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REVIEW

Advance on Engineering of Bacteriophages by Synthetic Biology

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Abstract: Since bacteriophages (phages) were firstly reported at the beginning of the 20th century, the study on them experiences booming-fading-emerging with discovery and overuse of antibiotics. Although they are the hotspots for therapy of antibiotic-resistant strains nowadays, natural phage applications encounter some challenges such as limited host range and bacterial resistance to phages. Synthetic biology, one of the most dramatic directions in the recent 20-years study of microbiology, has generated numerous methods and tools and has contributed a lot to understanding phage evolution, engineering modification, and controlling phage-bacteria interactions. In order to better modify and apply phages by using synthetic biology techniques in the future, in this review, we comprehensively introduce various strategies on engineering or modification of phage genome and rebooting of recombinant phages, summarize the recent researches and potential directions of phage synthetic biology, and outline the current application of engineered phages in practice.

Keywords: phage, synthetic biology, engineering, rebooting, phage therapy, phage application

Introduction

Bacteriophage (phage) is a class of widespread viruses exclusively infecting bacteria, playing a key role in ecosystems.[1–3] Since phages were discovered over a hundred years ago, many techniques and reagents, such as DNA polymerase, restriction endonuclease, ligase, and CRISPR/Cas system, have been developed from phages, which have together accelerated the development of modern biology. In addition, although the genetic and functional diversity of phages remains unclear, they provide an extremely rich library of genetic elements and toolkits for synthetic biology.[4–7] From an application perspective, phages are believed to have great potential in antimicrobial agent, phage therapy, biosensor, and delivery vector.[8,9] In particular, as a potential alternative to antibiotics, phages are considered as the white hope in solving the problem of the emergence and spread of antimicrobial resistance (AMR). So far, even though abundant phages have been isolated and characterized worldwide, many aspects about phage genes and life cycles are still mysterious, which limiting direct and extensive use of natural phages in clinical medicine.

Synthetic biology refers to the rational design, transformation, and even de novo synthesis of organisms according to specific goals under the guidance of engineering, which is a good way to overcome some limitations of natural bacteriophages.[10–12] The efficiency of phage infection can be enhanced by adding functional genes to the phage genome. The virulence genes and genes with non-essential functions are removed as far as possible, and then the chassis genome of phage is used to achieve the purpose of highly controllable biosafety.[13–15] New synthetic biology strategies and methods for bacterial genomes, such as high-throughput sequencing technology and large DNA fragment synthesis technology, can no doubt accelerate the ability of phage genome design and construction, and further, explore the

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potential of phage in all aspects. Advances about phage display are well summarized in other reviews.[16,17] In this review, we focus on strategies on engineering or modification of phage genome and rebooting of recombinant phages, narrate and discuss the recent research and potential directions of phage synthetic biology, and summarize the current application of engineered phages in practice.

Engineering Strategies for Phage Genome

Genetically Engineered Phage

Although phages have application potential in many aspects, such as phage therapy, precision medicine, and bacterial prevention and control, native lytic phages remain an underutilized option due to challenges such as regulation, limited host range, bacterial resistance to phages, manufacturing, and side effects of bacterial lysis and delivery.[18] These limitations can be potentially overcome by genetically engineered phages. In this section, we mainly discuss the kinds of means and methods used to modify lytic phages.

Currently, various methods of phage genetic engineer with advantages and disadvantages have been developed, in order to modify their host range, improve safety and antimicrobial activity.[19] Homologous recombination (HR) in vivo is an early popular approach of phage genome editing, which allows gene insertion, replacement, and deletion (Figure 1a).[20] By this means, linear dsDNA phages, such as *Mycobacterium smegmatis* phage L5 and *Pseudomonas aeruginosa* phage PaP1, can be modified.[21,22] It is worthy to note that HR is feasible only when the host strains are competent for the donor DNA and restriction-deficient. However, the recombination efficiency of HR is always unsatisfactory, sometimes as low as 0.1%, requiring intensive labor to select recombinant phages. A selection marker inserted into the donor DNA is helpful.[23,24] One example is *trxA* gene, which is essential for phage replication but not essential for host growth. The screening efficiency of T7 recombinants was higher in *E. coli* Δ*trxA* mutant compared with the wild-type *E. coli* strain.[24,25] In addition, overexpression of heterologous recombinant proteins, such as λ red system (Exo, Gam and Beta) and RecE/RecT-like proteins in the host can improve the recombination efficiency.[20,26,27] Mycobacteriophages Che9c *gp60* and *gp61* encode homologs of both RecE and RecT, which could substantially enhance *mycobacterial* recombination frequencies.[20] For example, they facilitated the construction of *M. smegmatis* phage BPs gene knockout and replacement mutant, with efficiency up to 15%.[28]

