

Bacteriophage Therapy: Exploiting Smaller Fleas

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Although bacteriophages have been used for the treatment of patients with bacterial infections in some regions of the world for >9 decades, adequate clinical trials of the safety and efficacy of the treatment have not been reported. The increasing problem of antibiotic resistance has, however, rekindled interest in this approach to therapy. Although potentially significant obstacles to systemic administration of phages exist, topical and oral administration of phages and/or phage products, such as lysins, are feasible in the short term. In addition to exploitation of the effects of native phages and phage products, bioengineering of phages will allow directed specificity and their use as delivery systems for antimicrobial and antivirulence molecules. This brief overview of the history and status of phage therapy, along with speculation about its future, provides a background for understanding of this imminent therapeutic modality.

Hobbes clearly proves, that every creature
Lives in a state of war by nature.

.....

So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed ad infinitum.

—Jonathan Swift, “On Poetry: A Rhapsody”

Giorgi Eliava, having been declared an enemy of the state, was executed by the security apparatus of the Union of Soviet Socialist Republics on 9 July 1937 [1]. Eliava met his fate despite the fact that he had previously been a star of the Soviet scientific firmament, having founded an institute that exists to this date in eponymous form. The lethal turn of events may have resulted from Eliava's reported injudicious flirtation

with an object of the affection of Lavrenty Beria, the Chief of the state security apparatus.

The focus of the Eliava Institute of Bacteriophage, Microbiology, and Virology, in Tblisi, Georgia, was and remains the therapeutic use of bacteriophages [2]. The use of phages for therapy of bacterial infection has its origin in an observation reported in 1896 by Ernest Hankin [3] of the presence of heat-labile, filterable antibacterial activity capable of killing *Vibrio cholerae* in the waters of the Ganges and Jumna Rivers. This observation lay fallow until the 1915 report by Frederick Twort [4] that described “glassy transformation” of micrococci on agar plates; he suggested that this was the result of an “acute infectious disease” of the bacteria and that a virus may have been the culprit [5]. These observations were soon extended and interpreted by Felix d'Herelle [6], who used the word “plaque” to

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Table 1. Selected commercial companies involved in the development of clinical uses of phages.

Company	Location	Web site
Biophage Pharma	Montreal, Quebec, Canada	http://www.biophage.com
Enzobiotics/New Horizons Diagnostics	Columbia, Maryland	http://www.nhdiag.com
Gangagen	Bangalore, Karnataka, India; San Francisco, California; Ottawa, Ontario, Canada	http://www.gangagen.com
Intralytix	Baltimore, Maryland	http://www.intralytix.com
Novolytics	Coventry, United Kingdom	http://www.novolytics.com
Omnilytics	Salt Lake City, Utah	http://www.omnilytics.com
Phage Biotech	Tel Aviv, Israel	http://www.phage-biotech.com
Phico Therapeutics	Cambridge, United Kingdom	http://www.phicotherapeutics.co.uk

describe the areas of clearing on agar and coined the word “bacteriophage” to indicate the presence of a “virus parasitic on bacteria.” He observed, using plaque counts, that an increase in stool phage titers correlated with recovery from dysentery and typhoid, and he concluded that bacteriophages were “exogenous agents of immunity” [1, 6]. The view that these agents were particulate, viral or otherwise, was strongly challenged by Jules Bordet, who was a formidable scientific adversary as a consequence of his having received the Nobel Prize in 1919, and by others who believed that the activity of the agents represented enzymatic or other soluble substance activity; the issue was not settled until the visualization of bacteriophage by electron microscopy in the 1940s [5]. In the meantime, d’Herelle commercialized bacteriophage preparations as therapy for infectious diseases. These products—Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage, and Bacté-staphy-phage—were marketed by the Société Française de Teintures Inoffensives pour Cheveux (Safe Hair Dye Company of France; now L’Oréal) [7]. Bacteriophage therapy entered the public imagination with the 1925 publication of the Sinclair Lewis book *Arrowsmith* [8] (subsequently made into a motion picture), which told the story of a young, heroic physician, Martin Arrowsmith, who attempts to cure plague with phages. Several US pharmaceutical companies became involved in commercial phage production, including Eli Lilly, which marketed phage-lysed, bacteriologically sterile broth cultures of several target organisms [7]. The drive for commercialization of bacteriophage therapy faded, however, with the introduction of sulfonamides in the 1930s and penicillin in the following decade. Bacteriophage therapy, nonetheless, continued to be offered through the Eliava Institute and, later, by others, such as the Hirschfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland [9]. Academic and commercial interest in the potential of the therapeutic use of phages has recently grown in response to the widespread emergence of resistance to antibiotics (table 1).

