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Opinion

Steering Phages to Combat Bacterial Pathogens

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A critical issue facing humanity is the rise in antimicrobial resistance. Although increasing research effort is spent on developing novel drugs, few alternatives reach the clinic. Phage therapy is an old idea, long practiced in eastern European countries and gaining serious attention in the western world. In this Opinion piece, we outline a strategy that harnesses the ability of bacteriophages (phages) to impose strong selection on their bacterial hosts: instead of avoiding resistance, this therapy *relies* on the target organism resisting the phage. By using phages to both kill bacterial cells and 'steer' survivors towards resistant but more compromised phenotypes, we can potentially improve treatment outcomes.

Antimicrobial treatments work by reducing the number of viable pathogens, and their persistent application in a population of infected individuals invariably selects for resistant strains [1–3]. Antimicrobial resistance is a growing clinical challenge, reducing the ability of physicians to care adequately for patients [4]. Compounding the problem, the discovery of novel antimicrobial compounds has decreased over recent decades [5]. These two interrelated challenges have led to the emergence of untreatable multidrug-resistant microbes and associated increases in morbidity and mortality [6–8]. In light of this crisis, there are growing calls to revitalize research into alternative and complementary treatments such as antivirulence drugs [9], vaccines [10], anti-resistance adjuvants [11], diagnostic-informed treatments [12], and the topic of this article – phage therapy [13].

Phage therapy has a long history of great successes and perplexing failures [13,14]. Although the exact causes of failure are often unclear and barring inappropriate choice, the most likely mechanism is the evolution of resistance to phage [15]. Given the pervasive risk of resistance evolution and the possible worsening conditions should a therapy fail, phages and the strategies used to apply them need to be chosen very carefully. Several candidates have been proposed to combat resistance evolution, including phage cocktails and combinations [16], phage–antibiotic combinations [15], phage training [17,18], phage engineering [19], and approaches targeted at structural resistance such as biofilms [20].

Another approach that we advocate here is to *deliberately* use the evolution of resistance to attain therapeutic objectives. The basis of this idea relies on trade-offs between resistance to phage and other bacterial traits, namely virulence factors and antibiotic resistance. Such approaches have been previously discussed [13,21–23], and it is our aim to unite these into a single concept that we call 'phage steering'. Phage steering is similar to other strategies by its immediate aim of killing phage-sensitive bacteria. The novelty of phage steering is that remaining bacterial cells will be phage resistant, but somehow more vulnerable to other antimicrobials or with reduced virulence. In either case, target bacteria not eliminated by the phage persist but either cause far less damage to the host or are more sensitive to antibiotics. The notion of 'steering' has previously been discussed in the context of preventing or guiding the emergence of drug resistance in microbial pathogens [24,25] and cancers [26]. Phage steering differs by a focus on actively selecting for specific phage resistances that are correlated with improved infection outcomes. It is thus arguably the phage that steers the infection and not directly the practitioner.

Highlights

Antibiotic resistance is a global issue that currently causes increased morbidity and mortality. It is crucial to expand and explore alternative strategies to combat the crisis.

The use of bacteriophages, the viruses of bacteria, is one of the many alternative strategies being considered and developed.

Bacteriophage therapy conventionally works by reducing the number of bacteria at a given site in a manner analogous to antimicrobials.

Bacteriophage therapy also shares the same problem as conventional antimicrobials: the rapid emergence of resistance.

A well reported phenomenon of bacteriophage interactions is the reduction of virulence of phage-resistant bacteria, and recent studies have shown that resistance to phage can also reduce resistance to antimicrobials.

Phage therapy could be used as a selective force against pathogenic surface factors, manipulating the evolution of the pathogen in favour of the infected patient.

Phage Steering

Bacterial surface factors are integral to disease phenotypes. These factors have diverse functions in different environments, mediating attachment, secretion, or nutrient uptake [27–29]. In a pathogen context, surface factors are often described as virulence factors or antibiotic-resistance mechanisms, as they can mediate attachment and damage to hosts, and antibiotic efflux, respectively. For

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example, the opportunistic pathogen *Pseudomonas aeruginosa* has 306 described virulence factors of which 45% are predicted by PSORTb to be localized to the cell membrane [30].

