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Trends in Biotechnology

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Review

Approaches for bacteriophage genome engineering

Marina Mahler [1,2], Ana Rita Costa [2,3], Sam P.B. van Beljouw [2,3], Peter C. Fineran [1,4,5] and Stan J.J. Brouns [2,3,*]

In recent years, bacteriophage research has been boosted by a rising interest in using phage therapy to treat antibiotic-resistant bacterial infections. In addition, there is a desire to use phages and their unique proteins for specific biocontrol applications and diagnostics. However, the ability to manipulate phage genomes to understand and control gene functions, or alter phage properties such as host range, has remained challenging due to a lack of universal selectable markers. Here, we discuss the state-of-the-art techniques to engineer and select desired phage genomes using advances in cell-free methodologies and clustered regularly interspaced short palindromic repeats-CRISPR associated protein (CRISPR-Cas) counter-selection approaches.

Highlights

Clustered regularly interspaced short palindromic repeats-CRISPR associated protein (CRISPR-Cas) systems are efficient at counter-selecting mutant phages generated by homologous recombination-based approaches, but are still limited by spacer efficiency and CRISPR-evading strategies of phages.

RNA-targeting CRISPR-Cas systems, such as type III and VI, can provide a more robust selection tool, especially for phages that evolved strategies to protect their DNA from targeting. Furthermore, these systems avoid the enrichment of CRISPR escape mutants.

Ex vivo phage genome engineering strategies assemble and reboot synthetic phage genomes and allow for increased flexibility in the design of the phage genome.

Rebooting of synthetic phage genomes is facilitated in cell-free systems by the exclusion of the cell membrane barrier, but it is currently restricted to a very small number of bacterial species.

Bacteriophages in biotechnology

Viruses of bacteria, known as bacteriophages or phages, were discovered more than a century ago [1]. Since then, research into phages and their interactions with bacteria has had an immense impact on our understanding of biology. For example, the study of phages provided the evidence that DNA is the genetic material [2], established the triplet nature of the genetic code [3], and provided numerous paradigms for gene regulation, including the organization of functionally related genes in operons whose transcription is controlled as a unit [4]. Phage-centered research was also at the foundation of molecular biology. For example, bacteria were found to encode restriction enzymes to protect from phage infection by cleavage of specific DNA sequences [5]. By combining this property of restriction enzymes with the ability of phage T4 DNA ligase to join DNA molecules together, it was possible to create a molecular cut and paste approach for DNA assembly. This technique represented the start of the golden age of recombinant DNA by allowing the cloning of genes for functional studies [6]. Additionally, phage DNA polymerases have been essential to the development of sequencing technologies [7,8] and, more recently, the antiphage clustered regularly interspaced short palindromic repeats-CRISPR associated protein (CRISPR-Cas) system has enabled a genome-editing revolution [9]. Many other exciting discoveries are likely awaiting the study of phage–bacteria interactions and phage genomes. However, most protein-encoding genes on phage genomes are still of unknown function and lack homology to other sequences in databases, thus calling for experimental approaches to uncover gene function.

The development of phage-based antibacterial approaches has also observed a resurgence of interest in the past decade due to the antibiotic resistance crisis. Multiple successful **compassionate use** (see Glossary) cases have proven the utility of phage therapy [10–12], but also highlight that the application of phage therapy using natural phages may be limited by issues such as narrow host range, possible development of phage resistance, or instability of phage particles.

Further advances in phage research and subsequent biotechnological developments can be propelled by our ability to manipulate phage genomes to study gene function and alter phage

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properties. Modified phages have already found applications in multiple fields (Box 1) and are expected to continue contributing to biotechnological innovation in years to come. Manipulation of **temperate phage** genomes that integrate into the bacterial chromosome as **prophages** is made possible by the same techniques used to manipulate bacterial genomes (reviewed in [13]). However, modification of **virulent phages** that replicate by killing their host cell is more complex, mainly due to the inability to easily select for genetically modified phages and the time available for recombination to occur. In this review, we will discuss the current status of virulent phage genome engineering, aiming at providing a guide to researchers considering engineering a phage genome. We summarize and compare the multiple different approaches that have traditionally been used for phage engineering and highlight the most recent developments. These include innovations in **homologous recombination**-based methodologies, the adaptation of different CRISPR-Cas systems, and advances in cell-free methodologies.

In vivo phage engineering

Most initial efforts to develop phage engineering technologies focused on modifying existing phages, either by treating the phage particles with **mutagenic agents**, or by exploring homologous recombination-based approaches. These techniques usually require follow-up screening to identify recombinant phages.

Random mutagenesis

The simplest way to obtain mutant phages is by exposure to conditions that stimulate random mutagenesis, such as ultraviolet light [14] or chemical mutagens (e.g., alkylating agents) [15]. The pool of mutant phages can be screened for the desired phenotype and selected mutants further investigated by whole genome sequencing to identify genes responsible for the observed phenotypic changes. An adaptation of this method uses selective pressure to enrich for mutant

Box 1. Applications for engineered phages

Phage genomes have been engineered for multiple purposes. A common goal of phage engineering is to broaden the narrow host range of phages for therapeutic, detection, and **transduction** purposes, by modifying their receptor-binding proteins (RBPs) [26,62,89]. It is also possible to engineer phages to increase their efficacy at targeting specific cells, as those in biofilms. This has been achieved by introducing genes that code for biofilm-degrading depolymerases [90] or enzymes that interfere with **quorum-sensing** [91]. The quick development of phage resistance in bacteria has also been tackled with phage engineering. Preadapted mutant phages selected *in vitro* for increased infectivity and reduced capacity to provoke phage resistance, were used to treat a **pan-drug resistant** *K. pneumoniae* infection in combination with antibiotics [21]. Moreover, temperate phages of *Enterococcus faecalis* have been engineered to become fully lytic by removing genes related to **lysogeny** [92]. This reduces the risk of phages transducing virulence genes between bacterial strains, making them more suitable for therapeutic applications. Such engineered phages were recently used to treat a *Mycobacterium abscessus* infection in a 15-year-old cystic fibrosis patient [10], setting up a milestone for phage therapy with the first ever use of modified phages in humans.

Phage engineering has also permitted the use of phages as vehicles for the delivery of payloads (e.g., CRISPR-Cas systems) that will alter antibiotic susceptibility [93–95], neutralize virulence gene expression [96], self-target the host chromosome [97], or alter gene expression *in situ* [98].

Phage engineering has expanded the therapeutic potential of phages to eukaryotic cells. A well-known example is the **phage display** technology, in which phage tail fiber or capsid proteins are altered to display motifs targeting eukaryotic cells (e.g., cancer cells) [99]. These approaches are being explored for the targeted delivery of drugs [100] or photosensitizers to cancer cells [101] and for the development of phage-based vaccines that display antigens from human pathogens [102], including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [103]. Importantly, the ability of phages to cross the blood–brain barrier has been explored for the delivery of small drug cargos to the brain [104].

Additionally, phages have been modified to carry or deliver reporter genes such as fluorescent proteins [105], luciferases [106,107], or hydrolyzing enzymes (e.g., β-galactosidase) [108,109] for sensitive detection of bacterial pathogens in clinical samples, contaminated food products, and water supplies, or for use as reporters in research [98].

