Approaches to optimize therapeutic bacteriophage and bacteriophage-derived products to combat bacterial infections

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Received: 28 May 2019 / Accepted: 18 January 2020 / Published online: 8 February 2020
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Abstract
The emerging occurrence of antibiotic-resistant bacterial pathogens leads to a recollection of bacteriophage as antimicrobial therapeutics. This article presents a short overview of the clinical phage application including their use in military medicine and discusses the genotypic and phenotypic properties of a potential “ideal” therapeutic phage. We describe current efforts to engineer phage for their improved usability in pathogen treatment. In addition, phage can be applied for pathogen detection, selective drug delivery, vaccine development, or food and surface decontamination. Instead of viable phage, (engineered) phage-derived enzymes, such as polysaccharide depolymerases or peptidoglycan-degrading enzymes, are considered as promising therapeutic candidates. Finally, we briefly summarize the use of phage for the detection and treatment of “Category A priority pathogens”.

Keywords
Antibiotic resistance · Biofilms · Category A priority pathogens · Genetic engineering · Military medicine · Phage application · Phage-derived enzymes · Therapeutic phage

Introduction
The idea to use phage as treatment option for bacterial infections came up immediately after the discovery of bacterial viruses (bacteriophage or phage) one century ago. As early as in the 1920s, an institute for bacteriophage was founded in Tbilisi, Georgia (former Soviet Union). In the following “era of antibiotics”, the potential significance of phage as therapeutics became underestimated. Under the pressure of global emergence of antibiotic-resistant bacteria and supply shortages for certain antibiotics, the idea of phage therapy has been revitalized during the last years; many institutions and commercial companies are currently engaged in this field. There are case histories describing successful phage therapy of single patients; however, the number of controlled clinical studies, particularly those demonstrating efficacy to modern standards, is still limited. Besides the treatment of antibiotic-resistant bacterial pathogens, phage can be applied as biological control agents for pathogen detection and identification or for food and surface decontamination. Molecular engineering of phage or phage-derived enzymes could improve its practical use for these applications. Certainly, detection and treatment approaches of Category A priority pathogens will benefit from these developments. Figure 1 summarizes main targets for phage application studied currently.

Antibiotic-resistant bacteria have emerged globally, mainly due to antibiotic overuse and misuse, resulting in increased morbidity rates, mortality rates, and healthcare costs. On reviewing medicinal literature and experts’ opinion, Bassetti et al. stated that in the last decade, all attempts...
of infection prevention and control were unable to circumvent the rapid spread of resistant bacterial pathogens. The authors estimate that this antibiotic resistance crisis will cause worldwide 10 million deaths per annum in 2050 [1]. Therefore, the work on alternative strategies to reverse this trend and to provide a means to treat these pathogens is indispensable [2–6]. Phage therapy is one promising route.

Bacteriophage are viruses of bacteria and as obligate cellular parasites have mainly two options to interact with the bacterial host: a lytic or a lysogenic growth cycle. During the lytic way, phages use the cellular processes and resources to produce tens to hundreds of progeny phages, which exit the host cell with or without killing. During the lysogenic way, the phage integrates its genome into the bacterial chromosome and is replicated as part of it (in some cases the phage keeps an extrachromosomal form) [7]. Under certain intracellular and/or environmental conditions, this process is reversible and the phage genome can be excised and can switch back to the lytic cycle. These so-called temperate phages can decide in every infection cycle whether to replicate and lyse their host or to lysogenize and keep the host alive.

Clinical use of phage

Short overview

Among all scientists more than one century ago who described lytic effects in bacterial colonies, which could have been caused by an agent called bacteriophage today (cp [8]), the Franco-Canadian Felix d’Herelle, in 1917, forwarded the most conclusive description of this multiplying agent. From the beginning, he forced the use of phage for therapeutic measures to kill pathogenic bacteria. In the twenties and thirties of the twentieth century, when he helped to establish the bacteriophage institute in Tbilisi, Georgia, he even wrote a book “Bacteriophage and the phenomenon of recovery” in Russian language [9]—see Fig. 2. Since then, this institute is a leading institution in the production and use of therapeutic phage. However, it has been argued that only few systematic studies were performed in Tbilisi and the clinical trials did not comply with internationally approved standards. This appraisal might have not considered those facts as military secrecy in Soviet Union, loss of documentation, and language barriers. Therefore, the Tbilisi colleagues published conclusive overviews demonstrating the methods and results of their studies [8, 10, 11].