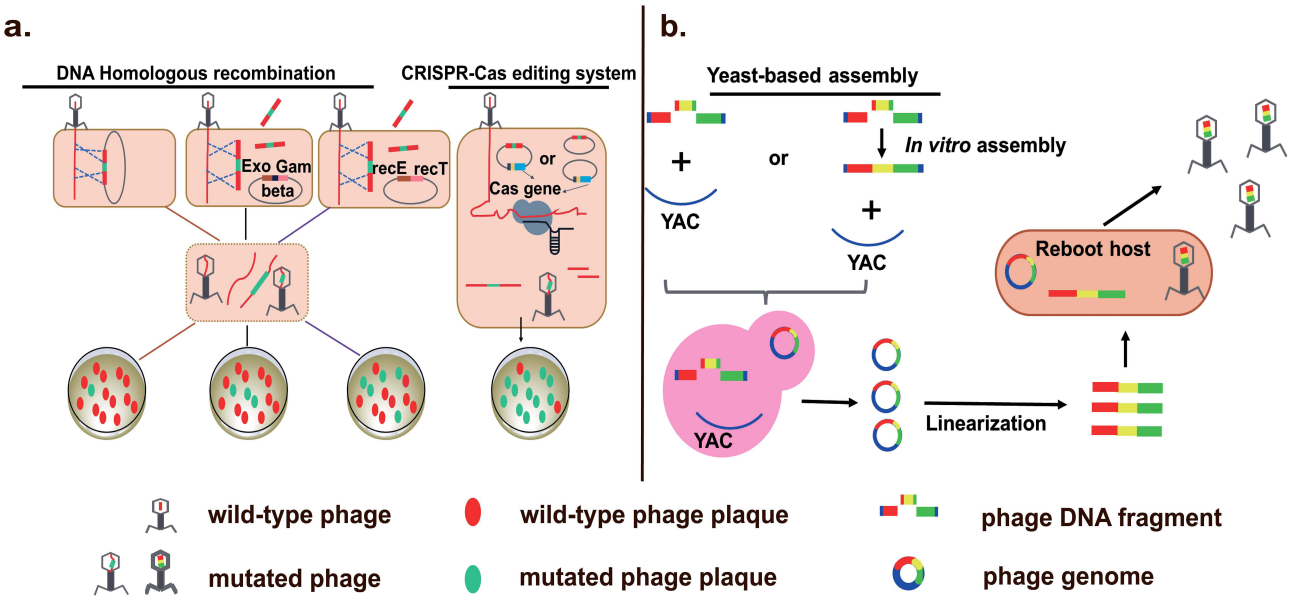

Figure 1 Strategies of genetic engineering for phage genomes. (**a**) Phage genome can be edited by homologous recombination (HR) in vivo and CRISPR-Cas system. Exo, Gam and Beta are three components of the lambda red recombineering system. YAC: yeast artificial chromosomes. (**b**) Phage DNA fragments are assembled to become complete genome in the yeast, followed by transformation into the reboot host to produce activated phages.

CRISPR Cas9 system is widely used for gene knockout, insertion, and site-directed mutation of phage genome (Figure 1a).[29–32] Martel lab confirmed that it can be used for mutation and large fragment deletions of *S. thermophilus* phage 2972 genome. They successfully achieved phage recombination, and all the tested plaques contained recombinant phages that had the desired mutation.[33] In 2017, Aslan lab used natural CRISPR Cas10 system of *S. epidermidis* to engineer *staphylococcal* lytic phages Andhra, achieving silent mutation efficiency up to 100%.[34] Besides, 11 kb to 29 kb of phage T4 genome was deleted by utilizing CRISPR Cas12a system, and all plaques were mutant.[35] Of course, type I-E CRISPR Cas systems are also powerful tools for phage genome editing. With them, *Vibrio cholerae* phage ICP1_2011_A's 33 bp gene deletion and 2670 bp gene deletion efficiency were 100% and 58%, respectively.[36] Editing efficiency of CRISPER system is influenced by homologous-arm size of donor fragment, and the size of target sequence. For example, a 500 bp fragment in the *Klebsiella* bacteriophage phiKpS2 was replaced with 923 bp using homologous arms of 40 bp, 50 bp and 60 bp, the success rate was 41.7%, 60.4%, and 87.5%, respectively.[31] An editing efficiency of 76.7% and 56.7% for deletion of a 1 kb and 2 kb fragment, respectively, could be obtained with a 40 bp homologous arm.[37] Taken together, CRISPR-Cas system makes phage gene editing simple and efficient, which can also be used to identify unknown functional genes, and expand our understanding of phage–host interactions.

The technology of artificial design and synthesis of multiple or large DNA fragments has been constantly progressed, such as Gibson assembly and Transformation-Associated Recombination (TAR).[38–41] Gibson assembly is developed by Dr. Daniel Gibson et al, which allows for the successful assembly of multiple DNA fragments and the flexible and suitable construction of large DNA in vitro.[42] In 2019, Nugen lab inserted a NanoLuc luciferase expression cassette into the T7 phage using Gibson assembly in vitro.[43] TAR is a frequently used phage genome modification technology, which allows recombination of multiple large DNA segments in yeast artificial chromosomes (YAC) containing yeast selectable marker (HIS) and yeast centromeric locus (CEN/ARS) in yeast.[44] Based on the assembly and capture of synthetic genomes into YAC, researchers have artificially modified and rebooted various Gram-positive and Gram-negative bacterial phages such as *E. coli* phage T3 and T7, *Klebsiella* phage K11, *L. monocytogenes* phage P35, and *P. aeruginosa* phages (Figure 1b).[45–47] These strategies provide weapons and support for the artificial design and rebuilding of phages (Table 1).

