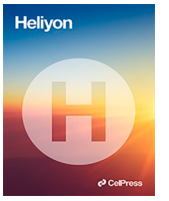
Heliyon 10 (2024) e34333

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Review article

Engineered bacteriophages: A panacea against pathogenic and drug resistant bacteria

Anuja Kakkar [a,1], Garima Kandwal [a,1], Tanmayee Nayak [a], Lav Kumar Jaiswal [a], Amit Srivastava [b], Ankush Gupta [a,*]

[a] Molecular Microbiology Laboratory, Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, UP, 221005, India
[b] University of Jyväskylä, Nanoscience Centre, Department of Biological and Environmental Science, 40014, Jyväskylä, Finland

ARTICLE INFO

Keywords:
Antimicrobial resistance
Phage engineering
Endolysins
Phage therapy

ABSTRACT

Antimicrobial resistance (AMR) is a major global concern; antibiotics and other regular treatment methods have failed to overcome the increasing number of infectious diseases. Bacteriophages (phages) are viruses that specifically target/kill bacterial hosts without affecting other human microbiome. Phage therapy provides optimism in the current global healthcare scenario with a long history of its applications in humans that has now reached various clinical trials. Phages in clinical trials have specific requirements of being exclusively lytic, free from toxic genes with an enhanced host range that adds an advantage to this requisite. This review explains in detail the various phage engineering methods and their potential applications in therapy. To make phages more efficient, engineering has been attempted using techniques like conventional homologous recombination, Bacteriophage Recombineering of Electroporated DNA (BRED), clustered regularly interspaced short palindromic repeats (CRISPR)-Cas, CRISPY-BRED/Bacteriophage Recombineering with Infectious Particles (BRIP), chemically accelerated viral evolution (CAVE), and phage genome rebooting. Phages are administered in cocktail form in combination with antibiotics, vaccines, and purified proteins, such as endolysins. Thus, phage therapy is proving to be a better alternative for treating life-threatening infections, with more specificity and fewer detrimental consequences.

1. Introduction

From ancient times until today, mankind has faced serious health concerns due to several bacterial infections, such as tuberculosis, typhoid, syphilis, diphtheria, and cholera [1]. Discovered in the 1930s, antibiotics became the primary treatment modality for these infections and were highly effective in the initial stages. However, the widespread use of antibiotics soon led to the emergence of antibiotic resistance. Natural causes of antibiotic resistance include rapid mutations in genes involved in antibiotic transport and metabolism, horizontal gene transfer, and selective pressures from the uncontrolled and inappropriate use of antibiotics [2]. Approximately 4.95 million deaths occurred due to bacterial antimicrobial resistance in 2019, with the maximum resistance caused by *Escherichia coli (E. coli)*. In addition, *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Streptococcus pneumonia* (*S. pneumonia*), *Klebsiella pneumonia* (*K. pneumonia*), *Acinetobacter baumannii* (*A. baumannii*), *Mycobacterium tuberculosis* (*Mtb*),

* Corresponding author.
E-mail address: ankushgupta@bhu.ac.in (A. Gupta).
[1] Equal contribution by both authors.

https://doi.org/10.1016/j.heliyon.2024.e34333
Received 11 April 2024; Received in revised form 18 June 2024; Accepted 8 July 2024
Available online 9 July 2024

Enterococcus faecalis (*E. faecalis*), *Enterococcus faecium* (*E. faecium*), and *Streptococcus agalactiae* (*S. agalactiae*) (group B *Streptococcus*) are also responsible for higher mortality rates [3]. In 2016, the United Nations General Assembly graded the problem of AMR as the "greatest and most urgent global risk," aiming to search for better alternatives for treating deadly bacterial infections [4]. In this search, probiotics, nanobiotics, antibody–antibiotic conjugates, vaccines, stem cell-based antimicrobial peptides, CRISPR-Cas editing machinery, and phage therapy are the currently most widespread options to combat AMR. Among these, phage therapy is considered the most effective in the treatment of persistent bacterial infections that are globally prevalent [5]. Phages are self-replicating, highly specific to their host, and quite resistant to environmental changes such as pH and temperature, making them suitable candidates for combating ongoing AMR [6].

Phage therapy utilizes phages, which are the natural predators of bacteria that hijack their machinery to reproduce by the process commonly known as transduction. Phages consist of nucleic acid (DNA/RNA) encapsulated in a protein capsid. They attach to the bacterial surface via specific receptors, insert their genetic material, and complete their lifecycle through either the lytic cycle (resulting in the release of new phage progeny by lysis of the bacterial cell) or the lysogenic cycle (integrating the phage genome into the bacterial genome) [7]. Felix d'Herelle was the first person to propose the idea of using phages therapeutically, with the clinical application of phages to treat four pediatric instances of bacterial diarrhea in 1919 at the Hôpital des Enfants-Malades in Paris [8]. However, with continued efforts in the early 20th century, d'Herelle advanced phage therapy by treating cholera, bubonic plague, and dysentery using a network of phage therapy facilities in Europe and India [9]. Phage therapy in India was first carried out in the Punjab region for the treatment of cholera, where the mortality rate was reduced by 90 % in the experimental group compared to that in the control group [8].

Phages should possess specific characteristics that include a strict lytic lifecycle, absence of toxic genes, broader host range, good transduction, and virulence potential [10]. Phages co-evolve with their bacterial hosts, resulting in a rich diversity of genetic elements in their genomes to combat bacteria, such as tail-fiber/spike proteins for host recognition and holin–endolysin machinery for cell lysis [11].For example, in a particular genus such as *Mycobacterium*, pathogenic (*Mtb*) and non-pathogenic (*Mycobacterium smegmatis* mc^2155 (*M.smeg*)) species differ only in the composition of the sugar moieties present in their cell wall that imparts specificity in their phage binding [12]. In addition, phages and their respective hosts have co-evolved, also leading to the emergence of phage resistance, limiting their antimicrobial efficiency and host range [13]. Therefore, phages isolated from the natural environment may require modifications for successful therapeutic use [14].

Genetic engineering of phages can enhance their therapeutic potential. A diverse phage library against a bacterial host provides opportunities for generating desired genetically engineered phages. Phage engineering can produce host range mutants via tail fiber mutations, exclusively lytic phages from lysogenic ones, non-toxic phages through gene deletions, and diagnostic phages by incorporating reporter genes. This review is divided into two sections: the first section explains in detail the various methods that have been successfully applied for the generation of genetically engineered phages, and the second section explains the importance of genetically engineered phages in the treatment of bacterial infections.

2. Methods for phage genetic engineering (PGE)

The various methods that are employed in phage genome engineering for better therapeutic outcomes are as follows:

2.1. Conventional homologous recombination (HR)

The most conventional method employed for PGE is homologous recombination (HR) of two wild-type parental phages inside the host cell. This process is also known as phage cross, in which bacterial host cells are co-infected with wild-type parental phages carrying different phenotypes, as demonstrated in Fig. 1A [15]. Although rare, once inside the host cell, the phage genome can either opt for its own or its bacterial host recombination system. Bacterial recombination machinery cannot carry out recombination between divergent genome sequences because phages with divergent genome sequences are likely to avoid the bacterial recombination machinery and preferably precede their own genome-encoded recombination functions. There are three major super families of phage

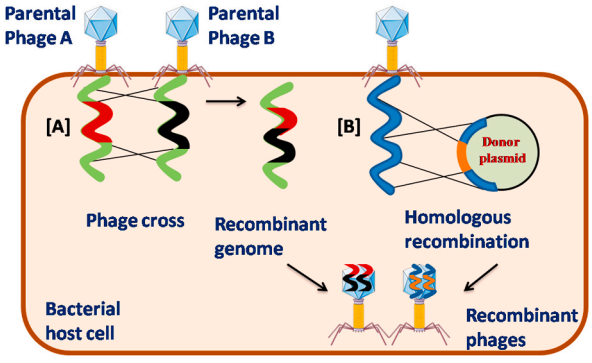


Fig. 1. Schematic representation of [A] Phage Cross and [B] Homologous recombination (HR) in phage genetic engineering.

recombinases, which are divided into Rad51, Rad52, and Gp2.5-like proteins, among which Rad52 is the most divergent and the largest family of phage recombinases [16]. Modifications of the phage genome via HR usually do not occur at a specific position, limiting the use of this method. HR between the phage genome and a plasmid carrying the desired mutation flanked by the corresponding sequence in the phage genome is required to overcome this shortcoming, as illustrated in Fig. 1B. In this method, a plasmid containing the desired mutation is first transformed into the host bacterium, followed by infection of the phage genome to be modified, and mutants with insertions, deletions, or gene replacement can be generated [17]. The recombination frequency for some phages was approximately 5×10^{-3}, which was much higher than that of the conventional phage crossing method. However, in general, the recombination frequency is relatively low, limiting its use [18]. Thus, HR is a long-drawn method that makes it difficult to screen recombinants with lower recombination frequencies [19].

2.2. Bacteriophage recombineering of electroporated DNA (BRED)

Recombineering is a genetic engineering technique that is based on HR [20,21]. Initially, HR systems such as the red system of bacteriophage λ and the RecE/RecT system of the Rac prophage were used for genetic engineering. The red system of lambda phage primarily encodes three proteins, Exo, Beta, and Gam, whereas Rac-prophage encodes two proteins, RecE and RecT [22,23]. Rec E and Exo have 5′–3′ dsDNA exonuclease activity that cleaves one strand of dsDNA to generate a single-stranded DNA (ssDNA) substrate, whereas RecT and Beta are single-strand binding proteins (SSBs) that encourage the annealing of one strand of the DNA substrate to its recombination target in the phage genome. The λ phage Gam protein binds to the host *E. coli* RecBCD exonuclease complex and SbcD enzymes to inhibit their activities from preventing the degradation of the linear dsDNA substrate [24,25].

Recombineering requires co-electroporation of the substrate DNA and phage DNA template into a recombinant bacterial strain that expresses the phage recombineering proteins via inducible plasmid to promote HR [26–28]. Substrate DNA is designed on the basis of the required genome alterations to be made in phages that are flanked by homologous sequences of the phage region where the mutation has to be incorporated, which leads to HR between the phage genome and the substrate DNA as illustrated in Fig. 2. Recombination is thought to occur only after phage genome replication begins [27]. Plaques produced by phages contain both wild-type (non-recombinant) and mutant (recombinant) phages. Phage particles containing the desired mutation can then be re-trieved by plating transformed cells, followed by screening these plaques using Polymerase Chain Reaction (PCR). Therefore, several rounds of plating and PCR are required to isolate recombinants [27,28]. This recombineering technique, when used in phages against *Mycobacterium* species, is known as BRED [25,29]. This technique was first devised by Marinelli et al. for mycobacteriophages and utilized a recombination system encoded by mycobacteriophages with an enhanced frequency of HR [30].

Homologs of RecE and RecT are rare among mycobacteriophages [31]. However, recombinant proteins from the mycobacteriophage Che9c, gp60, and gp61, which are distant relatives of RecE and RecT, respectively, have been identified and isolated [32]. BLAST analysis and biochemical characterization confirmed these functions. BLAST analysis depicted that Che9c gp60 protein shares 28 % identity with λ RecE C-terminus, while Che9c gp61 shares 29 % identity with λ RecT (contains a motif common to the fam-ily of ssDNA annealing proteins) [26,33,34]. Hence, these genes are introduced into an inducible plasmid (named pJV53) that is widely used to engineer mycobacteriophages. However, there is a need to identify a comparable recombineering system encoded by mycobacteriophages because the *E. coli*-derived proteins do not produce or function properly in mycobacteria because of the high mycobacterial GC content [33]. BRED has been applied to mycobacteriophages to construct chromosomal gene knockouts, gene deletions, base substitutions, heterologous gene insertions, and specific gene replacements [26,27,35]. Examples of such modifications include the deletion of the repressor gene (Δ45) in ZoeJ, the integrase gene (ΔHTH33) in BPs, and both the repressor and integrase gene in Adephagia (Δ41Δ43) and Fionnbharth (Δ45Δ47). In addition, the insertion of the Egfp gene (D29 Phsp60-egfp) has been performed in the D29 phage [26,27,32,34].

BRED also has certain drawbacks, such as the co-transformation of substrate DNA and phage DNA into the same cell is generally

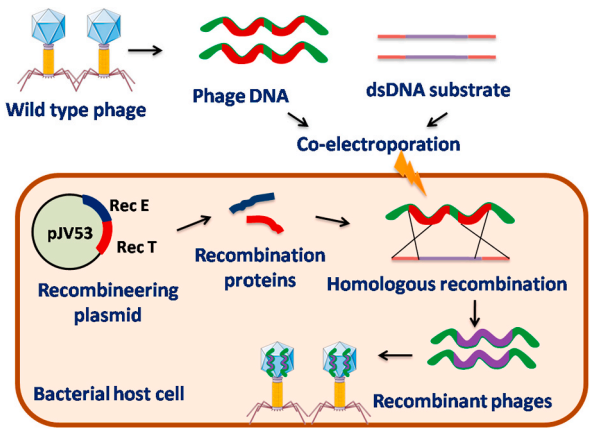


Fig. 2. Schematic representation of Bacteriophage Recombineering of Electroporated DNA (BRED) in phage genetic engineering.

low. Hence, it is particularly challenging to apply this technique to Gram-positive bacteria that exhibit low transformation efficiencies [19].

