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Developing Phage Therapy That Overcomes the Evolution of Bacterial Resistance

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Abstract

The global rise of antibiotic resistance in bacterial pathogens and the waning efficacy of antibiotics urge consideration of alternative antimicrobial strategies. Phage therapy is a classic approach where bacteriophages (bacteria-specific viruses) are used against bacterial infections, with many recent successes in personalized medicine treatment of intractable infections. However, a perpetual challenge for developing generalized phage therapy is the expectation that viruses will exert selection for target bacteria to deploy defenses against virus attack, causing evolution of phage resistance during patient treatment. Here we review the two main complementary strategies for mitigating bacterial resistance in phage therapy: minimizing the ability for bacterial populations to evolve phage resistance and driving (steering) evolution of phage-resistant bacteria toward clinically favorable outcomes. We discuss future research directions that might further address the phage-resistance problem, to foster widespread development and deployment of therapeutic phage strategies that outsmart evolved bacterial resistance in clinical settings.

1. INTRODUCTION

Widespread use of small molecule antibiotics in medicine and agriculture has exerted strong selection for bacterial species to evolve multi-drug resistance (MDR), prompting a global public-health antibiotic-resistance crisis (1–3). Worse, new antibiotics are discovered and developed at a slow pace (4), causing novel antibiotic development to lag significantly behind the emergence of MDR pathogens: bacteria resistant to multiple antibiotic classes (5). Although antibiotics still save countless lives each year, their waning potency for treating certain infections raises concerns that we are entering a postantibiotic era (6) and manifests the urgent need to develop new strategies (7, 8).

A promising alternative is the use of bacteriophages (phages), viruses that specifically target bacterial cells. In phage therapy, lytic phages are administered to patients to target and kill the bacterial pathogen(s) causing an infection (9–12). Phages present many advantages over traditional antibiotics: They are highly specific for their hosts, reducing off-target bacterial killing; phages are the most abundant biological entities on Earth, providing an enormous reservoir for phage therapy; they can self-amplify and disseminate in the body to encounter susceptible bacteria; phages are generally regarded as safe, based on extremely rare cases of toxicity in animals and patients (13); and finally, phages often target different bacterial mechanisms than antibiotics, possibly minimizing evolution of cross-resistance (14–16).

Despite the promising therapeutic potential of phages, phage therapy presents several limitations that are yet to be addressed (14–16). Two of the most significant obstacles to developing generalized phage therapy (beyond personalized treatment) are phage specificity or phages with narrow host range, and the emergence of phage-resistant bacteria. Mitigating the latter is the focus of this review. Like antibiotics, lytic phages impose strong selective pressures on their hosts, to evolve resistance and prevent virus attack. Selection and enrichment of phage-resistant genotypes in the target bacterial population might result in therapy failure. Hence, it is crucial that in modern development of phage-based treatments, phages and therapeutic strategies are carefully designed to mitigate evolution of phage resistance. Here, we begin by reviewing the various mechanisms that bacteria employ to resist phage attack.

2. MECHANISMS OF PHAGE RESISTANCE IN BACTERIA

Phages and bacteria have likely coevolved for over 3 billion years. Strong evolutionary selection pressure by phage predation is thought to be responsible for an impressive, growing list of bacterial defense mechanisms that span various stages of the phage infection cycle (17–19). The stages of lytic phage replication in bacteria include phage binding to receptor(s) on the cell surface; subverting cell metabolism to achieve transcription/translation of phage-encoded genes; and cell lysis (death), which releases progeny virus particles that can repeat this cycle (**Figure 1**).

As a proximate defense strategy, bacteria can prevent phage entry to the cell by altering surface phage receptors such as bacterial lipopolysaccharides (LPS), outer membrane proteins, cell-wall teichoic acids, and appendages such as flagella and pili (20, 21) (**Figure 1**). Bacteria can evolve to modify these receptors via spontaneous mutations or by phase variation, which permits alternative expression of genes for surface-exposed structures (22). Also, receptors can be shielded from phage binding via proteins or polysaccharide capsules that cover the cell surface. A recent study shows that capsular types of *Klebsiella pneumoniae* clinical isolates are the main determinants of phage host range when viruses attack these bacteria (23). Furthermore, bacterial populations can avoid lytic phage infection by capitalizing on the protective shield of a biofilm, a population or community of bacteria enclosed in a self-produced exopolysaccharide matrix that adheres to a biotic or abiotic surface (24).

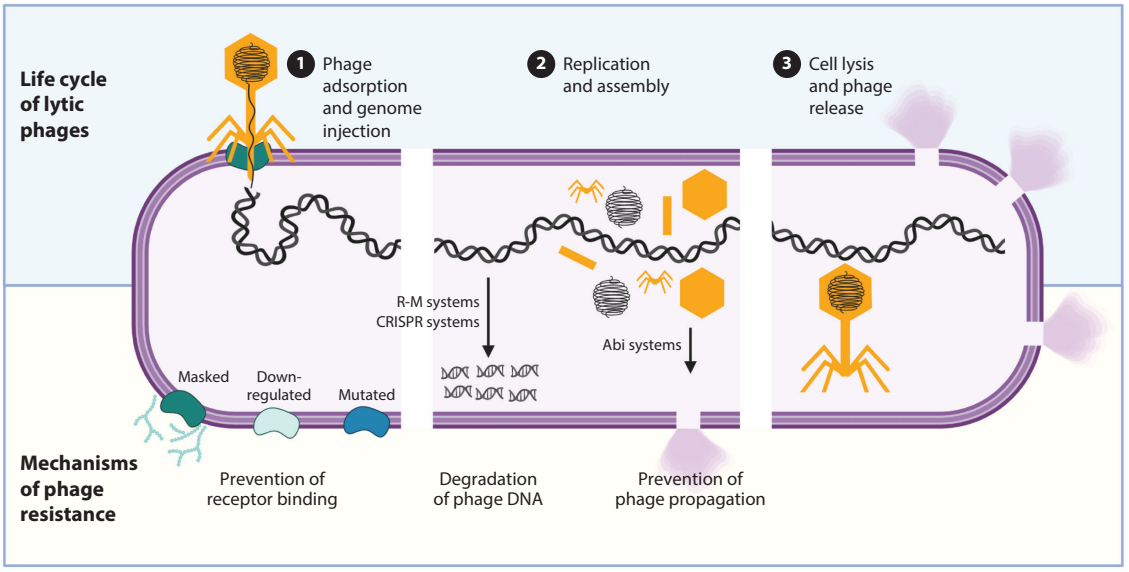

Figure 1

Replication cycle of lytic phages, with depicted examples of phage defense mechanisms that act at different stages of replication. ① A single phage particle (virion) attaches to specific phage receptors on the host surface, which is termed phage adsorption. After binding to the phage receptor(s), the genome is injected into the host. Bacteria can evolve to downregulate, mutate, or mask the receptor, thus preventing phage adsorption. ② If a phage manages to inject its genome into the host, it then uses the host's genome replication machinery to synthesize copies of various components of the phage, which assemble into new virions within the cytoplasm. R-M and CRISPR systems can degrade the injected phage genome, representing mechanisms of bacterial innate and adaptive immunity, respectively. Abi systems can recognize certain components of the phage and respond by inducing cell suicide before mature virions can form, which protects neighboring cells. ③ After complete assembly of new virions, the cell lyses and the released phages can initiate the infection cycle in neighboring phage-susceptible cells. Abbreviations: Abi, abortive infection; R-M, restriction-modification. Figure adapted from images created with BioRender.com.

If a phage manages to adsorb to the cell surface and successfully injects its genetic material (RNA or DNA) into the cell, bacteria can deploy defense mechanisms that prevent phage replication by targeting and degrading the phage nucleic acid (18) (**Figure 1**). These defenses are analogous to both innate and adaptive immune systems in eukaryotes. Innate immunity comprises restriction-modification (R-M) systems and other related mechanisms, such as BREX (bacteriophage exclusion) and DISARM (defense islands system associated with R-M). These defenses rely on recognition of DNA modifications to discriminate between bacterial DNA and foreign DNA (18). By contrast, adaptive immunity in bacteria can occur via CRISPR-Cas systems that recognize and cleave specific phage DNA or RNA sequences, which match short phage-derived DNA sequences (CRISPR spacers) in the host genome that reflect previous interactions with similar phages (25, 26).

Should intracellular phage replication proceed to later stages, bacteria can deploy additional defenses (**Figure 1**), such as abortive infection (Abi) systems and the newly discovered form of Abi, CBASS (cyclic oligonucleotide-based antiphage signaling system) (27, 28). Abi systems are activated when phage-specific components are detected within an infected cell. Once activated, Abi systems induce cell death or arrest bacterial metabolism to halt the phage replication cycle (18).

Unlike chemical antibiotics, phages are capable of evolving their own strategies to circumvent the bacterial resistance mechanisms they encounter when infecting bacterial cells. Phages can evolve to use a different receptor (29) and encode antidefense proteins to inactivate nucleic acid–targeting defense mechanisms (R-M and CRISPR-Cas defense systems) (18), among other counter-defense strategies (30–35).

Next, we briefly discuss the occurrence of phage-resistant bacteria and the prevalence of bacterial resistance mechanisms when pathogenic bacteria are challenged with phage in laboratory conditions, animal models, and more importantly clinical settings.

3. OCCURRENCE OF BACTERIAL RESISTANCE IN VITRO, IN VIVO, AND IN THE CLINIC

Abundant evidence shows that bacteria tend to rapidly evolve resistance to phages in laboratory conditions. Typically, in vitro phage-host experiments are conducted in nutrient-rich, well-mixed environments where harmful effects of mutations can be buffered (36–40). In these conditions, the most frequently observed mechanism of phage resistance is modification of cell-surface receptors, such as LPS, outer membrane proteins, capsules, and flagella (20, 41), to prevent or reduce phage adsorption. Bacterial resistance due to new genetic innovations or via adaptive-immune mechanisms such as CRISPR-Cas constitutes a gain-of-function change that is unlikely to occur in the simple microcosms and short timescales of typical in vitro studies. Thus, the annotation and characterization of most bacterial defense systems and their implications in phage therapy remain largely underexplored through laboratory studies.

