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# Mitigation of evolved bacterial resistance to phage therapy

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## Abstract

The ease with which bacteria can evolve resistance to phages is a key consideration for development of phage therapy. Here, we review recent work on the different evolutionary and ecological approaches to mitigate the problem. The approaches are broadly categorised into two areas: *Minimising* evolved phage resistance; and *Directing* phage-resistance evolution towards therapeutically beneficial outcomes.

## Introduction

Phage therapy uses bacteriophages (phages; bacteria-specific viruses) to treat bacterial infections, offering a promising arsenal to combat the alarming global rise of pathogens resistant to multiple antibiotic drugs. Each year, an increasing number of patients are treated with these potentially useful viruses, in countries that historically have invested in this practice (e.g., Georgia, Poland) and in countries where it is relatively new [1]. Currently, the success or failure of treatments mostly lies on the timing of this last resort care of severely ill patients, and the availability of phages targeting the specific bacteria. However, it is increasingly reported that bacteria can evolve resistance to phages during therapy, a major concern that potentially jeopardises the wider use and future development of more sophisticated phage therapy [2–4]. Fortunately, the societal-level management of phage resistance will be aided by lessons learnt from our poor track record of antibiotic resistance management, and many have suggested that horizontal gene transfer of generalised phage resistance (a major issue for antibiotic resistance spread) is probably unlikely [5]. Here, we highlight recent research exploring how to mitigate the within-patient evolution of bacterial resistance to phages during the course of therapy. We organise the different strategies for overcoming bacterial resistance to phages in two complementary approaches: Minimizing evolved phage resistance, and Directing phage-resistance evolution in bacteria (Figure 1).

## Minimizing phage-resistance evolution

A simple but effective strategy is to maximise the rate of phage killing, with the aim of minimising the population size of the target bacteria. Small population sizes limit the genetic backgrounds on which the most-beneficial phage-resistance mutations will spontaneously arise, hence reducing the probability that such changes will reach high frequencies or spread to fixation in the target

bacteria. Under these circumstances, even if some phage-resistance evolution occurs, other factors such as the action of the patient immune system or that of co-administered antibiotics could help clear the reduced load of surviving bacteria. This reduction of potentially phage-resistant bacteria can be achieved via several approaches.

An elevated phage dose and the use of highly efficient lytic phages can neatly reduce bacteria numbers. For example, the low phage doses ( $10^2$  pfu/mL) used in the PhagoBurn clinical trial concerning burn wounds infected by *Pseudomonas aeruginosa*, are proposed as one factor explaining why phage-resistant bacteria were isolated from patients and phage treatment clinically failed [6]. Large burst sizes, short latent periods and high adsorption rates are among the phage traits contributing to their high virulence (relative ability to lyse host bacterial cells), classically observed in lab studies where phages quickly overwhelm their host bacteria; but a cautionary note is that *in vivo* phage-bacteria interactions may differ from *in vitro* observations [3,7,8].

Wide host range and high efficiency of plating (EOP) in therapeutic phages are desirable traits for depleting as many clinical variants of bacteria as possible, especially when targeting genotypically diverse bacterial taxa. This is also commonly attempted by employing phage cocktails. Phage cocktails are of long-standing interest in phage therapy and offer a major strategy to reduce resistance evolution in bacteria. Multiple phages that target different receptors in bacteria should diversify which counter-defenses must be used by bacteria, likely diminishing or preventing the evolution of phage-resistance [9,10]. For instance, a combination of 5 phages based on maximising host range of *Mycobacterium tuberculosis* strains resulted in reduced persistence and reduced phage-resistance evolution, thus enhancing survival of the *Galleria mellonella* insect model [11]. Another study supports the intuition that high phage functional diversity in cocktails maximizes phage efficacy, after *in vitro* testing 827 unique phage combinations of 1 to 12 phages against *P. aeruginosa* [12]. However, it is important to emphasise that phage cocktails are not always synergistic: the net *in vitro* efficacy of phage cocktails against *Escherichia coli* O157:H7 were sometimes synergistic, but also neutral or antagonistic [13].

