Reinventing phage therapy: are the parts greater than the sum?

Vincent A Fischetti, Daniel Nelson & Raymond Schuch

Although whole phage continue to generate interest as an alternative to antibiotics, focus is shifting to the use of purified phage components as antibacterial agents.

When phages were first discovered nearly a century ago[1], it was immediately apparent that their potent bactericidal capacity should be harnessed to treat human infections. The industrialization of antibiotics in the 1940s, however, changed the focus of anti-infective research and development in the West away from phage treatments and toward natural products and their semisynthetic derivatives with antibacterial effects. Now, over 60 years later, interest again is turning to phage as adjunct therapies in the control of bacterial pathogens that have emerged as resistant to some, and in certain cases all, clinically approved antibiotics. This increased incidence of antibiotic-resistant pathogens is particularly worrisome in the hospital setting, where it is resulting in a significant upturn in morbidity and mortality. While several research groups continue to develop whole phage as alternative treatments, the isolation and optimization of purified phage components as antibacterials opens up new opportunities in the fight against intractable infections.

Natural born killers

Phage—that is, bacteriophage (the viruses of eubacteria)—are the most abundant biological entities on earth by a factor of at least ten. Studies emerging over the past 10 years (reviewed in refs. 2,3) imply a staggering impact for phage in areas as diverse as global carbon cycling to the structure and maintenance of all bacterial populations to the evolution of bacterial genomic structure and adaptive behavior.

Most significantly, phage predation destroys an estimated half of the world bacteria population every 48 hours[4]. This has indeed been a dynamic process, occurring for hundreds of millions of years, in all ecosystems, with bacteria countering the phage onslaught by several strategies, including the development of intrinsic resistance to steps of the phage lytic cycle. During their encounters with bacteria, the phage have in turn accumulated genetic loci in their limited genomes, perhaps of ancestral bacterial origin, that in some way (generally

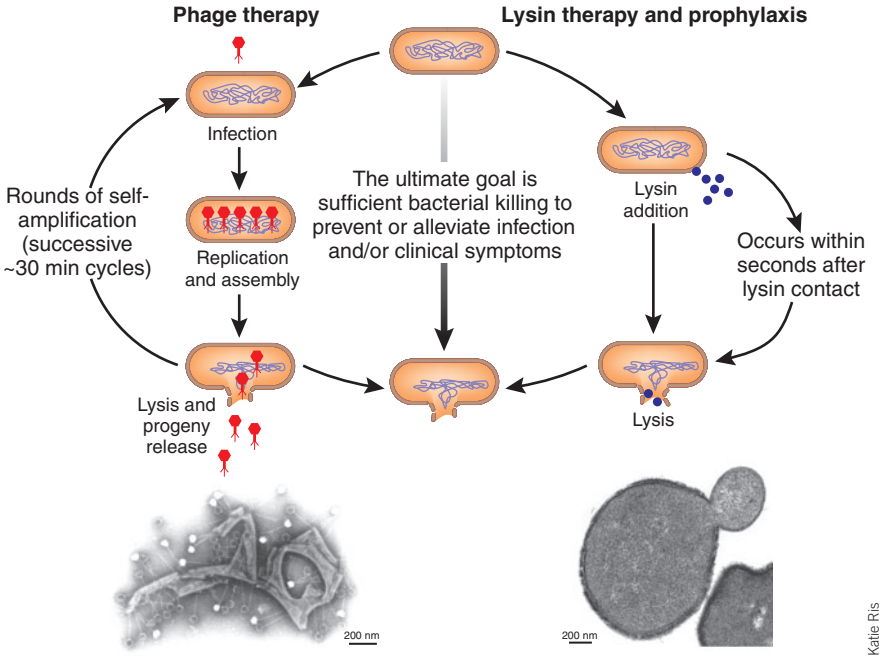

Figure 1 Steps to bacterial lysis in phage and lysin therapy. Phage therapy (on the left) exploits a natural phage lytic cycle, which occurs over 30 min and is divided into three major steps, including the release of new virions (in red) into the environment. Subsequent infection of new hosts illustrates the process of self-amplification. The electron micrograph depicts phage particles adhering to the debris of a lysed streptococcal cell. In comparison, lysin therapy and prophylaxis (on the right) is defined by only two steps, in which purified lysin (in blue) binds to, and rapidly kills through osmotic lysis, the target pathogen. The electron micrograph depicts a cross-section of *Bacillus anthracis* treated with the purified PlyG lysin showing an externalized cytoplasmic membrane just before lysis. Scale bar, 200 nm.

