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Understanding the Impacts of Bacteriophage Viruses: From Laboratory Evolution to Natural Ecosystems

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Abstract

Viruses of bacteria (bacteriophages or phage) have broad effects on bacterial ecology and evolution in nature that mediate microbial interactions, shape bacterial diversity, and influence nutrient cycling and ecosystem function. The unrelenting impact of phages within the microbial realm is the result, in large part, of their ability to rapidly evolve in response to bacterial host dynamics. The knowledge gained from laboratory systems, typically using pairwise interactions between single-host and single-phage systems, has made clear that phages coevolve with their bacterial hosts rapidly, somewhat predictably, and primarily by counteradapting to host resistance. Recent advancement in metagenomics approaches, as well as a shifting focus toward natural microbial communities and host-associated microbiomes, is beginning to uncover the full picture of phage evolution and ecology within more complex settings. As these data reach their full potential, it will be critical to ask when and how insights gained from studies of phage evolution in vitro can be meaningfully applied to understanding bacteria-phage interactions in nature. In this review, we explore the myriad ways that phages

shape and are themselves shaped by bacterial host populations and communities, with a particular focus on observed and predicted differences between the laboratory and complex microbial communities.

INTRODUCTION

Viruses are key players in shaping the evolution and ecology of life on Earth. This is in large part due to their own high rates of evolution, ability to integrate into the genomes of other organisms, and relentless selection for mechanisms of host resistance/immunity. Much of our understanding of viral ecology and evolution comes from work on bacteriophage viruses (phages), in large part because of their ease of use as model systems in the laboratory (1). However, phages are far more than model systems; these diverse and ubiquitous viruses have the potential to alter competitive dynamics within microbial communities, shape bacterial community diversity and function, and drive nutrient cycling and ecosystem function (2). Because of this, there has been increasing interest in understanding bacteria-phage interactions and phage evolution within natural contexts (recently reviewed in 3). Moreover, the recent explosion of work focusing on host-associated microbiomes has emphasized the need to better understand the role of phages in shaping these microbial communities to fully explain diversity, stability, and function (4, 5).

Beginning with the codiscovery of bacteriophages by Frederick Twort and Felix d'Herelle over a century ago (6, 7), most research on bacteria-phage interactions has been undertaken in classic microbiological high-nutrient media in laboratory environments. These in vitro results likely do not reflect bacteria-phage interactions/coevolution in the natural environment or within a host, yet they provide a necessary foundation for understanding how phages can evolve and how they can shape bacterial population dynamics and evolution. These simplified laboratory environments can also be used to carefully manipulate single factors (such as population size, nutrient availability, spatial structure, or gene content) to uncover causal relationships (8). As we gain information about how bacteria-phage dynamics are shaped by particular environmental and genetic features of a given system, we can begin to piece together the puzzle to predict how these factors may combine to shape bacteria-phage interactions in nature. However, these predictions should be seen as starting points that require further validation through both empirical testing and comparisons to observational data from nature. For example, understanding of two factors independently does not necessarily allow for successful predictions about their combined impacts (i.e., there are likely to be nonadditive effects within complex systems). As we move forward, therefore, it is just as important to highlight the cases in which observed natural dynamics could not be predicted from data gleaned in vitro as it is to highlight those in which results nicely align.

Translating our understanding of bacteria-phage interactions from the laboratory setting to complex communities requires careful consideration of how these environments generally differ. Among the features of natural environments that are typically not taken into account in experimental studies in vitro are:

1. Longer (evolutionary) timescales allowing for codiversification of phages and hosts and eco-evolutionary dynamics;
2. Spatial structuring of bacterial populations and phage dispersal limitation maintaining coexistence and changing local selection pressures;
3. Variation in bacterial growth phases occurring over time, especially in response to seasonality or eukaryotic host state;
4. Large and/or fluctuating population sizes and metapopulation/metacommunity dynamics;

5. Diversity of phages and bacterial strains and species co-occurring within communities; and
6. Additional ecological complexity and selection pressures that might impact the strength and dynamics of the interaction.

In this review we explore how each of these features might meaningfully change our understanding of phage evolution in natural settings. We highlight recent empirical work where ecological complexity has been incorporated to understand when each factor can impact the outcome of bacteria-phage dynamics, but we also highlight the wealth of theoretical knowledge that exists to help predict how evolution might proceed under particular sets of conditions. Wherever possible, we then compare the predicted outcomes to observed patterns from natural populations and communities. In this way, we hope to guide further work allowing for a more predictive understanding of bacteria-phage dynamics in nature.

OVERVIEW OF BACTERIA-PHAGE INTERACTIONS

Bacteriophages (viruses that infect bacterial cells) have genomes that can be either DNA or RNA, and either double stranded or single stranded. It is generally assumed that all bacteria on Earth are host to phages (although it is a challenging idea to test). The two best-described modes of phage replication within bacterial cells are the lytic and lysogenic cycles. The lytic cycle involves phage adsorption to a bacterial receptor, injection of phage genetic material, assembly of phage virions, and lysing of the host cell to release the assembled phage particles into the environment. The lysogenic cycle involves an additional step after injection of phage DNA into the host cell, where the phage genome will integrate into the host genome and be vertically transmitted during bacterial replication (after which it is considered a prophage). Prophages typically remain embedded within the host genome until the host experiences stressful conditions/DNA damage, at which point the phage will excise, produce proteins, reassemble into virions, and lyse the cell. Less well-studied modes of phage lifestyles include pseudolysogeny (a period of stalled development prior to integration of the phage genome) and filamentous phages (which can be seen as a chronic infection of cells, allowing for viral shedding without cell lysis), and some prophages are able to stably replicate across bacterial generations while remaining extrachromosomal, similar to plasmids (9).

