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Genetically Engineered Phages: a Review of Advances over the Last Decade

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SUMMARY

Soon after their discovery in the early 20th century, bacteriophages were recognized to have great potential as antimicrobial agents, a potential that has yet to be fully realized. The nascent field of phage therapy was adversely affected by inadequately controlled trials and the discovery of antibiotics. Although the study of phages as anti-infective agents slowed, phages played an important role in the development of molecular biology. In recent years, the increase in multidrug-resistant bacteria has renewed interest in the use of phages as antimicrobial agents. With the wide array of possibilities offered by genetic engineering, these bacterial viruses are being modified to precisely control and detect bacteria and to serve as new sources of antibacterials. In applications that go beyond their antimicrobial activity, phages are also being developed as vehicles for drug delivery and vaccines, as well as for the assembly of new materials. This review highlights advances in techniques used to engineer phages for all of these purposes and discusses existing challenges and opportunities for future work.

INTRODUCTION

Bacteriophages (phages) are among the most abundant biological particles on earth. They are also highly versatile and adaptable to a great number of applications. Phages are viruses that infect bacteria; their self-replication depends on access to a bacterial host. Phages were discovered independently by Frederick Twort in 1915 (1) and by Félix d'Hérelle in 1917 (2), and they were used early on as antimicrobial agents. Although the initial results of phage therapy were promising (3, 4), poorly controlled trials and inconsistent results generated controversy within the scientific community about the efficacy and reproducibility of using phages to treat bacterial infections (5–7). The discovery of penicillin in 1928 and the subsequent arrival of the antibiotic era further cast a shadow on phage therapy (5, 6). As a result, phage therapy was discontinued in Western countries, even as its use continued in Eastern Europe and the former Soviet Union (8–10).

Despite the important success of antibiotics in improving human health, antibiotic resistance has emerged with steadily in-

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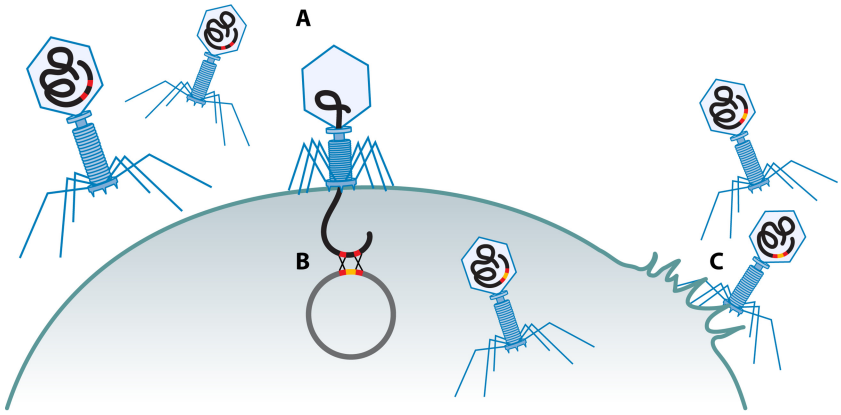

FIG 1 Phage engineering via homologous recombination. Upon phage infection (A), the injected DNA recombines with plasmid DNA carrying regions of homology (loci in red) to the phage genome (B), resulting in new, recombinant phage particles (fragments in orange) (C).

creasing frequency, rendering many antibiotics ineffective (11–14). Multidrug-resistant bacteria currently constitute one of the most widespread global public health concerns (15–17). More than 2 million people are sickened every year in the United States alone as a result of antibiotic-resistant infections, resulting in at least 23,000 deaths per year (16). The rising tide of antibiotic resistance coupled with the low rate of antibiotic discovery (17, 18) has revived interest in phages as antibacterial agents (19–21).

Unlike most antibiotics, phages are typically highly specific for a particular set of bacterial species or strains and are thus expected to have fewer off-target effects on commensal microflora than antibiotics do (22). The self-replicating nature of phages and the availability of simple, rapid, and low-cost phage production processes are additional advantages for their use as antimicrobials (22). Phages have been used not only to treat and prevent human bacterial infections (9, 23–25) but also to control plant diseases (26–29), detect pathogens (30–33), and assess food safety (34–37).

Notwithstanding their antimicrobial potential, some major concerns remain about the use of phages in clinical medicine. The specificity of phages means that they can target bacterial strains precisely; however, because a single phage type is unlikely to target all strains within a given species, cocktails combining various phages are often necessary to be broadly applicable for treating the wide range of bacteria that can cause clinical infections. Obtaining regulatory approval for the therapeutic applications of such cocktails can be challenging because of the significant diversity of phages in terms of structure, life cycle, and genome organization (22, 38). Like certain antibiotics, phages can cause rapid and massive bacterial lysis and the subsequent release of cell wall components (e.g., lipopolysaccharides [LPS]), which can induce adverse immune responses in the human host (39, 40). Bacteria frequently live in biofilm communities surrounded by extracellular polymeric substances (EPS), which can act as a barrier to phage penetration (41). Furthermore, as bacteria evolve, they can develop resistance mechanisms to avoid phage infection (38, 42, 43). By genetically engineering phages, it may be possible to overcome many of these limitations (44). The engineering of specific phages and components has been facilitated by the ever-growing abundance of fully sequenced phage genomes in public databases (45, 46) and by research into elucidating the structures of phage components (47–51) and the interactions between phages and their host bacteria (52–54). This review focuses on advances made in phage engineering techniques and applications in the past decade. Specifically, we discuss the use of phages in pathogen control and detection, as well as their broader application in other research areas, including targeted drug delivery and materials engineering.

TECHNIQUES FOR ENGINEERING SYNTHETIC PHAGES

Homologous Recombination

One of the most commonly used and well-established methods for engineering phage genomes is homologous recombination in their bacterial hosts, which can occur between two homologous DNA sequences as short as 23 bp (55, 56). Homologous recombination is a naturally occurring phenomenon. It enables cells to recombine heterologous DNA introduced into cells with their own genomic DNA when both sequences share regions of homology (57, 58). This mechanism can also be co-opted to incorporate foreign genes into a phage genome (Fig. 1). The generation of gene insertions, replacements, or deletions by homologous recombination with phage DNA follows principles similar to those that apply to the bacterial counterparts. As with all of the phage genetic engineering techniques described below, phage genome sequencing is important for the successful design of constructs to modify the phages. In brief, the gene to be introduced into the phage genome, flanked by two regions of homology with the phage genome, is first cloned into a replicative plasmid. The homologous regions determine where in the phage genome the foreign gene will be incorporated (59). The bacterial host harboring the donor plasmid is then infected by the phage to be engineered. Homologous recombination occurs between the plasmid and the phage genome, allowing the heterologous gene to be integrated into the phage genome and eventually packaged within the phage particle (59, 60). However, often only a small fraction of the progeny phage will be recombinant. Reported recombination rates range from 10^{-10} to 10^{-4} (59–61), though this frequency can be higher and may depend on the phage and the genes being manipulated. Without an efficient phage screening method, finding the desired clone is labor-intensive at best. Therefore, a reporter gene, usually encoding luciferase or a fluorescent protein, is commonly cloned along with the gene of interest to facilitate the identification of mutant phages by detecting the reporter (59, 62–65). Because the recombination rates obtained with this technique are low, it is improbable that targeting multiple loci at the same time will result

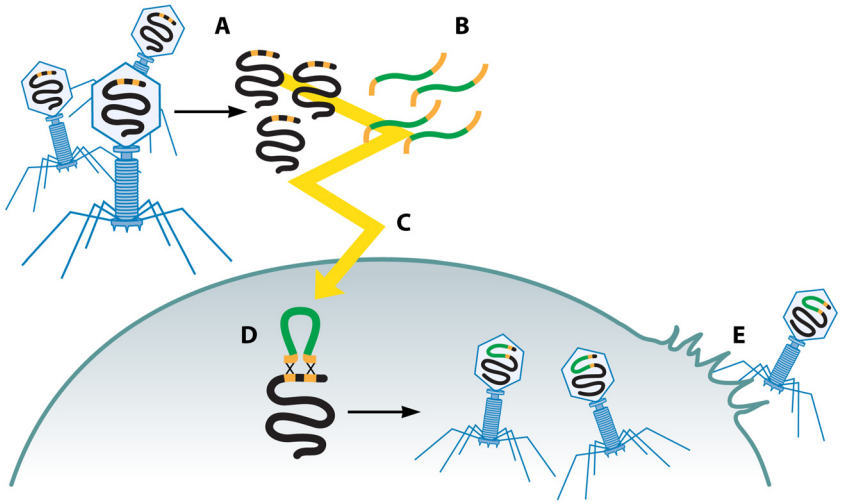

FIG 2 Bacteriophage recombineering of electroporated DNA. Purified phage DNA (A) and dsDNA recombineering substrates (B) are coelectroporated into cells (C). Recombination between their homologous regions (in orange) (D) results in recombinant phage particles (containing DNA fragments in green) (E).

in an organism carrying all the desired mutations. Thus, when the aim is to obtain multiple mutations, each mutation is often made independently in a sequential fashion, which is a time-consuming process.

Bacteriophage Recombineering of Electroporated DNA

Another frequently used strategy for the engineering of phage genomes is bacteriophage recombineering of electroporated DNA (BRED) (Fig. 2) (63, 66). This technique was first applied by Marinelli et al. to modify mycobacteriophages (66) and has since been expanded to modify phages that target bacterial hosts other than mycobacteria for which recombineering systems are available, such as *Escherichia coli* and *Salmonella enterica* (67, 68). BRED can be used to delete, insert, and replace genes, as well as to create point mutations in phage genomes. It consists of coelectroporating the recombineering substrates, i.e., phage DNA and double-stranded DNA (dsDNA), into electrocompetent bacterial cells carrying a plasmid that encodes proteins promoting high levels of homologous recombination, such as the RecE/RecT-like proteins (63, 66). The dsDNA substrate comprises the DNA fragment to be inserted along with regions of homology to the loci immediately up- and downstream of the region of the phage genome to be modified (66, 69). After electroporation, the bacterial cells are recovered, mixed with the wild-type bacterial host, and plated. The plates are then checked for the presence of phage plaques. Individual plaques, indicative of bacterial cell lysis, are then screened by PCR for the correctly mutated phage genome (66). By using this method, modified phages have been obtained at high frequencies (10 to 15%), thus enabling putative mutants to be screened by a small number of PCRs, without the requirement for further selection (66). This technique requires highly competent bacterial hosts.

