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Revisiting phage therapy: new applications for old resources

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The success of phage therapy is dependent on the development of strategies able to overcome the limitations of bacteriophages as therapeutic agents, the creation of an adequate regulatory framework, the implementation of safety protocols, and acceptance by the general public. Many approaches have been proposed to circumvent phages' intrinsic limitations but none have proved to be completely satisfactory. In this review we present the major hurdles of phage therapy and the solutions proposed to circumvent them. A thorough discussion of the advantages and drawbacks of these solutions is provided and special attention is given to the genetic modification of phages as an achievable strategy to shape bacteriophages to exhibit desirable biological properties.

Phage therapy

Bacteriophages (phages) were independently discovered at the beginning of the 20th century by Frederick Twort (1915) and Félix D'Hérelle (1917). Since then, these bacteria-infecting viruses of high specificity have significantly contributed to the evolution of many fields of science, in particular the areas of molecular biology and bacterial genetics [1,2]. They have also been pursued as antimicrobial agents, but lack of knowledge of phage biology and the advent of antibiotics in the 1940s resulted in the disregard of this application. However, the alarming rise of multidrug-resistant bacteria and the consequent need for antibiotic alternatives has renewed interest in this application in the West. Despite the numerous successful therapeutic outcomes reported in Eastern European countries, phage therapy remains disregarded in the West. This is mainly due to the lack of a specific regulatory framework that meets the requirements of a flexible and patient-tailored model of phage therapy [3] and the demand for large-scale *in vivo* trials that provide efficacy and safety evaluation of a standardized phage product [4]. Due to these hurdles and the difficulty to obtain intellectual property rights for therapeutic phage products [5], large pharmaceutical companies remain reluctant to invest in phage therapy. In April 2014, the European Parliament proposed a motion for the resolution of antibiotic resistance asking member states of the Council of Europe to prioritize the development of phage therapy as a complement to antibiotic therapy [6]. This was an important sign of good will, but the impact is unknown and will probably take some time to have an effect.

The current limitations of phages, whether in terms of biological properties or patentability, may be circumvented by synthetic biology approaches. In this review we discuss the possibility of applying genetic manipulation of bacteriophage genomes to shape these viruses to exhibit desirable biological properties for therapeutic applications. As a patentable product, we believe this strategy would be appealing to the pharmaceutical industry and would attract potential investors to the field.

Bacteriophage properties, advantages, and limitations

Phages have many advantageous properties over antibiotics as antimicrobial agents; however, some of those properties can also be limiting in certain applications, as detailed below.

The main characteristic of phages is their high specificity of infection, typically recognizing a limited range of bacterial strains. This reduces the damage caused to the normal microbial community of the host but it also requires identification of the specific target pathogen and the selection of an effective phage [7,8], which may delay the treatment. Also, as the propagation of phages depends on their host, they replicate only at the site of infection, are self-limiting and self-dosing, and do not persist when their specific bacterial pathogen becomes absent.

Another advantage of phages is their general lower propensity to induce resistance and the absence of cross-resistance to antibiotics [9,10]. This makes phages an effective solution against multidrug-resistant bacteria and biofilms [11]. However, the development of phage-resistant bacteria may occur and some resistance mechanisms have already been identified. These include blocking of phage adsorption due to loss or mutation of the bacterial receptor [7,12] as well as horizontal acquisition of a restriction-modification system or development of adaptive immunity by interfering clustered regularly interspaced short palindromic repeats (CRISPR) sequences, both resulting in degradation of the injected phage DNA [12,13].

Another concern is that phages may carry antibiotic-resistance genes or other bacterial virulence factors, which can be transferred to the bacteria through generalized transduction [14,15]. As this is more common in phages able to infect bacteria lysogenically, only obligatory-lytic, non-transducing phages are used as therapeutics and even these should be propagated on hosts lacking virulence

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genes [16]. However, lytic phages can also be problematic, as the rapid lysis of a large number of bacteria *in vivo* may lead to the release of endotoxins and superantigens that may induce an inflammatory response, potentially causing serious side effects [7,17].

There is a conceivable concern about phage immunogenicity and consequent *in vivo* efficacy. Phages are perceived by the immune system as invaders and can be rapidly removed from systemic circulation, making it hard to sustain an effective phage concentration [17]. A recent study by Łusiak-Szelachowska *et al.* demonstrated induction of antiphage antibodies after phage therapy, with the activity being dependent on the route of administration and phage type [18]. Nevertheless, the authors considered that the detection of antiphage activity during and after phage therapy does not exclude a favorable result of the treatment [18].