Bacteria-phage interactions are the result of and result in constant coevolution, whereby lytic phages select for resistant bacterial strains and species, and resistance evolution drives subsequent selection for newly infective phage types. Bacteria can evolve various resistance mechanisms to escape phage predation (recently reviewed in 10), including the inhibition of phage adsorption to cell-surface receptors, recognition and cleavage of phage DNA with restriction-modification or CRISPR-Cas systems, or by inducing cell death upon infection before phage particles can be assembled. While the breadth of bacterial resistance mechanisms to phages continues to expand through discovery and more high-throughput approaches (11, 12), so too do the mechanisms by which phages can overcome resistance. These include recognition of new or mutated receptors, hydrolyzing compounds that otherwise would prevent access to receptors, induction of methylation of phage DNA to avoid restriction-modification systems, cleavage of bacterial DNA (with their own CRISPR systems) or production of proteins that prevent CRISPR action, or the prevention of infection abortion through production of antitoxins (13, 14). Such counteradaptations also shape the movement of prophages across hosts. Recent evidence from *Staphylococcus*-associated phages shows that alternative integration sites can rapidly be acquired, allowing prophage integration in novel hosts or those that have evolved resistance to previous phage types (15).

In the laboratory, bacteria-phage coevolution can be readily observed by coculturing a single bacterial strain and a single phage type in nutrient broth over many generations and measuring changes in bacterial resistance and phage infectivity over time. Here, bacterial resistance and

phage sensitivity phenotypes can be attributed to mutational change within lineages, and phenotypic change can be examined at the genomic level through whole genome sequencing (16). This relatively straightforward approach not only allows for the measurement of the speed and mode of coevolution but also can be expanded to incorporate specific factors of interest, such as migration among populations (17) or resource availability (18), and to compare dynamics across different phage types (19). Laboratory experiments show that for CRISPR-based immunity, acquisition of just a few spacers is sufficient to stabilize a bacterial population against lytic phage (20, 21). However, these studies also show that phages can rapidly evolve to evade this specificity-based resistance (22) and that CRISPR immunity can drive fixation of mutations specifically in the targeted phage genome regions (23). These experimental studies have been invaluable in uncovering the wealth of mechanisms underlying bacteria-phage coevolution, the speed at which phages can select for resistance and counteradapt to resistance, and the phenotypic consequences of resistance evolution and phage adaptation.

Moving beyond the laboratory to nature, it is clear that phages and bacteria are also locked in a relentless battle of coevolution, but data from these complex and diverse settings are harder to gather and interpret. For example, in the phyllosphere of horse chestnut trees, culturable bacterial strains from the leaf microbiome were found to be more resistant to phages from the same trees but collected earlier in the growing season and less resistant to phages collected later in the growing season, suggesting phage-mediated selection of bacterial resistance month to month (24). More recently, these effects have been confirmed across multiple growing seasons, whereby bacteria were shown to be more resistant to phages collected from the same tree in previous years but less resistant to those phages collected from future years, suggesting an even longer-term coevolutionary dynamic in this system (25). However, as in many natural community settings, it is unclear whether the observed dynamics are explained by ecological species sorting (whereby resistant bacterial strains/species in the community have an evolutionary advantage and outcompete sensitive strains/species) or by selection favoring resistance mutations and counteradaptations within particular bacterial and phage lineages (or, for that matter, both processes simultaneously). Genomic analyses of populations of the fish pathogen *Flavobacterium columnare* from an aquaculture setting elegantly confirm that observed increases in resistance over time, and counteradaptation by phages, can indeed occur within natural populations via mutational change within lineages (26). Here again, bacteria were found to be more resistant to phages from previous years and less resistant to those from future years, but in this case the individual molecular mechanisms were examined. Phage evolution was demonstrable through detection of nonsynonymous differences in putative phage tail and structural proteins, while bacterial resistance evolution seemed to occur primarily through CRISPR spacer acquisition and mutation. Similar work has demonstrated genomic signatures of phage-mediated selection and evolution in wastewater treatment plants (27) and within the human gut (4, 28).

WHO INFECTS WHOM?

Ultimately, the impact of phages across ecological and evolutionary scales will be primarily determined by their host range, i.e., the strains and species of bacterial hosts in which the phages are able to successfully replicate. Phage host range, however, is constantly reshaped by the coevolutionary process as resistance evolution shrinks the range of any given phage while phage counteradaptation expands it. The vast majority of work exploring phage host range has come from studies performed in vitro, and we still have little understanding of when this will translate into infectivity in natural settings (29). Indeed, it seems likely that the breadth of host range for most phages in nature is far wider than would be predicted from laboratory studies (30). This can

be easily understood when we consider how host range is typically determined. When a given phage is characterized, it is generally tested against a panel of bacterial strains in order to measure lytic activity. This introduces two biases: First, host range estimates will be confined to the bacterial species and strains used in the screen and therefore will by definition be more narrow than possible (i.e., a vast underestimate of true host range); second, host range estimates will typically be made on strains and species isolated at different times and/or from different environments than the phage being tested. This latter point is problematic because phage evolution is driven by counteradaptations against local host resistance, and thus phages are less likely to infect bacterial populations with which they have not recently been interacting/coevolving. Indeed, observations that phages are locally adapted to bacteria from their own population/community have held true across many natural systems (31–33), suggesting that estimated phage host range could be very different (even for the same set of bacterial species being screened) depending on whether strains are isolated from the same sample/environment as the hosts being tested. Finally, while using sympatric bacterial panels could move us closer to estimating the true host range, even in this case bias will still exist as a result of both laboratory infection outcomes (e.g., plaque formation) that differ from infection outcomes occurring under natural conditions and the restriction of these assays to culturable bacterial hosts.

In recent years, and in part due to increasing interest in the microbiome, newer technologies and approaches are allowing for more realistic measurements of host range by focusing not on infection in the laboratory but on quantification of infected cells in the natural environment. For example, single-cell sequencing allows observations of active infections in natural settings by capturing lytic phages that are within or attached to host cells (34, 35), and metagenomic data sets are giving unprecedented insight to the wealth of both lytic and prophages within microbial communities (36, 37). Our increasing ability to measure phage host range in situ and to track changes in host range over time within natural communities will fill gaps in our understanding of how phages impact microbial diversity, stability, and function. Given the importance of phage host range in horizontal gene transfer among bacteria, these new data could help us gain much-needed predictive ability for movement of genes within and among bacterial species (38–40). Finally, as we move closer to fully embracing phage therapy in clinical settings, phage host range will become a key predictor of both therapy success and off-target effects within the broader microbiome.