In Vivo Recombineering

The *in vivo* recombineering method uses phage λ as a tool for the engineering of other, less well-studied *E. coli* phages (Fig. 3) (70). Briefly, *E. coli* cells carrying a defective λ prophage and the *pL* operon are infected with the phage to be engineered at a multiplicity of infection (MOI) of 1 to 3 and allowed to adsorb for 15 min. The *pL* operon, which is involved in general and site-specific recombination (71), is under the control of a temperature-sensitive repressor. Following phage infection, the λ Red recombination functions are induced by heating the mid-log-phase bacterial culture to 42°C. At this point, the cells are electroporated with the dsDNA or single-stranded DNA (ssDNA) substrate (69, 70). The phage lysate is subsequently recovered and checked for incorporation of the desired DNA (70). The yield of recombinant phages obtained by using this technique is about 0.5 to 2%, which is higher than the yield obtained by homologous recombination but still low, so screening for the mutant phages remains challenging (63, 66, 70). This technique can potentially be adapted to other phages and other bacterial species by introducing the λ Red system via plasmids (without the rest of the λ phage), or another recombination machinery, into host bacteria that can be targeted by the phage to be engineered.

CRISPR-Cas-Mediated Genome Engineering

Clustered regularly interspaced short palindromic repeats (CRISPR) in combination with *cas* (CRISPR-associated) genes form an "adaptive" immune system in bacteria and archaea, protecting microbial cells from invading foreign DNA, such as DNA delivered by invading phage genomes (72, 73). The CRISPR-Cas systems consist of two main components: the Cas proteins, which work as the catalytic core of the system and are responsible for cleaving DNA, and the CRISPR locus, which functions as the genetic memory that directs catalytic activity against foreign DNA (74). CRISPR loci are typically composed of several noncontiguous direct repeats separated by short stretches of variable DNA sequences, called spacers, acquired from extrachromosomal elements (72, 75, 76). CRISPR-Cas systems are currently divided into three major types (I, II, and III) characterized by distinct sets of *cas* genes, with a further division into several subtypes (77, 78). The mode of action of CRISPR-Cas systems comprises three main processes, namely, CRISPR adaptation, RNA biogenesis, and CRISPR-Cas interference, which are further reviewed by Westra et al. (74) and Makarova et al. (77).

Recently, Kiro et al. described a method to enhance the engi-

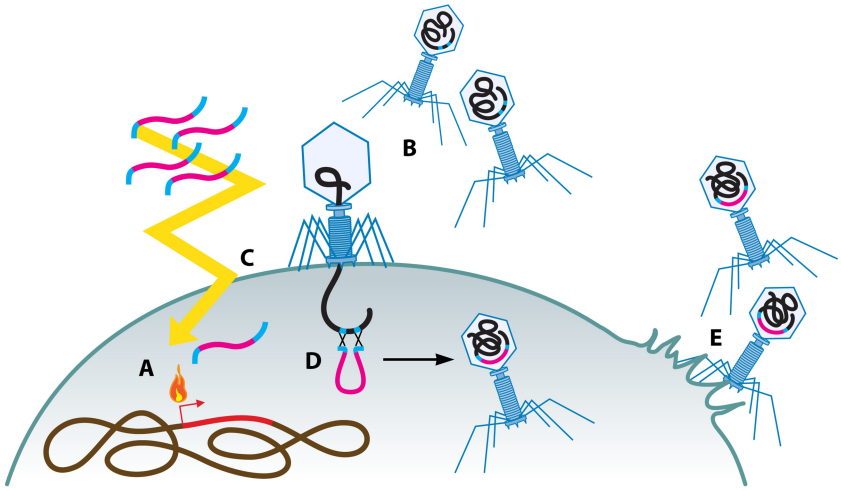

FIG 3 *In vivo* recombineering. Bacterial cells carrying a defective λ prophage and the pL operon under the control of a temperature-sensitive repressor (in red) (A) are infected with the phage to be manipulated (B) and subsequently transformed with dsDNA or ssDNA (C). Recombination then occurs between the phage genome and the dsDNA/ssDNA (homologous loci in blue) (D), after which recombinant phage particles (carrying the DNA fragments in pink) are recovered (E).

neering of the T7 phage genome by using the type I-E CRISPR-Cas system (Fig. 4) (79). Homologous recombination was first used to delete a nonessential T7 gene (gene *1.7*). Specifically, the T7 phage was propagated in a bacterial host harboring a plasmid carrying regions of homology to the upstream and downstream regions of the phage gene *1.7*, such that recombination would delete this gene. The phage population resulting from this reaction contained recombinant phages lacking gene *1.7* as well as nonrecombinant wild-type phages. In order to enrich for the desired recombinant phages, a CRISPR-based counterselection system was used (79). The recombinant phage lysate was plated on host bacteria carrying 3 plasmids encoding the components required for CRISPR-Cas activity: the targeting *cascade* complex, the *cas3* degradation machinery, and the CRISPR spacer targeting gene *1.7*. The result was selective cleavage of the nonrecombinant phage genomes, which contained gene *1.7*, but not of the recombinant phage genomes, which lacked gene *1.7*, thus inhibiting replication of the former phages and enriching for the latter phages. This method thus overcomes the issue of having to fish out a very small percentage of recombinant phages from a large pool of wild-type phages (79). Similarly, the *Streptococcus thermophilus* type II-A CRISPR-Cas system has been used to select for *Streptococcus* phage 2972 recombinants that have undergone point mutations, small and large DNA deletions, and gene replacements (80). It remains to be seen how generalizable this technology will be to other phage families and bacterial species.

Rebuilding/Refactoring Phage Genomes *In Vitro*

Phage genomes can be manipulated and edited *in vitro* before they are introduced into their bacterial hosts. For example, in 2005, Chan et al. redesigned the genome of T7 phage by eliminating overlaps between genetic segments in a process known as refactoring (Fig. 5) (81). These segments, of which there were 73, were grouped into 6 sections. Bracketing restriction sites were added to the DNA sequence of each section so that the DNA could be altered within each section without affecting the other sections. Al-

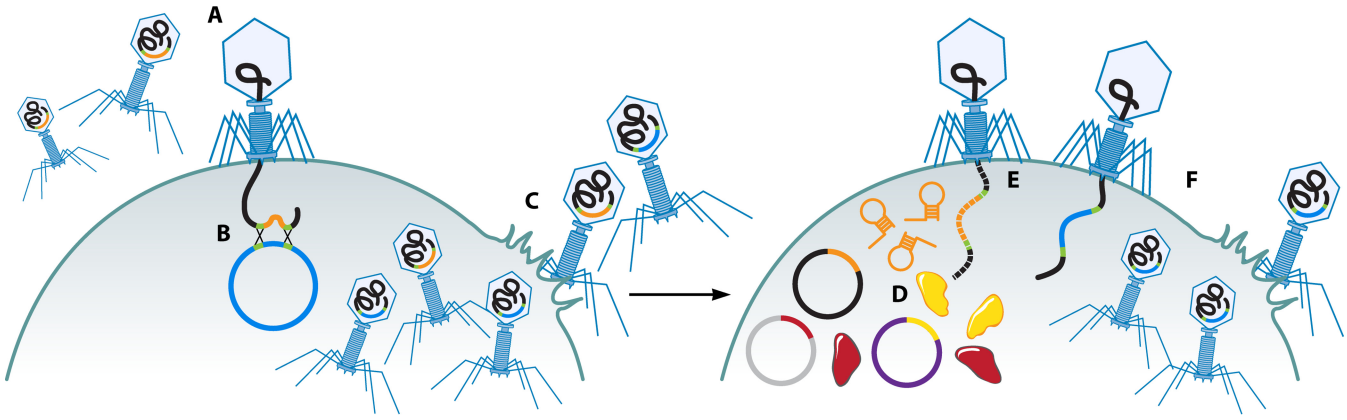

FIG 4 CRISPR-Cas-mediated phage engineering. Upon phage infection, homologous recombination occurs between phage DNA (A) and plasmid DNA (B), such that a phage gene (in orange) is deleted. The resulting phage population is mixed (phages containing fragments in blue or orange) (C), but by using the CRISPR-Cas system (single guide RNA [sgRNA] is shown in orange and Cas proteins in red and yellow, encoded on separate plasmids) to target the gene retained in the wild-type particles (D), it is possible to counterselect the wild-type phage population (fragment in orange in genome) (E) and to retain the recombinant version (phage containing the blue-colored fragment) (F).

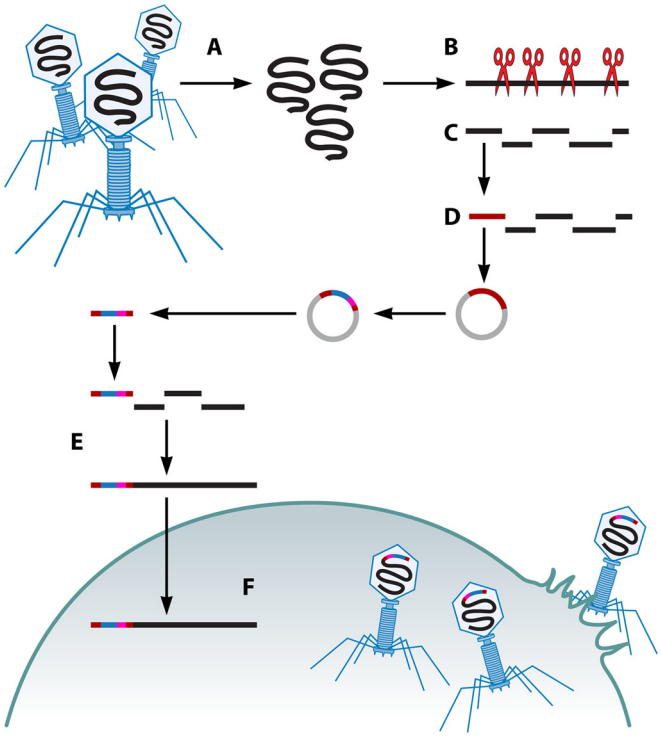

FIG 5 Rebuilding/refactoring of phage genomes *in vitro*. Once the phage DNA has been purified (A), it is digested using native restriction sites (B), and independent pieces (in burgundy) (C) can be subcloned and further manipulated (represented by the DNA fragments in burgundy, blue, and pink) (D). Once released from the vector, the recombinant section is ligated to the rest of the phage genome (E) and electroporated into the phage host for recovery of engineered phage particles (F).

tered genomes were then assembled *in vitro* from these sections by molecular cloning. Chan et al. built three chimeric T7 phage genomes made up of different engineered sections and then generated viable phages out of these refactored genomes by transforming the bacterial host with the engineered phage genomes. However, many of the recombinant phages produced considerably smaller plaques than the wild-type phage, suggesting that fitness was adversely affected by these efforts (81). Bottlenecks associated with this engineering approach include the difficulty of working with large DNA fragments *in vitro* and the need to transform bacteria with the engineered genomes in order to recover viable phages, which can be an inefficient process, especially for nondomesticated bacteria.

