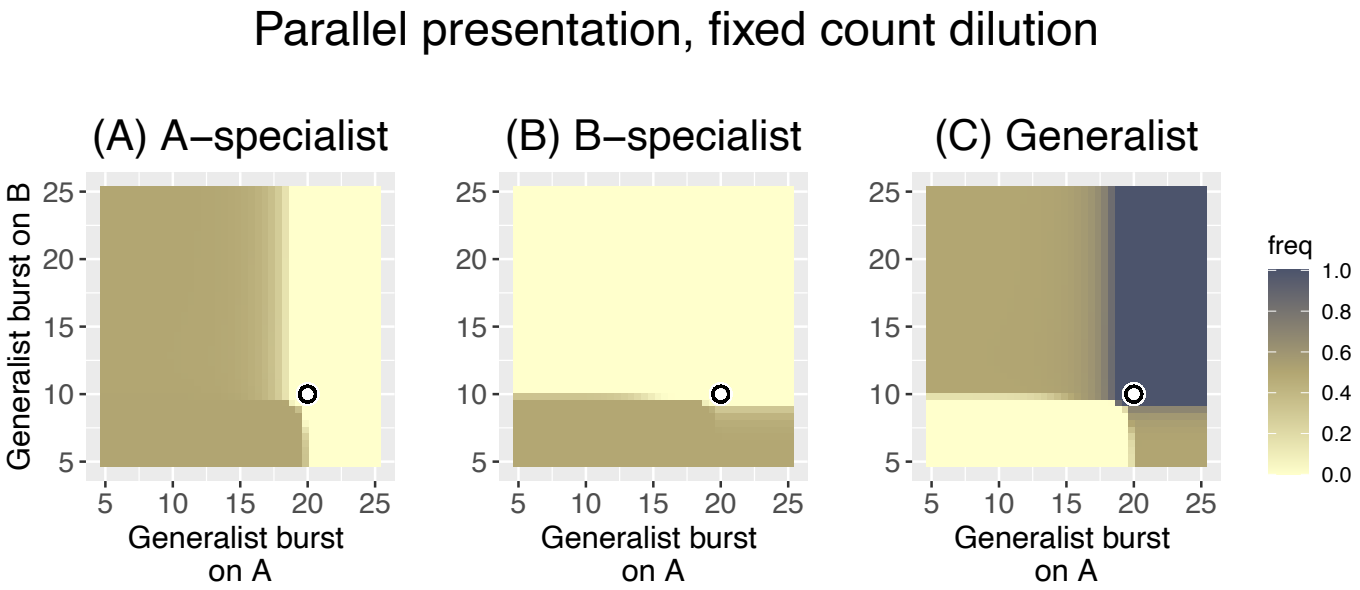

Fig. 2. Heat maps of Parallel presentation with unequal burst sizes of the two specialists (20, 10, as indicated by the coordinates of the black ring). The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. As in Fig. 1, the generalist is maintained on either or both hosts according to whether its burst size exceeds that of the specialist on that host, and there are large zones in which two phages are maintained. Each panel shows, for the phage indicated in the title, its frequencies at time 1000 based generalist and specialist burst sizes. Each specialist is lost when the generalist burst size exceeds that of the specialist. The generalist is lost only when its burst sizes are less than those of the respective specialists on both hosts. Parallel presentation is thus a 'fair' arbiter of phage retention. Again, there is a slight advantage to being a generalist in that the generalist displaces the specialist even when the generalist bursts are both slightly less than that of the respective specialist. There is no obvious 'tyranny' of generalists on the good host here. However, a tyranny is not necessarily expected because the bacterial growth rate is so high (0.3) that the poor host (B) reaches a high density before the poor phage overwhelms it – resulting in a large $N_{B|B}$ despite its small burst. Bacterial growth rate is 0.3 and dilution reduces phage density to 1000 every 20 time steps. The key is the same as in Fig. 1.

We next consider how these outcomes depend on details within Parallel presentation. The trials in Fig. 1 assumed a bacterial growth rate of $r = 0.3$ and a fixed-count dilution to density 1000 every cycle. As phage density often reached 10^9 or more, this dilution allowed phage growth by many logs every cycle. These previous analyses were modified in either or both of two ways: (i) bacterial growth rate was reduced to 0.1, and (ii) the pool was diluted by a fixed volume (5% of the phage density). The time between phage pools remained the same as before, at 20 steps. Only the asymmetric case was considered (b_A =20, b_B =10).

