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Viruses versus bacteria—novel approaches to phage therapy as a tool against multidrug-resistant pathogens

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Bacteriophage therapy (the application of phages to treat bacterial infections) has a tradition dating back almost a century, but interest in phage therapy slowed down in the West when antibiotics were discovered. With the emerging threat of infections caused by multidrug-resistant bacteria and scarce prospects of newly introduced antibiotics in the future, phages are currently being reconsidered as alternative therapeutics. Conventional phage therapy uses lytic bacteriophages for treatment and recent human clinical trials have revealed encouraging results. In addition, several other modern approaches to phages as therapeutics have been made *in vitro* and in animal models. Dual therapy with phages and antibiotics has resulted in significant reductions in the number of bacterial pathogens. Bioengineered phages have overcome many of the problems of conventional phage therapy, enabled targeted drug delivery or reversed the resistance of drug-resistant bacteria. The use of enzymes derived from phages, such as endolysin, as therapeutic agents has been efficient in the elimination of Gram-positive pathogens. This review presents novel strategies for phage-related therapies and describes our current knowledge of natural bacteriophages within the human microbiome. Our aim is to provide an overview of the high number of different methodological concepts, thereby encouraging further research on this topic, with the ultimate goal of using phages as therapeutic or preventative medicines in daily clinical practice.

Keywords: bacteriophages, phage therapy, human virome, engineered phage, endolysin

Introduction

Bacterial infections are known to cause high mortality worldwide, however, there has been little successful development of new drugs against multidrug-resistant pathogens.[1] Of particular concern are antibiotic-resistant opportunistic pathogens in hospital settings, where they pose an increasing threat to immunocompromised patients. The bacterial species in question have previously been referred to by the (somewhat ironic) acronym ESKAPE, which includes Gram-positive *Enterococcus faecium* and *Staphylococcus aureus*, as well as Gram-negative *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species.[2] The lack of effective antibiotics makes it increasingly difficult to control these pathogens, and consequently alternative antibacterial strategies are urgently needed. One potential alternative could be bacterial viruses (bacteriophages, colloquially referred to as 'phages'), which are probably the most abundant biological entity on our planet.[3] Soon after their discovery in the early 20th century, researchers began using phages for the treatment of infectious diseases. After the discovery of antibiotics in the 1940s, research into phage therapy slowed in the West, while it continued in the countries of the former Soviet Union.[4] However, as we are now faced with a constantly increasing number of antibiotic-resistant bacterial infections, there has been a re-emergence of interest in Western countries in phages as potential therapeutic agents. In addition to the principal possibility of filling an important gap in medical therapeutic practice, the striking advantage of phage therapy is its high target specificity, which leaves the natural human microbiome virtually unaffected; this is a situation rarely seen when using antibiotics.[5]

This review discusses recent approaches to phage therapy, with a focus on infections caused by the most important multidrug-resistant bacteria (i.e. the ESKAPE organisms). Our aim is to provide an overview of the high number of innovative methodologies, thereby encouraging further research on this topic with the ultimate goal of using phages therapeutically or preventatively in daily clinical practice. The spectrum of strategies described here ranges across natural and engineered phages, combinations of phages with other antimicrobial substances, the use of lytic enzymes derived from phages and the phage-mediated prevention of antibiotic resistance (Figure 1).

Conventional phage therapy

The traditional concept of human phage therapy is the administration of naturally isolated virulent phages (mostly from an environmental source) directly to the patient with the goal of lysing a bacterial pathogen deemed to be responsible for an acute or a chronic infection. To distinguish this from other forms of phage application, we hereafter refer to this as 'conventional phage