BACTERIOPHAGE BIOLOGY

Bacteriophages, of which there are currently 13 families and 30 genera, are believed to be the most abundant life form on the planet, suffusing the biosphere with a predicted 1×10^8 species [10] comprised of an estimated total of 1×10^{30} to 1×10^{32} phage particles; if gathered, these particles would weigh $\sim 1 \times 10^9$ metric tons [11]. Phages are ubiquitous, with, for example, an estimated 1×10^6 particles per drop of seawater and as many as 1×10^8 particles per g of soil. Phage predation destroys an estimated one-half of the bacterial population worldwide every 48 h. As a consequence, bacteriophages have played a critical role in the evolution of the bacterial biosphere, in part by accelerating bacterial mutation rates [12]. This antagonistic coevolution results from the constant emergence of countermeasures by which bacteria resist phages, while phages, mutating at a more rapid rate than their prey, find means to overcome this resistance [13, 14]. The billions of years of evolution emanating from this predator-prey relationship has made bacteriophages a potentially rich resource for the discovery of candidate antibacterial agents [15, 16].

These obligate parasites of bacteria contain a core nucleic acid, usually double-stranded DNA (dsDNA), within a protein or lipoprotein capsid [17, 18]. More than 95% are tailed and interact with various bacterial surface receptors through fibers extending from the tail (figure 1). This interaction dictates an affinity that is usually specific for groups of bacteria (which may include strains from >1 species) but, in some cases, is specific for a single strain. This strain specificity has led to the use of phages in a variety of bacterial typing systems. When irreversible attachment to the surface receptor occurs, the phage genome is injected into the bacterial cell and transcribed by host cell RNA polymerase. Two potential phage life cycles, lytic (“virulent”) or lysogenic (“temperate”), may then ensue. Lysogenic phages remain quiescent as prophages, only replicating together with the host genome, unless mobilized. In contrast, virulent phages, having replicated and assembled into complete

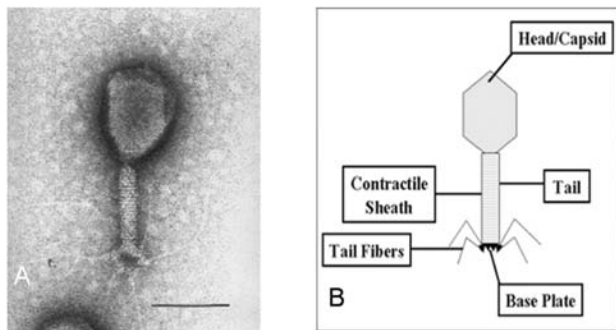


Figure 1. Typical bacteriophage morphology. A, Negative stain electron micrograph of T4-like *Aeromonas* phage, a typical tailed phage (reprint permission granted for Dr. H.-W. Ackerman). B, Structure of a T4 phage.

virions, cause rapid lysis and death of the bacterial cell, with release of 10–100 virions per phage; these virions then find more prey. Bacterial killing, in the case of dsDNA phages, is usually accomplished with activation of a 2-component lysis system, consisting of small hydrophobic pore-forming proteins (holins) and murein-degrading enzymes (endolysins or lysins) [19, 20]. The membrane-disrupting effect of holins allows access of lysins to the bacterial peptidoglycan layer. Of interest, lysis is not necessary for bacterial cell death; lysis-deficient phages cannot exit the cell but are nonetheless lethal to it [21].