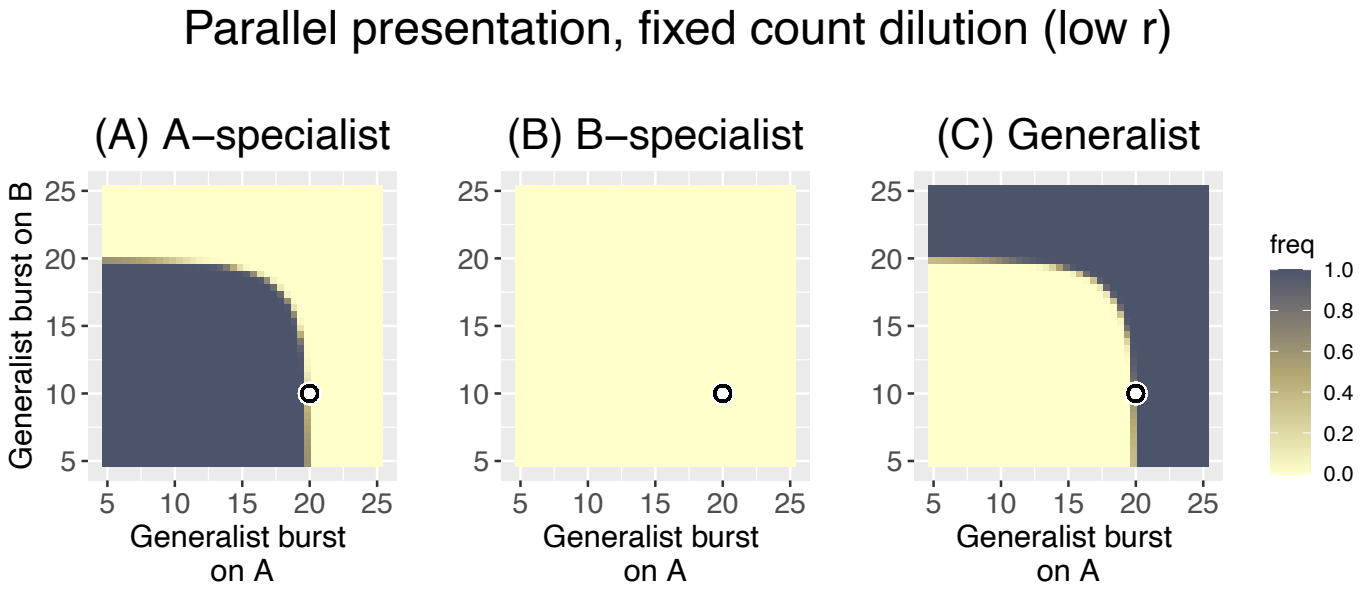

Fig. 3. Heat maps of Parallel presentation with unequal burst sizes of the two specialists (20, 10, as indicated by the black ring). The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. The trial conditions are the same as in Fig. 2 except that the bacterial growth rate has been lowered to $r = 0.1$. With this change, the pattern is radically different from before. Now only one phage is ever maintained, and the B-specialist is lost everywhere. Although the specialist burst sizes are unequal (given by the black circle), the pattern of phage maintenance is symmetric, the A-specialist being the only phage maintained up to the point that the generalist burst on either host exceeds 20 (20 is the burst size of the A-specialist). As in Fig. 1, dilution reduces phage density to 1000 every 20 time steps. The key is the same as in Fig. 1.

Merely lowering the bacterial growth rate (with no change in the dilution protocol) has a large effect on the outcome: only the A-specialist is retained in much of the space, and its effect at purging the generalist is symmetric despite the asymmetry in specialist burst sizes (Fig. 3). That is, despite the presence of two phages that can grow on B, both are lost in much of the space, and the B-specialist is lost everywhere. This radical change is necessarily due to the reduction in bacterial growth rate, which has the unexpected effect that none of the phages grows fast enough to exhaust their hosts by cycle's end (Fig. 4A versus 4B). Now, the fastest-growing phage (whether on host A or B) sets the dilution limit, and all other phages are diluted more than they grow, hence they never catch up. Thus, once the burst size of the generalist exceeds 20 – on either host – it now displaces the A-specialist for the same reason the A-specialist was displacing the other phages when it had the superior burst.

This radical change in outcome is due to demographic effects of the protocol rather than to evolution. Ironically, reducing bacterial growth rate not only limits phage growth but also prevents slow-growing phages from exhausting their hosts in a fixed-duration protocol. (Phage growth depends on the product $P{\cdot}C$, so phage growth increases with bacterial density and more quickly exhausts the cells. With lower bacterial growth, phage numbers of the more slowly-growing phages never get high enough to exhaust cells by cycle's end.) As bacterial growth rate -- or cycle length -- is increased, eventually the fastest-growing phage runs out of hosts, allowing slower-growing phages (on the other host) to exhaust their host and catch up to the dilution limit. Indeed, increasing the cycle to 40 steps while maintaining $r = 0.1$ restores the pattern to that of Fig. 2.

This case reveals that non-independence of phage growth on different hosts can arise through the dilution protocol (also dependent on culture conditions). This outcome is not due to a change in the *relative* advantage of one phage over another (i.e., not due to selection) but rather stems from the demographic consequences of the dilution protocol. Phages can be lost because they cannot grow fast enough to maintain themselves, even though they may be 'evolutionarily' superior to their competitors. The host-presentation protocol thus has ramifications for evolution and, separately, for demography.