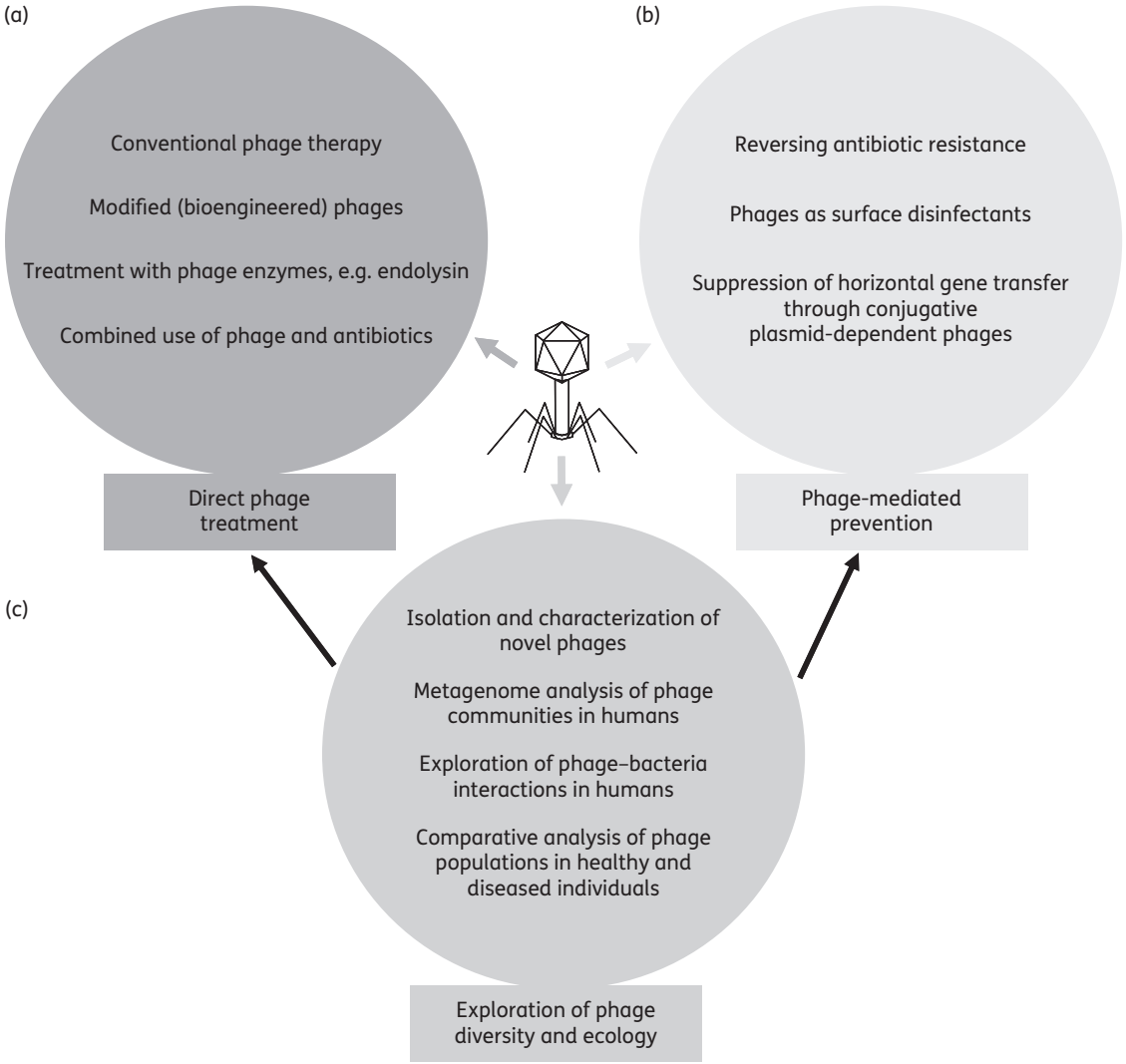

Figure 1. Envisioned connections between phage applications and research. (a) Direct treatment of bacterial infections, (b) phage-mediated prevention of infection and (c) exploration of phage diversity in environmental and human ecological niches as a basis for improving strategies in (a) and (b) and to provide novel concepts for phage application against bacterial pathogens.

therapy'. Principally, the use of strictly lytic phages for therapy is the method of choice, because the possible transfer of virulence genes into the genome of the host bacterium, as is the case with temperate phages, is rather unlikely.[6] Due to the relatively high number of reviews that have been published on conventional phage therapy, this section focuses on the most recent human studies that have met high standards (i.e. by considering biological safety issues and measuring the efficacy of phage application in controlled clinical trials). For a comprehensive overview of reported phage therapies, including animal trials, the reader is referred to the recent literature.[7] For a detailed insight into the history of the discovery of bacteriophages and the early clinical studies with phages, other work in the literature can be consulted (e.g. Sulakvelidze *et al*.[8]).

Although phage therapy has been intensively studied in the Western world over the last few decades and before that in Eastern European countries, there are currently no phage applications for humans that have either been approved or are in Phase III clinical trials in the European Union or the USA.[9] However, several placebo-controlled clinical human trials have demonstrated phage therapy to be safe. For instance, in a 1 month study of 15 adults taking *Escherichia coli* phage T4 in their drinking water, only five mild adverse reactions were noted. No antibodies against T4 were observed.[10] In a recent follow-up study, the oral administration of a mixture of phages (a 'phage cocktail') of nine different T4 phages against *E. coli* diarrhoeal strains to 15 healthy adults in Bangladesh also registered no adverse effects even though the dosage was 100 times higher than in the aforementioned trial. The phages given did not amplify inside the healthy humans, apparently due to the absence of target bacteria.[6] Hence, the administered phages were harmless to the tested healthy individuals. However, a prediction of the definite outcome regarding safety requires testing patients suffering from *E. coli* diarrhoea, since the level of emerging phages in the gut can be expected to be several orders of magnitude higher in the presence of their bacterial host.[6] In a different report, a cocktail of three lytic

phages against *P. aeruginosa* and *S. aureus* infecting burn wounds had no side effects when administered topically. Efficacy has not been described as clinical trials are still in progress.[11] Another controlled human Phase I trial demonstrated acceptable safety but a poor efficacy of phage cocktails in wounds.[12]

Conversely, a comprehensive randomized, double-blind, placebo-controlled Phase I/II clinical trial supported the potential of phage therapy. While 25% of patients with chronic otitis (infection of the ear) caused by antibiotic-resistant *P. aeruginosa* had an almost complete recovery, the vast majority of patients improved after treatment with a cocktail of six lytic phages. Phage replication continued as long as the target bacteria were common, after which phage levels decreased.[13] Promising results of phage therapy (described as clinical improvement up to the eradication of bacterial pathogens) were achieved in 40% of 153 treated patients suffering from various infectious diseases according to the most recent retrospective analysis.[14] Except for six patients, who had to discontinue treatment owing to adverse reactions possibly correlating with the phage therapy, no further safety risk could be evaluated. The most convincing results were achieved with oral or intrarectal application. The best outcomes were obtained when treating infections caused by pathogens of the genus *Enterococcus*; the highest healing rates were achieved for the treatment of infections of the urinary/genital tract, and the lowest for respiratory tract infections.[14] Overall, these results can be interpreted as encouraging, considering that all the medicated patients were suffering from infections that were untreatable using common antibiotics. In other words, a 40% healing efficacy is better than zero, and it can be anticipated that success with phage therapeutics will steadily grow with an increasing body of experience in this field.

