BioDrugs (2021) 35:255–280
https://doi.org/10.1007/s40259-021-00480-z

LEADING ARTICLE

Engineered Bacteriophage Therapeutics: Rationale, Challenges and Future

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Accepted: 29 March 2021 / Published online: 21 April 2021

Abstract
The current problems with increasing bacterial resistance to antibacterial therapies, resulting in a growing frequency of incurable bacterial infections, necessitates the acceleration of studies on antibacterials of a new generation that could offer an alternative to antibiotics or support their action. Bacteriophages (phages) can kill antibiotic-sensitive as well as antibiotic-resistant bacteria, and thus are a major subject of such studies. Their efficacy in curing bacterial infections has been demonstrated in in vivo experiments and in the clinic. Unlike antibiotics, phages have a narrow range of specificity, which makes them safe for commensal microbiota. However, targeting even only the most clinically relevant strains of pathogenic bacteria requires large collections of well characterized phages, whose specificity would cover all such strains. The environment is a rich source of diverse phages, but due to their complex relationships with bacteria and safety concerns, only some naturally occurring phages can be considered for therapeutic applications. Still, their number and diversity make a detailed characterization of all potentially promising phages virtually impossible. Moreover, no single phage combines all the features required of an ideal therapeutic agent. Additionally, the rapid acquisition of phage resistance by bacteria may make phages already approved for therapy ineffective and turn the search for environmental phages of better efficacy and new specificity into an endless race. An alternative strategy for acquiring phages with desired properties in a short time with minimal cost regarding their acquisition, characterization, and approval for therapy could be based on targeted genome modifications of phage isolates with known properties. The first example demonstrating the potential of this strategy in curing bacterial diseases resistant to traditional therapy is the recent successful treatment of a progressing disseminated *Mycobacterium abscessus* infection in a teenage patient with the use of an engineered phage. In this review, we briefly present current methods of phage genetic engineering, highlighting their advantages and disadvantages, and provide examples of genetically engineered phages with a modified host range, improved safety or antibacterial activity, and proven therapeutic efficacy. We also summarize novel uses of engineered phages not only for killing pathogenic bacteria, but also for in situ modification of human microbiota to attenuate symptoms of certain bacterial diseases and metabolic, immune, or mental disorders.

1 Introduction

Until recently, the human population had been decimated for ages by epidemics of bacterial diseases and wound infections, infections during childbirths, food poisoning, and diarrhea. In 1900, the average lifespan was 31 years, and even in the richest countries, it did not exceed 50 years. The discovery of antibiotics and their introduction into medical practice in the first half of the 20th century is estimated to have increased the average lifespan by 23 years and is considered the greatest medical breakthrough of the last century [1]. However, the wide use of antibiotics in medicine, veterinary practice, animal production, and agriculture has brought about the emergence and spread of antibiotic-resistant bacterial pathogens, leading to a rising frequency of incurable bacterial infections and causing an estimated 700,000 deaths each year worldwide [2–4]. The most promising candidates for agents that can replace antibiotics, support their action, or revert the antibiotic resistance phenotypes are bacteriophages (phages)—viruses that specifically infect bacteria and are harmless to eukaryotic cells [5, 6]. They have

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Key Points

Recent progress in phage genetic engineering enables deliberate modifications of natural phage isolates to make them more suitable for therapeutic applications by modifying their host specificity and/or improving other properties on demand.

Panels of genetically engineered phages with improved therapeutic properties, increased safety and differentiated specificity, based on the genomic scaffolds of selected well characterized phage isolates, and the ability to acquire them rapidly at a relatively low cost may soon lead to therapeutic applications.

Progress in the understanding of the phage–bacteria interactions in the human/animal microbiome and their influence on health opens up possibilities for the application of genetically engineered phages to modify the microbiome composition and thereby attenuate symptoms of certain bacterial diseases as well as selected metabolic, mental, or immune disorders.

been regaining interest in recent years, not only due to their proven potential in controlling bacteria, but also owing to their contribution to bacterial adaptation to diverse environments [7–9], their abundance and unprecedented diversity [10–12], their major impact on nearly all environments and forms of life [13–17], and the recently expanded possibilities of their targeted modification [18, 19].

The bacterial killing potential of phages, which was appreciated at the time of their discovery, is also the major driving force that has rejuvenated interest in phages today. However, the viral nature of phages makes regulations developed for chemically defined antibacterial drugs inapplicable to phages without substantial changes approved by authorized institutions [20–24]. This requires time. Despite that, the constantly growing incidence of prolonged, chronic, or incurable infections by antibiotic-resistant bacteria, coupled with the amassing experimental and clinical data proving the antibacterial activity and safety of phages in vivo, even upon intravenous application, urges studies on phages by themselves [25–34]. But first of all, it prompts the development of new solutions that would allow humanity to benefit from the antibacterial activity of phages in medical practice. Moreover, recent genomic and metagenomic discoveries and progress in genetic engineering and nanotechnology allow one to see the possibility of therapeutic phage applications much broader than previously anticipated.

Phages are the most abundant entities on Earth, with an estimated number of nearly 10^{31}, exceeding that of bacterial cells by about one order of magnitude [12, 35, 36]. However, only recently has a comprehensive insight into the phageomes of various environments, including human and animal skin, gastrointestinal tract, and other body niches, become possible [17]. During that time, the number of sequenced phage genomes has been rising nearly exponentially [37]. According to the most recent classification of the International Committee for Taxonomy of Viruses, bacterial viruses are divided into 19 families [38–40]. The natural phage isolates selected for therapeutic use belong to the order *Caudovirales* (tailed phages), which currently includes ten families [38, 39]. Their virions consist of a head (capsid), packed with linear double-stranded DNA (dsDNA), and a tail. The end of the tail contains proteins allowing phage adsorption to its specific host bacterium and its penetration through the bacterial cell envelope to introduce the phage DNA [41].

All phages can be seen as delivery vectors. Any genetic material packed within their capsid can be delivered to the phage-specific host and initiate the development of progeny phages and cell lysis or participate in recombination, transcription, or other processes leading to a phenotype change or death of the infected cell. This opens up various possibilities of phage genome modifications that either do not interfere with the natural processes of phage propagation and packaging or require in vitro packaging of the phage DNA. Some changes in the phage DNA may be designed to modify the phage capsid so that it gains affinity to certain bacterial or eukaryotic cells that normally are not the parental phage targets [42–44]. If capsids of such phages are additionally packed with cytotoxin-encoding genes or filled with other cytotoxic compounds or even a recombinant eukaryotic virus genome, they can serve as delivery vectors of their content when, e.g., engulfed by their new target cells [45–51]. Finally, the external structure of a phage virion, which in the simplest phages is composed of only a few types of proteins, enables the use of phages as molecular scaffolds for chemical modifications to conjugate them with already approved drugs (e.g., [52]; reviewed in [53]). An additional advantage of some phages is their ability to penetrate epithelial cell layers and to cross the intestine–blood or blood–brain body barriers, making them promising drug delivery platforms [54–59]. The methods of phage genome modification differ in complexity and depend on the developmental strategies of the phage of interest (depicted in Fig. 1).

2 Engineered Phages as Future Therapeutic Options: Rationale and Perspectives

Traditionally, phages are considered potential antibacterial agents that are an alternative to antibiotics. Their advantages over antibiotics include a narrow specificity range preventing the undesired killing of commensal microbiota, the ability

to multiply at the site of their host location as long as the host persists, the ability to evolve in response to host evolution, and the general lack of adverse effects on human health (reviewed in [29, 60, 61]). Most importantly, phages can cure infections with antibiotic-sensitive as well as antibiotic-resistant bacterial strains and can also be successfully used in combination with antibiotics [62]. Metagenomic data on the human and animal microbiota expand the possibilities of the therapeutic use of phages even further by demonstrating the previously unsuspected influence of the phageome on the quantitative and qualitative microbiome composition, which is critical for health and may lead to autoimmune, metabolic, or mental diseases when disturbed [63–73].

