Trends in Microbiology

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Review

Engineering Bacteriophages as Versatile Biologics

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Viruses of bacteria (bacteriophages or phages) are highly evolved nanomachines that recognize bacterial cell walls, deliver genetic information, and kill or transform their targets with unparalleled specificity. For a long time, the use of genetically modified phages was limited to phage display approaches and fundamental research. This is mostly because phage engineering has been a complex and time-consuming task, applicable for only a few well characterized model phages. Recent advances in sequencing technology and molecular biology gave rise to rapid and precise tools that enable modification of less-well-characterized phages. These methods will pave the way for the development of modular designer-phages as versatile biologics that efficiently control multidrug-resistant bacteria and provide novel tools for pathogen detection, drug development, and beyond.

Highlights

The increasing prevalence of antibiotic-resistant pathogenic bacteria has sparked renewed interest in bacteriophage therapy.

Synthetic biology methods allow design, straightforward construction, and testing of engineered bacteriophages that target both Gram-positive and Gram-negative bacteria.

Phages with small genomes are easier to engineer using synthetic methods, while recombination-based approaches are currently the method of choice for larger phages.

The efficiency, safety, and therapeutic suitability of phage-based antimicrobials can be specifically tailored through targeted phage engineering.

In contrast to non-modified viruses, engineered phages offer intellectual property protection opportunities that may fuel the commercial implementation of phage therapy in many countries.

Bacteriophages as Antimicrobials

Phages are the most abundant biological entities in the environment [1–3], are relatively easy to isolate and propagate, and are highly evolved to kill specific bacterial strains, species, or sometimes even genera. Their antimicrobial and therapeutic potential was recognized immediately after their discovery, until the introduction of antibiotics displaced the application of phages shortly after World War II. In eastern Europe and the Soviet Union, **phage therapy** (see Glossary) was further developed, and it currently constitutes a standard medical practice in the Republic of Georgia [4]. More than 100 years after their discovery [5,6], the number of commercially available phage products is still limited, at least in Western countries. This is partly due to the availability of antibiotics as a cheap and effective alternative, to the lack of controlled studies on therapeutic phage efficacy, and to uncertainties with respect to intellectual property (IP) rights and the approval of phage-based treatments [7–9]. Nevertheless, the increasing incidence of infections with antibiotic-resistant bacteria and the decreasing rate of discovery of conventional antibiotics have revived a strong interest in antimicrobial phage applications [9–11]. In addition, undesirable side-effects associated with the antibiotic-mediated removal of commensal bacteria (e.g., gut **dysbiosis**) [12] demonstrate that pathogen-specific therapeutic options will be an important avenue for future drug development. Here, we outline the potential of novel phage engineering approaches to improve, customize, and market phage-based antimicrobials.

Enhancing the Properties of Natural Bacteriophages

In this section we discuss some of the inherent limitations associated with the clinical use of native, nonmodified phages and how they could be overcome by genetic engineering.

Avoiding Phage Resistance

Bacteria have evolved a large number of sophisticated phage-resistance mechanisms to prevent virus binding, infection, and replication [13–16] which can negatively affect phage

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antimicrobial efficacy. Each of these mechanisms can potentially be mitigated using engineered phage. For example, many bacteria evade phage predation through modification of cell-wall-associated receptors. This evolutionary arms race may partly explain the limited host ranges of most natural phage isolates, which represents a major limitation for potential therapeutic uses. For some phages, host ranges have been modified through targeted engineering or exchange of baseplate- or fiber-associated **receptor-binding proteins** (**RBPs**) [17–23]. While this approach holds great promise, it requires an in-depth molecular understanding of receptor–RBP interactions and is thus not yet broadly applicable. Bacteria that form biofilms or capsules are also associated with higher resistance to phage and antibiotics. Recent studies demonstrate that these cells can be targeted more efficiently using engineered phages that deliver specific biofilm/capsule depolymerases or **quorum-quenching enzymes** [24–26]. Restriction–modification and **CRISPR-Cas systems** confer intracellular resistance and abortion of infection through sequence-specific cleavage of incoming phage genomes. By introducing methyltransferase genes into phage genomes, phages can increase their infection efficiency on strains that encode a corresponding restriction enzyme [27]. The recent discovery of phage-encoded anti-CRISPR proteins suggests that a similar strategy may also be applicable to counteract CRISPR-Cas systems using engineered, anti-CRISPR-expressing phages [28,29]. Irrespective of the resistance mechanism employed, only few targeted cells become resistant and prevail. By equipping phages with mechanistically independent bactericidal payload proteins that are released upon host lysis, this limited number of resistant cells can be removed early before clonal expansion occurs [30]. Finally, through lysogenization, temperate phages quickly induce cells that are resistant to superinfection through a mechanism known as homoimmunity. Therefore, temperate phages are generally not suitable for use as antimicrobials. As a solution to this limitation, temperate phage genomes can be modified to create synthetic, strictly lytic (virulent) phage derivatives that kill host cells with much increased efficiency (a strategy described as 'virulent conversion') [30].

