

Current Topics

Challenges in the Control of Environmental Pathogenic Microbes

Review

Engineered Bacteriophages for Practical Applications

Javier Pizarro-Bauerle and Hiroki Ando*

*Department of Microbiology, Graduate School of Medicine, Gifu University;
1-1 Yanagido, Gifu 501-1194, Japan.*

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The diversity of advanced genetic engineering techniques that have become available in recent years has enabled a more precise manipulation of genes and genomes. Among these, bacteriophage genomes stand out as an interesting target due to their dependence on a host for replication, which previously complicated their manipulation, and due as well to the many possible fields in which they can be used. In this review, we highlight recent applications for which genetically modified bacteriophages are being employed: as phage therapy in medicine, animal industries and agricultural settings; as a source of new antimicrobials; as biosensors for research, health and environmental purposes; and as genetic engineering tools themselves.

Key words bacteriophage; synthetic biology; phage therapy; biosensor; directed-evolution

1. INTRODUCTION

Bacteriophages (phages) are a diverse group of viruses that infect bacteria. They were independently discovered just over a hundred years ago, by Felix d'Herelle and Frederick Twort.^{1,2)} Since their discovery, phages have been instrumental in landmark discoveries in genetics and molecular biology, and they remain to this day a fundamental piece of the geneticist's and molecular biologist's toolkit.

Phages are the most abundant biological entities on the planet, present in virtually any place that can harbor bacteria, with new phages being discovered and sequenced at a staggering rate. Phages essentially offer an infinite number of possibilities for genetic engineering and synthetic biology, given that phage diversity means that it is possible to find phages and/or phage-derived products able to perform multiple functions and under many different conditions. These functions can then be adapted, improved, and applied in many ways. Here, we review recent research in which engineered phages and phage-derived products are being used to improve human health, to increase food safety, and to develop new materials, among other applications.

2. PHAGE THERAPY

Given that phages are described as entities able to kill bacteria (even named after this capacity, from the Greek *phagein*, “to devour”), it should come as no surprise that their most well-known use is phage therapy, to combat bacterial infection. However, though their discovery quickly led to interest in treating human cases, the unknown nature of these phages, and the inability to study them further, led to mixed results. This, coupled with the discovery and subsequent widespread use of antibiotics, led to phage therapy being an almost completely unknown discipline for many years. However, with the growing menace of antibiotic resistance now looming over governments and health care institutions,^{3,4)} threatening mil-

lions of people worldwide, phage therapy has experienced a resurgence, with researchers across the globe getting involved.

However, while phages indeed have several advantages over antibiotics, there are important limitations to phage therapy, which are being tackled from different perspectives.

2.1. Broadening Host Range and Increasing Efficiency

One of the biggest hurdles faced by researchers in the development of phage therapies is that, given the constant struggle between phages and their host bacteria, it is not uncommon to find phages that can only infect one or a few strains of a certain species. This meant that, until recently, there were at least two approaches to phage therapy: first, to look for phages with broad activity in the hopes of maximizing the number of patients that could benefit from one uniform therapy; and second, to look for phages on a case-by-case basis, isolating the bacteria to be treated, and testing many different phages until finding the adequate candidate. Many potential therapies and applications in animal models have been published using these approaches. And recently, in what is probably one of the best known cases of phage therapy, total recovery was achieved in a patient with a multidrug-resistant *Acinetobacter baumannii* infection⁵⁾ through a phage cocktail specifically developed for the patient.

Another important obstacle to intervention with phage therapy is the fact that, unlike antibiotics, the detailed molecular composition of the phages is essentially unknown, especially those phages derived from environmental samples. This complicates the approval of phage therapy as a mainstream treatment option.

These hurdles have led scientists to evaluate the genetic engineering of phages as a feasible alternative to extend their host range. This could be advantageous for at least two related reasons. First, if the determinants of host range can be identified and manipulated, this could significantly reduce the amount of work needed to generate phages against specific bacteria. Second, if only a handful of selected, well studied phages are used as “scaffolds” for the generation of custom