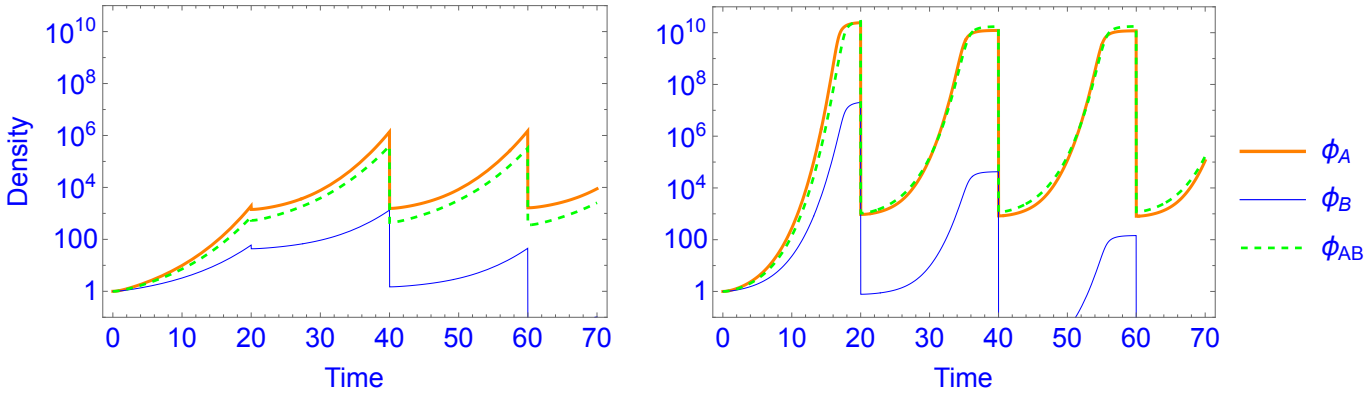

Fig. 4. Temporal, short-term dynamics of phage in Parallel-presentation trials detailed in in Figs. 3 (left panel) and Fig. 2 (right panel); the generalist has burst sizes of 17 on both hosts, representing a point in the heat map of Fig. 3 at which only the A-specialist is maintained but at which the A-specialist and generalist are maintained in Fig. 2. Comparison of these dynamics reveals why the two growth conditions yield such differences in patterns of phage maintenance between the different conditions of Figs. 2 and 3. With a low bacterial growth rate (r = 0.1, left) the phages never exhaust the bacteria before dilution; in essence, the phages are maintained in a constant state of exponential growth, and the fastest grower sets the threshold for dilution such that other phages are progressively diluted to extinction. Increasing the bacterial growth rate to r =0.3 has the unintuitive effect of allowing accelerated phage growth toward the end of a cycle; as cell densities reach high levels, phage growth accelerates, exhausting the cells. Even slowly growing phages can recover from low transfer densities and attain high densities by cycle's end. This exhaustion of hosts allows slowly growing phages on one host to be maintained along with fast-growing phages on the other host.

The second protocol modification considered here is to change the dilution mode while retaining the low bacterial growth rate of 0.1. Previously, with fixed-count dilution and low bacterial growth rate (Figs. 3 and 4A), the phage density reached just over 10^6 by cycle's end, so the dilution to 1000 phage was a 10^{-3}-fold reduction in phage density. With the fixed-volume dilution, each new cycle is started with 5% of the phage density in the phage pool. This means that any gains in total phage density in one cycle directly increase the density of phages transferred into the next cycle, which in turn results in even more phage at the end of the next cycle, and so on. This has the potential for phage concentration to become so high that bacterial density becomes limiting. Indeed, whereas phage density under fixed-count dilution ultimately reached and was maintained at just over 10^6 with transfers of 1000 phage, phage density reached almost 10^9 when transferring 5% of the pool, at which point hosts became limiting. When bacterial density becomes limiting, phages from host A no longer drive the demographic extinction of phages on host B. The patterns with fixed-volume dilution and low bacterial growth are now closer to those of fixed-count dilution with high bacterial growth (Fig. 5), although there are now also broad intermediate zones.