Fewer studies have looked at evolution of bacterial resistance to phages in animal models of bacterial infections, but these are environmental settings where bacterial resistance mechanisms may have a higher cost relative to laboratory habitats. In these studies, evolution of phage-resistant bacteria is observed in 50–80% of experiments and is dependent on the type of infection and animal model employed (20). Similar to observations in vitro, evolved phage resistance in animal models is typically linked to alteration of cell-surface features. While providing phage resistance, these changes often come at the expense of attenuated virulence, re-sensitization to antibiotics, easier recognition by immune system cells, and/or inferior competitive ability relative to native microbiome species. Although potentially inconsequential for growth in vitro, any of these trade-offs would hamper the ability of phage-resistant mutants to survive in animal models, as shown by Oechslin et al. (42) and in the classic studies by Smith and Huggins and colleagues (43–45). The occurrence of such evolutionary trade-offs has been leveraged to design phage therapies that address emergence of bacterial resistance, as we discuss later in this review.

In the clinic, findings to date are either conflicting or inconclusive (20). Some compassionate-use cases and pilot studies of phage therapy in humans report the emergence of phage-resistant bacteria (46, 47), while in others no phage-resistant bacteria were detected (48, 49). Interestingly, one of these case studies reported bacterial re-sensitization to antibiotics (46), which is in line with the virulence trade-offs observed in some animal studies. In the majority of clinical trials on phage therapy [e.g., PhagoBurn (50) and an acute pediatric *Escherichia coli* diarrhea trial (51)], evolution of bacterial resistance to phage therapy has been poorly studied and documented, leaving a clear need to better understand the mechanisms of bacterial resistance to phage therapy and their clinical significance. Recent efforts in compassionate-use cases of personalized phage therapy shed light on understanding phage-resistance phenotypes in clinical settings (52).

Fortunately, it seems unlikely that widespread development of phage therapy would recapitulate the antibiotic resistance crisis because bacteria do not seem to easily acquire phage defense mechanisms via horizontal genetic transfer, unlike multi-drug resistance genes that easily spread

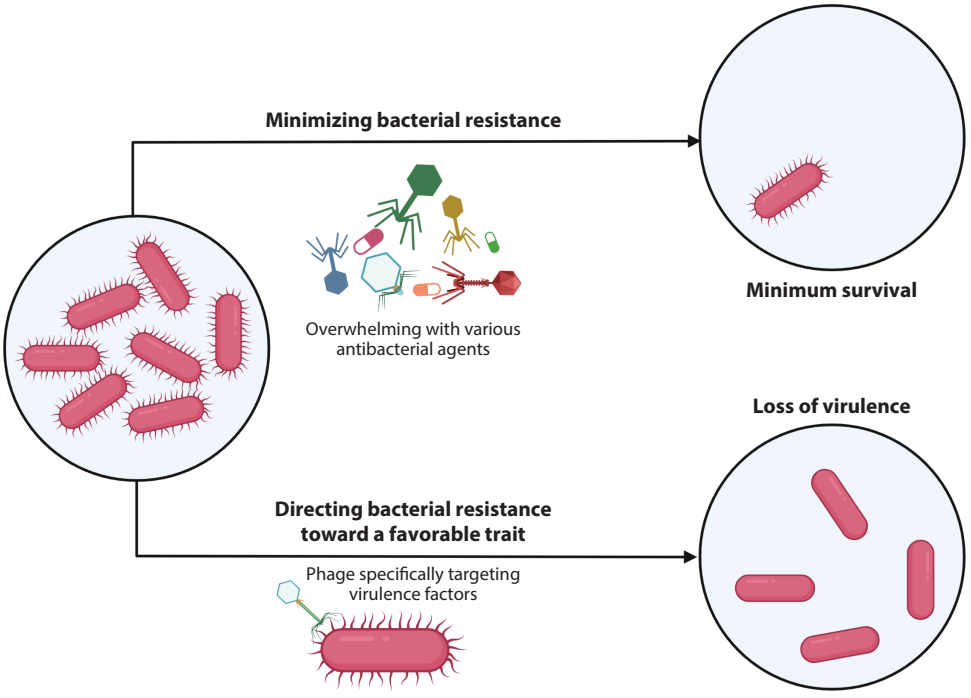

Figure 2

Mitigating bacterial resistance during phage therapy. We discuss two major strategies to address the evolution of phage resistance in bacteria. (*top*) The first strategy is to minimize the emergence of bacterial resistance. This can be achieved, for example, by using multiple therapeutics (e.g., phages, antibiotics) together, which can collectively kill diverse genotypes of target bacteria, thereby minimizing the size of the bacterial population and reducing its evolutionary potential. (*bottom*) The second strategy is to direct the evolution of phage resistance toward a favorable medical outcome. For example, using a therapeutic phage that binds to a bacterial virulence factor should exert selection to alter or delete this structure, thereby steering the remaining phage-resistant population to evolve lesser virulence or avirulence. Figure adapted from images created with BioRender.com.

via plasmid conjugation (53). Nonetheless, evolved bacterial resistance remains a major concern for the success of phage therapy. Mounting evidence in the literature suggests that this concern can be addressed (54). In the remainder of this review, we discuss the two major complementary approaches to mitigate bacterial resistance in phage therapy: minimizing the evolution of bacterial resistance and driving evolution of phage-resistant bacteria toward clinically favorable outcomes (**Figure 2**).

4. MINIMIZING BACTERIAL RESISTANCE

The straightforward approach to overcome bacterial resistance to phages is to design a phage therapy that maximizes the rate of phage killing across genotypes of target clinical isolates, thus restricting bacteria from growing to large population sizes. Reduced population size limits the ability for a bacterial population to overcome an environmental challenge (55), such as resistance to lytic phage attack. As spontaneous mutations occur randomly in genomes, population size must be above a certain threshold for these rescue mutations to arise and spread to fixation. Thus, minimizing the bacterial population size decreases the chance that bacteria can persist alongside lytic phages and therefore reduces the likelihood for evolved phage resistance. As an added benefit,

the patient's immune system and/or other administered antimicrobials can more easily clear the reduced population size (bacterial load) of survivors following initial phage deployment.

4.1. Choosing Highly Efficient Phages

Using efficiently propagating phages can contribute to reducing bacterial numbers. Some traits of the lytic phage replication cycle (56, 57) that may be desirable for maximizing therapeutic phage killing include adsorption kinetics, latent periods, and burst size. A phage that adsorbs strongly to one or more binding targets on the cell surface essentially maximizes rapid and irreversible adsorption to susceptible cells (58, 59). Latent period, the time between phage adsorption and release of new phages via cell lysis, should be minimized to achieve more rounds of infections per unit time (60). Large burst sizes are also beneficial because they result in generation of up to thousands of new offspring phage particles per cellular infection, thereby increasing the likelihood that the target bacterial population is overwhelmed by the presence of a smaller number of phages (60). Last, phage stability (e.g., avoidance of particle degradation and/or aggregation) is crucial for long-term storage of phages and may increase virus durability when administered to the patient.

All of these phage traits can be determined in the lab using standard microbiological techniques (61–64), where the aim could be to identify individual viruses with maximal performance traits or to combine different phages together that collectively exhibit optimal traits.

Here we describe various studies that address this overarching goal to minimize the evolutionary potential of target bacteria (**Figure 3**), using naturally occurring phages, those experimentally evolved in the laboratory, and viruses that are genetically engineered.

4.2. Phage Cocktails

Most naturally occurring phages exhibit narrow host ranges in laboratory settings, which may limit their usefulness in single (monophage) therapy due to frequent mismatches between a phage and the various genotypes (strains) of the infectious bacterium. To address the limited host range, multiple phages with complementary (nonoverlapping) host ranges can be combined into a cocktail preparation. Phage cocktails can be designed to target different genotypes of the same bacterial species or to attack multiple species. Thus, by spanning a broader host range, cocktails may be more effective at maximizing killing in a bacterial infection, especially when treating uncharacterized polymorphic or polymicrobial infections.

Phage cocktails have been the most popular and widely used strategies for phage therapy. Since the 1930s, phage cocktails have been commercially available as over-the-counter medications in many eastern European countries. These mixtures contain roughly 20–30 phages that target multiple bacterial species, and are periodically updated to ensure activity against emerging epidemiological strains. In the United States and Europe, there has been increasing use of phage cocktails in emergency and compassionate therapy cases, and within clinical trials testing phage safety and efficacy (12).

Traditionally, phage cocktails have been designed mainly to broaden host range, but this might not be sufficient to meet clinical needs. A major concern in devising cocktails is to choose wisely which phages are combined, such that therapy success is not undermined by easy ability for the bacteria to evolve resistance to one phage that permits cross-resistance to others present in the cocktail. Multiple studies show that rational design of phage cocktails may help to minimize evolution of phage resistance in target bacteria (65, 66) and to increase the chances of clinical success.

