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The next generation of bacteriophage therapy

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Bacteriophage therapy for bacterial infections is a concept with an extensive but controversial history. There has been a recent resurgence of interest into bacteriophages owing to the increasing incidence of antibiotic resistance and virulent bacterial pathogens. Despite these efforts, bacteriophage therapy remains an underutilized option in Western medicine due to challenges such as regulation, limited host range, bacterial resistance to phages, manufacturing, side effects of bacterial lysis, and delivery. Recent advances in biotechnology, bacterial diagnostics, macromolecule delivery, and synthetic biology may help to overcome these technical hurdles. These research efforts must be coupled with practical and rigorous approaches at academic, commercial, and regulatory levels in order to successfully advance bacteriophage therapy into clinical settings.

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Introduction

Antibiotic-resistant pathogens constitute a worsening global health problem exacerbated by interconnected travel, antibiotic overuse, horizontal gene transfer, and bacterial evolution. New classes of antimicrobials are needed to treat these pathogens but the drug development pipeline is dry [1]. As a result, there has been a renewed interest in alternative antimicrobial treatments, including bacteriophages, antimicrobial peptides and proteins, and nanoparticles. The discovery of bacteriophage particles that seemed to 'eat bacteria' is generally attributed to Twort [2] and d'Herelle [3] in the early 20th century. The therapeutic potential of 'phages' – members of the kingdom of viruses and obligate predators of bacteria – was recognized soon thereafter and applied for several decades before the discovery and widespread adoption of antibiotics [4]. A range of commercial products were distributed by companies in France (Laboratoire de Bacteriophage), Germany (Antipol), the UK (Medico-Biological Laboratories), and the US (Eli-Lilly, Swan-Myers, and Squibb) [5]. However, mixed therapeutic results, poor understanding of phage biology, and the advent of broad-spectrum antibiotics led to the decline of phage therapy in the Western world [4,6]. In the meantime, phage therapy continued to be studied and used in Eastern Europe and the Soviet Union.

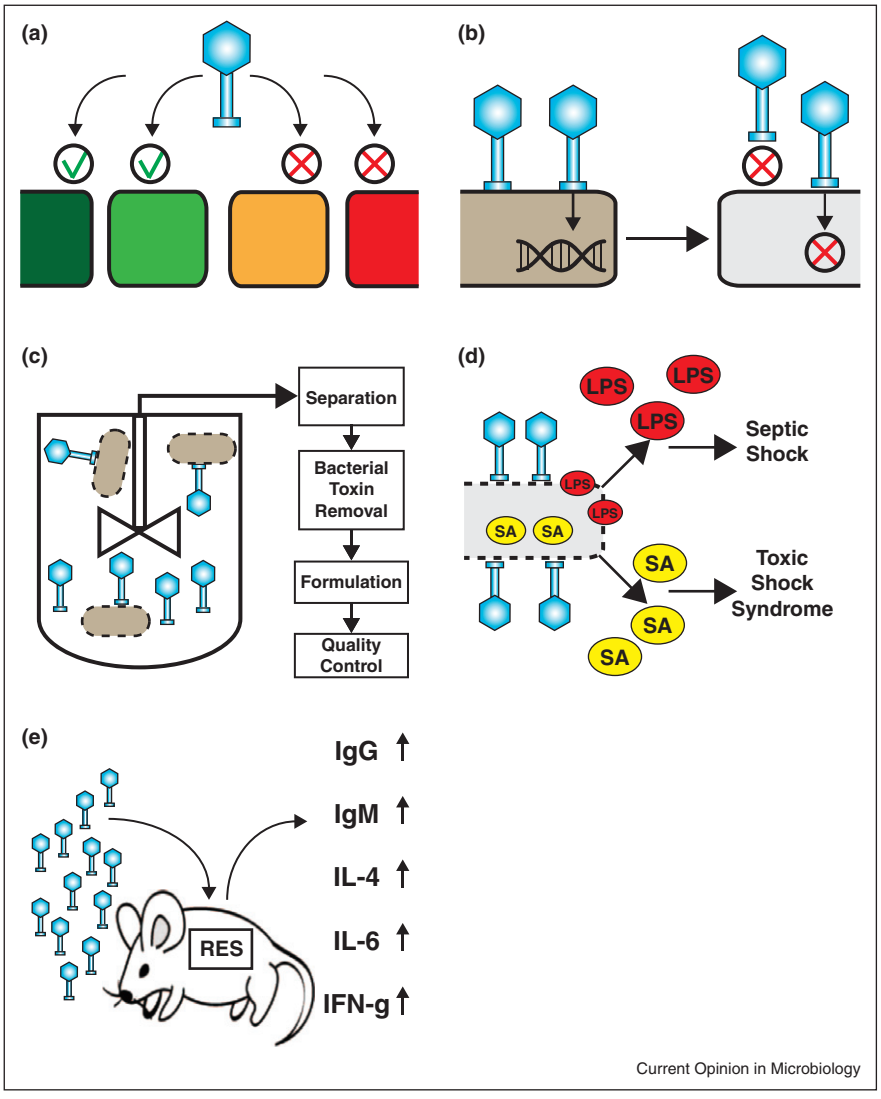
The reintroduction of bacteriophage therapy into modern-day Western medicine faces numerous hurdles. These challenges include skepticism about the rigor of prior phage therapy studies, strict regulatory constraints placed on new clinical therapeutics such as phages, limited phage host ranges, the evolution of bacterial resistance to phages, manufacturing challenges, systemic side effects of phage therapy, and delivery (Figure 1) [7]. Here, we shall present an overview of the literature to illustrate these hurdles and the approaches that have been used to address them (Figure 2). Furthermore, we will highlight recent research advances that confer additional benefits to phage therapy and discuss breakthroughs that are needed to achieve broad clinical applicability.