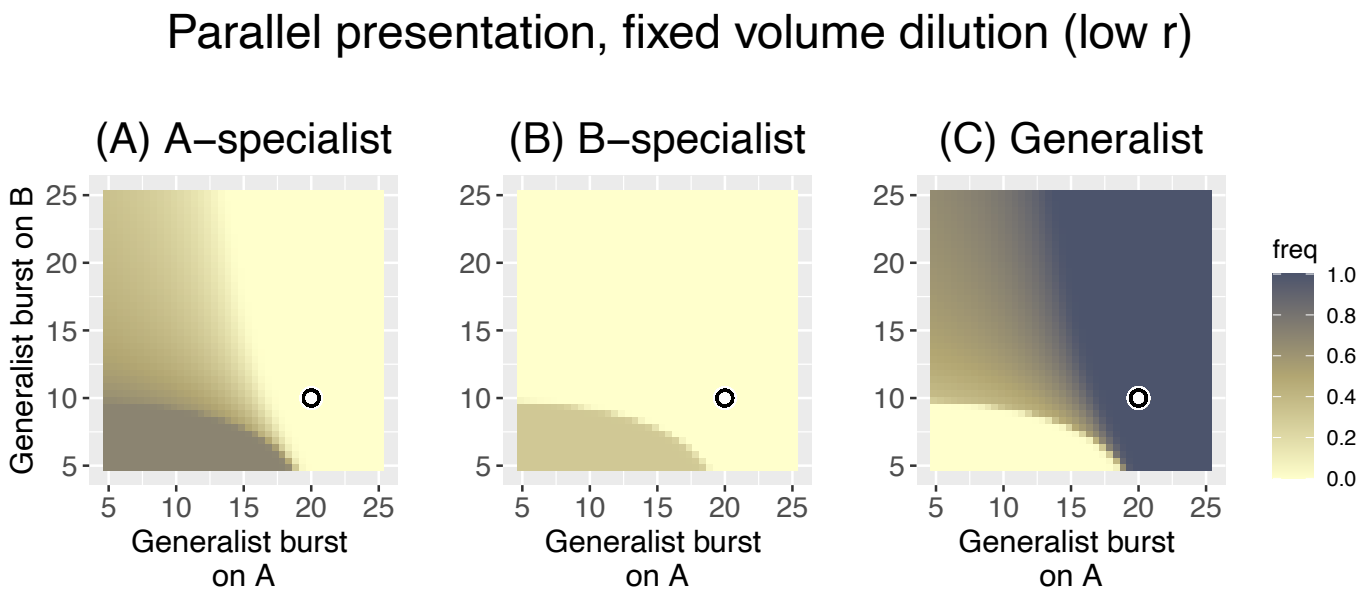

Fig. 5. Parallel presentation with fixed volume dilution and low bacterial growth rate (r=0.1). The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. Trials are the same as detailed in Fig. 3 except that more phage are transferred every cycle: here, 5% of the phage pool at the end of a cycle is added to each new culture instead of 1000 phage. Fig. 4 showed that total phage density reached just over 10^6 per cycle with fixed-count dilution, whereas here over 50,000 phage would have been transferred. The pattern is now much closer to that of Fig. 2 (r = 0.3, fixed-count dilution) than that of Fig. 3: two phages can be maintained over much of the space. Yet there are also differences from the patterns in these other two cases – the generalist is maintained over a wider set of burst size values. The key is the same as in Fig. 1.

Although the change to a fixed-volume dilution protocol (combined with low bacterial growth) somewhat restored the pattern in Fig. 2, the patterns in Figs. 2 and 5 have a striking difference: the generalist drives specialist extinction over broader parameter ranges in Fig. 5. In Fig. 5, the generalist is extinguishing specialists that are decidedly superior on the respective host. The reason for this change in outcome is not immediately clear. We conjecture that the difference lies in the lesser phage growth per cycle in Fig. 5 due to a combination of low bacterial growth rate and fixed-volume dilution. Thus, the dilution in Fig. 2 allowed approximately 1000-fold phage growth per cycle, whereas the dilution in Fig. 5 allows approximately 20-fold phage growth per cycle. This reduced growth per cycle has the effect of reducing the impact of burst size differences on the $N_{i|j}$ in the heuristic formulae (2). With smaller $N_{i|j}$, the arithmetic advantage of the generalist looms ever larger.

This change in outcome (from fixed-count dilution to fixed-volume dilution, both with low r) is readily seen to stem from a restoration of favorable demography – phages are no longer being diluted to extinction. Avoiding demographic extinction is merely a matter of growing the phages long enough relative to dilution, an outcome that can be achieved either by longer growth or lesser dilution. There are various ways to achieve this outcome, and a less obvious one is presented next.

These few variations on the Parallel presentation protocol point to manifold complexity stemming from protocol details such as cycle duration, bacterial growth rate and dilution. They likewise motivate ways to avoid ‘unfair’ recovery of phages. One modification that could ensure

maintenance of phages on each host despite differences in bacterial growth rate is to set the dilution separately for each host and then pool the samples. This protocol would also mitigate a tyranny from the generalist and prevent phage loss due to poor growth on one host even with high dilution rates (Fig. 6 for comparison to Fig. 3).