Given the critical involvement of surface factors in disease phenotypes, we argue that phages binding to these factors would reduce the number of bacteria and, importantly, select against these factors – leading to reductions in virulence or antibiotic resistance among surviving bacterial populations. Accomplishing this requires accounting for the evolution of surface factor interactions between phages and bacteria over short evolutionary time scales, that is, during treatment of a patient.

The main idea promoted here is that bacterial evolution of phage resistance can generate negative feedbacks for bacterial fitness (i.e., trade-offs) on key traits associated with disease. Several *in vitro* studies indicate these trade-offs (see [23] for examples of virulence reduction driven by phage, and [31] for reduction in antibiotic resistance). Moreover, in a promising recent clinical case, an appropriate phage was identified by screening for the desired effect of steering a bacterial phenotype and successfully administered to a patient [32]. We propose that a range of future therapies can be designed that capitalize on phage steering.

Several factors limit the current prospects for phage therapy; improvements or investment in regulation, supply chains, and basic biology need to occur before any routine use in the clinic (see Box 1 for discussion of Practice and Safety). It is with this in mind that we describe the scenarios under which we believe phage steering can contribute and hope that our discussion will help to illuminate the field and spark further interest and research into these ideas. Although there are many ways in which steering can be employed, we focus on the two most understood and most likely to be put into practice: reduced expression/activity of bacterial surface factors contributing to virulence (Figure 1A,C), and reduced expression/activity of bacterial surface factors contributing to antimicrobial resistance (Figure 1B,D).

Box 1. Practicality and Safety

Phage therapy (in the West) is typically performed as a compassionate care/release study, where phages that can target the focal pathogen are either selected from library stock or isolated *de novo* from the environment, normally within a limited time window. Choice of candidates for phage steering would be similar to other phage therapy strategies [62], with the notable exception that the former would require identification of targets that correspond to trade-offs that, when selected, would somehow compromise (e.g., either with reduced virulence or increased antibiotic sensitivity) the population of bacteria remaining after the lysis of sensitive cells.

Given the above, identifying steering phages will be challenging, but certainly possible given the enormous diversity of phages in the environment. Moreover, an unexplored but plausible alternative to phage discovery is to engineer them. Because engineering constructs can be patented, whereas natural phages cannot easily, this may promote regulation and spur investment.

Safety is a major consideration in phage therapy [63], and given its relatively recent history, considerations for phage steering have not yet been discussed in any detail. Because lysogenic phages may carry antibiotic-resistance genes, transfer toxins such as those of the *Vibrio cholerae* phage (CTXΦ) [64], and are likely to be less effective at selecting for resistance than lytic phages – outside of being engineered – they will not be candidates for steering.

Likewise, safety considerations for other phage therapies also apply to steering phages. These include avoiding phages that increase virulence [36], induce biofilm formation [65] or prophage activity [66], or select for the emergence of mutators [67]. For example, chronic infections of *P. aeruginosa* in CF patients may be associated with hypermutators [68] and with increased antibiotic resistance [69]. Although selection for compensatory mutations is a potential concern in phage steering, this is unlikely, due to both the sparse cell numbers remaining after phage introduction [70], and (as discussed in Box 3) the screening for phages that limit the ascension of phenotypically resistant bacteria.