Glossary

Anti-CRISPR protein: small protein used by phages and other MGEs to prevent the activity of CRISPR-Cas systems.
Compassionate use: treatment option that allows the use of an experimental medicine when no satisfactory authorized therapy is available.
Escape mutant: phage variant that encodes mutation(s) that allow the phage to escape targeting by a defense system.
Gibson assembly: molecular cloning method that employs the activities of an exonuclease, a DNA polymerase, and a DNA ligase to assemble multiple DNA fragments in a single reaction.
Golden Gate assembly: molecular cloning method that employs type IIS restriction enzymes and a DNA ligase to assemble multiple DNA fragments in a single reaction.
Homology-directed repair: repair of double-stranded DNA breaks using an endogenous or exogenous fragment of homologous DNA as a template.
Hypermodification: secondary modification of modified nucleotides already incorporated in the DNA.
Jumbo phage: tailed phage with a genome larger than 200 000 bp.
L-form: strain of bacteria that lacks the cell wall.
Lysogeny: life cycle in which the phage genome stably integrates into the cell chromosome and replicates in concert with it.
Mobile genetic element (MGE): segment of genetic material that can move within or between genomes or can be transferred between cells/species.
Mutagenic agent: substance that causes irreversible and heritable changes in the DNA sequence.
Non-homologous end joining: error-prone mechanism to repair double-stranded DNA breaks by direct ligation of the broken ends, often generating small deletions/insertions at the site of the lesion.
PAM: protospacer adjacent motif recognized by DNA-targeting CRISPR-Cas systems to discriminate invading DNA from self CRISPR locus.
Pan-drug resistance: nonsusceptibility to all agents in all antimicrobial categories.
Phage display: display of small proteins or peptides of interest fused to phage coat proteins, for the study of protein interactions.

phages with a desired phenotypic property [16]. This was successfully applied to select heat-tolerant *Escherichia coli* phages T3 and T7 and *Salmonella enterica* phages NBSal001 and NBSal002. Further investigation of the sequence and crystal structure of the mutated proteins from the phages allowed for identification of specific mutations in structural genes that led to heat tolerance [16]. While not an engineering technique *per se*, random mutagenesis is a simple method to obtain phages with desired phenotypes by applying selective pressure. However, it typically also results in the accumulation of mutations of unknown effect elsewhere in the phage genome.

Homologous recombination

Recombination between two homologous DNA sequences is one of the most commonly applied mechanisms for genetic engineering [17]. In phage research, homologous recombination between related phages was used in one of the earliest methods to modify phage genomes, known as phage crosses [18]. Co-infection of host bacteria with two phages showing different phenotypic characteristics results in recombination between the two parental phage genomes and subsequent release of progeny phages with new combinations of the parental phenotypes (Figure 1A) [19–21]. Phage crosses have been commonly used to exchange and combine phage genes involved in receptor recognition, resulting in altered host range, or to study the interaction between phages and newly identified bacterial defense systems [19,21–23]. Phage crosses were also used for initial gene mapping strategies based on recombination frequencies, such as the mapping of the T4 *rII* loci responsible for the T4r phenotype of faster host degradation and bigger plaques [2]. The use of phage crosses requires markers and phenotypes to identify recombinant phages and is limited to combining already existing phage genomes. To overcome this limitation, donor plasmids have been generated to allow specific deletions, insertions, and substitutions of nucleotides in the phage genome [24,25] (Figure 1B). These donor plasmids usually encode the desired mutations (or randomized libraries [26]) flanked by the homologous phage sequences and are transferred into the host bacteria. During phage infection and replication on these host bacteria, homologous recombination between the phage genome and the donor plasmid can occur, resulting in release of recombinant phages. Homologous recombination with a donor plasmid offers endless opportunities to generate phages with desired characteristics. The recombination template can also be provided on retrons, as recently demonstrated. Retron-based recombination does not require long homologous flanks, allowing fast and easy cloning, but is not suitable for insertion of large fragments due to the small size of the homologous domain in the retron (75 bp) [27].

A major limitation of homologous recombination-based engineering approaches is the low recombination efficiency, which requires time-consuming and labor-intensive screening for the recombinant phages, due to an inability to use selectable markers (e.g., antibiotic resistance genes) during phage lytic growth. To facilitate screening, it is possible to incorporate marker genes that allow specific selection for mutated phages (e.g., bioluminescence [28]) or to apply a subsequent counter-selection method to eliminate the wild type phages (see the following sections).

Recombineering

The success rate of homologous recombination can be improved with the use of recombination proteins, such as proteins Exo, Beta, and Gam of coliphage lambda, and gp60 and gp61 of mycophage Che9c. These proteins vastly enhance the frequencies of homologous recombination by generating 3′ single-stranded overhangs in linear double-stranded DNA (dsDNA) substrates for efficient annealing to a complementary single-stranded DNA (ssDNA) target in the cell. The properties of recombination proteins were explored to modify the genomes of *Mycobacterium smegmatis* [29], *E. coli* [30], *Salmonella* [31], and *Klebsiella* [32] phages using a

Phage-inducible chromosomal island (PICI): mobile genetic element that parasitizes the life cycle of certain temperate phages to promote its own spread.
Prophage: phage genome that is incorporated into the bacterial chromosome or exists as an extrachromosomal plasmid within the cell.
Protospacer: DNA or RNA sequence targeted by a CRISPR-Cas system.
Quorum-sensing: regulation of gene expression in bacteria in response to cell population using signaling molecules.
Rebooting: reactivation of phage DNA by gene expression and virus assembly.
Receptor-binding protein (RBP): structural phage protein (e.g., tail fiber or tail spike) used to identify and interact with a specific target receptor on the host cell surface.
Temperate phage: phage that can follow either the lysogenic or lytic life cycle. In the lysogenic cycle, the phage genome integrates into the host chromosome. In the lytic cycle, the phage genome takes over the cell metabolism to replicate and generate new phages that are released into the environment by cell lysis.
Transduction: process by which a phage transfers genetic material between bacterial cells.
Virulent phage: phage that follows the lytic life cycle.