POTENTIAL CLINICAL USES OF PHAGES

The characteristics of lytic phages—target specificity, rapid bacterial killing independent of antibiotic resistance, and amplification at the site of infection—make them of interest as possible therapeutic agents and for other uses. The first reported therapeutic use of phages in humans appeared in 1921: direct intralesional injection appeared to improve outcomes in 6 patients with staphylococcal boils [22]. This was the same year that d’Herrele [23] reported success in a cluster randomized trial of prophylaxis of avian typhosis due to *Salmonella gallinarum*. d’Herrele also performed a phase I trial designed to evaluate safety in humans, as he describes, “First I ingested increasing quantities...from 1 to 30 cc, without detecting the slightest malaise. Three persons in my family next ingested variable quantities several times without showing the least disturbance. I then injected myself subcutaneously with 1 cc of a 40-day-old suspension. There was neither a local or a general reaction” [24, p. 540]. He also injected family members and coworkers with the preparations. He then administered phages to patients with bacillary dysentery, cholera, and bubonic plague. His report of successful treatment of plague [25] stirred the imagination of Sinclair Lewis, leading to the writing (with the scientific assistance of Paul deKruif, the author of the still popular *The Microbe Hunters*) of *Arrowsmith*. Unfortunately, although scientific understanding of phages has advanced to a

remarkable extent, understanding of the clinical benefit of phage therapy has not advanced because of a lack of published reports of results obtained from the performance of studies with scientifically acceptable clinical trial design.

The potential routes of administration of phages include topical, oral, rectal, and parenteral; topical administration to chronic wound infections is the most frequently reported route. In such infections, phage cocktails—combinations of a variety of phages—have been used. One product used at the Eliava Institute is PhageBioDerm, a biodegradable polymer wound dressing impregnated with ciprofloxacin, benzocaine, chymotrypsin, bicarbonate, and 6 lytic phages (Pyophage) with activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus* species, and *Proteus* species [26]. Intralytix has received a patent for PhageBioDerm and is planning clinical trials [27]. In the meantime, WPP-201, a combination of 8 lytic phages with the same spectrum as PhageBioDerm, is being evaluated in a prospective, randomized, double-blind, placebo-controlled trial involving 64 patients with infected chronic venous ulcers [28].

Other potential means of administration include sprays, aerosols, lozenges, mouthwash, suppositories, bandages, eye drops, and tampons. Intrapleural administration and bladder irrigation are also feasible. Oral administration for treatment of enteric infections, including *Clostridium difficile*-associated disease, is promising. Oral administration may, however, be associated with phage translocation and resultant systemic exposure [29]. The US Food and Drug Administration has, nonetheless, implicitly designated as “generally accepted as safe” orally administered phages in the form of LMP-102, a 6-phage cocktail specific for *Listeria monocytogenes* that can be sprayed on cheese, chicken, and processed meat to prevent infection with this organism [27]. This is simply an acknowledgement that we continually ingest phages in food and water and that our gastrointestinal tracts already contain enormous numbers of these ubiquitous viral particles.

The correct choice of antimicrobial is a critical element in the effective therapy of infection, and this principle is even more true with regard to phage therapy. Ideally, infecting pathogens would be screened against a library of phages, each of which will have been fully characterized, including their genome sequences, and they must have demonstrated safety and efficacy. The library might include phages that had been genetically manipulated to alter their host range and to produce potent antibacterial molecules. Because the evolutionary battle between predator and prey would continue at the site of infection, persisting bacteria would require additional phage susceptibility testing to determine whether the high phage mutation rate had failed to outpace the emergence of bacterial resistance and, if so, to identify alternative agents. The likelihood of this occurring depends, in part, on the inocula of the

antagonistic components but could potentially be overcome by use of a combination of phages with overlapping specificities, by their use together with antibiotics [30], and by the development of dosing regimens that reduce the likelihood of selection of resistant mutants [31].