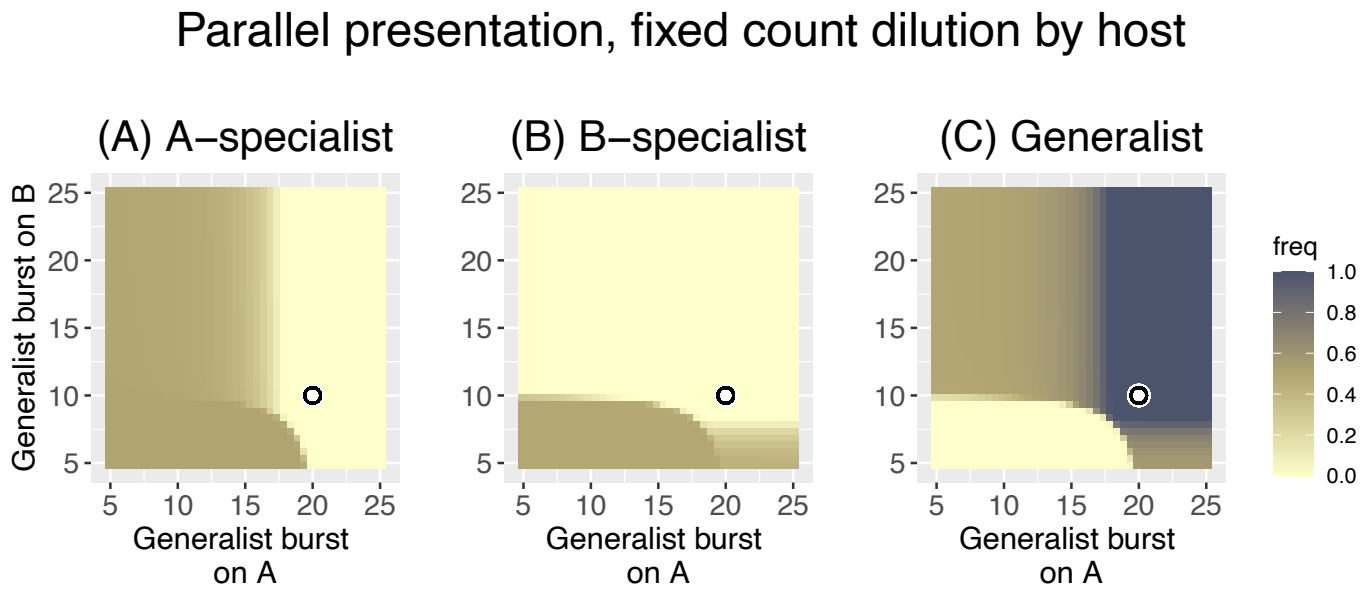

Fig. 6. Trials of Parallel presentation under the same conditions as in Fig. 3 (bacterial growth rate $r = 0.1$, fixed-count dilution), except that the phage pool from each host is diluted to density 1000 in each culture separately before being combined into a common pool. The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. In contrast to the pattern with fixed-count dilution and low bacterial growth in Fig. 3, the pattern with separate dilutions is now similar to that with high bacterial growth rate ($r = 0.3$, Fig. 2), although the generalist enjoys somewhat more of an advantage here than in Fig. 2. The key is the same in all figures.

3.2.2 Sequential presentation

The advantage of sequential presentation suggested by the heuristic analysis in (3) is equally evident from numerical analyses (Fig. 7). Thus, the Sequential protocol strongly favors the generalist even to the point of displacing far superior specialist phages.

Alternating hosts does not change phage properties *per se*, it merely selects a wider set of broad host range phages than does parallel presentation. Perhaps surprisingly, many of the issues with Parallel presentation are still present with sequential presentation. Thus, depending on dilution rates and bacterial growth, it's possible to purge all phages that grow on one of the hosts. And the 'tyranny' of generalists is now writ large because of the geometric advantage of the generalist over both specialists. But if the goal is to obtain a generalist, Sequential presentation is far better than Parallel.

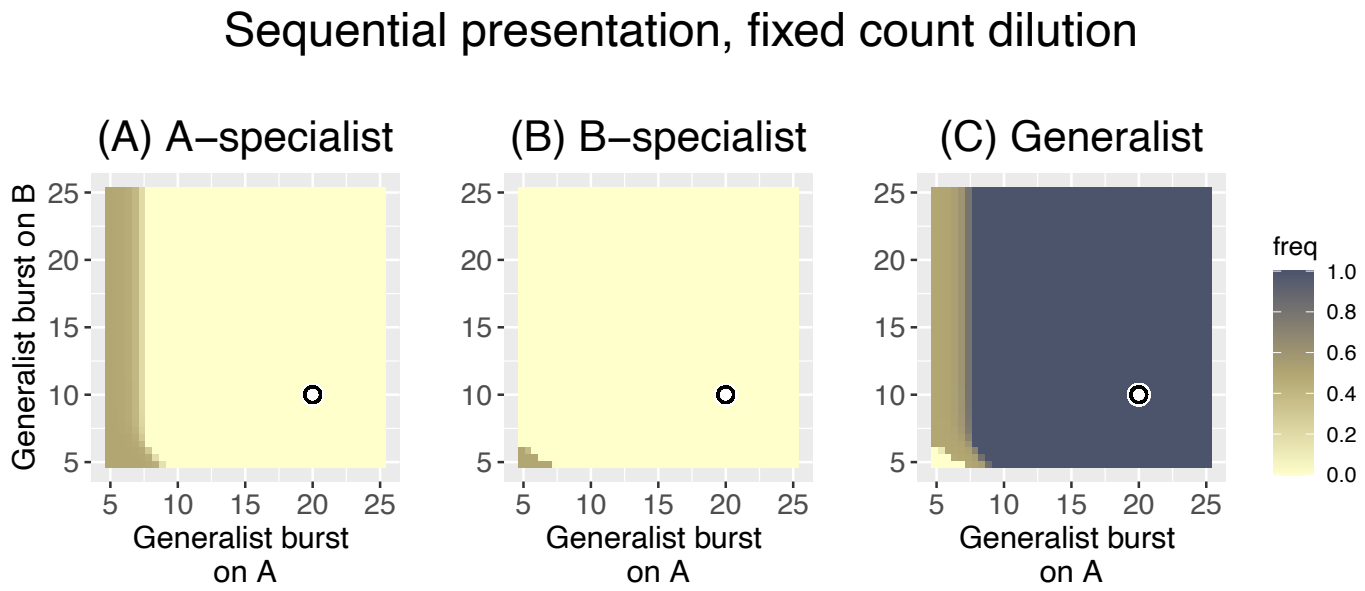

Fig. 7 Sequential host presentation provides a huge advantage for the generalist. The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. Trials were run under the same conditions as in Fig. 2 (r = 0.3, fixed-count dilution), but hosts were presented sequentially. The generalist is lost in only a tiny corner of the space, and both specialists are lost in most of the space. Furthermore, the generalist displaces both specialists throughout much of the space in which one or both specialists are superior on their respective hosts. The key is the same as in Fig. 1.