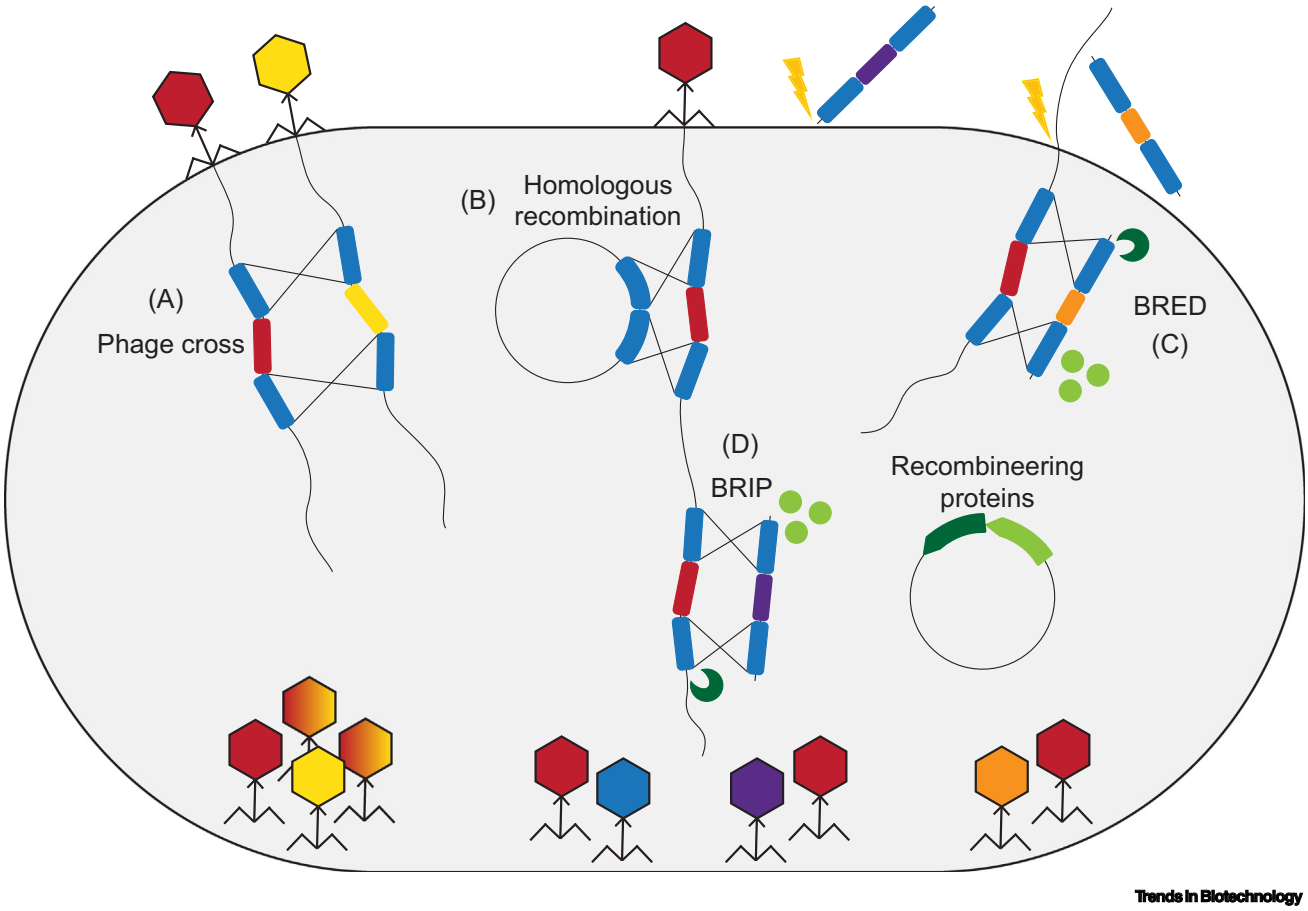

Figure 1. Recombination-based techniques for phage engineering. (A) In phage crosses, phages co-infecting a bacterium recombine, leading to the release of progeny with combined features of the parental phages. (B) In homologous recombination approaches, a donor plasmid is provided that includes the desired mutations flanked by sequences homologous to a phage; this facilitates recombination with the infecting phage, for the generation of recombinant phages. (C) Bacteriophage recombineering of phage DNA (BRED) makes use of recombineering proteins to enhance homologous recombination efficiency between electroporated phage DNA and a DNA substrate with the desired modification. (D) Bacteriophage recombineering with infectious particles (BRIP) is a variation of BRED, in which the desired modification is provided by electroporation of a DNA substrate and subsequent infection of the cell with the phage rather than transferring the phage DNA into the cell by electroporation.

technique known as bacteriophage recombineering of electroporated DNA (BRED, Figure 1C). BRED co-electroporates linear phage DNA and a DNA substrate containing the desired modification into bacterial cells that have been equipped with a plasmid for expression of the recombination proteins. The cells are plated as a bacterial lawn to obtain phage plaques that will contain a mixture of wild type phage and desired phage mutant. Because the success rate of BRED is relatively high (≈10% in *M. smegmatis*), phage mutants can be readily recovered and identified by PCR analysis of individual plaques, requiring no incorporation of additional markers into the phage genome or counter-selection methods. This provides the opportunity to generate phages that are edited but not recombinant and are therefore not considered to be genetically modified organisms. This facilitates the acceptance of edited phages for therapeutic use by regulatory agencies, which recently allowed the first therapeutic use of engineered phages in the UK [10].

Currently, BRED is mostly limited by difficulties in achieving high enough transformation efficiencies with the large phage genomes in most hosts. To overcome these limitations, variations of BRED have been employed in which the synthetic DNA substrate with the desired modification

is electroporated into the bacteria and the bacteria infected with the phage rather than electroporated with phage DNA [bacteriophage recombineering with infectious particles (BRIP); Figure 1D] [30,33]. In summary, recombineering-based techniques allow precise modification of phage genomes at much higher efficiency than classic homologous recombination and their application has the potential to be expanded to multiple bacterial species.

CRISPR-Cas-based methods for phage engineering by counter-selection

The development of CRISPR-Cas (Box 2) counter-selection methods has facilitated the time-consuming screening required to identify recombinant phages. Counter-selection with CRISPR-Cas (Figure 2A) can be performed after a first step of homologous recombination that results in a mixture of wild type and recombinant phages. This mixture of phages is subsequently propagated on a host strain expressing a CRISPR-Cas system that targets and depletes the wild type phage genomes, thereby enriching recombinant phages. The recombinants have lost the targeted **protospacer** sequence by successful homologous recombination and can therefore replicate on the CRISPR-Cas counter-selection strain [27,34,35]. Alternatively, recombination and counter-selection can be performed simultaneously in a one-step approach (Figure 2B) [36,37]. Different CRISPR-Cas systems have been reported to efficiently select against the wild type phages.

Type I systems successfully used for counter-selection of recombinant phages include type I-E and I-D. A plasmid-based type I-E CRISPR-Cas system heterologously expressed in *E. coli* allowed efficient targeting of wild type T7 phage and selected for deletion mutants obtained previously by homologous recombination. However, the targeting by type I-E also enriched for CRISPR-Cas **escape mutants** with mutations in the protospacer sequence [38]. Investigations with an endogenous type I-F system of *Pectobacterium atrosepticum* revealed that the only way to escape targeting by multiple spacers is through target site deletion, offering a way to increase selection of recombinant phages or drive natural deletions [39]. A separate study exploited the generation of phage escape mutants to characterize the sequence requirements of an introduced *Vibrio cholerae* type I-E CRISPR-Cas. The system could then be used for selection of recombination events between phages and an editing plasmid, resulting in precise gene deletion and insertions in phages [37]. Furthermore, the endogenous type I-D system of *Sulfolobus islandicus* was shown to efficiently select for recombinant rod-shaped archaeal viruses with desired mutations [40].

Type II systems are less abundant in bacterial genomes than type I, but are the most well studied CRISPR-Cas systems for engineering purposes. Similar to the type I-E system of *V. cholerae*, CRISPR-Cas escape mutant phages were analyzed to characterize newly identified endogenous type II systems in *Listeria ivanovii* and *Streptococcus thermophilus*. Both systems could subsequently be applied for efficient counter-selection of phage mutants obtained by homologous recombination [34,41]. Furthermore, the heterologous *S. thermophilus* Cas9 was used in a combined approach with BRED or BRIP (CRISPY-BRED/BRIP) to successfully increase efficiency of selection for recombinant *Mycobacterium* phages [33].