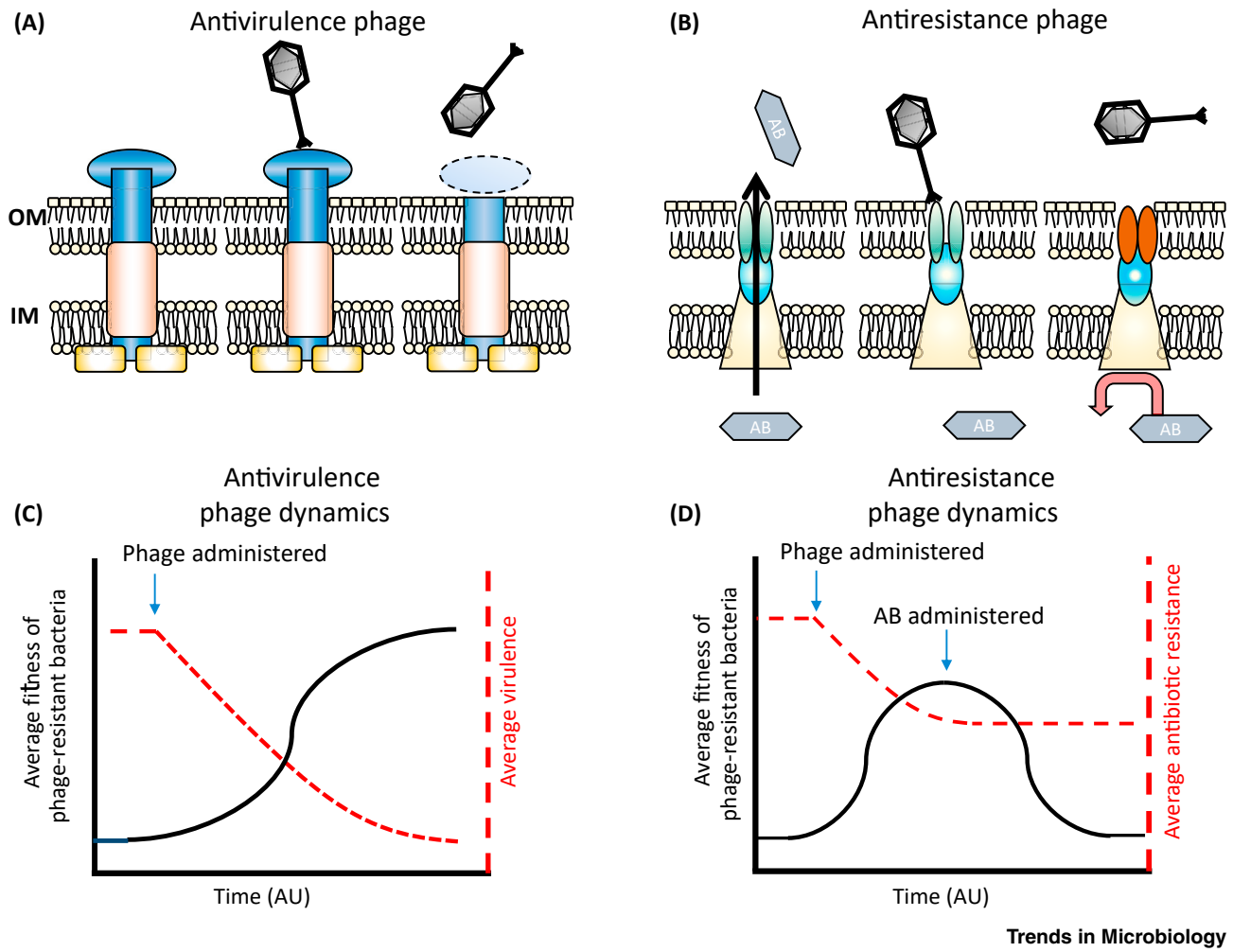

Figure 1. Phage Steering of Bacterial Virulence and Antibiotic Resistance.

Selection for bacterial resistance to phages at specific targets reduces bacterial virulence (A and C) or antibiotic resistance (B and D). As seen in C, the fitness of resistant bacteria (black line) increases due to phage pressure, while virulence (red line) declines. D shows a similar interaction, but this time focusing on antibiotic-resistance mechanisms. The fitness of phage-resistant bacteria (black line) increases as phage replicate, and there is coetaneous decline in average antibiotic resistance (red line). Subsequent administration of antibiotics would reduce the fitness of these now antibiotic sensitive cells.

Attenuating Virulence

Virulence is a widely used term with different definitions in different fields. Here we employ it to mean the *in vivo* expression of bacterial factors that damage the host [9], and bacterial life-history traits such as *in vivo* growth rate and competitiveness with commensals in the microbiome [33]. We focus below on virulence factors required for phage attachment and outline evidence of reduced virulence following phage exposure (Table 1).