2.3. CRISPR-CAS

To counter the invasion of phages, bacteria have developed a specific immunity mechanism popularly known as the CRISPR-Cas system, in which the nucleic acids of any foreign organisms are targeted and cleaved with the help of distinct nucleases [36]. It is very similar to the pattern recognition receptors (PRRs) of the mammalian innate immune defense mechanism [37]. The CRISPR-Cas system adapts to a short stretch of the phage genome of approximately 30–40 nucleotides (called "spacers") by binding and merging them into the CRISPR loci of the bacterial genome. Furthermore, these spacers are transcribed into CRISPR RNAs (crRNAs), which form effector complexes that combine with single or multiple Cas proteins. This effector complex further interferes with the infection of the phage genome, which is complementary to the crRNA, by recognizing and cleaving its nucleic acid (called "protospacers") and hence providing immunity to bacteria [38]. In light of all other phage engineering strategies, CRISPR-Cas systems in various bacteria have recently been utilized with remarkable effectiveness to aid phage genome engineering [39].

CRISPR-Cas has been classified into two classes based on the composition of cas genes. The Class 1 system is based on multiple subunits of the effector complex and has three types, I, III, and IV, whereas the Class 2 system is based on a single subunit of the effector complex and has three types, II, V, and VI [40]. Among the six types of CRISPR- Cas systems, type I, II, and III, have been efficiently applied for the engineering of phage genomes.

The type I-E system observed in *E. coli* is the most well-known instance of a Type I CRISPR-Cas system [41]. The endoribonuclease Cas6, also known as CasE or Cas6e, is essential for crRNA synthesis, as it detects and cleaves the precursor crRNA within each repetition. Cas6 and crRNA were further merged with Cas8 (large type component), Cas7 (six sets), CasB or Cse2 (two sets of small subtype subunits), and Cas5. In addition, a two to six-nucleotide protospacer-adjacent motif (PAM) must be present on the non-complementary DNA strand. After recognizing the target DNA by Cascade (a CRISPR-associated complex for antiviral defense), another protein helicase-nuclease, Cas3, cleaves the target DNA [42]. This type of I-E system has been successfully utilized to generate recombinant phages for *E. coli* [43] and *Vibrio cholera* [44]. Two non-essential genes were deleted from phage T7, which infects *E. coli* using this approach. The wild-type T7 phage was infected with an *E. coli* strain carrying a plasmid containing homologous phage sequences flanking the gene to be deleted. This step generated both wild-type and recombinant phages, which were further selected by propagating these phages onto the bacterial strain carrying plasmids encoding cascade, cas3, and spacer sequences, where the recombinant phages were automatically selected by cleaving the protospacer of wild-type phages, generating 38 % and 42 % recombinant phages for deleting two non-essential genes, respectively [43]. Using a similar approach, two deletions and one replacement were produced in the ICP1_2011_A phage infecting *V. cholera*, where approximately 50 % of the recombinants were discovered, whereas a small deletion of approximately 33 nucleotides generated 100 % recombinant phages [44].

The most commonly used CRISPR–Cas system in genome editing applications is CRISPR-Cas9 [45], which belongs to the type II–A CRISPR-Cas family typically found in *Streptococcus thermophilus* and *Streptococcus pyogenes* [38]. Here, a short trans-activating crRNA (tracrRNA) is necessary for crRNA production, having a corresponding region to repeat-derived sequences of crRNA. In this system, the 5′end of crRNA is modified using some unspecified nucleases while the 3′end of crRNA is trimmed by RNase III, a host-encoded nuclease when the complementary short *trans*-activating crRNA (tracrRNA) and progenitor crRNA binds with each other. This complex binds to Cas9, which recognizes and cleaves the targeted double-stranded DNA. The mechanistic action of the CRISPR-Cas type II system has been illustrated in Fig. 3. Similar to the CRISPR type I system, CRISPR-Cas9 requires PAM and a corresponding crRNA with a protospacer in the seed region to induce productive interference [46]. Some non-essential genes have been identified in phage 2972 against four different *S. thermophilus* strains, each carrying its own type II CRISPR-Cas system. In addition, with the help of donor DNA and plasmids carrying CRISPR-Cas9, point mutations and single gene deletions (100 % efficiency), two-nucleotide deletions (80 % efficiency), and gene exchange were efficiently performed in phage 2972 using this method [39]. CRISPR-Cas9 has also been used to engineer P2, a lytic phage, against *Lactococcus lactis*. Several short nucleotide insertions, point mutations, and single-gene deletions were introduced into the P2 phage using plasmid-encoded donor DNA and CRISPR-Cas9 [47].

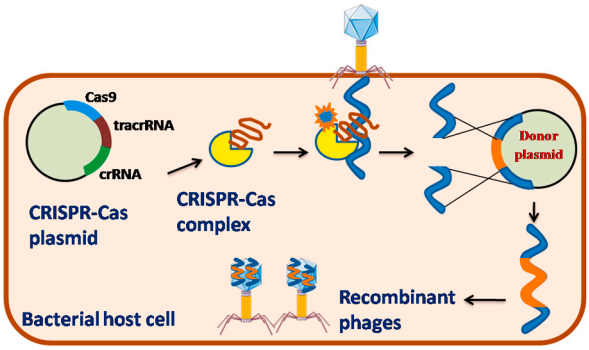


Fig. 3. Schematic representation of CRISPR-Cas Type II system in phage genetic engineering.

The type III-A system is well described in *Staphylococcus epidermidis*, showing a close similarity with the type I CRISPR-Cas system [48], which also depends on the Cas6 endonuclease for crRNA biogenesis at its 5′ end, while the 3′ end is modified by some non-Cas nucleases. The effector complex is composed of Cas10 (large type subunit), Csm2 (small type subunit), Cas5, and Cas7 (numerous homologs), which along with crRNA and Csm6, target and cleave foreign DNA and RNA [49]. The type III system functions in a transcription-dependent manner; hence, it cleaves the target molecule only after successful transcription. Therefore, it is difficult to engineer lysogenic phages using this system because the late genes of lysogenic phages are usually silent during this phase. However, lytic phages of *S. epidermidis* and *S. aureus* were efficiently engineered using the type III CRISPR-Cas system. Point mutations have been introduced in phage Andhra [50] against *S. epidermidis* and in phage ISP [51] against *S. aureus* with 100 % mutant phages.

Therefore, owing to the diversity of CRISPR-Cas in various bacterial species, it can be used to modify various phages using both host-encoded and plasmid-encoded CRISPR constructs. Some off-target cleavage can be performed by CRISPR nucleases; however, it can be easily screened once a desired recombinant phage is found [38]. One very important aspect of using CRISPR-Cas systems in phage engineering is their diverse role in different hosts, proving their efficacy once optimized properly.

2.4. CRISPY BRED and CRISPY BRIP

Phage engineering is mostly based on either a phage-encoded recombination system or host-derived CRISPR-Cas machinery. The engineering of mycobacteriophages [27] has been carried out by BRED, which is further implemented for phages of other bacterial species, including *E. coli* [35], *Klebsiella* [52], and *Salmonella* [53], whereas CRISPR-Casis readily used to engineer phages of *E. coli* [43], *V. cholerae* [44], and *S. thermophilus* [39]. When combined, these techniques can be used to efficiently carry out engineering known as CRISPY-BRED, where BRED or recombineering is used to carry out recombinations, and CRISPR is used to select recombinant phages. CRISPY-BRED has been used to modify various mycobacteriophages such as Alma, BPs, BuzzLysyear, LadyBird, Miko, PhiFW1, Fionnbharth, and Adephagia as mentioned in Table 1 [54]. The CRISPR system is encoded by a plasmid derived from pIRL53 containing an sgRNA (single guide RNA of approximately 20 bp in length, which is positioned 5′ to the PAM sequence used to target the gene to be modified), the cas9 gene17 of *S. thermophilus* [55], an *E. coli* replication origin, and a kanamycin resistance gene. The integration of this cassette into mycobacterial chromosomes contains an attP-Int site [56]. BRED contains the parental phage DNA to be modified and an artificial DNA substrate (150–250 bp long) that is homologous to the target phage DNA sequence. They are co-electroporated in *M. smeg* cells, which express recombination genes from the mycobacteriophage Che9c. Once the target phage DNA sequence, artificial DNA substrate, recombination genes, and CRISPR cassette are inside the *M. smeg* cell, the guide RNA cleaves the target DNA, followed by the recombination of the artificial DNA substrate with the targeted phage sequence using recombination genes. After one round of the lytic cycle, the recombinant phages are recovered by plating on the *M. smeg* sgRNA strain. Plaques are detected by PCR in the presence of mutant alleles. CRISPY-BRED is advantageous over BRED, as the former usually obtains homogenous mutant phages in its primary plaque screening, whereas the latter has the heterogeneity of both wild and mutant phages in primary plaque screening, which is further confirmed by secondary plaque screening. Therefore, CRISPY-BRED simplifies the screening procedure because the recombination efficiency is low in the case of BRED in *M. smeg* [54].

CRISPY-BRIP is another approach for phage engineering, which is similar to CRISPY-BRED in all aspects, except the co-electroporation of the target phage DNA sequence and the artificial DNA substrate. In CRISPY-BRIP, cells carrying the recombination genes are first electroporated with the artificial DNA substrate, followed by further infection with phage particles by natural route to release their DNA inside the host cell. This approach is useful for engineering phages with hosts that have low electroporation efficiency, such as Gram-positive bacteria, particularly *Mycobacterium* sp. Compared to CRISPY-BRED, CRISPY-BRIP is less efficient than replacing the repressor gene (gene 47) of the mycobacteriophage Fionnbharth with the mutant variant gene (gene 52) of mycobacteriophage Fruitloop, which yielded only two recombinants out of fourteen plaques (14 % efficiency), while similar replacement with CRISPY-BRED yielded twenty-two recombinants out of twenty-four plaques (~92 % efficiency) [54].

Owing to the higher efficiency and easier screening of recombinants, CRISPY-BRED has been efficiently used to introduce deletions and replacements in various mycobacteriophages, with a decent number of viable mutants. PAM site choice in Cas9 of *S. thermophilus* is restricted to 5bp, whose target is quite precise in case of deletion and replacement, but for targeted insertion and point mutation, PAM site choice is still restricted and requires other CRISPR-Cas systems with more specific PAM site choices. Recombineering remains a limitation for other phages and requires extensive screening of mutant phages. Therefore, CRISPY-BRED can be a better platform for

Table 1
CRISPY-BRED engineered phages [54].

Mycobacteriophage	Mutated Gene	Type of Mutation
Alma	Ori ncRNA (gp35)	Deletion
BPs HRM^{10}	Integrase (gp32), Repressor (gp33)	Deletion
BuzzLysyear	gp41,gp42,gp43	Deletion
LadyBird	Ori ncRNA (gp34)	Deletion
Miko	repA (gp36)	Deletion
PhiFW1	Capsid (gp14)	Deletion
Fionnbharth	Repressor (gp47) of Fionnbharth/F52mut3 of Fruitloop	Replacement
Fionnbharth	Integrase (gp45), gp46,Repressor (gp47)/mCherry (Fluorescent protein)	Replacement
BPs HRM^{10}	Integrase (gp32), Repressor (gp33)/mCherry (Fluorescent protein)	Replacement
Adephagia	Integrase (gp41), gp42, Repressor (gp43)/mCherry (Fluorescent protein)	Replacement

producing engineered phages, whereas CRISPY-BRIP can be readily used for engineering phages possessing a larger genome size and is unable to transfect its host organism efficiently [54].

2.5. Chemically accelerated viral evolution (CAVE)

Chemically accelerated viral evolution (CAVE) is based on a directed evolutionary strategy in which phages undergo a series of chemical mutagenesis cycles to evolve with better functional characteristics than the original wild-type form [57]. Ethyl methanesulfonate (EMS), an alkylating substance, is used to perform chemical mutagenesis in various *E. coli* phages, producing effective variants of these phages in vitro [58]. Here, a random mutation is inserted within the phage genome by chemical mutagenesis, which generates progeny of mismatched mutated phages upon replication in its host bacteria. Furthermore, these mutated phages are exposed to high temperatures, resulting in variants with enhanced thermal stability. These variants are multiplied and subjected to 30 cycles. Next-generation sequencing of these variants depicted that during the initial cycle of mutagenesis, mutations were restricted to fewer mutations, which further increased and saturated with the latter cycle of mutagenesis. The stability of mutated phages at higher temperatures increased by approximately 63 % compared to that of wild-type phages. Sequencing results demonstrated that all these mutations occurred within the coding regions of phages, especially in the structural and assembly protein subunits. In these mutations, the original amino acids were replaced with more hydrophobic amino acids than their corresponding wild-type counterparts; CAVE did not cause any secondary effects on the lytic activity or host range capability of these phages. CAVE can also be used to generate resilient phages at acidic pH without causing any significant changes in the structural biology of phages [59].

2.5.1. Genetic engineering vs. chemical modification: enhancing phage efficacy

Genetic engineering techniques offer distinct advantages over chemical modification methods in enhancing the efficacy of phages for therapeutic applications. These techniques allow introducing specific changes in phage DNA sequences, such as altering receptor recognition sites or enhancing lytic activity against antibiotic-resistant bacteria. It also facilitates the development of phages with expanded host ranges or increased stability under different environmental conditions, which are crucial for their effectiveness in diverse therapeutic settings.

In contrast, chemical modification methods typically involve surface alterations or conjugation of chemical moieties onto phage capsids or genomes. While these approaches can confer immediate changes to phage properties, they often lack the specificity and scalability of genetic engineering. Chemical modifications may not achieve the level of precision needed to finely tune phage interactions with bacterial targets or to overcome evolving bacterial resistance mechanisms effectively. Moreover, genetic engineering allows for the integration of sophisticated molecular tools, such as CRISPR-Cas systems, which enable real-time adaptation of phages to evolving bacterial threats through targeted genome editing.

Thus, genetic engineering emerges as a more advantageous method for advancing phage therapy by providing effective tools to customize phage characteristics precisely to therapeutic needs, enhancing their specificity, potency, and versatility in combating bacterial infections [60].