Until World War II, phage preparations were produced and used in Europe, Asia, and America [12]. One has to envision that, at this time, nobody had beheld these virus particles. By use of the newly developed electron microscope, Helmut Ruska, a medical doctor at the Charité Medical School in Berlin, visualized phage and phage-induced lysis of bacteria for the first time (cp. [13]).

Production of phage preparations for therapeutic use continued also after World War II but ceased during the next decennia because of the “triumphal procession” of antibiotics. Instead, phage became momentous experimental models contributing to the rapid development of molecular biology [14]. Scientific milestones such as the heredity role of DNA, the uncovering of the genetic code, the discovery of restriction–modification systems, and CRISPR-Cas in bacteria or the capability of viruses to integrate into the host genome are due to basic research on phage.

Phage preparation in France stopped as late as in the 1990s. This decision appears something paradoxically since this was the time of the beginning “antibiotics crisis”. However, French pharmaceutical legislation responding to the AIDS crisis forbade any quantities of ‘viruses’ in medicinal products [11]. From the discovery of bacteriophage at the beginning of the twentieth century till nowadays, they were applied to cure bacterial infections in the former Soviet Union and in Poland, but were more or less ignored in Western medicine [15].

Numerous excellent overviews have been published on the history and clinical use of phage therapy [2, 15–25]. Until today, the number of controlled clinical studies to
demonstrate the efficiency of therapy is limited and studies did not demonstrate credible efficacy in all cases. For major controlled studies, see references [26–30]. Older studies have been summarized by Abedon et al. [11].

Use of phage in military medicine

Even the discovery of bacteriophage by Felix d’Herelle had a military origin. 1915, during World War I, a group of soldiers in the outskirts of Paris suffered from a severe dysentery outbreak. Working with their fecal samples, d’Herelle observed an activity lysing bacteria, which could not be retained by bacterial filters [31].

During World War II, phages were used for the treatment of mainly intestinal and wound infections, which frequently occurred in the field, e.g., dysentery, gangrene, cholera, and infections by staphylococci. In the Soviet Union, the army was provided with huge amounts of phage in the form of vials and pills. Not only the institute in Tbilisi but also some other places in the Soviet Union were included in this mass production of phage, which were used for therapeutic and prophylactic purposes as well [12, 15, 32]. First use of phage in this war happened in November 1939 when Soviet troops started a campaign against Finland and undertook phage treatment of anaerobic wound infections leading to gangrene [31]. It has been reported that during the great battle of Stalingrad in 1942, when cholera outbreaks affected German and Soviet troops, phage was manufactured in a local Russian underground laboratory and has rescued the life of many soviet soldiers [33].

The German army used “Polyfagin,” produced by the “Behringwerke Leverkusen,” with the purpose to treat or prevent dysentery in soldiers. Polyfagin was a suspension containing phage specific for different Shigella strains. The fluid was given per os; to avoid phage inactivation during passage through the stomach, previous administration of a buffer substance (sodium bicarbonate) was recommended. The German army doctors observed some prophylactic and curative effect by Polyfagin. After their victory in Africa, the Allied troops confiscated some amount of Polyfagin from the Germans and used it—with limited success—to treat dysentery in a prisoner camp. Later one was found in the laboratory that the antibacterial activity of phage preparations was only low [12]. The US Army was also engaged in
the development of phage therapeutics but it seems that the general opinion was rather skeptic [12]. Prophylactic and therapeutic uses of phage by military forces continue till today as reported from Georgian army during the 1991 and 1992 battles in Abkhazia and in the conflict with Russia in 2008 [34].

Today, in consideration of lethal infections by so-called ESKAPE pathogens (antibiotic-resistant Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, Enterobacter species), which are of particular concern to injured soldiers, phage therapy is being re-evaluated also in military medicine and, moreover, even the generalized (intravenous) application of phage is accepted as treatment option. Recently, the US Navy was engaged in the successful treatment of a severely ill patient who consequently survived a highly critical Acinetobacter baumanii infection; the phage cocktail was given intravenously and percutaneously [35, 36].