*To whom correspondence should be addressed. e-mail: hiroando@gifu-u.ac.jp

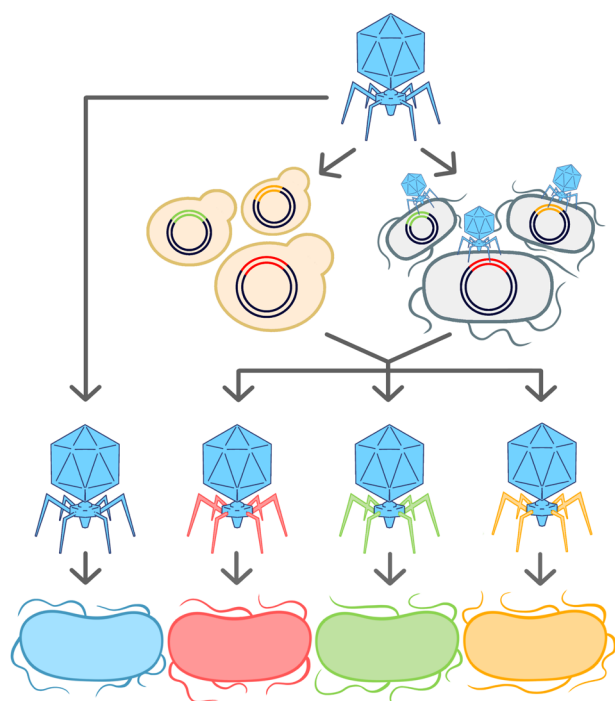


Fig. 1. Engineering Host Specificities

The host range of phages can be redirected by engineering a primary host determinant; in this figure, the tail fibers are used. A phage genome coding a different tail fiber gene can be designed *in silico*, assembled in a yeast cell, and rebooted in an appropriate bacterial cell. Also, a phage can infect host bacteria harboring a plasmid carrying a tail fiber gene with random mutations in relevant binding sites, then phages with mutated tail fibers are produced by recombination. (Color figure can be accessed in the online version.)

phages, this could enormously reduce the variability between treatments, making the translation to clinical applications more feasible.

In one example of this, well-studied phages were used as scaffolds, with their tail components manipulated in order to modify their host range⁶⁾ (Fig. 1). This was done by gap-repair cloning in yeast, where overlapping, synthetic fragments of a phage genome (with original or manipulated sequences) were provided, together with a suitable vector. All these were transformed into yeast, where the fragments were joined as determined by the overlaps and the genome was reconstructed. The resulting reconstructed product could be extracted and “rebooted” into a bacterial host to produce phages with the manipulated genome. This yielded, for example, phages that were able to infect new hosts, *e.g.*, a modified coliphage T3 able to infect *Yersinia pseudotuberculosis*, or a T7 phage able to infect *Klebsiella*. These phages, used as cocktails, also removed target bacteria from a mixed bacterial population.⁶⁾

This presented, however, a limitation: it is necessary to know the sequence of an infecting phage in order to engineer a “scaffold” phage to redirect it toward a target bacterium. This problem was approached in a following study, where the host-range determining regions (HRDRs) of phage T3 were identified and mutated, generating a high number of almost identical phages, but with different HRDRs (Fig. 1). This generated custom phages with extended host ranges without additional information requirements, and these phages suppressed the growth of target bacteria *in vitro* and in an *in vivo* mouse skin infection model.⁷⁾

Another approach involved the generation of transducing

particles, *i.e.*, phages lacking all elements necessary for producing progeny (Fig. 1). Among these elements are tail proteins that can be swapped for those of other phages, producing transducing particles with different host specificities. Then, the transduced sequences can be randomly mutated and retransduced, allowing the selection of mutants with increasing transduction efficiency. In this process, the particles, being non-self-replicating, cannot kill bacteria in the classical sense of phage therapy. On the other hand, this means that only plasmids containing the tail components need to be manipulated, instead of the whole genome. In addition to this, since the particles do not need to replicate within the target bacteria, this allows broader targeting options.⁸⁾ These transducing particles can be used instead to deliver genetic material that sensitizes antibiotic-resistant bacteria, as has been done with phages that deliver dominant antibiotic sensitive genes⁹⁾ or, more recently, clustered regularly interspaced short palindromic repeat (CRISPR)–Cas systems.^{10–13)}

Until now, the techniques described above had been developed for Gram-negative species; the characteristics of Gram-positive membranes make these techniques unsuitable for the species. However, a recent approach, proposed to solve this issue, is the use of L-form *Listeria* cells, which are devoid of a cell wall and are more amenable to transfection.¹⁴⁾ Using these cells, the authors were able to reboot not only *Listeria*-infecting phages, but *Bacillus*- and *Staphylococcus*-infecting phages as well, using both wild-type and modified genomes. One of the modifications introduced was the deletion of a region controlling lysogeny, making the phages strictly lytic, and therefore, more efficient at killing target cells.