Increasing Phage Safety

Many phages encode virulence factors or toxins and thus cannot be used in any antimicrobial application. This important safety issue can be resolved by precise removal of potentially hazardous genetic information from viral genomes. In addition, phage-induced lysis can lead to the release of bacterial toxins, lipopolysaccharide, and other pathogen-associated molecular patterns (PAMPs) that trigger innate immune responses, contribute to virulence, or cause other damage. Engineered, lysis-deficient phage derivatives that kill target cells without subsequent PAMP release [31–34] can be used to mitigate these potentially harmful effects. In general, the ability of phages to replicate at the site of infection is considered an important property that supports their efficacy. This self-amplification effect may mediate successful therapy outcomes, even when only few phages initially reach the site of infection [4]. Nevertheless, it may be desirable to use engineered, replication-deficient phages [35] for some applications because it enables precise calculations of applied doses and guarantees that engineered viruses are not released into the environment. Another safety concern is the inherent ability of transducing phages to contribute to the spread of antibiotic-resistance genes [36,37]. Generally, phages that recognize a highly sequence-specific ***pac* site** for genome packaging perform a sequence-specific **termination cleavage**, or completely degrade host DNA early during infection (such as T4) and are less likely to perform generalized transduction. It may be possible to engineer and optimize these features through targeted modification of **terminases** or through delivery of host-DNA degrading nucleases.

Glossary

CEM (CRISPR-escapemutant): A bacteriophage that was originally targeted by a CRISPR-Cas system, but has escaped based on mutation of its protospacer or protospacer-adjacent motif.

CRISPR-Cas system: clustered regularly interspaced short palindromic repeats and associated genes that constitute an adaptive bacterial defense system against invading DNA elements. Cas nuclease complexes are guided by a small CRISPR-RNA to cleave incoming DNA in a sequence-specific manner and thereby confer resistance to phages or plasmids.

Dysbiosis: a misbalance of the microbial composition of any ecological community of microorganisms. Can be associated with diseases such as inflammatory bowel disease, obesity, or cancer.

Gibson assembly: a cloning method for isothermal, sequence-specific assembly of multiple DNA fragments that is directed by terminal homologies of the fragments.

Homing peptides: peptides that mediate organ-specific delivery of drugs, DNA, chemicals, viruses, and more. Homing peptides are usually identified from *in vivo* phage display.

Lambda red system: a plasmid system for homologous recombination that is based on three phage lambda proteins (Gam, Exo, and Beta). Gam prevents degradation of linear DNA, Exo is a 5′ exonuclease, and Beta is an ssDNA-binding protein that facilitates annealing and thereby facilitates recombination.

***pac* site:** a specific DNA sequence recognized by phage packaging enzymes that is required for the initiation of DNA packaging into proheads.

Phage therapy: the therapeutic use of intact phages for the treatment of infections with pathogenic bacteria.

Quorum quenching enzymes: enzymes that interfere with bacterial cell-to-cell communication (quorum sensing) by inactivation of the quorum sensing signalling molecules.

RBPs (receptor-binding proteins): Phage structural proteins, typically located at the tip of fibers or baseplates, that mediate binding to

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Genome-specific Toxicity

By delivering sequence-specific CRISPR-Cas nucleases via engineered genomes or phagemids, phages can be programmed to cleave defined nucleotide sequences and genotypes, for example, host cells that carry antibiotic-resistance genes or specific virulence factors [38–41]. The concept of genome-specific toxicity would ultimately allow for the removal of disease-associated bacterial genotypes without disturbing the beneficial microbiota, an approach that seems particularly useful for opportunistic pathogens such as *Staphylococcus aureus* or *Escherichia coli*.

Reporter Phages for Pathogen Detection

Bacteriophages can be engineered to carry reporter genes that are expressed during infection of the target bacterium and subsequently detected. These reporter phages combine the highly specific phage–host interaction with the sensitivity of the employed reporter system, which ultimately allows for rapid detection of very few bacterial cells. Because of their superior sensitivity, luciferases are the most commonly used reporter genes [24,42–45], although several others have been published as well [46]. A key advantage of reporter phages is that a signal is only generated when viable target cells are present, a distinction not achieved by culture-independent diagnostic methods. In addition, reporter-phage assays are typically much faster than culture-dependent diagnostics, because enrichment procedures can be significantly shortened or omitted altogether. For more details on this subject, we refer the reader to recent review articles [46,47].