In Europe, a multicenter study, “PhagoBurn”, has been performed to evaluate phage therapy for the treatment of burn wounds infected with Escherichia coli and Pseudomonas aeruginosa; the French Ministry of Defense (through its Military Health Service and Percy Military Hospital) acted as Project Coordinator (https://www.phagoburn.eu/). Unfortunately, the trial had to be stopped because of insufficient efficacy of the treatment. Some reasons for this failure have been discussed [26, 27]. Nevertheless, the Queen Astrid Military Hospital in Belgium—also involved in the PhagoBurn study—plays an important role in the first routine treatments with phage in the West since the 1980s under the Magistral Framework [37].

Approaches for detection and treatment of “Category A priority pathogens”

The US National Institute of Allergy and Infectious Diseases (NIAID), together with the Centers for Disease Control and Prevention (CDC), has categorized dangerous pathogens. According to their definition, category A pathogens are those organisms/biological agents that pose the highest risk to national security and public health because they (i) can be easily disseminated or transmitted from person to person, (ii) result in high mortality rates and have the potential for major public health impact, (iii) might cause public panic and social disruption, and (iv) require special action for public health preparedness. The list of Category A pathogens includes some viruses (smallpox and other related pox viruses, hemorrhagic fever viruses as members of arena-, bunya-, hanta-, flavi-, and filoviruses) and the following bacteria: Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), and Francisella tularensis (tularemia) (https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens).

Most progress was made with Bacillus anthracis phage, which are able to interact with encapsidated, non-encapsidated, and spore forms of this pathogen [38]. Members of this phage group can be used for pathogen detection, spore decontamination, foodborne pathogen disinfection, and anthrax treatment [39]. Engineered B. anthracis reporter phages, which transduce bioluminescence markers to anthrax bacteria and their spores, were developed for pathogen detection [40]. Bacillus phage lysin was shown to act against vegetative cells and germinating spores of Bacillus anthracis and can be used as tools for treatment and detection of B. anthracis [41, 42]. Moreover, it was shown that a capsular depolymerase encoded by a B. subtilis phage improved phagocytic killing of encapsulated Bacillus anthracis both in vitro and in vivo [43, 44].

Very recently, a vaccine simultaneously protecting against infections by two category A pathogens, Bacillus anthracis and Yersinia pestis, was developed on the basis of a phage T4 platform. The bacterial antigens were fused with a small outer capsid protein of T4. In animal models, the vaccine provided protection against inhalational anthrax and/or pneumonic plague [45].

Yersinia pestis phages were used in first treatment experiments of plague. However, open questions remain, e.g., the susceptibility of bacteria intracellularly located in macrophages, the danger of endotoxic shock, and the introduction of an animal model better suited than mice [46]. Bioluminescent Yersinia pestis reporter phage can be used for rapid detection (and simultaneous antibiotic susceptibility analysis) of plague bacteria [40, 47].

The list of Category A pathogens-associated bacteriophage is mainly limited to B. anthracis and Y. pestis phage [48]. Progress in the practical use of Clostridium botulinum phage seems to be rather limited because cell killing of this endotoxin-containing bacterium can be dangerous for the organism [12]. For some forms of the disease, uptake of the botulinum toxin instead of bacterial infection is sufficient and, moreover, some toxins are encoded by prophage in the bacterium [49]. Even less is known about phage of Francisella tularensis and their practical use [50]: https://pcwww.liv.ac.uk/~hallison/EMBOVoM2016/data/abstracts/abstract_213.html).

Scientific challenges to select the “ideal” phage

In this chapter, we will try to define criteria for the selection of therapeutic phage. Since a complete genomic characterization of phage will be a necessary precondition for their approval in a regulatory framework [51, 52], we will also
discuss the use of genomic data for a putative prediction of relevant phenotypic properties of phage.

**Temperate vs. virulent character of phage**

It is generally accepted that phage for therapeutic use should be strictly virulent (lytic) without ability to lysogenize the target cell. In other words, all temperate phages have to be excluded from the collection of potential therapeutics. The lifestyle of a phage—virulent or temperate—can be analyzed by microbiological techniques [53].

Based on genome sequencing, the findings of repressor motifs putatively speak for a temperate phage. Detection of integrase or transposase motifs or genomic attachment (att) sites should also be important for the classification of a phage as being obligatory temperate. However, it is known that certain temperate phages do not need to integrate their genome into the cell chromosome, but are maintained in an episomal state. There are also attempts to distinguish virulent vs. temperate phage by in silico analyses of phage genome compositions including relative abundances of di-, tri-, and tetranucleotides from entire phage genomes [54, 55].