Finally, a similar genetic manipulation strategy was part of a highly publicized clinical case, the first in which engineered phages were used for phage therapy on a human patient. The patient, a 15-year old, double-lung transplant recipient with an antibiotic-resistant *Mycobacterium abscessus* infection, received cocktails of phages, including a lytic variant of a phage—that is, with a repressor gene removed. Subsequent to phage therapy, the patient showed remarkable clinical improvement,¹⁵⁾ and even though that phage therapy, as the authors indicate, cannot be unequivocally indicated as the cause for recovery, it is undoubtedly an incentive to continue with research geared toward safe and efficient phage therapies.

2.2. Addition of Novel Features to Phages Even though, in some cases, phages alone can be enough to resolve a bacterial infection, it is desirable to make the treatment as efficient as possible. One reason is to reduce the amount of phages needed for treatment, given the difficulty of manufacturing phages compared to some classical antibiotics. This can be achieved, for example, by arming phages with extra molecular tools. A pioneer study in this field used a conventional phage expressing an exogenous dispersin B, a biofilm-degrading enzyme, leading to an improvement in bacterial killing of about two orders of magnitude.¹⁶⁾ A similar approach was used in a previously mentioned study,¹⁴⁾ where the authors, in addition to generating lytic variants of phages, incorporated into the phage genome an endolysin with a broader spectrum than the endogenous endolysin, increasing the efficiency of the lytic variant (Fig. 2).

In a similar fashion, the systems of quorum-sensing used by bacteria to regulate their virulence and life cycles can be manipulated using modified phages. An example of this is

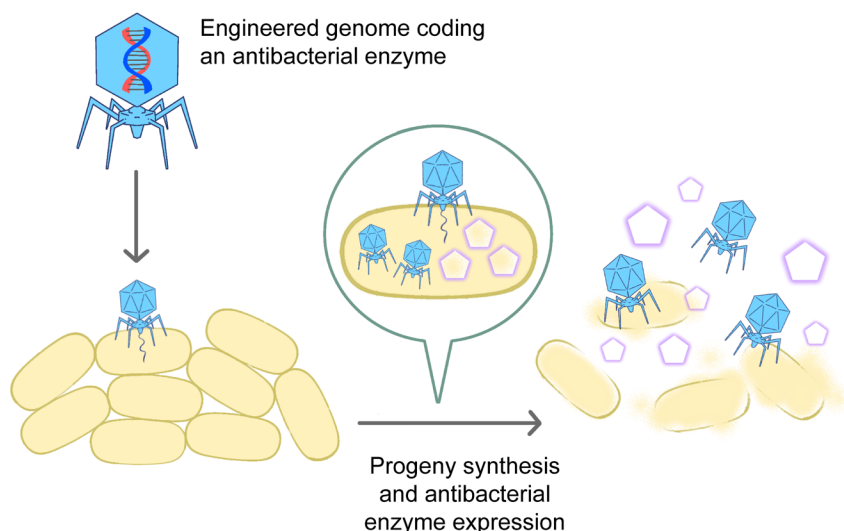


Fig. 2. Phages with Engineered Enzymatic Activity

After initial infection with an engineered phage carrying an enzyme with an activity detrimental to the host bacteria, the expression of this enzyme and the progeny phages cooperatively accelerate bacterial killing. The figure illustrates this process with a biofilm-degrading enzyme. (Color figure can be accessed in the online version.)

the use of a quorum-quenching enzyme to disrupt bacterial communication, leading to the inhibition of biofilm formation.¹⁷⁾ With the number of phages already sequenced, and the techniques available to improve enzyme function^{18–20)} and phage manipulation, these approaches could constitute a useful weapon to incorporate into future strategies.

2.3. Phage Therapy with Focus on Agriculture Phage therapy has been used in animals and plants²¹⁾ for several years now to improve growth rates, reduce mortality, and reduce antibiotic utilization, with several products currently available on the market.²²⁾ However, the use of genetic engineering in this capacity is just starting. A recent study focused on the pathogen *Erwinia amylovora*, the causative agent of fire blight, and an important threat to the production of several fruits. The authors introduced a depolymerase able to degrade the bacterial capsule into the genome of a phage able to infect a broad range of *E. amylovora* strains, significantly increasing its killing capacity.²³⁾

Another recent report screened a library of wild-type and recombinant peptidoglycan hydrolases for activity against *Staphylococcus aureus* in cow's milk,²⁴⁾ and found enzymes able to reduce the bacterial load in culture media and in milk. As mentioned before, currently available genetic modifications could take this approach even further, for example, by improving the activity of the hydrolases, or incorporating them into broad-spectrum phages.