The RNA-targeting type III CRISPR-Cas systems provide robust immunity against phages and are less affected by escape mutations in the protospacer sequence [42], representing an ideal tool to select for recombinant phage mutants. Replication of *Staphylococcus epidermidis* phages on a host strain encoding an endogenous type III-A system, a plasmid-encoded repeat-spacer unit, and a recombination template resulted in very efficient selection for the desired phage mutants using a one-step approach (Figure 2B) [36]. The heterologously expressed RNA-targeting type VI system was also successfully applied to counter-select for mutant phages in a two-step approach [43,44].

Similar to bacterial or mammalian engineering techniques, CRISPR-Cas systems, especially type II systems, have also been reported as engineering tools to induce precise double strand breaks in DNA. This has been proposed to stimulate **homology-directed repair** on phage genomes to obtain specific mutations, or to induce random mutations via **non-homologous end joining**

Box 2. CRISPR-Cas systems

CRISPR-Cas systems are adaptive prokaryotic immune systems that acquire genetic memories from past encounters with **mobile genetic elements (MGE)** like phages and plasmids [110,111]. A CRISPR-Cas system comprises the CRISPR array that encodes the genetic memories as unique spacers between repeat sequences [112] and the proximal *cas* genes [113]. The sequence-specific immunity provided by CRISPR-Cas is mediated by three stages: adaptation, expression, and interference [114] (Figure I).

During adaptation, new spacers are acquired by integrating short foreign DNA sequences into the CRISPR array [111]. To provide protection against the invader, the CRISPR array is expressed into a precursor CRISPR RNA (pre-crRNA), which is processed by Cas proteins into mature crRNAs [115,116]. Next, the effector proteins assemble around the mature crRNA to form an interference complex [115]. Interference takes place when the interference complex binds a protospacer sequence complementary to the crRNA and recruits and/or activates the nuclease component of the particular CRISPR-Cas system [115]. Different CRISPR-Cas systems encode distinct nucleases and interference can result in diverse consequences for the invading phage and the cell itself [114,117] (Table I). Based on the type of effector molecule responsible for the interference stage (multi-subunit or single effector proteins), CRISPR-Cas systems are classified in two different classes and further subdivided into six types and several subtypes, according to their *cas* genes [118] (Table I).

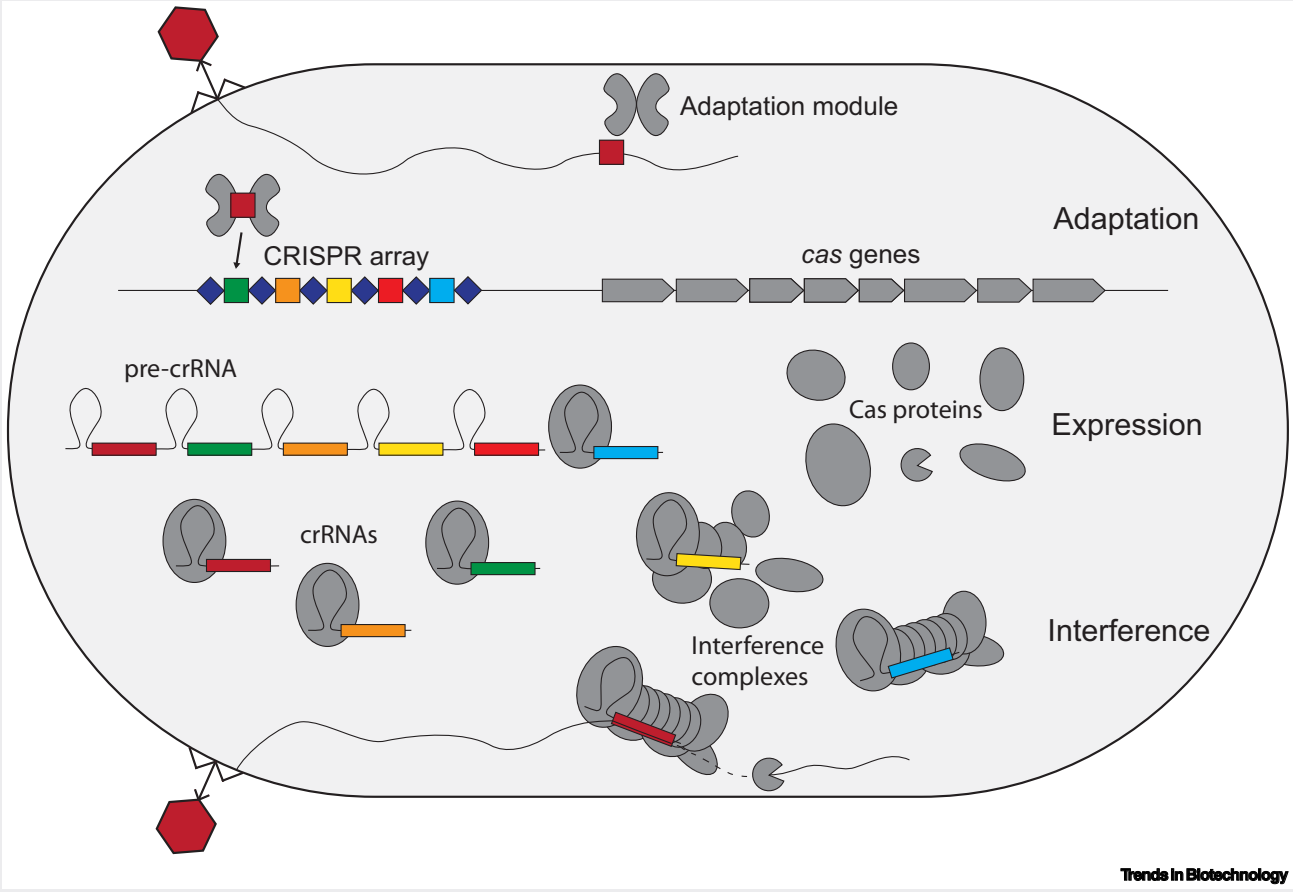

Figure I. The three stages of clustered regularly interspaced short palindromic repeats-CRISPR associated protein (CRISPR-Cas) immunity. In the adaptation stage, new spacers are acquired by the adaptation module (Cas1-Cas2) from invading foreign nucleic acids and incorporated into the CRISPR array. In the expression stage, the CRISPR array is expressed into a precursor CRISPR RNA (pre-crRNA) that is processed by Cas proteins into mature crRNAs. Effector Cas proteins then assemble around the mature crRNA to form an interference complex. In the interference stage, the interference complex binds a protospacer sequence complementary to the crRNA that it carries, resulting in nuclease activity targeting the invading nucleic acid.