Non-therapeutic applications of bacteriophages

The clinical use of phage therapy is faced with long product development and approval timelines in Western regulatory frameworks. As a result, many companies and researchers have pursued food safety, agricultural, industrial, and clinical diagnostic applications instead. Several companies have successfully developed phage-based products with EPA, USDA, and FDA approval. Such products have established a favorable regulatory precedent in which individual components of phage cocktails can be tailored towards bacterial targets. Products targeted at *Listeria monocytogenes* represent one of the first examples of phage cocktails to obtain Generally Recognized As Safe (GRAS) status from the FDA. These products are designed to be used as sterilizing agents for processed foods (ListShield™ and LISTEX™ P100). Another approved product treats crop pathogens such as *Xanthomonas campestris pv. vesicatoria* and *Pseudomonas syringae pv. tomato* (Omnilytics' Agriphage™). Further products are in development against other bacterial pathogens, including *Escherichia coli* strains (O157:H7) and *Salmonella enterica* [8].

In addition to being used to kill bacteria, phages have defined host specificities that can be exploited for detecting and typing bacterial infections. For example,

Figure 1



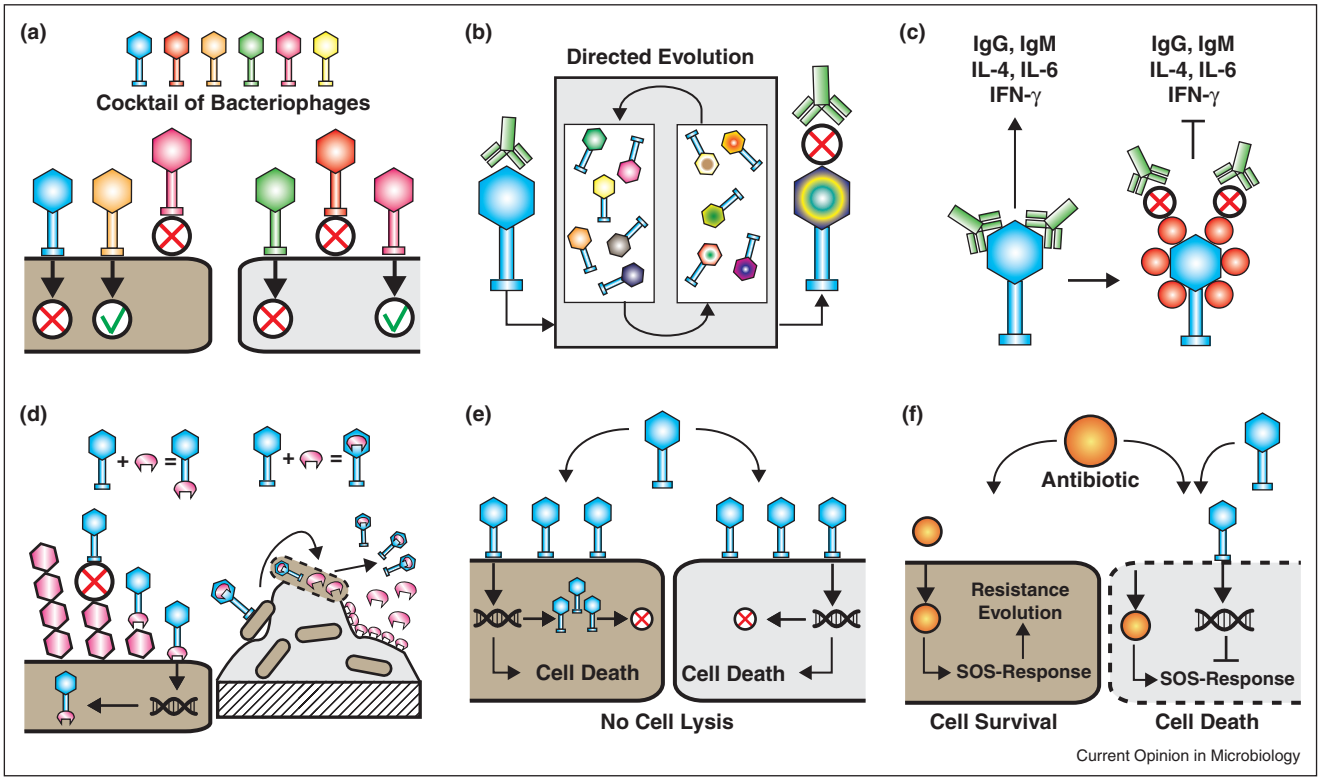
An overview of technical hurdles facing clinical phage therapy. **(a)** Individual phages are narrow-spectrum antimicrobial agents and thus cannot be broadly effective against a wide range of bacterial targets. **(b)** Bacteria can evolve resistance to phages through a variety of extracellular and intracellular mechanisms [42•]. **(c)** The bacteriophage manufacturing process requires multiple steps, including production, purification, formulation, and quality control. This process is complicated by the presence of endotoxins and other cellular toxins released during cell lysis and by the need for multi-phage cocktails. **(d)** The release of cellular toxins during cell lysis *in vivo* can potentially lead to systemic inflammatory responses and increased morbidity and mortality. **(e)** Bacteriophage delivery can be hampered by the inactivation and removal of phages by the host immune system, including the reticuloendothelial system (RES).

Microphage, Inc. recently received FDA approval for a blood culture test that uses phage infection to detect methicillin-resistant *S. aureus* [9]. Phage typing methods can involve the expression of luciferase genes delivered by modified phages, fluorescently labeled phages, and traditional plaque formation [10]. In addition to being useful as general diagnostics, these products can help lay the groundwork for effective phage therapy, which requires the rapid and accurate identification of bacterial targets and the determination of their susceptibility to specific phages [11–13].