An approach to avoid emergence of phage-resistant bacteria is to ensure that the phages in a cocktail target different bacterial receptors. This way, phage-resistant bacteria should be selected

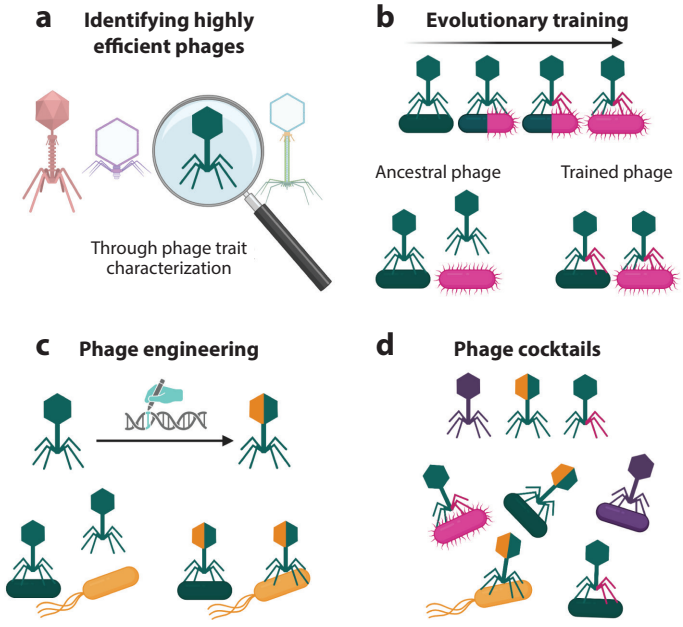

Figure 3

Phage therapy strategies to minimize evolution of bacterial resistance. (*a*) Naturally occurring phages with highly efficient growth traits can be identified through phage characterization and used to maximize the rate of phage killing, thus limiting the likelihood of evolved resistance. (*b*) Phages can also be trained or pre-adapted in the laboratory to efficiently kill resistant bacterial genotypes that are expected to evolve during phage therapy. (*c*) Phages can be engineered to alter their host range or other traits; e.g., genetic engineering has been used to turn temperate phages into their lytic derivatives, and to equip natural lytic phages with new functionalities [e.g., biofilm degradation (85)] to enhance therapeutic activity. (*d*) Natural, trained, or engineered phages can be combined into a single cocktail preparation to target multiple bacterial genotypes and thus minimize the potential of target bacteria to evolve phage resistance. Finally, phages can also be administered together with antibiotics to minimize the evolution of bacterial resistance. Figure adapted from images created with BioRender.com.

to evolve multiple rescue mutations in genes for two (or more) receptors independently, which should occur with a lower probability. For example, if the rate of bacterial mutation to phage resistance at a single locus is 10^{-6} mutations per bacterium per cell division, acquiring two mutations independently would occur at a rate of $10^{-6} \times 10^{-6} = 10^{-12}$ mutations per bacterium per cell division (67). However, choosing candidate phages that target different receptors requires prior characterization of said receptors, which may be laborious and time-consuming.

Aside from characterizing each individual phage's host range and receptor, developing phage mixtures also requires careful evaluation of their activity when combined as a cocktail. Niu et al. (68) demonstrated that the activity of individual phages does not necessarily predict their performance when they are mixed in a cocktail. The in vitro efficacy of some phage combinations was synergistic, whereas for others the effects were neutral or antagonistic (68). Also, as more phages are combined in a cocktail, the manufacturing process may become more time-consuming, complex, and expensive, and complex phage combinations are likely to cause pharmacokinetics and pharmacodynamics outcomes to be less predictable. Finally, the likelihood of eliciting a response from the patient's immune system is also expected to increase with a greater number or variety of phages. To limit these shortcomings and to increase the likelihood of clinical success, phage

cocktails must be rationally designed and characterized, with combinations of phages that are known to maximize the host range, act synergistically, and minimize the emergence of evolved phage-resistant bacteria.

4.3. Phage Training

A remarkable advantage of phage therapy relative to traditional chemical antibiotics is phage capability to evolve to overcome bacterial resistance. Phage training, also known as phage (pre)adaptation, exploits the natural potency for phage populations to quickly evolve to overcome bacterial defense mechanisms. In essence, the virus population is trained to anticipate how the target bacteria in the treated patient will evolve phage resistance. This approach allows the virus population to accumulate random point mutations or gene insertions/deletions to counter bacterial resistance. This way, phages can be evolutionarily trained to acquire expanded host ranges and to evolve traits that minimize the rise of phage-resistant bacteria during therapy.

One popular approach in phage training is to propagate viruses in vitro for serial rounds of infection on a nonevolving host strain. Morello and coworkers (69) employed this method for the first preclinical study demonstrating the benefits of trained phages in vivo. After five consecutive passages in liquid culture with a clinical *Pseudomonas aeruginosa* host strain, a trained phage population evolved to kill the host bacteria with a tenfold greater efficiency than the ancestral naturally isolated phage. This higher efficiency also translated into improved phage killing efficacy in a mouse model. The trained phages were able to kill an independent set of 20 *P. aeruginosa* clinical strains more efficiently than the ancestral phage. Therefore, phage training was successful in improving both growth rate on a target host and expansion of phage host range to encompass additional unselected bacterial strains (69).

Another phage training approach is based on allowing the host to coevolve with the phages during serial passage in the lab. For example, Borin and colleagues (70) coevolved a lytic phage with its *E. coli* host for 28 daily serial transfers. Initially, the host evolved to reduce the expression of the phage's receptor LamB. In turn, phages coevolved to gain the ability to infect through a secondary phage receptor, OmpF. This trained phage was found to suppress bacterial growth and minimize evolved phage resistance more efficiently than the ancestral phage strain (70). Because these trained phages recognize two cell-surface receptors, bacteria would likely have to acquire multiple mutations to evolve complete phage resistance. This outcome is less probabilistic than acquiring a single mutation and may impose a bacterial fitness cost, thus limiting the likelihood of bacteria to develop resistance against the trained phages. Similar results have been reported for trained phages targeting other pathogens such as *P. aeruginosa* (71).

Already, phage training has been leveraged successfully in the clinic to update commercial phage cocktails against emerging epidemiological strains of bacteria. For example, Ujmajuridze et al. (72) were able to increase the activity of a phage cocktail targeting multiple species of uropathogenic bacteria (*Staphylococcus aureus*, *E. coli*, *Streptococcus* spp., *P. aeruginosa*, *Proteus mirabilis*), from 41% to 75% coverage of clinical strains. The trained cocktail was then used to treat nine patients with phage-susceptible bacterial infections, and the observed bacterial loads decreased in six of the nine subjects (72). In principle, leveraging phage training to expand host range can reduce the number of phage types that must be combined in a cocktail to achieve broad killing and inhibit evolution of bacterial resistance, thus greatly simplifying the production process (73).

Despite promising lab studies and clinical relevance of phage training approaches, there are some notable caveats. Previous work warns that trade-offs between increased phage growth rate and decreased host range may occur during phage training (74, 75), and alternative approaches that address this problem are already emerging (75). The efficacy of phage training might be specific to phage and host strains, suggesting the need to determine the efficacy for each phage-host pair.

Importantly, the outcomes of long-term coevolution assays are not necessarily predictable, owing to many possible evolutionary trajectories (76). After coevolving phages and bacteria for ten passages, Betts et al. (77) reported variable outcomes, with some phages losing infectivity against their *Pseudomonas* hosts, which is certainly not the preferred outcome when harnessing phage training for therapeutic applications. In a separate study, four trained phages had similar variable effects on bacteria, which differed from strain to strain (71). This variability in outcomes highlights the importance of using well-designed evolution experiments to train phages and careful evaluations of the trained phage populations, in order to elucidate the extent to which (co)evolutionary trajectories of trained viruses are truly predictable versus highly stochastic, and whether this differs across certain phage-bacteria combinations.

4.4. Phage Engineering

Naturally occurring phages have been the focus of the majority of in vitro and in vivo studies as well as in clinical cases for over a century (78). However, there are some inherent limitations associated with using wild-type phages that can be overcome using a genetic-engineering approach. In the last decade, the synthetic biology revolution and development of new genetic-engineering technologies have paved the way for precise and rapid engineering of phage genomes, allowing the creation of novel designer phages (79, 80). Phage engineering has already been used to enhance properties of naturally occurring phages, including expanding their host ranges, reducing phage resistance in bacteria, increasing phage safety, and improving stability of phages and phage products (78, 81–83).

In 2019, Dedrick et al. (49) reported the first successful case of human phage therapy using engineered phages. Following a lung transplant, a 15-year-old cystic fibrosis patient acquired a disseminated *Mycobacterium abscessus* infection that was resistant to multiple antibiotics. The researchers screened hundreds of naturally isolated mycobacterial phages and identified a single lytic phage that efficiently killed the clinical strain as well as two temperate phages with poor killing activity. They genetically engineered the temperate phages by precisely removing their immunity repressor gene. The resulting lytic derivatives of the temperate phages showed enhanced killing activity against the target host strain. A cocktail composed of these three (one natural and two engineered) lytic phages was administered intravenously to the patient, which resulted in clinical improvement and alleviation of the infection (49). This landmark case study clearly exemplifies how genome engineering may be critical to expand the repertoire of phages suitable for clinical applications.

In addition to engineering temperate phages to turn them into their lytic derivatives, genetic engineering may also be used to enhance therapeutic activity of lytic phages. However, engineering of lytic phages is hampered by the fact that their genomes do not integrate into the chromosomes of host bacteria and thus cannot be manipulated with existing methods for bacterial genome engineering. Currently, the most popular phage engineering strategy relies on homologous recombination between the phage genome and a DNA-editing template plasmid transformed into host cells used in phage propagation. The recombination frequencies in this method are generally quite low, making it tedious and time-consuming to identify recombinant phages. To enhance the frequency of homologous recombination, recombineering-based methods exploit a phage-encoded recombination system. By heterologous expression of this system within the cell, the recombination template is protected from intracellular degradation and recombination frequency increases. A complementary downstream strategy to facilitate identification of engineered phages is to add a positive selection (e.g., fluorescence markers) or a negative selection (e.g., CRISPR-based system targeting wild-type phages).