3.2.3 Mixed presentation

This protocol is easy to implement but offers the greatest challenge for intuition. All three phages are growing in one culture, and the success of the specialist on one host directly impacts the success of the generalist growing on that host, which in turn affects the success of the generalist on the other host, then affecting the success of the specialist on the other host. Some of this interdependence has been true with the other presentation protocols. But with Mixed presentation, the generalist's success can no longer be separated between host A and B: its suppression by the phage specializing on host A affects its ability to compete with the phage specializing on host B and vice versa.

There is a further complication of Mixed presentation. When an individual phage has infected one host, it cannot also be infecting another host. This means that a generalist can be at a disadvantage when infecting a poor host if good hosts are in unlimited supply (Bull 2006); however, this effect disappears as the good host declines in abundance. Thus the cost-benefit to the generalist can change over the duration of a single culture, and a quantitative understanding of the implications defies intuition.

Fig. 8 shows results for Mixed presentation with high bacterial growth rate of r = 0.3 and fixed-count dilution of 1000. Compared to Parallel and Sequential host presentation, Mixed presentation is intermediate in favoring generalists: the generalist displaces somewhat superior specialists, but not to the degree as with Sequential presentation. The pattern for Mixed presentation with fixed-volume dilution is similar that in Fig. 8, although the generalist enjoys a somewhat greater advantage over the A-specialist.

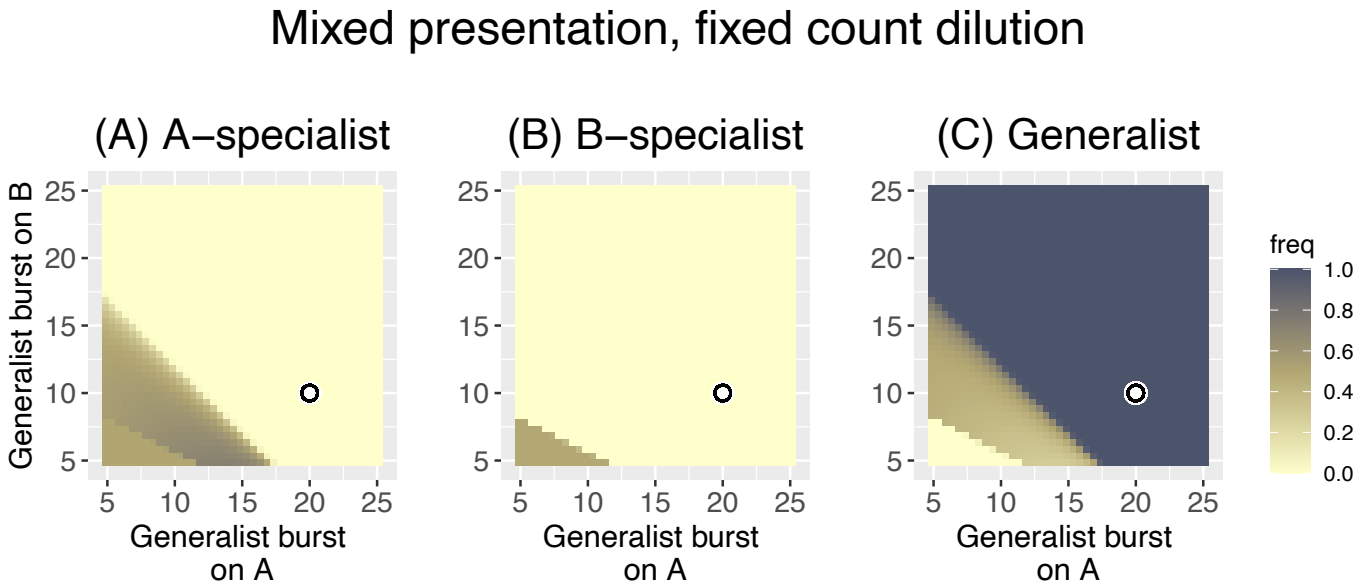

Fig. 8. Mixed presentation with fixed-count dilution and high bacterial growth (r = 0.3, otherwise as in Fig. 2). The three panels show the frequency of the A-specialist, the B-specialist, and the generalist. The outcome is intermediate between that of Fig. 2 (Parallel) and Fig. 7 (Sequential) in that the generalist displaces both specialists in much of the space where one or both specialists have higher burst sizes.

3.3 Other effects of protocol on evolution

Selection is not the only effect of protocol on evolution. The origin of genetic variation and stochastic loss of rare mutants are two properties to consider.

3.3.1 Recombination

Beyond the starting phage genomes, variation that is critical for evolution may be introduced during the protocol, as with mutagenesis or recombination. Studies of individual phages indicate that genetic changes are responsible for host range shifts (Hashemolhosseini, Holmes, et al. 1994; Hashemolhosseini, Montag, et al. 1994; Crill, Wichman, and Bull 2000; Meyer et al. 2012), so methods that enhance the variation beyond the starting pool are expected to accelerate the evolution (e.g., mutagenesis, Paff, Stolte, and Bull 2014).