It is generally believed that environmental phage population is so numerous and diversified that there exists a phage for each and every bacterial strain. However, to be considered for therapeutic applications, natural phage isolates must meet certain criteria [74–79]. For obvious reasons the choice is limited to phages that are obligatorily lytic to kill every infected bacterium, do not transfer bacterial DNA, and do not encode any toxins, virulence factors, or antibiotic resistance determinants. Additionally, each phage for therapy should be well characterized at the genomic and proteomic level, as well as at the level of interaction with its host(s) and the host's host. It should efficiently lyse the target bacteria, have the widest possible strain range within a pathogenic species, be sufficiently stable under storage conditions and at the sites of infection by the bacteria, be able to overcome at least some bacterial phage-resistance mechanisms, and cause no undesired immune reactions. Although hundreds of phages targeting bacterial pathogens have been isolated (e.g., [80–91]), none of them fulfills all these criteria, and in fact, none has been characterized with respect to all of them. First, many if not most DNA phages are temperate (Fig. 1) [72, 92–96]. They can stay in the infected bacterium in the form of DNA (a prophage) without causing its lysis and typically encode bacterial adaptive functions, among them toxins, virulence determinants, and regulators of bacterial pathogenicity [97–103]. Also certain obligatorily lytic phages encode distant homologs of virulence determinants whose functions must be determined before considering such phages for medical use (see, e.g., [104]). Second, the temperate and some obligatorily lytic phages can pack bacterial DNA to their capsids in place of or in addition to their own DNA and inject it into newly infected bacteria, thereby spreading the pathogenicity determinants [98, 105–109]. Third, even phages with a wide strain range within a pathogenic species typically do not infect all the strains of this species, and the variability of phage receptors and anti-phage defense systems among the bacteria make this one of the most challenging problems in the development of phage preparations for therapy [110–116]. Numerous anti-phage defense systems act after phage DNA injection and inactivate the infecting phage DNA by cleavage (e.g., restriction-modification systems and clustered regularly interspaced palindromic repeats [CRISPR]-CRISPR-associated proteins [Cas] systems) or activate a suicidal host response or dormancy to prevent the infection spread (e.g., toxin–antitoxin [TA] systems, abortive infection systems). There is a multitude of such systems, with different structural and functional variants, and the mechanisms of action of several of them await elucidation (reviewed in [115, 117]). Moreover, new discoveries constantly expand their list. Additionally, a phage treatment of sensitive bacteria may select for resistance, and there are no standard methods to prevent the development of resistance [115, 118–120]. What is more, the emergence of pathogenic strains among commensal bacteria, e.g., *Staphylococcus epidermidis* [121], requires the

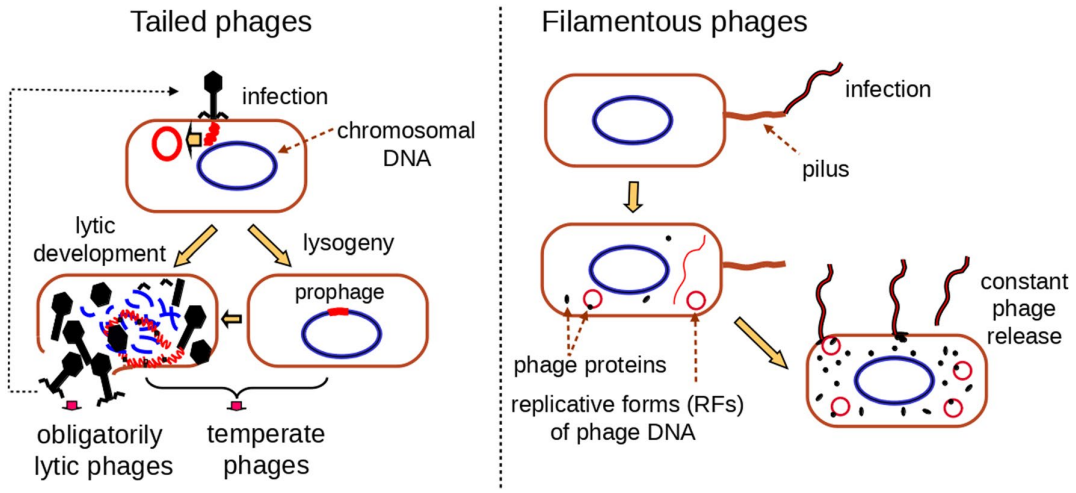

Fig. 1 Developmental strategies of tailed and filamentous phages. Objects in the scheme are not to scale

use of phages that can selectively kill these strains while being safe for the non-pathogenic ones. A major problem in the introduction of phages to medical use is also the limited knowledge of their coding potential and biology. Only a few phages infecting bacteria from about a dozen genera, mostly used for model studies, have been analyzed in detail with respect to their molecular features, physiology, and interaction with the bacteria, and even for these phages the functions of about half of their genes remain unknown [122, 123].

A reasonable strategy to deal with the problem of narrow strain specificity could be the development of well characterized, single-phage preparations targeting various strains of a pathogenic species and mixing them on demand, depending on the phage sensitivity profile of the infecting bacteria [124]. Such cocktails could be adapted to a given pathogenic strain or enriched with new environmental phage isolates when needed. Although such a strategy fits the current trend to personalize medicine and has not only been widely discussed but also successfully used [28], it is commercially unattractive, as custom-made phage preparations cannot be currently approved for medical use [21, 125, 126]. Moreover, even mixtures of phages of different specificity usually do not exhibit a universal strain range [21, 91, 127–129], and searching for natural phages of the missing specificity is tedious, time consuming, and often either fails or leads to the isolation of only temperate phages [21, 90]. Even the so called adaptation of phages to infect resistant strains by selection of natural phage mutants has limited potential in the acquisition of phages of new specificities [76, 120, 130–133].

In view of the aforementioned problems, it is obvious that the repertoire of natural phages suitable for therapy, the possibility of ensuring their antibacterial efficacy and safety in vivo, and licensing their medical use are limited. A strategy to overcome at least some of these problems could instead rely on the construction of desired therapeutic phages by engineering selected, well characterized, natural phage isolates [122]. The introduction of genetically engineered (GE) phages into medical practice could have several advantages (Table 1).

Moreover, the recent progress in the understanding of phages and in the methods of their genetic modification may soon make construction of engineered phages a routine procedure rather than a tedious scientific undertaking. Additionally, the new discoveries concerning the interaction of phages with bacteria inside the human body and the effect of phages on human health via their influence on the microbiome open up new possibilities of therapeutic application of modified phages not only to treat infections with bacterial pathogens, but also to improve the conditions of patients suffering from diverse disorders related to a dysfunctional microbiome, by in situ microbiome engineering [69, 71–73, 156–159].

3 Phage Genetic Engineering: Classical and Modern Technologies

The acquisition of a genetically modified phage comprises two steps: (1) in vivo or in vitro replacement of the entire phage genome or its fragment with a modified one, and (2) recovery of the modified phage. Several methods of phage genome modification have been developed, but each of them has limitations and cannot be used for each and every phage (Table 2).

In the case of filamentous phages whose replicative forms (RFs) are circular dsDNA plasmids, this can be done by in vitro manipulation of the RFs and transfection of the resulting DNA to relevant host cells (see Sect. 7 of this review). A similar approach was used in early studies to modify an integration-deficient mutant of the temperate phage lambda depleted of certain restriction sites and of a large dispensable fragment of its 49-kbp genome [160]. Two essential restriction fragments of the mutant were ligated with a heterologous DNA fragment. The ligation product served to produce modified progeny phages upon transfection of *Escherichia coli* cells. Lambda-derived vectors with a single cloning site or with two sites flanking the dispensable DNA segment to be replaced by an intended insert were constructed in a similar way [161–163]. They were used, e.g., to study functions of bacterial as well as phage genes [164]. Hybrids between lambda and *E. coli* phages P1, T4, and T5 or *Bacillus* phage SPP1 constructed in that way were used in studies on the gene functions of these phages [165–174]. Overcoming the problem of low transfection efficiency of engineered lambda derivatives became possible with the development of an in vitro lambda packaging system (reviewed by Chauthaiwale et al. [164]). In this system, the lambda *cos* sequences, which separate the phage genome units in a substrate for DNA packaging and are cut to form cohesive ends, can serve as in vitro packaging initiation and termination signals for any 38- to 51-kbp intervening sequence. The implementation of lambda in vitro packaging was broadened by the construction of cosmids—plasmids containing a drug resistance marker, two *cos* sequences, and a cloning site designed for the insertion of DNA fragments of 40- to 50-kbp [163, 175]. The first *E. coli–Mycobacterium* shuttle plasmid, which can replicate in *E. coli* as a plasmid and propagate in *Mycobacterium* as a phage, was constructed by the insertion of a cosmid into a non-essential ~ 50-kbp region of mycobacteriophage DNA [176]. A similar shuttle plasmid was used to construct an engineered mycobacterial phage carrying a luciferase reporter gene to detect mycobacteria [177, 178]. While this strategy allows the introduction