Chemically Modified Phages

With the development of gene sequencing and synthesis technology, more and more large DNA fragments have been magnificently de novo synthesized in vitro and vivo, including phage genomes (Figure 2a). De novo genome synthesis through chemically synthesized oligonucleotides (oligos) can generate completely novel phage genome. Phix174 genome

Table 1 Summary of Engineering Strategies of Phage Genomes

Phages	Year	Host Organism	Strategy	Editing	Efficiency	Reference
Andhra	2017	*S. epidermidis*	CRISPR-Cas10	Silent mutation	100%	[34]
AP205	2020	*Acinetobacter*	Synthesis and Gibson assembly	/	89.3%	[49]
BPs	2008	*M. smegmatis*	BRED strategy	200 nt deletion	15.8%	[28]
PaP1	2013	*P. aeruginosa*	In vivo recombination	Gene exchange	/	[22]
P2	2017	*L. lactis*	CRISPR-Cas9	Gene deletion	100%	[31]
phiKpS2	2018	*K. pneumoniae*	CRISPR-Cas9	1 kb deletion	100%	[32]
T4	2021	*E. coli*	CRISPR-Cas12a	11 kb deletion	100%	[35]
T7	2009	*E. coli*	Yeast-based platform	Gene exchange	>25%	[46]
T7	2014	*E. coli*	TypeI-E-CRISPR-Cas	Gene deletion	40%	[36]
T7	2019	*E. coli*	Gibson assembly	Gene insertion	100%	[43]
T7	2020	*E. coli*	λ Red-mediated recombination	Gene replacement	1.0%	[27]
ΦX174	2003	*E. coli*	Polymerase cycling assembly	/	/	[48]
ΦX174	2012	*E. coli*	Yeast-based platform	Full genome synthesis	44%	[57]
ICP1_2011_A	2016	*V. cholerae*	Type I-E CRISPR-Cas	2670 nt gene deletion	58%	[37]
2972	2014	*S. thermophilus*	CRISPR-Cas9	Point mutation	100%	[33]

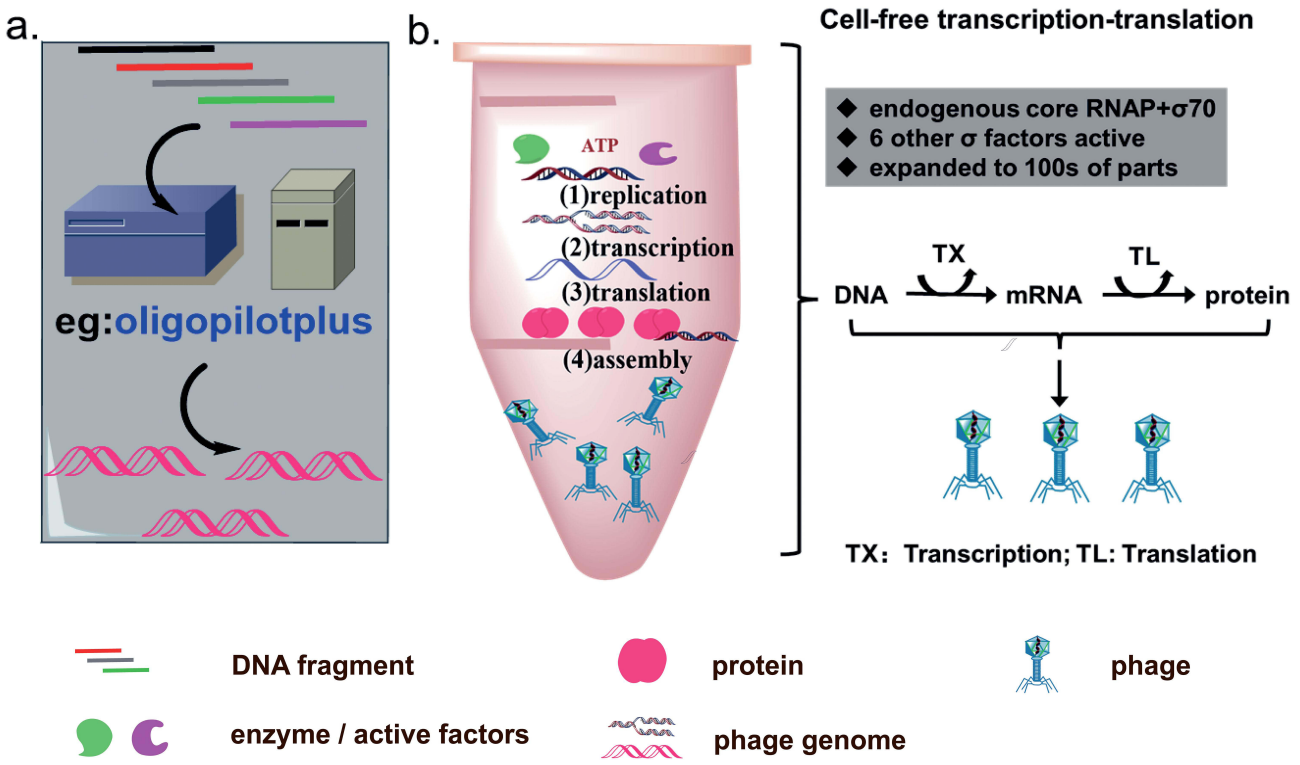

Figure 2 In vitro rebooting strategies for synthetic phage genome. (**a**) *De novo* phage genome synthesis depending on automated gene synthesis platform. (**b**) The synthetic phage genomes are rebooted via replication, transcription, translation, and assembly in a cell-free transcription-translation (TXTL) system.