Table I. Main features of the different classes and types of CRISPR-Cas systems used for phage engineering

	Class I		Class II		
Type	I	III	II	V	VI
Recognized target	DNA	RNA	DNA	DNA	RNA
Protection mechanism	DNA degradation (≈100 bp)	Specific RNA cleavage and collateral RNA or DNA degradation	Double-stranded DNA cut	Double-stranded DNA cut	Cleavage of target RNA and collateral RNA degradation
Interference complex	Multi-subunit	Multi-subunit	Single effector protein	Single effector protein	Single effector protein
Signature protein	Cas3	Cas10	Cas9	Cas12	Cas13

(Figure 2C). However, it is currently not possible to distinguish whether the recombinant phages resulted from stimulated recombination by CRISPR-Cas or just counter-selection. Direct evidence for the stimulated recombination would require performing quantitative experiments. Nevertheless, heterologous expression of a type II system targeting the gene to be engineered and the simultaneous supply of a homologous repair template resulted in the successful introduction and selection of desired mutant *Lactococcus lactis* [45], *Klebsiella pneumoniae* [46], *Bacillus subtilis* [47], and *E. coli* [48,49] phages. Similarly, the heterologous type V system of *Lachnospiraceae bacterium* was used in *E. coli* to engineer genomic DNA of phage T4 [50].

The application of CRISPR-Cas-based phage engineering methods is limited to bacteria that either encode a characterized native CRISPR-Cas system or are competent for transformation to enable expression of an active heterologous CRISPR-Cas system. This can be a major limitation for engineering phages of bacteria that are not genetically tractable. Additionally, phages have evolved multiple resistance mechanism against CRISPR-Cas targeting, such as masking their DNA by covalent modifications of the nucleotides or by employing **anti-CRISPR proteins** (Acrs), as reviewed in [51]. The well-characterized *E. coli* phage T4 was reported to possess glucosyl DNA **hypermodifications** and DNA recombination and repair mechanisms, which protect against the DNA-targeting type I and II CRISPR-Cas system, resulting in reduced efficiency in counter-selecting against wild type T4 [52–55]. Nevertheless, T4 could be successfully engineered by screening for effective type II spacers [49], or by combining Cas9-based genome editing with insertion of a luciferase gene for facilitated screening [48]. The type V CRISPR-Cas system, which is less affected by DNA modifications [50], and the RNA-targeting type VI system [44] were also efficiently applied to generate and select for mutant T4 phages. Both the one-step approaches with the DNA-targeting type II or V systems and the two-step approach with the type VI system eliminated the wild type T4 phages without resulting in enrichment of escape mutants [44,48–50]. Spacers resulting in a high enough targeting efficiency for counter-selection were readily found for type VI [44], while for type V [50] and type II [48,49] screening for an efficient spacer was required. Simultaneous expression of two type V spacers was shown to have a synergistic effect [53] and was used to obtain large deletions of up to 11 kbp on the T4 genome [50]. Interestingly, Acrs have been explored as an alternative CRISPR-Cas counter-selection strategy (Figure 2D) [40,43]. Recombination of the *acrID1* gene into the genome of *S. islandicus* virus SIRV2M allowed the mutant virus to evade targeting by the native host type I-D CRISPR-Cas system [40]. Combination of heterologously expressed Cas13a and insertion of the *acrVIA1* gene as selection marker was also reported as an approach to select for mutant phages that evaded recognition and targeting by the type VI-A CRISPR-Cas system [43]. This study demonstrated that RNA-recognizing CRISPR-Cas systems combined with Acrs can promote recovery of recombinant progeny of phages that have evolved strategies to evade DNA-targeting

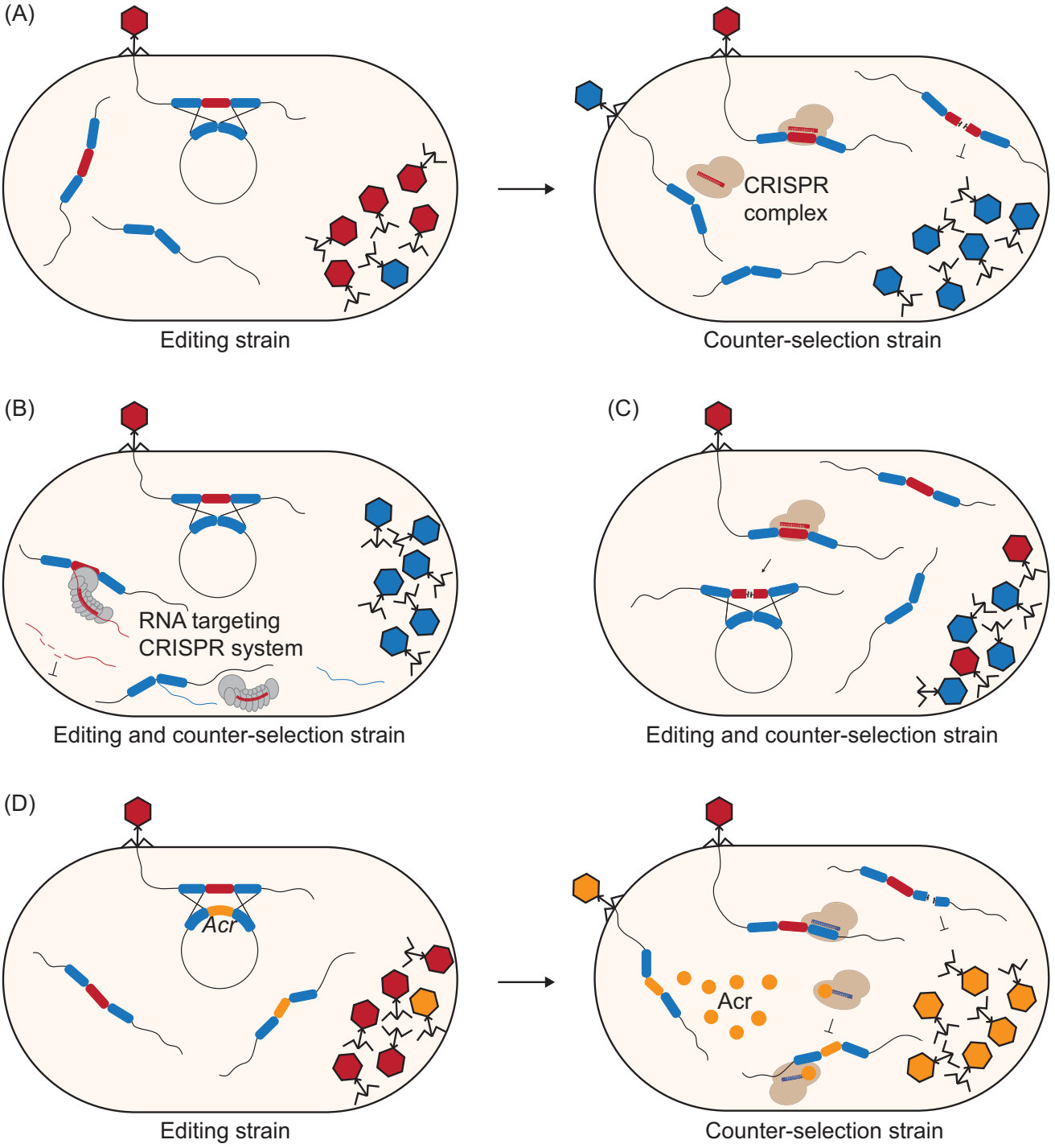

Figure 2. Clustered regularly interspaced short palindromic repeats-CRISPR associated protein (CRISPR-Cas)-assisted phage engineering. (A) CRISPR-Cas as a counter-selection method after homologous recombination (see Figure 1). The CRISPR-Cas system targets the wild type phage while the mutant phage is enriched. (B) Recombination and simultaneous counter-selection by RNA-targeting CRISPR-Cas systems. (C) CRISPR-induced stimulation of homology-directed repair. (D) Anti-CRISPR (Acr)-based counter-selection, in which an *acr* gene is recombined into the phage genome. The modified phage will express the Acr protein and escape CRISPR-targeting.