2.6. Rebooting a phage genome

The basic principle behind the rebooting approach is that the full-length phage genome containing the desired mutation is generated by artificial methods such as Gibson assembly or transformation-associated recombination (TAR). This synthetically generated phage genome is directly transformed into phage-specific host cells, which produce modified phages after the lysis of the host cell. This approach is helpful for certain phage gene products that are harmful to their host cells and was first applied to the phage genome of phiX174 (5386 bp). The phiX174 genome was assembled in vitro using artificial oligonucleotides by polymerase cycling assembly (PCA) and further transformed into yeast artificial chromosomes by the transformation-associated recombination (TAR) method in vivo. These fragments are then liberated by digestion with restriction enzymes and finally rebooted into their respective host cell, that is,*E. coli* [61].

Similar modifications have been applied to T7 phages to modulate their host range, particularly in Gram-negative bacteria. This shows that the tail fiber gene gp17 plays a significant role in determining the host range of T7 phages [62,63]. In addition, rebooting was successful in the *Salmonella* Myovirus FelixO1 strain [64], *P. aeruginosa* [65], and *Klebsiella* sp. [66]. Therefore, this method is more suitable for Gram-negative bacteria because it has greater transformation efficiency than Gram-positive bacteria.

For the rebooting of phages for Gram-positive bacteria, L-form cell wall-deficient bacterial strains are used, which can easily uptake the genomic DNA of the phage to be modified. This technique has been used to reboot the Gram-positive L-form of *Listeria monocytogenes* which has been applied to alter the host range of Listeria phages [67] in addition to generating reporter phages [68]. In addition to this, *Listeria* L-forms can also be employed to reboot genomes of other Gram-positive phages that do not infect *Listeria*. This includes TP21-L phage infecting *Bacillus cereus* and Bastille phage infecting *Bacillus thuringiensis,* which was rebooted inthe L-form of *Listeria* and caused successful infection in their respective hosts. The genome of phage 2638A and phage K against *Staphylococcus aureus* has also been rebooted using a similar approach [69]. Phage rebooting can also be performed outside the host cell using a cell-free transcription and translation (TXTL) system. This method utilizes phages with a higher self-assembly capacity, such as T4, T7, and phiX174. These phage particles are further grown in test tubes along with cell extracts of *E. coli* using the TXTL system [70]. Cell-free systems of genetic engineering can overcome the hurdle of bacterial species with inefficient transformation capacity and can be used to engineer their respective phages.

3. Applications of phage therapy

Phages assist in the treatment of bacterial infections at both the pre-infection (prophylaxis) and post-infection (therapy) stages.The findings that no major adverse side effects of phage therapy have been documented to date substantially support the safety of bacteriophage preparations. Most of the applications of phage therapy that have been employed clinically to date are most often personalized for a particular patient, and there is perhaps no report that describes its characterization or usage on a mass level. Several attempts have been made to aid in the selection of particular conditions and methods so that phage therapy experiments can be targeted most effectively in the future. Still, phages have been identified to have huge potential in therapy against several critical bacterial illnesses where antibiotics and (or) other treatment modalities are not very effective [71,72]. Phage therapy can be administered in several ways, some of which are listed below:

3.1. Engineered phages

In addition to their inherent antimicrobial properties, phage activity can be readily increased by genetically modifying them to carry genes that can code for antimicrobial proteins and (or) antimicrobial substances. The *DspB* gene of *Actinobacillus actinomycetemcomitans* was expressed by an engineered phage T7, which can be recognized by T7 RNA polymerase. As a result, it could considerably lower the number of bacteria in a single-species *E. coli* biofilm than in the T7 phage control [73]. Similarly, in order to prevent quorum sensing, the T7 phage was altered to express a lactonase enzyme which is crucial for the development of biofilms. Compared to the no-phage control, the resulting T7 phage decreased biofilm formation by 74.9 % and 65.9 % at 4 and 8 h after plating, respectively. However, after 4 and 8 h, the wild-type T7 phage decreased biofilm by only 23.8 % and 31.7 %, respectively [74].

In addition to targeting biofilms, phages can also be used to cleave antibiotic resistance genes either by delivering a specific antibiotic drug or a programmed CRISPR-Cas system [75,76]. For instance, phagemids encoding the CRISPR-Cas9 system were packaged in the staphylococcal phage ΦNM1 and designed to target the aph-3 kanamycin resistance gene [75]. Strong suppression of bacterial growth was observed when the recombinant ΦNM1phage was introduced into *S. aureus* RN4220 cells that had a kanamycin resistance gene. The ΦNM1phage, on the other hand, did not result in any appreciable suppression when combined with a non-targeting CRISPR-Cas system.

Artificial selection of useful traits to enhance the phage capability by phage engineering can be used to kill resistant bacteria more effectively than their wild-type forms. For example, engineered mycobacteriophages that contained host range mutants and exclusively lytic phages have been successfully used therapeutically to treat human infections in recent studies [77,78].After receiving bilateral lung transplantation, a 15-year-old patient with cystic fibrosis (homozygous for ΔF508) had non-tuberculous *Mycobacterium* (NTM) infections, including *Mycobacterium abscessus*, which was antibiotic-resistant. To investigate possible therapeutic phages, *M. abscessus* subsp. *massiliense* with a rough colony morphotype (named strain GD01) was isolated one-month post-transplantation. The GD01 strain was then used to screen 1000 phages. A phage cocktail was designed, and the patient underwent a single topical test of the sternal wound and continued intravenous (IV) therapy with a three-phage cocktail, consist of Muddy (Wild phage), Engineered (ZoeJΔ45) with deleted repressor gene and Engineered mutant (BPsΔ33HTH-HRM10) with deleted Integrase gene. The patient improved clinically with the healing of surgical wounds and skin lesions, and the lung function improved with no side effects [77].

Another successful clinical case was the treatment of *Mycobacterium chelonae* infection. Clinical manifestations of *M. chelonae* include localized skin or soft tissue infection, as well as extensive cutaneous disease. This rapidly proliferating non-tuberculous *Mycobacterium* infects immuno-compromised patients for a prolonged period. A 56-year-old man at a dermatology clinic complained of weight loss, sweats, and new nodular lesions on his left upper limb. The patient was diagnosed with *M. chelonae* infection. The strain (GD153) of *M. chelonae* was used to investigate possible therapeutic phages that might be helpful in the treatment of illnesses. Only Muddy and Muddy_HRM^{GD04}variations with a wider host range, demonstrated successful results in infecting GD153. In addition to medication and surgical treatment, a single bacteriophage was administered intravenously to the patient. The patient's illness improved steadily, and there were no signs of bacterial resistance to the phage [78].

The BRED technique has also been used to engineer phages from other strains, such as *Klebsiella* [52] and *E. coli* [35]. In *Klebsiella*, recombineering was used to demonstrate that the multi-host bacteriophage ΦK64-1 infects a different capsular strain of *Klebsiella*. Eleven of the genes in the bacteriophage ΦK64-1 had a sequence similar to that of tail fiber/spike or lyase. Eight of the 11 genes (S1-1, S1-2, S1-3, S2-1, S2-4, S2-5, S2-6, and S2-8) encoded capsule depolymerases, which allowed them to infect several *Klebsiella* capsular strains, including K1, K11, K21, K25, K30, K35, K64, and K69, as well as the novel capsular strains KN4 and KN5. The roles of these genes in phage infection were examined by deleting the gene followed by purifying mutant phages in the presence of hosts. Thus, mutant phages for the capsule depolymerase gene (ΔS1-2, ΔS2-2, ΔS2-3, and ΔS2-6) lost their ability to infect specific *Klebsiella* capsular strains (KN4, K25, K35, and K30/K69) respectively. This implies that capsule depolymerase is necessary for the infection to propagate in a specific host [52].

In coliphages, BRED was initially employed to eliminate a copy of the mobile element IS1 (transposon), which has been shown to be active, from the P1vir genome. The results demonstrated that the engineered phage with deleted IS1 (specific copy of lS1) did not directly contribute to lytic replication and displayed normal plaque morphology, burst size, phage titer, and capacity for generalized transduction. Therefore, P1virΔIS is a tool for genome engineering that is devoid of IS contamination [35].

Phage engineering is hampered by concerns about genetically modified phages being released into the environment because they may have unforeseen effects on the dynamics of bacterial communities. This needs to be rigorously validated and considered in phage genome engineering designs. Any natural product, either plant-based or bacteriophages, etc., cannot be patent protected, and hence, it becomes a major drawback for funding corporate organizations to invest and develop products for commercialization and large-scale

public use. Thus, genetic engineering to modify the phages to increase their fitness/value is a major factor that can lead to their patent protection and can attract funds from organizations for investment. Genetic alterations also provide an opportunity for different stakeholders to come up with newer and more potent phage products that can lead to effective competition, thereby reducing the cost of the products and providing sustainable options to the patients [79].

Some broader implications of engineered phages also include phage host range mutants (HRM) and temperature and pH-stable phages, which are listed below.

3.1.1. Phage host range mutants (HRM)

Phages are very specific to their host, and the major drawback of phage therapy is their limited host range [80]. It is nearly impossible to use a single phage type to target every strain of a species in a given genus. Through advancements in genetic engineering, receptor-binding proteins of phages can be swapped or modified to expand their host range in phage therapy. Changes in host specificity have been made by substituting receptor-binding protein genes across many strains, each of which targets a different host. For instance, switching the long-tail fiber genes of T2 and PPO1 phages leads to changes in the host of T2 from *E. coli*-K12 to *E. coli* O157:H7 [81]. Additionally, the T2 phage's long tail fiber gene was also switched with the IP008 phage tail fiber, which increased the T2 host range for additional *E. coli* strains [82]. By swapping heterologous receptor-binding genes between distant phages, even a phage intended to infect *E. coli* bacteria could possibly be capable of infecting *Klebsiella* bacteria and vice-versa [65]. Filamentous phages, such as fd and IKe, have minor pIII coat proteins that are responsible for their infectivity. The pIII coat protein of the fd phage, which infects *E. coli* containing F pili, was fused with the pIII coat protein (receptor-binding region) of the IKe phage that infects *E. coli* containing N or I pili that lead to widened host range of mutated fd phage as it can now infect either with N or even F pili [83]. The fd phage was also engineered to infect *V. cholera* by adding N-terminal 274 amino acids of the pIII gene of the filamentous phage CTXphi, which infects *V. cholera* through toxin-co-regulated pili [84]. As opposed to antibiotics, which have a broad spectrum activity, phages are more particular; however, some polyvalent (border host range) phages can also induce dysbiosis. To overcome this limitation, more testing on the host is necessary to prevent these side effects. On the other hand, phages can trigger prophages of the gut microbiome that can kill their respective hosts [85].

3.1.2. Temperature and pH stable phages

CAVE can prove to be an efficient tool for carrying out phage engineering with a directed evolutionary approach, as this strengthens the functional attributes of phages with a variety of criteria for phage selection [59]. CAVE has developed engineered phages with increased thermal stability at 60 °C and acidic pH resistance, and the mutations occurred within the structural and assembly protein subunits of phages listed in Table 2. Furthermore, CAVE can be employed for engineering phages with much better phenotypes, which are usually a constraint in wild-type phages, and these engineered phages can be effectively used in phage therapy and other antimicrobial purposes.

3.2. Phage endolysin derived enzybiotics as potential therapeutics

The term "enzybiotics" is derived from two words "enzyme" and "antibiotics" [86]. Enzybiotics are enzymes or, in some cases, non-enzymatic derivatives of phages that have been extensively utilized for their antibacterial and antimicrobial properties. The foundation of enzybiotic research is the hydrolytic enzyme class known as endolysins (or lysins), which are particularly successful in eradicating a variety of bacterial infections [87,88]. Endolysins, also known as murein hydrolases, cleave the bacterial host cell wall towards the end stage of the lytic cycle. Endolysins require a group of proteins known as holins to make pores/gaps in the cytoplasmic membrane from inside so that they can access the bacterial peptidoglycan layer. However, some endolysins can use signal sequences for their transport, such as pneumococcal phage SV1 and mycobacteriophage Ms6 lysin [89,90]. Only when the holin's concentration exceeds a predetermined level does this closely controlled chain of events begin. Endolysins may now access the peptidoglycan and break it down, which kills the bacterial cells [91]. The schematic representation of the endolysin mechanism has been demonstrated in Fig. 4.

Phage endolysins have been studied as potential medicines for the treatment of bacterial infections in both humans and animals ever since it was discovered that their exogenous application causes lysis of the host bacteria [92,93]. In 2001, Nelson et al. released the first study that demonstrated an endolysin's effectiveness *in vivo* [86]. As the phage and their hosts have co-evolved, there is far less

Table 2
CAVE-induced mutations in various structural proteins of T3 and T7 phage of *E.coli* [59].