3. SOURCE OF NEW ANTIMICROBIALS

Given that the complexity of therapy using whole phages was realized early on, there has been plenty of research using isolated phage products that show a capacity to kill bacteria. One of the most studied families of this type of molecule is lysins,²⁵⁾ one of which has completed a phase 2 clinical trial in humans. Even though this approach has involved a level of genetic manipulation from almost the beginning, some recent studies have tried to fine-tune the properties of the molecules studied. One early example of this is the fusion of pesticin, a toxin produced by *Yersinia pestis*, with the N-terminus of

T4 lysozyme. *Y. pestis* strains that produce pesticin protect themselves by producing a protein that blocks the action of pesticin. However, said protein is not capable of blocking the action of the T4 lysozyme domain of the fusion product, which can kill any cell expressing the pesticin receptor. This receptor is also a virulence factor, so this fusion preferentially targets virulent strains.²⁶⁾ A recent preliminary report, using a similar strategy, generated a fusion between a phage receptor binding protein and an endolysin, allowing passage through the outer membrane and killing *Escherichia coli*.²⁷⁾

Other studies, also looking to bypass the external membrane of Gram-negative pathogens, relied on the modification of specific amino acids in an endolysin to enable it to lyse *E. coli* from the outside of the cell,²⁸⁾ or in the fusion of endolysins with membrane-destabilizing peptides to generate molecules termed artilysins, active against antibiotic-resistant *A. baumannii* and *E. coli*.^{29,30)}

Antibodies have also been harnessed to work alongside lysins through the generation of hybrid molecules termed lysibodies. These consist of a carbohydrate binding domain, derived from either phage lysins or bacterial bacteriocins, bound to the Fc portion of an antibody. This connects the specificity of the lysins with the effector functions of the antibodies, since the obtention of strong, specific antibodies against carbohydrates is difficult using traditional methods. These lysibodies can, through the effector functions of the Fc fragment, protect animals from significant bacterial challenges.^{31,32)}

All this evidence seems to indicate that lysins and derived molecules will maintain or increase their roles as important components of the arsenal against infectious diseases, as reflected in the products currently undergoing clinical trials.^{33,34)}

4. BIOSENSORS

Given the high capacity of some phages to discriminate, even between different strains of the same species, it does not come as a surprise that phages have long been used in genetic studies and the characterization of clinical isolates, in a technique known as phage typing (Fig. 3). However, with the

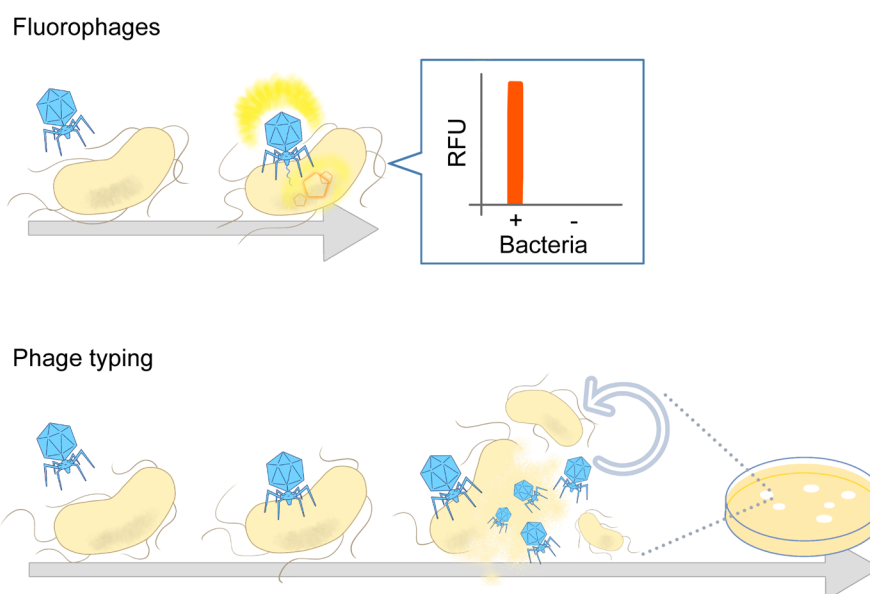


Fig. 3. Phages as Biosensors

Rapid and sensitive detection of target bacteria is possible by means of engineered phages able to generate a fluorescent signal upon successful infection (fluorophages). Traditional phage typing is useful, but it takes more time to detect slow-growing bacteria such as *M. tuberculosis*. (Color figure can be accessed in the online version.)

advancement of molecular biology techniques, this specificity has been improved in clinical settings, and has also been co-opted to detect a great diversity of organisms and substances, with practical applications in both the food industry and environmental science.