Clinical trials for bacteriophage therapy

The aforementioned non-therapeutic products stand in contrast to phage therapeutics, for which there are no current approvals in the Western world. In order to achieve clinical use, rigorous trials to validate safety and efficacy need to be established. Animal studies have generally supported the utility and safety of bacteriophage therapy against bacterial pathogens, such as *Pseudomonas aeruginosa* [14,15], *Staphylococcus aureus* [16], vancomycin-resistant *Enterococcus faecium* [17], *Clostridium difficile* [18], and *Klebsiella pneumoniae* [19]. Such

Figure 2



An overview of potential solutions for overcoming the technical hurdles facing clinical phage therapy. **(a)** Traditionally, phage cocktails have been the major way of addressing limited phage host ranges and the evolution of phage-resistant cells. The complexity of phage cocktails is limited by issues such as regulation and manufacturing. **(b)** *In vitro* and/or *in vivo* evolution can produce phages with enhanced properties such as decreased clearance by the host immune system [53]. **(c)** Drug-delivery technologies, such as polymer-based coatings, can enhance systemic phage delivery and reduce phage inactivation and clearance [55]. **(d)** Enzymatic bacteriophages can be engineered to degrade barriers to phage adsorption (left) [40] and disrupt the structure of bacterial biofilms (right) [57]. **(e)** Non-lytic and/or non-replicative phages can reduce the release of bacterial endotoxins while continuing to have antimicrobial efficacy [50,51]. **(f)** Bacteriophages can be engineered to target intracellular defense pathways, which protect bacteria against antibiotics [61••]. The resulting antibiotic-adjuvant phages can potentiate antibiotic killing against wild-type and antibiotic-resistant bacteria. Moreover, the co-application of phages and antibiotics can reduce the evolution of resistance to both modalities.

research has culminated in preclinical and veterinary trials, such as the application of a phage cocktail to treat *P. aeruginosa* otitis in dogs. This trial successfully met its primary safety endpoint and its secondary short-term efficacy endpoint [20•].

The bulk of experience with human phage therapy has been concentrated in Eastern Europe and the Soviet Union. Western skepticism of phage therapy is partly owing to the fact that many of the reports describing phage therapy from these countries are not accessible in English and those that are accessible often do not describe well-controlled studies [4,21]. Recently, more rigorous work has begun to be published, including ones focused on feasibility and efficacy [22–26], the cost of bacteriophage therapy compared to antibiotic treatment [27], the bioavailability and pharmacokinetics of bacteriophage treatment [28,29] and treatment of complicated infections in humans [30]. Nonetheless, on their own, such studies are not sufficient to meet the regulatory demands of countries with a strict emphasis on evidence-based medicine.

In the Western world, increasing interest in phage therapy has led to several safety and efficacy trials in humans [31–34]. These clinical trials have used between one and eight bacteriophages at levels between 10^5 to 3×10^9 plaque-forming-units, which translates into doses in the low nanogram to microgram range [32]. Human safety trials have included Staphylococcal phage lysate (SPL) and cocktails targeted against *P. aeruginosa*, *Staphylococcus aureus*, and *E. coli* [21,32]. For example, *S. aureus* phages in SPL were administered intranasally, topically, orally, subcutaneously and intravenously with only minor side effects [35]. In fact, SPL was licensed for use in humans until the 1990s, when its production for clinical applications was halted [21]. A formal safety study conducted in Switzerland demonstrated no safety concerns when bacteriophages targeting *E. coli* were orally administered to human volunteers [36]. Similarly, an

FDA-approved Phase I physician-led trial was completed at a wound care center in Lubbock, Texas using a mixture of bacteriophages targeting *P. aeruginosa*, *S. aureus* and *E. coli* [37••]. This study revealed no increase in adverse reactions associated with application of phage cocktails.