Bacteriophage	Gene no.	Gene name	Mutation rate
T3	gp37	Head-to-tail joining protein	0.998
T3	gp45	Internal virion B	0.713
T3	gp48	Tail fiber protein	0.197
T7	gp47	Tail tubular protein B	0.951
T7	gp51	Internal virion protein D	0.935
T7	gp42	Head-to-tail joining protein	0.930
T7	gp57	DNA maturation protein	0.877
T7	gp17	DNA binding protein	0.838
T7	gp43	Capsid assembly protein	0.720

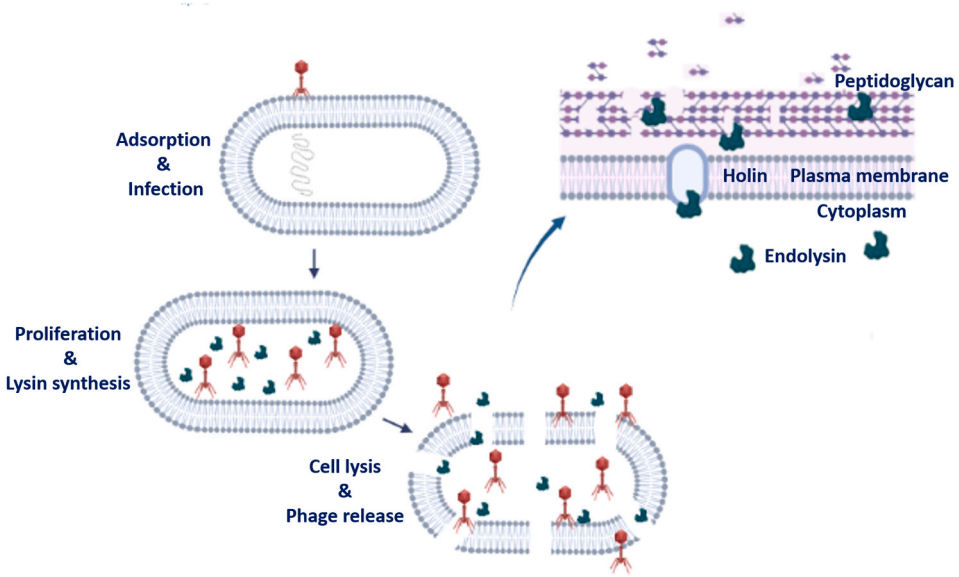


Fig. 4. Schematic representation of the action of phage-derived endolysins in phage therapy.

risk of resistance to endolysins than to antibiotics [94]. Over the past 20 years, research on these enzymes has advanced from straightforward *in vitro* characterization to sophisticated protein engineering methods, including state-of-the-art pre-clinical and clinical testing [95,96]. Despite these advancements, there are still certain problems that need to be addressed with the systemic use of endolysins. They include immunogenicity and circulation half-life, as well as characteristics that target and penetrate particular cells and tissues [97,98].

Owing to the distinct cell wall topologies of these two important bacterial groupings, endolysins that target Gram-positive, Gram-negative, and mycobacteria frequently have different structures. Most endolysins are composed of a polypeptide chain that is divided into two parts: a catalytic domain (CD) at the protein's N-terminus and a cell wall-binding domain (CBD) at the C-terminus connected by a brief, bendable linker between these two components [99,100].

Nelson et al. demonstrated in 2001 that the streptococcal phage lysin PlyC could both prevent and treat group A streptococcal upper respiratory colonization in mice (then known as C1 lysin). The test shows that lysin is a unique murein hydrolase that rapidly eliminates group A streptococci both in vitro and in vivo while having no impact on the other native microorganisms being studied. Using this broad approach, streptococcimay be decreased or eradicated from carriersor sick individualsupper respiratory mucosal epithelium, hence reducing the associated disease [101].

3.2.1. Gram positive bacteria-targeting endolysins

Most Gram-positive bacteria have a continuous, somewhat thick cell wall that is mostly composed of peptidoglycan and ranges in thickness from 30 to 100 nm [102]. A single type of endolysin is produced by bacteriophages that attack Gram-positive bacteria that have evolved a modular structure that typically separates enzymatic activity and cell wall recognition into different domains and connects them with flexible linkers [103]. The endolysin's cell wall-binding domain (CBD) enables it to make a specific, targeted connection to the target bacterial cell wall, whereas its enzymatically active domains (EAD), which give its catalytic activity, often reside at its N-terminus [104,105].

The PlyG endolysin from the *Bacillus anthracis* phage, a chemical significant with regard to biowarfare, has demonstrated remarkable results in addition to treating streptococcal infections. Mice that were intra-peritoneally injected with *Bacillus* cells and given buffer died after 2 h, whereas mice that were given PlyG lived for 70–80 % of the 72 h of the experiment [106]. The treatment of staphylococcal infections in animal models, particularly Methicillin Resistance *Staphylococcus aureus* (MRSA), has been the subject of numerous articles. In the first study from 2007, MRSA was eliminated from mouse nares using the MV-L endolysin from phage MR11. When the same enzyme was supplied intravenously at 0, 30 and 60 min following systemic MRSA infection, respectively, 100 %, 100 %, and 60 % of the mice survived the test [107]. *S. aureus* frequently causes osteomyelitis, a bone infection that is difficult to treat because the bacteria are resistant to medications and persist inside the cells. To treat experimental osteomyelitis in rats, Karau et al. gave endolysin CF-301 intravenously one week after infection, and the localized *S. aureus* bacteria in the bone were greatly reduced by this systemic injection. However, compared to the control, the impact was only about a 0.48 log decrease [108].

3.2.2. Gram negative bacteria-targeting endolysins

Gram-negative bacteria are shielded from the environment by an outer membrane made of lipopolysaccharides that surrounds the peptidoglycan cell wall [109]. Gram-negative bacteria have thin cell walls that are between 20 and 30 nm in thickness [110]. While endolysins that attack Gram-negative bacteria are typically small, single-domain globular proteins (molecular mass between 15 and

20 kDa), they can also be found in other structural forms [111,112].

Artylysins are altered endolysin structures that destabilize the lipopolysaccharide of Gram-negative bacteria. Artilysins are able to pass through the outer membrane and reach the peptidoglycan, where they exert their action. In one study, novel artilysins (designated as AL-3AA, AL-9AA, and AL-15AA) were developed with antimicrobial-peptide SMAP29 fusion at the N-terminal of LysPA26 and utilized them. The findings demonstrated the significant bactericidal activity of artilysin AL-3AA; even 0.05 mg/mL AL-3AA could kill 5.81 log units of *P. aeruginosa* without EDTA in 60 min. AL-3AA significantly reduced *P. aeruginosa* biofilms and prevented the development of *P. aeruginosa* PAO1 biofilms. Additionally, it may have had broad-spectrum activity against *K. pneumoniae* and *E. coli*, two susceptible Gram-negative bacteria most often found in hospitals [113,114]. The Gram-positive *Bacillus amyloliquefaciens* phage IAM 1521 endolysin (Lys1521) is the most extensively researched example. Lys1521 (40 μg/mL) externally applied to Gram-negative bacteria like *E. coli* W3110 and *P. aeruginosa* decreased the number of cells by 98 % (1.90 log) and 99.78 % (2.66 log) in 10 min, respectively [115]. The SPN9CC endolysin worked by cleaving the glycosidic linkages of peptidoglycan, demonstrated lytic activity by exogenous applications, and exclusively had antibacterial action against Gram-negative bacteria. It is interesting to note that without EDTA, a permeabilizer of the outer membrane, the SPN9CC endolysin could still lyse intact Gram-negative bacteria [116]. LysAB54, a novel endolysin with limited homology to other well-known related endolysins from bacteriophage p54, was cloned, expressed, and characterized. LysAB54 has demonstrated considerable bactericidal action in the absence of outer membrane permeabilizers against multidrug-resistant *A. baumannii* and other Gram-negative bacteria, such as *P. aeruginosa*, *K. pneumoniae*, and *E. coli,* making it a promising therapy option for Gram-negative superbugs that are multidrug-resistant [117].

3.2.3. Mycobacteriophage endolysin

Mycobacteriophages, viruses that infect mycobacterial hosts, face significant obstacles because of the unusual structure of the mycobacterial cell wall, which consists of a mycolic acid-rich mycobacterial outer membrane attached to an arabinogalactan layer connected to the peptidoglycan. The mycobacterial cell wall has a complex structure that makes it unique and sets it apart from both Gram-positive and Gram-negative bacteria [118]. Its exceptionally high lipid content (up to 60 % of the cell wall is made of lipids) accounts for the highly hydrophobic cell surface properties, leading to a naturally impermeable cell wall, resistance to many antibacterial drugs, and exceptional inflammatory activity, playing a key role in virulence [119].

While the arabinogalactan-peptidoglycan complex is covalently attached to the outer membrane rich in mycolic acid, lysis must not only remove the mycolic acid layer but also penetrate it in order to split the peptidoglycan layer. Consequently, two different endolysins are produced by mycobacteriophages: Lysin A (LysA), which hydrolyzes peptidoglycan, and Lysin B (LysB), a novel mycolylarabinogalactan esterase, which cleaves the mycolylarabinogalactan bond to release free mycolic acid [120–122].

Mycobacteriophage Ms6 endolysins, Lysin384 and Lysin241, were found to inhibit the growth of mycobacterial species like *M. smegmatis*, *M. aurum*, and *M. fortuitum* when exogenously applied. In a different investigation, mycobacteriophage BTCU-1 lysin A and lysin B demonstrated alteration of the morphology of *M. smegmatis* and its increased capacity to eradicate intracellular *M. smegmatis* [123,124].

There is an advantage over using complete mycobacteriophages, as only a few numbers of phages have been demonstrated to infect tubercle bacilli thus far. Therefore, the large number of isolated mycobacteriophages represents a vast reservoir of various endolysin-degrading enzymes that have the potential for numerous therapeutic uses. The major drawback of endolysin engineering procedures is labor and time extensive. However, numerous attempts have been made in the designing of endolysin derivatives that are capable of penetrating the outer membrane to reach the peptidoglycan layer of Gram-negative bacteria but have achieved patchy success [79].

The applications of microbial recombinant enzymes have increased the therapeutic possibilities for humans; however, challenges such as high immunogenicity, protein instability, brief half-lives, and low substrate affinity still need to be overcome [125]. Enzymes with higher activity and fewer side effects, as well as those that can be genetically changed, are all still being sought after. Endolysins have a lot of promise as potential alternatives or complements to traditional antibiotics. When endolysins are administered exogenously to some bacteria, cell lysis occurs quickly [126]. We can develop novel endolysins with higher stability, specificity, and lytic action, which can increase the potential of endolysins. To successfully create and use endolysins, it is essential to thoroughly comprehend how their biochemical, biophysical, and bacteriolytic characteristics interact with one another.

3.3. Phage cocktail/cocktail+antibiotic

Phage treatment is appealing since it can be applied to eliminate antibiotic-resistant bacteria [127]. There is no cross-resistance between antibiotics and phages because the processes by which phages and antibiotics eliminate bacterial pathogens are fundamentally different from one another. To be used for phage therapy,the newly isolated phage should be structurally (using TEM), functionally (MOI, one-step growth curve, and resistance to environmental stresses), and bioinformatically (genome assembly, annotation, and sequencing) characterized. The conventional cocktail is formed based on the idea of including more phages that have different target receptors than a common receptor, such as bacterial LPS, and the infectivity of the cocktail should be assessed in contrast to individual phages. Crucial lab parameters, such as a shorter latency period, larger burst size, broader antibiotic resistance spectrum, absence of toxic genes, and lytic nature of the phage, are desirable features. A cocktail is advised because of its higher infectivity than individual phages over a range of pH and temperature conditions as well as a wider host range and fewer chances of resistance [128]. However, this can be overcome by using a phage cocktail. A combination of six phages has been demonstrated to effectively treat sepsis in *Galleria mellonella* models and respiratory *P. aeruginosa* infections in mice [129]. In theory, phages can lyse both antibiotic-sensitive and resistant bacteria with equal effectiveness. In addition, phages and antibiotics can be used in conjunction to treat bacterial infections [130]. Research has also demonstrated that the co-administration of phages and antibiotics restores

Table 3
Clinical Trials of Phage therapy for various infectious diseases [142,143].

S. No.	Disease	Target Bacteria	Model organism	Bacteriophages	(Administration route)	Concurrent antibiotic treatment (Administration route)	Efficacy and Safety
1.	Bone Infection	*Proteus mirabilis, Morganella morganii* and *Staphylococcus aureus* (MDR)	Human	14-1, PNM, and ISP	Intralesional (IL)	Ciprofloxacin (IV), rifampicin (IV), and linezolid (IV)	Osteomyelitis resolved, targeted bacteria eradicated
2.	Bone Infection	*P. aeruginosa* (MDR)	Human	14-1, PNM, and ISP	Topical (bandages soaked with bacteriophages)	Ciprofloxacin (oral) and rifampicin (oral)	Targeted bacteria eradicate and clinical improvement observed
3.	Lower respiratory tract infection and urinary tract infection	*P. aeruginosa* (XDR)	Human	Acibel004, Acibel007, 14-1, PNM, and ISP	Nebulisation	Colistin (IV) and ceftolozane/ tazobactam (IV)	Patient transferred for revalidation and clinical improvement observed
4.	Upper respiratory tract infection	*S. aureus* and *A. xylosoxidans* (MDR)	Human	ISP and JWDelta	Nasal spray		Less sputum, less fatigue, sleeps better
5.	Lower respiratory tract infection	*P. aeruginosa* (MDR)	Human	14-1, PNM, and PT07	Nebulisation	Ceftazidime/ avibactam (IV), ciprofloxacine (IV), colistin (IV), and tobramycin (aerosol)	No more *P. aeruginosa* isolated from samples
6.	Lower respiratory tract infection	*P. aeruginosa* (usual drug resistance-UDR)	Human	PNM	Nebulisation		Less exacerbation, but a new bacterial species (*K. pneumoniae*) emerged post bacteriophage treatment
7.	Skin and soft tissue infection	*P. aeruginosa* (XDR)	Human	Acibel004, Acibel007, 14-1, PNM, and ISP	Topical		Wounds completely closed, patient regained ambulation and was discharged
8.	Skin and soft tissue infection	*Acinetobacter baumannii, K. pneumoniae, Staphylococcus haemolyticus, and P mirabilis*	Human	Acibel004, Acibel007, 14-1, PNM, and ISP	Topical	Cotrimoxazole (IV) and meropenem (IV)	Complete wound healing, patient discharged
9.	Erysipelas with skin necrosis	*S. aureus*	Human	ISP	Topical	Meropenem (IV)	After *S. aureus* eradication, *P. aeruginosa* (not sensible to the bacteriophages) emerged in the wound bed
10.	Other infection (Prostatitis)	*Escherichia coli* (MDR)	Human	PyoPhage	Rectal	Fosfomycin (oral)	No more clinical signs of prostatitis
11.	Other infection (Bloodstream infection)	*P. aeruginosa* (UDR)	Human	14-1 and PNM	Intravenous (IV)	Ceftazidime (IV) and ciprofoxacin (IV)	Negative blood cultures
12.	Other infection (Bloodstream infection)	*S. aureus* (UDR)	Human	ISP	Intravenous (IV)	Flucloxacillin (IV)	Clinically improved
13.	Abdominal infection and Chron's disease with anal fistula	*P. aeruginosa* (MDR)	Human	14-1, PNM, and PT07	Intralesional (draining system)		Wounds completely closed
14.	Abdominal infection (AbdI), Severe chest and abdominal infection after separation of conjoined twins	*K. pneumoniae* (XDR)	Human	M1	Intralesional (draining system)	Meropenem (IV), colimycin (IV), amikacin (IV), and fluconazole (IV	*K. pneumoniae* no longer isolated from the wound, while still present in the patient's stool
15.	Orthopedic prostheses infection and Chronic prosthetic joint	*S. epidermidis* (XDR)	Human	ISP	Intralesional (pigtail catheter)		Painless and functional shoulder and no discharge