**Presence of virulence, toxin, or resistance genes**

Phage genomes should be screened to avoid the presence of these potentially dangerous genetic determinants. However, in rare cases, genomes of virulent phage contain essential genes, which seem to be homologs of pathogenicity-associated bacterial genes. Still, it has been argued that they might be considered as therapeutic phage [56].

**Transduction ability**

Therapeutic phage should not have the ability to transduce bacterial genes, which could include virulence, toxin, and antibiotics resistance genes. Therefore, potential therapeutic phage should be investigated for their general ability to perform transduction. Bothering genes being transduced could be originate from bacteria in the organism of the patient or from cells used for phage production in the laboratory. Therefore, bacterial strains for phage production should undergo control to show the absence of those genes. However, especially for some target bacteria, it may not be possible to find production strains that are both functional and plausibly clean [52, 57].

In generalized transduction any fragment of the bacterial genome can be packaged into phage capsid and transferred to another bacterial cell. This process can occur with members of both virulent and temperate phage. The ability of generalized transduction appears to depend on the genome packaging mechanism of phage. Phage with packaging initiation from non- or low-specific DNA sequences are considered to accept also foreign genetic material during maturation. However, some phages, as classical T3 and T7 [58], require specific sequences in their genomes to initiate DNA packaging; therefore, they should be unable to attach and insert foreign genetic material. Moreover, phages that degrade the DNA of the infected cell and use it as building blocks for their own genomes should be considered as non-transducers [53]. Generalized transducing phages can be predicted by microbiological techniques (testing the ability to transfer easily selectable markers between bacterial strains) or by PCR-based techniques (testing the presence of bacterial DNA in purified phage particles). In addition to the use of sequencing and bioinformatic prediction programs, one can remember classical phage techniques to assess the transduction ability of a phage [53].

In specialized transduction, induction of a prophage can lead to additional packaging of neighboring bacterial genes into the phage particles. Therefore, specialized transduction can be only performed by temperate phage, which, however, should be excluded from therapeutic use. To predict the ability of a phage for special transduction, one can also search for motifs of integrase-encoding genes or attachment (att) regions.

**Narrow vs. broad host range, monospecific phage vs. phage cocktails**

Regarding the host range of bacteriophage, one has to find a compromise between very near and very broad. A host range focused on a particular pathogenic bacterial strain has the disadvantage that even other (pathogenic) strains of the same species might be not affected. On the other side, phage with a broad host range could also kill beneficial bacteria, which are part of the normal microbiome in the organism. Probably optimal are phages, which affect all members of a bacterial species but are without cross-activity against other species [53, 59].

To execute a “personalized medicine” one could define the pathogenic strains in an individual and chose specific phage for their treatment. The personalized medicine model involves the creation of a large bank of phage manufactured in advance as “Active Pharmaceutical Ingredients” (APIs), the members of this bank are then tested for activity against a patient’s clinical isolate using some form of companion diagnostic device, and active phages are then formulated into a medicinal product that is unique to the patient. This model being worked on in both the US and Europe is in opposition to a fixed drug model.

In the “fixed drug model,” use of phage cocktails encompassing phage of different specificity allows the treatment of mixed infections. This could be advantageous for clinical purposes [60]. The broader the host range of the phage
involved, the more economically advantageous the therapy will be. However, the development of phage resistance in each specific phage–pathogen dyad should not be reduced by the presence of other phage in a cocktail.

**Emergence of phage-resistant bacteria**

Under the selective pressure of phage therapy, phage-resistant bacteria can emerge. While certain bacterial strains may remain susceptible to a given phage for tens of generations, cells resistant to the same phage may appear in populations of other strains of the same species after a few generations [53]. To reduce this risk, the use of multispecific phage cocktails is recommended since the times of Felix d’Herelle; however, this principle might have some weakness (see previous chapter).

It has been argued that antibiotic-resistant bacteria, when becoming also phage-resistant, could regain their antibiotic sensitivity. However, the markers of antibiotic and phage sensitivities usually are not coupled—this is the basis of the phage therapy concept. Rather exceptionally, a *Pseudomonas* phage was described, which uses an outer membrane protein of a multidrug efflux system as receptor-binding site; development of phage resistance inhibited the drug efflux and increased the antibiotic sensitivity of the cells [61]. Likewise, phages that bind structural virulence factors such as capsular antigen can select phage-resistant bacterial mutants that lack the capsule and are less virulent because they can be more easily recognized by phagocytes [62].