As a conclusion, well-designed human clinical trials suggest that infections that are not susceptible to common antibiotics can be treated with phages. There are no recent reports on patients treated with phages that were injected intravenously. Although such studies would be an important step towards the treatment of systemic infections, a major hurdle to overcome is the requirement of intravenous phage solutions to be as pure as any other chemical drug. Clearly, rigorous trials to validate safety and efficacy need to be conducted in order to meet the challenges inherent in phage therapy. Further issues to overcome are the narrow host range of phages, the potential of bacteria to quickly develop resistance against phages, challenges in phage manufacturing, systemic side effects (especially endotoxin release), the undesired reduction in the number of phages by the immune system and phage delivery in general (as described by Lu and Koeris[15]).

Modified phages

The recent literature describes several examples of successful modifications of bacteriophages in order to overcome some of the obstacles mentioned above. For instance, chemical PEGylation (i.e. attaching the non-immunogenic substance monomethoxypolyethylene glycol to the phage's surface) enhanced the circulation time of phages in mice newly encountering this phage.[16] Although no such effect could be observed in already immunized animals, the authors argue that PEGylation results in a higher number of infective phages by delaying immune responses, thereby increasing the overall efficacy of the phage therapy. However, some trade-off must be noted, because the authors also observed that the infectivity of the phages decreased with increasing PEGylation.[16] Even more efficient was a single amino acid substitution in the major lambda phage capsid (E) protein in a different study, which resulted in an up to 1000-fold longer circulation time.[17]

Another important goal of genetic engineering is to develop phages with a broader host range (e.g. phages with the capacity to infect many if not all the virulent strains of a pathogenic species). A step towards this goal has recently been achieved by the generation of engineered virulent phage banks against *E. coli*[18] and through the recombination of two distinct phages.[19,20] In this way, the homologous recombination of long tail fibre genes enabled phage T2 to gain the broader host range of phage IP008 while retaining its own strong lytic activity.[20]

These examples are encouraging and show that phages can, in principle, be tailored to improve antibacterial therapy. A plethora of novel approaches can be expected in the near future, as we find ourselves in the age of 'synthetic biology' in which even whole phage genomes can be constructed,[21–23] leading to entirely artificial virions that are capable of infecting bacterial pathogens.[22,23]

All of the approaches described so far involve phages that directly kill bacterial pathogens by host cell lysis. However, lysis of the bacteria holds the risk of releasing toxic substances, e.g. endotoxin in the case of Gram-negative bacteria (a side effect also seen with many antibiotics). The use of lysis-deficient phages circumvents this issue. In fact, higher survival rates of mice with infections caused by either *P. aeruginosa* or *E. coli* were demonstrated when the mice were treated with engineered non-lytic phages. This effect was apparently due to lower levels of endotoxin release and resulting decreases in inflammatory reactions.[24,25] Lysis-deficient phages have also recently been applied against *S. aureus*. To this end the endolysin gene that encodes the peptidoglycan hydrolase relevant for bacterial cell degradation and the subsequent release of phage progeny was inactivated. As a result the successful treatment of an infection caused by methicillin-resistant *S. aureus* (MRSA) in mice was possible based on the sole activity of holin, which destroys the cytoplasmic membrane, followed by bacterial cell death without lysis.[26,27]

Another interesting approach is to use phages for the targeted delivery of lethal substances or genes to the site of infection. Filamentous phages in particular are well suited to this as they are generally non-lytic.[28] Recently, filamentous phages have been engineered to deliver protein-coding killing genes to their target bacterium. Successful results were noted for phage-delivered lethal genes coding for modified holin,[29] restriction endonucleases (which degrade the bacterial genome),[24,29] modified lethal catabolite gene activator protein (a lethal transcription regulator)[30] and addiction toxins, which cause programmed cell death.[31]

A disadvantage of the use of non-replicating phages compared with the use of replicating ones is that, similar to antibiotics, resistance is more likely to occur. Bacteria resistant to filamentous phages emerge by either the alteration[29] or the deletion of pili,[30] which are the binding structures of those phages. Therefore, in order to decrease the development of phage-resistant mutants, the target of the phages should ideally be a bacterial virulence factor or another highly important structure so that mutations in these structures are likely to cause a decrease in bacterial fitness.[29]

Similar to the above, phages can also function as carriers of antibiotics, which are either incorporated into the phage[32] or attached to its surface.[33,34] The evident benefit is that non-specific drugs such as chloramphenicol, an antibiotic not usually used *in vivo* because of its haemolytic side effects, can be employed due to a target-specific action at the site of infection. So far, targeting has been made possible by using either bacteria-specific antibodies linked to the phage or target-specific peptides from phage libraries. The antibiotic was linked exogenously to the phage coat protein through an aminoglycoside bond and labile linker subjects that may be cleaved by serum esterases in the blood wherever needed.[34] Higher efficiency and better solubility compared with the free drug were noted. A murine model confirmed this route of drug delivery as causing a lower antibody reaction and slower elimination from the blood than free phage particles.[33] Another advantage of this novel approach is that as pharmaceutical companies have low motivation for designing new antibiotics, already approved medications may be used. What remains to be elucidated *in vivo* is whether or not serum esterases in the blood also split the labile linker before the drug reaches the target site. Furthermore, the issue of the emergence of resistance remains, which has not been the subject of any of these studies.