(*continued on next page*)

Table 3 (*continued*)

S. No.	Disease	Target Bacteria	Model organism	Bacteriophages	(Administration route)	Concurrent antibiotic treatment (Administration route)	Efficacy and Safety
	infection of the left shoulder						
16.	Orthopedic prostheses infection (OPI), Chronic osteomyelitis of the femur	*P. aeruginosa* (XDR)	Human	14-1, PNM, and ISP	Intralesional (pigtail catheter)	Linezolid (IV) and ceftazidime/ avibactam (IV)	Osteomyelitis resolved as observed through MRI
17.	Urinary tract infection	*Cronobacter turicensis*	Mice		Intraperitoneal		Bacterial load reduced by 70 %
18.	Urinary tract infection	*Salmonella enteritidis*	Mice	Single phage	Intraperitoneal		Prevent 40 % of the mice fatal illness
19.	Eye and ear infections	*P. aeruginosa*	Dog	Single phage	Topical		After 48 h significantly decrease & No additional adverse effect
20.	Skin and Soft tissue infection	*P. aeruginosa, S. aureus and A. baumannii*	Pig	6 phage cocktail	Topical		Result varied depending on the species but debridement had positive effect
21	Skin and Soft tissue infection	*S. aureus*	Rat (Wister)		Intramuscular		After 12hr of infection, trasnfersome entrapped phage cocktail protected test animal
22.	Gastrointentestinal tract infection	*Vibiro cholerae*	Rabbit	Phage cocktail	Oral		Reduce the bacterial load
23.	Bacteremia	*E. coli*	Rat (Sprague Dawley rat pups)		Intraperitoneal or subcutaneous		Sepsis and meningitis model was used with single phage dose with suruvival 100 and 50 % respectively
24.	Gastrointentestinal tract infection (GIT)	*Clostridium difficle*	Hamster (Syrian golden)	Phage	Oral		Reduce the bacterial colonization after 36hr
25.	Soft and Soft tissue infection	M. ulcerans	Mice (BALB/c)	D29	Subcutaneous		Reduced pathology and prevented development of ulcers

antibiotic sensitivity [131]. In a case study, phage and ceftazidime antibiotics were administered directly to an aortic prosthetic graft patient that had been infected with *P. aeruginosa*. The infection was successfully treated and could potentially be eradicated [132]. According to Kirby's research, treating *S. aureus* with gentamicin and phage SA5 together is more effective than using gentamicin or SA5 alone [133]. Similarly, when phage LUZ7 and streptomycin were administered together rather than individually, *P. aeruginosa* titers were reduced [134]. Same results were observed in a mouse model for diabetes where *S. aureus* was infected on each mouse's hind paw. When phage MR-10 and linezolid were used to treat the infections simultaneously, the most significant decrease in bacterial titer was observed [135]. It has been demonstrated that the combined therapy employing ciprofloxacin and phage KPO1K2 can effectively halt the establishment of resistant variants in vitro, in addition to eradicating *K. pneumoniae* biofilms [136]. Researchers have discovered OMKO1, a lytic phage that attacks *P. aeruginosa* by attaching to its outer membrane porin M (OprM) receptor. The *P. aeruginosa* antibiotic efflux pump contains an OprM channel. As a result of phage OMKO1 infection, OprM mutations that compromised its efflux function were selected to restore antibiotic sensitivity in *P. aeruginosa*. Recent research has discovered a phenomenon known as phage-antibiotic synergy (PAS), which causes host bacteria to produce more phages when treated with a phage plus sub-lethal dosages of specific antibiotics [137,138]. Phage cocktail has some major drawbacks also. Firstly, the criteria for choosing a therapeutic phage are not clearly defined. The majority of findings to date have concentrated on the host range of phages; however, other aspects, such as the phage's capacity to infect stationary phase bacteria, its enzymes, mutation rate, and stability to serum inactivation, have been demonstrated to be significant but have not been studied yet. Secondly, there are insufficient public phage libraries (including engineered phages), which is a major problem for therapeutic phages [85].

3.4. Phage therapy advances for intracellular bacterial infections

Intracellular bacterial burden poses a significant challenge in infections, yet phage therapy offers a promising approach to address this issue. Studies have demonstrated phage-mediated killing of *E. coli* strain EV36 by phage K1F in urinary bladder epithelial cells and human cerebral microvascular endothelial cells ex vivo. Phage activity against multidrug-resistant *S. aureus* strains in fibroblasts, epithelial cells, osteoblasts, and peritoneal macrophages is also reported. Phage P1 has proven effective against *S. aureus* in murine models, while antimycobacterial phage D29 has shown efficacy against *M. tuberculosis* in macrophage cell lines. Phage TM4 has killed M. *tuberculosis* and *M. avium* inside murine macrophages both ex vivo and in vivo. Additionally, ex vivo phage activity has been demonstrated against *Chlamydia* spp., *K. pneumoniae*, *Salmonella enterica*, and *P. aeruginosa* [139].

Despite these promising results, clinical efficacy against intracellular pathogens like *P. aeruginosa*, *M. abscessus*, and *S. aureus* has not been conclusively proven, and the mechanisms of intracellular action remain unclear. Phages targeting intracellular bacteria must penetrate the host cell membrane and reach specific compartments, with entry efficiency influenced by both cell type and phage to be tested. Comprehensive studies are required to identify factors like phage concentration, cell type, and modifications that affect phage movement within cells. Challenges such as inactivation and destruction within eukaryotic cells reduce effective phage-bacterium interactions and pathogen clearance. Enhancing phage delivery and efficacy is essential to fully exploit their therapeutic potential against intracellular infections [139].

Phage engineering has enabled the development of phages that interact with human cells and bacteria. Enhancements such as incorporating mammalian cell surface receptors (e.g., EGF, FGF2) or cell-wall penetrating peptides from HIV into phage capsids have improved phage uptake into human cells and reduced bacterial loads. However, these modifications have yet to demonstrate infection clearance in vivo or progress to mammalian trials, highlighting the need for further animal studies before clinical application [140].

4. Addressing gene transfer concerns in phage therapy

Antibiotic resistance in bacteria typically arises through either horizontal transfer or vertical transfer of antibiotic resistance genes. Horizontal transfer can be facilitated by bacteriophages, while vertical transfer occurs among the progeny of bacteria or via certain chromosomal mutations. Bacteriophages typically do not contribute to antimicrobial resistance (AMR), but there are some reports suggesting their involvement in AMR [141]. However, this limitation can potentially be overcome through the engineering of phages. Phage engineering employs various advanced genetic techniques to effectively combat antimicrobial resistance (AMR). These methods include homologous recombination, which allows precise modifications to phage genomes, ensuring they specifically target and destroy antibiotic-resistant bacteria. BRED and CRISPY BRED/CRISPY BRIP enable the introduction of specific genetic alterations to enhance phage efficacy. CRISPR-Cas systems facilitate the removal of resistance genes from bacterial genomes, preventing horizontal gene transfer. Additionally, CAVE accelerates the adaptation of phages to new bacterial targets, while rebooting of phage genomes allows for the synthesis of phages with desired traits. These engineered phages can enhance their lytic activity, broaden their host ranges, and work synergistically with antibiotics to restore their efficacy or deliver genetic tools that reverse resistance mechanisms. Overall, these advanced techniques provide a targeted and sustainable solution to manage AMR.

5. Phage therapy in clinical trials

Phage therapy is a promising treatment for persistent bacterial infections and is undergoing clinical trialshas been listed in Table 3.

6. Ethical challenges of phage therapy

Phage therapy, whether utilizing natural or engineered bacteriophages, raises diverse ethical challenges across scientific, regulatory, and societal domains. The main concerns include the absence of intellectual property protections for natural phages, as they are not patentable under current IPR guidelines. This complicates investment recovery and introduces uncertainty to profitability in therapy development. Ethical challenges emerge from the complex and unpredictable risks of phage therapy, making rigorous patient consent processes essential because of the varying levels of comprehension among healthcare providers and patients. There are also concerns about phage therapy potentially contributing to antibiotic resistance through horizontal gene transfer and the appropriate use of lytic versus lysogenic phages, since lysogenic phages can induce resistance in bacteria. Phage therapy is currently used for topical treatments, food sterilization, and as a last-resort option post-antibiotic failure, given the unexplored nature of this field and associated risks. It demands increased research investment and robust clinical trials to enhance efficacy, develop precise diagnostics, and ensure equitable global access to this promising medical treatment [144].

7. Conclusions

Phage therapy offers promise for combating resistant bacterial strains, but it comes with certain advantages and challenges. Wild phages often do not meet therapeutic requirements and pose risks like lysogeny and the transfer of virulence or antibiotic resistance genes. Effective phage therapy necessitates extensive scientific and clinical research to validate its safety and dispel misconceptions about phages causing human infections. Regulatory frameworks, particularly those in practice the European Union, are essential to ensure safe phage introduction in environments such as farm, animal, water or feed.

For personalized medicine, comprehensive phage biobanks are crucial to match specific bacterial strains. Major challenges include

high commercialization costs, regulatory hurdles, and patenting difficulties. Technological advancements in genetic engineering, such as sequencing, CRISPR/Cas-based phage engineering, homologous recombination, and phage genomic DNA assembly, can create phages that target antibiotic-resistant bacteria without harming the patient's normal flora. These engineered phages can be patented and commercialized more easily. Rich phage libraries from global research teams and PT centers could ensure the availability of phage products. Diagnostic testing is pivotal in advancing bacteriophage therapy, yet there remains a critical need for further refinement and enhancement to maximize its efficacy. Currently, diagnostic protocols primarily involve monitoring the in vivo emergence of bacteriophage resistance, evaluating bacteriophage-antibiotic interactions in vitro, and assessing bacteriophage immune neutralization through patient serum. However, to improve the precision and impact of phage therapy, future efforts should focus on developing more sophisticated diagnostic tools.

Researchers are also optimizing phage therapies. For example, Locus is developing CRISPR-Cas3-based phage therapeutics, which enhance bacterial killing by shredding bacterial DNA. This ability to modify phages enhances their potential in combating antimicrobial resistance (AMR) by enabling more effective bacterial eradication compared to regular phages. While phage engineering methods can be time-consuming, labor-intensive, and may require some modifications for improved screening of recombinant phages, but they provide us with valuable insights into the methods that are currently being utilized for PGE and the possibility for their future modifications/upgrades for improvement. Engineered phages have shown effective results, bridging the gap from research to clinical applications [145].

Clinical applications of phage therapy have demonstrated its efficacy in treating bacterial infections. In Georgia, the Eliava Institute offers over-the-counter phage preparations, while Poland's Hirszfeld Institute provides personalized phage products to medical practitioners. Phages exhibit good primary pharmacodynamics (antibacterial effectiveness), minimal secondary pharmacodynamics (low adverse effects on humans), and effective pharmacokinetics (ability to reach target bacteria).

To advance phage therapy, collaboration among researchers, medical professionals, and regulatory organizations is essential. They must address regulatory issues, develop treatment guidelines, and organize public phage libraries. The priorities for phage therapy include extensive research and clinical trials, establishing regulatory frameworks, maintaining phage biobanks, better diagnostic testing, advancing technological methods, and ensuring quality control [146].

Data availability statement

All data to support the conclusion have been either provided or are otherwise publicly available.

Funding

This review received no external funding.

CRediT authorship contribution statement

Anuja Kakkar: Writing – original draft, Data curation. **Garima Kandwal:** Writing – original draft, Data curation. **Tanmayee Nayak:** Writing – original draft, Data curation. **Lav Kumar Jaiswal:** Writing – review & editing. **Amit Srivastava:** Writing – review & editing. **Ankush Gupta:** Validation, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The work described in this article has not been published previously, it is not under consideration for publication elsewhere, its publication is approved by all the authors and tacitly or explicitly by the responsible authorities where the work was carried out, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

References

[1] T.T. Yoshikawa, Antimicrobial resistance and aging: beginning of the end of the antibiotic era? J. Am. Geriatr. Soc. 50 (2002) 226–229. https://agsjournals.onlinelibrary.wiley.com/doi/abs/10.1046/j.1532-5415.50.7s.2.x.

[2] D. Yong, M.A. Toleman, C.G. Giske, H.S. Cho, K. Sundman, K. Lee, T.R. Walsh, Characterization of a new metallo-β-lactamase gene, bla NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India, Antimicrob. Agents Chemother. 53 (2009) 5046–5054. https://journals.asm.org/doi/abs/10.1128/aac.00774-09.