Using three phages and a Parallel presentation protocol (i.e., the Appelmans protocol), Burrowes, Molineux and Fralick (2019) evolved a broad-range *Pseudomonas* phage that was a manifold recombinant between two of the starting phages. Furthermore, they commented that minimal success at host range expansion was observed when initiating the Appelmans protocol with single phages, for which the main source of variation would have been point mutations arising at intrinsic rates. Extrapolating from these results, Burrowes, Molineux and Fralick (2019) proposed that recombination greatly facilitates the evolution of broad host range in phages. An appreciation of the evolutionary benefit of recombination in phages is not without precedent: Botstein (1980) proposed that evolution of phages in the wild was highly ‘modular,’ a

process that necessarily relies on recombination among otherwise possibly divergent phages. Likewise, the field of directed evolution has experimentally demonstrated the value of recombination or 'DNA shuffling' over mutation (Stemmer 1994).

Otherwise, recombination has rarely been argued to be important in the directed evolution of phage host range, and few protocols are designed to encourage it (although protocols to engineer host range expansion libraries rely on it, Yehl et al. 2019; Zhang et al. 2022; Latka et al. 2021). The Appelmans protocol is an exception (Burrowes, Molineux, and Fralick 2019). It has two design features that should enable high levels of natural recombination: (i) multiple starting phages, and (ii) hosts individually permissive to more than one of the initial phages. Beyond this, there are additional design properties that can influence recombination. One is the similarity among the different phages. As (homologous) recombination relies on sequence similarity, starting phages that have regions of similar sequence should be most prone to recombine, provided they can infect a common host. However, the novel variation generated by recombination is likely greater the more that the parent sequences differ. Therefore, there may be an optimal level of sequence divergence among the phages for recombination to be useful in host range evolution. The broad host-range phage evolved by Burrowes, Molineux and Fralick (2019) was a mosaic of two starting phages that were 99% similar, suggesting that even high sequence similarity does not preclude the generation of novel phenotypic variation via recombination. Yet the evolved phage had 48 identified recombination segments (contrasted with only a single unambiguous point mutation), a number that seems extraordinarily high. It was not known how many of those exchanges were critical in the host range evolution, but the large number could reflect the intrinsically small phenotypic variation that arises via recombination when there is low genetic diversity between the parents. We are largely ignorant about how to facilitate recombination while enhancing variation for host range expansion.

For a protocol to encourage recombination, the different phages must coinfect. Co-infection requires a common host in the protocol but also requires high, approximately equal abundances of the different phages. A common host is easily employed in any of the three host-presentation protocols, but ensuring ongoing high abundances of different phages is not. Even when the protocol is deliberately initiated with equal numbers of the different phages, relative abundances will usually change rapidly because different phages will typically not have equal growth rates on common hosts. The better growing phages will thus quickly dominate the pool (Korona and Levin 1993; Bull et al. 2004). To ensure the continued opportunity for recombination, therefore, the evolving phage pool could periodically be supplanted with a stock in which all the phages are equally abundant and in which recombinants are already present. Again, this method can be employed with any of the three presentation protocols. A simple empirical method for creating a pool of recombinants is to cross streak the different phages on a plate seeded with a common host, allow phage growth, and recover the phages from the zone of overlap (Nguyen et al. 2012).

3.3.2 Initially, most mutations are likely to be lost

Virtually any protocol that amplifies phages on a permissive host will then dilute those phages before exposing them to selective conditions. This dilution has consequences for single mutants that have just arisen in the phage pool: a single mutant in the pool may not even enter the next round of cultures, much less be exposed to a host on which it can grow. For example, the Appelmans protocol generates a phage pool by combining all cultures that lyse with some cultures that do not lyse; but it then adds only a fraction of this pooled volume to the next round of cultures. In Fig. 1 of Burrowes, Molineux and Fralick (2019), 37 culture volumes are collected