Recently, new phage genome engineering methods that do not rely on homologous recombination have been described (79, 80). These approaches generate in vitro phage genomic DNA containing the desired genetic changes and reboot these genomes to produce infectious phage particles. There are two approaches to introduce genetic alterations into phage genomes in vitro: by modifying a genome extracted from a phage lysate or by printing and assembling synthetic DNA fragments into complete phage genomes. The assembly techniques can be accomplished within bacterial cells or in vitro. Then, the fully assembled engineered phage genome is rebooted via transformation of the DNA into bacterial hosts or using cell-free transcription-translation systems.

Phage engineering has been successfully used to increase therapeutic activity of natural lytic phages. For some viruses, host ranges have been modified through engineering of receptor-binding proteins. Yehl et al. (84) utilized a structure-guided approach to identify a specific host-range determining region in the gene encoding the tail fiber (filamentous protein involved in host recognition and binding) in phage T3. Then, they used targeted mutagenesis to generate phage libraries with vast genetic diversity in this region that translated into altered host ranges. Out of this library, they then chose a defined cocktail of 10 phage mutants. The cocktail was able to suppress emergence of phage resistance in *E. coli* bacteria for 1 week in vitro, whereas bacteria infected with the wild-type phage evolved resistance after merely 12 h. Finally, the study demonstrated that a single phage mutant was able to suppress bacterial growth in vivo in a mouse wound-infection model, which suggested that this phage engineering could be used to minimize evolution of phage resistance during therapy (84).

Bacterial resistance to phage infection does not exclusively arise from receptor mutations. For example, bacterial encapsulation or biofilm formation can restrict phages from accessing their binding target(s) and therefore limit their therapeutic efficacy. Lu et al. (85) engineered a T7 phage to express a biofilm-degrading enzyme (dispersin B) during host infection. When added to an *E. coli* biofilm model, the engineered T7 phage reduced biofilm-associated cell counts much more efficiently (~2 orders of magnitude lower biofilm cell counts) relative to wild-type T7 phage. In a different approach that was not constrained to a single target species (i.e., multi-species biofilms), T7 phage was engineered to deliver an enzyme that inactivates a quorum-sensing molecule produced by *P. aeruginosa* and is involved in biofilm formation. In a coculture of *E. coli* and *P. aeruginosa*, presence of the engineered T7 phage effectively inhibited biofilm formation, compared to the wild-type virus (86). These studies demonstrate that biofilm-forming bacteria can be targeted more efficiently using engineered phages, which is highly promising given the tendency for chronic bacterial infections to be associated with biofilm-forming pathogens.

Despite the advances in phage genome-editing methods described above, phage engineering research is still in its infancy. Most of these methods have been developed only for established model phages, and engineering non-model phages can be a lot more complex. Furthermore, many engineering approaches require domesticated bacterial hosts and/or an in-depth molecular understanding of cellular mechanisms and, thus, are not yet broadly applicable for emerging bacterial diseases. Further technological development is still required to provide broadly applicable, efficient, and inexpensive engineering methods.

To increase the efficacy of phage therapies, naturally occurring phages, trained phages, and engineered phages can be combined into a cocktail preparation as opposed to phage monotherapy and can also be co-administered with other antimicrobials (e.g., small molecule antibiotics) to further overwhelm bacteria and reduce their potential to evolve resistance (**Figure 2**, top).

Given the powerful ability for bacterial populations to defend against lytic phages and to readily evolve increased phage resistance, it may not be possible to always minimize bacterial evolution. Therefore, an equally powerful alternative is to assume that the bacteria will evolve to resist

therapeutic phages and devise phage therapies where this evolution is predicted and steered in a biomedically favorable direction that may foster its success.

5. DRIVING BACTERIAL EVOLUTION TOWARD FAVORABLE TRADE-OFFS

Bacterial resistance to phage therapy may be inevitable, especially for large bacterial populations with rapid generation times. One possible strategy is to direct the evolution of bacterial resistance toward clinically useful outcomes (87–89) (**Figure 4**). Genetic trade-offs occur during evolution by natural selection when an advantageous trait evolves to solve an environmental challenge (e.g., phage attack), at the expense of worsened performance in some other unselected trait (90). For example, evolution of brightly colored plumage might help a male bird attract mates and sire progeny, but these colorful feathers also make the bird more easily spotted by possible predators and therefore increase predation risk (91). Similarly, evolution of phage resistance can protect against lethal virus attack but often can impose costs on bacterial fitness (89). In a classic example, competitive growth measurements by Lenski (92) revealed that *E. coli* mutants that developed resistance to phage T4 had lower fitness relative to the ancestral bacteria.

The frequent occurrence of evolutionary trade-offs can be leveraged to design more effective phage therapies. Therapeutic phages can be chosen based on their ability to steer bacterial evolution toward trade-offs of biomedical significance. Below, we discuss several examples of phage-driven favorable trade-offs, including evolution of phage resistance in bacteria that coincides with increased sensitivity to antibiotics and reduced virulence.

5.1. Driving Re-Sensitization to Antibiotics

In some bacterial pathogens, a key mechanism of resistance to toxic molecules, such as antibiotics, is the active export of these antimicrobials out of the cell through transmembrane efflux pumps (93). This drug resistance mechanism may spread across bacterial populations via horizontal gene transfer, raising major concerns for evolution of MDR bacterial infections. However, numerous phages are known to use efflux pumps as host surface receptors. Thus, evolution of bacterial resistance to phage attack often should result in downregulation or even deletion of genes encoding efflux pumps. Such lytic phages can exert selection to re-sensitize bacteria to antibiotics because the evolved phage-resistant bacteria can be impaired in active transport of antibiotics out of the cell.

This idea was popularized in a study using the *P. aeruginosa*–targeting phage OMKO1. This phage likely interacts either directly or indirectly with the outer membrane porin M (OprM), which is a component of the multi-drug efflux systems MexAB and MexXY. The evolution of *P. aeruginosa* resistance to this phage may change OprM expression or cause gene deletions that modify efflux pump function, generally compromising antibiotic export from the cell. Clinical isolates of *P. aeruginosa* that developed resistance to OMKO1 had up to 50-fold higher sensitivity to ciprofloxacin, tetracycline, ceftazidime, and erythromycin antibiotics, observed in vitro and in experiments where phage protected against lethal bacterial infection of *Galleria mellonella* moth larvae (94, 95). By this logic, phage OMKO1 was predicted correctly to synergize with ceftazidime antibiotic in emergency therapy against MDR *P. aeruginosa* when treating a patient with an infected aortic arch replacement; the phage killed the bacteria while also steering phage resistance that coincided with re-sensitization to the ordinarily useless antibiotic (96).

Evolution of resistance against some phages may indirectly affect efflux pump function, seemingly without phage binding to pump proteins. ØS12-3, another lytic phage of *P. aeruginosa*, targets the O-antigen on the polysaccharide. Bacterial mutants resistant to this phage carry a deletion in

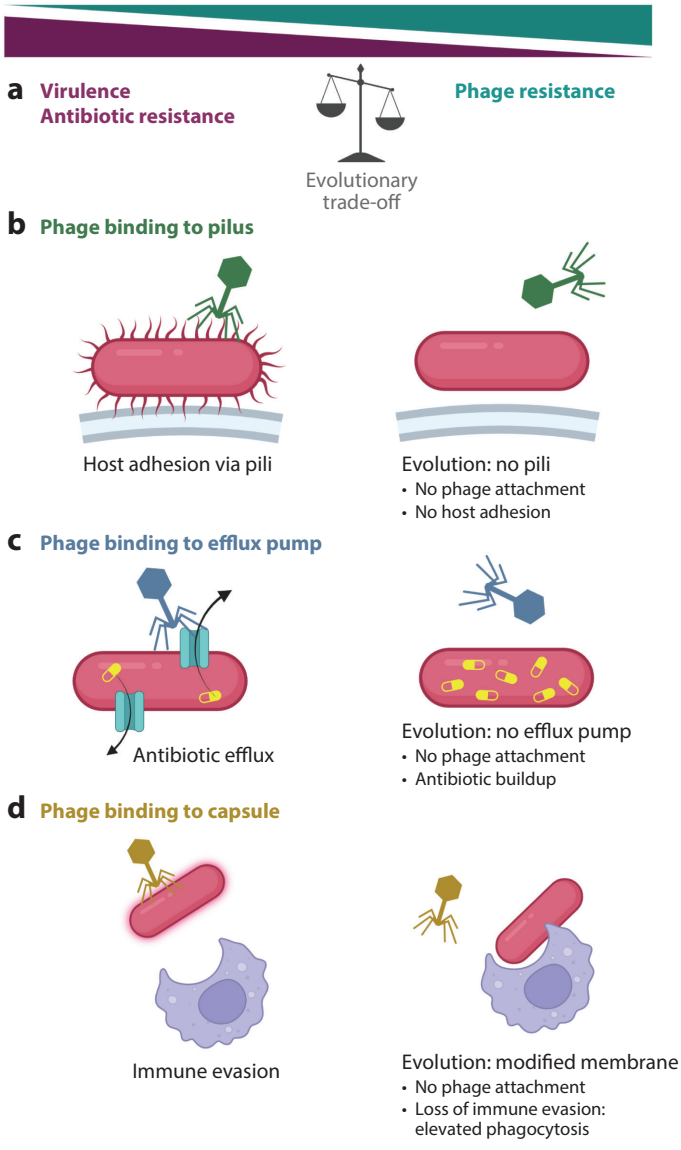

Figure 4

Evolutionary trade-offs can be used to direct phage-resistant bacteria toward a favorable biomedical outcome, such as reduced virulence or decreased antibiotic resistance. (*a*) Phage resistance may trade off with antibiotic resistance and virulence in bacteria. Such trade-offs can be leveraged by using phages that kill bacteria while selecting for phage-resistant mutants that are less virulent or re-sensitized to antibiotics. Examples are depicted in panels *b*–*d*. (*b*) Pili are virulence factors that allow certain pathogenic bacteria to adhere to host cells. Phages that bind to pili can drive the evolution of reduced (or deleted) pili expression, causing phage resistance to coincide with reduced host-adhesion capability. (*c*) One function of efflux pumps is to export antibiotics from the cell, thus availing antibiotic resistance that can span different drug classes. Phages that bind to efflux pumps can select for phage resistance that negatively impacts the efflux pump expression, allowing antibiotics to build up within the cell to achieve bacterial sensitivity at lower drug concentrations. (*d*) Phages that bind to bacterial capsules can drive the evolutionary trade-off toward modified capsular synthesis, whereby phage-resistant mutants become more easily recognized by host macrophages, important factors in the human immune system. Figure adapted from images created with BioRender.com.