Whole-Genome Synthesis and Assembly from Synthetic Oligonucleotides

Complete phage genomes can also be assembled from synthetic oligonucleotides *in vitro*. The entire genome of phage ΦX174 (5,386 bp) has been assembled in this manner (Fig. 6) (82, 83). The synthesized oligonucleotides were gel purified, phosphorylated, annealed, and assembled *in vitro* by polymerase cycling assembly (PCA). The full-length genome was amplified by PCR, digested with a restriction enzyme, gel purified, and circularized by ligation (phage ΦX174 has a closed circular genome). The assembled ΦX174 genome was then electroporated into *E. coli*, followed by plating to check for phage plaques. The synthetic phage DNA showed a lower infectivity than that of the natural phage DNA, a difference that was attributed to PCR-generated mutations (1 per 500 bp) introduced by this method (83). Nonetheless, viable phages were recovered. This approach is likely to be limited to relatively small phage genomes owing to the challenges of manipulating large DNA molecules *in vitro* and the potential introduction of mutations via the PCR-based process. However, *in vitro* DNA synthesis and assembly enable arbitrary genetic alterations to be introduced into phage genomes more easily than the case with recombineering-based approaches (83).

Yeast-Based Assembly of Phage Genomes

Propagating phage genomes in a bacterial host can be toxic for the host, thus limiting the efficiency of phage genome engineering with methods such as homologous recombination, BRED, and *in vivo* recombineering. This issue can be overcome by using *Saccharomyces cerevisiae* rather than bacteria as an intermediate host for genetic manipulation. Homologous recombination is particularly efficient in *S. cerevisiae*, and phage genomes do not cause toxicity

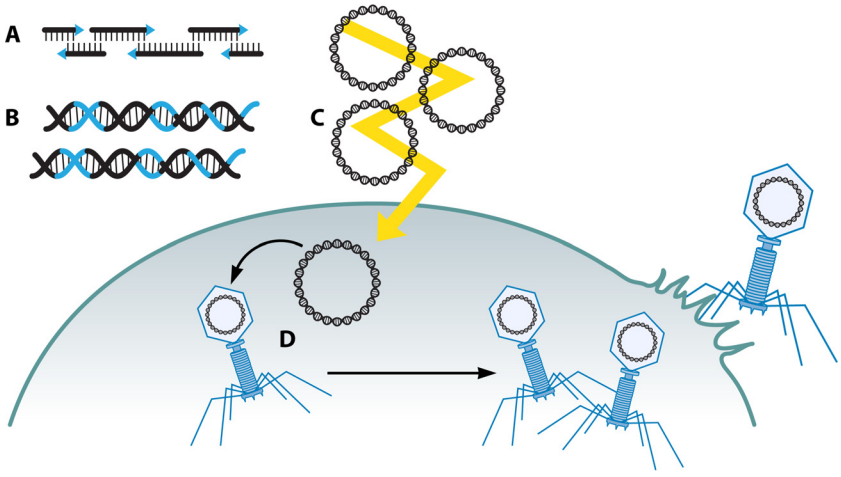

FIG 6 Synthesis and assembly of phage genomes from synthetic oligonucleotides. Synthetic oligonucleotides (A) are annealed and assembled by PCA, followed by ligation (B). *E. coli* is subsequently transformed with the full circular genome molecules (C), and phage particles are recovered (D).

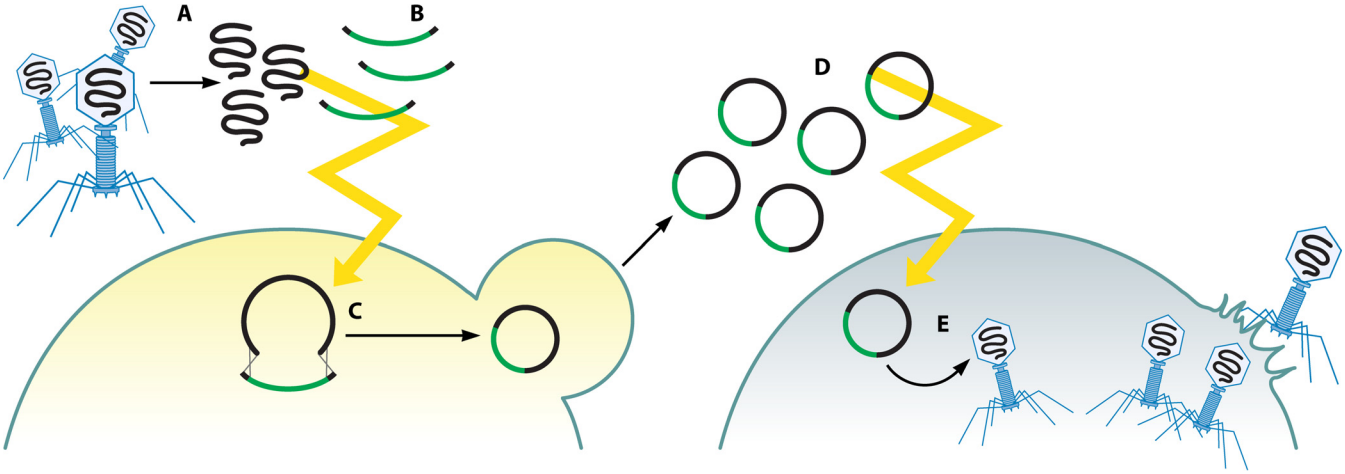

FIG 7 Yeast-based assembly of phage genomes. Purified phage DNA (A) is electroporated into *S. cerevisiae* together with linear YAC molecules with overhangs (in black) homologous to the 5′ and 3′ ends of the linear phage genome (B). Recombination in the yeast cell enables genomic subcloning (YAC backbone in green) (C), which upon YAC purification and electroporation (D) allows the recovery of phage particles (E).

in yeast and can be maintained stably (Fig. 7) (84). With this method, the phage genome is captured in an *S. cerevisiae*-bacterial shuttle vector. The main requirement is that the shuttle vector must contain overhangs homologous to the ends of the phage genome so that the vector and the phage genome can join via recombination. Phage genomes assembled, modified, and propagated in yeast have been isolated and introduced into bacteria to generate functional phage particles (84). This technique has been used to capture and genetically modify the genomes of the coliphages T3 (38,208 bp) and T7 (39,937 bp) (84, 85) as well as the *Klebsiella* phage K11 (41,181 bp) (85). It was further used to capture and archive the genome of fully refactored phage ΦX174 (6,302 bp) (86). This strategy requires the extraction of the phage genome from yeast and its introduction into bacteria, and thus its efficiency is restricted by bacterial transformation efficiencies.

Cell-Free Transcription-Translation Systems

One of the major advantages of using *in vitro* or yeast-based genome modification is that phage genomes can be engineered without causing toxicity to the host. To create functional phage particles from phage genomes modified *in vitro* or in yeast, researchers have generally relied on transformation as the means of getting phage genomic DNA back into the host bacterium, where phage particles are then "booted up" (i.e., viable phage particles are produced from the genomic DNA). However, this process requires high transformation efficiencies, especially for large phage genomes. While highly efficient transformation protocols have been devised for some bacteria (e.g., *E. coli* and *Pseudomonas aeruginosa*), many other bacterial species are extremely difficult to transform. This poses a bottleneck in the throughput and efficiency of *in vitro* and yeast-based systems for phage genetic engineering.

Cell-free transcription-translation systems offer a potential solution to this problem. For example, such systems have been used to replicate, synthesize, and assemble the T7 phage genome (Fig. 8) (87). In this case, as little as 1 nM phage genomic DNA, combined with a TX-TL cell-free system prepared from *E. coli* BL21 Rosetta2, resulted in the assembly of approximately 0.1 to 1 billion infectious T7 phage particles/ml of reaction mixture within a few hours of incubation. This method has also been used to boot up

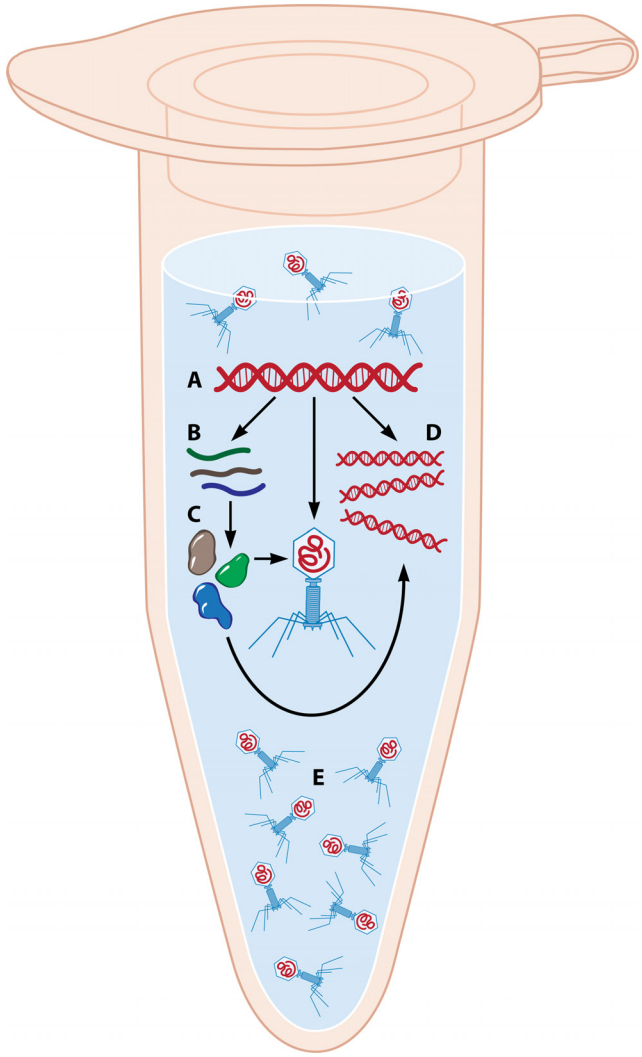

FIG 8 Cell-free systems for assembly of recombinant phage particles. Purified phage genome DNA is combined with cell-free expression systems (A) that enable gene transcription (B), translation (C), DNA replication (D), and assembly of whole phage particles (E).

phage ΦX174 to create approximately 1 million particles/ml of reaction mixture (87). In future work, it will be important to test this strategy for a wider range of phage genomes to see whether it is universally applicable.