4.1. Detection of Pathogens in Clinical Settings and Food Industry One of the areas where phage-mediated detection has interesting possibilities is in the sensitive detection of pathogenic bacteria from clinical and food samples. Even though phages are very good at binding and infecting bacteria, the output of this process had usually involved long culture times, specific media, and the results could be misleading. Now, with the help of molecular biology tools, the detection is becoming easier, faster and more reliable.

For example, the classic diagnosis for tuberculosis detects *Mycobacterium tuberculosis* positive cultures, which requires 3–6 weeks of culture. Even though faster methods are available, the complexity and cost of these make their use difficult in many countries where tuberculosis is prevalent. Thus, faster, simpler and cheaper methods are needed. With the development of luciferase reporters, *M. tuberculosis*-infecting phages able to deliver these reporters were developed,³⁵⁾ and after some iterations^{36,37)} they have evolved into powerful tools (Fig. 3). For example, they can detect *M. tuberculosis* in sputum samples with 91.98% sensitivity and 98.96% specificity compared to the reference method, with a sample recovery time of only 96h (compared to 3–6 weeks), and with an estimated cost of 2 USD, using infrastructure already available in most laboratories working with *M. tuberculosis*.³⁸⁾ Since this method detects only metabolically active bacteria, it can detect resistance phenotypes that might not be detected by sequencing, which is used in most of the current fast-diagnostic techniques.³⁸⁾

Phage-based detection of pathogenic bacteria in the food industry has followed a similar path. For example, in the case of *Listeria*, luciferase-based reporters were also created,^{39,40)} and these reporters, coupled with the high affinity of phage lysins

for *Listeria* cell wall carbohydrates,^{41,42)} were used to construct a robust assay, enabling detection limits of 0.1–1.0 cfu/g *Listeria* in less than 24h, versus 96h by the standard method. As mentioned for tuberculosis, even though there are PCR-based assays faster than the standard method, they cannot differentiate among live and dead cells, which is critical for relevant results, and it is solved with this assay.⁴³⁾ Similar techniques have been also reported for the detection of *Salmonella*,^{44,45)} *E. coli*,⁴⁶⁾ and *Clostridium*^{47,48)} in different types of samples.

Also, the capacity of phages to pack exogenous genetic material has been used to manufacture “armored RNA,” where a pathogen’s RNA genome is packed using the MS2 system to generate particles that serve as a stable positive control for pathogen detection.⁴⁹⁾ In a recent study, an improvement of this technique was obtained using phage Q β , generating particles with increased thermal stability in a norovirus detection model.⁵⁰⁾

This evidence, together with the explosive increase in smartphone-based biochemical detection systems,^{51–55)} might point to a future where inexpensive, fast and easy detection of pathogens, assisted by phages, could become a daily occurrence.

4.2. Detection and Capture of Biological and Chemical Substances As mentioned before, specific manipulation of the binding specificity of phages is becoming more and more feasible. This means that the binding specificity can be reoriented to detect molecules completely unrelated to those relevant to the phage. Phages have been used for a technique known as phage display (subject of a Nobel Prize in 2018), where proteins or peptides of interest are expressed on the phage surface, and then those with the desired activity (e.g., binding, enzymatic activity) can be selected and studied (Fig. 4). A recent example of an application for this technique was the identification of phages expressing peptides able to bind gallium.⁵⁶⁾ Gallium is a metal of high importance for the manufacture of electronics, but its future availability is uncertain, so new techniques are aiming to recover gallium from