Enhancing Phage Therapy in Humans

When translating from *in vitro* experiments to treating systemic and local infections, a number of additional factors will ultimately determine success, including: particle antigenicity, phage-clearance kinetics, tissue targeting, and the ability to target intracellular pathogens. Some of these challenges have already been addressed experimentally: For example, lambda phages that show an increased circulation half-life in the bloodstream contain specific mutations in capsid genes [48]. This desirable feature could potentially be engineered into other phages to be used for the treatment of systemic infections. In another recent study, Nobrega *et al*. engineered a T7 derivative with increased resistance to pH, a strategy that may prove useful for oral phage delivery via acidic compartments of the gastrointestinal tract [49]. Tissue targeting is an important strategy to increase local phage concentration and potentially reach intracellular bacteria [50]. While targeting has primarily been studied for delivery of therapeutic cargo (drug and gene delivery) [51], the lessons learned from these studies could also be adapted for the design of tissue-specific phage therapeutics. Intracellular and/or tissue-specific targeting is achieved by panning phage-display libraries or by directly testing previously described cell-penetrating peptides or tissue-specific **homing peptides**. Some phages feature the surprising ability to cross the blood–brain barrier [52,53]. Engineering these phages as therapeutic vectors opens avenues for the treatment of neurological conditions, for example, Alzheimer's disease or multiple sclerosis [54–56]. This has been achieved in mouse models through phage-mediated delivery of therapeutic antibodies [54] or through phage-mediated depletion of autoantibodies [56], respectively. In addition, pathogens that invade the central nervous system, such as *Listeria* or *Borrelia*, could potentially be targeted directly within neurological tissue. The use of phages for targeted modulation of the gut microbiota has been suggested multiple times [57–59]. Certain phages are able to interact with the gut mucus layer, thereby increasing phage concentrations within mucosal surfaces and facilitating phage retention and prey encounter [60,61]. In T4-like phages, this adherence phenotype is mediated by the dispensable capsid decoration protein Hoc [61]. Mucus-adherence phenotypes might potentially also be engineered and tuned in other phages to be used for gut microbiome therapy/engineering.

host receptors. RBPs are important host-range determinants.
TAR cloning: a cloning method based on transformation-associated recombination in yeast. Upon transformation, DNA fragments with homologous ends are efficiently recombined in yeast, which enables capture and assembly of multiple, large DNA fragments.
Terminase: the DNA translocation machine that encapsidates phage genomes during morphogenesis. Terminases consists of two subunits, TerS and TerL, which mediate recognition and translocation/cleavage, respectively.
Termination cleavage: the second cleavage that occurs when phage proheads are full of DNA. It terminates the DNA packaging process.
Transcription–translation systems: cell-free systems that combine a bacterial or eukaryotic cell extract with an RNA polymerase to enable both the transcription and translation of exogenously added DNA templates.

The examples mentioned so far demonstrate some of the clinical potential of genetically engineered phages. For a more comprehensive overview on established applications, the reader is referred to a recent review [62]. Finally, phages likely provide the largest pool of genes with unknown function [63], and efficient tools for the deletion or modification of phage genes will ultimately deepen our understanding of these versatile, self-replicating nanomachines. In the next sections, we discuss and compare some of the recent methodological developments that facilitate the construction of engineered phages.

Generating and Isolating Genetically Modified Bacteriophages

The substrate of every phage engineering effort is the nucleotide sequence. Approximately 2000 phage genome sequences are currently available on the NCBI genome database, and next-generation sequencing techniques enable rapid and reliable sequencing of novel isolates. Thus, the availability of sequence information no longer constitutes a major limitation for most host genera [64]. Two basic experimental strategies can be employed to generate engineered phages: (i) wild-type genomes can be recombined with a DNA-editing template in infected cells, or (ii) full-length synthetic genomes can be assembled from smaller fragments and subsequently reactivated to produce progeny. These two concepts are illustrated in Figure 1 (Key Figure) and discussed in the next sections.

Generating Engineered Phages by Homologous Recombination

Homologous recombination of phage genomes with a cytosolic recombination template is currently the most popular approach to generate genetically modified phages. There are two

Key Figure

Basic Phage Genome Engineering Strategies

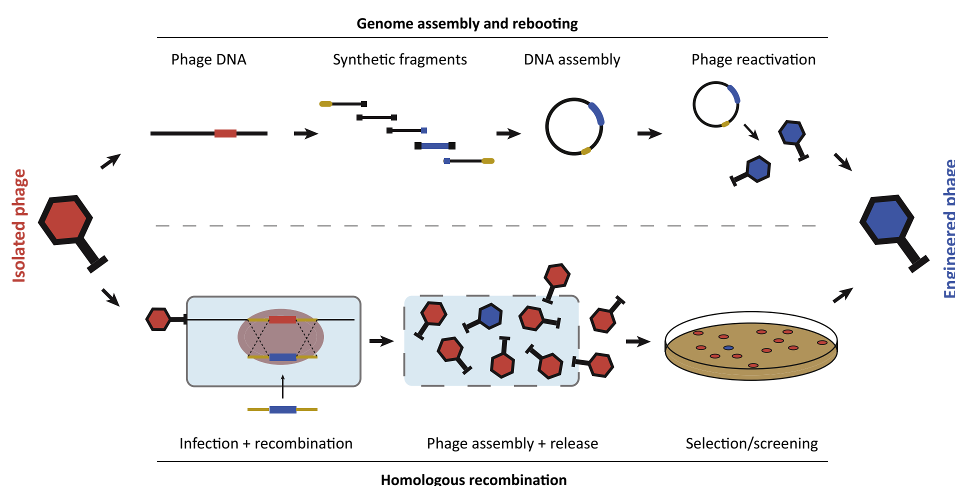

Figure 1. *Top.* Synthetic methods use phage genomes that have been assembled from smaller, overlapping DNA fragments, either using transformation-associated recombination cloning in yeast, or *in vitro* Gibson assembly. Synthetic genomes are subsequently reactivated (rebooted) in *Escherichia coli* (Gram-negative hosts), or cell-wall-deficient L-form bacteria (Gram-positive hosts). *Bottom.* Recombination-based engineering methods use editing templates provided in the infected host cell that recombine with the replicating phage genome and produce a mixture of wild-type and recombinant phages. Screening and/or selection of correct genotypes is required.