Vincent A. Fischetti, Daniel Nelson and Raymond Schuch are at the Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University, New York, New York 10021, USA.
e-mail: vaf@rockefeller.edu

unknown) interact with host physiology or structure to ensure phage survival. In fact, nearly half of all genes found in phage genomes have no correlate in current databases and may directly serve processes of host parasitism.

The therapeutic potential of phage was recognized very early in the twentieth century; indeed, in the 1930s and 1940s, several phage preparation products were marketed in the United Stated by such pharmaceutical companies as Eli Lilly (Indianapolis, IN, USA) and E.R Squibb and Sons (Princeton, NJ, USA). With the advent of penicillin and sulfa drugs, however, the focus of Western academic and company researchers shifted to antibiotic development, leaving such countries as the former Soviet Union to continue actively isolating phage from the environment and using them to treat serious infections[5]. This process has continued (with political interruptions) to this day, particularly at the Eliava Institute of Bacteriophage in Tbilisi, Georgia and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland. At the same time, interest has grown in the application of phage to environmental uses, such as agriculture[6], aquaculture[7,8] and wastewater treatment[9]. Given concerns over the use of antibiotics with livestock, phage may prove useful for the treatment of food-borne pathogens that are common contaminants of large animals, and phage cocktails have already shown modest efficacy against pathogens in initial trials in poultry[10] and cattle[11].

In this context, a major breakthrough for phage therapy occurred in August this year, when the US Food and Drug Administration (FDA) approved a cocktail of six individually purified phages as a treatment for *Listeria monocytogenes* contamination of ready-to-eat meat and poultry products (http://www.cfsan.fda.gov/~dms/opabacqa.html). Although phages are already approved in the United States for use in agriculture, this is the first time the FDA has regulated the use of a phage preparation as a food additive, which may open consideration to other human applications.

Therapy with intact phage

Some whole-phage products have already been used in the clinic to treat topical infections of patients with antibiotic-resistant bacteria; for example, biodegradable patches composed of polymeric scaffolds impregnated with phage (licensed in the Republic of Georgia as PhageBioDerm) have been used in Tbilisi to treat patients with chronic skin infections unresponsive to antibiotic therapy. One advantage of using whole phage as a therapy is the 'amplification factor'; that is, the infective cycle whereby one phage enters a bacterium and replicates, releasing 10 to 100 phage particles after lysis, which then go on to enter and replicate in other bacteria and so on (see **Fig. 1**).

On the other hand, an overriding constraint on this type of phage therapy is the rapid development of resistance to phage attachment—one of the defensive tools that bacteria have developed to survive in the phage-infested environment. Phage therapy scientists have countered this by developing a cocktail of phages designed to circumvent resistance. This approach has proven successful in limited clinical conditions and may prove to be a viable strategy[5]; however, it presents challenges for regulatory oversight—developing composite mixtures of phages with lot-to-lot consistency that are acceptable to the FDA is unlikely to be straightforward.

Other oft-cited potential drawbacks include the possibility of toxic shock after bacterial lysis[12], and the potential for the generation of neutralizing antibodies upon extended or repeated treatment of the same individual[13]. Although phage types with decreased immunogenicity can be selected by serial passage in mice[14], this approach may not be practical for cocktails composed of dozens of different phages. Once delivered intravenously, however, phages are found in nearly all organs, making them suitable for treatment of several types of infections[13].

> Will companies invest millions of dollars for a product that may have limited patent protection?

From a business standpoint, a factor that requires consideration is the intellectual property status of whole phage as a therapy. Will companies invest millions of dollars for a product that may have limited patent protection? A technology's patent portfolio is its ticket to development, without which financing becomes problematic. Decades of literature on the use of phage for a variety of applications, including therapy, decreases the novelty of whole phage therapy and thus its patentability. In spite of this, there are numerous companies commercializing phage therapies[15], and to prevent reverse engineering of their products, firms are adopting intellectual property protection strategies, such as patenting specific phage sequences in their products.

Furthermore, the past several years have seen an explosion of new whole phage therapy concepts. One strategy involves using phage deficient in their lytic system[12]. Lysis-deficient phages can infect, replicate within, and kill a target bacterium, but cannot egress into the environment. Macrophages then quickly clear the phage-filled bacterial ghosts. A second type of approach relies on whole phage as transport vehicles for delivery of lethal genes[16] or chemically linked antibiotics[17] to target bacteria.