Another approach based on the same principle is the phage-targeted delivery of photosensitizers (light-activated antibacterials). This procedure combined with subsequent irradiation with red light gave rise to a much greater elimination of *S. aureus* isolates, including MRSA and vancomycin-intermediate *S. aureus* (VISA), than either the phage or the light-exposed photosensitizer alone. Targeting enabled the use of low concentrations and light doses so that the side effects on human epithelial cells were minimized.[35] In addition, there is no need for the phage to infect the target bacterium, only for it to bind to it. However, the typical target specificity of the phage is not affected, as shown in a different experiment by the same group in which a conjugate of *S. aureus*-specific phage φ11 and a photosensitizer was able to kill only *S. aureus* isolates, but not *E. coli* cells.[36] Nevertheless, further *in vivo* studies to clarify target specificity and effectiveness are needed, although a topical application of this combination might be a potential success.

As skill in the engineering of phages is steadily increasing, all of these methods may eventually lead to the development of optimized phage therapeutics. Clearly, before genetically modified organisms can be used against human infections, regular (natural) phage therapeutics need to find general acceptance and must be approved and experienced in everyday therapy.

Treatment with enzymes derived from phages: endolysin

As an alternative to the application of entire phages (natural or modified), the use of single phage-encoded enzymes seems to be promising in combating multidrug-resistant bacterial infections. Endolysins in particular, which degrade the cell wall peptidoglycan and of which five different groups have been classified,[27] are immediate and strong bacteriolytic agents at a low dosage. Like the phages themselves, endolysins exhibit high target specificity in the case of Gram-positive bacteria, such that fewer side effects can be expected compared with antibiotics.[37] For instance, several endolysins with a high bactericidal activity against various strains of *Enterococcus faecalis* and *E. faecium*, including vancomycin-resistant enterococci, have been identified *in vitro*.[38–40] Interestingly, one endolysin even showed broad lytic activity against several streptococci from groups A, B and C.[40] Likewise, numerous *in vitro* studies have shown different staphylococcal endolysins, such as MV-L,[41] LysK[42,43] and the chimeric endolysin ClyS,[44] to be active against *S. aureus* infections including MRSA and vancomycin-resistant *S. aureus*. In a recent *in vivo* study Pastagia *et al.*[45] based an ointment on ClyS as active ingredient in a mouse model of skin infection. The therapeutic effect for both MRSA and methicillin-susceptible *S. aureus* was significantly higher than that of mupirocin, which is commonly used for the treatment of skin infections.[45] Furthermore, *in vivo* animal studies on three different staphylococcal endolysins against nasal MRSA colonization led to significantly decreased numbers of MRSA compared with the control group when the endolysins were applied intranasally either 60 h[41] or 24 h post-infection.[44] Similar results were achieved with the truncated endolysin CHAP(k), showing that native enzymes can be tailored for optimization.[46] Even systemic MRSA infections in mice were cured by a single intraperitoneal dose of diverse staphylococcal phage endolysins administered either 1 h[47] or 3 h post-infection.[44] The subcutaneous injection of phage endolysins was found to be highly effective in eliminating >99% of multidrug-resistant *S. aureus* in the spleens of treated mice. No adverse reactions were observed. However, only 17 out of 28 tested *S. aureus* strains were susceptible to the medication.[48]

Apart from the small target spectrum of most endolysins against Gram-positive pathogens, the endolysin PlySs2 (from a phage infecting *Streptococcus suis*) exhibits an exceptionally broad spectrum against Gram-positive bacteria including MRSA, VISA, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus sanguinis* and even *Listeria* spp.[49] Mice co-infected with MRSA and *S. pyogenes* have been successfully treated with this single endolysin.[49] A comparably broad lytic activity has also been observed when constructing chimeric enterococcal phage endolysins. In this case lytic activity ranged from several *E. faecalis* and *E. faecium* strains and *S. aureus*, including MRSA, up to group A streptococci.[50] Mixing endolysins with different sites of interaction at the peptidoglycan layer seems to create a synergism as indicated by higher killing rates.[50–52] Synergistic effects of antibiotics together with endolysin have also been reported.[41,44]