With the background of phage ecology and evolution in place, we next explore the primary ways in which natural settings will predictably differ from laboratory ones, with the hope of motivating further work to help determine when in vitro findings will translate to the natural world.

BACTERIA-PHAGE DYNAMICS OVER ECOLOGICAL AND EVOLUTIONARY TIMESCALES

The power of experimental (co)evolution is that it allows researchers to observe dynamical phenotypic and genetic changes over short timescales (often days to weeks). This necessarily limits the observed dynamics to ecological and microevolutionary processes, even when experiments are run for longer time periods. For bacteria-phage coevolution observed within a test tube, this often means that mutations of large effects are those driving phenotypic patterns, typically occurring as a series of selective sweeps. How these short-term patterns relate to ongoing coevolutionary dynamics and (co)speciation in nature is unclear (**Figure 1**). In cases where in vitro experiments have been drawn out for longer, it does indeed seem that the initial interaction dynamics can shift. For example, experimental coevolution between *Pseudomonas fluorescens* and phage SBW25Φ2 over the course of 120 days uncovered a saturating effect of time on overall host resistance and phage infectivity, as well as amino acid substitution rate within the phage tail fiber gene (41). While early

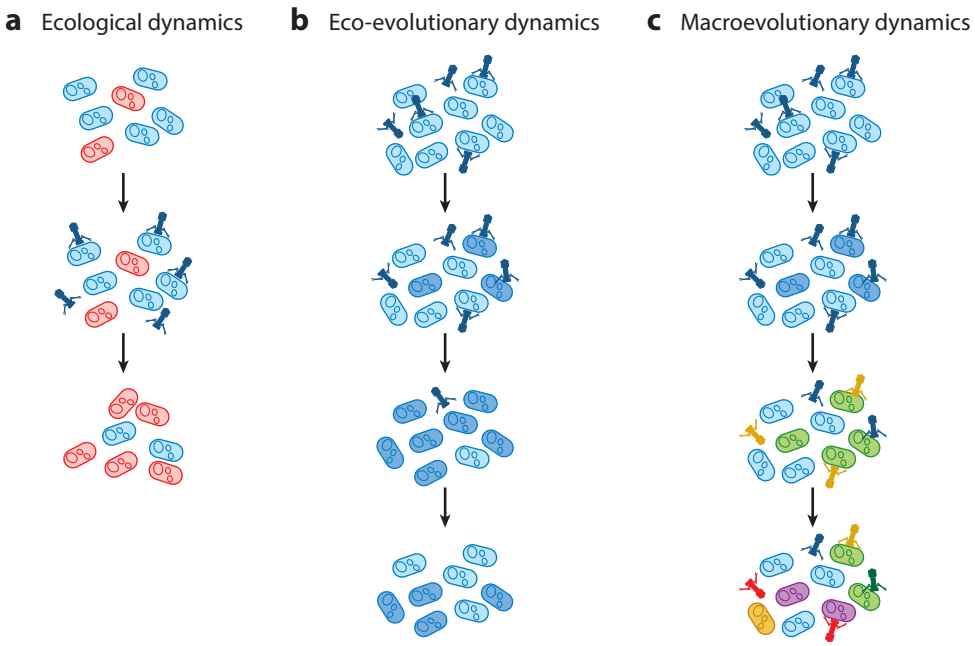

Figure 1

Examples of bacteria-phage interactions as we move across timescales. (*a*) Illustration of ecological dynamics in a community with two bacterial species, where one is a better competitor and more dominant (*blue cell*) in the absence of phage, and the other (*red cell*) gains a competitive advantage in the presence of phage. (*b*) Example of eco-evolutionary feedbacks, where the starting population is phage sensitive (*light blue cell*), and phages select for resistant mutants in the population (*dark blue cell*). As resistance spreads through the population, phage density (and thus phage-mediated selection) decreases, enabling the phage-sensitive strain to again increase in frequency in the population. (*c*) Illustration of macroevolutionary processes where an initially sensitive population (*light blue cell*) diverges into two types [resistant (*dark blue cell*) and sensitive]. As phages counteradapt to resistance, they also diversify (*yellow phage*, *blue phage*), eventually leading to coexistence of multiple bacterial (*blue cell*, *green cell*, *purple cell*, *yellow cell*) and phage types/species (*yellow phage*, *blue phage*, *green phage*, *red phage*).

interactions in this system were characterized by arms race dynamics, where host resistance is increased against all previous phages and phage infectivity is increased against all previous host types, later interactions were more in line with fluctuating selection dynamics, where newly infective phages evolve that are unable to infect previous host types. Indeed, temporal data sets from natural systems often uncover long-term coexistence of resistant and susceptible bacterial types that suggests lack of consecutive selective sweeps (27, 42).

Over ecological timescales, phages can and do alter bacterial densities and therefore can act as important drivers of bacterial evolution. However, the overall impact of phages on bacterial population size and growth rate can be positive, negative, or neutral depending on the type of phage (e.g., 43) and the other growth-limiting factors (44) such as competitor species (45) or resource availability (46). Even lytic phages, as obligate killers of their hosts, can have unexpectedly positive effects on bacterial growth (47, 48). For phages integrated into the host genome, the fitness of the phage is necessarily coupled with that of the bacterial host. As such, the general assumption is that prophage integration should increase bacterial growth rate/fitness (or at least be neutral). In principle, growth rate could be impaired through virtue of added genetic content imposing an added metabolic burden (49), but integrated prophages typically provide fitness benefits to their

bacterial host by, for example, protecting the host from lysis by related (50) and even unrelated (51) phages or by altering bacterial virulence and/or antibiotic sensitivity (52). Again, however, much of our understanding of phage impacts on bacterial growth and density comes from the laboratory setting. This is an important distinction because, in natural settings (unlike the laboratory), phages are just one of myriad selective forces acting on a given bacterial population. As such, phage-mediated selection is unlikely to be as important of a factor at any given time, relatively speaking, as it is observed to be in an isolated microcosm (e.g., 46, 53).