As early as 1931, d'Herelle noted that reduction in virulence was a hallmark of successful phage therapy, stating that bacterial modifications following phage treatment could lead to attenuated virulence if the bacteria are not completely eradicated. He included reduction in virulence as one of the three principles of phage therapy: first, the action of the phages in reducing the number of bacteria; second, the reduction in virulence; and third, an increase in phagocytosis due to lysins acting to opsonize pathogens [34]. Almost a full century later we are still reporting virulence reduction as an interesting yet unexploited phenomenon.

Table 1. Bacterial Virulence Factors with Defined Phage That Can Potentially Act as Steering Agents

Target	Description
Porins	A clear example in a natural system was shown in recent work by Seed and colleagues [83], where phage predation on the pathogen *V. cholerae* led to a reduction in virulence in humans. Resistance to the phage ICP2 can occur in *Vibrio cholerae* by modification of a major outer membrane porin OmpU. OmpU is linked with resistance to bile and anionic detergents [84]. The receptor OmpU has previously been demonstrated to be important for bacterial virulence in infections [85–87] and reduction in the receptor expression level leads to reduced virulence and colonization of *V. cholerae* in an animal model. The authors' findings for cases of cholera where the phage ICP2 was present were consistent with dampened virulence benefitting the patient.
Type IV pili	Phages can select for less-virulent bacteria via alteration in type IV pili [88]. Changes in the pili reduce phage attachment, but may also impair other functions, namely those involved in initiating bacterial infection [89]. Thus, trade-offs involved in selection for phage resistance via alteration of pili could result in reduced virulence [90]. Consistent with this idea, Betts *et al.* (2016) showed that exposure to type IV pili phage selected for altered bacterial *pilF* gene [91]. However, it remains to be determined whether this resistance leads to lower virulence in a host. Among the virulence genes identified by Turner *et al.* in *Pseudomonas aeruginosa*, the *pilF* gene was not essential in a cystic fibrosis (CF) sputum medium or CF sputum [41], implying that loss of this factor will not be strongly counterselected. This suggests that phages capable of attaching and initiating an infection via type IV pili are strong candidates for antivirulence therapies in CF patients infected with *P. aeruginosa*, provided that pili facilitate virulence in the CF lung environment (see Figure 1A for a schematic). If this is the case, we predict that bacteria evolving resistance to such phage will lose the ability to fully express type IV pili and exhibit attenuated virulence.
Lipopolysaccharides	Bacterial surface lipopolysaccharides (LPS) are used by a wide range of phages to initiate infection [92–94]. LPS is also associated with virulence as an endotoxin in many Gram-negative bacteria [95–97]. It can stimulate the Toll-like-receptor 4 immune response, which leads to increased cellular damage in infections [98]. Modification of LPS can achieve phage resistance and reductions in virulence [99,100]. Chart and colleagues were among the first to show a link between phage and LPS virulence reduction [99]. Using a range of *Salmonella enteritidis* phages (40 phages in total), they demonstrated that LPS modifications following phage infection lead to reduced bacterial virulence in mice [99]. Santander and Robeson similarly showed that phage resistance induced the loss of the O-polysaccharide in the LPS structure, resulting in reduced virulence of *S. enteritidis* in *Caenorhabditis elegans* [100]. In most Gram-negative bacteria, the LPS is an essential structure, but modifications are common [101]. For example, isolates of *P. aeruginosa* from CF patients commonly have altered LPS structures [97,102], but it is unclear whether this is due to increased fitness within the lung environment, phage predation, or some other interaction with the human host [44,97,102].
Siderophores	Bacteria require iron for metabolism, and many bacterial pathogens are adapted to scavenge for iron when the latter is limiting. These bacteria produce siderophore molecules, which strip iron from other ligands. Some phages use iron-binding receptors (e.g., TonB) on bacterial membranes as attachment sites [103] (see [13,23] for list of phage targets identified as virulence factors). In chronic infections like CF, where available iron is abundant but typically in the Fe^{3+} form, siderophores can chelate the iron to the usable Fe^{2+}; however, over time (during the long-term course of disease) the majority of iron in the lung becomes ferrous, which is readily available without

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Table 1. *Continued*

Target	Description
	siderophores. During persistent infections, siderophores might still contribute to virulence by further disrupting iron homeostasis but provide little to no fitness benefit for the pathogen [104]. Phages that select against these receptors could reduce harm to patients, corresponding to the category where phage-resistant bacteria, having lost or modified TonB, suffer little or no fitness consequences as iron is readily available.