In the realm of vaccines, phage can be used to display peptide antigens by means of classical phage display techniques or as recombinant fusions with coat proteins (reviewed in ref. 18). This technology takes advantage of the fact that phages are naturally immunogenic; as a result classic adjuvants, like tetanus toxoid or alum, are not required for immunization. Finally, phage-based DNA vaccines are being developed by John March's group[19,20] at the Moredun Research Institute (Midlothian, Scotland, UK). In their approach, an antigen gene in a eukaryotic expression cassette is incorporated into the genome of a generic bacteriophage, such as λ. Because of the inherent immunogenicity of phage, the virions are quickly engulfed by antigen-presenting cells (APCs) and broken down, allowing release of the DNA vaccine. The vaccine protein(s) are expressed within the APC and are subsequently presented on its surface.

Therapies based on phage components

Phage are, essentially, obligate intracellular pathogens of eubacteria and, as such, must interact with and subvert critical bacterial metabolic processes and structural components to ultimately produce progeny virions. On the basis of studies from several phage systems, it is clear that part of this 'hijacking' requires specific viral proteins that target bacterial RNA and DNA polymerase complexes during lytic proliferation to redirect activities toward viral gene expression and viral DNA replication. The manipulation of other critical aspects of host metabolism also occurs (especially considering long-term lysogenic interactions and the presence of prophage in the genomes of most sequenced bacteria), as do processes necessary to pierce the bacterial host envelope. By identifying such 'anti-host' viral proteins and elucidating the vulnerable cellular targets, we can exploit the 'high-throughput' screens continually performed by phage through the millennia to identify inherent vulnerabilities of bacterial physiology and structure. A greater understanding of these vulnerabilities and the phage elements that target them may provide a boon to anti-infective research, both in the identification of novel bacterial targets for drug discovery and in the development of specific antibacterial phage proteins.

One of the most promising phage components currently under development is a class of cell wall hydrolases termed lysins. Late in the viral infection cycle and upon a specific trigger

event, lysins translocate into the bacterial cell wall, bind the major structural polymer—peptidoglycan—and cleave bonds required for stability, resulting in hypotonic lysis and progeny release.

Fortuitously, the killing effect of lysins can also be elicited when their purified recombinant forms are applied directly to sensitive Gram-positive bacteria from without (**Fig. 1**). At this time, lysins have not been identified that are capable of traversing the outer membrane of Gram-negative bacteria, but they are likely to exist. The effective lytic spectrum for current lysins is usually narrow (species- or strain-specific) and is constrained by peptidoglycan-associated ligands, often carbohydrates, that serve as lysin-binding targets and are themselves distributed in species- or strain-specific manners. Binding is also strong, occurring in the nanomolar range, with affinity constants of $3–6 \times 10^{-8}$ M (in the same range as affinity-matured antibodies)[21]. The tight binding and potent lytic activity places the lysins in a category of irreversible, 'single-hit' inhibitors[22] and promises, at least in principle, a high efficacy compared with classic antibiotics. Furthermore, because they target peptidoglycan-associated carbohydrates as binding epitopes, the evolution of resistance to lysins may be precluded, as these moieties are often essential for viability (e.g., choline in *Streptococcus pneumoniae*, polyrhamnose in group A streptococci and a neutral polysaccharide in *Bacillus anthracis*).

The catalytic activities of lysins generally fall into two classes based on bond specificity within the peptidoglycan: the glycosidases that hydrolyze linkages within the aminosugar moieties and the amidases that hydrolyze amide bonds of cross-linking stem peptides. Significantly, these bonds are invariant in all bacterial cell walls, and are thus likely to be resistant to any structural alterations that could block phage infection cycles and select for phage-insensitive populations. As such, these bonds are vulnerabilities targeted by the phage lysins, possibly to guarantee progeny release. This is one cue, among several, that suggests an importance to pursuing lysin development as an anti-infective strategy.

A very important feature of the lysins, with respect to their development as antibacterial agents, concerns their structural versatility. With one exception[23], the functions of peptidoglycan hydrolysis and surface carbohydrate binding are encoded in two distinct functional lysin domains. These domains can retain activity after separation and can be fused to complementary domains of other lysins to redirect binding, alter catalytic function or both[24]. In this manner, a limited number of distinct lysin types can be recombined to generate a highly variable pool.

A basic application now envisioned for the lysins concerns their prophylactic use in clearing mucous membranes of colonizing pathogens responsible for nosocomial and community-acquired infections. Toward this end, our group[25,26] has used purified streptococcal phage lysins to rapidly clear group A streptococci (the agent of scarlet fever, rheumatic fever and necrotizing fasciitis) and *Streptococcus pneumoniae* (a significant cause of morbidity and mortality in healthcare settings), respectively, from the upper respiratory mucosal epithelium (the only niche for these organisms) of experimentally colonized animals. Similarly, we have used a purified lysin, PlyGBS, to significantly reduce the carriage of *Streptococcus agalactiae* (a dangerous agent of neonatal infections) from the vagina and oropharynx in a mouse model of colonization[27].