major limitations associated with this strategy. First, for recombination to occur, the editing template has to be transformed into the host bacterium. Many host species are not well characterized, and efficient transformation protocols are not available, notably for many Gram-positive bacteria. Second, recombination frequencies are usually very low [20,21,65], unless the phage brings its own recombination enzymes (e.g., phage lambda). The isolation of modified phage from such a recombination experiment is a daunting task that can often only be achieved through the incorporation of a marker gene such as β-galactosidase, luciferase, green fluorescent protein, or other more phage-specific marker genes [66,67] to facilitate plaque screening.

Recombineering

To increase recombination frequencies between phage DNA and the editing template, several laboratories made use of heterologous recombination proteins (recombineering). Bacteriophages can be engineered by coelectroporating the genomic phage DNA and the editing template into a recombineering strain, a strategy known as BRED (bacteriophage recombineering of electroporated DNA) [68,69]. The recombineering strain expresses proteins that confer high levels of recombination, such as the **lambda red system** (typically using the pKD46 vector [70]) [71] or RecE/RecT-like proteins [69]. Alternatively, recombineering-proficient cells can be infected with intact phage particles either before [72] or after [73] transformation of the editing template, a strategy known as *in vivo* recombineering. Both methods significantly increase recombination frequencies and allow for the isolation of engineered phages through a manageable amount of plaque screening. However, most bacterial species and genera are excluded from this approach because plasmid-based recombineering systems or transformation protocols are not available. A recombineering workflow is depicted in Figure 2A.

Selecting Recombinant Phages Using Sequence-specific Nucleases

As an alternative to recombineering, engineered phages can be enriched and isolated by actively counter-selecting the nonmodified genomes in homologous recombination experiments (also reviewed in [74]). Negative selection requires a sequence-specific intervention, such as a nuclease that can selectively remove wild-type genomes while allowing the replication of recombinant phage. Since 2014, nine studies have described the application of CRISPR-Cas systems for phage editing [27,75–81]. A general workflow for these approaches is depicted in Figure 2B. Martel and Moineau employed an endogenous type II-A CRISPR-Cas system from *Streptococcus thermophilus* to generate a point mutation, gene deletion, and gene exchange in *S. thermophilus* phage 2972 [27]. To this end, they used a plasmid-based mini-CRISPR to target the nuclease activity towards wild-type phage genomes. Others have successfully transferred *Streptococcus pyogenes* CRISPR-Cas to *Lactococcus lactis* [78], *E. coli* [80], *Klebsiella pneumoniae* [81], and *Bacillus subtilis* [79] and used it as a heterologous system to modify phages of the respective host strains. Even though *S. pyogenes* CRISPR-Cas activity was very weak in *L. lactis*, several p2 mutants were created (although editing efficiencies were not reported). Aside from using previously characterized type II-A systems, Hupfeld *et al*. have identified a novel CRISPR-Cas system from *Listeria ivanovii* that was then adapted and used for genetic engineering of large, nonintegrating phages in *Listeria monocytogenes* [59]. In addition, type I-E CRISPR-Cas systems from *E. coli* and *Vibrio chloerae* have been used to enrich modified phages. As for the type II-A systems, more than 50% of plaques formed in the presence of CRISPR-selection typically featured the correct genotype. If used as a heterologous system, type I-E systems require many plasmids for the expression of crRNA, *cascade*, *cas3*, and editing template [77], which limits use in bacteria where plasmid availability is restricted. Finally, Bari *et al*. have used a type III-A system to modify virulent *Staphylococcu*s