Table 1 Benefits of genetic engineering of phages for therapy

Benefits	Supporting arguments	References
Enrichment of phages with selected lethal or regulatory genes from other phages or from bacteria and/or removal of undesired genes could potentiate the lytic and/or therapeutic efficacy and stability of phages, make them safer for use, and endow them with additional desired properties	No single natural phage combines all the features required of an ideal therapeutic agent	[122]
The development of GE derivatives of already well characterized phages approved for therapy could help shorten the procedures of new therapeutic phage acquisition and approval significantly and decrease their cost	The development of a therapeutic phage preparation, from the isolation of a desired phage to its approval for therapy in humans, is a time-consuming, costly, multistep process. All these steps have to be conducted anew for each natural isolate	[23, 75, 77, 134– 136]
The ongoing analysis of existing therapeutic phage candidates with the use of targeted mutagenesis may soon lead to the completion of their functional genomic maps and the identification of genes that should be removed/modified to produce GE phages with desired properties and infecting particular hosts	Phages targeting clinically relevant bacteria and meeting the criteria for therapeutic use usually belong to a few taxa only at the level of genus or subfamily. Several of them are related to model phages. Related phages differ mostly in genome regions required for the host-specific interaction	[80, 81, 87, 91, 119, 127, 137– 139]
The phage that infects a bacterium of a given species should be metabolically compatible with all strains of this species. Thus, if well characterized, it could serve as a universal scaffold to construct its derivatives differing, e.g., in strain specificity, stability, or other properties	The core genomes of diverse bacterial strains of a given species, which determine their basic metabolic functions, are conserved	[140 –142]
The Earth phageome, even if incompletely characterized, can serve as a source of genes or gene modules that can be analyzed functionally when incorporated to the genomes of well characterized phages and provide a much broader repertoire of functions than that encoded by the few well characterized phages	The enormous diversity of phages, reflected, e.g., by their genome size range and differences in morphological and physiological properties, limits the possibility of a phage pan-genome functional analysis. This excludes poorly characterized phages with potentially desired properties from implementation in therapy in a reasonable time span	[10, 11, 17, 87, 122, 143– 146]
The identification of genes of highly lytic phages responsible for their low therapeutic efficacy could allow genetic alterations, making such phages useful in therapy	Not all phages highly effective in bacterial lysis in the laboratory are therapeutically effective	[147– 150]
Modification of temperate phage genomes to remove lysogeny modules and undesired genes (e.g., encoding virulence factors) should allow benefit from the potential of these phages to infect and kill certain bacteria resistant to virulent phages	Certain strains of pathogenic bacteria are resistant to available virulent phages, while temperate phages infecting those strains are available or can be acquired by prophage induction	[22, 90, 151, 152]
Arming a temperate phage with genes encoding regulators of bacterial metabolism or functions disabling certain genes could allow such a phage to be used to modify human or animal microbiota by disarming pathogenic bacteria, re-sensitizing them to antibiotics, or selectively killing pathogenic strains of commensal bacterial species	Phages, especially temperate ones, are efficient regulators of human/animal microbiome composition and hence have an impact on human/animal health	[153– 156]

GE genetically engineered

Table 2 Advantages and disadvantages of different strategies of phage genetic engineering

Modification strategy	Advantages	Disadvantages
In vitro modification of phage DNA and transfection of host cells	Phage DNA modification as simple as in the case of plasmids	Applicable mainly to phages of small genomes whose replicative form is circular dsDNA
	Only GE phage production by transfected cells if the phage is functional	Requires an R-M-deficient and transfection proficient bacterial host
	No need for screening or selection	Requires highly competent host cells for transfection with large phage genomes
Host-mediated homologous recombination between bacteriophage DNA and donor DNA cloned in a plasmid	Simplicity	Occurs only with plasmid-cloned DNA fragments as donors for recombination
	Modification of prophages as efficient as modification of chromosome	Requires an R-M-deficient and transformation proficient bacterial host
	Upon induction of prophage lytic development, PCR screening of < 100 plaques typically sufficient to recover recombinants	Recombinant recovery of obligatorily lytic phages so low that requires selection or screening of large number of plaques
As above, but supported by counterselection of unmodified phages with the use of CRISPR-Cas	High efficiency of GE phage recovery	Requires an R-M-deficient and transformation proficient bacterial host
	Single-step introduction of traceless changes in phage DNA	Requires the presence of active components of CRISPR-Cas system with an appropriate spacer in phage host
	No need for selection of GE phages	Modification of various phage genome regions with donor DNA each time requires cloning of relevant spacer in the CRISPR-Cas source plasmid
	Suitable for engineering of obligatorily lytic phages, even those that degrade host DNA	
Recombineering with the use of lambda Exo and Beta proteins or their homologs	Occurs with linear dsDNA or ssDNA fragments as donors for recombination	Requires an R-M-deficient and transformation proficient bacterial host
	Homologous end fragments as short as 50 bp suffice for recombination	Requires the presence of a source of appropriate phage recombination proteins in phage host
	Single-step introduction of traceless changes in phage DNA	Low recombinant recovery of obligatorily lytic phages that degrade host DNA
	Recombinants of lytic phages that do not degrade host DNA recoverable by PCR screening of plaques	
BRED	As above, but efficiency of modified phage recovery up to 20%	Requires an R-M-deficient bacterial host highly competent for transformation
	Suitable for engineering obligatorily lytic phages or lytic variants of temperate phages	Requires a source of appropriate phage recombination proteins in phage host
Phage genome rebooting in yeast	Allows introduction of multiple changes to phage DNA or construction fully synthetic phage genomes	Complex methodology and difficulty to assemble large phage genomes
	Allows independent preparation of various modified phage genome segments for in vivo recombination if they have overlapping ends	Recovery of modified phages requires an R-M-deficient bacterial host highly competent for transformation
		Obtaining GE phages impossible if phage genomes recovered from yeast cells are non-functional

Table 2 (continued)

Modification strategy	Advantages	Disadvantages
Phage genome rebooting in L-forms of bacteria with Gibson method as an intermediate genome assembly step	Allows modified or fully synthetic phage genomes to be assembled in vitro	Requires R-M-deficient and transformation proficient L-forms of the bacteria supporting intracellular development of phage to be modified
	Allows the recovery of engineered phages of differing specificities from L-forms of bacteria metabolically compatible with the phage host but not necessarily sensitive to infection with any of the phages	Requires hypotonic lysis of L-forms to release GE phages
Synthesis of engineered phages in a cell-free transcription and translation system (TXTL)	Allows GE phage production in a test tube, independent of phage host	Extremely low efficiency of complete phage synthesis
	Low cost	Requires high infection efficiency of the host by synthesized phage to allow efficient phage recovery

BRED bacteriophage recombineering of electroporated DNA, *Cas* CRISPR-associated proteins, *CRISPR* clustered regularly interspaced palindromic repeats, *dsDNA* double-stranded DNA, *GE* genetically engineered, *PCR* polymerase chain reaction, *R-M* restriction-modification, *ssDNA* single-stranded DNA

of modified mycobacterial phage DNA into *E. coli* cells by infection with in vitro-packed lambda virions and allows it to be isolated as a plasmid, it has serious limitations and thus has not been widely used. The recovery of a functional phage from the target bacterium requires the target to be efficiently transformed with large DNA molecules, and only functionally active phages could be recovered. Additionally, the possible expression of bactericidal and bacteriotoxic phage genes by such constructs excludes the use of this strategy for phages of bacteria more closely related to *E. coli* than *Mycobacterium*. Moreover, the use of lambda virions as delivery vectors for heterologous phage DNA for *E. coli* requires the availability of the lambda in vitro packaging system and experience in its use, which may be problematic in some laboratories.