**Ability to overcome defense systems of the bacterial cell**

After invasion of the bacterial cell, the phages have to overcome various defense systems of the cell; these countermeasures can work on the levels of the individual cell or the bacterial population [63]. Phage genomes can be attacked by systems as the classical DNA restriction/modification or CRISPR-Cas; however, in their evolution, the phages have developed multiple mechanisms to overcome these defense systems [64–66]. Whereas “defense islands” in the prokaryotic genomes can be characterized [67], it is too early to predict the existence of general anti-restriction properties from the nucleotide sequence of the phage.

**Lysis or lysis-free killing of bacteria**

Simultaneous killing of endotoxin-producing cells was traditionally assumed to result in an endotoxic shock in the affected individual. The construction of non-replicative and/or lysis-deficient phage may circumvent this problem [68–71]. However, those phage would be unable to replicate in the cell; this fact contradicts a crucial advantage of phage therapy—the phage enrichment at the site of bacterial infection in the organism. Moreover, at least to our knowledge, cases of the postulated endotoxic shock have not been reported. One could speculate that phages lyse bacteria in a more “gentle” way than bacteriolytic antibiotics known to induce endotoxic shock.

**Use of viable phage vs. phage-derived enzymes**

Figure 3 compares some important characteristics of viable phage vs. purified phage enzymes as antimicrobials. The pros and cons seem to be rather uniformly distributed. Unwanted risks of phage use concern rapid resistance development in the bacteria and genetic instability of phage as replicating pathogens. In contrast, purified phage enzymes as stable proteins could have better chances to get approval by official authorities. However, antimicrobial activity of endolysins in vitro was reported to be dependent on multiple parameters such as pH range, buffer conditions, temperature, and ionic strength nicely reviewed by Oliveira et al. [72]. The authors emphasize that virion-associated lysins (VALs, see below) may have even greater therapeutic potential than endolysins because, as component on the surface of the virion, they have to maintain activity in different environmental conditions. Altogether, comparisons between phage and phage-derived enzymes should attract more attention. Probably, only the combination of different antimicrobial

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![Fig. 3 Pros and cons in the use of viable phage vs. phage enzymes for therapeutic approaches.](image-url)
treatment options (including antibiotics) will increase the repertoire to combat multiresistant bacterial pathogens. For further details, see chapter 8 of this review.

Ability to treat bacteria in biofilms

A particular challenge is the treatment of pathogenic, antibiotic-resistant bacteria, which are parts of biofilms in the organism. Biofilms are complex bacterial communities kept together by an extracellular polymer matrix of exopolysaccharides, proteins, nucleic acids, and lipids. Biofilms represent an effective bacterial survival strategy because bacteria therein reach highly increased protection against antimicrobial agents compared to their planktonic counterparts [73–76].

Phage can be used to reduce biofilms on medical devices in the organism, as maintenance catheters or implants [77]. Some review papers have addressed the possible susceptibility of biofilm to phage and phage-derived enzymes, e.g., [74, 78–81]. For these studies, a critical point is the quality of the in vitro models used and how realistically they are able to simulate the complex in vivo conditions [74, 78–81]. Especially, phage-coded depolymerases that disassemble superficial polysaccharides on bacteria into smaller units are advantageous as they can weaken biofilms and make them vulnerable for an antibiotic treatment.

Phage engineering for pathogen treatment

Phage engineering is an area of research that is attracting intense interest and has great potential to enhance phage antimicrobial activity for pathogen treatment. Several original papers and reviews present techniques successfully applied to engineer phage [82–94]. Companies in several countries already take advantage of the methods described in the following chapter. Sophisticated molecular biological techniques, developments in synthetic biology, and fast and low-cost DNA sequencing methods provide technical preconditions to optimize phage for their increased antimicrobial effectiveness and efficiency [95].

Very recently, the first use of engineered phage to treat a severe disseminated Mycobacterium abscessus infection in a 15-year-old cystic fibrosis patient was reported. Lytic phage derivatives, which effectively killed the pathogen, were developed by Bacteriophage Recombineering of Electroporated DNA (BRED) to eliminate a phage repressor gene and by genetic selection of host range mutants. Intravenous therapy using a three-phage cocktail, conducted for 32 weeks, was well tolerated and was associated with significant clinical improvement [96].