(5386 bp) was the first phage synthesized by this way in 2003.[48] The synthesized oligonucleotides were gel purified, phosphorylated, annealed, and assembled in vitro, then electroporated into *E. coli*, followed by phage plaques checking . Even though this approach is not likely to apply to relatively large DNA molecules, it exhibits much more convenience than recombineering-based approaches. On this basis, Huiran Yeom et al presented a cell-free, low-cost, de novo gene synthesis technology called Sniper assembly for phage genome construction, and successfully obtained *Acinetobacter* phage AP205 genome and *E. coli* phage T7 genome with 89.3% and 83% success rate, respectively.[49]

Phages can be modified by chemical modification to improve the efficiency of sterilization or bacteria detection.[50,51] Phage can be visualized through labeling its genome with fluorescence dye, such as YOYO, Cl-YO, Cl-YO-Et, Cl-YO-Bu, and the labeled phages are observed by flow cytometry and microscope.[52,53] Since fluorescence dye may affect the ejection of the phage genome, dye selection is important. For example, when it was used to label phage LG1 to detect *E. coli* O157:H7, YOYO-1 can cross the capsid protein and bind with nucleic acids, forming the "halo-like" appearance on the cell surface rather than inside the cell.[54] This is because that phage nucleic acids labeled by YOYO-1 could not be injected into bacterial cytoplasms spontaneously, unless under external force.[55] SYBR-labeled phage DNA can enter the cytoplasm. In addition, dependent on nucleic acid type, available fluorescent dye is different. For example, DAPI can bind to dsDNA phage, SYBR gold binds to RNA, ssDNA, and dsDNA, and YOYO-1 binds to ssDNA and dsDNA.[56]

Rebooting Strategies for Engineered Phage

Phage rebooting refers to the acquisition of activated virions from the phage genome. Nowadays researchers generally rely on two ways. Firstly, phage genomic DNA is transformed into hosts or transitional hosts. Secondly, phage DNA is rebooted via cell-free transcription-translation (TXTL) systems (Table 2). The first rebooting strategy is traditional and frequently used. Phage genome is transformed into host competent cells through electro-transformation and incubated on a double-layer medium until phage plaques appear. Owing to the different cell structures between Gram-negative organisms and Gram-positive organisms, the methods are different.

Table 2 Rebooting Strategies of Recombinant Phage Genomes

Phages	Year	Genome Size (kb)	Original Host	Rebooting Strategy	Reference
E. coli phage Φ174	2012, 2016	5.3	*E. coli* HF 4704	TXTL reactions	[67,69]
E. coli phage T7	2012, 2016	39.9	*E. coli* Host B	TXTL reactions	[67,69]
E. coli phage MS2	2016	3.6	*E. coli* C-1	TXTL reactions	[67]
E. coli phage T4	2018	169	*E. coli* Host B	TXTL reactions	[66]
B. siphovirus TP21-L	2018	37.5	*B. cereus*	*L. monocytogenes* L-form bacteria "Rev2"	[47]
L. siphovirus P35	2018	35.8	*L. monocytogenes*	*L. monocytogenes* L-form bacteria "Rev2"	[25]
L. siphovirus P70	2018	67.2	*L. monocytogenes*	*L. monocytogenes* L-form bacteria "Rev2"	[47]
L. myovirus A511	2018	137.6	*L. monocytogenes*	*L. monocytogenes* L-form bacteria "Rev2"	[47]
S. aureus phage 2638A	2018	41.3	*S. aureus*	*L. monocytogenes* L-form bacteria "Rev2"	[47]
S. aureus phage K	2018	127.4	*S. aureus*	*L. monocytogenes* L-form bacteria "Rev2"	[47]

In regard of Gram-negative bacterial phages, a direct chemical or electrical transformation of synthetic phage genomes into host cells requires high transformation efficiency, especially for large phage genomes. Efficient conversion protocols have been designed for certain bacteria such as *E. coli, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.[45,46,57,58] As we know, some host genes are essential for successful phage lysis. One example is *trxA* in *E. coli*, which encodes thioredoxin, the processivity factor for T7 DNA polymerase. This gene is essential for phage replication but not essential for bacterial growth. The efficiency of plating (EOP) of T7 phage on the *E. coli* Δ*trxA* mutant was 10^{10}-fold lower than the wild-type strain. Therefore, *trxA* gene could be used as a screening maker, just like an antimicrobial marker gene used in the gene knockout experiment. After recombination in vitro, if *E. coli* Δ*trxA* mutant is chosen as rebooting host, only the recombinant phage containing *trxA* could form plaque. This is much easier to get recombinant phage.[23] Moreover, in order to produce more phage particles, the bacteria with specific restriction endonuclease gene deleted or recombinase gene expressed, sometimes could be used as a surrogate host for phage rebooting.[27] This reveals that we could engineer bacterial genome to prepare the phage rebooting host when necessary. In terms of Gram-positive bacterial phages, previously universal rebooting host organisms were less, mainly restrained by DNA transformation efficiency. Recently, *Listeria monocytogenes* and *Staphylococcus aureus* cross-genus reactivation platform was developed. Kilcher et al rebooted *Bacillus cereus* phage TP21-L, *Bacillus thuringiensis* phage Bastille and *Staphylococcus aureus* phage 2638A and phage K besides *Listeria* phages P70, A511, and B035 in *Listeria* L-form cells.[47,59] Nacyra Assad-Garcia et al rebooted Siphophage SA75, Myophage K, *E. faecalis* phages vB_EfaS_Ef5.1, vB_EfaS_Ef5.2, vB_EfaS_Ef5.3, vB_EfaS_Ef5.4, and vB_EfaS_Ef6.4.[60]