More common methods of engineering phages of linear dsDNA genomes and a complex virion structure use homologous recombination in vivo (Fig. 2). The earliest ones were based on phage infection of a bacterium with a fragment of the phage DNA (donor DNA) carrying a desired modification and cloned in a plasmid [18, 179–181]. Recombinants were subsequently recovered from among progeny phages. If any host specificity-determining gene was exchanged into one from a different phage, they could even be directly selected as forming plaques on the new host [182, 183]. However, the use of such methods is possible only when the phage host can be transformed with the plasmid and when a restriction-deficient host mutant is available, as in the case of, e.g., *E. coli* K-12 or *Pseudomonas aeruginosa* PAO1 and also *Mycobacterium smegmatis*. While temperate phages modified with this technique can be recovered by polymerase chain reaction (PCR) screening of single plaques following induction of lysogens, in the case of obligatorily lytic phages that degrade host DNA at infection, the frequency of recombination is extremely low. The recovery of recombinants may be facilitated by incorporating a marker gene into the donor DNA, allowing phenotypic differentiation of modified phage plaques [184], or better, a specific host gene if such is required for phage development [185–187]. In the latter case, an appropriate bacterial mutant has to be used to obtain only recombinant phages.

A technique that allows increasing the in vivo recombination frequency is based on the use of linear donor DNA and phage lambda recombination proteins Gam, Bet, and Exo, which protect linear dsDNA from host exonucleases (Gam) and efficiently promote homologous recombination between linear donor DNA and homologous target DNA [188]. This technique, known as recombineering and independent of host recombination functions, was initially applied to modify the *E. coli* chromosome, plasmids, and temperate phages [189–193]. It also increases the frequency of recombinant formation upon lytic phage infection of cells containing the donor DNA in a plasmid or in the form of a linear fragment,

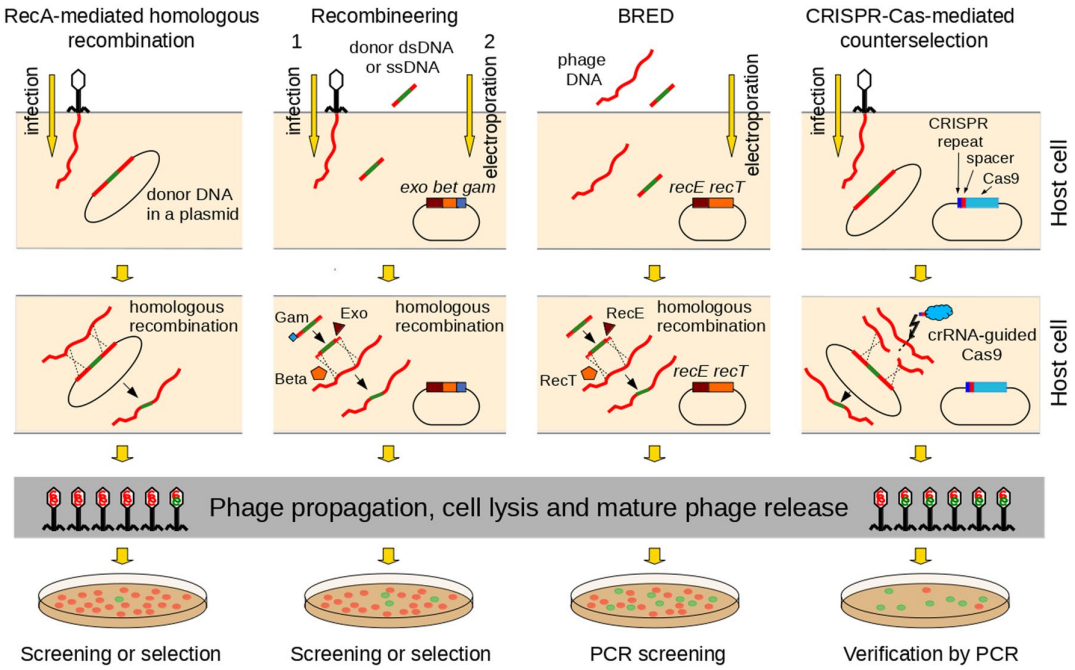

Fig. 2 In vivo homologous recombination-based methods of bacteriophage genome modification. Proteins depicted on the scheme of recombineering represent phage λ proteins that were originally used in this method [191]. Bet and its homologs/analogs are sufficient for recombineering with ssDNA substrates as donors (reviewed in [188]). Phage proteins depicted on the scheme of BRED represent *Escherichia coli* prophage Rac proteins RecE and RecT, whose mycobacterial phage Che9c homologs (gp*60* and gp*61*) were originally used in this method [198]. Recombination with the use of RecE and RecT does not require Gam (reviewed in [188]). *Bet* and *RecT* phage λ or prophage Rac single-strand annealing proteins, *BRED* bacteriophage recombineering of electroporated DNA, *Cas* CRISPR-associated proteins, *Cas9* nuclease Cas9, *CRISPR* clustered regularly interspaced palindromic repeats, *crRNA* CRISPR RNA, *dsDNA* double-stranded DNA, *Exo* and *RecE* phage λ or prophage Rac 5′-3’ exonucleases, respectively, that degrade dsDNA to expose the created 3’ single-stranded ends for binding of Bet or RecT (respectively), *Gam* phage λ protein increasing recombination frequency by inhibiting dsDNA substrate degradation by *E. coli* RecBCD, *PCR* polymerase chain reaction, *RecA* bacterial recombinase RecA, *ssDNA* single-stranded DNA

but a successful recovery of recombinants requires the development of their direct selection method [194, 195]. Recombineering allows the recombination to occur in *E. coli* with homologous DNA ends as short as 50 bp [196]. Its further modification, known as bacteriophage recombineering of electroporated DNA (BRED), was developed to facilitate the modification of obligatorily lytic phages and lytic mutants of temperate phages [197, 198]. In BRED, phage DNA and a linear fragment of homologous DNA with a desired change are electroporated together into a phage-sensitive host cell containing *E. coli* phage lambda or prophage Rac proteins (Exo and Bet, and RecE and RecT, respectively), or their homologs from other phages [199–201]. Recombinant phages are recovered upon cell lysis and infection of a sensitive host by PCR screening of plaques. However, the need for bacteria to be electroporated to carry a plasmid expressing the recombination-promoting proteins limits the application of this method to transformation-proficient bacterial strains for which relevant plasmids are available. BRED has been used for engineering of lytic phages infecting, e.g., *E. coli, M. smegmatis*, and *Salmonella enterica*, giving the recovery efficiency of recombinant phage of ca. 20%, which allowed for their identification by PCR plaque screening [199, 200, 202].

If the BRED method cannot be used and the duration of the phage and donor DNA co-existence in the cell is short, the problem of an extreme scarcity of recombinants can be circumvented by applying a counter selection of phages that did not acquire the intended modification. This can be done with the use of CRISPR-Cas systems [203–205]. These systems, identified in ~ 42% of bacteria, protect their native hosts from reinfection with foreign DNA [206, 207]. The CRISPR regions consist of 28- to 37-bp repeats of a conserved sequence interspersed with short spacers, which are a bank of immunological memory that stores DNA fragments from past invaders (plasmids or phages) [208]. The CRISPR regions are linked to *cas* genes encoding proteins responsible for RNA-guided recognition and cleavage of invading DNA sequences corresponding to the spacer regions. The guiding RNA fragments (CRISPR RNA [crRNA]) are

generated by chopping CRISPR-region transcripts into units including a spacer and an upstream repeat sequence, which serves to distinguish between host and non-host sequences [209–211]. To achieve the elimination of unmodified phages, the cell to be infected has to contain (1) a plasmid carrying a phage genome donor fragment with the desired modification and (2) a single CRISPR repeat followed by a spacer sequence representing the wild-type phage fragment to be replaced by the donor DNA and followed by the *cas* gene(s) of a given CRISPR-Cas system. [203, 212, 213]. The latter can be provided on a plasmid or on the chromosome. Only recombinant phages containing the donor sequence in place of the wild-type DNA, formed via homologous recombination by the host-encoded machinery, will be resistant to crRNA-guided degradation. A customized CRISPR-Cas system from *Streptococcus pyogenes*, which requires only one protein (Cas9) for crRNA processing and target recognition and cleavage [214], has successfully been used to introduce point mutations, deletions, or insertions to the genomes of obligatorily lytic phages infecting Gram-negative as well as Gram-positive hosts [187, 205, 213–218]. A customized CRISPR-Cas10 system from *S. epidermidis*, which requires a protein complex to perform its editing function and can target only actively transcribed sequences, has been used to modify the genomes of *S. epidermidis* and *Staphylococcus aureus* lytic phages ranging in size from 18 to 148 kbp [210, 219]. The CRISPR-Cas systems allow a single-step introduction of traceless changes in phage DNA with high efficiency (40–100%) without additional screening or selection procedures [213, 215, 220].