Phage display

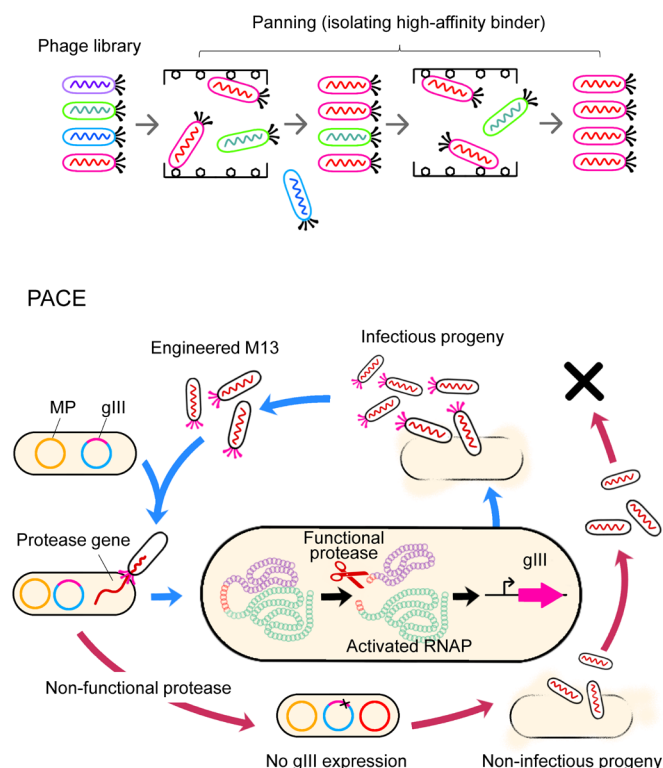


Fig. 4. Phage-Based Selection and Evolution of Molecules

In phage display, phages expressing candidate binding molecules are panned against target molecules, and iterations of this process allow the selection of desired molecules. Building on this principle, phage-assisted continuous evolution (PACE) can select and improve a molecule of interest. This requires a phage (*i.e.*, M13) where an essential gene (*i.e.*, a capsid gene, gIII) has replaced the gene of interest (the protease gene in this figure), as well as a culture of host bacteria able to supply the product of this gene *in trans*. However, the bacterial expression of this essential gene is engineered to be activated only if the molecule of interest encoded by the phage can perform the desired activity. Host bacteria also carry a mutagenic plasmid (MP) able to mutate the gene of interest during phage replication. Thus, only phages with the desired activity will replicate, and mutagenesis will generate variants that can be further selected and amplified, considerably improving the desired activity. (Color figure can be accessed in the online version.)

electronic waste.

Using a peptide also developed by phage display, able to bind the toxic herbicide paraquat, Lee *et al.*⁵⁷⁾ reported the improved sensitivity of a surface-enhanced Raman spectroscopy (SERS)-based paraquat detector, achieved by expressing the peptide on the surface of phage M13 and then using the obtained phages to functionalize the detector, obtaining a 3.9-fold increase in signal intensity.

Another innovative M13 phage-based application has been reported,⁵⁸⁾ where the phage surface, through amine residues on the gene 8 product, was functionalized with fluorescent dyes. This allows the phage to function as a “virus laser,” with tunable optical properties due to the repetitive arrangement of M13. The binding specificity can be modified to detect different molecules of interest, and the authors show, choosing a specific monoclonal immunoglobulin G (IgG) antibody as a target, a sensitivity of 90 fmol/mL without the need to previously immobilize the target on a surface, as is currently done in most routine techniques.⁵⁸⁾

5. VACCINE CARRIERS

Given their stability, and their ability to present molecules on their surface, another use that seems reasonable for phages is to serve as substance carriers, independently of their capacity to infect bacteria. Among the possibilities, their use in experimental vaccines as epitope carriers is probably the most prominent, with encouraging results in several animal models against bacteria, fungi, viruses, and cancer. For example, phage T4 was engineered to express *Bacillus anthracis* and *Y. pestis* antigens, to generate a double viral vaccine against anthrax and plague that generated a specific immune response and protected mice, rats and rabbits from bacterial challenge, even when administered simultaneous lethal doses of anthrax toxin and *Y. pestis*.⁵⁹⁾ Similarly, a *Candida albicans*-derived peptide expressed on a filamentous phage elicited an immune response and improved survival rates against a systemic candidiasis challenge.⁶⁰⁾ As for vaccines against viruses, there are recent examples for protection in mice against human papillomavirus (HPV) types associated with cancer, using MS2 virus-like particles (VLPs) expressing an HPV protein,⁶¹⁾ and for *in vitro* neutralization of the Zika virus by antibodies generated against different epitope-carrying VLPs.⁶²⁾ Finally, there have also been reports of phage-based vaccines against cancer, such as melanoma neoantigens expressed on phage T7 that elicited immune responses and antibodies that bound to melanoma cells,⁶³⁾ or phages carrying epitopes of a mutated version of HER2, able to break immune tolerance and provide protection in a breast cancer model in mouse.⁶⁴⁾ For a more in-depth review of vaccine-related uses for phages, the reader is referred to Bao *et al.*⁶⁵⁾