The examples in Table 1 of virulence-associated receptors targeted by specific phages demonstrate that, in principle, phages have the potential to reduce a bacterial pathogen's per capita virulence *in situ*. We suggest that, with appropriate development, phages could be used therapeutically to both reduce bacterial population size and the virulence of surviving phage-resistant cells [35]. However, care should be taken with generalizations, since not all phage-induced changes in the targeted bacterial receptors will affect virulence. Practitioners of phage therapies targeting lipopolysaccharides (LPS) and other surface factors will have to be mindful of whether any virulence attenuation is sufficient to meet therapeutic objectives. Moreover, increases in virulence after phage exposure have also been reported [36]. Key examples can be found in work using the filamentous phage Phi-RSS. Phi-RSS can increase the virulence of its host *Ralstonia solanacearum* (other phages have been shown to reduce virulence [37]). However, the alteration of virulence in these examples appears to be due to changes in the phage life cycle and not from selection against receptors to which the phage attach [38]. Obviously, such phages would be omitted from therapeutic consideration.

Co-opting Bacterial Resistance to Antibiotics

Another approach to improving therapeutic outcomes is for phages to *also* select against antibiotic-resistance mechanisms. For example, *Salmonella enterica* requires a TolC efflux pump for effective resistance against ciprofloxacin [39]. In screening for phages unable to bind to *tolC* knockout strains, Ricci and Piddock isolated a phage that uses this resistance mechanism as its attachment receptor [40]. Such cases where phages target structures providing resistance to antibiotics are of particular interest since they could extend the life of antibiotics that otherwise would have been discarded due to intrinsic or evolved resistance.

Phages that attach and infect via efflux pumps potentially alter the balance between selection for resistance to the phage and resistance to an antibiotic. Chan *et al.* isolated an outer membrane porin-dependent lytic phage of *Pseudomonas aeruginosa*, designated Omko1 [31]. Omko1 binds to the *oprM* subunit of the *mexXY* efflux pump responsible for resistance to a wide range of antibiotics in *P. aeruginosa* (see Figure 1B for a schematic). After selection with this phage, a significant reduction in *in vitro* resistance to a range of antibiotics was described by Chan *et al.* It is yet to be determined if phage-mediated selection can repeatedly produce the same effect *in vivo*. However, Omko1 was recently used successfully in two compassionate release studies, one of a long-term *P. aeruginosa* pericardial infection [32] and the second in a cystic fibrosis (CF) patient with a high abundance of *P. aeruginosa* (B. Chan, personal communication). In the latter case, the post-phage-treated isolates of *P. aeruginosa* showed a significant decrease in antibiotic resistance even though the patient continued to receive antibiotic treatment. This suggests that phage-mediated selection can be strong and sufficiently sustained to produce bacterial evolutionary responses during the course of patient treatment.

Interestingly, the *orpM* gene is nonessential in a range of clinical settings [41]. This might facilitate the evolution of phage resistance through modification of the efflux pump, and at the same time impose a substantial fitness cost on bacteria when they come into contact with antibiotics. Thus, a phage treatment may be a robust therapeutic approach even in the presence of drugs, with the desirable side-effect of selecting against antibiotic resistance. In explicit terms, used appropriately, this phage has the potential to revert existing antibiotic resistance in patients, thus an infection may be rendered treatable with antibiotics again.