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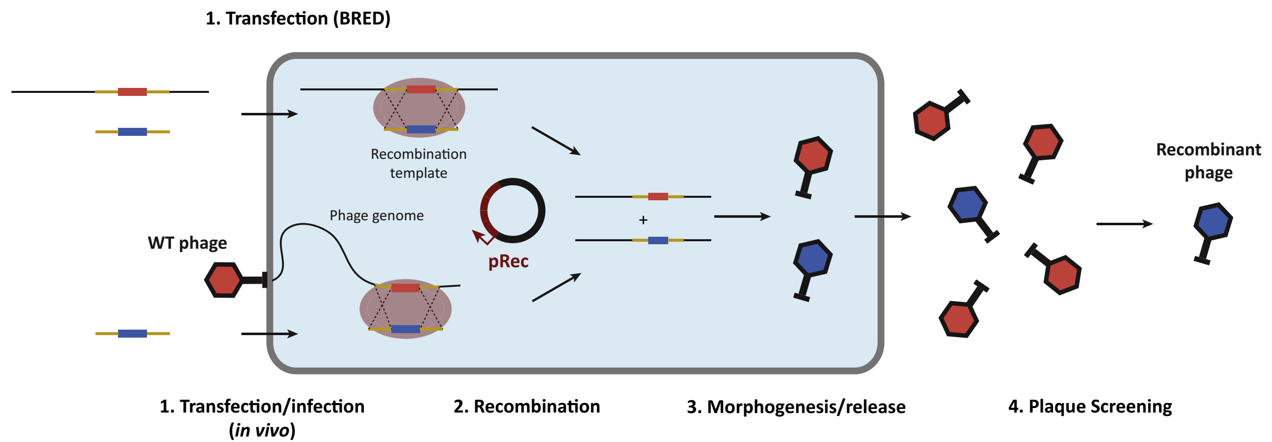

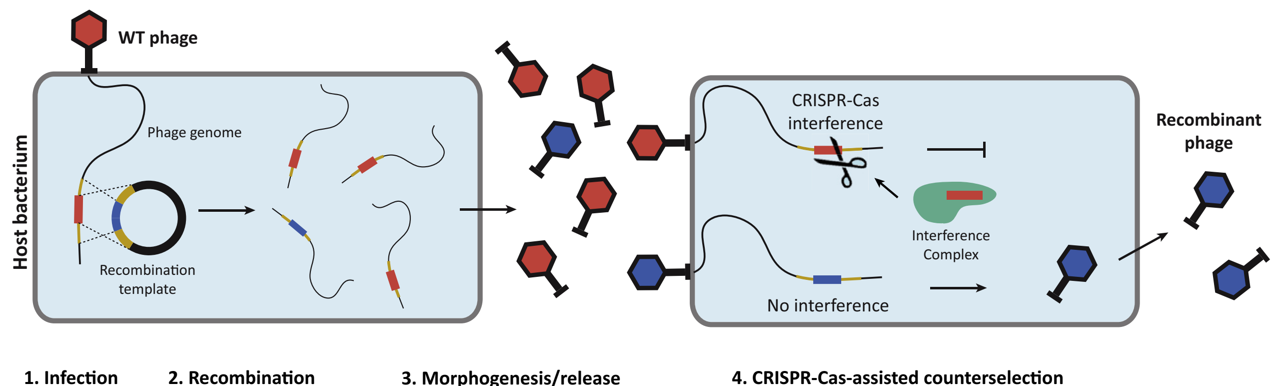

Figure 2. Recombination-based Phage Engineering. Homologous recombination frequencies between phage genome and recombination template are low, making the isolation of engineered phages a very labour-intense process. (A) To increase generation of desired recombinants, recombineering functions can be introduced into the host bacterium. Either the host is cotransformed with phage genomes and editing template (BRED), or a newly infected host is transformed with the recombination template (*in vivo* recombineering). (B) Alternatively, recombinant phages can be enriched using CRISPR-Cas sequence-specific nucleases that are programmed to cleave nonmodified genomes while allowing replication of recombinants. All approaches significantly reduce the amount of plaque screening required for phage isolation. BRED, bacteriophage recombineering of electroporated DNA; pRec, plasmid-encoding genes that mediate high-level recombination. WT, wild-type.

phages [75]. The type III-A system cleaves DNA and RNA in a cotranscriptional manner [82,83] and therefore acts exclusively against actively transcribed genes. This effectively limits the targetable sequence-space. On the other hand, type III-A systems do not feature a PAM or seed sequence and are tolerant to mismatches [84,85]. As a consequence, **CRISPR-escape mutants** (**CEMs**) do not arise with detectable frequency, and targeting leads to complete inhibition of phage replication, rendering it a very effective counterselection tool [75].

It can be concluded that CRISPR-Cas counterselection was quickly established as an important and very successful tool for scarless modification of nonintegrating phage genomes, and will likely be adapted to additional bacterial genera and phages in the near future. It will also

allow for targeting of toxic genes because short homology arms (50–150 bp) are sufficient to enrich phages above the background of naturally occurring CEMs [59,75,80]. On the downside, this approach requires an active endogenous or heterologous CRISPR-Cas system in the phage propagation strain, availability of plasmid systems, and a host that is relatively easy to transform. Because it requires cloning and plaque screening, the overall procedure is time-consuming, and multiple editing steps have to be performed sequentially.