6. PHAGES AS NEW GENETIC ENGINEERING AND MOLECULAR BIOLOGY TOOLS

Phages have been essential to genetics, molecular biology and genetic engineering. They have served as models, playing prominent roles in landmark studies such as the demonstration that the genetic material is DNA,⁶⁶⁾ the elucidation of the genetic code,⁶⁷⁾ and with the first genome ever to be sequenced belonging to phage ϕ X174.⁶⁸⁾ They have also been a source of many fundamental tools used in diverse techniques, such as T4 polynucleotide kinase, T4 ligase, T7 RNA polymerase, the Cre-Lox recombination system, and in using phages themselves as cloning vectors.

However, with an ever-increasing quantity of phage genomes available, researchers are looking for ways to expand the molecular biologist's toolkit. The use of phages in phage display technology was previously mentioned. Recently, a similar principle, coupling the specificity of phage display with the selection of the desired phenotype on host bacteria, allows for the phage-assisted continuous evolution (PACE) (Fig. 4) or non-continuous evolution (PANCE) of biomolecules.^{69,70)} In this method, the protein of interest replaces a phage protein critical for replication. Then, the phages are added to a medium with bacteria carrying a plasmid that can produce the missing phage protein, but only if the exogenous protein in the phage can perform the desired activity. This, coupled to a mutagenic plasmid in the same bacteria, prompts the appearance of variants that continuously improve, in order to compete for phage persistence and replication. A recent

example of this method is the evolution of a methanol dehydrogenase of *Bacillus methanolicus* in order to obtain variants with up to 3.5-fold improvements on their $V_{\max}$,⁷⁰⁾ opening up new possibilities for the conversion of methanol into other molecules of interest.

Other elements of phages being revamped are their integration systems; for example, a recent investigation used the integration system of phages Φ CD27 and Φ C31 to stably engineer a biosynthetic pathway for butyrate in the gas-fermenting bacteria *Clostridium ljungdahlii*. This organism can utilize carbon monoxide and carbon dioxide, but until now there were few genetic manipulation tools available.⁷¹⁾ In a similar fashion, these recombinases are being used to study different eukaryotes. For example, a mouse artificial chromosome was modified so it could be edited by three different phage integrases. The transient expression of one of said integrases allowed the specific editing of its target sequence without affecting the targets of the other integrases.⁷²⁾ One of these integrases was also shown to work in *Drosophila melanogaster*, adding to the classical tools available for this valuable model species.⁷³⁾ These results show that phages as a source of new tools for genetic engineering and molecular biology are far from being exhausted, with likely much more to come from yet unknown phages.

7. PHAGES IN MATERIAL SCIENCE AND INDUSTRY

Perhaps some of the most surprising and yet diverse applications of engineered phages are those found in material science and industry. A significant portion of these applications relies on the repetitive structures found on the surface of phages, and the possibility of introducing functionalization at specific points. One example of this is M13 phages engineered to express a peptide that binds graphene (a material with many applications in nanotechnology), but that also has negative charges. When interacting with graphene in aqueous media, the phages increased the stability of graphene, and the negative charges allowed the nucleation of cations of interest to generate stable, electricity-carrying nanostructures that performed better than those generated without phages.⁷⁴⁾ A similar strategy used phage-expressed peptides containing amino acids with side chains that can establish different types of interactions, enabling the assembly of phage-based nanoporous structures, which were then used as a scaffold for electronic applications.⁷⁵⁾ The same principle was used to construct M13 based aerogels, porous 3D structures with relevant mechanical features, such as elastic behavior,⁷⁶⁾ to construct phages able to self-assemble in order to originate structures with piezoelectrical properties,⁷⁷⁾ or to construct phages with an altered dipolar moment, allowing the generation of nanowires that can be pole-aligned *in situ*.⁷⁸⁾

Another emerging application is the use of engineered phages in the generation of biomaterials with novel properties, e.g. phages carrying peptides that stimulate tissue regeneration and the differentiation of cells toward a specific cell type, such as osteoblasts^{79,80)} or myocytes.⁸¹⁾ For a more in-depth review of this topic, the reader is referred to the recent work of Cao *et al.*⁸²⁾

As for industry, an interesting example is found in the work of Kim *et al.*,⁸³⁾ in which the authors, using a phage derived lysin, were able to control, to different degrees, the con-

tamination of ethanol-producing yeast cultures by undesired bacteria. Given the relevance of fermentation to industrial processes, any modification that can improve performance could have a significant impact on overall process efficiency and economic viability.