PHAGE ENGINEERING FOR PATHOGEN CONTROL

Natural Phage-Based Antimicrobials

The potential of natural lytic phages to act as antimicrobial agents against targets that include multidrug-resistant bacteria has been studied extensively both *in vitro* (88–91) and *in vivo* (92–98). Natural phages have been used therapeutically since 1919, when d'Hérelle successfully treated children suffering from severe dysentery with phages (9). Other studies were later conducted on the use of phages in clinical practice, such as for the treatment of surgical infections and suppurative lesions (3, 99, 100). Even after the discovery of antibiotics, phage therapy continued to be used in the former Soviet Union and Eastern Europe (7, 9). Although many studies have reported on the potential and safety of phage therapy to treat patients with bacterial infections (23, 92, 94, 101–104), the widespread use of phages in Western medicine is currently awaiting approval. In addition to the requirement for regulatory approval, other obstacles stand in the way of bringing phages into the clinical setting, including the development of bacterial resistance to phages, the narrow host ranges of phages, and concerns over the immunogenicity of phage therapy (44).

Bacteria can quickly evolve to counter phage infection. Just as bacterial exposure to antibiotics favors the emergence of antibiotic-resistant bacteria (105, 106), resistance to phages may appear spontaneously within a few hours post-phage treatment *in vitro* (91, 107–109). Bacteria can avoid being infected by phages in several ways (42), as follows. (i) Adsorption-blocking mechanisms prevent phages from binding to cellular receptors on bacterial cell surfaces. Adsorption can be blocked by the loss or change of bacterial phage receptors; by physical barriers, such as the EPS matrix, which hides phage receptor molecules; or by the production of molecules that bind to receptors, thus making them inaccessible to phages. (ii) Bacteria can impede the entry of phage DNA by superinfection exclusion systems. (iii) Bacteria can cleave phage nucleic acids by restriction-modification systems or CRISPR-Cas systems, which protect bacterial cells from invading foreign DNA. (iv) Abortive infection systems can lead to host cell death (38, 42). Phages, in turn, are capable of countering these resistance mechanisms, in part because of their genomic plasticity and fast replication (43). Phage diversity is generated by point mutations, genome rearrangements, and the exchange of genetic material with other phage particles or bacteria (43). The ability to engineer, mutagenize, and screen for new phages in high-throughput fashion may pave the way for the rapid creation of modified phages that can overcome bacterial resistance mechanisms. This is in contrast to chemical antimicrobials, for which it can be difficult to discover novel agents that can be applied against bacteria that have evolved antibiotic resistance.

There are indications that medical applications of phage-based products may be gaining greater acceptance. PhagoBurn, a clinical trial funded by the European Commission, aims to evaluate the efficacy of a topical application of a well-defined phage cocktail for the treatment of *E. coli* and *P. aeruginosa* infections in burn patients (110). PhagoBurn is setting a precedent for future therapies that may one day involve engineered phages. Although the initial small trial (9 patients) did not allow for an adequate evaluation of the efficacy of the phage cocktail used for treatment, no adverse effects or clinical abnormalities related to its application were observed (110). Similarly, no adverse effects were observed in a safety study described by Bruttin and Brüssow, in which 15 healthy human volunteers received the *E. coli* T4 phage orally (103). These clinical studies have used or are using natural phages, but the regulatory framework being laid down may help to facilitate future clinical development plans for engineered phages.

In addition, a number of studies have also focused on applying natural phages to agriculture (28, 29), food safety (34, 36, 37), or veterinary medicine (96, 111, 112). For example, several natural phage-based products have received regulatory approval for treating food products. ListShield, EcoShield, and SalmoFresh from Intralytix control the respective foodborne bacterial pathogens *Listeria monocytogenes*, *E. coli* O157:H7, and *S. enterica* in foods or food-processing environments. Salmonelex and Listex P100 from Micreos reduce contamination with *Salmonella* and *L. monocytogenes*, respectively, during food processing. AgriPhage from OmniLytics controls *Xanthomonas campestris* and *Pseudomonas syringae* on tomato and pepper plants.

Modifying Phages for Enhanced Antibacterial Activity

In addition to having direct antimicrobial activity, phages can be engineered for use in conjunction with other antimicrobial strategies. For example, phages can be modified to enhance the bactericidal activity of antibiotics (113). Lu and Collins modified the lysogenic phage M13mp18 to overexpress *lexA3*, a repressor of the SOS DNA repair system, to enhance antibiotic-induced killing of *E. coli* (113). The *in vitro* administration of this *lexA3*-producing phage together with a quinolone antibiotic (ofloxacin) significantly improved the bactericidal activity against wild-type *E. coli* EMG2, by as much as 2.7 (compared with ofloxacin plus wild-type phage) and 4.5 (compared with ofloxacin alone) orders of magnitude. The engineered phage also improved the bactericidal activity of other antibiotics besides quinolones, such as the aminoglycoside gentamicin and the β-lactam ampicillin. Enhanced bactericidal activity was observed as early as 6 h posttreatment. The engineered phage also increased the antibiotic-based killing of bacteria that had already acquired resistance to these antibiotics, as well as the killing of persister and biofilm cells, and it reduced the emergence of antibiotic-resistant mutants. This strategy was also effective in an *in vivo* mouse model, with an 80% survival rate for *E. coli*-infected mice that received the engineered phage plus ofloxacin (versus 50% for treatment with unmodified phage plus ofloxacin and 20% for treatment with ofloxacin alone) (113).

In another strategy to decrease the development of bacterial resistance to antibiotics, Edgar et al. engineered temperate phages to deliver genes encoding sensitivity to antibiotics into bacteria (114). More precisely, the dominant genes *rpsL* and *gyrA*, which confer sensitivity to streptomycin (an aminoglycoside) and nalidixic acid (a quinolone), respectively, were inserted into phage λ by homologous recombination. The authors generated resistant mutants by exposing *E. coli* K-12 to these antibiotics. Antibiotic-resistant strains were then lysogenized with the engineered phages (carrying *rpsL* or *gyrA*), and MICs were evaluated. After lysogenization with phage, the bacterial susceptibility to both antibiotics was restored: MICs decreased 8- and 2-fold for bacteria resistant to streptomycin and nalidixic acid, respectively (114).

In addition to enhancing antibiotic activity, phages are being

engineered as antimicrobial agents that are more effective than the corresponding natural phages to fight bacterial infections. Some bacteria, such as *Chlamydia trachomatis*, the most common cause of sexually transmitted infections, are obligate intracellular pathogens and therefore are inaccessible to phages added to the extracellular milieu (115, 116). To inhibit *C. trachomatis* infection, a phage was engineered to be endocytosed by eukaryotic cells. This was achieved by engineering the M13 phage to express two functional peptides: an integrin-binding peptide (RGD), expressed on the p8 major coat protein, and a peptide (PmpD) from a *C. trachomatis* protein, expressed on the p3 minor coat protein of the phage (115). RGD induced integrin-mediated endocytosis, which facilitates internalization into eukaryotic cells. On the other hand, the PmpD peptide interrupted *C. trachomatis* infection and propagation. The engineered phage was used to pretreat HeLa and primary endocervical cells or was added simultaneously with the bacterium. Fluorescence microscopy and measurement of inclusion-forming units showed a significant inhibition of *C. trachomatis* infection in both cell lines, although the effect of the phage was more pronounced when it was applied at the same time as the bacterium in primary endocervical cells (115).

Westwater et al. described the use of engineered phages as lethal-agent delivery systems (117). The lethal genes *gef* and *chpBK* were amplified from *E. coli* XL1-Blue MRF′ and cloned under the control of a LacI/IPTG (isopropyl-β-D-thiogalactopyranoside)-regulated promoter into a vector containing the intergenic region of phage f1. *E. coli* cells carrying this phagemid were then infected with the M13 helper phage R408, allowing for the preferential packaging of phagemid DNA over helper phage DNA: 95% of the resulting lysates comprised lethal-agent phagemid particles. The lysates were then used to infect *E. coli* cells, which were incubated in the presence of IPTG. After overnight incubation, viable cell counts were reduced 948-fold by the Gef-expressing phagemid and 1,579-fold by the ChpBK-expressing phagemid (117). The phagemids delivering the lethal agents also reduced bacterial titers in mice more than 90% 5 h after intraperitoneal injection (117).

Biofilms, which are commonly associated with persistent and chronic bacterial infections, are structured microbial communities with reduced metabolic activity, especially in the inner layers. Biofilms can be less susceptible than planktonic bacteria to antimicrobial agents, including phages and antibiotics (118). This effect is often attributed to biofilm matrices, which can limit the diffusion of molecules and particles, or to slowed bacterial metabolism (41, 119, 120). To overcome the reduced efficacy of phages against biofilms, Lu and Collins engineered a T7 phage to express the biofilm-degrading enzyme dispersin B (DspB) during phage infection (121). Specifically, the *dspB* gene from *Actinobacillus actinomycetemcomitans* was cloned downstream of the T7select415-1 *10B* capsid gene under the control of the T7 φ10 promoter. This engineered phage was efficient against *E. coli* TG1 biofilms, reducing biofilm cell counts by ~4.5 orders of magnitude after 24 h of treatment, a reduction that was ~2 orders of magnitude more than that caused by the wild-type nonenzymatic phage (121). In future work, this technology could be expanded to encompass new enzymes that can target the heterogeneous extracellular composition of biofilms to achieve more efficient biofilm destruction, since biofilms can be made up of many different bacteria producing a range of matrix components.

T7 phage has also been engineered to encode an enzyme that interferes with quorum sensing (122), a bacterial cell-cell communication process involved in biofilm formation (123, 124). This engineered phage (T7aiiA) was built by cloning the acyl-homoserine lactonase (AHL-lactonase) gene *aiiA* from *Bacillus anthracis* into the T7select415-1 phage vector (121, 122). This quorum-quenching enzyme inactivates acyl-homoserine lactone (AHL), a quorum-sensing molecule, by hydrolyzing its lactone bonds (125). In order to evaluate the effect that the quorum-quenching phage T7aiiA had on biofilm formation, *E. coli* and *P. aeruginosa* were mixed together and allowed to form biofilms in the presence of engineered or wild-type phage for 4 and 8 h (122). Phage T7aiiA reduced the biomass by 74.9% and 65.9% at 4 and 8 h, respectively, whereas control T7 phage caused only 23.8% and 31.7% reductions, respectively, in comparison to the control (no phage) (122).

Engineered Phages with Shifted or Broadened Host Ranges

Any individual phage typically infects a very limited range of bacterial strains within a given species. This specificity means that phages can act as precision antimicrobial agents, but it can also pose a major hurdle for phage therapy, as it is important to know whether a given bacterial target is susceptible to a particular type of phage prior to treatment. A combination of phages with different host ranges in a single cocktail is currently the most common approach taken to achieve a wider target spectrum, because a single therapeutic mixture can be applied to a broader range of bacterial infections than a single phage alone. Although this strategy is promising (94, 126–128), it remains difficult to target all bacterial strains in a given species, and the diverse assortment of phages per cocktail may unintentionally target bacteria outside the desired range. This is a challenge with both natural and current-generation engineered phages. Assembly of phage cocktails may also require optimization of phage proportions within the cocktails to improve performance (129, 130), which can be achieved based on experimental design methodologies. An alternative to using cocktails composed of a large and diverse group of phages, which can pose challenges for manufacturing, characterization, and engineering, may be to assemble a more uniform set of phages based on common scaffolds and host ranges that are shifted, expanded, or both.