These studies paved the way for the first fully regulated, placebo-controlled, double-blinded, randomized Phase II clinical trial for phage therapy in the Western world, which was recently completed by Biocontrol Limited [38••]. Wright *et al.* reported improved outcomes and decreased *Pseudomonas* loads in adult patients with chronic otitis externa treated topically with bacteriophages [38••]. This study had a design inclusion criteria which mandated that bacterial infections had to be predominantly composed of *P. aeruginosa* that could be targeted by at least one of the phages in the cocktail [38••]. This study supports the hypothesis that successful bacteriophage therapy can be achieved if susceptible host bacteria are present at the site of application and emphasizes the importance of accurately identifying bacterial infections before phage treatment. This pioneering work will set a positive precedent for phage therapy in Western medicine if successful Phase III trials can be conducted. These results of such trials will also encourage continued research into novel strategies that can address the technical hurdles of phage therapy (Figures 1 and 2).

The challenge of bacteriophage host ranges

Each bacteriophage has a limited spectrum of infectivity against its bacterial targets, which must be understood in order to enable successful *in vivo* use (Figure 1A). Thus, the development and adoption of clinical assays to rapidly identify causative bacterial pathogens and their susceptibility to phages are necessary. Traditionally, a cocktail approach has been used to address the limited host range of any single phage (Figure 2A) [4]. However, the desire to increase coverage by adding more members to a phage cocktail must be balanced with the challenge of producing and testing well-defined multi-component mixtures for regulatory approval. Thus, with modern biotechnology and automation techniques, establishing high-throughput screening and engineering efforts to expand phage host range and potentially reduce the number of bacteriophage necessary in a given cocktail are important. For example, multi-species enrichment protocols can enable the selection of natural phages with broader infective spectra [39]. Scholl *et al.* designed bacteriophage that could exhibit extended host ranges by degrading barriers to phage adsorption and infection [40]. Specifically, they found that the K1 capsule of *E. coli* acts as an effective barrier to T7 infection. Thus, they engineered T7 bacteriophage to express an endosialase that degrades the K1 capsule and found that it was able to productively infect *E. coli* K1 strains (Figure 2D, left). In another strategy, Marzari *et al.* showed that grafting the g3p phage protein of one filamentous phage (IKe) to another (fd) enabled an extension of host range [41].

The challenge of bacterial resistance to bacteriophages

Bacteria can evolve resistance to phages through a variety of different mechanisms, including blocking phage adsorption, inhibiting the injection of phage genomes, restriction-modification systems, and abortive infection systems (Figure 1B) [42•]. In *in vitro* monoculture studies, phage resistance can evolve on the order of hours to days. One question that deserves more study is whether the evolution of phage resistance *in vitro* is relevant to *in vivo* conditions where bacteria may be replicating more slowly and challenged with a greater set of environmental conditions. For example, Capparelli *et al.* found an average resistance frequency of 1.3×10^{-8} for *S. aureus* A170 treated with phages *in vitro*, although they were unable to isolate any phage-resistant *S. aureus* strains from mice treated with phages [43]. Phage mixtures constitute the traditional method used to deal with this problem but have shown mixed results (Figure 2A) [4]. Abuladze *et al.* reported no instances of resistant *E. coli* O157:H7 from food treated with a cocktail of three phages [8]. O'Flynn *et al.* found resistance frequencies of 1.2×10^{-6} to 3.3×10^{-4} for single-phage treatments and 1.1×10^{-6} to 1.5×10^{-6} for double-phage or triple-phage cocktails against *E. coli* O157:H7 *in vitro* [44]. Tanji and colleagues showed that although cocktails can delay the evolution of phage-resistance, bacteria and phages eventually reach coexistence [45,46]. Thus, new techniques may be needed to further reduce the evolution of resistant bacteria. These include using phages in combination with other antimicrobials, such as antibiotics (Figure 2F), cycling through different phage mixtures, and engineering phages to directly target phage-resistance mechanisms [42•].