A second major application for the lysins concerns their use in treating systemic bacterial infections. Our group has now described lysins for anthrax[28,29], pneumococcal septicemia[30] and enterococcal septicemia[31]. Although the hurdles facing systemic treatments are significant (though no more so than those facing other phage therapies), several factors do favor the lysins, including the apparent absence of lysin-neutralizing antibodies in hyperimmunized rabbits, the absence of toxic side effects associated with either topical or systemic use and the inability to detect lysin resistance either after serial passage in the presence of lysin or in chemically mutagenized cells (that is, under conditions that favor high-level antibiotic resistance)[26,28].

> It is increasingly clear that the future of lysin development will also focus on agbiotech and food biocontrol applications.

So far, the proof of concept that purified lysins can indeed act as antibacterial agents has been provided. Our studies have shown that lysin-mediated killing is not only efficient (often requiring only a single dose), but can be directed toward clinically relevant antibiotic-resistant strains and antiphagocytic capsule-producing strains, and is highly specific for targeted pathogens, with no activity toward commensal flora. Although these findings alone warrant further preclinical testing, the case for further attention is bolstered by new and exciting findings that lysins can be used in the following ways: first, in combination (that is, a glycosidase and an amidase) for synergistic lethal effects both *in vitro* and *in vivo*[32]; second, to treat the often antibiotic-resistant bacterial biofilm structures that form on medical implants (catheters and other devices)[33]; third, to kill the slow- or non-growing 'persistor' organisms that are increasingly viewed as significant contributors to chronic bacterial infections and may be naturally resistant to antibiotic and phage treatment[34]; and fourth, as mutagenized derivatives (via deletions, point mutations, etc.) with improved catalytic efficiency.

It is increasingly clear that the future of lysin development will also focus on agbiotech and food biocontrol applications. One example is secretion of recombinant lysins from *Lactococcus lactis* starter cultures, used in the fermentation of milk, to combat *L. monocytogenes*, which can contaminate dairy products[35]. Similarly, *Lactococcus* starter strains have been engineered for leaky expression of antilactococcal lysins that then autolyze the lactococci, releasing intracellular enzymes that accelerate cheese ripening[36]. Another example is the creation of transgenic cattle that secrete a recombinant lysin-like hydrolase, lysostaphin, into their milk to protect against *Staphylococcus aureus* mastitis (a disease with significant economic impact)[37]. Finally, lysins have been used to protect plants against phytopathogenic *Erwinia* spp., either produced in transgenic potatoes[38] or expressed recombinantly and applied to the surface of pears[39].

Although the majority of discussion here has focused on the lysins, we must also point out that other phage components may, directly or indirectly, lead to valuable new therapeutic agents. For example, the lysis systems of certain 'small genome' viruses, such as Qβ and ϕX174, encode alternative non-lysin lytic agents that block the enzymes responsible for early steps in bacterial peptidoglycan biosynthesis. The development of these as therapeutics has been proposed[40,41]. Alternatively, Liu *et al.*[42] have used antihost phage components in an entirely different manner, whereby a phage-derived protein (that inhibits bacterial growth) is used to identify its bacterial target (DnaI, a DNA replication protein), which in turn allows the development of a novel high-throughput screen to identify small molecules that inhibit growth by the same mechanism. In this manner, the myriad of antihost phage proteins can be used to map bacterial vulnerabilities that can then serve as targets in novel drug discovery.

Conclusions

The past few years have seen a significant resurgent interest in the old concept of using phage as a therapeutic tool. No doubt reports of declines both in antibiotic efficacy and in pharmaceutical company interest in developing new agents

is fueling the drive for antibiotic alternatives. Although they are not without their constructive critics[43], whole-phage approaches may certainly become valuable anti-infective tools in certain therapeutic applications. That said, we and others envision an alternative use for phage that, although less heralded, focuses on their specific antibacterial components, rather than the infective virion, as the active killing agent. With the lysins emerging as the most promising antibacterial candidate, defined by their potent and specific lytic activities, we are proceeding with lysin development. Whether used topically or systemically, in humans or agriculture, as purified proteins or as forms expressed from transgenic animals or bacterial secretory systems, an increasing body of data validates the potential utility of lysins.

Even beyond the lysins, phage are professional parasites of bacteria, and as such, employ an arsenal of agents to subvert host function and structure. It is only logical that a comprehensive search for new antibacterial agents would attempt to mine this vast viral pool of antibacterial functions. If it is at all possible that the 'parts are greater than the sum' for phage, this work is justified.

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