The above methods are not applicable to phages infecting only bacteria with active restriction systems or bacteria for which efficient transformation procedures have not been developed. Additionally, none of them can be used to simultaneously edit multiple unlinked loci in a phage genome. The methods that were developed recently and revolutionized the strategy of phage genome modifications use wall-less bacteria or yeast cells to reboot the genomes of obligatorily lytic phages constructed from DNA fragments [122, 221, 222]. Jaschke et al. [222] assembled from PCR-amplified or chemically synthesized DNA fragments an engineered 5.4-kbp genome of phage φX174. The engineered DNA was ligated with a yeast plasmid vector and transformed to yeast cells. Transformants selected for the presence of the recombinant plasmid were used to isolate large quantities of plasmid DNA, which was then restriction digested to recover the phage genome. The obtained phage DNA was circularized by ligation and used to transfect cells of an *E. coli* strain that is normally resistant to φX174 infection, but can propagate the phage. The progeny phages could infect a φX174 sensitive host. Ando et al. [221] extended this strategy to phage T7 and its relatives whose genomes are in the range of 37–45 kbp. They used the natural ability of yeast cells to assemble linear dsDNA fragments with homologous termini into a single molecule by recombination [223]. Complete phage genomes inserted into a yeast mini-chromosome (YAC) were obtained upon transformation of yeast cells with several PCR-amplified phage DNA fragments of up to 11 kbp overlapping at the ends, and with PCR-amplified YAC DNA carrying regions of homology to the ends of the phage DNA to be assembled [221]. Swapping the genomic fragments encoding receptor-binding proteins (RBPs) or their fragments between T7 and related phages specific for different bacterial hosts allowed the construction of hybrids with a single genomic scaffold and small regions encoding specificity determinants and with different host specificity. A functional *Salmonella* phage FelixO1 of an 86.2-kbp genome could also be recovered from assembled genomic fragments cloned in a YAC in yeast cells [224].

Although the use of yeast cells for the recovery of engineered phage genomes has several advantages over other methods and allows constructing fully synthetic phage genomes, its disadvantage is the necessity to transform bacteria with the phage genomic DNA isolated from yeast cells to acquire functional phages. This limits the use of this method to bacteria that can be made highly competent for the uptake of large DNA molecules.

A strategy to overcome this obstacle has been developed by Kilcher et al. [225] and used to engineer temperate as well as obligatorily lytic phages with genomes of up to 154 kbp infecting *Listeria monocytogenes* or related Gram-positive hosts. The genomes with designed modifications were assembled enzymatically in vitro into circular forms from PCR-amplified and/or synthetic DNA fragments with overlapping ends with the Gibson method [226]. They were then used to transform so called L-forms of specific *L. monocytogenes* strain cells, which are deprived of the cell wall, can grow in osmotically stabilized media, and can be transformed with large DNA molecules [225]. Phages produced in the L-form cells were released by hypotonic lysis and used to infect their specific bacterial host to propagate. Surprisingly, the L-forms of *L. monocytogenes* could be used not only to reboot efficiently the synthetic genomes of *Listeria* phages, but also those of *Bacillus* and *Staphylococcus* phages, indicating a general applicability of L-form bacteria for phage genome engineering.

While the propagation of a complete engineered phage can be initiated by a single infection of a sensitive host, the dependence of such phage acquisition on living cells poses a problem. These obstacles have been overcome recently by the development of new-generation cell-free transcription and translation systems (TXTLs) that enable the synthesis of complete phages (virions) in the test tube using a suitable phage genome as a template and *E. coli* cytoplasmic extract enriched with certain components [227–229]. *E. coli* phages of diverse genomes, such as MS2 (3.6 kb, RNA), φX174

(5.4 kb, single-stranded DNA [ssDNA]), and T7 (40 kbp, dsDNA), could be synthesized in this way, with efficiency reaching about 0.5, 0.6, and 2.2 phages per genome copy, respectively [230]. This method appeared to be suitable even for the synthesis of the large lytic phage T4 with a 170-kbp genome, albeit with a trace efficiency only (0.001 phages per genome). Although a cell-free T4 reconstruction has so far been demonstrated from a wild-type T4 genome only, the method is suitable for the acquisition of engineered phages as well, provided that the engineered variants are functional and can be recovered upon infection of the phage host. A recently developed cell-free, de novo phage genome assembly technology from chemically synthesized oligonucleotides, which was shown to allow the assembling of a 4.3-kbp *Acinetobacter* phage genome at a cost below $0.015 per bp [231], may soon enable various variants of engineered phage genomes to be obtained on demand and at a low price. High throughput systems to transform such synthetic genomes into appropriate cells to recover functional phages (when possible) may soon speed up the functional analysis of phage genes as well as the acquisition of phages with desired properties for therapeutic use.

4 Phage Genetic Engineering to Modify Phages for Traditional Phage Therapy

The validity of the idea of using genomes of well characterized obligatorily lytic phages as scaffolds to construct their derivatives with desired properties with the help of genetic engineering has been proven in several studies. Numerous features of phages constructed based on such scaffolds appeared to be modifiable, with no or just slight interference with other phage properties. For instance, an engineered phage T7 expressing the biofilm-degrading enzyme dispersin reduced biofilm cell counts with an efficacy two orders of magnitude higher than the wild-type phage [232]. An engineered virulent *Erwinia amylovora* phage Y2 with a wide strain range that was enriched with a gene encoding the exopolysaccharide depolymerase of phage L1, showed increased infectivity and killing efficacy and the ability to reduce the *E. amylovora* contamination of flowers as compared to the wild-type phage [233]. Phage T7 engineered to display lipids on the capsid showed a significantly increased stability in simulated gastrointestinal tract conditions, indicating possible directions to obtaining therapeutic phages for oral delivery [234]. Phage T7 enriched with a gene encoding lactonase, an enzyme that degrades acyl homoserine lactone (a quorum-sensing signaling molecule required for biofilm formation by bacteria of many species), inhibited the biofilm formation when added to mixed-species biofilms containing *E. coli* and *P. aeruginosa* [235].

The toxin release caused by the lytic action of a phage may cause serious side effects during phage therapy. Therefore, a phage eliminating target bacteria without causing their lysis would alleviate these concerns. Paul et al. [236] developed a recombinant endolysin-deficient staphylococcal phage unable to cause lysis, but still lethal to methicillin-resistant *S. aureus*. The recombinant phage was highly efficient in rescuing mice infected with a lethal dose of *S. aureus*. In similar experiments, lysis-deficient phages infecting *P. aeruginosa* or *E. coli* provided better protection to mice from a lethal dose of these bacteria than did their wild-type counterparts [237, 238]. In all the above cases, the phages suitable for therapy could be efficiently propagated in hosts complementing their endolysin function to obtain high titer lysates.

One of the most challenging features of known phages suitable for antibacterial therapies is their limited host range. The mechanisms determining the susceptibility of bacteria to a given phage are highly differentiated, and the list of known ones is constantly expanding [112, 115, 239–241]. The initiation of phage infection requires recognition and binding to surface receptors on the host cell. Mapping of the variable domains of phage RBPs, which form tail fibers or tail spikes [242, 243], allows targeted mutagenesis of their genes or shuffling between phages of different host specificities to generate phage variants infecting a wider or changed repertoire of hosts without compromising their lytic activity [182, 221, 243–247]. Moreover, the determination of intradomain structures of RBPs allows an antibody-engineering-like approach to change the structure of their fragments interacting with the host receptors to fit them to, e.g., mutationally changed receptors conferring to bacteria resistance to a given phage [248]. Selected phages infecting *E. coli*, *Klebsiella pneumoniae*, *Yersinia pseudotuberculosis*, *P. aeruginosa*, or *Acinetobacter baumannii*, the model *E. coli* phages T2, T4, and T7 among them, have already been used as scaffolds to construct variants with modified host specificity [18, 241]. Engineering the RBPs may be especially useful for rapid acquisition of variants of well characterized phages capable of infecting emerging pathogens of new serotypes. For instance, a derivative of phage T2 infecting *E. coli* O157:H7 was constructed by replacing genes encoding the tip of the T2 long tail fiber with those of a new phage isolate specific for this *E. coli* serotype [246].