Over evolutionary timescales, the impact of phage on bacterial growth will depend on the capacity of the bacterial populations to evolve resistance, the fitness costs paid for such resistances, and the subsequent effects of any compensatory evolution to mitigate these costs. There is strong evidence across systems and mechanisms that bacterial resistance to phages comes at a significant cost (54–57). These costs can drive eco-evolutionary dynamics, whereby the spread of resistance alleles in the population reduces selection for those very same alleles as a result of decreasing phage prevalence (58). Indeed, in the previously discussed case of experimental coevolution between *P. fluorescens* and phage SBW25Φ2, the shift to fluctuating selection dynamics observed later in the experiment was attributed in part to selection against the more costly general bacterial resistance in favor of less costly but more specialized resistance mechanisms (41). These costs of resistance can lead to coexistence of phage-sensitive and -resistant cells over time (59), and recent empirical evidence and theoretical models support the idea that constant reversion to sensitivity can maintain sufficient phage densities within the system to select for resistance and allow long-term coexistence of bacteria and phage (60, 61).

Fitness costs associated with increased bacterial resistance and/or phage infectivity can be reduced over time as a result of compensatory mutations (62). Very little work to date has explored the role of compensatory evolution in bacteria-phage interactions, and those studies that have tested for compensation in vitro have been mixed in their findings (63, 64). This is perhaps not surprising given the short timescales of these studies, and further work will be required to understand how important compensatory evolution is in shaping viral evolution over longer timescales in nature. For example, in activated sludge biological wastewater treatment plants, where bacterial CRISPR-Cas systems and their corresponding spacer targets were monitored over 1.5 years, the mean time between first detection of a mobile genetic element (including phage) and integration of new spacers into the CRISPR array was 9.5 weeks (65), longer than most laboratory studies.

Macro-evolutionary patterns of codiversification and cospeciation are often used to study species interactions over longer timescales. Patterns whereby host phylogenetic relationships are mirrored (at least to some degree) by those of their parasites/pathogens are typically used to support the possibility of long-standing coevolution among the partners and/or the importance of one species in driving divergence and speciation of the other. Alignments of phylogenies can also highlight cases of host switching, host escape, or parasite host range expansion events. Despite this common method across the coevolution field, very few studies have taken this approach to explore macroevolutionary patterns of bacteria and phages. Those that have, however, find intriguing results. For example, analysis of head and tail genes from T4-type phages infecting different genera of enterobacteria uncovered some evidence that host relatedness predicts gene-level phylogeny of the phage (66). Further, cophylogenetic analyses of *Paraburkholderia* species and their associated prophages found overall low concordance of the host and phage tree topologies but were able to uncover evidence for abundant host-switching events as well as some cases of cospeciation (67). The paucity of studies of bacteria and phage at macroevolutionary scales is due in large part to the difficulty in resolving viral phylogenies more generally as a result of their rapid evolution, smaller genome sizes, lack of conserved genomic regions, and high level of genomic rearrangements/reassortments (68). New methods for comparing relatedness among phages will likely

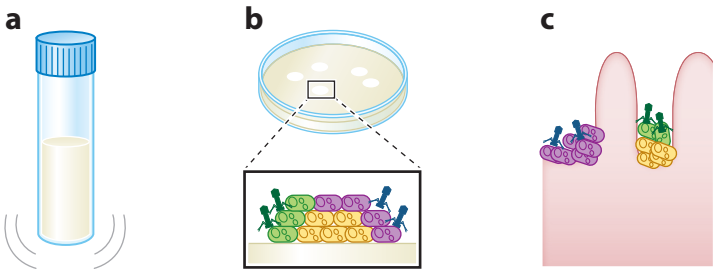

Figure 2

Examples of differences in spatial structuring moving from in vitro to natural environments. (*a*) Liquid cultures where shaking leads to dispersal of cells and mass action–like transmission dynamics. (*b*) Solid media, where colony growth and/or biofilm formation create a structure of cells that can act as barriers to dispersal. (*c*) The gut environment, whereby cilia create physical barriers to dispersal, which can be further structured by growth of cells.

pave the way for more informative macroevolutionary comparisons among phages and their hosts (69). However, given evidence that phage evolution and recombination rates differ across phage lifestyle and host types (70), it may be that this cophylogenetic approach will never be particularly informative.

THE ROLE OF SPATIAL STRUCTURE IN BACTERIA-PHAGE DYNAMICS

In the laboratory, bacteria-phage interactions are commonly studied in liquid cultures. This environment is likely characterized by mass action transmission dynamics, especially in cases where cultures are placed on a shaker (71). Here, cells will interact with phages at a rate dependent upon the density of phage particles in the environment, and the rate will be, on average, the same for all host cells in the test tube. Of course, the vast majority of natural environments will not meet these assumptions due to spatial structuring of host cells and/or viral particles (72) (**Figure 2**). This spatial structuring can be extrinsically generated as a result of the physical environment (73, 74) or intrinsically generated as a result of growth and/or local mutational spread (75). Among the clearest examples of such structuring is through biofilm formation (recently reviewed in 76). Indeed, in systems where biofilm formation is examined in vitro, it is often observed that phages are unable to infect and lyse all sensitive bacteria due to the spatial refuges generated by resistant cells (77, 78). As resistant cells spread through a spatially structured population, phage reproduction is blocked and a physical barrier can be formed that results in a phage-free refuge where resistance is not selected for, and thus sensitive cells continue to reproduce. These spatial refuges result in coexistence of bacterial types over short timescales, and in the case of multi-species communities, likely result in very different phage-mediated selection and phage evolution than would be expected in a well-mixed planktonic setting.

