

Different approaches for using bacteriophages against antibiotic-resistant bacteria

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Bacterial resistance to antibiotics is an emerging threat requiring urgent solutions. Ever since their discovery, lytic bacteriophages have been suggested as therapeutic agents, but their application faces various obstacles: sequestration of the phage by the spleen and liver, antibodies against the phage, narrow host range, poor accessibility to the infected tissue, and bacterial resistance. Variations on bacteriophage use have been suggested, such as temperate phages as gene-delivery vehicles into pathogens. This approach, which is proposed to sensitize pathogens residing on hospital surfaces and medical personnel's skin, and its prospects are described in this addendum. Furthermore, phage-encoded products have been proposed as weapons against antibiotic resistance in bacteria. We describe a new phage protein which was identified during basic research into T7 bacteriophages. This protein may serendipitously prove useful for treating antibiotic-resistant pathogens. We believe that further basic research will lead to novel strategies in the fight against antibiotic-resistant bacteria.

Bacteria have evolved to overcome a wide range of antibiotics, and resistance mechanisms against most conventional antibiotics have been identified in at least some bacteria.¹ The accelerated development of newer antibiotics is counteracted by the rate of bacterial-resistance development. Ultimately, the emergence of a multidrug-resistant pathogen that spreads efficiently from host to host may pose a significant health problem. This increasing threat has revived studies on the

efficacy of bacteriophage (phage) therapy in the West, and boosted research in some of the former Soviet Union countries and Poland, in which phage therapy has been practiced for many decades. Lytic phages have been considered a potential treatment against bacterial pathogens because they evolved to propagate optimally in bacteria and then kill them. They have been experimentally tested in the last few decades against dozens of human pathogens, such as *Staphylococcus aureus*² and *Pseudomonas aeruginosa*.³

Phage therapy on external and mucosal tissues such as the skin, upper respiratory tract, gastrointestinal tract, urogenital tract, eyes, and ears may prove useful in the future. Indeed, a clinical phase I and II control trial has been completed successfully for the treatment of chronic bacterial ear infections in humans caused by *P. aeruginosa*.⁴ Nevertheless, despite the optimistic outlook on the prospects of phage therapy in these tissues, there are still many doubts as to their ability to replace antibiotics in internal tissues.

First, it has been shown that most of the phages entering the bloodstream are sequestered within minutes by the spleen and liver.⁵ This problem was overcome in phage lambda by selecting mutant phages which are not sequestered by those organs.⁵ However, this problem is still a major barrier for therapy using other phages. Second, frequent phage usage may cause a significant antibody-neutralizing response, eliminating its effectiveness. Third, due to their relatively large size compared with antibiotics, most phages cannot diffuse into all of the infected tissues that the bacterial pathogen actively

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penetrates, and thus cannot always eradicate the pathogen. Fourth, the narrow host specificity of individual phages renders them useful only against a narrow range of pathogens. Nevertheless, this specific drawback also entails an advantage in the sense that the phages kill only one type of bacterium, causing minimal disturbance to the natural microbial flora. Lastly, bacterial resistance to phages evolves quickly, rendering phage therapy useless in many cases. Resistance can be achieved by modifying the phage receptor on the bacterial membrane,⁶ producing a capsule,⁶ modifying bacterial metabolic pathways,⁷ acquiring phage-specific DNA into the CRISPR arrays,⁸ or other means.

The pros and cons of phage therapy are discussed in detail elsewhere (e.g., ref. 9). It is clear that more phage-based approaches to fighting antibiotic resistance can be developed. Temperate phages can be used therapeutically to transfer genetic material to bacteria through their lysogenic cycle. In addition, phage-encoded proteins can be used to target bacterial biosynthetic pathways. In this addendum, we describe some of the recent developments in the use of temperate phages and phage-encoded products as weapons in the never-ending fight against antibiotic-resistant bacteria.

Temperate phages infect host bacteria via two pathways. In productive, lytic pathways, they produce new virion particles and then kill their host. In the lysogenic pathway, they stay dormant, often by integrating their DNA into the host chromosome, until the shift to the lytic cycle. The lysogenic cycle can thus be used as means to transfer desired DNA into bacteria. Edgar et al. used this capability as a proof of principle for the delivery of genes by temperate phages that may help in the fight against antibiotic resistance.¹⁰ In that study, temperate phages targeted bacteria residing on surfaces as a prophylactic measure, rather than directly targeting pathogens infecting host tissues. As opposed to the use of lytic phages to kill pathogens, Edgar et al. used temperate phages to reverse a pathogen's resistance to antibiotics by restoring its sensitivity to antibiotics.¹⁰ The temperate phages transferred specific genes into bacteria and integrated them into the bacterial genome by lysogenization. The integrated genes

conferred sensitivity to two types of antibiotics, streptomycin and quinolones. The sensitization was achieved simply by virtue of dominance of the sensitive allele over the resistant one. These sensitizing genes were linked to a gene conferring resistance to the toxic compound tellurite, and thus sensitized bacteria could be selected for in the presence of tellurite.