Temperate phages have generally been seen as unsuitable for traditional phage therapy due to their adaptive effects on the bacteria they infect (including pathogens) and their participation in horizontal gene transfer [74]. However, animal studies indicate that such phages can also have therapeutic potential [249, 250]. Moreover, phages of an obligatorily lytic phenotype derived from temperate phages by spontaneous mutations have been identified among environmental isolates or upon induction of lysogens [251–253]. The use

of temperate phages in therapy need not be considered when lytic phages of a desired host specificity are available. If this is not the case, obligatorily lytic phages of the missing specificities can be obtained from appropriate temperate phages by the removal from their genomes of genomic modules essential for lysogeny and, if present, also those encoding virulence determinants [225, 250, 254, 255]. Such modified phages, although not the first choice, can be further engineered to enhance their lytic potential or broaden the host range. An advantage of temperate phages is the easiness of their genetic modifications in the lysogenic state, when their DNA is integrated with the bacterial chromosome. Several temperate phages modified to deprive them of the ability to lysogenize bacteria appeared to be effective as targeted antibacterials in in vitro and in vivo experiments [255]. For instance, the temperate *Burkholderia* siphovirus KS9 converted to a lytic variant by disrupting the phage repressor gene rescued *Galleria mellonella* infected experimentally with *Burkholderia cenocepacia* [250]. A recombinant of a temperate *Enterococcus faecalis* phage with an *E. faecalis* prophage, which was converted to a lysogeny-deficient variant insensitive to the phage repressor by the removal or modification of lysogeny-associated genome modules, markedly decreased the residual *E. faecalis* population and the biofilm biomass of both vancomycin-sensitive and resistant strains in extracted human dentin root segments [254, 256, 257]. A derivative of *L. monocytogenes* PSA phage depleted of genes essential for lysogeny and enriched with an additional endolysin gene from a different *Listeria* phage formed larger plaques than the original phage and also could lyse cells of a PSA-resistant serovar of *Listeria* [225].

The first case of a human phage therapy with GE phages was described by Dedrick et al. (2019) [152]. A cystic fibrosis patient underwent lung transplantation, which was complicated by *Mycobacterium abscessus* infection resistant to multiple antibiotics administered intravenously. Of the hundreds of mycobacterial phages tested, only one efficiently killed the infecting strain, and two others were temperate and inefficient in the infection. A lytic derivative of one of the latter obtained by genetic engineering gained high lytic activity against the infecting *M. abscessus* by the acquisition of a host range mutation, and an efficient lytic derivative of the second one was constructed with BRED to remove its phage repressor gene precisely [152, 258]. The three-phage cocktail (including two temperate phages engineered to become effective lytically) was administered every 12 h intravenously for 32 weeks. The treatment was well tolerated, with no major side effects, evidence of organ toxicity, or significant increase in the level of pro-inflammatory cytokines, allowing the patient to be discharged home while still on phage therapy. The therapy resulted in significant clinical improvement and eradication of infection. Phages could be detected in the patient's serum 1 day after therapy initiation, reaching a titer of $\geq 10^9$ PFU/ml, and became undetectable 6 days later. Although no formal proof of the phage therapy effectiveness has been provided (it was an uncontrolled study, during which the patient was also receiving other treatments, e.g., antibiotics, which could have contributed to the final outcome), the results of this experimental use of GE phages in human treatment indicated that (1) a prolonged intravenous administration of GE phage is possible without harm to the patient, while producing high therapeutic phage levels in the serum, suggesting phage multiplication in vivo, and (2) even intravenous phage administration in a human patient does not provoke antibody production that could interfere with its therapeutic efficacy. The experiment also confirmed earlier data from patients who received wild-type phages by oral or topical administration [259].

An important question is whether phages can kill intracellular bacteria. Phage genetic engineering may help to resolve this problem. Møller-Olsen et al. [260] used CRISPR-Cas-based selection to obtain a GE derivative of a T7-like phage, K1F, equipped with a gene for green fluorescence protein (GFP). The GFP-labeled phage showed some delay in infection as compared to wild-type K1F [261]. However, tracing K1F-GFP by fluorescence microscopy demonstrated its ability to penetrate human urinary bladder epithelial cells and human cerebral microvascular cells. Moreover, the phage was able to kill inside human cells a hybrid between *E. coli* strains K12 and K1, the latter being a nosocomial pathogen responsible for urinary tract infections, meningitis, and sepsis [260, 261].

5 Future Possibilities to Extend the Host Range of Phages by Genetic Engineering

Attempts to construct phages with extended host range by genetic engineering have been focused on manipulations with phage genes encoding RBPs, as summarized in Sect. 4 [244]. However, the phage host range depends not only on the phage's ability to penetrate bacterial extracellular polymers or to interact with a bacterial cell receptor and inject the phage DNA, but also on phage-encoded mechanisms overcoming the post-infective bacterial anti-phage defense. Their multitude seems to correspond to the multitude of bacterial anti-phage defense mechanisms [115]. The anti-restriction and anti-CRISPR mechanisms are the best known. The former operate by avoiding relevant restriction sites in phage DNA, titrating out restriction enzymes, physically protecting phage DNA from host restrictase access, modifying the phage DNA, or stimulating the host DNA-modifying enzymes to make the DNA resistant to restriction [115, 262–269]. The anti-CRISPR defense systems act by inducing the degradation of Cas nucleases, disruption of

DNA binding, or inhibition of target cleavage [270–273]. Numerous anti-CRISPR (Acrs) proteins identified so far comprise at least 46 families, and a machine-learning in silico approach allowed the identification of at least 2500 additional candidates [274–277]. When a gene encoding an Acr protein(s) is present in a phage or cloned in a plasmid in a cell with its target CRISPR-Cas system, it can suppress the action of this system [274, 277]. This opens wide possibilities to construct GE phages containing appropriate *acr* genes able to overcome specific types of acquired CRISPR-Cas-mediated immunity and may counteract the immunity to the phage acquired by bacteria during phage therapy [271]. Similarly, the incorporation into the phage genome of genes encoding specific anti-restriction proteins could extend the host range of such GE phages to the hosts whose restriction-modification systems would otherwise prevent the infection when the ratio of infecting phages to bacteria is not high enough to break the restriction-modification barrier.

Some phages encode homologs/analogs of antitoxins of certain bacterial TA systems [119, 278, 279]. They were shown or predicted to replace bacterial antitoxins whose supply upon phage infection could stop, leading to the death or dormancy of the infected cell. The enrichment of GE phages with genes encoding antitoxins of their host TA systems could increase the therapeutic efficacy of such phages by enabling their lytic development and facilitating their spread at the infection site.

Bacterial anti-phage defense genes including those that encode restriction-modification, TA, and CRISPR-Cas systems tend to cluster in so called defense islands [280, 281]. The systematic, ongoing analysis of other genes of these islands has brought about the discovery of new anti-phage defense systems of surprisingly differentiated mechanisms of action and high protective efficacy [117, 282–287]. Some of them stem from the same evolutionary origin as anti-viral systems of eukaryotic cells and can be complemented by relevant eukaryotic genes [287–290]. The design of therapeutic GE phages of wide strain range will benefit from future identification of phage strategies to evade these newly discovered anti-phage defense systems.