Spatial structure can alter our predictions about bacterial and phage evolution, in terms of both the rate of spread of new mutations and the mechanisms by which resistance and infectivity are gained. For example, experimental studies of CRISPR-based immunity in liquid culture revealed that most bacteria evolve resistance via acquisition of a single spacer, whereas in solid media the bacteria surviving phage infection are found in colonies displaying complex morphologies with heterogeneous compositions of multiple spacers (79). Similarly, bacterial cell death upon infection was observed to be a more common defense in spatially structured than well-mixed populations (80), and reduced expression of phage receptors was found to be a key phenotypic resistance

mechanism favored under spatial structure, while genetic resistance was the primary mechanism observed in well-mixed populations (81). In terms of phage evolution, spatial structure is predicted to affect phage growth parameters, such as latent period, burst size, and adsorption rate (82) as well as selection for phages in the lytic versus lysogenic cycle (83). For example, host-integrated (prophage) phage λ was able to outcompete the lytic version of the phage in spatially structured environments due to prevention of superinfection but lost out to the lytic strain in well-mixed populations (83). And at a slightly larger, metapopulation scale, phage dispersal was found to increase phage host range relative to phages in isolated populations (84). How such patterns translate to natural settings is unclear given the dearth of knowledge on phage dispersal across the landscape, but recent work suggests that phage dispersal might be more common than expected even outside of aquatic habitats (85).

DYNAMICS OUTSIDE OF EXPONENTIAL GROWTH

Because most laboratory studies are run in batch culture, where a small amount of inoculum is added to nutrient broth to seed populations and then a small subset of the population is reinoculated into fresh medium after nutrient depletion, most of our understanding of bacteria-phage dynamics comes from replication and selection during exponential growth. In nature, however, this is unlikely to be the primary pattern of growth for most bacterial populations. For example, recent work from the phyllosphere suggests the stationary phase might be a more common lifestyle than typically considered (86). Understanding how bacteria-phage interaction outcomes might differ across growth phases will be key to expanding our knowledge beyond the laboratory (**Figure 3**), especially as exponential growth in the laboratory has been linked to loss of costly

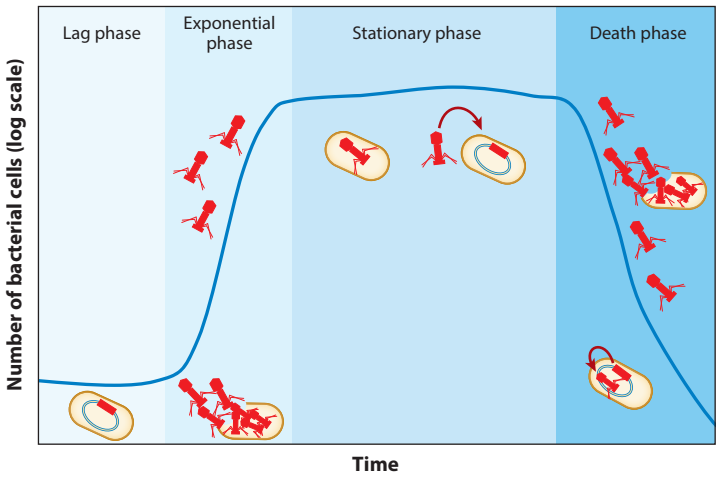

Figure 3

Example of how phage infection might differ across bacterial growth phases. In the lag phase, free phages might be degraded through exposure to UV or harsh environmental conditions, resulting in primarily integrated prophages within the bacterial population. As bacteria begin reproducing, for example after a pulse of resources arrives into the environment, prophage might spontaneously enter the lytic cycle. Bacterial cells and newly arriving lytic phage will be able to actively reproduce and lyse host cells during growth. Although less is known about the dynamics of phages in stationary phase, evidence suggests that they can continue to adsorb to and infect cells, sometimes entering a carrier state, and integrated prophage can remain embedded in host genomes. Finally, as bacterial populations enter the death phase, prophages can be actively induced to enter the lytic cycle, resulting in a pulse of free phage.

protein expression (87) and long-term stationary phase is known to be linked with elevated mutation rates (88) and the emergence of specific mutations (89). Although lytic phage reproduction is typically considered to be limited by bacterial reproduction, phage infection does occur in stationary phase (90) and—in some cases—new modes of infection (i.e., hibernation) have been described for bacteria in this state (91). Moreover, prophage induction and cell lysis have in some cases been specifically linked to the stationary growth phase as a result of cell stress response (92). Recent work on soil microbial communities suggests that lytic phage abundance is higher in shallow soil, where active growth is occurring, and decreases with depth faster than does bacterial abundance, while inducible prophages become relatively more abundant with increasing soil depth (93).

Moving our understanding from laboratory to nature will require both better understanding of bacteria-phage dynamics outside of the exponential growth phase and new models to describe growth kinetics across phases (94). This is especially true in light of seasonal dynamics, which have been predicted and observed to alter bacteria-phage dynamics across multiple systems (65, 95, 96). For example, temporal data from the Red Sea suggest that even modest seasonal changes in temperature can affect both bacterial abundance and the relative abundance of inducible prophages, with higher prophage abundance observed in lower abundance populations occurring at lower temperatures (97). At much finer scales, daily fluctuations in bacterial growth have been linked to diel patterns in phage infectivity and abundance (98). It remains unclear if and how longer periods of stationary phase impact phage evolution, but evidence from strictly lytic phage inoculated into stationary phase populations of their hosts found evidence that adsorption in these conditions was detrimental to phage fitness, and that phage extinction was prevented by phages with extremely low adsorption rates (99). Environments with primarily metabolically inactive cells and/or low bacterial abundances might therefore select for lytic phages with high environmental persistence/low particle decay rates. Although relatively unexplored, there is compelling evidence that phage decay rates can be related to other key traits, including host range (100). Whether due to long-term seasonal shifts or short-term resource pulses, incorporation of resource heterogeneity across space and time (and therefore shifts in bacterial growth phases) will also shed light on natural dynamics. Resource enrichment in chemostat cultures was found to increase both phage and bacterial densities, but also to destabilize the bacteria-phage dynamics (101). Resource fluctuations have also been observed in experimental settings to reduce bacteria-phage coevolution, especially when rapid, due to interruption of selective sweeps (102).