Since the outer membrane of Gram-negative bacteria protects the peptidoglycan from potential contact with exogenous endolysin, reports about the successful use of endolysins against Gram-negative pathogens are scarce in the literature. One of the few examples is a report in which the lytic potential of five endolysins from bacteriophages of Gram-negative bacteria (e.g. *E. coli* and *K. pneumoniae*) was tested *in vitro*. All endolysins exhibited a broad lytic activity on the peptidoglycan of several Gram-negative bacteria when the outer membrane was initially removed. However, no lysis occurred on intact cells, apparently due to the inability of the endolysins to penetrate the outer membrane.[53] The broad spectrum of lytic activity against these Gram-negative bacterial cells lacking their outer membrane may be easily explained. In contrast to endolysins targeting Gram-positive pathogens, those against Gram-negative bacteria do not necessarily need to have a specific bonding to the peptidoglycan of their host. Since they cannot work exogenously through

the outer lipid layer membrane, there is no risk of accidentally lysing further host cells that are needed for the phage progeny. The outer lipid layer membrane may be a barrier for most endolysins. However, a notable exception to the rule is *A. baumannii* phage endolysin LysAB2.[54] This had broad *in vitro* antimicrobial activity against a variety of Gram-negative and Gram-positive bacteria including a multidrug-resistant *A. baumannii* strain, MRSA and *E. coli*. The authors suggest that this broad lytic feature is due to a C-terminal domain containing amphipathic helices, which interfere with the outer cell membrane, thereby enhancing permeability.[54] This finding is highly interesting, since low membrane permeability is one intrinsic resistance mechanism of Gram-negative pathogens. Orito *et al*.[55] described a similar activity of *Bacillus amyloliquefaciens* phage endolysin which expressed bactericidal effects against *P. aeruginosa* strain PA01 when given externally. As *P. aeruginosa* is intrinsically resistant to many classes of antibiotics due to its low outer membrane permeability,[56] increasing its permeability would be a major step towards curing infectious diseases caused by multidrug-resistant *P. aeruginosa*. There is now a need for *in vivo* experiments to verify the dual function of these endolysins (i.e. membrane permeabilization and cell wall lysis) against Gram-negative species. In addition, it has to be considered that, due to the relatively broad lytic activity, the beneficial microflora might also be affected. Hence, those endolysins are probably not suitable for everyday therapeutic use. Even an interference with mammalian lipid layers may be possible, so experiments with human cell cultures are required to rule out any future risk. Furthermore, resistance might evolve since peptidoglycan is not the only target structure of those endolysins.

In summary, endolysin therapy against Gram-positive pathogens in particular is promising and holds several advantages over the use of phages for therapy. Endolysins are effective immediately (there is no lag phase as in the case of lytic phages), and there is no risk of the transduction of virulence factors. In addition, the most compelling advantage is that, to our knowledge, no resistance to endolysin has so far been observed.[45,49,57–59] This might be the result of a long-term co-evolution between phages and bacteria such that endolysin targets components of the cell wall that cannot be easily altered by the bacterium.[45] For instance, the pneumococcal bacteriophage Cp-1 encodes an endolysin that has been shown to be evolutionarily related to the host bacterium's autolysin.[60] This means that mutation of the host in order to prevent the action of endolysin would therefore inevitably also affect the autolysin and ultimately the proliferation of the host cell.[57]

Combination therapy with phage and antibiotics

An increased antibacterial effect of the combined use of antibiotic and phage compared with use of each agent alone has been observed in multiple studies.[61–67] If anything, resistance evolved less frequently with dual therapy,[64,68,69] because a strain non-susceptible to one agent could be eliminated by the second.[70]

Furthermore, the eradication of *K. pneumoniae* biofilms was successful, apparently due to the phage-encoded enzyme depolymerase, whose polysaccharide-degrading activity paved the way for the antibiotic to penetrate the biofilm.[61,67,71]

The overall synergistic effect of the phage and the antibiotic could be increased by genetic engineering. Lu and Collins described such an enhancing effect of dual therapy that was based on a modified non-lytic filamentous phage as an antibiotic adjuvant.[72] The aim was to make *E. coli* more susceptible to antibiotic therapy by manipulating structures inside the cell that enhance the effect of the drug. Repression of the SOS response of *E. coli* through phage-encoded genes resulted in reduced selection pressure and therefore decreased antibiotic resistance. Killing efficacy was several orders of magnitude higher than with regular dual therapy. No recovery of the treated bacteria after a period of either 12 or 24 h was detected. However, since not all strains were eliminated by this treatment,[72] there must have been bacteria resistant to both the phage and the antibiotic.

A special form of the combined use of phages and antibiotics, termed phage–antibiotic synergy (PAS), is based on the observation by Comeau *et al*.[73] that sublethal concentrations of certain antibiotics can strongly stimulate the production of virulent phages in *E. coli* cells. An altered physiology of the host bacterium due to these low doses of antibiotic seems to be the cause.[73] Understandably, it is essential for the concentrations to be sublethal, as keeping bacterial cells alive is obligatory for phage replication. It has been observed that PAS occurs only with some phages and only with certain antibiotics. Successful antibiotics were β-lactams (e.g. cefotaxime),[73,74] quinolones, mitomycin C[73] in the case of *E. coli* and gentamicin, tetracycline, chloramphenicol and carbenicillin[75] in the case of *P. aeruginosa*.