The proposed use of these temperate phages consists of two steps. The first sensitizes nosocomial pathogens on nosocomial surfaces, as well as natural bacterial flora residing on the skin and hands of hospital personnel (a major contamination source for patients), using the phages encoding sensitizing DNA elements. This sensitization step occurs *ex vivo*, prior to antibiotic administration to an infected patient, and thus there is no negative selection against harboring the sensitizing cassette. The phages are intended for spraying in hospitals, thus gradually reversing the occurrence of drug-resistant pathogens on hospital surfaces and replacing the resistant population with a sensitive one. The fact that this step is performed *ex vivo* bypasses the aforementioned toxicity and immunogenicity issues that often restrict conventional phage therapy. In the second step, the “new” hospital-residing pathogens, which now contain genes conferring sensitivity to antibiotics of choice, become treatable by antibiotics in the infected patient. The two-step approach allows applying extended selective pressure, *ex vivo*, to introduce the sensitizing genetic elements, which are linked to a resistance marker. This enrichment of sensitive pathogens also reduces the ability of newly introduced resistant pathogens to propagate because their ecological niche is occupied by these sensitive pathogens. Thus, most of the nosocomial infections will become treatable due to enrichment of antibiotic-sensitive pathogens in the hospital. Broad use of such a spray, in contrast to antibiotics or phage therapy, could potentially shift the nature of nosocomial infections toward susceptibility rather than resistance to antibiotics.^{11,12} The above described study demonstrates that phages can be used as gene-delivery vehicles to resensitize resistant pathogens to antibiotics. This proof of concept could

be developed against resistant pathogens, and against a broader spectrum of drugs.

Aside from temperate phages' ability to transfer DNA into potential pathogens, phage-encoded products can also be used to kill pathogens directly. The advantage of using specific phage products rather than the entire phage to kill bacteria lies mainly in the enhanced penetration ability of the purified component compared with an entire phage. Whereas the latter is filtered by the patient's organs, a purified component may be less detectable, and diffuse more efficiently into the tissues (e.g., phage lysins—as elaborated below). The advantage of using phage-derived components over synthetic or other natural products is the co-evolution of the former with their targets for billions of years, resulting in optimized target inhibition and increased specificity toward bacterial targets. Co-evolution often shapes inhibitors to match their target in a way that is hard to resist.

One example of such phage products is lysins, which are enzymes that hydrolyze bacterial cell walls. These enzymes have been suggested as an antibacterial weapon, and their use has been studied for the last decade.^{13–17} Phage lysins are considered relatively safe and efficient against bacteria. Indeed, in a hallmark study, it was shown that resistance against lysins is less frequent than that against antibiotics.¹⁵ Several phage lysins have been successfully tested in animal models against Gram-positive pathogens including *Streptococcus pneumoniae*,¹³ *S. pyogenes*,¹⁴ *Bacillus anthracis*,¹⁵ *Enterococcus faecium*,¹⁶ and *S. aureus*.¹⁷ However, to date, no phage lysin has been successfully applied against Gram-negative pathogens.¹⁸ Although the prospect of using phage lysins as an antibiotic seems plausible, the arsenal of weapons against resistant pathogens should be further expanded.

Other phage products have been proposed against bacterial pathogens and strategies to identify them have been reported (e.g., refs. 19–21). Recently, Kiro et al. conducted a study on the interactions between gene products of T7 coliphage and bacterial proteins.²² Interestingly, the aim of the study was simple: a basic understanding of an unknown gene product, with the overall goal of mapping all

phage–host interactions. Eventually, this mapping will provide a better understanding of host-machinery acquisition by the phage. The T7 phage gene product (Gp) 0.4 was found to directly interact with and inhibit FtsZ, a key component of the bacterial division ring. Inhibition of division confers a competitive advantage to the phage. To the best of our knowledge, this is the first time that such an advantage has been shown. Presumably, the phage prevents division in order to prevent the escape of one of the daughter cells, thus preserving all of the host's resources for production of its progeny. An additional possible explanation, raised by David Weiss (<http://f1000.com/prime/718173172>), is that unequal partitioning of phage structural proteins due to host division later in the infection cycle might result in suboptimal stoichiometries that reduce the efficiency of particle assembly.

Gp0.4 inhibition of cell division kills the cells, as they are not able to multiply. Consequently, Gp0.4 can potentially serve as a new weapon in the fight against antibiotic-resistant bacteria. From

a therapeutic point of view, FtsZ is a possible target for antibiotics as it is an essential bacterial protein, conserved across all known bacterial species, and absent in eukaryotes. Indeed, several research groups have shown that small molecules inhibiting FtsZ can potentially serve as antibacterial drugs (e.g., refs. 23–26). Further studies on Gp0.4 toward its use as an antimicrobial compound should determine the minimum effective peptide length for inhibition, its FtsZ inhibition capability across pathogenic bacterial species, its stability inside and outside mammalian tissues, and its ability to penetrate both the patient's tissues and the targeted bacteria. To the best of our knowledge it is the only division protein that the phage inhibits directly.

It seems only natural that FtsZ is the optimal target among over a dozen other known division proteins²⁷ because it is the one that arrives first at the cell center and initiates the entire division process; in its absence, none of the other division proteins are localized to the mid cell.²⁸ This example and others (e.g., ref. 20)

demonstrate that studying novel phage proteins not only potentially increases our arsenal of weapons, but may also reveal novel targets, or Achilles' heels of many biological processes, which are optimal for inhibition from the viewpoint of the phage, and most likely also from a therapeutic viewpoint. In our opinion, one of the most important lessons from this study is that it reemphasizes the notion that basic research can eventually lead to applicable products. The Gp0.4 study was initiated out of pure scientific curiosity to understand how phages take over their hosts. We believe that similar basic studies will reveal many more of nature's secrets, from which the next antibiotic can hopefully be formulated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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