[3] C.J. Murray, K.S. Ikuta, F. Sharara, L. Swetschinski, G.R. Aguilar, A. Gray, N. Tasak, Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, Lancet 399 (2022) 629–655. https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(21)02724-0/fulltext?ref=healthtips.kr.

[4] UN. At UN, global leaders commit to act on antimicrobial resistance: collective effort to address a challenge to health, food security, and development, Press release: high-Level Meeting on Antimicrobial Resistance, General Assembly of the United Nations, 21 September 2016. Available from: https://www.un.org/pga/71/2016/09/21/press-release-hl-meeting-on-antimicrobial-resistance/.

[5] M. Kumar, D.K. Sarma, S. Shubham, M. Kumawat, V. Verma, P.B. Nina, R.R. Tiwari, Futuristic non-antibiotic therapies to combat antibiotic resistance: a review, Front. Microbiol. 12 (2021) 609459. https://www.frontiersin.org/articles/10.3389/fmicb.2021.609459/full.

[6] B. Gibb, P. Hyman, C.L. Schneider, The many applications of engineered bacteriophages—an overview, Pharmaceuticals 14 (2021) 634. https://www.mdpi.com/1424-8247/14/7/634.

[7] D.M. Lin, B. Koskella, H.C. Lin, Phage therapy: an alternative to antibiotics in the age of multi-drug resistance, World J. Gastrointest. Pharmacol. Ther. 8 (2017) 162. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5547374/.

[8] N. Chanishvili, Phage therapy—history from Twort and d'Herelle through Soviet experience to current approaches, Adv. Virus Res. 83 (2012) 3–40. https://www.sciencedirect.com/science/article/pii/B9780123944382000013.

[9] A. Sulakvelidze, Z. Alavidze, J.G. Morris Jr., Bacteriophage therapy, Antimicrob. Agents Chemother. 45 (2001) 649–659. https://journals.asm.org/doi/abs/10.1128/aac.45.3.649-659.2001.

[10] P. Hyman, Phages for phage therapy: isolation, characterization, and host range breadth, Pharmaceuticals 12 (2019) 35. https://www.mdpi.com/1424-8247/12/1/35.

[11] T. Nayak, R.K. Singh, L.K. Jaiswal, A. Gupta, Bacteriophage encoded endolysins as potential antibacterials, J. Sci. Res 63 (2019) 39–48. https://www.researchgate.net/profile/Rakesh-Singh-38/publication/337974652_BACTERIOPHAGE_ENCODED_ENDOLYSINS_AS_POTENTIAL_ANTIBACTERIALS/links/5df8ca8e4585159aa4832f8a/BACTERIOPHAGE-ENCODED-ENDOLYSINS-AS-POTENTIAL-ANTIBACTERIALS.pdf.

[12] M. Sho, Identification and Characterization of Nutrient Transporters in *Mycobacterium Smegmatis*, Ph.D. Thesis, Cornell University, New York, NY, USA, 2011, https://ecommons.cornell.edu/bitstream/1813/29422/1/ms474thesisPDF.pdf.

[13] S. Meile, J. Du, M. Dunne, S. Kilcher, M.J. Loessner, Engineering therapeutic phages for enhanced antibacterial efficacy, Curr. Opin. Virol. 52 (2022) 182–191. https://www.sciencedirect.com/science/article/pii/S1879625721001607.

[14] T. Nayak, A. Kakkar, R.K. Singh, L. Kumar, A.K. Singh, L. Temple, A. Gupta, Isolation and characterization of a novel mycobacteriophage Kashi-VT1 (KVT1) infecting Mycobacterium species, Front. Cell. Infect. Microbiol. 13 (2023) 1173894. https://www.frontiersin.org/articles/10.3389/fcimb.2023.1173894/full.

[15] A. Lopes, J. Amarir-Bouhram, G. Faure, M.A. Petit, R. Guerois, Detection of novel recombinases in bacteriophage genomes unveils Rad52, Rad51 and Gp2.5 remote homologs, Nucleic Acids Res. 38 (2010) 3952–3962. https://academic.oup.com/nar/article-abstract/38/12/3952/2409217.

[16] M. De Paepe, G. Hutinet, O. Son, J. Amarir-Bouhram, S. Schbath, M.A. Petit, Temperate phages acquire DNA from defective prophages by relaxed homologous recombination: the role of Rad52-like recombinases, PLoS Genet. 10 (2014) e1004181. https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1004181.

[17] M. Namura, T. Hijikata, K. Miyanaga, Y. Tanji, Detection of *Escherichia coli* with fluorescent labeled phages that have a broad host range to *E. coli* in sewage water, Biotechnol. Prog. 24 (2008) 481–486. https://aiche.onlinelibrary.wiley.com/doi/abs/10.1021/bp070326c.

[18] Y. Tanji, C. Furukawa, S.H. Na, T. Hijikata, K. Miyanaga, H. Unno, *Escherichia coli* detection by GFP-labeled lysozyme-inactivated T4 bacteriophage, J. Biotechnol. 114 (2004) 11–20. https://www.sciencedirect.com/science/article/pii/S0168165604002743.

[19] Y. Chen, H. Batra, J. Dong, C. Chen, V.B. Rao, P. Tao, Genetic engineering of bacteriophages against infectious diseases, Front. Microbiol. 10 (2019) 954. https://www.frontiersin.org/articles/10.3389/fmicb.2019.00954/full.

[20] D. Yu, H.M. Ellis, E.C. Lee, N.A. Jenkins, N.G. Copeland, D.L. Court, An efficient recombination system for chromosome engineering in *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 5978–5983. https://www.pnas.org/doi/abs/10.1073/pnas.100127597.

[21] H.M. Ellis, D. Yu, T. DiTizio, D.L. Court, High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides, Proc. Natl. Acad. Sci. USA 98 (2001) 6742–6746. https://www.pnas.org/doi/abs/10.1073/pnas.121164898.

[22] Y. Zhang, F. Buchholz, J.P. Muyrers, A.F. Stewart, A new logic for DNA engineering using recombination in *Escherichia coli*, Nat. Genet. 20 (1998) 123–128. https://www.nature.com/articles/ng1098_123.

[23] M.M. Gottesman, M.E. Gottesman, S. Gottesman, G. Martin, Characterization of bacteriophage λ reverse as an *Escherichia coli* phage carrying a unique set of host-derived recombination functions, J. Mol. Biol. 88 (1974) 471–487. https://www.sciencedirect.com/science/article/pii/0022283674904963.

[24] S.K. Kulkarni, F.W. Stahl, Interaction between the sbcC gene of *Escherichia coli* and the gam gene of phage lambda, Genetics 123 (1989) 249–253. https://academic.oup.com/genetics/article-abstract/123/2/249/5998734.

[25] R. Court, N. Cook, K. Saikrishnan, D. Wigley, The crystal structure of λ-Gam protein suggests a model for RecBCD inhibition, J. Mol. Biol. 371 (2007) 25–33. https://www.sciencedirect.com/science/article/pii/S0022283607006730.

[26] L.J. Marinelli, G.F. Hatfull, M. Piuri, Recombineering: a powerful tool for modification of bacteriophage genomes, Bacteriophage 2 (2012) 5–14. https://www.tandfonline.com/doi/abs/10.4161/bact.18778.

[27] L.J. Marinelli, M. Piuri, Z. Swigoňová, A. Balachandran, L.M. Oldfield, J.C. van Kessel, G.F. Hatfull, BRED: a simple and powerful tool for constructing mutant and recombinant bacteriophage genomes, PLoS One 3 (2008) e3957. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003957.

[28] L.C. Thomason, A.B. Oppenheim, D.L. Court, Modifying bacteriophage λ with recombineering, in: Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions, Springer, Berlin/Heidelberg, Germany, 2009, pp. 239–251. https://link.springer.com/protocol/10.1007/978-1-60327-164-6_21.

[29] N. Marsić, S. Roje, I. Stojiljković, E. Salaj-Smic, Z. Trgovcević, In Vivo studies on the interaction of RecBCD enzyme and lambda Gam protein, J. Bacteriol. 175 (1993) 4738–4743. https://journals.asm.org/doi/abs/10.1128/jb.175.15.4738-4743.1993.

[30] N. Nafissi, R. Slavcev, Bacteriophage recombination systems and biotechnical applications, Appl. Microbiol. Biotechnol. 98 (2014) 2841–2851. https://link.springer.com/article/10.1007/s00253-014-5512-2.

[31] J.C. Van Kessel, G.F. Hatfull, Efficient point mutagenesis in mycobacteria using single-stranded DNA recombineering: characterization of antimycobacterial drug targets, Mol. Microbiol. 67 (2008) 1094–1107. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2958.2008.06109.x.

[32] J.C. van Kessel, G.F. Hatfull, Mycobacterial recombineering, Chromosom. Mutagen. 435 (2008) 203–215. https://link.springer.com/protocol/10.1007/978-1-59745-232-8_15.

[33] J.C. van Kessel, G.F. Hatfull, Recombineering in *Mycobacterium tuberculosis.nat*, Methods 4 (2007) 147–152. https://www.nature.com/articles/nmeth996/briefing/signup/.

[34] L.J. Marinelli, M. Piuri, G.F. Hatfull, Genetic manipulation of lytic bacteriophages with BRED: bacteriophage recombineering of electroporated DNA, in: Bacteriophages: Methods and Protocols, vol. IV, Springer, Berlin/Heidelberg, Germany, 2019, pp. 69–80. https://link.springer.com/protocol/10.1007/978-1-4939-8940-9_6.

[35] T. Fehér, I. Karcagi, F.R. Blattner, G. Pósfai, Bacteriophage recombineering in the lytic state using the lambda red recombinases, Microb. Biotechnol. 5 (2012) 466–476. https://ami-journals.onlinelibrary.wiley.com/doi/abs/10.1111/j.1751-7915.2011.00292.x.

[36] P. Tao, X. Wu, V. Rao, Unexpected evolutionary benefit to phages imparted by bacterial CRISPR-Cas9, Sci. Adv. 4 (2018) eaar4134. https://www.science.org/doi/abs/10.1126/sciadv.aar4134.

[37] U. Müller, P. Vogel, G. Alber, G.A. Schaub, The innate immune system of mammals and insects, Trends Innate Immun 15 (2008) 21–44. https://karger.com/Article/Abstract/135684.

[38] A. Hatoum-Aslan, Phage genetic engineering using CRISPR–Cas systems, Viruses 10 (2018) 335. https://www.mdpi.com/1999-4915/10/6/335.

[39] B. Martel, S. Moineau, CRISPR-Cas: an efficient tool for genome engineering of virulent bacteriophages, Nucleic Acids Res. 42 (2014) 9504–9513. https://academic.oup.com/nar/article-abstract/42/14/9504/1283784.

[40] E.V. Koonin, K.S. Makarova, F. Zhang, Diversity, classification and evolution of CRISPR-Cas systems, Curr. Opin. Microbiol. 37 (2017) 67–78. https://www.sciencedirect.com/science/article/pii/S1369527417300231.

[41] S.J. Brouns, M.M. Jore, M. Lundgren, E.R. Westra, R.J. Slijkhuis, A.P. Snijders, J. Van Der Oost, Small CRISPR RNAs guide antiviral defense in prokaryotes, Science 321 (2008) 960–964. https://www.science.org/doi/abs/10.1126/science.1159689.

[42] H. Deveau, R. Barrangou, J.E. Garneau, J. Labonté, C. Fremaux, P. Boyaval, S. Moineau, Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*, J. Bacteriol. 190 (2008) 1390–1400. https://journals.asm.org/doi/abs/10.1128/JB.01412-07.

[43] R. Kiro, D. Shitrit, U. Qimron, Efficient engineering of a bacteriophage genome using the type IE CRISPR-Cas system, RNA Biol. 11 (2014) 42–44. https://www.tandfonline.com/doi/abs/10.4161/rna.27766.

[44] A.M. Box, M.J. McGuffie, B.J. O'Hara, K.D. Seed, Functional analysis of bacteriophage immunity through a type IE CRISPR-Cas system in *Vibrio cholerae* and its application in bacteriophage genome engineering, J. Bacteriol. 198 (2016) 578–590. https://journals.asm.org/doi/abs/10.1128/JB.00747-15.

[45] J.A. Doudna, E. Charpentier, The new frontier of genome engineering with CRISPR-Cas9, Science 346 (2014) 1258096. https://www.science.org/doi/abs/10.1126/science.1258096.

[46] J.E. Garneau, M.È. Dupuis, M. Villion, D.A. Romero, R. Barrangou, P. Boyaval, S. Moineau, The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA, Nature 468 (2010) 67–71. https://www.nature.com/articles/nature09523.

[47] M.L. Lemay, D.M. Tremblay, S. Moineau, Genome engineering of virulent lactococcal phages using CRISPR-Cas9, ACS Synth. Biol. 6 (2017) 1351–1358. https://pubs.acs.org/doi/abs/10.1021/acssynbio.6b00388.

[48] L.A. Marraffini, E.J. Sontheimer, CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA, Science 322 (2008) 1843–1845. https://www.science.org/doi/abs/10.1126/science.1165771.

[49] P. Samai, N. Pyenson, W. Jiang, G.W. Goldberg, A. Hatoum-Aslan, L.A. Marraffini, Co-transcriptional DNA and RNA cleavage during type III CRISPR-Cas immunity, Cell 161 (2015) 1164–1174. https://www.cell.com/cell/pdf/S0092-8674(15)00444-4.pdf.

[50] K. Cater, V.S. Dandu, S.N. Bari, K. Lackey, G.F. Everett, A. Hatoum-Aslan, A novel Staphylococcus podophage encodes a unique lysin with unusual modular design, mSphere 2 (2017) e00040, 17, https://journals.asm.org/doi/abs/10.1128/mSphere.00040-17.