On the other hand, Cell-free TXTL system is a major improvement for phage rebooting in vitro (Figure 2b). This technique has become a suitable platform, which consists of a cell lysate or purified transcription/translation machinery and a buffer/energy mix optimized to express genes from template DNA.[61–63] At first, researchers use cell extract preparation to reboot phage genome in vitro. For example, the *B. subtilis* phage Φ29 and T7 genome have completed the assembly in vitro utilizing the extract preparation of *B. subtilis* SpoA12 and *E. coli* extract preparation, respectively.[64,65] Phage T4 was also rebooted in this cell-free reaction system.[66] In recent years, Noireaux lab has successfully developed three versions of cell-free toolboxes that can reboot phage genome in vitro. At first, they completed the synthesis and rebooting of phage T7 (dsDNA, 40 kb) and ΦX174 (ssDNA, 5.4 kb) in the first version of cell-free reactions (CFRs), composing of one-third crude extract and two-thirds of water, genomes and buffer.[67,68] Secondly, Version 2.0 included *E. coli* MazF interferase or ClpXP AAA+ proteases at the basis of Version 1.0, which can respectively degrade mRNA and high protein in order that the version has a much wider range of rates compared to Version 1.0. Rebooting of MS phage (ssRNA) was completed in this system.[69] On the basis of version 2.0, two major changes of version 3.0 were made: (1) the *E. coli* cells were grown at 40°C instead of 37°C, (2) 60 mM maltodextrin and 30 mM d-ribose were carbohydrate source rather than only maltodextrin in cell-free reactions.[70] At last, Vogele applied the cell-free sDNA (small DNA) technique (CF-sDNA) to express the native T7 phage genome in the context of cell-free protein expression by inhibiting the production of the major capsid protein of phages.[71]

Successful genome rebooting depends on the reaction incubation time and the potential yield of the system. Phage T4 was rebooted in cell-free reaction by optimizing biochemical settings, like concentrations of genome, Mg^{2+}, K^{+} and PEG8000. Meanwhile, DNase I, EDTA, pyrophosphatase, and ATP analogs adenosine 5'-[α, β-methylene] triphosphate and adenosine 5'-[β, γ -methylene] triphosphate is crucial for bacteriophage synthesis in vitro.[64,72] As a whole, the development of TXTL system not only promotes phage synthesis in vitro but also offers unique possibilities to interrogate quantitatively the links between phage gene expression, self-assembly and metabolism in the future study.

Applications of Engineered Phages

Expanding Phage Host Range

In the process of phage infection, it is necessary to recognize and bind with specific receptors on cell surfaces, which vary from bacteria to bacteria.[73,74] Most of natural phages have narrow lytic host range, limiting the practical application in phage therapy.[10] Therefore, it is important to broaden or alter the host range of phages by changing their genetic modules (Figure 3a). Synthetic biology techniques enable modification of phages to target different hosts by engineering and mutating phage receptor-binding proteins (RBPs), which generally are tail fibers (TFs) or tail spike proteins (TSPs).[13,75,76]

In 2005, Mahichi et al exchanged *gp37* and *gp38* at the tip of the long tail fiber of the T2 phage with the counterpart of *E. coli* O157:H7 specific phage PP01. The antibacterial spectrum of recombinant phage T2ppD1 was the same as that of PP01, instead of T2, and the adsorption rate was weaker than PP01.[77,78] In 2015, *E. coli* phage T7, whose major host determinant is the tail fiber (*gp17*), was engineered by modular swapping of its tail component,