Phage-antibiotic synergism (PAS) has been proven to limit selection for phage-resistance mutations, although the underlying mechanisms often remain unclear. The use of temperate phages coupled with antibiotics is a recent approach. Here, the synergy relies on a phenomenon that is different from the classic PAS involving virulent phages, and is particularly efficient [14]. The eradication of lysogens by antibiotic lysis induction is the most likely explanation for the pronounced decrease in bacterial population size caused by temperate PAS (tPAS). This method is proposed for temperate phages with known integration mechanisms that do not pose a risk for the transduction of virulence traits between bacterial cells [14]. Combining antibiotics with phage-derived enzymes such as lysins, holins or depolymerases, can also boost the antimicrobial effect and significantly constrain resistance [15]. A depolymerase specific to *Acinetobacter baumannii* in addition to antibiotics, decreased bacterial population sizes, treatment-resistance frequency and biofilm formation *in vitro* [16]. Resistance frequency in bacteria against these phage-derived proteins had never previously been recorded [17]. However, as with phage cocktails, antibiotic-phage combinations are not necessarily synergistic.

For example, recent work shows antagonistic effects of some phage and antibiotic paired combinations, such as aminoglycosides inhibiting phage infection by blocking an early step of the phage infection cycle [18,19]

The use of therapeutic temperate phages has the potential to be effective in the absence of antibiotics. Recently discovered phage-encoded molecules can also trigger the lytic cycles of prophages. The “Rap $\phi$ -Phr $\phi$ ” peptide-based communication systems have been used to control the lysis-lysogeny decision in *Bacillus*-infecting phages [20]. New molecular details and evolutionary predictions on these phage-bacteria density-dependent systems anticipate their potential therapeutic use when inducing host lysis by resident prophages [20–22]. Additionally, as prophages do not automatically kill cells, and even temporarily protect host cells owing to superinfection-exclusion mechanisms, these “programmed host death” mechanisms will likely impose relatively weak selection for bacterial resistance. Further experiments could test whether the frequency of bacterial resistance to these ‘Trojan-horse’-like prophages is indeed as low as expected.

Experimental evolution of phages is an important strategy to improve many of the above-mentioned phage capabilities. So called “trained” phages have been used in human treatments conducted at the George Eliava Institute of Bacteriophage in Tbilisi, Georgia for decades, and their properties are being extensively re-analysed in academic and clinical research labs [23]. Phages recovered after *in vitro* co-evolution with their bacterial hosts rapidly improve the capacity to infect resistant co-evolved bacterial clones and further reduce the growth of host bacteria, compared to ancestral (wild-type) phages [24,25]. A recent study found that trained phages which evolved to recognise two bacterial receptors instead of one, were more-efficient at inhibiting bacterial growth and avoiding or delaying significant evolution of phage resistance [25]. In this and other analyses of phage training, variation generated via phage-phage recombination apparently accelerated evolutionary changes [25,26].

Trade-offs are an important consideration in trained phages. For example, the evolution of phages that could infect resistant genotypes of the fish pathogen *Flavobacterium columnare*, resulted in correlated reductions in phage adsorption rates to host cells [27]. However, it also may be possible to experimentally evolve populations of phage that overcome trade-offs important for therapy. Populations of phage alternately evolved on ancestral and co-evolving populations of *Pseudomonas fluorescens in vitro* overcame a commonly reported trade-off between broad host range and poor growth rate, although this may itself have traded off against other unknown phage traits [28].

Trained phages could reduce the number of viruses needed for a cocktail, assuring efficiency of such treatment strategies while simplifying their production and reducing selection for multi-phage resistant bacteria [3]. A 5-phage cocktail containing one trained phage, delayed the evolution of phage-resistant *P. aeruginosa in vitro* in the short-term, which required mutations in multiple loci [29]. A cocktail of 3 trained phages inhibited the evolution of resistance in *Staphylococcus aureus* after 20 co-evolutionary passages *in vitro* [30]. Sequential use of different cocktails of trained phages depending on

bacterial susceptibility and evolution may be an effective tool to minimize bacterial resistance.

### Directing phage-resistance evolution

If phage resistance is unavoidable, a second option is to manage it through directed evolution. In other words, to use a specific phage treatment that selects for a certain type or frequency of mutation [31]. For instance, it has been repeatedly proven that phage resistance can impose costs for other bacterial traits that are therapeutically beneficial. Decreased virulence, antibiotic re-sensitisation, and host-colonization deficiencies in target bacteria are examples of these biomedically useful fitness trade-offs.