Finally, from a development point of view, phages have the advantages of rapid isolation, lower development costs than antibiotics, and versatility of formulation and application [11,16]. However, although strictly lytic phages are easily obtained for major bacterial pathogens (e.g., *Escherichia coli*, *Salmonella*, *Campylobacter*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), their isolation for certain bacterial species has proven to be difficult (e.g., *Mycobacterium tuberculosis*, *Clostridium difficile*) [17,19,20].

The few hundred phages currently described in public datasets represent a 'drop in the ocean' of the estimated 10^{31} virions present in nature. Such diversity confers them potential as one of the most promising therapeutic strategies identified to date. It is therefore expected that, with the identification of more phages, the application of phage therapy will become successful.

Strategies to overcome phage limitations

Many strategies are being pursued to overcome the limitations of phages as therapeutic agents. Among them, phage cocktails were the first to be considered. Using a cocktail of phage types of different but complementary features, the limited host range of a single phage may be circumvented, also allowing the use of presumptive (before pathogen identification) phage therapy [16,17,21]. However, this may result in a greater impact on non-targeted bacteria and higher costs [16]. Another advantage of phage cocktails is that having different types of phage infecting the same species and strains reduces the probability of emergence of phage-resistant bacteria [21,22]. Nevertheless, resistance to phage cocktails may eventually emerge, as was demonstrated by Tanji *et al.* [23].

A more recent strategy explores the antimicrobial synergy between phages and antibiotics [24,25]. Studies have shown that sublethal concentrations of certain antibiotics (typically cell division inhibitors) increase the biosynthetic capacity of bacteria, which the phage explores to increase its own production. This hastens cell lysis and ultimately allows the phages to spread more quickly [24,25].

Another approach uses phage gene products instead of the whole virion; for example, endolysins, which are considered a promising alternative in several applications [26–28]. Their use eliminates the risk of phages imparting toxic properties to bacteria [29] and reduces the risk of development of resistance [27,28,30]. However, like phages, endolysins that target Gram-positive pathogens are highly specific, whereas endolysins for Gram-negative bacteria, in their native state, are nonspecific, with the related advantages and disadvantages, and have an inherent limited application due to the impermeable outer membrane of Gram-negative bacteria. Moreover, endolysins for Gram-positive bacteria have been shown to stimulate a fast immune response resulting in a short half-life [31].

Also relevant is the use of drug-delivery technologies, such as polymer-based coatings, to enhance the systemic delivery of phages and reduce their inactivation and clearance by the immune system [32]. This enhancement was observed after the chemical modification of phages by conjugation of monomethoxy polyethylene glycol (mPEG) to its proteins. However, it also resulted in the loss of phage infectivity, proportionally to the degree of modification [32].

The strategies presented have indeed shown positive outcomes. However, they aim to circumvent specific phage limitations instead of eliminating or correcting their detrimental properties. To specifically address this point, we propose the genetic manipulation of phage genomes as a way to shape bacteriophages into safe and efficient biocontrol agents.

The dawn of a new era: bacteriophage genomic engineering

A range of genetic tools that have been used to study phage biology and function and to shape phages' biological properties toward the improvement of their antimicrobial effect.

Until recently, efficient targeted modification of phage genomes has been hindered by a lack of broadly applicable techniques that can be used for both temperate and virulent phages. Fortunately, a new *in vivo* technology was developed to introduce genetic changes in bacterial genomes – recombineering – which has been adapted for the efficient manipulation of temperate and lytic phages in bacteriophage recombineering of electroporated DNA (BRED). This technique was developed by Marinelli *et al.* for *Mycobacterium* phages [33] but is adaptable to other phages. For example, it has been applied by others for the modification of *E. coli* phages [34,35]. BRED uses bacterial overexpression of plasmid-encoded recombination genes to enhance the frequency of homologous recombination between phage DNA and the targeted DNA substrate [34,35]. The recombination systems used for BRED are typically those encoded by phage lambda and Rac prophage. The lambda Red system comprises three proteins: Exo, Beta, and Gam. Exo degrades one strand of double-stranded DNA, generating a single-stranded substrate that is annealed to the chromosomal target by the DNA-pairing enzyme Beta. Gam prevents the degradation of the double-stranded DNA by inhibiting the *E. coli* RecBCD and SbcD enzymes [36,37]. The Rac prophage system comprises RecE and RecT, which are functionally equivalent to lambda Exo and Beta, respectively [38,39]. Similar recombination systems exist for other