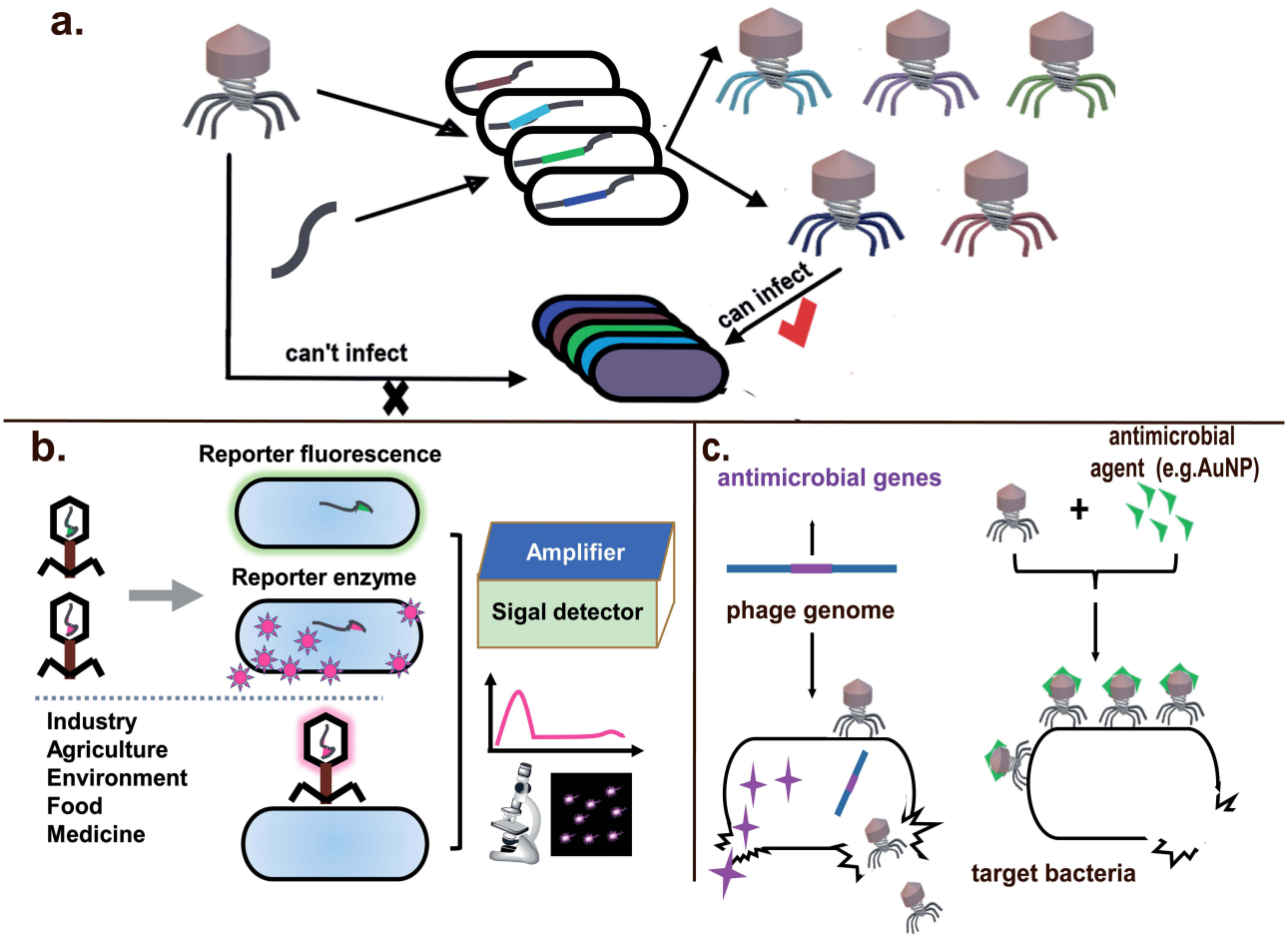

Figure 3 Applications of engineered phages. (**a**) The host range of phages could be broaden or changed by modification of receptor-binding proteins. (**b**) Phages with reporter fluorescence or enzyme can be used to detect bacteria in the fields of medicine, food industry and environmental science. (**c**) Phages could be used as a delivery vector to carry antimicrobial genes or bactericidal agents to enhance bacterial killing.

targeting different hosts including *E. coli, Yersinia* and *Klebsiella* bacteria.[46] The strategy is easier to achieve in well-studied phages. Even though the T7 phage cannot propagate in some hosts, it can package and transduce plasmids to those hosts. Yosef and G. Goren established a platform that extends the host range of T7 phage for DNA transduction by displaying various phage tail/tail fiber proteins. Specifically, they first respectively transformed 15 plasmids that encode different tail fiber genes that carry homologous arm with T7 phage tail fiber gene to *E. coli* hosts to produce various host *E. coli*. Then, when T7 phage infected those host *E. coli*, a plasmid expressing tail fiber gene could change the original tail fiber gene to assemble new phages that target their host to DNA transduction.[79] This approach significantly extends the host range of phages and paves the way for advanced genetic manipulations and analyses, and provides a reference for other phage modifications. In 2019, Kevin Yehl et al developed a powerful high-throughput strategy to mutate T3 phage tail fiber protein that was identified as host-range-determining regions (HRDRs) through site-directed mutagenesis. This strategy created a huge diverse "phagebody" library and could suppress bacterial resistance.[80]

Besides, the phage genome containing the diaminopurine (Z) base is called Z-genome that can evade most restriction enzyme attacks of hosts. Zhou et al have reported a multienzyme system of Z-genome synthesis in *Acinetobacter* phage SH-Ab 15,497.[81] Hence, it may expand the host spectrum when the Z-genome biosynthetic enzymes incorporate into the engineered phage genome. In brief, the engineered phages with expanded host range are able to effectively suppress bacterial resistance and may be very useful to boost phage applications.[80,82]

Detecting Bacterial Pathogen

Combining with phage specificity and reporter genes such as green fluorescent protein (*gfp*), luciferase-expressing gene (*lux* and *luc*), and bacterial *ice* nucleation (*inaW*), phages can be more easily, faster, and more reliably employed to detect organisms and substances with practical applications in medicine, food industry and environmental science (Figure 3b).[83–85] For example, *E. coli* phage PP01 was labeled by fusing GFP with the small outer capsid proteins (SOC) and used for detection of *E. coli* O157:H7 in the sewage water. Adsorption of the GFP-labeled PP01 phages to the host cell surface made cells visualized under a fluorescence microscope.[86] Due to the background natural fluorescence of biological samples, bioluminescence, a process that produces light through the enzymatic oxidation of chemical substrates is chosen as an optional substitute. Pulkkinen et al inserted a reporter luciferase enzyme Nano Luc (Nluc) into the T7 phage and used it to detect the presence of *E. coli*.[43] A biosensor platform based on T4 phage encoding luminescent reporter enzymes allowed for detection of <10 cfu/100 mL of viable *E. coli* within 7 h.[87] Additionally, with the advancement of molecular biology techniques, phages can be easily used to display foreign peptide sequences or materials on their coat proteins. Huan Peng et al modified the capsid of M13KE phage to display the receptor-binding proteins from different phages that naturally target the desired bacteria including *E. coli, P. aeruginosa, V. cholerae*, and *Xanthomonas campestris*.[88] On the other hand, as the result of phage lysis, the released bacterial intracellular components, such as adenosine triphosphate (ATP), adenylate kinase (AK), and β-glucosidase, have also been used as cell markers for detection purposes.[89–91] Hussain et al summarized the advantages and limitations of whole phage-based bacterial detection.[92]