[51] K. Vandersteegen, W. Mattheus, P.J. Ceyssens, F. Bilocq, D. De Vos, J.P. Pirnay, R. Lavigne, Microbiological and molecular assessment of bacteriophage ISP for the control of *Staphylococcus aureus*, PLoS One 6 (2011) e24418. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0024418.

[52] Y.J. Pan, T.L. Lin, C.C. Chen, Y.T. Tsai, Y.H. Cheng, Y.Y. Chen, P.F. Hsieh, Y.T. Lin, J.T. Wang, Klebsiella phage ΦK64-1 encodes multiple depolymerases for multiple host capsular types, J. Virol. 91 (2017) e02457, 16, https://journals.asm.org/doi/abs/10.1128/JVI.02457-16.

[53] H. Shin, J.H. Lee, H. Yoon, D.H. Kang, S. Ryu, Genomic investigation of lysogen formation and host lysis systems of the Salmonella temperate bacteriophage SPN9CC, Appl. Environ. Microbiol. 80 (2014) 374–384. https://journals.asm.org/doi/abs/10.1128/aem.02279-13.

[54] K.S. Wetzel, C.A. Guerrero-Bustamante, R.M. Dedrick, C.C. Ko, K.G. Freeman, H.G. Aull, G.F. Hatfull, CRISPY-BRED and CRISPY-BRIP: efficient bacteriophage engineering, Sci. Rep. 11 (2021) 6796. https://www.nature.com/articles/s41598-021-86112-6.

[55] J.M. Rock, F.F. Hopkins, A. Chavez, M. Diallo, M.R. Chase, E.R. Gerrick, S.M. Fortune, Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform, Nat. Microbiol. 2 (2017) 16274. https://www.nature.com/articles/nmicrobiol2016274.

[56] M.H. Lee, L. Pascopella, W.R. Jacobs Jr., G.F. Hatfull, Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis, Mycobacterium tuberculosis*, and bacille Calmette-Guérin, Proc. Natl. Acad. Sci. USA 88 (1991) 3111–3115. https://www.pnas.org/doi/abs/10.1073/pnas.88.8.3111.

[57] N. Maheshri, J.T. Koerber, B.K. Kaspar, D.V. Schaffer, Directed evolution of adeno-associated virus yields enhanced gene delivery vectors, Nat. Biotechnol. 24 (2006) 198–204. https://www.nature.com/articles/nbt1182.

[58] D.R. Krieg, Ethyl methanesulfonate-induced reversion of bacteriophage T4rII mutants, Genetics 48 (1963) 561. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1210494/.

[59] A.H. Favor, C.D. Llanos, M.D. Youngblut, J.A. Bardales, Optimizing bacteriophage engineering through an accelerated evolution platform, Sci. Rep. 10 (2020) 13981. https://www.nature.com/articles/s41598-020-70841-1.

[60] T. Luong, A.C. Salabarria, D.R. Roach, Phage therapy in the resistance era: where do we stand and where are we going? Clin. Therapeut. 42 (9) (2020) 1659–1680. https://pubmed.ncbi.nlm.nih.gov/32883528/.

[61] P.R. Jaschke, E.K. Lieberman, J. Rodriguez, A. Sierra, D. Endy, A fully decompressed synthetic bacteriophage øX174 genome assembled and archived in yeast, Virology 434 (2012) 278–284. https://www.sciencedirect.com/science/article/pii/S0042682212004618.

[62] L.Y. Chan, S. Kosuri, D. Endy, Refactoring bacteriophage T7, Mol. Syst. Biol. 1 (2005), 2005–0018, https://www.embopress.org/doi/abs/10.1038/msb4100025.

[63] S. Kosuri, *Simulation, models, and refactoring of bacteriophage T7*. https://dspace.mit.edu/handle/1721.1/35864.

[64] S. Kilcher, M.J. Loessner, Engineering bacteriophages as versatile biologics, Trends Microbiol. 27 (2019) 355–367. https://www.cell.com/trends/microbiology/fulltext/S0966-842X(18)30202-6?sf209551104=1.

[65] D.P. Pires, R. Monteiro, D. Mil-Homens, A. Fialho, T.K. Lu, J. Azeredo, Designing *P. aeruginosa* synthetic phages with reduced genomes, Sci. Rep. 11 (2021) 2164. https://www.nature.com/articles/s41598-021-81580-2.

[66] H. Ando, S. Lemire, D.P. Pires, T.K. Lu, Engineering modular viral scaffolds for targeted bacterial population editing, Cell Syst 1 (2015) 187–196. https://www.cell.com/cell-systems/pdf/S2405-4712(15)00111-8.pdf.

[67] M. Dunne, B. Rupf, M. Tala, X. Qabrati, P. Ernst, Y. Shen, S. Kilcher, Reprogramming bacteriophage host range through structure-guided design of chimeric receptor binding proteins, Cell Rep. 29 (2019) 1336–1350. https://www.cell.com/cell-reports/pdf/S2211-1247(19)31259-8.pdf.

[68] S. Meile, A. Sarbach, J. Du, M. Schuppler, C. Saez, M.J. Loessner, S. Kilcher, Engineered reporter phages for rapid bioluminescence-based detection and differentiation of viable Listeria cells, Appl. Environ. Microbiol. 86 (2020) e00442, 20, https://journals.asm.org/doi/abs/10.1128/AEM.00442-20.

[69] S. Kilcher, P. Studer, C. Muessner, J. Klumpp, M.J. Loessner, Cross-genus rebooting of custom-made, synthetic bacteriophage genomes in L-form bacteria, Proc. Natl. Acad. Sci. USA 115 (2018) 567–572. https://www.pnas.org/doi/abs/10.1073/pnas.1714658115.

[70] B.C. Bundy, M.J. Franciszkowicz, J.R. Swartz, *Escherichia coli*-based cell-free synthesis of virus-like particles, Biotechnol. Bioeng. 100 (2008) 28–37. https://onlinelibrary.wiley.com/doi/abs/10.1002/bit.21716.

[71] D.D. Rhoads, R.D. Wolcott, M.A. Kuskowski, B.M. Wolcott, L.S. Ward, A. Sulakvelidze, Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial, J. Wound Care 18 (2009) 237–243. https://www.magonlinelibrary.com/doi/abs/10.12968/jowc.2009.18.6.42801.

[72] L. Fernández, D. Gutiérrez, P. García, A. Rodríguez, The perfect bacteriophage for therapeutic applications—a quick guide, Antibiotics 8 (2019) 126. https://www.mdpi.com/2079-6382/8/3/1260.

[73] T.K. Lu, J.J. Collins, Dispersing biofilms with engineered enzymatic bacteriophage, Proc. Natl. Acad. Sci. USA 104 (2007) 11197–11202. https://www.pnas.org/doi/abs/10.1073/pnas.0704624104.

[74] R. Pei, G.R. Lamas-Samanamud, Inhibition of biofilm formation by T7 bacteriophages producing quorum-quenching enzymes, Appl. Environ. Microbiol. 80 (2014) 5340–5348. https://journals.asm.org/doi/abs/10.1128/AEM.01434-14.

[75] D. Bikard, C.W. Euler, W. Jiang, P.M. Nussenzweig, G.W. Goldberg, X. Duportet, L.A. Marraffini, Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials, Nat. Biotechnol. 32 (2014) 1146–1150. https://www.nature.com/articles/nbt.3043.

[76] R.J. Citorik, M. Mimee, T.K. Lu, Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases, Nat. Biotechnol. 32 (2014) 1141–1145. https://www.nature.com/articles/nbt.3011.

[77] R.M. Dedrick, C.A. Guerrero-Bustamante, R.A. Garlena, D.A. Russell, K. Ford, K. Harris, H. Spencer, Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*, Nat. Med 25 (2019) 730–733. https://www.nature.com/articles/s41591-019-0437-z.

[78] J.S. Little, R.M. Dedrick, K.G. Freeman, M. Cristinziano, B.E. Smith, C.A. Benson, G.F. Hatfull, Bacteriophage treatment of disseminated cutaneous *Mycobacterium chelonae* infection, Nat. Commun. 13 (2022) 2313. https://www.nature.com/articles/s41467-022-29689-4.

[79] S. Ryu, Grand challenges in phage biology, Front. Microbiol. 12 (2021) 715039. https://www.frontiersin.org/articles/10.3389/fmicb.2021.715039/full.

[80] F.L. Nobrega, M. Vlot, P.A. de Jonge, L.L. Dreesens, H.J. Beaumont, R. Lavigne, S.J. Brouns, Targeting mechanisms of tailed bacteriophages, Nat. Rev. Microbiol. 16 (2018) 760–773. https://www.nature.com/articles/s41579-018-0070-8.

[81] M. Yoichi, M. Abe, K. Miyanaga, H. Unno, Y. Tanji, Alteration of tail fiber protein gp38 enables T2 phage to infect *Escherichia coli* O157: H7, J. Biotechnol. 115 (2005) 101–107. https://www.sciencedirect.com/science/article/pii/S0168165604004559.

[82] F. Mahichi, A.J. Synnott, K. Yamamichi, T. Osada, Y. Tanji, Site-specific recombination of T2 phage using IP008 long tail fiber genes provides a targeted method for expanding host range while retaining lytic activity, FEMS Microbiol. Lett. 295 (2009) 211–217. https://academic.oup.com/femsle/article-abstract/295/2/211/585969.

[83] R. Marzari, D. Sblattero, M. Righi, A. Bradbury, Extending filamentous phage host range by the grafting of a heterologous receptor binding domain, Gene 185 (1997) 27–33. https://www.sciencedirect.com/science/article/pii/S0378111996006233.

[84] A.J. Heilpern, M.K. Waldor, pIIICTX, a predicted CTXϕ minor coat protein, can expand the host range of coliphage fd to include *Vibrio cholerae*, J. Bacteriol. 185 (2003) 1037–1044. https://journals.asm.org/doi/abs/10.1128/jb.185.3.1037-1044.2003.

[85] S. Divya Ganeshan, Z. Hosseinidoust, Phage therapy with a focus on the human microbiota, Antibiotics 8 (2019) 131. https://www.mdpi.com/2079-6382/8/3/131.

[86] D. Nelson, L. Loomis, V.A. Fischetti, Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 4107–4112. https://www.pnas.org/doi/abs/10.1073/pnas.061038398.

[87] K.M. Danis-Wlodarczyk, D.J. Wozniak, S.T. Abedon, Treating bacterial infections with bacteriophage-based enzybiotics: in Vitro, in Vivo and clinical application, Antibiotics 10 (2021) 1497. https://www.mdpi.com/2079-6382/10/12/1497.

[88] V.A. Fischetti, Development of phage lysins as novel therapeutics: a historical perspective, Viruses 10 (2018) 310. https://www.mdpi.com/1999-4915/10/6/310.

[89] M.J. Frias, J. Melo-Cristino, M. Ramirez, Export of the pneumococcal phage SV1 lysin requires choline-containing teichoic acids and is holin-independent, Mol. Microbiol. 87 (2013) 430–445. https://onlinelibrary.wiley.com/doi/abs/10.1111/mmi.12108.

[90] S. Hassan, A. Dusthackeer, B. Subramanyam, C. Ponnuraja, N.S. Gomathi, V. Kumar, Lytic efficiency of mycobacteriophages, Open Syst. Biol. J. 3 (2010) 21–28. https://eprints.nirt.res.in/1046/.

[91] R.Y. Young, Bacteriophage lysis: mechanism and regulation, Microbiol. Rev. 56 (1992) 430–481. https://journals.asm.org/doi/abs/10.1128/mr.56.3.430-481.1992.

[92] M.J. Loessner, Bacteriophage endolysins—current state of research and applications, Curr. Opin. Microbiol. 8 (2005) 480–487. https://www.sciencedirect.com/science/article/pii/S1369527405000767.

[93] J.R. Clark, J.B. March, Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials, Trends Biotechnol. 24 (2006) 212–218. https://www.cell.com/trends/biotechnology/fulltext/S0167-7799(06)00074-6?large_figure=true&code=cell-site.

[94] M.U. Rahman, W. Wang, Q. Sun, J.A. Shah, C. Li, Y. Sun, S. Wang, Endolysin, a promising solution against antimicrobial resistance, Antibiotics 10 (2021) 1277. https://www.mdpi.com/2079-6382/10/11/1277.

[95] P. Domingo-Calap, J. Delgado-Martínez, Bacteriophages: protagonists of a post-antibiotic era, Antibiotics 7 (2018) 66. https://www.mdpi.com/2079-6382/7/3/66.

[96] V.A. Fischetti, Lysin therapy for Staphylococcus aureus and other bacterial pathogens, in: Staphylococcus aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis, Springer, Berlin/Heidelberg, Germany, 2017, pp. 529–540. https://link.springer.com/chapter/10.1007/82_2015_5005.

[97] Y. Briers, M. Walmagh, V. Van Puyenbroeck, A. Cornelissen, W. Cenens, A. Aertsen, R. Lavigne, Engineered endolysin-based "Artilysins" to combat multidrug-resistant Gram-negative pathogens, mBio 5 (2014) e01379, 14, https://journals.asm.org/doi/abs/10.1128/mbio.01379-14.

[98] A.M. Pinto, M.D. Silva, L.M. Pastrana, M. Bañobre-López, S. Sillankorva, The clinical path to deliver encapsulated phages and lysins, FEMS Microbiol. Rev. 45 (2021) fuab019. https://academic.oup.com/femsre/articlepdf/doi/10.1093/femsre/fuab019/40527425/fuab019.pdf.

[99] H. Gerstmans, B. Criel, Y. Briers, Synthetic biology of modular endolysins, Biotechnol. Adv. 36 (2018) 624–640. https://www.sciencedirect.com/science/article/pii/S0734975017301611.