gene *galU*, resulting in removal of the O-antigen (97). Nakamura and coworkers (98) recently investigated ØS12-3-resistant mutants of Pa12 host bacteria, a veterinary isolate of *P. aeruginosa*. They discovered that as a consequence of large chromosomal deletions in the region surrounding the *galU* gene, multi-drug efflux genes *mexXY* were also deleted. Because MexXY mediates quinolone efflux removal, the phage-resistant mutants evolved to be more sensitive to fluoroquinolones (98). Thus, phages that do not use efflux pumps as receptors per se can still indirectly affect the function of these protein complexes and may be potentially useful in re-sensitizing MDR bacteria to antibiotics.

Components of the bacterial membrane (e.g., polysaccharide layers) provide a frontline barricade to foreign molecules such as antibiotics. Mutations in these components may also render bacterial cells sensitive to certain antibiotics. An example comes from phage U136B, which uses both the multi-drug efflux pump protein TolC and LPS components as coreceptors on host *E. coli* cells. Several U136B-resistant bacteria presented mutations in *tolC* and as a result became re-sensitized to various antibiotics such as tetracycline, which is effluxed by TolC (39). However, some phage-resistant bacteria harbored mutations in the genes encoding the LPS, rather than in *tolC*. These changes in LPS made the phage-resistant cells sensitive to antibiotics such as colistin, which are not removed by TolC efflux pumps. Therefore, phage U136B drives separate trade-offs in the same *E. coli* bacterial host: Cells harboring mutations in *tolC* were more sensitive to tetracycline, while LPS mutants became more sensitive to colistin.

In the previous section, we mentioned the potential of combining phages and antibiotics to expand overall host range and minimize bacterial resistance. Evolutionary trade-offs between increased phage resistance and antibiotic re-sensitivity suggest another benefit of these combinations: In clinical settings, phages that drive antibiotic sensitization can be delivered together with currently ineffective antibiotics. During therapy, phages will kill the target bacteria while selecting for phage-resistant mutants in the bacterial population that become re-sensitized to the antibiotic, increasing the possibility for reduced bacterial load and infection clearance. This type of interaction has been termed phage-antibiotic synergy. A recent example comes from murine models of *Acinetobacter baumannii* bacteremia, where Gordillo Altamirano et al. (99) reported that phages targeting the bacterial capsule resulted in emergence of phage resistance in 96% of test animals. All phage-resistant bacterial isolates lost their ability to synthesize the capsule, which is the identical phenotypic outcome of evolved phage resistance previously observed by these researchers in vitro (100). Capsule-deficient mutants became susceptible to other antimicrobial agents, including two antibiotics (and one alternative phage) (99).

5.2. Steering Evolution Toward Other Reduced-Virulence Traits

Beyond antibiotic resistance, other virulence factors aid bacterial infection and pathogenicity in humans, such as adherence to host cells, invasion of host tissues, and evasion of host defenses. The cell structures associated with these factors include membrane-embedded proteins and various appendages, as well as polysaccharides forming the outer cell layers such as membranes, cell walls, and capsules. For example, flagella and pili are critical for the formation of bacterial biofilm (24), which protects bacteria from environmental stressors such as phages and antibiotics, and enable bacterial persistence through dormancy in chronic infections. Phages that use virulence factors as receptors, e.g., phages binding to flagella (101–103) or pili (104–107), can select for evolution of resistance that negatively affects functions of these structures, thus reducing bacterial virulence (87).

Phages that drive trade-offs with reduced virulence have already been successfully used in the clinic. Evidence suggests that phage-directed trade-offs that compromise synthesis of the bacterial capsule, a major virulence factor that provides protection from environmental stressors, likely played a crucial role in a well-known case of intravenous phage therapy in the United States. Here,

a life-threatening infection of the opportunistic pathogen *A. baumanii* was resistant to all antibiotic options for the patient. Through emergency treatment approval, several iterations of cocktails composed of different phages were administered to the patient intravenously and eventually led to their full recovery. Follow-up in vitro work revealed that the phages in the cocktail targeted bacterial capsules, the polysaccharide cell envelopes (108). Characterization of patient isolates before and after phage administration suggested that *A. baumanii* cells with evolved resistance to the administered phages traded off with capsule biosynthesis (108). Notably, this case and the aforementioned use of OMKO1 against MDR *P. aeruginosa* (96) helped popularize the power of phage therapy in Western medicine.

Next, we provide examples of in vitro and in vivo studies of phages targeting very diverse pathogenic bacteria that drive trade-offs toward reduced virulence.

5.2.1. Reduced bacterial adherence and colonization. Phages can direct evolution of resistance toward impaired ability of bacteria to adhere to and colonize mammalian host cells. For example, *Listeria monocytogenes*, an intracellular pathogen, requires a complex glycosylation pattern acquired through teichoic acids in its cell wall for successful infection. Acquiring resistance to a phage specifically recognizing this pattern resulted in loss of these sugars from the bacterial cell surface. The phage-resistant mutants of *L. monocytogenes* were unable to target the putative receptors on host mammalian cells and, hence, were unable to adhere and infect the host tissue (109). Notably, the phage used in this study was originally a temperate phage that was engineered to be fully lytic. Through this engineering approach, it is evident that temperate phages that drive bacterial evolution in a favorable direction may also provide promising candidates for therapy. Another example of phage-directed modulation of the host-colonization capability of bacteria was reported for *Enterococcus faecalis*–targeting phages. *E. faecalis* mutants resistant to a cohort of 19 phages evolved to acquire mutations in the enterococcal polysaccharide antigen (*epa*) gene cluster, a known virulence factor in enterococci. These *epa* mutants exhibited altered cell-surface properties (which also made them more susceptible to daptomycin and vancomycin, antibiotics that target the cell wall). Due to reduced fitness of the altered membranes, the mutants were deficient in intestinal colonization and transmission in mice (110).

5.2.2. Decreased host invasion. Another way phages can steer bacterial evolution toward reduced virulence is by limiting bacterial invasion and spread to additional cells within the infected host. To spread between cells in the human intestine, pathogen *Shigella flexneri* requires outer membrane protein A (OmpA) as well as the O-antigen (a component of LPS). *Shigella* phages A1-1 (38) and Sf6 (111) require OmpA for entry into the cell. In a recent study, five spontaneous *S. flexneri* mutants resistant against A1-1 were isolated (38). Sequencing revealed that two mutants had a deletion in *ompA*, while the other three genotypes featured mutations in LPS. The phage-resistant mutants, unlike the ancestral wild-type bacteria, were unable to spread between eukaryotic host cells in a tissue-culture pathogenicity model. It was concluded that the phage-resistant mutants had lost their ability to move between eukaryotic cells, indicating that evolved phage resistance compromised bacterial pathogenicity.

5.2.3. Impaired ability to evade host immune system. Phage resistance also has been shown to trade off with reduced virulence by impairing the bacteria's ability to evade the host immune system. Cai and coworkers (112) found that *K. pneumoniae*, when subjected to selection by phage GH-K3, evolves to downregulate three glucosyltransferase-encoding genes involved in capsule synthesis. The phage-resistant mutants, defective in capsule synthesis, were less virulent, as measured by mortality counts in murine infection models. Immunofluorescence assays revealed

that while a very small fraction of the wild-type *K. pneumoniae* cells became internalized within macrophages, the phage-resistant mutants had a much higher probability of being endocytosed by macrophages. The authors suggested that the bacterial capsule likely helps in avoiding phagocytosis by macrophages, whereas phage-resistant mutants were less able to evade phagocytosis. Thus, phage resistance may trade off with evasion of host defenses, a topic that deserves further scrutiny.

It is promising that regardless of many species-characteristic differences in surface morphology, host-invasion mechanisms, or virulence capabilities, phages that steer bacterial evolution toward lower virulence can be found. These phages should help in managing the disease progression by ESKAPE pathogens [emerging MDR pathogens involved in hospital-acquired infections: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (113)] and other bacteria where antibiotic resistance is on the rise.

5.3. Cautionary Tales from Evolution: Trade-Offs Are Favorable, Trade-Ups Are Not

As emphasized many times in this review, caution is warranted when choosing a phage or phage combination for use in therapy. For example, some phage(s) may drive a deleterious trade-up that increases bacterial resistance to one or more antibiotics, as opposed to steering a desired trade-off, which is obviously not a favorable outcome for the patient (114). For instance, coliphages T6 and U115 bind to Tsx porins, which also permit cell entry of the antibiotic albicidin. Bacteria that evolved resistance against each of the two phages or to the antibiotic harbored mutations in Tsx, resulting in increased cross-resistance to all three antimicrobial agents (37). This example highlights that careful characterization of phages is needed before deploying them for therapeutic use. Accurately predicting phage-driven trade-offs requires determination of phage receptor binding and would benefit from high-throughput screening of phage candidates to identify those that select for trade-offs useful in the clinic.