EXPANDING BEYOND THE (SMALL) POPULATION

The vast majority of experiments on bacteria and phage in the laboratory are run using an initially homogeneous population of both bacteria and phage. As such, selection is able to result in evolutionary change only once genetic variation is introduced via mutation. This is in stark contrast to natural populations, which—unless having recently undergone an extreme bottleneck event, for example after colonization of a new environment/host—harbor considerable standing variation at any moment in time. Experimental evolution as a method works because even small laboratory populations that are seeded by a single cell (i.e., started through isolation of a single bacterial colony) are able to rapidly generate variation by mutation. For example, recent in vitro work using *Staphylococcus aureus* and its lytic phage used deep sequencing after experimental evolution to uncover striking evidence for genetic polymorphism of minor alleles, which Zhang et al. (103) argue could predispose the rapid evolution of resistance and counteradaptation of these populations moving forward. Within homogeneous populations, the number of reproducing individuals still matters given mutational target size. Larger populations will have higher numbers of mutations and more efficient selection. Recent in vitro work demonstrates the dramatic impact

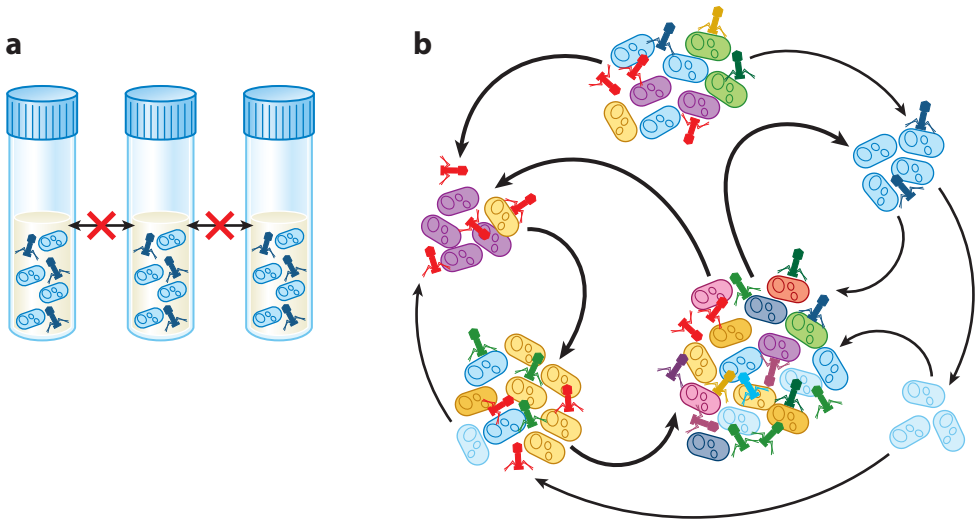

Figure 4

Considering bacteria-phage dynamics within the metapopulation and metacommunity framework. (*a*) In the laboratory, experiments typically focus on small, initially homogenous populations within closed environments. (*b*) In contrast, natural populations are variable in both size and diversity, and populations/communities are connected via dispersal. The additional variation generated by higher mutation and higher migration in these large, interconnected populations should accelerate coevolution, but dispersal can also disrupt coevolutionary dynamics.

that bacterial mutation rate can have on the mechanism of phage resistance, as CRISPR-based resistance switched from being the predominant mechanism in the wild-type background to very rarely evolving in the high mutation rate strains of *Pseudomonas aeruginosa* (104). Environmental conditions that alter population sizes of either bacteria or phage therefore have the potential to dramatically alter the efficacy of selection and tempo of coevolution. For example, the lower population sizes achieved in the presence of bile salts were shown to reduce the strength of coevolution in vitro (105).

Dispersal among populations not only fuels genetic variation upon which selection can act, thereby increasing the effective population size and tempo of coevolution (106), but also leads to metapopulation and metacommunity dynamics that can drive the maintenance of diversity (107) (**Figure 4**). When individual experimental microcosms are purposefully connected by dispersal, for example, the outcome of coevolution is considerably altered (e.g., 17, 108). Overall, when populations are interconnected, genetic variation is shaped by migration, and mutations favored in one population can fuel adaptation of connected populations. This can result in dynamics whereby individual populations each contain small subsets of universally observed alleles at any given time, as has been elegantly described in CRISPR arrays from within and among cystic fibrosis patients (109). In addition to shaping and being shaped by the evolution of their local hosts (110), phages can also shape the colonization success of dispersing bacteria, both positively and negatively (e.g., 111, 112), making bacteria-phage interactions a key component of our understanding of metacommunity dynamics in nature. Work from two geographically remote sludge bioreactors found that phage-mediated selection resulted in specific local bacterial adaptations despite global migration of the bacteria (110), suggesting that phage-mediated selection in this system is strong relative to dispersal. Similarly, phage adaptation to their local *Rhizobium* communities was recently demonstrated across natural populations in Mexico and Argentina (33), again indicating the importance

of bacteria-phage coevolution in shaping metapopulation dynamics. Moreover, for phages evolving outside of the laboratory, comparative genomic evidence consistently suggests that horizontal gene transfer/gene acquisition is a large driver of diversification and evolution (68, 69, 113), suggesting that most laboratory studies, which generally exclude genetic input/exchange, are potentially vastly underestimating the ability of phages to rapidly adapt to new or mutated hosts.

MOVING BEYOND PAIRWISE INTERACTIONS

Species interactions and coevolution are most easily measured within pairwise interactions, and thus it is no surprise that most studies of bacteria-phage interactions in the laboratory have been performed at this scale. However, bacteria-phage interaction networks in nature are rarely observed to be pairwise; phage host range can and does expand beyond single strains/species, bacteria are likely sensitive to many different phage types within the environment, and multiple infections are common, especially when prophages are considered. Recent evidence from single-cell sequencing of the human gut suggests that phage host range in this environment is generally constrained to a single strain/species (35), a result that was paralleled in a recent metagenomic study (37). However, studies using whole metagenomes (rather than viral metagenomes) or based on capture and sequencing of bacterial cells for inference of phage diversity are likely more informative about the host range of prophages than of lytic phages, and previous analyses across environments suggest more intertwined infection networks between lytic phages and their hosts in nature (114). Recent analysis of ^{13}C-enriched metagenomic DNA to track the transfer of carbon from hosts to their associated viruses in situ exemplifies the complex network of these interactions (115). In the case where hosts are susceptible to multiple phage types, multiple infections are possible and can have important impacts on phage evolution. Single-cell sequencing of marine bacteria found viral coinfection to be common (34), and experimental evidence suggests that such within-host competition impacts phage fitness (116). Moreover, although overlap in killing abilities of phages infecting marine *Vibrio* bacteria was found to be minimal, phage genome analyses suggest rampant recombination within phage species indicative of coinfections (117). This recombination has the potential to accelerate phage evolution, as was elegantly demonstrated in phages infecting *Streptococcus thermophilus* (23).