CRISPR-Cas systems (e.g., **jumbo phages** [56,57]). Even though this CRISPR-Cas- and Acrs-based selection has been shown to be very efficient, the insertion of an *acr* gene in the phage genome limits the use of the same *acr* gene for selection of further mutations.

Generally, both DNA- and RNA-targeting CRISPR-Cas systems provide efficient counter-selection, but their applicability for phage engineering can be limited by the identification of a spacer providing strong protection against the wild type phage. This limitation can usually be overcome by screening for efficient spacers before they are used for engineering [36,43,45,46,48–50]. Additionally, site selection for spacer design may be limited by available protospacer adjacent motifs (**PAM**) in the phage sequence. However, the extensive Cas9 engineering has expanded PAMs and removed PAM selection in some cases [58,59], and if site choice is limited with one CRISPR-Cas subtype, other subtypes can be used for which a site with the desired PAM is available.

Selection and screening methods

Methods other than CRISPR-Cas have been developed for selection and screening of recombinant phages after homologous recombination. Selection can be facilitated by using bacterial host genes essential for phage replication but not essential for bacterial growth. These genes can be introduced in the phage genome during homologous recombination followed by selection for recombinant phages on a host with a knockout of the same gene [60]. Several essential host genes for *E. coli* phage T7, such as *trxA*, *cmk*, and *waaC*, have been identified and successfully used as marker genes for selection of recombinant T7 [60–62]. However, using host genes essential for phage replication as marker genes requires extensive knowledge of the host and phage requirements for phage replication, which is usually only the case for well-studied model phages like T7. Another option to facilitate selection involves the insertion of genes encoding fluorescent proteins or luciferases to allow selection for recombinant phages based on fluorescence [33] and luminescence [63]. In some cases, mutant phages can easily be selected for their desired function, such as a changed host range [26] or resistance to a certain bacterial defense system [23,40,43].

To circumvent the limitation of having to insert a marker gene and for facilitated recovery of the recombinant phages, enrichment- and PCR-based screening protocols were developed for CRISPR-Cas insensitive phages, such as a T7-like cyanophage and *E. coli* phage T5 [25,27]. In both screening methods, the lysate obtained after homologous recombination was divided in subsamples to allow phage enrichment on the host in microtiter plates. Detection of recombinant phages was based on mismatched amplification mutation assay PCR, which is PCR amplification with a primer binding to a unique sequence introduced during recombination, offering the opportunity to screen for specific point mutations. Wells showing positive PCR bands were subsequently plated to obtain individual plaques for further purification. These recombination, enrichment, and PCR-based screening protocols resulted in efficient recovery of desired phage mutants [25,27].

Overall, screening methods are labor intensive but facilitate the efficient recovery of mutant phages, especially if recombination frequency is low and no other counter-selection method is available.

Ex vivo phage engineering

Phage engineering approaches performed inside a living bacterium have proven useful, but come with various limitations. Intracellular phage engineering often: (i) requires host-engineering (e.g. introducing a plasmid), which is limited to genetically tractable hosts; (ii) is dependent on low-chance events (e.g., homologous recombination) that require complex selection and screening procedures; and (iii) has reduced efficiency in phages that degrade the host and plasmid

DNA, or that kill the cell quickly, before engineering events can occur. Alternatively, modified phage genomes can be obtained using fully synthetic approaches. This typically involves PCR amplification of the phage genome in overlapping fragments that include one or more segments containing tailored mutations or random mutant libraries (Figure 3) [64]. The fragments are subsequently joined in **Gibson assembly** [63] or **Golden Gate assembly** [64] reactions, or in yeast cells [65]. The obtained synthetic phage genome can be reactivated into infectious phage particles by transformation into host cells or **L-forms**, or by *in vitro* transcription and translation in cell-free expression systems, in processes known as **rebooting**.

Yeast-based genome assembly

The assembly of phage genomes can be efficiently achieved in yeast cells [65–67]. In this approach, the phage genome is amplified by PCR in multiple overlapping regions of approximately 4–12 kb each, with the first and last fragments having overhangs homologous to a linear yeast artificial chromosome (YAC). All fragments are cotransformed into *Saccharomyces cerevisiae*, where the native yeast recombination machinery efficiently assembles the phage genome and YAC fragment via gap repair [65]. The resulting YAC vector containing the assembled phage genome is extracted from the yeast cells and transformed into bacterial host cells for rebooting functional phages. Phage plaques obtained are amplified and sequenced to confirm the introduction of the desired modification in the phage genome. Yeast-based phage engineering has been successfully employed for the modification of *E. coli* [65], *Klebsiella* sp. [65], and *Pseudomonas aeruginosa* [67] phages of up to 44 kb so far, with rebooting happening either in *E. coli* first or directly in the host strain, and was also adapted to engineer **phage-inducible chromosomal islands (PICI)** [68].

YAC-assisted assembly of modified genomes is highly efficient, but rebooting of the phage genomes has relied solely on transformation of host bacteria [65–67], which restricts the applicability of this engineering strategy to highly transformable bacteria. Alternatively, it may be feasible to reboot assembled phages via strategies such as the bacterial L-forms and cell-free methods as described next (Figure 3).

Rebooting in bacterial L-forms

The generally low competence of Gram-positive bacteria for transformation is a major limitation for rebooting engineered phages infecting these hosts. This limitation can be circumvented by replacing the thick-walled Gram-positive host cells with L-form bacteria to reactivate the synthetically assembled phage genome (Figure 3). L-form bacteria are wall-deficient cells, which are still metabolically active and engaged in cell division. The generation of L-form cells usually results from prolonged cultivation in an osmotically stabilized media containing antibiotics actively affecting cell wall synthesis, such as penicillin, and it is thought that a wide range of Gram-positive and -negative bacteria can enter the L-form state. [69,70]. The lack of a cell wall allows the L-form bacteria to be transformed with large DNA molecules (up to 154 kbp so far) like synthetic phage genomes [69]. An L-form derivative of *Listeria monocytogenes* has been reported to efficiently reboot phages following polyethylene glycol-assisted transformation of purified genomic phage DNA, or synthetically engineered and assembled phage genomes [71]. Rebooted progeny phages are released by disruption of the osmotically stabilized L-form cells and can be further amplified by replication on their native host. Therefore, rebooting in L-forms is not only independent of receptor recognition and adsorption by the phage, but also phage-mediated lysis of infected cells, providing the opportunity for cross-genus reactivation of phages. This has been successfully demonstrated for *L. monocytogenes* L-forms capable of rebooting diverse *Staphylococcus* and *Bacillus* phages [71]. Rebooting of synthetically engineered and assembled phage genomes in L-forms was applied for host range expansion by structure-guided modifications of the **receptor-binding proteins**