To demonstrate that it is possible to switch or extend the host ranges of phages, several studies have exploited the fact that host range is linked to tail fiber composition for some phages. Yoichi et al. (131) genetically modified a T2 phage by swapping the long tail fiber genes (*gp37* and *gp38*) with those from phage PP01, which specifically targets *E. coli* O157:H7. The exchange was done by homologous recombination between the genome of phage T2 and a plasmid carrying two regions of homology, flanking the *gp37* and *gp38* genes of PP01. The recombinant phage T2ppD1, carrying the PP01 genes *gp37* and *gp38*, had the same host range as phage PP01 but had lost the capacity to infect the original host of phage T2, *E. coli* K-12 (131). An identical approach was used by Mahichi et al. to expand the host range of phage T2. Because phage IP008 has a broader host range than that of phage T2 (infecting 33% versus 7%, respectively, of environmental *E. coli* isolates), the tail fiber genes *gp37* and *gp38* from phage T2 were exchanged, by recombination, with their homologous counterparts in phage IP008, resulting in an engineered T2 phage with a host range identical to that of IP008 (61).

A hybrid T3 and T7 phage (T3/7) was also devised in which part of the tail fiber gene of T3 (*gp17*) was replaced with that of phage

TABLE 1 Synthetic phages engineered by Ando et al., with respective genotypes and phenotypes[a]

Phage	Genotype[b]	Phenotype
$T7_{T3(C\text{-}gp17)}$	T7WT gene *17* (1–447)-T3 gene *17* (448–1677)	$>10^8$-fold-reduced efficiencies of plating on *E. coli* BW25113 and *E. coli* MG1655
$T7_{T3(gp17)}$	T7WT Δgene *17*, T3 gene *17*	$>10^8$-fold-reduced efficiencies of plating on *E. coli* BW25113 and *E. coli* MG1655
$T7_{13a(C\text{-}gp17)}$	T7WT gene *17* (1–450)-13a gene *17* (451–1677)	Forms plaques on *E. coli* ECOR16; infects *E. coli* BW25113 and MG1655
$T7_{13a(gp17)}$	T7WT Δgene *17*, 13a gene *17*	Forms plaques on *E. coli* ECOR16; does not infect *E. coli* BW25113 and MG1655
$T3_{T7(C\text{-}gp17)}$	T3WT gene *17* (1–447)-T7 gene *17* (448–1662)	Efficiencies of plating similar to those of T7 phage on *E. coli* BW25113 and *E. coli* MG1655
$T3_{T7(gp17)}$	T3WT Δgene *17*, T7 gene *17*	Efficiencies of plating similar to those of T7 phage on *E. coli* BW25113 and *E. coli* MG1655
$T3_{R(gp17)}$	T3WT Δgene *17*, R gene *17*	Infects *Yersinia pseudotuberculosis* IP2666 and YPIII as well as *E. coli* BL21
$T7_{K11(gp11\text{-}12\text{-}17)}$	T7WT Δgenes *11-12-17*, K11 genes *11-12-17*	Infects *Klebsiella* sp. 390 but not *E. coli*
$K11_{T7(gp11\text{-}12\text{-}17)}$	K11WT Δgenes *11-12-17*, T7 genes *11-12-17*	Infects *E. coli* but not *Klebsiella* spp.

[a] Based on data from reference 85.
[b] WT, wild type.

T7. The T3/7 recombinant phage exhibited a broader host range and a better adsorption efficiency than those of either of the wild-type phages, i.e., T3 and T7 (132). Le et al. demonstrated that host specificity in *P. aeruginosa* phages is also tail fiber dependent (60). They first isolated a spontaneous mutant phage that exhibited a broader host range than that of the parental phage, JG004. Analysis of the sequence encoding the putative tail fiber gene (ORF84) in the spontaneous mutant revealed a single point mutation in this gene. The authors replaced the homologous gene (ORF69) in phage PaP1 with the tail fiber gene (ORF84) from phage JG004 by homologous recombination, which resulted in a chimeric phage capable of inducing plaque formation in the host strain of phage JG004 (*P. aeruginosa* PAO1) but not in the host strain of wild-type phage PaP1 (*P. aeruginosa* PA1) (60).

Marzari et al. increased the host range of the filamentous coliphage fd by adding a receptor-binding domain from the filamentous phage IKe, which encodes a receptor specific for N-pili, to the N terminus of the fd gene 3 protein (*g3p*) (133). The chimeric fd phage was able to infect *E. coli* strains bearing N-pili (133). The filamentous coliphage fd, which normally infects *E. coli*, was also engineered to recognize *Vibrio cholerae* (134). This was achieved by fusing the minor coat gene *pIII* from fd with a sequence of the *orfU* gene encoding the N-terminal 274 amino acids of a putative minor coat protein gene from CTXΦ, another filamentous phage (134). The recombinant phage fd-$pIII^{CTX}$ was able to infect *V. cholerae*, but its ability to infect *E. coli* was not affected (134).

Using a yeast-based platform, Ando et al. engineered phage genomes to modulate their host ranges (85). Since *E. coli* phages T7 and T3 share high homology with each other, the authors exchanged the tail fiber gene *17*, or fragments thereof, with its counterpart from the other phage, as indicated in Table 1. The authors also demonstrated that gene swapping between more distant phage relatives could enable a genetically modified *E. coli* phage to target *Klebsiella* bacteria and a genetically modified *Klebsiella* phage to target *E. coli* bacteria (Table 1). This work further showed that synthetic phage cocktails composed of phages with the same scaffold but different tail components can be used to target mixed bacterial populations and to selectively remove specific bacterial species from them (85).

Although these strategies have modulated the host ranges of several types of phage, the systematic, efficient, and high-throughput construction of phages with desired host ranges has yet to be achieved. We envision that improved technologies for phage genome engineering, DNA synthesis, and DNA sequencing will make it possible to establish such platforms and facilitate the construction of well-defined phage cocktails with tunable host ranges. In the future, host range determinants may be mined from sequence databases, synthesized, and swapped with portions of well-characterized phage scaffolds to potentially identify novel phages with desired target spectra.

Engineered Phages with Reduced Impacts on Mammalian Systems

One of the concerns associated with the use of phages in the treatment of bacterial infections is the capacity of the human immune system to neutralize them due to their immunogenicity (39). Hodyra-Stefaniak et al. demonstrated that in murine models of the systemic inflammatory response, there is a decrease in the availability of active phages in circulation and in numerous tissues due to the action of phagocytes, antibodies, and the serum complement system (135). To avoid the problem of phage elimination by the host defense system, particularly by the reticuloendothelial system (RES), Merril et al. adopted a serial passage technique (136). This technique consisted of serial injections of phages into mice to search for phage mutants capable of remaining in the circulatory system for longer times (136). Using this method, the authors isolated "long-circulating" mutants of the *E. coli* phage λ. The two λ variants, isolated after passaging phage λ through 10 selection cycles, had 16,000- and 13,000-fold higher capacities to evade RES clearance 24 h after intraperitoneal administration than that of the parental λ phage. Compared to the parental λ phage, both variant phages contained identical mutations in the major capsid protein E, consisting of the replacement of a glutamic acid with a lysine residue, and one of them had an additional mutation in the capsid D protein (136). This technique was also used to isolate long-circulating mutants of *S. enterica* serovar Typhimurium phage P22, suggesting that the method can be generalized to obtain other phages capable of evading the RES (136).

In addition, treatment with lytic phages can cause massive bacterial lysis, and the subsequent release of bacterial components and toxins may trigger an immune response (9, 22). In order to circumvent this problem, phages have been engineered as nonrep-

licative or lysis-deficient mutants. For example, to minimize endotoxin release, Hagens and Bläsi engineered the lysogenic phage M13 in two ways to express lethal but nonlytic proteins (137). The engineered phage M13R encodes the restriction enzyme BglII, which induces double-strand breaks in the bacterial chromosomal DNA. The engineered phage M13S105 encodes a holin from phage λ (S105), which causes cytoplasmic membrane lesions. Each of these engineered phages reduced *E. coli* cell counts by ca. 2 orders of magnitude in just 2 h (137). The endotoxin levels present in the supernatants 4 h after *E. coli* infection with phages M13R and M13S105 increased only 7- and 6-fold, respectively, which was much lower than the 27-fold increase observed for λ*c*I$^-$ phage, a lytic lambda phage used as a control (137). However, regrowth of the bacterial cells was observed 120 to 300 min after infection, suggesting the emergence of phage-resistant *E. coli* mutants (137).

The same authors genetically modified the *P. aeruginosa* filamentous phage Pf3 to become a nonlytic, nonreplicative lethal variant (Pf3R) by replacing an export protein gene in the phage genome with the BglII endonuclease gene (40). *In vitro* studies showed that Pf3R was lethal to *P. aeruginosa* PAO1, reducing the number of CFU by 99% after 90 min of infection, while endotoxin release was minimal. To evaluate its *in vivo* therapeutic efficacy, the engineered phage was administered to mice 45 min after *P. aeruginosa* infection (40). Treatment with Pf3R resulted in about 75% survival of mice, while the Pf3-treated and untreated mice died. The levels of inflammatory markers, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), were almost twice as high after phage infection of mice with the original lytic phage (Pf3) as those after phage infection of mice with the recombinant phage, Pf3R (40). These results demonstrated that the engineered Pf3R phage could effectively treat *P. aeruginosa* infections in mice, while endotoxin release was kept to a low level (40).

Similarly, Matsuda et al. (138) showed that treating *E. coli* peritonitis in mice with the lysis-deficient phage T4*LyD* significantly increased the survival rate (81% survival at 48 h) compared to that of mice treated with the wild-type phage (52% survival), mice treated with the β-lactam moxalactam sodium (33% survival), or control untreated mice, which died within 20 h (138). At the same time, T4*LyD*-treated mice had lower endotoxin and cytokine (TNF-α and IL-6) levels 12 h after infection than the other groups, indicating that the systemic immunological side effects of phage therapy had been attenuated (138). Similar to the results obtained with M13S105 and Pf3R, the effectiveness of T4*LyD* suggests that converting lytic to nonlytic phages is a fruitful strategy for reducing their immunological effects.