Phages are biological entities, with the expected potential to evolve greater phenotypic diversity over time, if they undergo genetic changes during patient treatment. Mounting evidence suggests that nongenetic phenotypic variation (physiological differences) can accumulate in phage populations, as recently reviewed by Igler (115). Such phenotypic variation in phages may complicate the interactions between bacteria and phages. Furthermore, the evolution of bacterial resistance in response to differing phages may take myriad evolutionary paths, and changes in bacterial diversity are difficult to predict (114). Variation in target bacteria populations raises similar concerns. For example, differing expression or underlying mutations in *E. coli* efflux pump gene *tolC* can drive pleiotropy between phage resistance and antibiotic resistance for phages that use TolC as a cellular receptor. In an example we discussed above, while a majority of the mutants resistant to the TolC-targeting phage U136B became more susceptible to antibiotics, a few variants were observed to become more drug resistant, likely due to synergistic pleiotropy (39). Thus, complexities of genotypic and phenotypic variation in both phage and bacteria—arising spontaneously as well as via their interactions—deserve much greater attention. This is important in order to refine our predictions for phage therapies designed to steer bacterial evolution and to minimize surprising pleiotropic effects and outcomes.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The nearly inevitable evolution of bacterial resistance to phage attack during patient treatment will continue to pose challenges for widespread development of therapeutic phages. It is remarkable

that phage therapeutic approaches have historically worked safely and effectively despite abundant knowledge gaps regarding the evolution of phage resistance in clinical settings. The rules of engagement between lytic phages and their bacterial hosts, studied using in vitro and in vivo models in the laboratory, can sometimes translate to accurate predictions of phage utility in human patients, as observed during some recent cases of emergency personalized treatment (96, 108, 116). These observations suggest that phage-bacteria interactions may obey (at least some) general rules that are robust across disparate environments, ranging from well-oxygenated liquid growth medium housed in shaking culture flasks to the relative black box of oxygen-limited biofilm-structured infections in the human body. However, these supposed rules may reflect constraints such as the biophysics of phage attachment to binding receptors on host-cell surfaces or ability of phages to hijack key nodes in cellular metabolic networks to achieve intracellular replication despite environmental differences. We should thus avoid naïve assumptions regarding consistencies of phage-bacteria interactions and acknowledge that crucial details of bacterial resistance to phages in clinical settings remain largely unknown.

To fill this knowledge gap, we recommend that attempts to isolate and characterize phage-resistant bacteria should be an increasing focus of clinical trials testing safety and efficacy of phage treatments (52). For example, bacteria harbor a wide variety of phage defense mechanisms (18, 19, 27, 30–34, 117, 118), but it is unclear which of these should be most impactful in contributing to treatment failures in the clinic. Moreover, if phage-bacteria coevolution occurs in the treated patient, it is uncertain how the dynamics and type of interaction could alter the course of treatment success. The spatial distribution of infecting bacteria (e.g., biofilm structures that may protect susceptible cells against collisions with phages) should influence the relative importance of certain defenses. Genotypes of target bacteria may also play crucial roles. For instance, bacteria may harbor prophages that prevent lytic phages from completing their intracellular replication (119). The relative frequencies of these prophage-carrying cells in a bacterial population are difficult to gauge accurately. These and many other factors can determine whether phage particles can gain access to host cells and successfully infect cell variants that may spatiotemporally differ in genotype, metabolism, and/or physiology. Therefore, characterizations of evolved phage-resistant bacteria in the clinic present vital topics for developing trusted strategies in phage therapy. Mitigating bacterial resistance during phage therapy is an exemplar of evolutionary medicine goals that seek to harness evolution thinking to improve the understanding and treatment of infectious diseases (120).

The tools used in microbiology research have advanced tremendously since the discovery of phages and the early work that first used these viruses in attempts to cure infections in humans and other animals. These pioneering efforts occurred when laboratory techniques were crude relative to modern advances in culture technologies, molecular biology, high-throughput genetic sequencing, and microscopy. A goal for phage therapy is to characterize details of phage traits and interactions with host bacteria using modern methods that recapitulate the environments of clinical settings. Examples include laboratory microcosms such as pathogenicity models in tissue culture, cell systems with tissue differentiation (e.g., air-liquid interface culture), and animal models with relevant biotic and abiotic features (e.g., robust innate and adaptive immunity that mimic potential synergies between host defenses and therapy strategies) (121). Without these efforts, we face perpetual mismatches between presumed microbial fitness and (co)evolution in culture flasks versus actual outcomes in relevant circumstances. Importantly, these data should bring greater reality to studies of evolved phage resistance, with the possibility of accumulating knowledge that hastens our abilities to make clinical treatment decisions to benefit patient health and therapy outcomes. Given the power for target bacteria to leverage existing cellular defenses against virus attack and to rapidly evolve increased phage resistance, it is crucial to utilize experiments and

analyses that address these perpetual challenges, in order to develop therapies that minimize the potency for bacteria to evolve and/or steer evolved phage resistance along favorable paths for treatment success.

DISCLOSURE STATEMENT

P.E.T. is a cofounder of Felix Biotechnology, Inc., a company that seeks to develop phages for human therapy. A.O.-B. is employed at Felix Biotechnology, Inc. Yale University has an institutional conflict of interest related to this project.

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LITERATURE CITED

1. Maragakis LL, Perencevich EN, Cosgrove SE. 2008. Clinical and economic burden of antimicrobial resistance. *Expert Rev. Anti-Infect. Ther.* 6(5):751–63
2. de Kraker MEA, Davey PG, Grundmann H, BURDEN Study Group. 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. *PLOS Med.* 8(10):e1001104
3. Cosgrove SE. 2006. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clin. Infect. Dis.* 42(Suppl. 2):S82–89
4. Sommer MOA, Munck C, Toft-Kehler RV, Andersson DI. 2017. Prediction of antibiotic resistance: time for a new preclinical paradigm? *Nat. Rev. Microbiol.* 15(11):689–96
5. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, et al. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18(3):268–81
6. Streicher LM. 2021. Exploring the future of infectious disease treatment in a post-antibiotic era: a comparative review of alternative therapeutics. *J. Glob. Antimicrob. Resist.* 24:285–95
7. World Health Organ. 2014. *Antimicrobial Resistance: Global Report on Surveillance*. Geneva: World Health Organ.
8. Cent. Dis. Control Prev. 2019. *Antibiotic Resistance Threats in the United States*. Atlanta: US Dep. Health Hum. Serv.
9. Sulakvelidze A, Alavidze Z, Morris JG. 2001. Bacteriophage therapy. *Antimicrob. Agents Chemother.* 45(3):649–59
10. Chan BK, Abedon ST. 2012. Phage therapy pharmacology: phage cocktails. *Adv. Appl. Microbiol.* 78:1–23
11. Chan BK, Abedon ST, Loc-Carrillo C. 2013. Phage cocktails and the future of phage therapy. *Future Microbiol.* 8(6):769–83
12. Gordillo Altamirano FL, Barr JJ. 2019. Phage therapy in the postantibiotic era. *Clin. Microbiol. Rev.* 32(2):e00066-18
13. Liu D, Van Belleghem JD, de Vries CR, Burgener E, Chen Q, et al. 2021. The safety and toxicity of phage therapy: a review of animal and clinical studies. *Viruses* 13(7):1268
14. Loc-Carrillo C, Abedon ST. 2011. Pros and cons of phage therapy. *Bacteriophage* 1(2):111–14
15. Nilsson AS. 2014. Phage therapy—constraints and possibilities. *Upsala J. Med. Sci.* 119(2):192–98
16. Principi N, Silvestri E, Esposito S. 2019. Advantages and limitations of bacteriophages for the treatment of bacterial infections. *Front. Pharmacol.* 10:513
17. Rostøl JT, Marraffini L. 2019. (Ph)ighting phages: how bacteria resist their parasites. *Cell Host Microbe* 25(2):184–94
18. Bernheim A, Sorek R. 2020. The pan-immune system of bacteria: antiviral defence as a community resource. *Nat. Rev. Microbiol.* 18(2):113–19
19. Vassallo CN, Doering CR, Littlehale ML, Teodoro GIC, Laub MT. 2022. A functional selection reveals previously undetected anti-phage defence systems in the *E. coli* pangenome. *Nat. Microbiol.* 7(10):1568–79