The combined impacts of multiple phages on bacterial evolution are not particularly well explored, but evidence from experimental coevolution suggests that resistance costs can be higher for bacteria evolving in a multi-phage environment (118), and that phage diversity can increase host genomic evolution and divergence among populations (119). Phage diversity can also impact the mechanisms of resistance evolved; CRISPR-based immunity was found to be more likely to evolve under experimentally low phage diversity, while receptor modification was more likely to evolve as a defense in high phage diversity environments (120). This latter idea was then tested within natural communities using metagenomic analyses across diverse ecosystems, and again it was found that CRISPR-Cas prevalence was higher in high virus abundance but low viral diversity environments (121). How bacterial diversity impacts phage evolution is even less well studied (reviewed in 122), but recent evidence suggests that, although phages can rapidly overcome bacterial resistance mechanisms when grown in pairwise interactions in the laboratory, this process is significantly slowed when bacterial diversity is introduced into the system (123). Although phage-phage coinfection and competition are relatively underexplored, and interactions among phages have likely been underestimated as a result of pairwise studies, these competitive dynamics (whereby one phage excludes infection by another) are increasingly recognized as a key part of the (co)evolutionary process (50). Finally, the evolution of bacterial resistance to phage has been shown to differ in a natural community setting due to the horizontal transfer of mobile phage

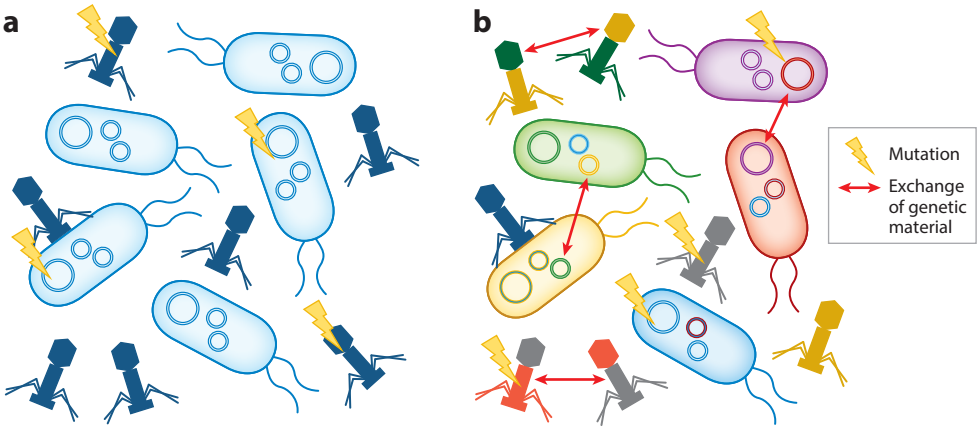

Figure 5

Mechanisms of genetic diversity generation in pairwise interactions and beyond. (*a*) Pairwise interactions, consisting of (initially clonal?) bacteria and phage species in isolation, will rely on mutation as the mechanism of diversity generation that evolution can act on. (*b*) In communities of multiple species, both mutation and exchange of genetic material (e.g., recombination, horizontal gene transfer) will dominate as mechanisms of genetic diversity generation that evolution can act on.

defense elements among bacterial taxa (124), further exemplifying how coevolution in natural populations is likely to differ from the laboratory (**Figure 5**).

INCREASING ECOLOGICAL COMPLEXITY

Natural bacteria-phage interactions are embedded within complex webs of ecological interactions. Although the vast majority of laboratory studies exclude these additional selection pressures, the growing body of experimental work that does include a third (or more) organism type has demonstrated that they can have drastic impacts on bacteria-phage ecological dynamics and selection pressures. For example, work comparing dynamics of multi- and single-enemy communities demonstrated that a specialist phage could drive its host to extinction only in the absence of grazing by a generalist protist predator (126). Protists can also graze on viruses as a nutrient source (127), suggesting that the presence of protists in an environment could reduce bacteria-phage contact rates. Moreover, both phage lysis and protist grazing have larger-scale consequences for the way resources move through ecosystems (128), making our understanding of these complex interactions key to predicting ecosystem function. Additionally, the impact of fungi on bacteria-phage interactions has been underexplored, although they were recently shown to play a possible role in phage dispersal (129), with hyphae enabling the codispersal of bacteria and phages in dry environments (85). Phages can also directly impact bacterial-fungal interactions, for example through inhibition of fungal biofilms (130). While examples of specific mechanisms remain limited, any organism that directly or indirectly causes changes in bacteria and/or phage growth, death, or contact rates would impact their ecological dynamics (**Figure 6**), and there are likely many novel mechanisms to uncover.