The challenge of bacteriophage manufacturing

Issues associated with bacteriophage manufacturing for clinical use include the removal of endotoxins and pyrogens released during phage-induced lysis and the development of stable formulations (Figure 1C). Concurrent with the advancement of biotechnology, phage manufacturing has increased in sophistication is capable of producing clinical-grade bacteriophage preparations [47–49]. Merabishvili *et al.* recently described a complete protocol for the isolation, characterization, manufacturing, purification, and quality control of bacteriophages for clinical use [49•]. This protocol included the use of a commercially available endotoxin removal kit and was able to achieve sufficient purity for use in a European clinical trial. Such processes will continue to be optimized and scaled if more commercial entities enter into phage therapy and positive regulatory precedents are set.

The challenge of systemic side effects of bacteriophage therapy

A concern with any lytic bacterial treatment is that the rapid and massive destruction of bacteria *in vivo* may release endotoxins and superantigens that stimulate an inflammatory response that can cause significant morbidity (Figure 1D). In order to reduce the risk of this happening, phages have been selected or engineered to be lysis-deficient and/or non-replicative (Figure 2E). These approaches can significantly decrease the levels of endotoxin and inflammatory mediators generated during phage therapy and thus improve survival. For example, Hagens and Blasi engineered filamentous phages to express restriction endonucleases and holins in *E. coli* [50]. These phages were toxic to bacteria but did not cause cell lysis and thus released minimal levels of endotoxin [50]. They also designed phages that were non-lytic and non-replicative phages against *Pseudomonas aeruginosa* [51]. They found that the increased survival of infected mice treated with non-lytic phages compared with lytic phages was correlated with decreased levels of inflammation. Matsuda *et al.* reported similar results in a murine peritonitis model; they showed significantly greater survival within the lysis-deficient treatment groups at 6 h and 12 h post-treatment, along with concurrently lower TNF-α and IL-6 inflammatory marker levels at 12 h [52].

The challenge of bacteriophage delivery

Removal by the immune system is a major hurdle confronting the delivery of bacteriophages as antimicrobial agents, especially via the bloodstream (Figure 1E). As a result, many studies to-date have focused on treating non-systemic diseases, such as wound infections, gastrointestinal infections, and ear infections [4]. Others have sought to address the issue of systemic delivery through evolutionary and rational approaches. Merril *et al.* described a technique for serially passaging phage through animals to isolate mutants that circulate for longer periods of time *in vivo* (Figure 2B) [53]. Most isolates contained mutations in the major phage head protein that were postulated to enable escape from clearance by the reticuloendothelial system (RES) [54]. In addition, drug delivery technologies, such as polymers used for drug delivery, constitute an underexplored but potentially powerful source of solutions for systemic phage delivery (Figure 2C). For example, to decrease the immune response, Kim *et al.* conjugated phages to polyethylene glycol (PEG) [55]. PEG-phage constructs had longer circulation times and generated lower levels of T-helper type 1 (T_h1) immune reactions compared with natural phages. However, PEG conjugation did not protect phages from rapid neutralization in animals that were pre-immunized with phages, suggesting that there is still much more room for better chemical-based delivery strategies for phages.

Advantages of natural and engineered bacteriophages

Despite the numerous hurdles, both natural and engineered phages have several beneficial properties that justify continued research and development. The isolation and characterization of novel phages can usually be achieved more rapidly and cheaply than small-molecule antibiotics. Owing to their host specificity, phages may be used to achieve the targeted destruction of bacterial pathogens with minimal effects on the beneficial microbial flora. This feature will become increasingly important as our understanding of the human microbiome improves and antibiotic-associated overuse diseases, such as *C. difficile* infections, become more prevalent.

Moreover, phages can be used to target bacterial states that are difficult to address with conventional antimicrobials, including biofilms, persisters, and antibiotic resistance. For example, many antimicrobial agents are ineffective against biofilms owing to the thick extracellular matrix and the dormancy of biofilm cells. May *et al.* reported that natural filamentous phages can be used to reduce biofilm formation but are not effective at breaking up mature biofilms [56]. Lu and Collins showed that phages could be engineered to disrupt existing bacterial biofilms by expressing biofilm-degrading enzymes during infection (Figure 2D) [57]. Specifically, they inserted a genetic module that overexpresses Dispersin B into T7 phage and showed that the engineered T7 construct was ~100-fold better than control phage at disrupting biofilms.