20. Oechslin F. 2018. Resistance development to bacteriophages occurring during bacteriophage therapy. *Viruses* 10(7):351
21. McCallin S, Oechslin F. 2019. Bacterial resistance to phage and its impact on clinical therapy. In *Phage Therapy: A Practical Approach*, ed. A Górski, R Międzybrodzki, F Borysowski, pp. 59–88. Cham, Switz.: Springer
22. Phillips ZN, Tram G, Seib KL, Atack JM. 2019. Phase-variable bacterial loci: how bacteria gamble to maximise fitness in changing environments. *Biochem. Soc. Transact.* 47(4):1131–41
23. Beamud B, García-González N, Gómez-Ortega M, González-Candelas F, Domingo-Calap P, Sanjuan R. 2023. Genetic determinants of host tropism in *Klebsiella* phages. *Cell Rep.* 42(2):112048
24. Wong GCL, Antani JD, Lele PP, Chen J, Nan B, et al. 2021. Roadmap on emerging concepts in the physical biology of bacterial biofilms: from surface sensing to community formation. *Phys. Biol.* 18(5):051501
25. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, et al. 2020. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nat. Rev. Microbiol.* 18(2):67–83
26. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Soria E. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J. Mol. Evol.* 60(2):174–82
27. Cohen D, Melamed S, Millman A, Shulman G, Oppenheimer-Shaanan Y, et al. 2019. Cyclic GMP-AMP signalling protects bacteria against viral infection. *Nature* 574(7780):691–95
28. Tal N, Morehouse BR, Millman A, Stokar-Avihail A, Avraham C, et al. 2021. Cyclic CMP and cyclic UMP mediate bacterial immunity against phages. *Cell* 184(23):5728–39.e16
29. Meyer JR, Dobias DT, Weitz JS, Barrick JE, Quick RT, Lenski RE. 2012. Repeatability and contingency in the evolution of a key innovation in phage lambda. *Science* 335(6067):428–32
30. Hampton HG, Watson BNJ, Fineran PC. 2020. The arms race between bacteria and their phage foes. *Nature* 577(7790):327–36
31. Samson JE, Magadán AH, Sabri M, Moineau S. 2013. Revenge of the phages: defeating bacterial defences. *Nat. Rev. Microbiol.* 11(10):675–87
32. Birkholz N, Fineran PC. 2022. Turning down the (C)BASS: Phage-encoded inhibitors jam bacterial immune signaling. *Mol. Cell* 82(12):2185–87
33. Costa AR, van den Berg DF, Esser JQ, Muralidharan A, van den Bossche H, et al. 2022. Accumulation of defense systems drives panphage resistance in *Pseudomonas aeruginosa*. bioRxiv 2022.08.12.503731. **https://doi.org/10.1101/2022.08.12.503731**
34. Hobbs SJ, Wein T, Lu A, Morehouse BR, Schnabel J, et al. 2022. Phage anti-CBASS and anti-Pycsar nucleases subvert bacterial immunity. *Nature* 605(7910):522–26
35. Mendoza SD, Nieweglowska ES, Govindarajan S, Leon LM, Berry JD, et al. 2020. A bacteriophage nucleus-like compartment shields DNA from CRISPR nucleases. *Nature* 577(7789):244–48
36. Kortright KE, Chan BK, Evans BR, Turner PE. Arms race and fluctuating selection dynamics in *Pseudomonas aeruginosa* bacteria coevolving with phage OMKO1. *J. Evol. Biol.* 35(11):1475–87
37. Kortright KE, Doss-Gollin S, Chan BK, Turner PE. 2021. Evolution of bacterial cross-resistance to lytic phages and albicidin antibiotic. *Front. Microbiol.* 12:658374
38. Kortright KE, Done RE, Chan BK, Souza V, Turner PE. 2022. Selection for phage resistance reduces virulence of *Shigella flexneri*. *Appl. Environ. Microbiol.* 88(2):e01514-21
39. Burmeister AR, Fortier A, Roush C, Lessing AJ, Bender RG, et al. 2020. Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *PNAS* 117(21):11207–16
40. Pires DP, Melo LDR, Azeredo J. 2021. Understanding the complex phage-host interactions in biofilm communities. *Annu. Rev. Virol.* 8:73–94
41. Gordillo Altamirano FL, Barr JJ. 2021. Unlocking the next generation of phage therapy: The key is in the receptors. *Curr. Opin. Biotechnol.* 68:115–23
42. Oechslin F, Piccardi P, Mancini S, Gabard J, Moreillon P, et al. 2017. Synergistic interaction between phage therapy and antibiotics clears *Pseudomonas aeruginosa* infection in endocarditis and reduces virulence. *J. Infect. Dis.* 215(5):703–12
43. Smith HW, Huggins MBY. 1982. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *Microbiology* 128(2):307–18

44. Smith HW, Huggins MBY. 1983. Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. *Microbiology* 129(8):2659–75
45. Smith HW, Huggins MB, Shaw KMY. 1987. Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *Microbiology* 133(5):1127–35
46. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, et al. 2017. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob. Agents Chemother.* 61(10):e00954-17
47. Zhvania P, Hoyle NS, Nadareishvili L, Nizharadze D, Kutateladze M. 2017. Phage therapy in a 16-year-old boy with Netherton syndrome. *Front. Med.* 4:94
48. Khawaldeh A, Morales S, Dillon B, Alavidze Z, Ginn AN, et al. 2011. Bacteriophage therapy for refractory *Pseudomonas aeruginosa* urinary tract infection. *J. Med. Microbiol.* 60(11):1697–700
49. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, et al. 2019. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat. Med.* 25(5):730–33
50. Jault P, Leclerc T, Jennes S, Pirnay JP, Que YA, et al. 2019. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect. Dis.* 19(1):35–45
51. Sarker SA, Sultana S, Reuteler G, Moine D, Descombes P, et al. 2016. Oral phage therapy of acute bacterial diarrhea with two coliphage preparations: a randomized trial in children from Bangladesh. *EBioMedicine* 4:124–37
52. Chan BK, Stanley GL, Kortright KE, Modak M, Ott IM, et al. 2023. Personalized inhaled bacteriophage therapy decreases multidrug-resistant *Pseudomonas aeruginosa*. medRxiv 2023.01.23.22283996. **https://doi.org/10.1101/2023.01.23.22283996**
53. Cohan FM, Zandi M, Turner PE. 2020. Broadscale phage therapy is unlikely to select for widespread evolution of bacterial resistance to virus infection. *Virus Evol.* 6(2):veaa060
54. Torres-Barceló C, Turner PE, Buckling A. 2022. Mitigation of evolved bacterial resistance to phage therapy. *Curr. Opin. Virol.* 53:101201
55. Burch CL, Chao L. 1999. Evolution by small steps and rugged landscapes in the RNA virus ϕ6. *Genetics* 151(3):921–27
56. Bull JJ, Pfennig DW, Wang IN. 2004. Genetic details, optimization and phage life histories. *Trends Ecol. Evol.* 19(2):76–82
57. Abedon ST. 1989. Selection for bacteriophage latent period length by bacterial density: a theoretical examination. *Microb. Ecol.* 18(2):79–88
58. Moldovan R, Chapman-McQuiston E, Wu XL. 2007. On kinetics of phage adsorption. *Biophys. J.* 93(1):303–15
59. Dennehy JJ, Abedon ST. 2021. Adsorption: phage acquisition of bacteria. In *Bacteriophages*, ed. DR Harper, ST Abedon, BH Burrowes, ML McConville, pp. 93–117. Cham, Switz.: Springer
60. Dennehy JJ, Abedon ST. 2021. Phage infection and lysis. In *Bacteriophages*, ed. DR Harper, ST Abedon, BH Burrowes, ML McConville, pp. 341–83. Cham, Switz.: Springer
61. Dennehy JJ, Turner PE. 2004. Reduced fecundity is the cost of cheating in RNA virus ϕ6. *Proc. R. Soc. B* 271(1554):2275–82
62. Chao L, Rang CU, Wong LE. 2002. Distribution of spontaneous mutants and inferences about the replication mode of the RNA bacteriophage ϕ6. *J. Virol.* 76(7):3276–81
63. Stent GS. 1963. *Molecular Biology of Bacterial Viruses*. San Francisco: Freeman
64. Clokie MRJ, Kropinski AM, eds. 2009. *Bacteriophages.* Totowa, NJ: Humana
65. Yang Y, Shen W, Zhong Q, Chen Q, He X, et al. 2020. Development of a bacteriophage cocktail to constrain the emergence of phage-resistant *Pseudomonas aeruginosa*. *Front. Microbiol.* 11:327
66. Forti F, Roach DR, Cafora M, Pasini ME, Horner DS, et al. 2018. Design of a broad-range bacteriophage cocktail that reduces *Pseudomonas aeruginosa* biofilms and treats acute infections in two animal models. *Antimicrob. Agents Chemother.* 62(6):e02573-17
67. Abedon ST, Danis-Wlodarczyk KM, Wozniak DJ. 2021. Phage cocktail development for bacteriophage therapy: toward improving spectrum of activity breadth and depth. *Pharmaceuticals* 14(10):1019