The combined use of a phage and even lower (i.e. subinhibitory) concentrations of ceftriaxone also led to a decrease in growth of the bacteria.[76] The effect was, however, observed for only one phage out of a variety tested. In agreement with Comeau *et al*.[73] cell elongation and enhanced filamentation were observed in those cases. It seems that cell elongation fosters the synergistic effect of the phage and the antibiotic.[76]

A possible drawback of PAS *in vivo* is that sublethal concentrations of an antibiotic may provoke resistance in bacteria that are not the intended target of the phage. Consequently, phages and low doses of antibiotics should only be applied together when their effective spectrum is of similar range. Conversely, PAS could be considered advantageous when the concentration of the antibiotic is significantly below the MIC as no selection pressure is then exerted on most bacteria. In conclusion, the type of antibiotic and phage and the order of their application influence the success of dual therapy. As long as experimental designs have not ruled out the possibility of these sublethal doses of antibiotic causing resistance within the natural human microflora, it is disputable whether or not this treatment should be viewed as a reasonable alternative to other discussed therapies.

'A stitch in time saves nine': phage-mediated prevention

It is well recognized that temperate phages can transfer foreign genes into their host bacteria, including genes that confer resistance to a given antibiotic.[77] It is therefore conceivable that temperate phages could be used for exactly the opposite, namely to retransfer susceptibility genes into bacterial strains that have become resistant to certain antibiotics. The intriguing benefit would be that infections caused by multidrug-resistant bacteria could then be treated with well-established drugs with which the physician is familiar. Proof-of-principle was previously

provided by Edgar *et al*.,[78] who demonstrated that resistance to the antibiotics streptomycin and nalidixic acid could be reversed in *E. coli* by using engineered temperate phage λ, which inserted the dominant-sensitive wild-type genes *rpsL* and *gyrA* into the bacterial genome. *rpsL* codes for the bacterial 30S ribosomal subunit and is mutated in many cases of streptomycin resistance.[79,80] Mutations in *gyrA*, which result in an altered gyrase, can cause quinolone resistance.[81] Drastic decreases in the MICs of both streptomycin and nalidixic acid were observed after the successful integration of the engineered phage into the *E. coli* genome. This means that bacterial susceptibility to both antibiotics was restored despite the continued presence of the resistant alleles in the same bacterial cell. This concept of reversing resistance could be a cornerstone for prophylaxis, which means that bacterial pathogens are being attacked prior to the event of an infection, with a prepared phage solution acting as a quasi 'surface disinfectant'.[78] To this end the phages would be dispersed on critical hospital surfaces where they would infect the nosocomial multidrug-resistant pathogens that inevitably colonize the surfaces over time. Instead of being killed (minimizing selection pressure against phage resistance), bacteria would become susceptible to one or more antibiotics. The co-introduction of a tellurite-resistance gene by the temperate phage would provide a selective advantage over non-infected pathogens, thereby ensuring a gradual replacement of resistant bacteria by lysogenic (i.e. resensitized) bacteria on hospital surfaces upon treatment with the antimicrobial agent tellurite.[78,82] The compelling advantage of such an elaborate system would be that it would not be necessary to administer phages directly to the patient, which would circumvent many problems associated with conventional phage therapy. Clearly, this approach is only possible in cases where the drug susceptibility genes are dominant over the resistance genes. Therefore, only certain antibiotics come into consideration, such as streptomycin, nalidixic acid or trimethoprim.[78] In conclusion, further dominant susceptibility genes for antibiotics, which are more applicable against ESKAPE-related infections, need to be identified.

In a similar research study Bohnert *et al*.[83] used λ phage-based homologous recombination to change the resistance phenotype of a multidrug-resistant *E. coli* strain. While reversal of resistance was not the initial focus of the study, they were able to show that a single mutation in an efflux pump could result in very different antibiotic susceptibility phenotypes. The engineered strain was generated from an *E. coli* E12 strain by selection with increasing concentrations of levofloxacin. Two levofloxacin-resistant strains emerged, one with the wild-type efflux pump gene *yhiV* and one with a single mutation in this efflux pump that caused multidrug resistance. Both had different susceptibility phenotypes (different MICs) for a variety of antibiotics. Similar to the examination by Edgar *et al*.[78] λ phage-based homologous recombination caused inactivation of the mutated efflux pump gene and the reintroduction of a wild-type efflux pump into the cell. The fact that engineered strains had phenotypes similar to that of the second levofloxacin-resistant strain with the wild-type *yhiV* rather than to the original *E. coli* E12 strain[83] shows that the evolution of resistance is a complex field in which more than one pathway can be affected in altered drug resistance phenotypes. Overall, as efflux pumps contribute in large part to multidrug resistance,[84] there may be great potential in further research in this field. Of course, pathogens should not carry fitness costs when being made resusceptible.

Another strategy for phage-mediated prevention could aim to inhibit the spread of antibiotic resistance genes in bacterial communities. This could be achieved with the use of conjugative plasmid-dependent phages, such as PRD1, which only infect bacterial cells containing conjugative plasmids.[85] Given that many resistant bacteria harbour conjugative plasmids encoding antibiotic resistance genes, their selective elimination would strongly suppress horizontal gene transfer and thus the rise of antibiotic resistance. Successful restriction based on phage PRD1 of an antibiotic resistance gene transfer between a susceptible and a multiresistant *E. coli* strain has previously been demonstrated even under conditions of non-lethal antibiotic selection.[86] Hence, unlike in conventional phage therapy, plasmid-dependent phages could be used to assist an empirical antibiotic treatment and prevent non-lethal antibiotic concentrations promoting the transfer of antibiotic resistance genes within the human microbiome.