In another report of changing a lytic phage to a nonlytic phage, a *Staphylococcus aureus* temperate phage, P954, was modified by homologous recombination to inactivate the gene coding for the endolysin responsible for bacterial cell lysis (139). *In vitro* studies showed that the endolysin-deficient P954 phage had as much bactericidal activity as the wild-type phage, and *in vivo* studies revealed that administration of the engineered phage fully rescued mice from fatal methicillin-resistant *S. aureus* (MRSA) infection, though the immune response was not characterized (139).

The aforementioned approaches were undertaken independently to lower the immunogenicity of phage therapy by finding mutations that reduce phage clearance by the RES and by inhibiting massive bacterial lysis. It remains to be determined whether these mechanisms can be integrated into a single phage to achieve high efficacy at treating infections while simultaneously reducing immune responses.

Engineering Phages To Create DNA Sequence-Specific Antimicrobials

One of the major problems of antibiotics is their broad spectrum of activity, which leads to the killing not only of targeted pathogens but also of nontargeted commensal bacteria (140). This off-target activity can alter the microbiome and result in antibiotic-associated infections, such as those caused by *Clostridium difficile* (11, 140). Phages can be engineered to kill bacteria based on their genetic signatures, resulting in much more precisely aimed antimicrobial activity. Building on CRISPR-Cas technology, Citorik et al. and Bikard et al. developed antimicrobials whose spectrum of activity can be programmed against specific DNA sequences, enabling the killing of only those bacteria carrying targeted DNA, such as that encoding antibiotic resistance or virulence (141, 142). This approach, based on the CRISPR-Cas system, consists of delivering RNA-guided nucleases (RGNs) on conjugative vectors or phagemids into bacterial cells, where they target specific DNA sequences for cleavage. Once introduced into bacterial cells, the RGNs seek out a specific genetic sequence, where they induce a double-strand break, leading to cell death or plasmid loss. In the absence of the target sequence, the RGNs have no effect on bacterial viability (141). Thus, this approach enables selective pressure to be exerted against bacteria at the level of genes. These authors designed RGN constructs to target $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-18}}$, encoding extended-spectrum resistance and pan-resistance to β-lactam antibiotics, respectively. The *in vitro* treatment of *E. coli* EMG2 carrying the resistance plasmid pNDM-1 ($bla_{\text{NDM-1}}$) or pSHV-18 ($bla_{\text{SHV-18}}$) with the respective phagemid-packaged RGN (ΦRGN) at an MOI of 20 reduced the number of viable cells by 2 to 3 orders of magnitude, with no significant reduction of counts for cells lacking the target sequences. Furthermore, the authors tested the system against a quinolone-resistant strain of *E. coli* in which quinolone resistance was due to a single-nucleotide mutation in DNA gyrase (*gyrA*). The ΦRGN targeted against $gyrA_{\text{D87G}}$ was cytotoxic for the *E. coli* strain harboring the chromosomal mutation but not for the otherwise isogenic parental strain. This system was further tested *in vivo*, in a *Galleria mellonella* infection model, to determine whether it could target a virulence factor of enterohemorrhagic *E. coli* O157:H7 (EHEC) chromosomally encoded by the *eae* gene. *G. mellonella* larvae were infected with EHEC, and a ΦRGN construct targeting *eae* (ΦRGN*eae*) was administered at an MOI of 30. The survival of ΦRGN*eae*-treated *G. mellonella* was significantly higher than that of controls (buffer treatment only or treatment with a ΦRGN construct targeting an absent DNA sequence) (141).

The same approach was used by Bikard et al. to selectively kill antibiotic-resistant and virulent *S. aureus* strains (142). Using the CRISPR-Cas system, they first created a phagemid to target the *aph-3* kanamycin resistance gene in *S. aureus*. After treatment with a staphylococcal phage (ΦNM1) carrying this phagemid at an MOI of 20, the number of viable kanamycin-resistant *S. aureus* CFU was reduced by 4 orders of magnitude, while nontargeted cells remained unaffected (142). *In vivo* experiments were also performed with a mouse skin colonization model. After 24 h of treatment with the CRISPR-Cas9 phagemid directed against *aph*, the population of kanamycin-resistant *S. aureus* declined from

50% to 11.4% (142). The same method was also effective against an *S. aureus* clinical isolate. In that case, the *mecA* and *sek* genes, encoding methicillin resistance and an enterotoxin, respectively, were targeted by the phagemid (142).

Sequence-specific nucleases delivered by phages or phage-derived particles thus have the potential to be developed as novel antimicrobial agents effective against emerging and well-established virulent and antibiotic-resistant pathogens. The major hurdle for the future use of this technology is to achieve efficient delivery of RGNs into a broad range of target bacteria. The continued development of phage-based gene delivery vectors with tunable host ranges may be able to address this challenge.

PHAGE-DERIVED ANTIMICROBIALS

In addition to harnessing phages for their antimicrobial (lytic) properties or as delivery vehicles for antimicrobial agents, phage proteins can be used on their own as direct antimicrobials (143–145). Once phages have infected bacteria and undergone replication, phage-encoded endolysins degrade the peptidoglycan of the bacterial cell wall from within the cell. Endolysins thus come into play at the terminal stage of the phage replication cycle, causing host cell lysis (146). Endolysins can be effective when applied to the outside of the bacterial cell, even though they are naturally active from the inside. Experimentally, these enzymes have been expressed, purified, and used mostly against Gram-positive bacteria, which are more susceptible to lysis than Gram-negative bacteria because they lack an outer membrane (147, 148). Endolysins added externally to Gram-positive bacteria can result in rapid lysis (147, 149). Furthermore, they have successfully prevented or treated infections caused by Gram-positive bacteria in animal models (150–155).

Phage endolysins have also been engineered. For example, one study reported the construction of four chimeric phage endolysins (Cpl-711, Cpl-771, Cpl-117, and Cpl-177) by shuffling and combining the structural elements (catalytic domain, linker, and cell wall-binding domain) of two pneumococcal phage endolysins, Cpl-1 and Cpl-7S (a synthetic variant of Cpl-7 with improved bactericidal activity) (156). The bactericidal activity of the new chimeric endolysins against *Streptococcus pneumoniae* was evaluated, and Cpl-711 was found to be the most efficient chimera. This chimera was composed of the catalytic domain of Cpl-7S and the linker and cell wall-binding domain of Cpl-1. At a concentration of 0.01 μg/ml, Cpl-711 reduced the number of *S. pneumoniae* cells by 2 orders of magnitude after 1 h of treatment, whereas Cpl-1 reduced cell viability by only 15%. At 1 μg/ml, the chimeric enzyme Cpl-711 reduced the number of viable cells in pneumococcal biofilms by 4 orders of magnitude after a 2-h treatment, which was an improvement over the approximately 1.5 orders of magnitude of killing by the parental proteins, Cpl-1 and Cpl-7S (156). In *in vivo* assays, mice infected intraperitoneally with an *S. pneumoniae* suspension and treated 1 h after bacterial challenge with Cpl-711 had about 50% greater survival than those treated with Cpl-1 (156). This study demonstrated the flexibility with which new and improved phage endolysins can be engineered. Because phage endolysins are diverse, engineering them may provide a pipeline of novel antimicrobial agents. In fact, similar work has been performed on phage endolysins isolated from *Listeria* spp. (157), *Streptococcus* spp. (158, 159), and *Staphylococcus* spp. (158–161).

Gram-negative bacteria are difficult to lyse because the outer membrane blocks access of the endolysin to the peptidoglycan. Endolysin-mediated lysis of Gram-negative bacteria has been achieved, however, through the use of permeabilizing agents (162). There are two classes of outer membrane permeabilizers: (i) polycationic agents, such as polymyxin and its derivatives, which interact with phospholipids in the cell membrane (163, 164), or lysine polymers, which adsorb to the cell surface and block growth (164, 165); and (ii) chelators, such as EDTA, which remove ions from the outer membrane, leading to its disintegration (163, 164), or weak organic acids, which penetrate the cell wall and interfere with bacterial physiology (163, 164). Nonetheless, it is important to highlight that the *in vivo* toxicity of the outer membrane permeabilizers might limit the applicability of this approach. For example, both EDTA and citric acid were found to have cytotoxic effects on macrophages *ex vivo* (166).

Briers et al. combined endolysin EL188 from *P. aeruginosa* phage EL with outer membrane permeabilizers and evaluated the antibacterial activity against *P. aeruginosa* strains. The permeabilizers tested were polymyxin B, poly-L-lysine, EDTA, and citric acid (164). *In vitro* antibacterial assays revealed that EDTA was the best permeabilizer: the combination of the endolysin and EDTA reduced the number of *P. aeruginosa* PAO1 cells in mid-log phase by more than 4 orders of magnitude in just 30 min (164). Similarly, another study in which EDTA was used as a permeabilizer reported reductions of up to approximately 3 orders of magnitude of *P. aeruginosa* PAO1 cell counts after 30 min of incubation with globular endolysins encoded by phages infecting Gram-negative bacteria (162). Oliveira et al. reported the lethality of a *Salmonella* phage endolysin (Lys68) combined with organic acids against Gram-negative bacteria (167). The best results were achieved against *Pseudomonas* cultures: reductions of *Pseudomonas aeruginosa* cells of approximately 2.4, 1.5, and 3.3 orders of magnitude and reductions of *Pseudomonas fluorescens* cells of approximately 1.6, 1.4, and 5.4 orders of magnitude were observed 30 min after applying Lys68 in combination with EDTA, citric acid, and malic acid, respectively (167). Determining the best combination of permeabilizer and lysin for a given target bacterium currently appears to be performed empirically.

To circumvent the problem of outer membrane permeability, Briers et al. engineered endolysins to contain LPS-destabilizing peptides. The resulting endolysins, called Artilysins, penetrate the bacterial outer membrane, which is something that the original endolysins are not capable of doing (168). With this approach, the LPS ion-based membrane stabilization is disrupted by the physicochemical properties of the synthetic peptides coupled to the endolysins, enhancing their killing effect (168). Thus, the fusion of a polycationic nonapeptide (PCNP) to the OBPgp279 endolysin (from *P. fluorescens* phage OBP) enhanced the bactericidal activity of the native endolysin against *P. aeruginosa* PAO1 from 1.10 to 2.61 orders of magnitude of reduction, even in the absence of permeabilizers. Although the PCNP-fused endolysin was found to be effective without permeabilizers, its activity was enhanced by EDTA: viable cell counts were reduced by 5.38 orders of magnitude and 4.27 orders of magnitude for *P. aeruginosa* PAO1 and multidrug-resistant *P. aeruginosa* Br667, respectively, within 30 min (168).