[100] L.Y. Low, C. Yang, M. Perego, A. Osterman, R. Liddington, Role of net charge on catalytic domain and influence of cell wall binding domain on bactericidal activity, specificity, and host range of phage lysins, J. Biol. Chem. 286 (2011) 34391–34403. https://www.jbc.org/article/S0021-9258(20)73982-7/abstract.

[101] D. Nelson, R. Schuch, P. Chahales, S. Zhu, V.A. Fischetti, PlyC: a multimeric bacteriophage lysin, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 10765–10770. https://www.pnas.org/doi/abs/10.1073/pnas.0604521103.

[102] M. Rohde, The Gram-positive bacterial cell wall, Microbiol. Spectr. 7 (2019), 7-3, https://journals.asm.org/doi/abs/10.1128/microbiolspec.gpp3-0044-2018.

[103] V.A. Fischetti, Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens, Int. J. Med. Microbiol. 300 (2010) 357–362. https://www.sciencedirect.com/science/article/pii/S1438422110000275.

[104] V.A. Fischetti, Bacteriophage lysins as effective antibacterials, Curr. Opin. Microbiol. 11 (2008) 393–400. https://www.sciencedirect.com/science/article/pii/S136952740800129X.

[105] V.S. Gondil, K. Harjai, S. Chhibber, Endolysins as emerging alternative therapeutic agents to counter drug-resistant infections, Int. J. Antimicrob. Agents 55 (2020) 105844. https://www.sciencedirect.com/science/article/pii/S092485791930305X.

[106] R. Schuch, D. Nelson, V.A. Fischetti, A bacteriolytic agent that detects and kills *Bacillus anthracis*, Nature 418 (2002) 884–889. https://www.nature.com/articles/nature01026.

[107] D. Gutiérrez, L. Fernández, A. Rodríguez, P. García, Are phage lytic proteins the secret weapon to kill *Staphylococcus aureus*? mBio 9 (2018) e01923, 17, https://journals.asm.org/doi/abs/10.1128/mBio.01923-17.

[108] E.A. Masters, R.P. Trombetta, K.L. de Mesy Bentley, B.F. Boyce, A.L. Gill, S.R. Gill, G. Muthukrishnan, Evolving concepts in bone infection: Redefining "biofilm", "acute vs. chronic osteomyelitis", "the immune proteome" and "local antibiotic therapy". Bone Res. 7 (2019) 20. https://www.nature.com/articles/s41413-019-0061-z.

[109] O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E.T. Rietschel, D.H. Shaw, Lipopolysaccharides of gram-negative bacteria, in: Current Topics in Membranes and Transport, vol. 17, Academic Press, Cambridge, MA, USA, 1982, pp. 79–151. https://www.sciencedirect.com/science/article/abs/pii/S0070216108603093.

[110] L. Pasquina-Lemonche, J. Burns, R.D. Turner, S. Kumar, R. Tank, N. Mullin, J.K. Hobbs, The architecture of the Gram-positive bacterial cell wall, Nature 582 (2020) 294–297. https://www.nature.com/articles/s41586-020-2236-6.

[111] H. Yang, J. Yu, H. Wei, Engineered bacteriophage lysins as novel anti-infectives, Front. Microbiol. 5 (2014) 542. https://www.frontiersin.org/articles/10.3389/fmicb.2014.00542/full.

[112] M. Schmelcher, D.M. Donovan, M.J. Loessner, Bacteriophage endolysins as novel antimicrobials, Future Microbiol. 7 (2012) 1147–1171. https://www.futuremedicine.com/doi/abs/10.2217/fmb.12.97.

[113] H. Gerstmans, L. Rodríguez-Rubio, R. Lavigne, Y. Briers, From endolysins to Artilysin® s: novel enzyme-based approaches to kill drug-resistant bacteria, Biochem. Soc. Trans. 44 (2016) 123–128. https://portlandpress.com/biochemsoctrans/article-abstract/44/1/123/65410.

[114] T. Wang, Y. Zheng, J. Dai, J. Zhou, R. Yu, C. Zhang, Design SMAP29-LysPA26 as a highly efficient artilysin against *Pseudomonas aeruginosa* with bactericidal and antibiofilm activity, Microbiol. Spectr. 9 (2021) e00546, 21, https://journals.asm.org/doi/abs/10.1128/Spectrum.00546-21.

[115] M. Morita, Y. Tanji, K. Mizoguchi, A. Soejima, Y. Orito, H. Unno, Antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin without holin conjugation, J. Biosci. Bioeng. 91 (2001) 469–473. https://www.sciencedirect.com/science/article/pii/S1389172301802759.

[116] W.C.B. Lai, X. Chen, M.K.Y. Ho, J. Xia, S.S.Y. Leung, Bacteriophage-derived endolysins to target Gram-negative bacteria, Int. J. Pharm. 589 (2020) 119833. https://www.sciencedirect.com/science/article/pii/S0378517320308188.

[117] F.M. Khan, V.S. Gondil, C. Li, M. Jiang, J. Li, J. Yu, H. Yang, A novel *Acinetobacter baumannii* bacteriophage endolysin LysAB54 with high antibacterial activity against multiple Gram-negative microbes, Front. Cell. Infect. Microbiol. 11 (2021) 637313. https://www.frontiersin.org/articles/10.3389/fcimb.2021.637313/full.

[118] D. Chatterjee, The mycobacterial cell wall: structure, biosynthesis and sites of drug action, Curr. Opin. Chem. Biol. 1 (1997) 579–588. https://www.sciencedirect.com/science/article/pii/S1367593197800555.

[119] K.A. Abrahams, G.S. Besra, Mycobacterial cell wall biosynthesis: a multifaceted antibiotic target, Parasitology 145 (2018) 116–133. https://www.cambridge.org/core/journals/parasitology/article/mycobacterial-cell-wall-biosynthesis-a-multifaceted-antibiotic-target/BFAD214219E98634B6B7CE602E5E8354.

[120] M.J. Catalão, M. Pimentel, Mycobacteriophage lysis enzymes: targeting the mycobacterial cell envelope, Viruses 10 (2018) 428. https://www.mdpi.com/1999-4915/10/8/428.

[121] K.M. Payne, G.F. Hatfull, Mycobacteriophage endolysins: diverse and modular enzymes with multiple catalytic activities, PLoS One 7 (2012) e34052. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0034052.

[122] A.M. Gigante, C.M. Hampton, R.S. Dillard, F. Gil, M.J. Catalão, J. Moniz-Pereira, M. Pimentel, The Ms6 mycolyl-arabinogalactan esterase LysB is essential for an efficient mycobacteriophage-induced lysis, Viruses 9 (2017) 343. https://www.mdpi.com/1999-4915/9/11/343.

[123] M.J. Catalao, C. Milho, F. Gil, J. Moniz-Pereira, M. Pimentel, A second endolysin gene is fully embedded in-frame with the lysA gene of mycobacteriophage Ms6, PLoS One 6 (2011) e20515. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0020515.
[124] M.J. Lai, C.C. Liu, S.J. Jiang, P.C. Soo, M.H. Tu, J.J. Lee, K.C. Chang, Antimycobacterial activities of endolysins derived from a mycobacteriophage, BTCU-1, Molecules 20 (2015) 19277–19290. https://www.mdpi.com/1420-3049/20/10/19277.
[125] E. Murray, L.A. Draper, R.P. Ross, C. Hill, The advantages and challenges of using endolysins in a clinical setting, Viruses 13 (2021) 680. https://www.mdpi.com/1999-4915/13/4/680.
[126] M.J. Love, G.S. Abeysekera, A.C. Muscroft-Taylor, C. Billington, R.C. Dobson, On the catalytic mechanism of bacteriophage endolysins: opportunities for engineering, Biochim. Biophys. Acta (BBA)-Proteins Proteom. 1868 (2020) 140302. https://www.sciencedirect.com/science/article/pii/S1570963919301888.
[127] T.M. Viertel, K. Ritter, H.P. Horz, Viruses versus bacteria—novel approaches to phage therapy as a tool against multidrug-resistant pathogens, J. Antimicrob. Chemother. 69 (2014) 2326–2336. https://academic.oup.com/jac/article-abstract/69/9/2326/2911275.
[128] L. Chen, S. Yuan, Q. Liu, G. Mai, J. Yang, D. Deng, Y. Ma, In Vitro design and evaluation of phage cocktails against Aeromonas salmonicida, Front. Microbiol. 9 (2018) 1476. https://www.frontiersin.org/articles/10.3389/fmicb.2018.01476/full.
[129] F. Forti, D.R. Roach, M. Cafora, M.E. Pasini, D.S. Horner, E.V. Fiscarelli, D. Ghisotti, Design of a broad-range bacteriophage cocktail that reduces *Pseudomonas aeruginosa* biofilms and treats acute infections in two animal models, Antimicrob. Agents Chemother. 62 (2018) e02573, 17, https://journals.asm.org/doi/abs/10.1128/aac.02573-17.
[130] C. Torres-Barceló, M.E. Hochberg, Evolutionary rationale for phages as complements of antibiotics, Trends Microbiol. 24 (2016) 249–256. https://www.cell.com/trends/microbiology/fulltext/S0966-842X(15)00302-9?innerTabvideo-abstract_mmc1=.
[131] C. Liu, Q. Hong, R.Y.K. Chang, P.C.L. Kwok, H.K. Chan, Phage–Antibiotic therapy as a promising strategy to combat multidrug-resistant infections and to enhance antimicrobial efficiency, Antibiotics 11 (2022) 570. https://www.mdpi.com/2079-6382/11/5/570.
[132] B.K. Chan, P.E. Turner, S. Kim, H.R. Mojibian, J.A. Elefteriades, D. Narayan, Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*, Evol. Med. Public Health (2018) 60–66. https://academic.oup.com/emph/article-abstract/2018/1/60/4923328.
[133] A.E. Kirby, Synergistic action of gentamicin and bacteriophage in a continuous culture population of *Staphylococcus aureus*, PLoS One 7 (2012) e51017. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0051017.
[134] C. Torres-Barceló, F.I. Arias-Sánchez, M. Vasse, J. Ramsayer, O. Kaltz, M.E. Hochberg, A window of opportunity to control the bacterial pathogen *Pseudomonas aeruginosa* combining antibiotics and phages, PLoS One 9 (2014) e106628. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0106628.
[135] S. Chhibber, T. Kaur, S. Kaur, Co-therapy using lytic bacteriophage and linezolid: effective treatment in eliminating methicillin resistant *Staphylococcus aureus* (MRSA) from diabetic foot infections, PLoS One 8 (2013) e56022. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0056022.
[136] V. Verma, K. Harjai, S. Chhibber, Characterization of a T7-like lytic bacteriophage of Klebsiella pneumoniae B5055: a potential therapeutic agent, Curr. Microbiol. 59 (2009) 274–281. https://link.springer.com/article/10.1007/s00284-009-9430-y.
[137] A.M. Comeau, F. Tétart, S.N. Trojet, M.F. Prère, H.M. Krisch, Phage-antibiotic synergy (PAS): β-lactam and quinolone antibiotics stimulate virulent phage growth, PLoS One 2 (2007) e799. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0000799.
[138] F. Kamal, J.J. Dennis, Burkholderiacepacia complex phage-antibiotic synergy (PAS): antibiotics stimulate lytic phage activity, Appl. Environ. Microbiol. 81 (2015) 1132–1138. https://journals.asm.org/doi/abs/10.1128/aem.02850-14.
[139] A. Fajardo-Lubian, C. Venturini, Use of bacteriophages to target intracellular pathogens, Clin. Infect. Dis. 77 (Supplement_5) (2023) S423–S432. https://academic.oup.com/cid/article/77/Supplement_5/S423/7334982.
[140] J.M. Lewis, J. Williams, A.P. Sagona, Making the leap from technique to treatment—genetic engineering is paving the way for more efficient phage therapy, Biochem. Soc. Trans. (2024) BST20231289. https://pubmed.ncbi.nlm.nih.gov/38716972/.
[141] Q. Chen, T. Dharmaraj, P.C. Cai, E.B. Burgener, N.L. Haddock, A.J. Spakowitz, P.L. Bollyky, Bacteriophage and bacterial susceptibility, resistance, and tolerance to antibiotics, Pharmaceutics 14 (7) (2022) 1425. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9318951/.
[142] J.P. Pirnay, S. Djebara, G. Steurs, J. Griselain, C. Cochez, S. De Soir, T. Glonti, A. Spiessens, E. Vanden Berghe, S. Green, J. Wagemans, Personalized bacteriophage therapy outcomes for 100 consecutive cases: a multicentre, multinational, retrospective observational study, Nature Microbiology (2024) 1–20. https://pubmed.ncbi.nlm.nih.gov/38834776/.
[143] S.L. Karn, M. Gangwar, R. Kumar, S.K. Bhartiya, G. Nath, Phage therapy: a revolutionary shift in the management of bacterial infections, pioneering new horizons in clinical practice, and reimagining the arsenal against microbial pathogens, Front. Med. 10 (2023). https://pubmed.ncbi.nlm.nih.gov/37928478/.
[144] G. Verbeken, I. Huys, J.P. Pirnay, S. Jennes, N. Chanishvili, J. Scheres, A. Górski, D. De Vos, C. Ceulemans, Taking bacteriophage therapy seriously: a moral argument, BioMed Res. Int. 2014 (1) (2014) 621316. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4020481/.
[145] B. Zalewska-Piątek, Phage therapy—challenges, opportunities and future prospects, Pharmaceuticals 16 (12) (2023) 1638. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10747886/.
[146] S.T. Abedon, P. García, P. Mullany, R. Aminov, Phage therapy: past, present and future, Front. Microbiol. 8 (2017) 274123. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2017.00981/full.