After sequencing of bacterial mutants resistant to 19 enterococcus-specific phages, a study showed that mutations in the enterococcal polysaccharide antigen (*epa*) gene cluster were the main drivers of phage resistance [32]. *E. faecalis epa* mutants harboured altered cell-surface properties and were deficient in intestinal colonization and transmission in mice, and less resistant to cell-wall-targeting antibiotics [32]. Likewise, modifications in cell-wall teichoic acids of Gram-positive *Listeria monocytogenes* bacteria after evolved phage resistance, were associated with decreased virulence of this pathogen in human cells [33]. Motility (pili and flagella) systems and capsules are frequently modified in bacteria in order to achieve phage resistance, and are often associated with reduced bacterial virulence [34,35]. Environmental conditions can favour these reduced-virulence resistance mechanisms over alternative solutions. For example, the costs of resistance associated with type-IV pilus modification compared with CRISPR-mediated resistance (which has less impact on virulence), as a function of competition and the phage encounter rate, both of which are affected by the presence of competitors [36,37].

Phage resistance can increase susceptibility to other antimicrobials. In a phage that used two host receptors related to antibiotic resistance, the lipopolysaccharide (LPS) and an efflux pump protein, phage-resistant mutants were sensitized against antibiotics in most cases [39]. Yet, a few phage-resistant mutations potentiated antibiotic resistance in *E. coli in vitro*, demonstrating that multiple pleiotropic interactions are possible and should be investigated [38]. Conversely, antibiotic resistance can sensitize for phage lysis, as in several clinical *E. coli* strains tested *in vitro*. These bacteria overexpressed AmpC, a  $\beta$ -lactamase for ampicillin resistance, and displayed higher levels of OmpA, a receptor used by some phages, resulting in increased lysis [39]. Resistance to phages can also increase susceptibility to other phage. Some phage-encoded protein can inhibit bacterial quorum sensing, with phage-resistant mutants displaying altered growth and quorum sensing abilities, rendering them more susceptible to other phages (quorum sensing regulates many phage defenses) and potentially less virulent as pathogens [40].

The genetic background of the bacterial strains or species implicated in infections is a crucial element to understand the potential and direction of phage-resistance mutations. After studying the interaction of a group of genetically diverse phages with different *P. aeruginosa* strains, it was shown

that the genetic response of bacteria to infection varied between bacterial genotypes, at least *in vitro* [10]. Similarly, parallel but also differential susceptibility in phage resistance was identified using two model *E. coli* strains and 14 genetically different phages [41].

In another recent study, a pipeline was developed to analyse 20,000 bacterial genomes to infer presence or absence of all known phage-defense systems [42]. 105 systems were identified, and more than two defense systems were found to be encoded by 75% of prokaryotes. In general, anti-phage system distribution in bacteria is varied and impacted by genomic location and phylogeny [42]. It is still unclear how contingent is evolution based on genetic background, and how well we can predict evolution by natural selection in phages and their host bacteria. Encouragingly, *in vitro* and *in vivo* resistance evolution that led to reduced virulence and dynamics were broadly similar in both cases, raising the possibility of predicting the evolutionary dynamics between specific isolates of bacteria and their phage from simple *in vitro* experiments [43].

### Conclusion

Significant progress has been made in the development and application of phages to both minimise the evolution of resistance in target bacteria, and/or direct it in a way that reduces infection severity. This has been achieved by careful choice of wild-type phages, experimentally evolving them to improve therapeutic efficacy, and combining them in specific cocktails. The ever-increasing biodiversity of novel discovered phages, and our growing understanding of bacteria-phage ecological, evolutionary and molecular interactions, should continue to open many possibilities for successfully tackling the complexities of bacterial infections. Additionally, elucidating complexities of phage-bacteria interactions in the contexts of patient microbial communities and immunobiology, suggests that phage therapy may necessitate precise approaches for directing the evolution of phage resistance in target bacteria.

### Acknowledgements

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### Figure Caption

**Figure 1.** Two complementary strategies for overcoming bacterial resistance to phages represented with an example: Minimizing evolved phage resistance with a highly efficient phage cocktail (left), and Directing phage-resistance evolution by selecting impaired flagellar motility in bacteria (right).