Lood et al. built a genomic library based on prophages induced from the Gram-negative organism *Acinetobacter baumannii* to screen for genes encoding antibacterial endolysins (169). They identified and isolated several endolysins active against *A. baumannii*. *In vitro* studies showed that phage lysin PlyF307, the one

with the greatest activity, reduced exponentially growing cultures of *A. baumannii* clinical isolates by >5 orders of magnitude within 2 h. *In vitro* treatment of *A. baumannii* biofilms with PlyF307 for 2 h reduced the number of cells by 1.6 orders of magnitude. Furthermore, the lysin also rescued mice from lethal *A. baumannii* bacteremia: while 90% of the buffer-treated mice died within 2 days, 50% of PlyF307-treated mice survived the lethal dose of *A. baumannii*. This was the first study to use native endolysins without additional factors for the treatment of Gram-negative infections in mice (169).

PHAGE ENGINEERING FOR BACTERIAL DETECTION AND DIAGNOSTICS

Most of the methods used to detect and identify bacterial pathogens in food, hospital, and industrial environments are time-consuming, in part because they require enrichment steps for increased sensitivity and/or specificity (170, 171). Traditional plating techniques not only are laborious but also often fail to detect pathogens present in samples at low levels (170–172). Other methods, such as antibody-based ones, do not usually perform well for complex samples without enrichment to amplify the bacterial targets (173). Techniques such as PCR or hybridization-based assays can be very sensitive but are not able to discriminate between live and dead cells without bacterial enrichment, and they also require the careful design of primers to avoid off-target hits and the misidentification of species (171, 173). Recent advances in genetic engineering and synthetic biology, particularly the development of phage-based tools for pathogen detection, have made it possible to overcome such limitations.

Loessner et al. described a rapid, easy, and sensitive method for using engineered phage to detect *Listeria monocytogenes* in contaminated food (59). This method consisted of inserting, by homologous recombination, a *Vibrio harveyi luxA* and *luxB* gene fusion (*luxAB*) downstream of the major capsid protein gene of *Listeria* phage A511 (59). Upon infection of the targeted bacteria, this engineered phage generated light. Detectable luminescence was generated rapidly, within 2 h of application, even on food contaminated with as few as 5×10^2 *L. monocytogenes* cells/ml (30). When an enrichment step was included, levels of <1 CFU/g could be detected by use of the engineered phage (59). Sarkis et al. described a similar method for detecting live mycobacteria. They cloned a luciferase gene into the tRNA region of the genome of the L5 mycobacteriophage and used the recombinant mycobacteriophage to identify *Mycobacterium smegmatis* cells. Aliquots of cultures with hundreds of *M. smegmatis* cells produced a positive signal (above the background) in just a few hours. Even samples with as few as 12.2 and 2.7 CFU/100 μl produced positive signals, though only after 2 and 3 days, respectively (62).

Phages expressing green fluorescent protein (GFP) have been proposed as a fast and accurate method for detecting *E. coli* (64, 65, 174). The *gfp* gene, originally carried on a plasmid, was inserted by homologous recombination into the genomes of phages T4 (wild type), T4e$^-$ (a gene *e* amber mutant phage) (64), and PP01 (65) such that it was displayed on the small outer capsid (SOC) of these phages, resulting in phages T4wt/GFP, T4e$^-$/GFP, and PP01-SOC/GFP (GFP introduced into the C terminus of SOC) or PP01-GFP/SOC (GFP introduced into the N terminus of SOC), respectively. The *gfp* gene was also inserted into phages IP008 and IP052, within the *e* gene, which encodes a phage lysozyme, resulting in phages IP008e-/GFP and IP052e-/GFP, which exhibited suppressed lytic activity (174). Incubating T4wt/GFP with *E. coli* K-12 led to increased fluorescence intensity during the initial stages of infection but then resulted in cell lysis, which made it difficult to identify phage-infected cells by fluorescence microscopy. On the other hand, *E. coli* incubated with the engineered phage T4e$^-$/GFP exhibited fluorescence, the intensity of which increased with the infection time (64). The GFP-labeled PP01 phage was able to specifically detect *E. coli* O157:H7, and fluorescence could be observed by microscopy after as little as 10 min of incubation (65). Engineering phages to express multiple *gfp* genes can enhance the detectable signal. For example, *E. coli* B^e cells infected with phage IP008e-/GFP or IP052e-/GFP exhibited low fluorescence intensity. When *gfp* was additionally fused to the *soc* gene in phages IP008e-/GFP and IP052e-/GFP, resulting in IP008e-/2xGFP and IP052e-/2xGFP, the fluorescence intensity was stronger and increased with incubation time (174). The detection limit of this approach has not yet been evaluated.

Edgar et al. proposed a biodetection system that combines *in vivo* biotinylation of an engineered phage followed by conjugation of the phage to streptavidin-coated quantum dots (QDs), semiconductor nanocrystals that give a fluorescence signal (175). The T7 coliphage was engineered to display a small biotinylation peptide on its major capsid protein. After propagation of the recombinant phage in the bacterial host, the biotinylated progeny phage could be detected by the fluorescence of the streptavidin-coated QDs. If the host was not present, biotinylated phage were not produced, and the functionalized QDs did not bind and were washed away. This method is fast, sensitive, and specific: as few as 10 and 20 *E. coli* cells/ml were detected by fluorescence microscopy within 1 h for experimental and environmental samples, respectively (175).

Piuri et al. genetically engineered the mycobacteriophage TM4 to carry a fluorescence-encoding reporter gene, namely, *gfp* or *ZsYellow* (176). The engineered mycobacteriophages detected *Mycobacterium tuberculosis* by delivering the reporter genes into the cells; the fluorescence could then be monitored by microscopy or flow cytometry (176). With this rapid and sensitive method, fewer than 100 cells present in the 5-μl aliquots used for microscopy could be detected, and bacterial antibiotic susceptibility could be determined in less than 24 h, as fluorescence was suppressed only in rifampin- or streptomycin-susceptible cells when the corresponding antibiotics were added (176, 177).

Diagnostic phage technologies are being translated into actual use outside research labs (173). For example, the first enrichment-free pathogen diagnostic system for *Listeria* was recently released for commercial applications (178). In addition, for phages to be used as personalized antimicrobials in the era of precision medicine, rapid and accurate diagnostics are needed to identify pathogens and determine which phages are most suitable for therapy. We envision that phage-engineering technologies will play an important role in a wide range of settings where rapid microbial detection is desirable.

PHAGE ENGINEERING FOR DRUG DELIVERY SYSTEMS

In addition to the delivery of engineered DNA as described above, phages can be adapted for targeted drug delivery to both prokaryotic and eukaryotic cells, including cancer cells. Most of the studies done so far rely on phage display, a powerful screening process by which peptides that specifically bind to the target cells of inter-

est are selected from a library of phage particles expressing a wide range of functional peptides on the phage coat surface (179).

Enhancing Antibiotic Activity

Yacoby et al. used filamentous phages (fd and M13) to target *S. aureus* either by displaying target-specific peptides on the major coat protein or by antibody-mediated targeting. The latter involved linking immunoglobulin G (IgG) antibodies to the phages via an IgG-binding domain displayed on the minor coat protein of the phage. In both cases, the authors chemically conjugated the phages with chloramphenicol, a bacteriostatic antibiotic, through a labile linker that enabled controlled release (180). After bacteria were exposed to the phage-drug conjugates, phages bound to the target cells and chloramphenicol was released, retarding bacterial growth. However, inhibition of bacterial growth by this system was limited due to the hydrophobic nature of chloramphenicol, which restricted the loading capacity to fewer than 3,000 drug molecules/phage (180). This limitation was later overcome by using hydrophilic aminoglycoside antibiotics (e.g., neomycin) as branched, solubility-enhancing linkers (181). This new approach, in which chloramphenicol was conjugated via a neomycin linker, allowed the authors to load over 40,000 chloramphenicol molecules/phage (181). When this drug-carrying phage was tested *in vitro* by measuring the optical densities of the bacterial cultures, it was found to inhibit the growth of *S. aureus*, *Streptococcus pyogenes*, and *E. coli* almost completely (181). Moreover, this approach was nontoxic to mice and less immunogenic than the use of native, unconjugated phage particles; the authors surmised that drug conjugation may have prevented antibodies from recognizing phage (182).

Delivery of Anticancer Drugs

A drug delivery platform for cancer therapy based on the use of genetically modified and chemically manipulated fUSE5-ZZ filamentous phages has also been described (183). These phages were first engineered to display a ligand that conferred specificity to the target cancer cells on the major coat protein and then loaded with cytotoxic drugs (hygromycin or doxorubicin) by chemical conjugation. *In vitro*, these drug-carrying phages targeted ErbB2-overexpressing human breast adenocarcinoma SKBR3 cells. Once endocytosed, the phages were degraded, releasing the drug inside the cancer cells and resulting in about 50% inhibition of target cell growth, a >1,000-fold improvement in the potency of hygromycin compared with that of free-drug treatments lacking the phage and the ligand (183).

Similarly, Du et al. coupled phages that displayed a hepatocarcinoma-specific binding peptide to doxorubicin in order to create a drug delivery strategy for hepatocellular carcinoma (184). The binding peptide was selected by repeated panning of phage display libraries inside mice to identify peptide motifs that directed phages to tumors (184). *In vivo* antitumor activity tests showed that all mice treated with the drug-loaded phages survived during the 25 weeks of the experiment, whereas only 40% of the mice treated with free doxorubicin survived (184).

The phage library f8/8 was screened for a highly specific peptide targeting the metastatic prostate cancer cell line PC-3M (185). A prostate cancer-specific phage was isolated and then converted to a phagemid which encoded the expression of emerald GFP under the control of a cytomegalovirus promoter (185). The *in vitro* delivery and production of emerald GFP by the phagemid were observed in PC-3M cells by fluorescence microscopy, indicating that this technique may potentially be used to deliver therapeutic genes to prostate cancer cells. Wang et al. (186) screened a phage library to identify a breast cancer MCF-7 cell-specific peptide fused with a phage coat protein. These phage particles were coupled with doxorubicin and tested *in vivo*. Antitumor activity was enhanced in mouse models compared to results obtained with the nontargeted formulations, and there was no detectable hepatotoxicity (186).