Changes in ecological dynamics via a third species can in turn impact the strength of phage-mediated selection and potentially coevolutionary dynamics. In one experimental evolution study, bacteria evolved in the presence of both protists and phages reached lower levels of defense against each ancestral enemy than when evolved with only a single partner, resulting in the breakdown of bacteria-phage coevolution in the presence of the predator (131). In another study using the same

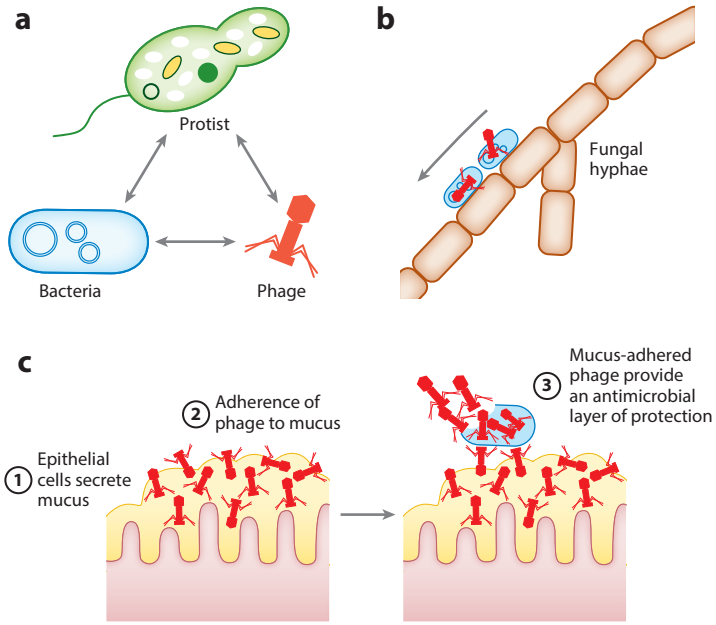

Figure 6

In natural ecosystems the interactions of phage will be part of a more complex web of interactions across trophic levels. (*a*) Laboratory work incorporating a third species (e.g., protists) into bacteria-phage experiments has revealed insight into how increasing ecological complexity can influence the interactions and impacts of phage. (*b*) Experiments increasing ecological complexity have revealed, for example, that the hyphae of fungi can play a role in the codispersal of phage and bacteria in dry environments (85). (*c*) If we look further in vivo, phage can provide a nonhost-derived immunity through their adherence to epithelial cell-derived mucus. Phage are shown to be enriched in mucus through specific binding interactions, providing layered protection against bacterial invasion (125).

bacteria and protist genotypes, but a different phage, bacteria that were experimentally evolved in the presence of both enemies showed increased phage defense compared to populations that were experimentally evolved with phages alone, suggesting that the correlation between enemy defenses is strain specific (132). More generally, if the addition of a third species alters the responses of bacteria and phage to selection, this results in diffuse, rather than pairwise, coevolution. Diffuse coevolution can lead to evolutionary changes that are not predictable from individual interactions, and the extent to which this process shapes natural bacteria-phage interactions is an open and critical area for future study (133, 134). In natural environments with diverse phage types, the influence of a third species could also select for phages with different life history strategies. For example, lysogeny can shape the outcome of phage competition with other bacterial antagonists, and prophage-derived exotoxins can function as broad antiprotist defenses, ensuring the proliferation of bacterial populations and their associated prophages in the face of protist predation (135).

For host-associated microbes, the environment itself is a third species, and the host-associated environment—with all of its abiotic complexity and resident microbiome—is drastically different from the in vitro one. These key differences can impact coevolutionary dynamics, for example by shaping the costs of phage resistance (54). These differences can impact the evolution of bacterial resistance, as has been shown in a plant system where both phage replication and resistance evolution were dramatically reduced in planta relative to in vitro (53). Similarly, limited phage

replication and no evidence of evolution of phage resistance were also observed in the murine gut, in this case due to the mucosa serving as a spatial refuge for bacteria (73). The impact of mucus has been an emerging theme in the study of gastrointestinal bacteria-phage interactions, as it has been shown that some phages can bind to and have enhanced activity in mucus (with others inhibited), thereby preventing bacterial movement across these barriers (125, 136). Importantly, synergies between phage and host immunity in reducing bacterial densities have also been well described (137).

Understanding if and how bacteria-phage dynamics might differ in the plant and animal host environment relative to the laboratory is critical not just to predicting natural dynamics but also to predicting the success of phage therapy in the control of bacterial pathogens or restructuring of microbiomes (138). Recent works exploring bacteria-phage dynamics within animal hosts have uncovered factors that may limit phage therapy, including the uptake and movement of phages through epithelial cells and the inactivation of phages by the immune system (139–141). Luckily, despite key differences, relevant outcomes of bacteria-phage interactions may still be predictable and phages that suppress bacterial densities in vitro have in many cases successfully controlled bacterial infections in therapeutic settings as well (142, 143). A recent study used bacteria and phage strains from a phage therapy patient as part of in vitro evolutionary simulations and observed the evolution of costly resistance in both environments (144). There has also been success with the design of cost-directed phage therapy, which involves choosing phages (such as those that bind to antibiotic efflux pumps) that should select for costly resistance mechanisms (145–147). In summary, host-associated bacteria-phage interactions can sometimes reflect those in vitro, but when there is incongruence in their ecology and evolution across environments, further investigation of the many factors that might be responsible for the difference is warranted. Based on emerging themes in the literature, these factors may include spatial structure, the resident host microbiome, and the host immune system.

CONCLUDING REMARKS

Our understanding of viral evolution and bacteria-phage coevolution is built upon a strong foundation of in vitro work on model systems, allowing for repeatable and mechanistic evaluation of the underlying processes shaping observed dynamics. Recent incorporation of more ecological complexity into these experimental systems, as well as a strong body of observational work on bacteria-phage dynamics in nature, puts the field in an excellent position to begin predicting when and how ecological and evolutionary dynamics in natural systems can be predicted based on knowledge gained from laboratory systems. In this review, we have highlighted key factors that might differ between typical laboratory experimental systems and natural populations/communities and identified how each factor might specifically alter bacteria-phage interactions. In addition to those outlined, however, there are a number of recently discovered or underappreciated aspects of phage biology that might also be critical in translating laboratory-based findings to natural communities or therapeutic settings (148). These include communication among phages (149), interactions between phages and the eukaryotic immune system (150, 151), and the role of phages in microbiome assembly (152). Moreover, as we move further into leveraging synthetic products and engineered phages (14), it will be critical to apply our understanding of these ecological and evolutionary processes to build durable and safe treatments. For example, work is needed to understand the impact of phage-derived products on bacteria-phage interactions during and after phage therapy, and to predict the longer-term ecological and evolutionary implications of engineered phages. Overall, the field has a wealth of theoretical and empirical knowledge that must be synthesized as we fully incorporate phages and their evolution into our understanding of the microbial world.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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