Enhanced antimicrobial activity

To enhance the bactericidal effect, phage can be altered either to enter the target cell or to damage the cell after entry more efficiently. To this end, Scholl et al. designed a T7 construct with the K1-5 endosialidase gene cloned downstream of the major capsid gene of T7 (T7end). The endosialidase was released into the lysate after T7end infection and lysis of the host cells. T7end in contrast to wild-type T7, allowed the phage to infect also capsul-producing E. coli cells [97]. Lu and Collins equipped phage T7 with gene dspB from Actinobacillus actinomycetemcomitans that confers biofilm-degrading activity to T7 by Dispersin B [98]. The engineered T7 phage reduced bacterial biofilm cell counts by two orders of magnitude when compared with wild-type T7.

An alternative approach is to introduce genes into the phage genome, the products of which severely interfere with functional cellular networks. The same authors engineered the non-lytic filamentous phage M13mp18 to produce the Lex3 repressor of the SOS response [99]. Antibiotic treatment induces hydroxyl radical formation that leads to DNA, protein, and lipid damage and eventually to cell death. DNA damage in turn induces the SOS response resulting in DNA repair. By preventing the repair process, the engineered M13mp18 enhanced antibiotic-induced killing of Escherichia coli in vivo and in vitro.

In another study, phage T7 was engineered to express a lactonase enzyme with broad-range activity to disturb quorum-sensing [100]. Most bacteria use this biochemical communication process to coordinate the behavior of individual bacterial cells in a group by small molecules (acyl homoserine lactones, AHL). Quorum sensing is a pre-requisite for biofilm formation that displays an important pathogenicity marker. T7 lactonase produced by a recombinant T7 variant degrades essential small AHL autoinducer molecules and inhibited biofilm formation by a mixture of Escherichia coli and Pseudomonas aeruginosa [100]. A further route is to introduce “lethal genes” into bacteria that (i) restore bacterial susceptibility to antibiotics [101], (ii) reduce the bacterial population [68, 69, 102], or (iii) achieve endocytosis of engineered phage into eukaryotic cells to combat intracellularly replicating pathogens [103]. Other studies showed how phage therapy becomes more effective by encapsulating phage in liposomes delivered to treat intracellular bacteria [104].

Altered host range

Currently, the most common approach to achieve a wider bacterial spectrum is the combination of phage with different host range. An alternative to use such cocktails may be to change and/or expand the host range of a phage by
genetic engineering. There are several examples in the literature where the host range of phage was altered by adding or swapping of receptor-binding domains [105–110]. Improved technologies for phage engineering, DNA synthesis, and DNA sequencing might allow the construction of defined phage cocktails with variable host ranges.

**Development of DNA sequence-specific targeting antimicrobials**

One critical issue with antibiotics is their lack of specificity, which leads to the killing not only of targeted pathogens, but also of non-targeted commensal bacteria. This effect can alter the composition and the balance of a microbiome and result in antibiotic-associated severe infections. To avoid negative side effects, the development of sequence-specific antimicrobials based on controllable CRISPR-Cas nucleases seems a valuable option. Bikard et al. selectively attacked virulence genes of *Staphylococcus aureus* by RNA-guided Cas9 nuclease and thereby killed the virulent, but not avirulent bacteria [87]. The efficiency of this method was confirmed in vivo in a mouse model. Citorek et al. used the same tool to induce cell death or plasmid loss upon detection of genetic signatures derived from virulence or antibiotic resistance genes in carbapenem-resistant *Enterobacteriaceae* and enterohemorrhagic *Escherichia coli* [86]. Yosef et al. used an engineered temperate phage delivering the CRISPR-Cas system together with an engineered lytic phage to re-sensitize bacteria to antibiotics and selectively kill antibiotic-resistant bacterial cells [89].