726 to create the phage pool, but less than 4.5 of those volumes would be used in the next round
727 (12%). Any individual specialist that survives this bottleneck then has only a 1/8 chance of being
728 placed in a culture with the non-permissive host on which it grows or a 1/8 chance of being
729 placed in the culture with the permissive host (for an overall net rate of 3% that a single mutant
730 will survive the dilution and be placed with a permissive host in the next cycle).
731
732 Other protocols may also use only an aliquot of the phage pool for the next round. In all cases,
733 the numbers are subject at least partly to experimental control. Thus, the odds are improved
734 under the Appelmans protocol by using fewer non-permissive hosts and by pooling fewer of the
735 cleared wells (as per Mapes et al. 2016). Mixed presentations would not face the problem that a
736 new mutant might fail to be exposed to the host on which it grows. Thus, several of these risks
737 of mutant loss are protocol-specific. Concentrating the phage in the pool before the next round
738 of host exposure would likely reduce loss in any protocol. Beyond these problems, there are
739 further random processes that can lead to loss of single or even low-copy, mutant phages
740 (Patwa and Wahl 2008). Of course, if host range mutations or recombinants arise at high
741 frequency, individual losses will not matter.
742
743
744 **4. Discussion and Conclusions**
745
746
747 The suite of bacterial strains and species infected by any phage tends to be narrow, at least
748 compared to the spectrum of hosts impaired by single antibiotics. Applications of phage therapy
749 would benefit from broad host range phages, if merely to simplify treatment and phage
750 preparation. Fortunately, the host range of a phage is not a fixed feature, and there is a long
751 history of 'directing' the evolution of phages in the laboratory to change and even expand host
752 range. These methods for directed evolution of broad host range all expose phages to a 'new'
753 bacterial hosts, with the goal of evolving the phages to grow on those hosts.
754
755 Here, we considered the quantitative consequences of different protocols for the directed
756 evolution of phage host range. We distinguished three protocols that differ in the way hosts are
757 'presented' to the evolving phage pool, all of which have been used in prior work: Parallel,
758 Sequential, and Mixed. There were qualitative differences among these protocols in their
759 selection of generalist versus specialist phages. Sequential presentation strongly favors
760 generalists over specialists, Mixed presentation less so, and Parallel presentation (with an
761 appropriate dilution protocol) provides little or no intrinsic advantage to generalists. However,
762 the generalists evolved under Sequential presentation can be far inferior on individual hosts
763 than the specialists they displace, so generalism can have drawbacks. Other protocol details
764 can have modest effects. The dilution protocol should not be so extreme as to cause
765 demographic extinction of phages.
766
767 Selection is not the only evolutionary dimension influenced by the protocol. It influences the
768 origin of novel variation (via recombination and mutation) and also influences the fate of rare
769 mutations. These topics were given less attention here than selection, but some qualitative
770 suggestions were offered.
771
772 We can offer a few conclusions coupled with recommendations.
773
774 1) Choose an appropriate presentation protocol. Although the protocols vary in how deferential
775 they are to generalist phages, the utility of generalist over specialist phages will depend on the

application. To take advantage of the variety of host-presentation protocols available, the protocol could be tailored to the application. For example, sequential presentation of hosts is unlikely to exist in infections (except possibly if host resistance evolves following treatment), whereas both Parallel and Mixed protocols could directly match infections. In turn, this would suggest that a Sequential protocol was not the best choice for evolving phages to treat infections. Phages evolved under Mixed presentation might be better suited to treating a Mixed infection than would phages evolved under other protocols, and so on.

2) Recombination. Although much remains to discover the general importance of recombination to host range evolution, there may be little lost by encouraging recombination in a protocol. Recombination is promoted by using multiple parental phages and ensuring they are maintained at moderate frequencies. However, including different phages at the start may offer only a transient boost to recombination. The propagation of different phages on a permissive host will typically result in numerical dominance by one phage (Korona and Levin 1993), so the opportunities for recombination may dissipate quickly. Periodically supplementing the phage pool with the original phage stock will maintain opportunities for recombination. Furthermore, the phage pool itself may be created with recombination so that the pool is not only supplemented with parental phages but also supplemented with recombinants among the parents. Recombination rates also depend on the sequence similarities among starting phages.

3) Early losses. If mutations are limiting, it may be beneficial to modify the protocol to reduce phage dilution and avoid phages being trapped on an inappropriate host – the latter a greater problem with Parallel presentation than, say, Mixed presentation. In the Appelmans protocol, for example, this would involve reducing the number of non-permissive hosts. Concentrating phages at the end of a cycle would reduce dilution. Alternatively, the protocol may be modified to increase the number of recombinants and mutants (as with mutagens), so that early loss is inconsequential.

4) The initial pool. A diversity in the initial pool of phages will surely increase the range of possible outcomes. As noted above, the initial pool will affect opportunities for recombination. We yet can offer little insight on whether a diverse starting pool will affect the outcome toward generalists or specialists. Starting with a single phage might seem to ensure the evolution of a generalist, but selection is agnostic to phage origins, and a single phage might well diversify into a suite of specialists.

5) Dilution. There is a risk from too high dilution in a protocol – demographic extinction of phages that would otherwise evolve. But low dilution has its own problems. First, low dilution slows evolution merely because there is less phage growth per cycle and thus less opportunity for fitness differences to manifest. Low dilution could thus greatly increase the empirical work associated with evolving phage host range. Second, low dilution promotes phage growth at high multiplicity. This can facilitate the evolution of phages that are good at within-host competition at the expense of independent growth (Turner and Chao 1999; Karki, Bull, and Krone 2022). High dilution will usually be desirable, and any demographic effects can be offset by extending the culture duration.

Are there limits to the evolution of broad host ranges in phages? The works of Burrowes, Molineux and Fralick (2019) and of Mapes et al. (2016) suggest that host ranges can be extended across multiple strains of the same species. Mapes et al. (2016) even found that expanded host-range phages could grow on strains not included in the protocol, whereas Burrowes, Molineux and Fralick (2019) found the opposite. The ease of evolving expanded host

range phages (and even cocktails) may well depend on the basis of bacterial resistance (Hyman and Abedon 2010; Labrie, Samson, and Moineau 2010). The limits to phage host ranges is a matter that looms large in the future of phage therapy.

Data Accessibility: C code files, R code files, and Mathematica files are available at https://github.com/bull71/host_range .

Acknowledgments: H.A.W. was funded by R01-GM076040 and the work was facilitated by P20-GM104420 from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions: J.J.B. and H.A.W. conceived of the problem. J.J.B. and S.M.K. developed the general approach and were responsible for analytical work. Code was written by J.J.B. and checked by S.M.K. The manuscript was written by all authors.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analy

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