Box 2. Alternative Resistance Mechanisms and Indirect Targets

Adaptation of the surface receptor is not the only mechanism by which bacteria can evolve phage resistance. Considerable recent research has investigated how bacteria resist phage attack (for a review see [71]). This has yielded very promising biotechnology applications, such as CRISPR-Cas [72], and multiple other resistance mechanisms, such as restriction modification systems [73]. It will be important to know how these mechanisms contribute to the short-term evolution of resistance to phage selection, and whether they are involved in coevolutionary arms races [49]. What little *in vitro* evidence exists suggests that coevolution gradually shifts from ever-escalating arms race dynamics to fluctuating selection dynamics [74,75], indicating increasing costs of accumulating attack and defence mechanisms. Such costs (of bacterial defence) are of particular interest in the context of phage therapy since they could reduce the ability of the bacterial pathogen to respond to demanding environments, or to actively modify their environments. Recent work on community dynamics of phage therapy employing a virulent lysogenic phage *in vitro* has shown that the form of resistance could change depending on the local bacterial community [76]. We discuss the impacts of phage steering on community dynamics in Box 3.

Given their importance to bacterial fitness, virulence and antibiotic resistance expression are expected to be tightly regulated. Phage steering could apply selection to a wide range of the pathogenic mechanisms by causing down- and/or dysregulation of master regulators. An example is the interaction between the bacteria *Bordetella* spp. and the phage BPP-1. BPP-1 utilizes the pertactin autotransporter (Prn) for secretion of pertactin, which is used in adhesion to epithelial cells [77]. Prn is under the genetic control of the Bvg two-component system [78]. Bvg also controls the expression of multiple virulence determinants, such as the type III secretion system [79]. Thus, if the evolution of resistance to phage BPP-1 involves mutations in the Bvg cassette, this may cause negative pleiotropic effects on the expression of one or more of the many virulence factors under its control. This example illustrates how virulence can be managed indirectly through phage-induced selection on genes that do not directly interact with the phage but control the expression of the phage attachment site.

Considerations of Phage Steering as a Therapeutic

Current approaches to treating bacterial pathogens with antibiotics select for resistance [1,3,42,43]. Employing phages as stand-alone agents or in combination with other antimicrobials shows great promise, but discussion and study have been limited to only a few of the many possible strategies. Our argument is that phages can be employed to do more than their primary objective of reducing bacterial density. Antivirulence phages can select for a range of bacterial phenotypes, resulting in reductions in disease severity on both an individual and public health scale – thereby reducing the need for antibiotic applications and conserving antibiotic sensitivity. Antiresistance phages can drive antibiotic sensitivity in otherwise antibiotic-resistant bacterial pathogens, and when used in combination with antibiotics, result in treatment success. However, given the complexity of phage–bacteria interactions, several considerations are important in developing effective phage steering therapies for clinical use.

First, the availability and phenotypic impact of bacterial surface-factor mutants requires consideration. Not all mutations in surface factors will lead to desirable patient outcomes, and, similarly, not all pathogenic phenotypes involve surface factors. For example, a mutation in a surface factor that reduces phage binding may have little or no effect on virulence. Although LPS endotoxins are virulence factors, structures like lipid A and O antigens within LPS can be changed (e.g., sugar modification [44]), reducing the absorption of phages, but have little effect on disease progression or severity [45].

Second, the phages need to impose and maintain selection on the target surface factor for steering to be most effective. Maintaining this selection might be disrupted if the phage and bacteria coevolve [46–48]; thus, selection on a target surface factor may change through time. Moreover, resistance to phage can be generated by a number of mechanisms other than receptor modification. For example, under certain conditions receptor modification is predicted to maintain phages in the presence of active CRISPR-Cas immunity (Box 2) [49]. Even less appreciated is how bacterial virulence and antibiotic resistance are impacted over short evolutionary timescales – that is, the duration of treatment.

Bacterial escape mutants, such as those that form biofilms, may maintain unmodified surface factors and still contribute to virulence [50]. This could be compounded by phages switching surface factors, that is, evolving to use novel receptors [51]. This suggests more generally that, for steering to be successful, the range of resistance mechanisms employed by the bacterium need to be understood in order to minimize the risk of unanticipated treatment failures.

A third consideration is the impact that phage steering has on the fitness of the selected, resistant bacterial pathogen. Changes in receptors or structures with essential functions (such as LPS in most Gram-negative bacteria) are unlikely to involve gene deletions, and those that do will likely have higher fitness costs when compared to mutants that make modifications to motifs or reduce the expression of the receptor [52]. This difference in fitness would lead to the mutants with slight modifications outcompeting the full gene deletion strains. In contrast, receptors with nonessential functions may be lost or downregulated due to phage selection without lowering bacterial fitness, and in fact, as seen in Tn-seq experimentation [41,53], could *increase* bacterial fitness, yet still have the desired outcome of lowering either virulence or antibiotic resistance [54]. Box 3 provides an overview of what makes a 'good' phage target.