**Prolonged circulation time in the macroorganism**

Another concern is that the human immune system neutralizes the therapeutic phage, particularly if administered systemically. In a murine model of systemic inflammatory response, the count of viable phage decreased in circulation and in numerous tissues due to the action of phagocytes, antibodies, and serum complement [111]. There are two ways to counteract this problem: (i) the selection of long-circulating phage and (ii) the use of growth- and/or lysis-deficient phage. To avoid the problem of phage elimination by the reticuloendothelial system (RES) of the host, Merril et al. [112] serially injected phage (*E. coli* phage Lambda and *S. typhimurium* phage P22, resp.) into mice to search for phage mutants capable of remaining in the circulatory system for longer times. The isolated long-circulating phage mutants after ten selection cycles had approximately five orders of magnitude higher capacity to evade RES clearance than the wild-type phage tested 24 h after intraperitoneal administration. Lambda phage mutant obtained after selection had a relevant mutation in the major capsid protein E [112]. By using genetic engineering, Vitiello et al. introduced just this single mutation in the major capsid gene and obtained a similar longer circulating phage mutant [113]. Very recently, Nan et al. demonstrated that lytic phage T7, mutated by the expression of CD47-derived peptides, showed prolonged blood circulation and thereby dramatically increased antibacterial activity in mice in vivo [114].

Another idea to aid therapeutic phage to longer circulate in the organism is the use of phage which express orphan DNA methyltransferases (MTases) [115]. These DNA MTases exist solely, without a corresponding restriction endonuclease. Phage genomes with mono- or multispecific orphan DNA MTases confer protection against different restriction endonucleases of their host cell and thereby may prolong the effectiveness of phage therapy [115].

**Phage engineering for pathogen detection**

Phage engineering is also a versatile means for rapid detection and diagnostics of bacterial pathogens. Phages were altered genetically to express transgenes that after replication in the respective host produce luminescence [116, 117] or fluorescence signal [82, 118–121] that easily and quickly can be detected. If the pathogen searched for is absent, no signal appears. Especially important are these manipulated phage if they can detect Category A bacterial pathogens [39, 47, 122–124].

**Phage in selective drug delivery and vaccine development**

Filamentous phages were adapted for selective drug delivery to both prokaryotic and eukaryotic cells. Goals were (i) to amplify the potential of low-efficient drugs by creating a high concentration around the targeted bacterial cell (releasing the drug and tethering the phage), (ii) reduction of general toxicity because the drug is inactive as long as it is linked to the phage, and (iii) re-introduction of non-selective toxic substances as drug candidates against drug-resistant bacteria [125–128]. Very recently, engineered phage T4 was successfully used as vaccine against Category A pathogens. The authors consider phage T4 as a suitable nanoparticle platform to produce multivalent vaccines against high-risk pathogens [45].

**Phage in food and surface decontamination**

Numerous studies focused on the importance of applying phage for food and surface decontamination. Several phage-based products have received regulatory approval for treating food products. ListShield, EcoShield, and SalmoFresh
from Intralytix control the respective foodborne bacterial pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* in foods or food-processing environments. Salmononelex and Listex P100 from Micreos reduce contamination with *Salmonella* and *L. monocytogenes*, resp., during food processing. Agriphage from OmniLytics controls *Xanthomonas campestris* and *Pseudomonas syringae* on tomato and pepper plants [129]. Several attempts were described applying phage in food-producing animals to reduce colonization of the animals with bacterial pathogens, e.g., *E. coli*, *Salmonella* spp., *Campylobacter* spp., and *Listeria* spp.[22, 129, 130].

### Engineering of phage enzymes

Besides complete phage used as direct antimicrobials, also phage-derived enzymes as polysaccharide depolymerases and peptidoglycan-degrading enzymes are promising therapeutic candidates [131–140]. Many bacteria produce high-molecular weight polysaccharides present on the surface of the cell. These polysaccharides contribute to pathogenicity, protect bacteria from phagocytosis, can increase biofilm production, and are physical barriers to phage preventing access to their receptors. Phage have evolved to use polysaccharide structures as their primary receptors by having depolymerization activities in their neck, base plate, and/or tail fibers that degrade polymers to smaller subunits [133]. Phages with this property produce haloed plaques due to the production of free depolymerase enzymes that remove polysaccharides of the neighboring cells thereby conferring a great advantage in biofilm prevention or removal. Depolymerases do not lyse bacteria but sensitize them against antibiotics or other antimicrobials [141–143]. Pires et al. [133] have collected information about all depolymerases encoded by fully sequenced phage. They found 160 putative depolymerases that remove polysaccharides in 143 double-stranded DNA phage infecting 24 genera of bacteria.