Systemic administration of phages, which are essentially self-replicating nanoparticles, would necessitate knowledge of the pharmacokinetics and pharmacodynamics of each phage. Phage pharmacokinetics and pharmacodynamics are complex because of the nature of the predator-prey relationship at the site of infection, in which both elements are replicating while the target bacteria are also undergoing lysis [31]. Phages are rapidly removed from the circulation by cells of the reticuloendothelial system and may also be subjected to removal by neutralizing antibody, if present. The former, however, has been overcome in a murine model by selection of virus persisting in the circulation after serial phage passage [32]. Organ and tissue targeting may be further complicated by the fact that bacteriophages are within the size range of nanoparticles (9–15 nm). Nanoparticles in biological fluids, such as blood, acquire a corona, a “cloud” of proteins and other molecules, the composition of which depends on particle size and charge, and the corona would affect the trafficking of the nanoparticles [33]. Computer modeling suggests that the timing of administration relative to the onset of infection is likely to be important [34, 35]. In addition to an understanding of the pharmacokinetics and pharmacodynamics, the safety of phage therapy will also require strict attention. Administered phages should be demonstrated to be free of genes that encode factors that enhance bacterial virulence—a requirement that is relatively easy to address with modern gene sequencing techniques. Recently, Kwan et al. [36] determined the genomes and proteomes of 27 individual *S. aureus* bacteriophages, including the first discovered phage, phage Twort. Therapeutic phages would also require demonstration of stable viability, preferably without the necessity of maintenance of an intact cold chain.

PHAGES AS THERAPEUTIC DELIVERY SYSTEMS

The specificity of targeting by phages may be exploited for their use as a delivery system for antibacterial molecules. As an example, US patent 6759229 (issued in 2004) describes a “toxin-phage bacteriocide antibiotic and uses thereof” [37], consisting of a modified phage-encoding TPB peptide toxin A, which is bactericidal only when it is located intracellularly. Similarly, nonlytic phages engineered to produce antimicrobial proteins were effective in a systemic mouse model of *Escherichia coli* infection [38]. Yacoby et al. [39, 40] demonstrated in vitro activity against *S. aureus* of filamentous phages modified to display target-specific peptides or antibody, with chloramphenicol attached to the phages through a labile linker mole-

cule. The antibiotic is only active when it is released, and it is only released at the target site; therefore, its concentration is maximized at the site of infection, and the systemic toxicity is minimized. Although phages alone can disrupt biofilm colonies of target organisms, such as *Staphylococcus epidermidis* growing on silicon catheters [41, 42], phages have also been bioengineered to attack biofilm. An *E. coli* phage, T7, was modified to express dispersin B, an enzyme that degrades β -1,6-N-acetyl-D-glucosamine (an important component of biofilm) in such a way that the enzyme was released into the extracellular milieu during bacterial cell lysis [43]. The dispersin B-expressing phage reduced the biofilm bacterial load by $\sim 99.997\%$ ($-4.5 \log_{10}$), to a level ~ 2 orders of magnitude lower than that achieved with phages alone.

Targeted gene deletions have produced phages that are capable of binding to their target receptors and injecting their DNA but that do not replicate and do not lyse bacteria [44]. Such modified phages have been further altered to encode small acid-soluble proteins that bind to DNA (chromosomal and plasmid) in a non-sequence-specific manner, causing a shift in conformation and halting DNA replication and gene expression [44–47]; this potentially results in inhibition of toxin production and in bacterial killing. Exposure of methicillin-resistant *S. aureus* to this construction results in a $>99.9\%$ kill in 5 min with an inoculum of 1×10^5 organisms and a $>99.9\%$ kill in 10 min with 1×10^7 organisms [45–48].