68. Niu YD, Liu H, Du H, Meng R, Sayed Mahmoud E, et al. 2021. Efficacy of individual bacteriophages does not predict efficacy of bacteriophage cocktails for control of *Escherichia coli* O157. *Front. Microbiol.* 12:616712
69. Morello E, Saussereau E, Maura D, Huerre M, Touqui L, Debarbieux L. 2011. Pulmonary bacteriophage therapy on *Pseudomonas aeruginosa* cystic fibrosis strains: first steps towards treatment and prevention. *PLOS ONE* 6(2):e16963
70. Borin JM, Avrani S, Barrick JE, Petrie KL, Meyer JR. 2021. Coevolutionary phage training leads to greater bacterial suppression and delays the evolution of phage resistance. *PNAS* 118(23):e2104592118
71. Friman VP, Soanes-Brown D, Sierocinski P, Molin S, Johansen HK, et al. 2016. Pre-adapting parasitic phages to a pathogen leads to increased pathogen clearance and lowered resistance evolution with *Pseudomonas aeruginosa* cystic fibrosis bacterial isolates. *J. Evol. Biol.* 29(1):188–98
72. Ujmajuridze A, Chanishvili N, Goderdzishvili M, Leitner L, Mehnert U, et al. 2018. Adapted bacteriophages for treating urinary tract infections. *Front. Microbiol.* 9:1832
73. Sáez Moreno D, Visram Z, Mutti M, Restrepo-Córdoba M, Hartmann S, et al. 2021. ε2-Phages are naturally bred and have a vastly improved host range in *Staphylococcus aureus* over wild type phages. *Pharmaceuticals* 14(4):325
74. Poullain V, Gandon S, Brockhurst MA, Buckling A, Hochberg ME. 2008. The evolution of specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage. *Evolution* 62(1):1–11
75. Zhang QG, Chu XL, Buckling A. 2021. Overcoming the growth–infectivity trade-off in a bacteriophage slows bacterial resistance evolution. *Evol. Appl.* 14(8):2055–63
76. Bono LM, Draghi JA, Turner PE. 2020. Evolvability costs of niche expansion. *Trends Genet.* 36(1):14–23
77. Betts A, Kaltz O, Hochberg ME. 2014. Contrasted coevolutionary dynamics between a bacterial pathogen and its bacteriophages. *PNAS* 111(30):11109–14
78. Pires DP, Cleto S, Sillankorva S, Azeredo J, Lu TK. 2016. Genetically engineered phages: a review of advances over the last decade. *Microbiol. Mol. Biol. Rev.* 80(3):523–43
79. Guan J, Oromí-Bosch A, Mendoza SD, Karambelkar S, Berry JD, Bondy-Denomy J. 2022. Bacteriophage genome engineering with CRISPR–Cas13a. *Nat. Microbiol.* 7(12):1956–66
80. Adler BA, Hessler T, Cress BF, Lahiri A, Mutalik VK, et al. 2022. Broad-spectrum CRISPR-Cas13a enables efficient phage genome editing. *Nat. Microbiol.* 7(12):1967–79
81. Kilcher S, Loessner MJ. 2019. Engineering bacteriophages as versatile biologics. *Trends Microbiol.* 27(4):355–67
82. Lenneman BR, Fernbach J, Loessner MJ, Lu TK, Kilcher S. 2021. Enhancing phage therapy through synthetic biology and genome engineering. *Curr. Opin. Biotechnol.* 68:151–59
83. Azam AH, Tan XE, Veeranarayanan S, Kiga K, Cui L. 2021. Bacteriophage technology and modern medicine. *Antibiotics* 10(8):999
84. Yehl K, Lemire S, Yang AC, Ando H, Mimee M, et al. 2019. Engineering phage host-range and suppressing bacterial resistance through phage tail fiber mutagenesis. *Cell* 179(2):459–69.e9
85. Lu TK, Collins JJ. 2007. Dispersing biofilms with engineered enzymatic bacteriophage. *PNAS* 104(27):11197–202
86. Pei R, Lamas-Samanamud GR. 2014. Inhibition of biofilm formation by T7 bacteriophages producing quorum-quenching enzymes. *Appl. Environ. Microbiol.* 80(17):5340–48
87. Mangalea MR, Duerkop BA. 2020. Fitness trade-offs resulting from bacteriophage resistance potentiate synergistic antibacterial strategies. *Infect. Immunity* 88(7):e00926-19
88. León M, Bastías R. 2015. Virulence reduction in bacteriophage resistant bacteria. *Front. Microbiol.* 6:343
89. Bohannan BJM, Lenski RE. 2000. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. *Ecol. Lett.* 3(4):362–77
90. Garland T, Downs CJ, Ives AR. 2022. Trade-offs (and constraints) in organismal biology. *Physiol. Biochem. Zool.* 95(1):82–112
91. McQueen A, Kempenaers B, Dale J, Valcu M, Emery ZT, et al. 2019. Evolutionary drivers of seasonal plumage colours: Colour change by moult correlates with sexual selection, predation risk and seasonality across passerines. *Ecol. Lett.* 22(11):1838–49
92. Lenski RE. 1988. Experimental studies of pleiotropy and epistasis in *Escherichia coli*. I. Variation in competitive fitness among mutants resistant to virus T4. *Evolution* 42(3):425–32

93. Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, et al. 2018. Multidrug efflux pumps: structure, function and regulation. *Nat. Rev. Microbiol.* 16(9):523–39
94. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. 2016. Phage selection restores antibiotic sensitivity in MDR *Pseudomonas aeruginosa*. *Sci. Rep.* 6(1):26717
95. Gurney J, Pradier L, Griffin JS, Gougat-Barbera C, Chan BK, et al. 2020. Phage steering of antibiotic-resistance evolution in the bacterial pathogen, *Pseudomonas aeruginosa*. *Evol. Med. Public Health* 2020(1):148–57
96. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. 2018. Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. *Evol. Med. Public Health* 2018(1):60–66
97. Le S, Yao X, Lu S, Tan Y, Rao X, et al. 2014. Chromosomal DNA deletion confers phage resistance to *Pseudomonas aeruginosa*. *Sci. Rep.* 4(1):4738
98. Nakamura K, Fujiki J, Nakamura T, Furusawa T, Gondaira S, et al. 2021. Fluctuating bacteriophage-induced galU deficiency region is involved in trade-off effects on the phage and fluoroquinolone sensitivity in *Pseudomonas aeruginosa*. *Virus Res.* 306:198596
99. Gordillo Altamirano FL, Kostoulias X, Subedi D, Korneev D, Peleg AY, Barr JJ. 2022. Phage-antibiotic combination is a superior treatment against *Acinetobacter baumannii* in a preclinical study. *EBioMedicine* 80:104045
100. Gordillo Altamirano F, Forsyth JH, Patwa R, Kostoulias X, Trim M, et al. 2021. Bacteriophage-resistant *Acinetobacter baumannii* are resensitized to antimicrobials. *Nat. Microbiol.* 6(2):157–61
101. Guerrero-Ferreira RC, Viollier PH, Ely B, Poindexter JS, Georgieva M, et al. 2011. Alternative mechanism for bacteriophage adsorption to the motile bacterium *Caulobacter crescentus*. *PNAS* 108(24):9963–68
102. Samuel ADT, Pitta TP, Ryu WS, Danese PN, Leung ECW, Berg HC. 1999. Flagellar determinants of bacterial sensitivity to χ-phage. *PNAS* 96(17):9863–66
103. Esteves NC, Scharf BE. 2022. Flagellotropic bacteriophages: opportunities and challenges for antimicrobial applications. *Int. J. Mol. Sci.* 23(13):7084
104. McCutcheon JG, Peters DL, Dennis JJ. 2018. Identification and characterization of type IV pili as the cellular receptor of broad host range *Stenotrophomonas maltophilia* bacteriophages DLP1 and DLP2. *Viruses* 10(6):338
105. Chibeu A, Ceyssens PJ, Hertveldt K, Volckaert G, Cornelis P, et al. 2009. The adsorption of *Pseudomonas aeruginosa* bacteriophage φKMV is dependent on expression regulation of type IV pili genes. *FEMS Microbiol. Lett.* 296(2):210–18
106. Holland SJ, Sanz C, Perham RN. 2006. Identification and specificity of pilus adsorption proteins of filamentous bacteriophages infecting *Pseudomonas aeruginosa*. *Virology* 345(2):540–48
107. Gutierrez-Rodarte M, Kolappan S, Burrell BA, Craig L. 2019. The *Vibrio cholerae* minor pilin TcpB mediates uptake of the cholera toxin phage CTXϕ. *J. Biol. Chem.* 294(43):15698–710
108. Liu M, Hernandez-Morales A, Clark J, Le T, Biswas B, et al. 2022. Comparative genomics of *Acinetobacter baumannii* and therapeutic bacteriophages from a patient undergoing phage therapy. *Nat. Commun.* 13(1):3776
109. Sumrall ET, Shen Y, Keller AP, Rismondo J, Pavlou M, et al. 2019. Phage resistance at the cost of virulence: *Listeria monocytogenes serovar* 4b requires galactosylated teichoic acids for InlB-mediated invasion. *PLOS Pathog.* 15(10):e1008032
110. Chatterjee A, Johnson CN, Luong P, Hullahalli K, McBride SW, et al. 2019. Bacteriophage resistance alters antibiotic-mediated intestinal expansion of enterococci. *Infect. Immunity* 87(6):e00085-19
111. Parent KN, Erb ML, Cardone G, Nguyen K, Gilcrease EB, et al. 2014. OmpA and OmpC are critical host factors for bacteriophage Sf6 entry in *Shigella*. *Mol. Microbiol.* 92(1):47–60
112. Cai R, Wang G, Le S, Wu M, Cheng M, et al. 2019. Three capsular polysaccharide synthesis-related glucosyltransferases, GT-1, GT-2 and WcaJ, are associated with virulence and phage sensitivity of *Klebsiella pneumoniae*. *Front. Microbiol.* 10:1189
113. World Health Organ. 2017. *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics*. Geneva: World Health Organ.
114. Burmeister AR, Turner PE. 2020. Trading-off and trading-up in the world of bacteria–phage evolution. *Curr. Biol.* 30(19):R1120–24

115. Igler C. 2022. Phenotypic flux: the role of physiology in explaining the conundrum of bacterial persistence amid phage attack. *Virus Evol.* 8(2):veac086
116. Castledine M, Padfield D, Sierocinski P, Soria Pascual J, Hughes A, et al. 2022. Parallel evolution of *Pseudomonas aeruginosa* phage resistance and virulence loss in response to phage treatment in vivo and in vitro. *eLife* 11:e73679
117. Doron S, Melamed S, Ofir G, Leavitt A, Lopatina A, et al. 2018. Systematic discovery of antiphage defense systems in the microbial pangenome. *Science* 359(6379):eaar4120
118. Cury J, Bernheim A. 2022. CRISPR-Cas and restriction–modification team up to achieve long-term immunity. *Trends Microbiol.* 30(6):513–14
119. Bondy-Denomy J, Garcia B, Strum S, Du M, Rollins MF, et al. 2015. Multiple mechanisms for CRISPR–Cas inhibition by anti-CRISPR proteins. *Nature* 526(7571):136–39
120. Natterson-Horowitz B, Aktipis A, Fox M, Gluckman PD, Low FM, et al. 2023. The future of evolutionary medicine: sparking innovation in biomedicine and public health. *Front. Sci.* 1:997136
121. Rodriguez-Gonzalez RA, Leung CY, Chan BK, Turner PE, Weitz JS. 2020. Quantitative models of phage-antibiotic combination therapy. *mSystems* 5(1):e00756-19

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