Expanding knowledge of naturally occurring phages in healthy humans

It is common knowledge that phages are present in the environment, such as in soil and water,[3] but little is known about phage diversity within the human virome. At present, only a small number of studies have explored the different anatomical sites in humans for the presence of phages. In addition, these studies were performed to address different questions and consequently different techniques were used, making the current picture of human phage diversity rather sketchy. Promising elaborate techniques, such as metagenomic analysis, nowadays enable the identification of phages without the need for their cultivation. Nonetheless, cultivation studies are still important in order to gain insight into the host range of newly identified phages and into the environmental requirements for infecting a bacterial host. The following examples therefore include both metagenomic and cultivation-based studies on phage diversity within humans (Figure 1c).

According to two different reports, phage diversity on human skin seems fairly restricted. Based on a metagenomic sequencing approach, Foulongne *et al*.[87] found only a small proportion of phage DNA within the human skin microbiome. *Microviridae* and *Siphoviridae* phages of common skin-colonizing bacteria were mainly identified. Their proportional fraction was comparable to that of their bacterial host.[87] This agrees with a cultivation-based study in which 11 siphoviral lytic *Propionibacterium acnes* phages were characterized from the sebaceous follicles of donors. A genome analysis of isolated phages revealed high similarities between isolates from geographically distinct individuals.[88] However, it was notable that these phages were very efficient in the lysis of a broad range of *P. acnes* strains, making them interesting candidates for therapeutic purposes.[88]

In another cultivation-based study no lytic phages against *S. aureus* were found in the anterior nares of 202 individuals including patients and medical staff, regardless of whether or not they were *S. aureus* carriers.[89] Lack of phage detection in this study was related to the fact that only one particular *S. aureus* strain was used for the plaque assay technique. This raises the possibility that individuals may nonetheless harbour specific phages capable of infecting only particular *S. aureus* strains. Likewise, by testing the same set of individuals, lytic phages

against *S. epidermidis* were found in only 5.5% of subjects.[90] Again, only one reference host strain of *S. epidermidis* was used, bearing the risk of an underestimation of the true phage population. The overall occurrence of phages in the nares might in fact be substantially higher considering that they primarily exist as temperate phages.[91]

Based on metagenomic analysis, respiratory tract samples were demonstrated to contain high phage diversity. Phages of *S. pneumoniae*, *Haemophilus influenzae*, lysogenic *Brucella melitensis* and staphylococci had the highest prevalence of detection and it was speculated that these bacteria were most likely air-borne.[92]

Healthy individuals were the focus of two further metagenomic analyses of either oropharyngeal swabs[93] or saliva.[94] Viral communities consisted almost entirely of phages, many of which carried virulence genes. Both temperate and extracellular phages were found. Unrelated persons living together had more similarities, but still each virome was found to be unique.[94] At least 10% of phages were evaluated as being definitely temperate, as a result of containing integrase genes.[94] In essence, it can be concluded that phages are abundant and permanent members of oral microbial communities. On the other hand, Hitch *et al.*[95] assumed that the oral ecosystem is not heavily influenced by interactions between bacteriophages and their hosts, a finding based on the low recovery of oral phages by isolation techniques. Again, cultivation based methods may strongly underestimate the true diversity of lytic phages; moreover, temperate phages are entirely overlooked.[95,96]

Several recent examinations have revealed the presence of phages of Firmicutes, Proteobacteria and Bacteroidetes in the human gut virome.[97–101] Virus-like particles (VLPs) were discovered,[99–101] although a majority of phages were temperate.[97,100–102] These findings have led to the assumption that temperate phages are more important than lytic phages for the functioning of the gut microbiota.[98,101,103] However, low numbers of VLPs may not necessarily reflect the true extent of phage-mediated bacterial lysis in the human gut as VLPs may be constantly removed by the immune system.

Clearly, temperate phages can generate a selective advantage for their host. For example, antibiotic resistance genes coding for β-lactamases have been confirmed in gut phages,[101] which implies constant selection as long as β-lactam antibiotics are ubiquitously present, e.g. in food.[104,105] When considering temperate phages as therapeutics, the increase in fitness of the targeted bacterial host is therefore an important issue.

Metagenomic analyses of the VLPs and total DNA of faeces samples have shown that each study participant keeps a stable and unique phage profile over time.[101,106] In addition, a non-negligible proportion of phages is shared between persons from close (78% shared bacteriophages between Europeans) and even geographically further distinct (12.4%–16.3% shared bacteriophages between Europeans and US citizens) populations.[101] In addition, the human gut viromes of related persons[100] and individuals on the same diet[99] are more alike.[99,100] Overall, the high degree of interpersonal diversity of human gut bacteriophages may reflect the recognized interpersonal diversity of bacterial host populations, but may also be influenced by rapid within-individual viral evolution.[99]

Finally, it is important to note that the microbiome of faecal samples differs from the microbiome of the intestinal mucosa[107]—and the same may apply to the gut virome. Therefore data obtained from faecal samples have to be viewed with caution as they may not reflect the virome of the intestinal mucosa.