Peptidoglycan-degrading enzymes are phage-encoded enzymes that hydrolyze peptidoglycan layers in the bacterial cell wall, one to three layers in gram-negative and up to 40 layers in gram-positive bacteria [72, 144]. These enzymes can be located in the virion, often referred to as virion-associated lysins (VALs), and act during viral DNA entry. These enzymes attack the cell wall from outside and promote the injection of phage DNA. In contrast, endolysins normally accumulate in the cytoplasm and, via pores formed by phage-encoded holins, they reach the peptidoglycans and disrupt them [72, 145]. Endolysins and holins are required for the programmed host cell lysis during progeny phage release in the end of a productive phage replication. Purified lysins also can lyse bacterial cultures in vitro. VALs and endolysins are categorized according to the peptidoglycan bond they cleave into different classes. The enzymatic diversity of these enzymes is remarkable [72].

To exploit endolysins as efficient antimicrobials against gram-negative pathogens with an impermeable outer membrane, they were combined with lipopolysaccharide-destabilizing peptides that render these “artilysins” able to pass the outer membrane [131, 146–148]. Artilysin® Art-175 is very effective also against highly resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [149]. Generally, they are species-specific thereby allowing selective killing of a given pathogen saving accompanying microflora [138]. The effect of the degrading activity of endolysins can be demonstrated in seconds as osmotic lysis of the targeted cell [135]. For an improved antimicrobial activity, endolysins were also combined with holin [150] or depolymerase [151] against *Staphylococcus aureus/S. suis* and *S. aureus* biofilms, respectively. Moreover, the right combination of phage lytic proteins and antibiotics or bacteriocins significantly increased survival in animal experiments [134, 152–154]. Application of phage-derived enzymes in various model systems was excellently summarized recently [134, 135].

Modular organization of phage endolysins in distinct functional domains—catalytic and cell wall binding domain(s) connected by linker sequences—provides the proteins with two useful properties: a remarkable substrate specificity and it allows protein engineering in order to design new proteins with enhanced antimicrobial activities [133, 134, 138, 147–149, 155]. Design of new enzymes by deletion and shuffling of domains, synthesis of truncated proteins, and production of chimeric enzymes by combining domains from different lytic proteins (“chimeolysins”) have shown good results with regard to the development of improved lytic proteins [134, 155, 156]. This includes not only the ability to change binding and catalytic specificity, but also protein solubility and thermostability [138]. Sobieraj et al. reported the fusion of a staphylococcal endolysin to an albumin-binding domain that increased the half-life of the proteins [158]. Wang et al. combined an endolysin from a *Staphylococcus aureus* phage with a cell-penetrating peptide that facilitates crossing of the eukaryotic membrane by the lytic protein and thereby can kill intracellular infections by *S. aureus* [157]. A first product containing an endolysin for use in humans is available on the market (Staphefekt, Gladskin brand). This product is recommended for the treatment of early stages of *S. aureus*-related skin infections [134]. Resistance acquisition to lysins has not been observed so far. This may be due to the fact that their targets in the peptidoglycan molecule are essential for bacterial viability and mutations would be too harmful to the bacteria [134, 159, 160].
A great advantage of phage-derived enzymes over traditional antibiotics is their ability to lyse bacteria in biofilms even if they are non-replicative and are also active against persister cells (see above) [134]. Genetic screens [132, 161] or metagenomic sequences of uncultured viral populations are sources to search for new active phage lytic enzymes [162].

Outlook

In the era of emerging antibiotic resistances, there is a high need to force developments in the therapeutic use of phage and phage-derived enzymes. More controlled clinical studies are necessary to evaluate the usefulness of this strategy and efforts are required to guarantee defined formula, titer, stability, and purity of phage preparations. On this basis, regulatory issues have to be solved—this might be easily manageable for defined standard preparations but requires also clues for “individualized” preparations using phage specifically selected to treat the diagnosed pathogens of a particular patient.

Acknowledgements The authors thank Deutsche Forschungsgemeinschaft for long-term support of their work on bacteriophage and bacterial restriction endonucleases. We are grateful to Bob Blasdel (Leuven) and Udo Bläsi (Vienna) for critical reading of the manuscript and helpful suggestions. Nevertheless, the authors are responsible for all putative weaknesses of the paper.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the authors.

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