Generating Engineered Phages by Rebooting Synthetic Genomes

Large DNA molecules can be assembled from smaller fragments either using transformation-associated recombination (**TAR cloning**) in yeast cells [86,87], or by enzymatic *in vitro* assembly (**Gibson assembly**) [88]. These methods allow for the design of synthetic phage genomes on the drawing board, followed by synthesis of required individual fragments, and rapid assembly into synthetic full-length genomes. Assembled genomic DNA can then be reactivated inside a suitable host cell or potentially using cell-free systems in a process referred to as phage genome 'rebooting'. Infectious particles are subsequently isolated from rebooting reactions and amplified on suitable phage-propagation strains. A clear advantage of the rebooting approach is the absence of wild-type phages, removing the need for plaque screening. Depending on the efficiency, these methods should also be applicable for the generation of mutant phage libraries directly from DNA libraries. In the next section, we discuss how synthetic approaches may be applied for the generation of engineered phages that infect either Gram-negative or Gram-positive bacteria. A general workflow is shown in Figure 3, and synthetic methods are compared to recombination-based approaches in Table 1.

Yeast-based Genome Assembly and Rebooting in E. coli

The first report of yeast-based phage genome assembly was published by Jaschke *et al*. [89]. The 5386 bp wild-type genome of phiX174 was captured in a yeast artificial chromosome (YAC) using TAR, released from the YAC by restriction digestion, and electroporated into *E. coli* cells for rebooting. Using synthetic fragments, the authors also constructed a heavily engineered, fully decompressed version of this phage. In a later study, Ando *et al*. used a similar approach to reboot several T7-like phages that infect various Gram-negative hosts [17]. This approach also worked for the large *Salmonella* Myovirus FelixO1 [90], suggesting that it may be more broadly applicable. In these experiments, *E. coli* was used as a (surrogate) host for rebooting of YAC-phage DNA because it can easily be transformed with large DNA molecules. Rebooted phages were subsequently released by chloroform-induced lysis, and amplified on their natural propagation strains. The host range of T7 phages is largely dictated by the conserved tail fiber gene gp17 [91]. Ando *et al*. were able to modulate T7 host range across genus barriers, simply by swapping gp17 genes or domains when building synthetic genomes from PCR fragments. Altogether, it can be assumed that variations of this rebooting approach will be broadly applied to engineer many phages that infect Gram-negative organisms, as long as a suitable host for surrogate transformation and rebooting is available. Unfortunately, phages that infect Gram-positive bacteria cannot be rebooted in *E. coli,* but instead require a Gram-positive host for transformation. Due to the presence of the very thick peptidoglycan layer however, transformation of large DNA molecules is severely restricted or even impossible in most Gram-positive bacteria.

In vitro *Genome Assembly and Rebooting in L-form Bacteria*

To enable transformation and rebooting in Gram-positive cells, we have recently developed a phage-engineering platform that employs bacterial L-form cells as rebooting compartments [30]. L-forms are cell-wall-deficient bacteria that divide and grow in osmotically stabilized media [92,93]. They have been isolated from many different Gram-positive bacteria, generally