Phages can infect persister cells, which exhibit high phenotypic tolerance to antibiotics, and lyse these cells when they exit from dormancy [58]. Natural filamentous phages can also inhibit conjugation, which is a mechanism involved in the horizontal transfer of antibiotic-resistance genes [59]. Antibiotic-resistant infections can be treated more effectively by combining phages with antibiotics (Figure 2F) [60]. A recent article from the Eliava Institute of Bacteriophages stated that clinical trials were conducted in the former Soviet Union in the 1970s comparing the treatment of staphylococcal sepsis with natural phage alone, antibiotics alone, or phages with antibiotics [21].They found 41%, 23%, and 78% complete recovery in response to the three treatment classes, respectively.

Moreover, phages can be directly engineered to have enhanced efficacy when used in combination with antibiotics (Figure 2F). For example, Lu and Collins engineered lysogenic phages to overexpress proteins that improve antibiotic killing of wild-type and antibiotic-resistant bacteria [61••]. These proteins were identified via a systems-biology analysis as important contributors to a common pathway by which bactericidal antibiotics cause cell death [62]. Combination therapy with engineered phages and bactericidal antibiotics (from the

quinolone, aminoglycoside, and β-lactam classes) were found to be several orders of magnitude more efficacious than antibiotics alone or control phages at killing bacteria [61••]. Moreover, combination therapy killed antibiotic-resistant *E. coli* more effectively than antibiotics alone and improved the survival of infected mice to 80%, up from 20% with antibiotics alone [61••]. This work demonstrates that antimicrobial targets can be rapidly identified and translated into phage-based antibiotic-adjuvants using systems biology and synthetic biology techniques.

The majority of phage-engineering efforts to-date have been proof-of-concept studies focused a single phage or a small number of phages. For these studies to be useful for clinical applications against a broad range of bacterial targets, high-throughput techniques for modifying phages need to be established. Several techniques have been described for recombineering phages, although these techniques have been largely restricted to specific bacterial hosts. For example, Bacteriophage Recombineering of Electroporated DNA (BRED) can be used to construct mutant mycobacterial phages [63]. This strategy simultaneously introduces phage DNA and targeting substrates into mycobacterial cells for homologous recombination. We expect that host-agnostic, modular, and synthetic methods for engineering phages will result from the emergence of synthetic biology as an engineering discipline [64,65]. With DNA sequencing and synthesis capabilities advancing exponentially each year, our ability to design and build engineered phages is steadily increasing [66]. These technologies will enable a complete and rapid bacteriophage design cycle, encompassing isolation, characterization, engineering, and evolution, leading to the assembly of libraries of optimized therapeutic agents for clinical use.

Conclusions

Bacteriophage therapy is a promising yet challenging antimicrobial therapy that has regulatory and technical hurdles to overcome in order to achieve clinical use in Western medicine. Continued investments in research, development, and clinical trials from the public and private sectors are needed but are hampered by the lack of any approved phage-based therapeutics as precedents as well as systemic issues that plague antimicrobial development in general [67]. Clarity on the regulatory status of natural and engineered phages will allow researchers, companies, and investors to map out a clearer path to real-world use. In addition, well-controlled trials in countries with long-standing experiences in phage therapy will be important in guiding development, acceptance, and approval elsewhere. Technical challenges associated with phage therapy will require new strategies for addressing limited phage host ranges, the evolution of phage-resistant bacteria, phage manufacturing, systemic side effects, and phage delivery. Building upon the strategies described to-date, we are confident that the development of high-throughput methods for isolating, characterizing, engineering, manufacturing, and delivering phages using techniques from modern biotechnology, synthetic biology, and drug delivery will play a major role in advancing phage therapy.

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