A refactored M13 phage was used for tumor cell imaging and drug delivery to prostate cancer cells *in vitro* (187). First, the genome of phage M13 was redesigned to separate the regulatory elements and coding regions of *gene VII* and *gene IX*, which overlap naturally, so that these genes could be manipulated independently. For example, this redesign allowed the authors to modify the N-terminal end of the p9 protein without affecting p7. Three peptides were displayed on different phage components of the refactored M13 phage: the SPARC (secreted protein, acidic and rich in cysteine) binding peptide (SBP) on the p3 phage minor coat protein encoded by *gene III*, a DFK amino acid motif on the p8 phage major coat protein encoded by *gene VIII*, and a biotin acceptor peptide (BAP) on the p9 minor coat protein encoded by *gene IX*. The modified phage was designated M13-983. SPARC is a matricellular glycoprotein overexpressed in many cancers (188, 189), while the DFK peptide sequence is recognized by cathepsin B, a lysosomal cysteine protease overexpressed in prostate cancer (190, 191). Doxorubicin was then conjugated to the aspartic acid residue of p8. In order to further functionalize M13-983 for microscopic imaging, the p9 protein displaying BAP was enzymatically biotinylated and incubated with streptavidin-coated Alexa Fluor 488 dye, resulting in phage M13-983-Alexa-DOX (187). This phage was subsequently tested *in vitro* on human prostate cancer cell lines expressing SPARC at either higher (C42B) or lower (DU145) levels. This modified phage was shown to be an effective platform for targeted imaging, since the normalized fluorescence intensity measured by a plate reader was about 10 times greater for C42B cells than for DU145 cells, revealing efficient targeting of SPARC by the phage. Furthermore, M13-983-Alexa-DOX was ca. 100 times more cytotoxic than free doxorubicin for the target cells (187).

Antibody Delivery

Frenkel and Solomon engineered filamentous phages to mediate antibody delivery to the brain as a way to detect Alzheimer's disease (192). A filamentous phage was engineered to display antibodies to amyloid-β peptide (Aβ) as a probe to detect the accumulation in the brain of Aβ, a peptide thought to be involved in the progression of Alzheimer's disease (193–196). Phages displaying Aβ-specific antibodies were administered intranasally to transgenic mice. The resulting *in vivo* targeting of Aβ deposition confirmed that the engineered phages had reached the central nervous system, owing to the properties of the phage-based delivery vector, and had bound Aβ *in vivo*, owing to the antibody, with high specificity and no detectable toxicity. This binding was confirmed upon sectioning the brain and staining the sections by use of thioflavin-S and antiphage antibodies, followed by visualization under a fluorescence microscope (192). These results show that phages may play an important role in imaging in the future, particularly if the system can be adapted to include isotopes already used for *in vivo* diagnostic imaging in humans (192). In

addition, engineered phages may be used to achieve specific delivery of drugs to or into target cells. This localized action may decrease the concentration of the drug needed for a specific treatment and the concentration of free drug in the organism. Thus, there may be benefits in terms of reduced side effects and lowered costs. Nonetheless, more studies on the pharmacokinetics and immunogenicity of these drug-delivering phages are needed before their application can be extended toward clinical use.

Phages for Vaccine Development

Phages may also prove useful for vaccine development, as shown in preliminary studies of human immunodeficiency virus (HIV), anthrax, and foot-and-mouth disease virus (FMDV). By engineering phages to display various antigens on their surfaces, vaccines can be created that are adaptable to evolving medical or veterinary needs, such as new outbreaks.

An effective HIV vaccine may include multiple antigens and should generate broadly neutralizing antibodies (197, 198). Sathaliyawala et al. proposed a vaccine delivery system for HIV that used T4 phage (198). Up to three purified HIV antigens were displayed, individually or in combination, on the T4 phage capsid surface to generate a multicomponent HIV vaccine. The immunogenicity of the T4-displayed HIV antigens was tested in mice, and the phage-based vaccine was found to elicit strong antibody and cellular immune responses (198). The same group also used the T4 system to display combinations of antigens derived from anthrax toxin proteins (199). A T4 phage displaying three anthrax toxin antigens elicited strong immune responses in mice, in the form of anthrax-specific antibodies; furthermore, the sera alone could block the cytotoxicity of lethal toxin for a specific macrophage cell line (199).

Ren et al. used a T4 phage display system to generate a vaccine against the lethal virus FMDV (200). The FMDV-T4 phage recombinant vaccines proved to be effective in mouse model assays, affording up to 100% protection against the FMDV O serotype after oral or subcutaneous immunization (200). FMDV vaccination requires multivalent vaccine preparations to confer protection against the multiple serotypes of the virus. The putative advantage of this technology is that it would facilitate the development of tailor-made serotype and subserotype FMDV vaccines.

PHAGE ENGINEERING FOR MATERIALS SCIENCE

In addition to their applications in human health, veterinary health, and food safety, phages have been adapted for use in materials science. By combinations of phage display and genetic engineering techniques, phages have been used to build novel nanostructured materials with various applications, such as energy generation and storage (201–204), biosensing (205–207), and tissue regeneration (208–210). The well-defined shape of M13 and the possibility of displaying functional peptides on its surface have made it the phage of choice most often used for the assembly of new materials (201, 209, 211). Genetically engineered M13 phages have been adapted to assemble and arrange quantum dots (212, 213), build liquid crystals and films (214–216), and fabricate nanorings (217) and micro- and nanofibers (218).

In 2006, Nam et al. reported the use of M13 to synthesize and assemble nanowires of cobalt oxide for the fabrication of battery electrodes (202). M13 was first engineered to display gold-binding peptides with affinity for cobalt ions on its major coat protein (202). The engineered M13 phages were then used to form nanowires of gold-cobalt oxide, improving the storage capacity of lithium ion batteries (202). Later, the same group, using M13-based cobalt oxide nanowires, built and characterized microbattery electrodes with full electrochemical functionalities (charge storage capacity and performance rate) (219). Cobalt manganese oxide nanowires made by M13 phage-mediated synthesis have also been used to build high-capacity lithium-oxygen battery electrodes (220).

M13 phages have also served as templates for the integration of single-walled carbon nanotubes (SWNTs) into photovoltaic devices for highly efficient electron collection (221). This method stabilized the SWNTs while maintaining their electronic properties and increasing the power conversion efficiency in dye-sensitized solar cells (221).

Phage-based materials can also serve medical aims. M13-functionalized SWNTs have been used as effective probes for noninvasive fluorescence imaging of prostate tumors in mice (207), as well as to target SPARC and to visualize deep, disseminated tumors in mouse models of human ovarian cancers (222). By attachment of an antibacterial antibody to the p3 minor coat protein of the M13-SWNT complex, probes were made that could be used to image bacterial infections *in vivo* (223). *S. aureus* endocarditis in mice was visualized by this method, and deeply buried infections were detected with high contrast and high specificity (223). In order to image tumors in mice *in vivo*, M13 was modified to display a SPARC-binding peptide on the p3 minor coat protein and a triglutamate motif on the p8 major coat protein for the templated assembly of magnetic iron oxide nanoparticles (224). This strategy improved the magnetic resonance contrast of prostate cancer in mice compared with that of traditional nanoparticles used clinically, as each SPARC-targeting phage particle delivered a large number of detectable nanoparticles into the target cells (224).

Mao et al. synthesized phage-based fibers and coatings with antibacterial properties by engineering phage M13 to express negatively charged glutamic acid peptides on its p8 major coat protein. Silverized phage fibers were then created by the electrostatic binding of silver ions, which have antibacterial properties (225). *In vitro* studies showed that these phage-based fibers exhibited bactericidal activity against *Staphylococcus epidermidis* and *E. coli* strains, which was visualized by fluorescence-based live-dead staining and zones of inhibition. Silverized phage fibers may thus be useful as anti-infective materials (225).

Genetically engineered M13 phages have also been used to construct novel tissue-regenerating materials. Merzlyak et al. engineered the M13 phage to display cell signaling motifs (laminin peptides RGD and IKVAV) at the N terminus of the p8 major coat protein (209). These phage building blocks self-assembled into structurally aligned liquid crystalline-like matrices that could maintain the viability, proliferation, and differentiation of hippocampal neural progenitor cells, as well as control their directional growth (209). The same research group reported the use of engineered M13 phages to fabricate directionally organized two- and three-dimensional phage-based scaffolds, which showed good cytocompatibility and supported the directional growth and encapsulation of fibroblast cells (226). Similarly, Wang et al. assembled M13-based matrices that provided a biomimetic microenvironment with controlled biochemical and biophysical

cues for the directed differentiation of induced pluripotent stem cells (227).

CONCLUSIONS

The arrival of the synthetic biology era married with the prodigious diversity of phages has led to powerful applications for therapeutics, diagnostics, and materials science. The introduction of new genetic engineering technologies has led to a more precise and accelerated modification of phage genomes for basic science as well as engineering. Phages have already been used to create new anti-infective agents, diagnostics, drug delivery systems, and vaccines, as well as new materials for nanoscale devices, imaging, and tissue scaffolds.

Despite the advances described above, phage research is still in its infancy. The tremendous diversity of phage types and structures in nature (228, 229) has not yet been fully tapped. In fact, most naturally occurring phages have not yet been propagated in the lab. Of those that are known, many have not yet been characterized or are not yet amenable to genetic manipulation. Thus, phage engineering has so far involved only a small percentage of existing phage types. For example, most materials science applications have been based on the M13 phage, even though phages with other morphologies and sizes might extend the practical applications of phages in this field.

Next-generation sequencing technologies (230, 231) have the potential to deposit phage genomes or phage-derived sequences into bioinformatic databases in large quantity without the need to first isolate these phages in the lab. These sequences can then be mined either to recreate natural phages via direct digital-sequence-to-DNA synthesis or to engineer novel phages that combine parts derived from various phages. New technologies are further needed to accelerate the design-build-test cycle for creating specialized phages and to make it possible to translate proof-of-concept academic work into real-world use more efficiently. As described above, highly reliable and rapid strategies that can be generalized to a wide range of phages are still lacking. Many strategies for engineering phages require the ability to genetically modify their bacterial hosts or to efficiently deliver exogenous DNA into these hosts, which is still a challenge for many bacterial species. Thus, new tools for genetic manipulation or DNA transformation are needed. Ideally, it would be possible to introduce multiple genetic alterations into phage genomes with high efficiency and at precise locations.

Finally, the vast majority of the work described in this review has resulted in genetically modified phages that may have significant benefits for diagnosing and treating bacterial infections, for treating nonbacterial diseases, or for constructing new materials. Despite the potential benefits, the acceptance of genetically modified phages for real-world applications may vary across different regions of the world. Strategies for inactivating phages so that they cannot propagate outside the lab, for example, by deleting essential protein genes from the phage genome and supplying these in *trans* in production hosts, may help to address such concerns. In the case of human use, the choice of compelling areas of tremendous medical need (e.g., for use against Gram-negative pathogens that are highly resistant to antibiotics and other antimicrobials) and explicit demonstrations of safety will both be important. Furthermore, techniques to contain the use of genetically modified phages for diagnostic and materials science applications and to inactivate the phages after use may also help to mitigate these issues. In summary, phage engineering is an area of research that is attracting intense interest and has great potential utility, but it has yet to be fully exploited.

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