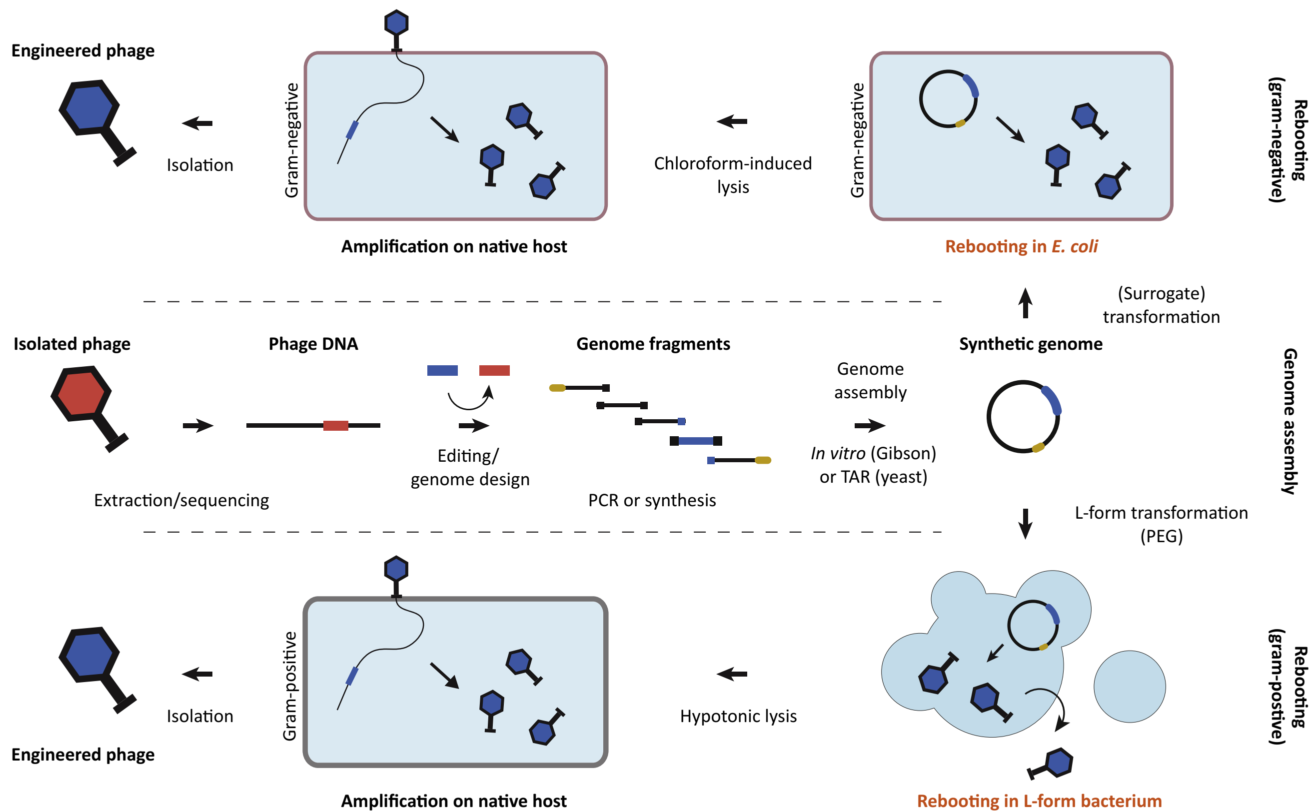

Figure 3. Synthetic Biology-based Phage Engineering. A workflow for the assembly (middle row) and rebooting (top and bottom rows) of synthetic phage genomes is depicted. Engineered genomes are assembled from smaller, overlapping fragments that can be generated by either *de novo* synthesis or PCR amplification. Any desired edits, such as fragment exchange, addition, removal, modification, or reorganization, is possible using this universal and versatile engineering approach. Assembly may be achieved in yeast cells using transformation-associated recombination (TAR) and capture in a yeast artificial chromosome. Alternatively, genomes can be assembled in a test tube using the Gibson method. Synthetic DNA is transformed into *Escherichia coli* cells for rebooting of phages that infect Gram-negative host cells (top row). Rebooted phages are released using chloroform-induced lysis followed by propagation on suitable hosts. Due to their multilayered cell wall, Gram-positive host cells cannot efficiently be transformed with synthetic phage genomes. To overcome this limitation, cell-wall-deficient L-form bacteria are used as rebooting compartments (bottom row) and engineered phages released by hypotonic lysis. A significant advantage is that these approaches do not require plaque screening. PEG, polyethylene glycol.

through prolonged cultivation in the presence of cell-wall-active compounds such as penicillin. Because they lack their native cell walls, L-forms can be transformed with large DNA molecules using long polyethylene glycol chains [92,94]. A specific L-form strain derived from *L. monocytogenes* reboots synthetic phage genomes that had previously been assembled *in vitro* using the Gibson method. This process is highly efficient for *Listeria* phage genomes and is also applicable for cross-genus rebooting of *Bacillus* and *Staphylococcus* phages and possibly other related genera, albeit at a somewhat reduced rebooting efficiency (i.e., the number of full-length genomes required to form one plaque-forming unit). Phage progeny produced within L-forms are released by simple hypotonic lysis and subsequently amplified on their native host cells (akin to the chloroform-mediated release of rebooted genomes in *E. coli*). This approach seems to work for many different phage types, independently of genome size or structure, phage morphology, and life-style. Therefore, it will likely be very broadly applicable. Expanding the platform to include more suitable L-form cell lines is currently ongoing and will hopefully encompass several additional important Gram-positive pathogens,

Table 1. Advantages and Limitations of Phage Engineering Techniques

	Advantages	Limitations
Homologous recombination + BRED/ CRISPR-Cas	Relatively simple protocol	Host transformation protocols required
	No marker genes required	Active recombineering/Crispr-Cas system required
	Engineering large genomes possible	Plaque screening required
		Labour-intensive protocol
		Cloning of potentially toxic recombination templates
		Protocol must be adapted to each host strain
		Moderate efficiency
Genome assembly + rebooting	Fast	Rebooting protocols not yet established for many host organisms
	No screening required	Large genomes are difficult to assemble
	Introduce multiple changes in one step	Relatively complicated protocols
	No cloning required	Genome sequence must be complete (including termini/packaging strategy)
	Efficient, enables generation of phage libraries	
	Rebooting possible across genus boundaries	

including spore formers and various cocci. Moreover, since the rebooting process was shown to be reasonably efficient, with as little as 2 pg of DNA required to form one infectious particle, it may be possible to construct and evaluate suitable libraries of mutant phage directly from DNA fragment libraries.

Rebooting in Cell-free Systems

Several studies have demonstrated that virus-like particles and even phages can be created directly from DNA in a test tube, employing *E. coli*-based cell-free protein synthesis systems. A clear advantage would be independence from transformation efficiency and the ability to work with gene sequences encoding products that are toxic to intermediate strains used for cloning. The first study demonstrating the feasibility of using *E. coli* **transcription–translation** (TX–TL) systems for the generation of virus-like particles (VLPs) was published by Bundy *et al*. [95]. The authors used a previously generated TX–TL system [96] to express a codon-optimized MS2 coat protein that self-assembles to form VLPs for nucleic acid encapsidation. Later studies by the Noireaux laboratory demonstrate that very high yields (10^{11}–10^{12} pfu/ml) of infectious T7, phiX174, and MS2 phages can be generated quickly using optimized reagents [97–99]. For T7, such systems can also replicate phage genomes and result in the production of more than two phage particles per input genome. Initially, it was assumed that cell-free rebooting is restricted to small phages of limited complexity. However, TX–TL-based production of the large, 170 kb Myovirus T4 was recently achieved, suggesting that even the most complex phages can potentially be produced with high yield [100]. It is likely that the currently available TX–TL systems are restricted to phages that infect *E. coli* or closely related genera. Therefore, it will be important to develop cell-free systems for other pathogenic bacteria, including Gram-positive hosts. Even though a formal demonstration is still missing, it is very likely that cell-free systems can actually be employed to generate engineered phages.