External application of purified phage-derived lysin rapidly kills gram-positive bacteria with species- or strain-specific activity [49, 50]. Gram-negative bacteria cannot, however, be targeted in this way, because the outer cell membrane of these organisms represents an impenetrable barrier, preventing access to cell wall target sites. Pathogens for which potential therapeutic lysins have been specifically identified include *Bacillus anthracis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae* [49, 50]. Potential uses include topical administration for therapy or the elimination of colonizing organisms, as well as systemic therapy. The latter is illustrated by evidence that the phage-derived enzyme Cpl-1 has had a significant antibacterial effect in rodent models of pneumococcal septicemia [51] and endocarditis [52] with intravenous administration and against meningitis with both intracisternal and intraperitoneal administration [53]. MV-L, a lysin cloned from *S. aureus* bacteriophage ϕ MR11, was active against methicillin-resistant *S. aureus*, vancomycin-resistant *S. aureus*, and some vancomycin-intermediate *S. aureus* in vitro, interacted favorably with vancomycin, and was active in a murine infection model [54].

Mayer et al. [55] determined the genome sequence of a *C. difficile* bacteriophage, subcloned the endolysin gene, and expressed the gene in *E. coli*. The endolysin was active against a diverse panel of 30 strains of *C. difficile*, including the epidemic

strain, ribotype 027 (B1/NAP1). The authors also expressed the gene in *Lactococcus lactis* MG1363, a potential delivery organism to the gastrointestinal tract. Similar to phages, lysins could be used in combinations of >1 enzyme or together with antibiotics. Sublethal concentrations of at least some quinolones and β -lactam antibiotics mobilize virulent phages, increasing bacterial killing [56].

WHAT ARE THE PROSPECTS FOR THERAPEUTIC USES OF PHAGES IN THE UNITED STATES?

Nonhuman applications of phages and phage lysins, some of which are already implemented in the United States, will represent the first and widest use of these agents. These include use in food safety, agriculture, animal husbandry and veterinary medicine, aquaculture, waste-water treatment, and environmental remediation. The initial approved uses of phages in humans will likely be for topical administration (e.g., for infected chronic wounds) or for elimination of colonization with organisms such as methicillin-resistant *S. aureus*. Phages may also be used in the United States for prevention and treatment of gastrointestinal infections, including those caused by enteric pathogens and, possibly, *C. difficile*.

The systemic administration of phages for the treatment of deep infections would likely meet significant regulatory obstacles in the United States and elsewhere. Phage therapy is reported to present European regulatory agencies with a conundrum [57], and this is also true for the International Conference on Harmonization [58]. There appears to be no published US Food and Drug Administration guidelines about the evaluation of the safety of phage therapy, but the following statement from the agency with regard to somatic cell therapy and gene therapy may possibly indicate a lack of great concern: "In the case of MCB's [Master Cell Banks] consisting of bacteria carrying plasmids of interest, testing for bacteriophage is not required but the possible presence of bacteriophage should be considered, since it could adversely affect stability and yield" [59].

In the absence of significant advancements in point-of-care diagnostics, the rational use of phages and some phage products would likely be limited, in the majority of instances, to circumstances in which the pathogen is known and, preferably, its in vitro susceptibility to the available phage(s) is also known. This implies the ready availability of in vitro testing methods that can be performed in a clinical microbiology laboratory, which is also important for topical phage therapies. Optimal targets may be multidrug-resistant pathogens and organisms that are difficult to eradicate, such as those growing in biofilm. In the absence of rapid and accurate point-of-care testing, the systemic use of phages will logically be limited to follow-up use when classical microbiology techniques provide support for choosing this method of antibacterial therapy. One advantage

of developing phage lysins for therapeutic use, rather than developing only phages, is the likely greater ease with which the lysins can move through the regulatory process. In any case, the path will be a difficult one, but reaching the goal has great potential benefit. With that goal in sight, attention may eventually be given to the potential usefulness of even smaller fleas in the form of viroplage, such as the recently described Sputnik, a viral parasite of the mimivirus-related mamavirus [60].

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