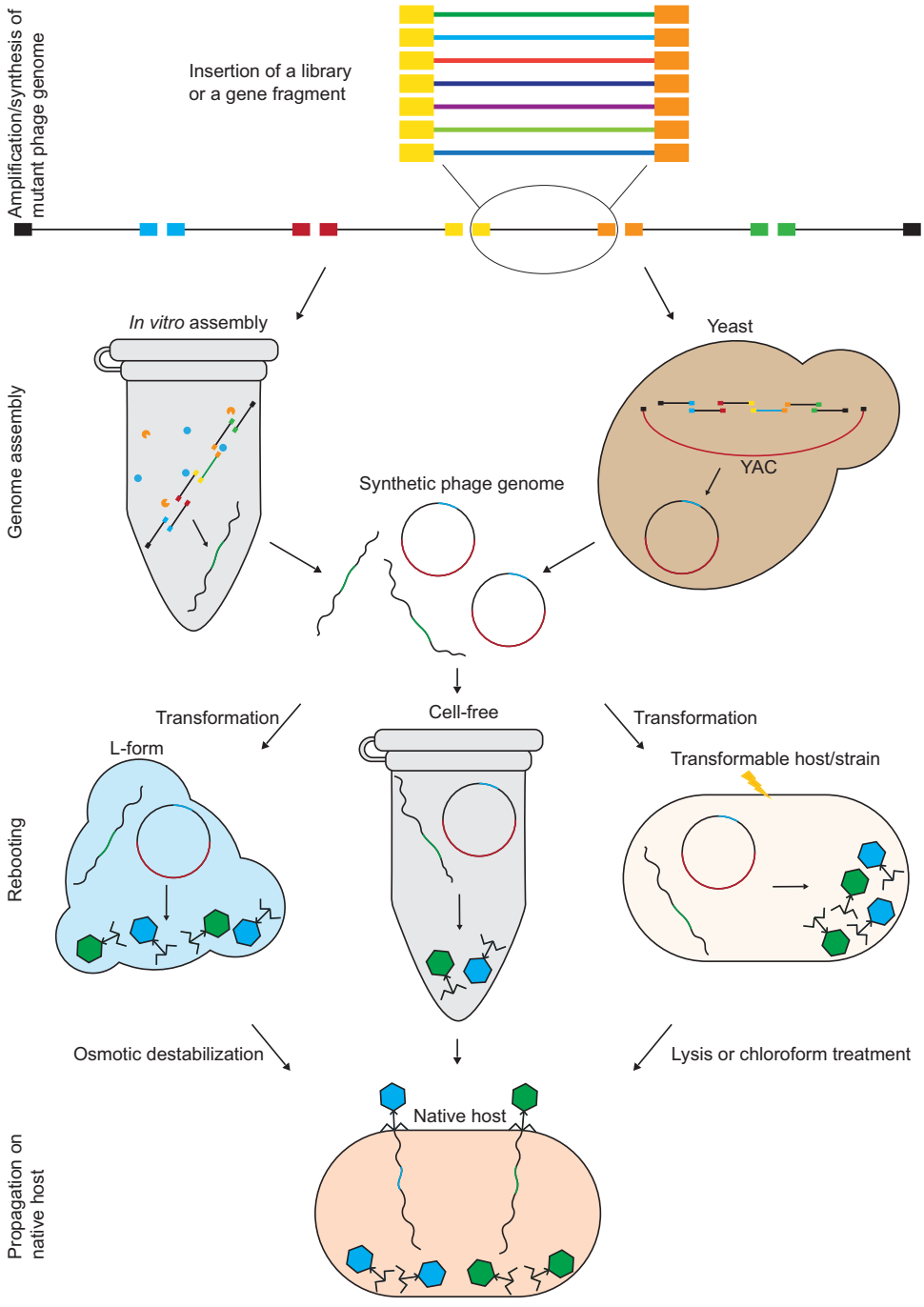

Figure 3. *Ex vivo* approaches to phage engineering. The phage genome is amplified or synthesized in fragments with homologous arms, with the desired modifications introduced as specific gene fragments or random gene libraries. The genome fragments are then assembled *in vitro* in Gibson or Golden Gate reactions, or *in vivo* in yeast cells. The resulting assembled synthetic genome is subsequently rebooted *in vivo* by transformation into host bacterial cells, L-form bacterial cells, or *in vitro* in cell-free expression systems. The rebooted phages are recovered and can be further propagated using the native host strain. Abbreviation: YAC, yeast artificial chromosome.

(RBP) [72]. This approach allowed randomization of the RBP as well as combination of compatible RBPs to produce phages with an extended host range [72].

Rebooting of phage genomes in bacterial L-forms has been demonstrated to be widely independent of the viral lifestyle, morphology, DNA packaging strategy, as well as genome ends (e.g., cohesive ends, terminally redundant, but all dsDNA) and size, offering an engineering platform for a wide range of phages [71]. Even though rebooting of engineered phage genomes in L-forms has only been demonstrated in Gram-positive cells, the possibility of generating L-forms of Gram-negative bacteria [73] suggests they can also be used as phage rebooting machineries. Still, it is so far unknown if L-forms can be successfully generated for all bacterial species.

Cell-free methods

Phage rebooting through direct transformation of a synthetic phage genome into the native host or L-form cells is limited by the maximum DNA size that the bacterial cell can take up. To escape the constraints of working with a living cell, phage engineering strategies performed entirely *in vitro* are being investigated.

The first extracellular virus experimentation was done by Sol Spiegelman in the 1960s, when he was performing evolution studies on the coliphage Qβ [74]. Instead of infecting cells with live Qβ particles, he purified the 4217-nt Qβ genomic RNA and introduced it into a solution with RNA replicase, free nucleotides, and salts. By doing so, Spiegelman was able to successfully replicate the Qβ genome entirely *in vitro*. A next milestone in cell-free phage biology was demonstrated in 2012, when viable T7 phage particles were synthesized in a test-tube directly from the 40-kbp genome [75]. For this, bacterial cell-free extractions containing all the native transcription-translation (TXTL) machinery were used [76], in which other coliphages (including MS2/RNA [77], ΦX174/ssDNA [77], and T4/dsDNA [78]) were shown to reboot as well. By incorporating the RNA polymerase sigma factor SigA of *B. subtilis* into the cell-free system, the *in vitro* generation of phages was extended to those targeting Gram-positive bacteria (*B. subtilis* phages Φ29 and Goe1 [79]). Also, phages against therapeutically relevant bacteria (CLB-P3 targeting EAEC, ΦA1122 targeting *Yersinia pestis*, and MUC-5 targeting *K. pneumoniae* [79]) were produced cell-free, demonstrating the potential of this approach for phage therapy.

Inspired by the principle of RNA interference in bacteria, addition of small antisense DNA (sDNA) was demonstrated to control protein expression levels during cell-free replication of phage T7. The major capsid protein of T7 was successfully repressed by providing 60-bp-long sDNA molecules complementary to the ribosome-binding site and the downstream sequence, resulting in highly reduced phage titers, but more efficient *in vitro* DNA replication [80]. Furthermore, coexpression of modified variants of the T7 minor capsid protein from a plasmid during cell-free phage production resulted in transient non-genomic modification of the phage particles, allowing fast and easy production of modified phages for single use [79].