Temperate phages were also found to be common in vaginal lactobacilli.[108,109] These temperate phages were eventually able to become lytic. A promising aspect is that temperate phages from several lactobacilli showed lytic activity towards another strain. This might be a common feature of phages to circumvent total elimination from their host, which would be disastrous for self-replication. Interestingly, lysogens were infected by phages—either by the same or by different ones.[109] This is puzzling, given the generally accepted theory that temperate bacteria cannot be infected by a second, genetically related phage.[4] However, since many phages possess antiphage defence systems,[110] superinfection may be a rare event and/or only relevant for certain phage species. More research on this issue is clearly warranted.

While all of these examples are not exhaustive, they clearly confirm the existence of a wide variety of phages in the human ecosystem. Even blood samples from healthy donors have been reported to contain phage DNA, related to, among others, *Chlamydia* phage φCPAR39 and *Methanobacterium* phage psiM2.[111] In addition, a low level of natural antiphage antibodies was discovered in patients who had not previously been treated with a phage preparation.[112] Interestingly, human anatomical niches seem to represent natural reservoirs of phages even for potential pathogenic bacteria such as *E. coli*,[113] *E. faecalis*[96] or MRSA.[91] Evidence exists that the ratio of natural phages to bacteria seems to have an impact on human health and might be unbalanced during infection.[94,114,115] Gaining more knowledge regarding natural phage diversity and phage–bacterium interactions within the human microbiome of healthy and diseased individuals might therefore lead to new concepts for treating or preventing endogenous infections.

Conclusions

Most of the evidence depicted above supports phage therapy as a promising alternative in the fight against multidrug-resistant pathogens, such as the ESKAPE organisms. As these pathogens seem to be of narrow genetic diversity, they may be the ideal target for phage therapy. For example, 75% of antibiotic-resistant childhood pneumonias are caused by only 10 different strains of *S. pneumoniae*,[116] and 70% of MRSA cases in countries around the world are caused by only five distinct *S. aureus* strains.[117] Currently, however, no phage applications are approved in the USA or European Union. Recent human studies confirm the safety of phage therapy in healthy individuals, while efficacy studies show mixed but encouraging results. The engineering of phages is steadily increasing so that designing optimal phage therapeutics may be an option in the near future. Engineered non-lytic phages hold the advantage of minimal endotoxin release while excluding the spread of genetically modified phage progeny. In addition, the use of phage enzymes, such as endolysin, is highly promising, particularly against Gram-positive bacterial infections, as no resistance and no neutralizing effects by antibodies have so far been observed. Likewise, dual therapy with a phage and antibiotics results in enhanced bactericidal effects with low rates of resistance. PAS is not likely to become established as antibacterial therapy, as it may lead to the emergence of antibiotic resistance

in non-targeted bacteria. In any case, the type of antibiotic, phage and order of application influence the success of this combinational therapy. As it is not yet possible to predict the definite outcome of dual therapy, other therapies should be preferred. Reversing resistance via phage-mediated gene delivery is an enterprising approach, although still in its infancy. This concept may more easily find general acceptance as it circumvents direct application of the phage to the patient. Although the general safety of direct phage application is supported by the fact that phages are ubiquitous commensals within the human ecosystem, there is a definite need for further studies that explicitly address this issue. A prime example is the comprehensive evaluation of an already-in-use Russian phage cocktail against *E. coli* and *Proteus*, which did not lead to allergic reactions or an increase in antibodies against the phage when applied orally to either healthy adults or children.[118] Whether or not a phage causes adverse effects in an individual is probably dosage dependent. Therefore, safety information is also needed from patients with infections, as the administered phages would multiply in the presence of the pathogenic host bacteria. Nonetheless, the testing of healthy individuals is the first step necessary. The development of resistance against phages is frequently raised as a concern. However, the term 'phage resistance' is somewhat rigid or misleading and cannot really be compared with the awkward situation of resistance against multiple antibiotics. The innovative potential of phages in order to conquer bacterial resistance is excellently illustrated in a recent example in which a phage simply 'turned the tables' by means of encoding its own CRISPR/Cas adaptive response (which is the so-called adaptive immune system response of bacteria), thereby efficiently evading the innate immunity of the host.[119]

In order to establish any form of phage therapy in the Western world, presumably the most important aspect is to learn much more about the true natural phage diversity within the human microbiome and about the interactions of phages with their bacterial hosts and with our own immune system. The relatively low recovery of phages from human samples by cultivation efforts might strongly reflect our ignorance of the correct host strain and the proper conditions required for infection. This gap in knowledge can be filled by future metagenomic analyses. The omnipresence of phages at high abundance within healthy humans strongly suggests that, from a global point of view, most phages are harmless commensals that shape bacterial populations and thereby probably even balance bacterial homeostasis.[94,114] This is supported not only by the fact that phage viromes differ between healthy and sick individuals,[92] but also by recent evidence of an evolutionarily stable symbiotic relationship between phages and their metazoan hosts providing a hitherto unrecognized antibacterial defence that actively protects mucosal surfaces.[120] This finding has led to the compelling hypothesis that animal cells including human cells use phages as weapons against invading bacterial pathogens.[120–122] From this perspective, nothing is more obvious than making use of the natural enemy of bacteria in the treatment of infections. Future research should aim to provide further confidence in the efficacy and safety of phages and to identify and characterize novel phages as tools against bacterial pathogens.

As a final remark, patients suffering from multidrug-resistant pathogenic infections should soon be allowed access to phage therapy to ultimately achieve an 'ESKAPE' from the threat of untreatable bacterial infections.

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