Engineered Phages as a Business Opportunity

Native phage isolates often cannot be patented because they have been known for many years, are ubiquitously available in the environment, and are relatively easy to isolate, with no innovative step required. However, genetically engineered phages with improved properties such as antimicrobial efficiency, safety, or novel functions can generate interesting opportunities for IP protection that will hopefully lead pharmaceutical companies to invest in this exciting new field [101,102]. So far, the only commercially available engineered phage products are reporter phages. Nevertheless, it is encouraging that several companies that feature engineered phages in their planned product portfolio have been founded in recent years and collectively attracted millions of dollars in venture capital. To attract more funding and interest from pharmaceutical companies, demonstrating the benefit of engineered versus native phage will be of pivotal importance.

Concluding Remarks

Even though phages have been studied for more than a century, refined genome-editing techniques have only recently become available. Within the decade, almost 1500 additional phage genomes were added to public databases [63], and it can be expected that efficient engineering approaches will become available for many of them in the near future. In terms of technology, it will be important to deliver simple and efficient protocols that can be implemented in many laboratories and enable editing of phages for relevant bacterial host genera. Based on their speed and simplicity, the authors believe that synthetic methods will soon replace recombination-based approaches. The major limitation of synthetic phage engineering is the assembly of large genomes. However, with plummeting gene synthesis costs and the availability of inexpensive high-fidelity long-range polymerases, this restriction will soon be history. Now is the time to move from developing techniques to developing engineering concepts that provide a desired added value to a given phage. We envision a scenario where few well characterized, safety-approved phage 'backbones' are enhanced through modular genome engineering. This may include the diversification or modification of existing phage genes, the incorporation of additional genetic payloads, and the deletion of nondesired functions (Figure 4; and see Outstanding Questions). Through modular engineering, phages are on their way to become extremely versatile biologics, fine-tuned to the requirements of each specific application.

Outstanding Questions

Will modular phage engineering enable construction of broadly applicable antimicrobial biologics based on few well characterized phage backbones?

Is it possible to modulate host ranges by targeted engineering of receptor-binding proteins?

How will regulatory agencies deal with biologics that are subject to replication and evolution?

What are the most important strategies to increase antimicrobial efficiency in a clinical setting?

Will we be able to use phages for the control of systemic infections?

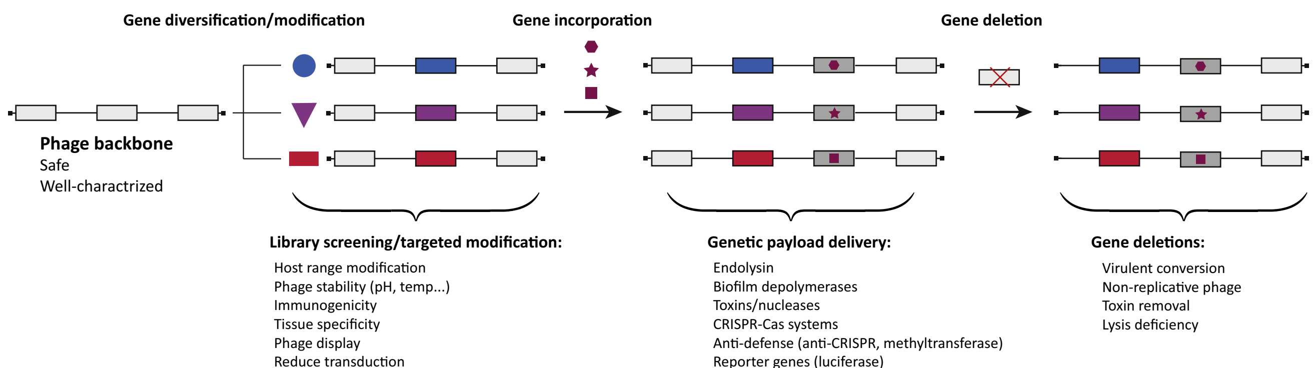

Figure 4. Modular Phage Engineering Produces Versatile Biologics. Well characterized phage backbones are optimized for each application through gene diversification, incorporation, and/or deletion. Examples for each class of modification are shown; library screening refers to the production of a phage library with diversified genes that are subsequently screened for improved function. This strategy is restricted to synthetic methods. Virulent conversion describes the deletion of genes that mediate the establishment and maintenance of lysogeny, creating a synthetic virulent phage.

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