The phage-based electrochemical biosensor is reported to detect various bacteria, such as *Y. pseudotuberculosis, B. cereus* and *Mycobacterium smegmatis*, and *E. coli* using metal ions like Hg (II), Na (I), Mn (II), Ca (II), Pb (II), and Zn (II).[87,93,94] For example, engineered phage M13 displaying five RBPs from other filamentous phages aggregates AuNPs to detect *E. coli, P. aeruginosa, V. cholerae*, and two strains of the plant pathogen *X. campestris*.[95] AuNPs act as a signal amplifier and detection of bacteria is achieved by testing shift in surface plasmon resonance (SPR) absorbance.[96]

Totally, phage-based biosensor opens the door to develop novel sensing devices such as detection of viruses and disease biomarkers or selective labeling systems for in vivo imaging, as well as identification of food pathogens. At present, the major problems of biosensors are sensitivity and repeatability. Although there are no commercially available phage-based sensing devices, multiple technologies have been patented.[97,98]

Enhancing Bactericidal Efficiency

To augment bactericidal activity of phage and combat antimicrobial resistance, various engineered phages are developed and applied (Figure 3c). Since phage-resistant bacteria often escape phage killing, some bacterium-killing genes originated from different organisms are integrated to phage genomes, which is the so-called "double insurance" strategy. James Cass et al have developed a SASPject platform which integrated small acid-soluble spore proteins (SASPs) encoding gene of *Bacillus* to phage genomes.[99] SASP gene is ordinarily expressed only during sporulation, when SASPs are used to coat and protect the spore DNA. However, in vegetative cells, the binding of SASPs with DNA prevents replication and transcription and causes cell death.[100,101] In vitro studies showed that the product SASPject PT1.2 killed 225 diverse isolates of *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA).[102] The engineered phage Y2::*dpo*L1-C was constructed by introducing the depolymerase gene (*dpo*L1-C) of *Erwinia amylovora* phage L1 into the genome of *E. amylovora* phage Y2, which significantly increased the bactericidal efficiency compared to phage Y2.[103]

As we know, biofilm formation is "a devastating complication", which can shield bacteria themselves from the host immune system and antimicrobial therapy and cause treatment inefficiency. The extraneous enzymes are a promising supplement to the lytic phages to withstand those complex, matrix-reinforced biofilms.[8,104,105] For example, DspB, an enzyme that is produced by *Actinobacillus actinomycetemcomitans*, hydrolyzes β-1,6-*N*-acetyl-D-glucosamine, which is a crucial adhesin for biofilm formation and integrity in *Staphylococcus* and *E. coli*.[106] Timothy K. Lu cloned the *dspB* gene into T7 genome under the control of the strong T7Φ10 promoter. The removal rate of *E. coli* biofilms by the engineered enzymatic phage was 99.997% that was about two orders of magnitude better than the original phage.[107] Additionally, quorum-sensing (QS) is a process of bacterial cell-to-cell chemical communication, which is essential for virulence production and biofilm formation.[108] A lactonase enzyme encoding gene was inserted into T7 phage genome, which endows the engineered phage capability of inhibiting biofilm formation of both *P. aeruginosa* and *E. coli* since this enzyme could quench QS system.[50]

In addition, synthetic M13 phagemid could be used as a delivery vector to upload genes for antimicrobial peptides (AMPs), or toxin proteins.[109] Recombinant phagemid DNA is first transformed into a production strain harboring a helper plasmid to amplify abundant functional phagemids.[110] Therefore, along with M13 infection against target bacteria, antimicrobial peptides (AMPs) or toxin proteins are expressed and inhibit intracellular processes, causing the death of nonlytic bacteria. For example, the platform expressing cecropin PR-39 or apidaecin Ia showed strong antagonistic ability against *E. coli* and could induce bacterial cell death effectively.[111] The toxin networks-based expression of two copies of cecropin PR-39, apidaecin Ia, and topoisomerase inhibitor *ccdB* genes resulted in the robust killing of target *E. coli*.[112] Mice treated by this recombinant phagemid had an average survival rate of 80% over the course of the experiment, compared to a survival rate of 27% in the untreated group.[112,113]

Besides phage genome engineering, phage proteins could also be modified to enhance bacterial killing efficiency. Ran et al developed a multi-functional antibacterial system (APNB) for the treatment of multi-drug resistant *Acinetobacter baumannii* and its biofilm, through coupling a Nile blue photosensitizer (NB) to the capsid protein of *A. baumannii* phage.[114] The phage provides specificity and the photosensitizer produces reactive oxygen species (ROS). It is worthy to mention an interesting class of fluorescent dyes, AIEgens, which refer to fluorogens with aggregation-induced emission. Since the concept of AIE was proposed in 2001 by Tang and teammates,[115] many AIEgens and AIEgens-based photodynamic therapy (PDT) approaches have been developed, such as tetraphenylethene (TPE), tetraphenylpyrazine (TPP),[116] quinoline-malononitrile (QM),[117] and a bacterial-based AIE molecule (TBP-2) delivery system.[118] Xuewen He et al developed a novel chemical modification strategy for phage, integrating AIEgens with bacteriophage to form a new class of antimicrobial bioconjugates (TVP-PAP).[119] When TVP-PAP is mixed with bacteria, TVP-PAP can be used for specific bacterial recognition and real-time fluorescent tracking in the absence of UV radiation, and host killing via TVP −PAP-guided ROS generation when in the presence of UV–vis spectra.[119]