The least known repertoire of genes that could extend the host range of GE phages when inserted to their genomes comprises phage genes whose products help to evade prophage-encoded anti-phage defense mechanisms that protect lysogens from superinfection by other phages, especially unrelated ones. While a few examples of such gene products have a long history of study, how numerous and diversified the mechanisms of action of the remaining ones are and how many various stages of phage infection and development they can interfere with is only beginning to be discovered (for an extensive review, see [115]). They are typically encoded by accessory genes of temperate phages and show no homology to proteins of known function, and often only the conserved location of their genes in the genomes of related phages can serve as a guide for their identification [291, 292]. While some of them provide protection against multiple phages, others have been found to be specific only for one of the phages tested [291–294]. Notably, lysogeny is common in clinically relevant bacteria [102, 295]. Therefore, discovery and understanding of the prophage-encoded anti-phage defense system and identification of phage-encoded mechanisms to overcome them may appear to be critical for successful development of GE phages with wide strain specificity highly effective therapeutically.

6 New Directions in Phage Genetic Engineering to Expand the Possibilities of Phage Therapy

Phage replication and bacterial cell lysis, which accompany a productive phage infection and are considered to be advantages of traditional phage therapy, can be considered to be disadvantageous as well. Lysis leads to the release of bacterial toxins and can potentially cause undesired inflammatory reactions, while phage replication during therapy may raise concerns about further uncontrolled phage spread. Thus, there is an increasing interest in the use of phages to deliver to bacteria a lethal or regulatory load inserted in the phage genome by genetic engineering to achieve a therapeutic effect while attenuating lysis of bacteria, preventing phage replication, or both. Temperate phages are naturally suited for this purpose. However, obligatorily lytic phages that are defective in lytic development in a non-laboratory host can be used as well.

Among the most promising lethal loads that can specifically eradicate bacteria of certain species or strains within complex microbial communities in a sequence-specific manner without causing their massive lysis are programmable CRISPR-Cas-based gene cassettes. In a pioneering experiment, Bikard et al. [296] used an engineered temperate *S. aureus* φMN1 phage derivative to deliver to its host bacteria a CRISPR-Cas module with spacers targeting certain *S. aureus* virulence genes. The GE phage selectively killed only the *S. aureus* strains carrying the targeted genes when used to infect a mixed *S. aureus* population of virulent and avirulent strains in laboratory culture or on the skin of infected mice. Similar φMN1 derivatives targeting antibiotic resistance determinants selectively killed antibiotic-resistant *S. aureus* strains in a mixed population. Additionally, by lysogenization of the antibiotic susceptible cells, the phage made them immune to the acquisition of the resistance-conferring genes. Park et al. [218] integrated a CRISPR-Cas9 system targeting the *nuc* gene, unique to *S. aureus*, into the genome of a temperate *S. aureus* phage. To broaden the host range of the GE phage, the strain for its propagation was

transformed with a plasmid carrying the tail fiber genes of a broad-range *S. aureus* phage. The GE phage produced in this strain showed high in vitro efficacy in eliminating *S. aureus* and was effective in vivo in reducing the bacterial burden of infected mouse skin. A similar approach gave promising results in a rat model of soft tissue infection where the reduction of *S. aureus* burden by a GE phage was similar to that obtained with a high-dose antibiotic treatment [297]. Likewise, a temperate *Clostridium difficile* phage was engineered by removing key lysogeny genes and arming it with a CRISPR module targeting a *C. difficile* genomic sequence [298]. The lytic phage obtained reduced the *C. difficile* burden in vivo and decreased disease severity.

In addition to targeted killing of bacteria without their lysis, the concept of using phages as delivery vectors of specifically designed genetic load opens up the possibility of modifying human or animal microbiota phenotypically, in a non-lethal way. With the increasing knowledge about the human microbiome, its association with health and diseases, and the molecular mechanisms of bacterial pathogenicity, microbiome modifications may turn out to be more effective in the long run than killing of the bacteria [299]. For instance, a temperate GE phage carrying a transcriptional repressor of Shiga toxin (Stx) gene was successfully used to repress Stx in an established *E. coli* population colonizing the gut in mice. The phage propagated in the gut and markedly reduced the fecal Stx concentration [300]. A novel approach to fight antibiotic-resistant bacteria with the use of a temperate GE phage was developed by Edgar et al. [301]. Lambda-derived phages carrying dominant alleles of respective genes causing sensitivity to streptomycin or nalidixic acid reverted the antibiotic resistance phenotypes caused by recessive mutations in those genes in the bacteria.

An innovative approach to deliver any programmed genetic load to bacteria by phages was proposed by Yosef et al. [302]. The method is based on the transducing capabilities of phages and not only prevents phage propagation in the target bacteria, but also allows the spectrum of target bacteria to be extended to those that can be infected but do not support phage propagation. Its utility was demonstrated with the use of T7-derived defective phages, whose certain essential functions, including tail fiber genes, were provided in trans from a plasmid carried by the phage-propagating host. The genetic load to be delivered to the target cells was provided in a separate plasmid, which also contained a site required to initiate DNA packaging to phage heads. Initial experiments on the delivery of an antibiotic resistance marker to evaluate the frequency of transduction demonstrated the great potential of this strategy for therapeutic applications upon its further development. Moreover, mutagenesis of the tail fiber genes present in the plasmid complementing the lacking functions of the phage could extend the phage host range. An additional extension of the host range for transduction can be achieved by epigenetic modifications of the DNA to be transduced to prevent its restriction in recipient cells [303].

7 Engineered Filamentous Phages in Therapies of Bacterial Infections

While the phages used in traditional phage therapy have a complex virion structure and encode from 20 to over 300 different functions, at least half of them unknown, genetic engineering has opened up the possibility of modifying phages of simple structure and smaller genomes for therapeutic use. Filamentous phages, which are workhorses in the phage display technology, appear to be the most promising candidates for this purpose [95, 304, 305]. They do not lyse bacteria, but are constantly secreted by infected cells, thereby decreasing the host growth rate (Fig. 1). Their virions, composed of a few proteins only, have the form of thin filaments 6–7 nm in diameter. Their length is not as tightly controlled as is the size of icosahaedral phage capsids, allowing the insertion of more additional DNA. The filamentous phage genome, typically comprising 11 genes, is an ssDNA molecule in the virion, but assumes a double-stranded (dsDNA) circular RF during phage propagation. It can be isolated from host bacteria by methods used for plasmid isolation, then engineered in vitro and introduced back to the cells by transformation. If it contains all the genes necessary for phage propagation and release, it can initiate the production of mature phages. Plasmids that contain an origin of dsDNA replication and an origin for ssDNA replication derived from a filamentous phage (so called phagemids) can be packed in the single-stranded form into filamentous phage virions if the other phage functions are provided in trans by a helper plasmid [306]. Filamentous phages adsorb to retractable bacterial pili and have a narrow host range [95, 305]. However, parts of their RBPs can be engineered to adsorb into bacteria of different species by replacement with suitable parts of RBPs from other filamentous phages infecting those species [307]. Alternatively, the specificity of filamentous phages can be redirected towards bacteria that are not their natural hosts by phage display-based selection of GE phages exposing peptides that bind those bacteria or that bind antibodies against them. Yacoby et al. [308] engineered M13-derived phages to bind *S. aureus* cells and conjugated them chemically with chloramphenicol. The *S. aureus*-killing efficacy of the phage-conjugated chloramphenicol was several orders of magnitude higher than that of free drug.

Filamentous phage capsids packed with phagemid DNA can deliver their genetic load to the infected cells to kill them or to modify their metabolism. For instance, M13-derived phagemids engineered to encode antimicrobial peptides (AMPs) and toxins disrupting diverse bacterial intracellular

processes caused rapid, non-lytic death of bacteria in laboratory culture and were effective in vivo in a murine peritonitis infection model [309]. Another M13-derived GE phagemid specifically killed its host *E. coli* cells by delivering genes encoding bactericidal proteins [310]. A filamentous phage of the *P. aeruginosa* PAO1 strain engineered by replacing its export protein gene with a restriction endonuclease gene could not replicate in the absence of a complementing export gene nor could lyse cells, but caused chopping of their chromosomes, leading to bacterial death without a massive release of endotoxin or a significant inflammatory response [311]. More importantly, it could rescue mice infected with a lethal dose of PAO1 [237].