Finally, as suggested above, phage steering is unlikely to be used as a stand-alone antimicrobial therapy. Steering phages work by killing sensitive bacteria and selecting for resistant strains that are otherwise vulnerable. To be a viable strategy, either the resistant strains need to be compromised so as to be avirulent, or need to be eliminated directly by additional treatments and/or by the immune system. These objectives are most readily achieved through combinations with other antimicrobial agents in the form of phage–phage or phage–antibiotic cocktails. In this context, phage steering plays the role of both primary treatment (bacterial killing) and an adjuvant (compromised survivors) to more inclusive therapies.

Concluding Remarks

Ecological interactions govern life and how it adapts and responds to changing environments. This applies to classic ecosystems as well as to disease ecosystems, and it would be short-sighted to not use this knowledge to its full potential [33]. Our understanding of phage ecology in an infection context is still in its infancy [55,56]. The classic view of infection as a simple interaction between the host and the pathogen is being replaced with an appreciation that there exists a complex community ecology [33,57,58]. Some pathogens actively alter their environments to foster growth, reproduction,

Box 3. What Is a Good Target for Phage Steering?

Depending on the environment, a number of outer membrane structures may actively contribute to virulence or antibiotic resistance [9,21,22]. For example, work by Turner *et al.* identified several virulence factors in *P. aeruginosa* that are potentially directly costly to the pathogen in a clinically relevant environment (i.e., the mutants were enriched *in vitro*) [41,53]. These genes would make ideal candidates for phage steering due to the apparent lack of counter-selection to maintain functionality. It is thus in the bacteria's 'best interest' to not express these genes if they are not under positive selection. If this is bolstered by the addition of phage that apply selection on these same genes, then resistant bacteria are expected to reduce the associated phenotype (i.e., virulence or antibiotic resistance) [23,31,80]. Given encouraging findings that certain phages may select for antibiotic sensitivity [31,80], future research should consider longer term effects where there is a coevolutionary response between the antagonists [48].

Chronic infections are often polymicrobial in nature and are receiving increasing attention [81]. Phages have been shown to affect the types of mutation that occur when bacteria compete in complex communities [76]. Surface factors that mediate interactions between the different species or with the environment are potential targets for phage steering. Given the complexity of even the simplest *in situ* microbial communities, while it might be possible to reduce pathogen burden by selecting against another species in the microbiome that facilitates the pathogen [82], in practice we currently lack the required understanding of complex community dynamics to make this a viable therapeutic option.

and colonization to the detriment of the host [59,60]. Understanding how these interactions can be disrupted will open the way for novel and effective therapeutics [33] (see also Outstanding Questions).

Phages will likely not completely supplant antibiotics. There is increasing effort in the search for novel antimicrobials [61], especially how antibiotic combinations increase both the efficacy of treatments and minimize the emergence and spread of antibiotic resistance. Nevertheless, phages augment the arsenal available to successfully treat certain bacterial pathogens [33]. Even if phages are not sufficient as a stand-alone alternative treatment to antibiotics, their ability to compromise bacterial virulence or alter bacterial vulnerability to antibiotics merits attention. If used in an evolutionarily rational way, phages can both improve treatment success and extend the useful lifespans of antibiotics [15]. Thus, counterintuitively, the selection for resistance may actually form an important part of the antimicrobial arsenal.

Outstanding Questions

What is the range of bacterial surface factors that steering phage can target?

To what extent is phage steering evolution-proof; is it robust to compensatory mutations?

Can phage steering work on a public health scale?

How does phage steering drive coevolution?

What is the impact of steering phage on integrated lysogenic phage?

How well would phage steering work under constant antibiotic pressure?

Can these principles be applied to other selective agents, such as porins or lysins?

How does phage target choice evolve under therapeutic conditions?

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