In total, phages inspire the development of various antimicrobial materials, which are the promising supplement to conventional treatment strategies or industrial applications.[8,104,105]

Altering Microbiota Composition

As microbiome modulators, phages can improve microbiome structure and suppress pathogens. Although it is infrequent in a clinic, researchers confirmed its authenticity in mice models and ecological microbiome.[120–124] Hsu et al constructed a mouse model carrying human gut commensal bacteria and tested the influence of lytic *E. coli* phage T4, *C. sporogenes* phage F1, phage *B. fragilis* B40-8, and *E. faecalis* phage VD13 on to the gut microbiome. The results showed that phages not only directly impacted targeted bacteria but also resulted in cascading effects on other bacterial species.[124] Zheng reported that *Fusobacterium nucleatum* lytic phage-guided nanoparticles reduced the side effects of chemotherapy drugs and promoted the proliferation of endogenic *Clostridium butyricum* in mouse models of colorectal cancer.[125] The strategy provides a new way for the development and application in cancer treatment. In addition, phages have also been utilized as a precise regulatory tool to control the natural rhizosphere microbiomes. Phages were used to decrease the incidence of *Ralstonia solanacearum* by up to 80% in tomatoes, which did not affect the existing rhizosphere microbiota.[126]

Conclusions and Perspectives

With the quick spread of AMR and the slow pace of new antibiotics discovery, the design and development of phage or phage functional elements, as novel and alternative antibacterial therapies, detection and diagnostic tools, have been proposed and implemented (Figure 4).[127–129] As well, developing a more convenient phage display technique is needed for protein engineering. In this new journey, the road ahead is still long. Considering the limitations of applications such as their narrow host range, immune toxicity, and low infection efficiency, it is time-consuming and laborious to select suitable phages from the natural phage library. Under "design-build-test-learn" theory, synthetic biologists manipulate phage genomes bottom-up by means of evolving molecular biology methods, such as recombination tools, large fragment cloning technology, CRISPR and base editing, cheap synthetic genes, and cell-free systems, to obtain engineered phages with specific functions to support pathogen defense, drug delivery, bacterial detection, and material science.

Engineering of phage is encouraging. The huge diversity of phage types and gene pools in nature is our valuable gift. So far, phage synthetic biology has only utilized a small part of the existing phage types, and the potential of the vast arsenal is far from being tapped. For commercial production and application of the engineered phages in "outside-The-lab" spaces in the future, challenges still remain, such as long-term stability, storage and production conditions, and production cost.[130] With the innovation of technology and the combination of multi-disciplines, it is believed that it will

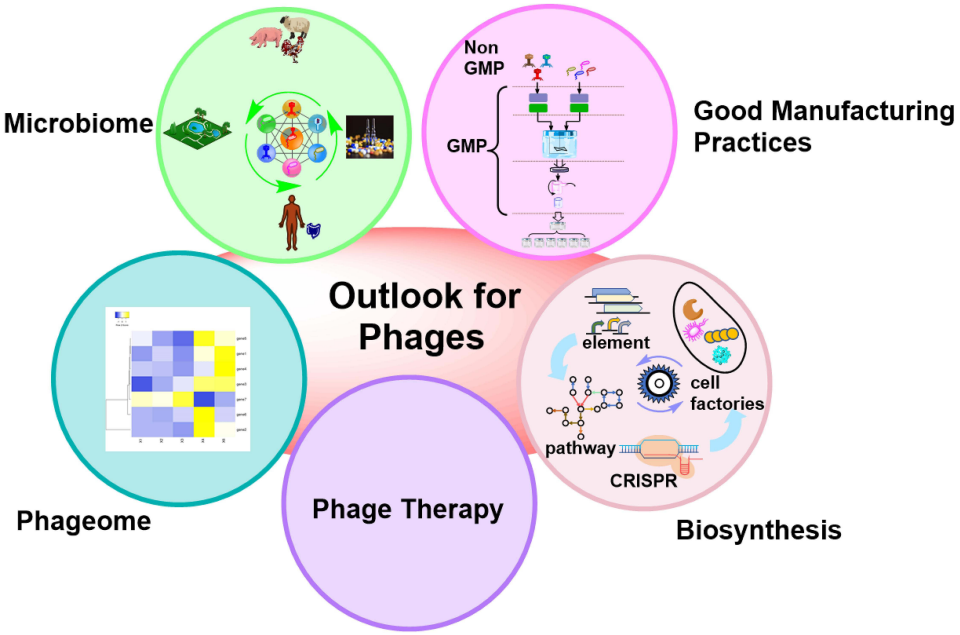

Figure 4 Perspectives of engineered phages. Development and improvement of phage therapy and Good Manufacturing Practices (GMP) provides possibilities for real-world applications. Phage is a powerful tool to shape microbiomes and also an abundant resource of genetic elements for biosynthesis.

come true to develop robust, cost-effective, safe, and efficacious platforms that can translate more and more suitable engineered phages in the lab into real-world applications.

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Disclosure

The authors declare no conflicts of interest.

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