Lu and Collins [312] engineered an M13-based phagemid to overproduce the repressor protein of the *E. coli* SOS response system, which activates the pathways of oxidative DNA damage repair, e.g., in response to certain bactericidal antibiotics such as quinolones, exerting their action by inducing DNA damage and leading to cell death. Inhibiting the *E. coli* SOS system with such a phage enhanced the bactericidal action of quinolones in vitro. Moreover, the phage increased survival of mice with an *E. coli* bacteremia. It also enhanced the elimination of antibiotic-resistant bacteria, biofilm cells, and persister cells and reduced the emergence of antibiotic-resistant cells in an antibiotic-treated bacterial population. Similar effects were achieved with phages overproducing regulators of certain non-SOS gene networks, indicating the efficacy of engineered phages targeting certain genomic networks as adjuvants of antibiotic therapies. To treat *Chlamydia trachomatis*, an obligate intracellular pathogen, a filamentous phage was engineered to express an integrin-binding peptide (inducing phage endocytosis) and a conserved peptide from this pathogen interfering with its propagation. The engineered phage significantly reduced *C. trachomatis* infection in HeLa cells and primary human endocervical cells [313].

Peng et al. [314] have applied photothermal ablation of bacteria as a modification of phage therapy. Chimeric *E. coli* filamentous phages engineered to display RBPs of filamentous phages targeting bacteria of other genera were conjugated to gold nanorods (to form so called phanorods) [307, 314]. Upon excitation with near-infrared light, such phanorods release energy, and the resulting heat kills targeted bacteria. Moreover, phanorod irradiation efficiently eliminated bacterial cells within a biofilm, causing only minimal damage to epithelial cells.

It has also been recognized that arming filamentous phages with specific peptides offers a novel antigen delivery system for the development of new vaccine formulations. Such phages engineered to target mouse dendritic cells can strongly activate the innate and adaptive immune responses excluding the need for adjuvants. A recent article provides an overview of potential uses of such "nature-made nanoparticles" [58].

8 Engineering Phages to Control Phage Pharmacokinetics

The control of phage pharmacokinetics by engineering relies on modifying phage interactions with the immune system as the major factor determining the phage fate in vivo [315–320]. Typical modifications involve making virions 'invisible' to the immune system, mostly to its innate part, which is capable of removing phages without their specific recognition. Chemical modifications and microencapsulation, for instance, PEGylation or lyposomal preparations, have been used to help the phage escape filtration and metabolism in the human/animal organism. This approach will not be presented here in more detail as it does not involve specific engineering of the phage; an expert review of this topic has been published recently by Malik et al. [321].

Compared to chemical modifications, site-directed engineering of phage proteins seems less straightforward in terms of its effect on phage pharmacokinetics. In fact, effective control of the phage circulation in vivo by molecular modifications has not been achieved so far. This is probably due to the complexity of phage virions, which consist of a multitude of proteins, each potentially interacting with the phage-treated organism and affecting phage pharmacokinetics. Phages are very large as compared to conventional drugs, and this seems to be a major factor determining phages' ability to circulate in vivo, rather than details of phage molecular structures [320]. Nevertheless, there are reports indicating that phage pharmacokinetics in animals and humans can in fact be modified (if not fully controlled) by molecular modifications of the phage capsid. Sokoloff et al. [322–324] used phage display to study interactions of an engineered T7 phage with the rat and human innate immunity system. Their study focused on the complement system, which is a major component of humoral (non-cellular) innate immunity. Due to its wide interactions with other elements of the immune system, both non-specific, like phagocytes, and specific, like IgM antibodies, modifying phage interactions with the complement system may affect its interactions with other types of immune response. The authors assumed that the amino acid composition of the phage surface can make the phage less or more reactive to the complement system. Indeed, they demonstrated that presenting peptides with the C-terminal lysine or arginine on the capsid changed the phage phenotype into *long-circulating* in rats [322]. That finding was consistent with earlier observations of Merril and coworkers who isolated from rabbits spontaneous *long-circulating* mutants of phage λ with the glutamic acid to lysine substitution in a part of the major capsid protein presumably located

at the solvent-exposed surface in mature virions [325, 326]. They proposed that the *long-circulating* phenotype could be linked to lower susceptibility of the mutated phage to be captured by the mononuclear phagocytic system (referred to by them as the reticulo-endothelial system). An alternative explanation could be that the *long-circulating* phage was less susceptible to inactivation by the complement system, since both Sokoloff et al. [322] and Vitiello et al. [326] found that the exposure of lysine or arginine on the phage head surface facilitated phage escape from the innate immune response.

It seems likely that at least some phages whose life cycles are bound with animal- or human-associated bacteria have adapted to the complement system by minimizing their recognizability. In such cases, further improvement of the phage resistance to the immune system via genetic engineering may prove challenging, as was found by Hodyra-Stefaniak et al. [327]. In that work, coliphage T4 was engineered by phage display to present specific tissue-homing peptides expected to induce phage accumulation in specific targeted tissues (organs). However, the concentration of the engineered phages in the targeted organs was not increased, and in some cases, it was even lower than that of the wild-type phage. An analysis of the systemic pharmacokinetics of the phages revealed that their low concentration in the targeted organs was due to a lowered systemic concentration. Thus, it turned out that the engineered phages were removed from the system more rapidly than the non-engineered one, i.e., they exhibited a *short-circulating* phenotype. Analysis of the modifications introduced revealed that all the peptides conferring the *short-circulating* phenotype lacked arginine, which was present in all the others. That correlation seemed to confirm the role of arginine in protecting the phage from rapid elimination, although a similar role of lysine was not observed in that study. Apparently, engineering destroyed evolutionarily optimized properties of the phage, making it more susceptible to the immune system. In general, a *short-circulating* phenotype is unfavorable in therapeutic applications of phages, since their rapid elimination from the treated organism may prevent them from combating the bacterial infection [320].

9 Limitations of Phage Genetic Engineering, Potential Risks, and Future Challenges

The increasing interest in the multifarious modifications making phages safer and more suitable for defined therapeutic applications on demand has contributed to the rapid progress in the development of phage genetic engineering. While technically, phage genome modifications should soon become possible in any laboratory, phage genetic engineering has its limits [18]. Some of them result from the varying nature of phages. First, in the case of most phages, except for those with filamentous virions, the size of the genome is limited by capsid capacity. This limits the modifications to incorporation of only small fragments of extra DNA or replacement of original genomic fragments with fragments of similar size. Second, certain changes in the phage genome sequence or organization, or in the virion structure, may negatively influence phage infectivity or development [328–331]. For instance, chemical PEGylation of surface proteins of two obligatorily lytic myophages infecting *Salmonella* or *Listeria* species, which prolonged blood circulation time of those phages in mice, also decreased their infectivity [332].

The use of GE phages in therapy has raised concerns about potential effects of their release into the environment [333]. Whether such concerns are justified has not been verified yet. GE phages could influence bacterial community dynamics, genome evolution, and biogeochemistry. However, the small scale of potential use of GE phages as compared to antibiotics limits the potential risks of their spread. Nair and Khairnar [333] have asked an important question of whether the concerns associated with GE microorganisms are also relevant for GE phages. The GE phages depleted of lysogeny-associated or other undesired genes can potentially recombine with other phages, but they cannot spread such genes in the environment. Additionally, modifications of phage genomes have been shown to be associated with a fitness cost that can lead to a loss of function in GE phages or to their outcompetition by native phages [328–331, 334, 335]. However, some modified phages of reduced fitness could recover the fitness of their original phage through compensatory evolution even in less than a hundred generations. An additional concern is posed by possible interactions of GE phages with eukaryotic cells. Contrary to earlier assumptions, data accumulating in the last few years suggest such a possibility, albeit its significance is unclear at this moment [336]. It is thus obvious that despite the highly encouraging results of experimental therapeutic applications of GE phages, further progress in the development of antibacterials based on GE phages as well as wild-type phages will depend on progress in the understanding of phage structure, biology, and interactions with their hosts and other organisms.

Acknowledgements The authors acknowledge support from the Polish Academy of Sciences and the National Science Foundation.

Declarations

Funding This work was supported by funds from the National Science Foundation, Grant no. 2019/33/B/NZ2/02006.

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials Not applicable.

Code availability Not applicable.

Author contributions MŁ: conceptualization, preparation of the major part of the manuscript and artwork, reviewing, and editing; KD: writing part of the manuscript; AG: writing parts of the manuscript and support and advice during manuscript preparation.

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