The next step is to reboot modified phage genomes: *in vitro* genome engineering combined with *in vitro* genome rebooting could establish platforms for fully synthetic phage generation. This bypasses the involvement of cells during the engineering process, increasing the attainable diversity as engineering is no longer restricted by the transformation efficiency. Cell-free approaches also shorten the design–build–test cycle, as they do not rely on growth and manipulation of a bacterial strain. Recently, T7 phage particles were generated in TXTL from a Gibson-assembled genome containing a modified tail fiber gene [81], aiming at expanding the host range (Box 1). *In vitro* phage tail engineering is currently limited by retaining the relation between genotype and phenotype during rebooting to enable tracing of a successful phage particle within a large library

of mutants. Performing individual TXTL reactions, for example, in droplets [82], would be a solution. More generally, expanding methods for *in vitro* genome engineering as well as bacterial species from which cell-free is available (currently limited to *E. coli*, *Vibrio natriegens* [83,84], *Streptomyces venezuelae* [85], and *B. subtilis* [86]) would greatly facilitate the development of *in vitro* phage engineering platforms.

Concluding remarks and future perspectives

Here, we summarized techniques currently available to engineer phage genomes, which have contributed invaluably to understanding phage biology and to the full development of biotechnological tools based on phages. However, some limitations are preventing their wide usage for phage genome engineering (Table 1, Key table).

Random mutagenesis and homology-based approaches have been the most commonly applied techniques for phage engineering efforts since researchers started to modify phage characteristics. While easy to implement, these are limited by the effort required to identify the desired mutants from a large pool of wild type phages. This issue was largely improved by increasing the efficiency of homologous recombination using recombineering approaches, such as BRED or BRIP. Furthermore, Argonaute proteins that were shown to enhance homology sequence-directed recombination in bacteria may also be applied to increase recombination efficiencies in future studies (see Outstanding questions) [87].

Due to the low efficiency of homologous recombination, CRISPR-Cas counter-selection to eliminate wild type phages has been implemented. All CRISPR-Cas types, except type IV, have been shown to efficiently deplete wild type phages to enrich the low abundant mutant phages. Among these, RNA-targeting systems are a promising choice, as they provide stronger protection than DNA-targeting systems, especially against phages that have evolved DNA targeting-evading strategies, such as DNA modifications. Additionally, RNA-targeting CRISPR-Cas systems are less affected by small mutations in the protospacer and have no PAM requirements, resulting in less escape mutants. While CRISPR-Cas systems have been mostly employed as a counter-selection strategy, some strategies also explore the ability of these systems to induce precise double strand breaks in DNA to stimulate recombination. In this context, base editing of phage genomes using CRISPR base editors [88] may be an interesting approach for direct introduction of specific point mutations without requiring dsDNA breaks and donor templates. However, the efficiency of specific CRISPR-Cas systems for counter-selection or direct genome engineering might be affected by the presence of corresponding Acrs on the phage genome and, as a recombination-based approach, it typically requires extensive cloning and optimization. These limitations resulted in the development of methods to assemble and reboot synthetic phage genomes. These approaches enable the introduction of multiple mutations simultaneously, providing greater freedom in synthetic phage design. Currently, *ex vivo* techniques still commonly require amplification or synthesis of DNA fragments that are subsequently assembled. In the future, it might be possible to synthesize whole phage genomes and even obtain libraries of complete phage genomes to investigate. Rebooting synthetic phage genomes currently requires transformation in host cells or L-forms, but rebooting of large synthetic genomes remains difficult. The generation of Gram-negative L-forms can assist with transformation of larger genomes into less genetically tractable Gram-negative strains, but cell-free processes are likely to become the method of choice. Cell-free systems remove the barrier of the cell membrane, allowing for unrestricted rebooting of phage genomes of all sizes. While still limited to a small number of species and strains, cell-free systems are expected to expand to other species.

Outstanding questions

Most phage genome engineering methods are optimized for DNA phages. Can these be used to engineer RNA phages or will new methods be developed?

Will L-form cells replace all nontransformable hosts for rebooting engineered phage genomes?

Most *in vivo* phage engineering strategies use plasmid-based approaches and therefore rely heavily on the genetic tractability of the host cell. Can L-forms be used to enable these strategies in less genetically tractable species and strains?

Will RNA-targeting CRISPR-Cas systems become the method of choice for engineering phages with evasion strategies for DNA-targeting systems?

When will advances in synthetic biology make it possible to synthesize complete phage genomes without the need for amplification and assembly?

Can the efficiency of homologous recombination approaches improve such that counter-selection is no longer required with the application of homologous recombination enhancing systems like Argonaute?

The extent of modification of a phage genome is limited by the genome size that can be incorporated into the capsid. Will progresses in structural biology coupled to genome research make it possible to overcome this limitation and adjust the phage capsid to the desired modified genome size?

Key table

Table 1. Main advantages and limitations of phage genome engineering methods

Method	Advantages and limitations
In vivo	
Random mutagenesis	+ Applicable to any phage or phage genome + No cloning required - Requires selection or screening for desired phenotype - Accumulation of unwanted mutations
Homologous recombination: phage crosses	+ No cloning required - Usually only applicable to phages with high sequence identity - Not applicable to generate specific gene modifications - Requires screening or selection for desired phenotype
Homologous recombination: plasmid template	+ Precise mutations - Requires host engineering - Requires genetically tractable host - Requires labor intensive screening due to low recombination efficiency
BRED	+ Precise mutations + More efficient than homologous recombination - Requires host engineering - Requires genetically trackable host
BRIP	+ Precise mutations + More efficient than homologous recombination + No highly transformable host required - Requires host engineering - Requires genetically tractable host
CRISPR-Cas	+ Efficient counter-selection + Can combine recombination and selection in one step + Possible in hosts containing a native CRISPR-Cas system - Requires an efficient spacer - Escape mutants can emerge (less relevant in RNA-targeting systems) - May require extensive host engineering - Requires a CRISPR-Cas system suitable for the host strain - Requires genetically tractable host when no native CRISPR-Cas exists - Can require a PAM, limiting target site choice in the phage genome
CRISPY-BRED/BRIP	+ Combined benefits of BRED/BRIP and CRISPR-Cas counter-selection - Requires genetically tractable host
Ex vivo	
In vitro or yeast-based assembly	+ Allows multiple simultaneous mutations + Libraries can be inserted easily - Requires PCR/synthesis of phage genome fragments - Efficiency reduces with increasing genome size
Rebooting	
Transformation	+ Independent of receptor recognition and adsorption - Requires highly transformable cells (very difficult in Gram-positive cells) - Efficiency reduces with increasing genome size
L-forms	+ Independent of receptor recognition, adsorption, and phage-mediated lysis + Allows transformation in Gram-positive cells + Highly independent of phage characteristics + Allows cross-genus rebooting - Not every strain can be made into L-forms - Labor-intensive, prolonged cultivation to obtain L-forms
Cell-free systems	+ Independent of receptor recognition, adsorption, and phage-mediated lysis + Not restricted by the transformation efficiency of the cells - Limited bacterial species from which cell-free is available - Difficult to retain genotype–phenotype association during rebooting when using mutant libraries

Overall, diverse methods are available for phage genome engineering, but the usability depends widely on the specific phage–bacterium pair. One thing is certain: the generation of engineered phages will become easier, faster, and cheaper due to the rapid advances in synthetic biology and will be assisted by our increasing understanding of phage biology and phage defense.

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Declaration of interests

No interests are declared.

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