8. FUTURE CHALLENGES AND PROJECTIONS

Aside from the obstacles previously mentioned, currently being tackled by researchers around the world, there are some additional practical concerns that reduce the efficiency of phage related applications.

For example, given the lytic capacity of phages, an important concern for phage therapy or the administration of phage-derived products is potential side effects. Even though phage administration is increasingly considered devoid of serious adverse side effects, phage therapy usually relies on the phage lysing the bacteria, thus, the release of endotoxins is an ever-present concern. The endotoxin can usually be removed to safe levels, but this usually complicates the manufacturing process and/or makes it more expensive. This has led to studies looking to use non-lytic, non-replicating versions of phages in order to control these parameters,⁸⁴⁾ but this also has limitations.⁸⁵⁾ Given the possibility that the phages escape from the subjects receiving therapy into the environment, it is be important to develop ways to prevent this escape, that is, by generating phages that can be easily contained.

Their foreign nature suggests that immune host responses could make the therapy less efficient or even dangerous, but in the limited cases in which phage therapy has been applied to humans, only modest anti-phage responses have been recorded. Moreover, the contribution of the host immune system may even synergize with the phage to make the therapy succeed, as suggested by Roach *et al.*⁸⁶⁾ However, phage clearance is indeed usually fast, which demands high-titer preparations. At least one report indicates that this problem might be more relevant in genetically manipulated phages.⁸⁷⁾ A similar phenomenon is seen with endolysins; however, a recent report indicates that the engineering of an endolysin in order to add an albumin-binding domain extended the half-life of the endolysin.⁸⁸⁾

Another area that could become relevant, if phage therapy advances toward widespread use, is the study of storage conditions and administration routes. For example, even though phages are considered relatively stable, being able to unequivocally guarantee their stability under different conditions would be useful. A study by Carrigy *et al.*⁸⁹⁾ reports phage stability for prolonged times at room temperature for a powdered preparation of a phage directed against *Campylobacter jejuni*; this low-cost process may be applied to other phages. Future studies might also assess the usefulness of genetic modification to improve phage stability. As for administration routes, early studies have centered on topical use, but this is limited to only some applications. The more recent, high-profile cases have relied mainly on intravenous administration, but this restricts its use in a daily context. One obvious choice, oral administration, is hindered by the obstacle of the hostile conditions found by phages in the digestive system. However, one study reports a genetic modification to phage T7 that enables the binding of lipids to its capsid, increasing phage stability in conditions simulating those found in different animals,⁹⁰⁾

and a recent case report shows the bacterial elimination of a drug-resistant *Klebsiella pneumoniae* strain by a combination of oral and intrarectal phage administration.⁹¹⁾ Nasal administration has also been considered, especially for bacteria affecting the respiratory system. Recently, a small phase I trial for *S. aureus*-associated chronic rhinosinusitis showed that the treatment is safe, and preliminary clinical improvement was observed.⁹²⁾ However, to the best of our knowledge, the studies related to administration routes have not used genetically manipulated phages, even though this could enable, for example, the targeting of phages to specific mucous membranes.

In another report, lysostaphin, delivered as part of a hydrogel in a fracture model, prevented infections, with better results than either antibiotics or soluble lysostaphin, while supporting fracture healing.⁹³⁾ A similar method was used to deliver phages against *P. aeruginosa*, where phage release was regulated by modulating the gel degradation; this method also reduced the bacterial load in a bone infection model.⁹⁴⁾ This, coupled to phage engineering techniques, could prove useful in developing new strategies to help with fractures, implants or surgical intervention sites, where bacterial infections are commonly encountered.

Finally, in the areas of phage engineering and synthetic biology, at least two areas for growth should be mentioned. First, given the use of phages as carriers of genetic information, and the fact that this sometimes requires the deletion of phage information, thereby limiting the size to be introduced, it could be helpful, as done with bacteria,⁹⁵⁾ to determine the minimal number of genes necessary for phage replication, and which genes can be removed without negative consequences; similarly, if the goal is to manipulate individual components of phages, it could be useful to have “decompressed” phages, as done for phage ϕ X174,⁹⁶⁾ where overlapping genes were separated in order to make individual modification easier. Second, for a long time, phage generation has inevitably required the use of a bacterial host, especially when big numbers of phages are required, as is the case in phage therapy. This has recently started to change with the application of cell-free systems in phage production, with a report describing the synthesis of functional T4 phage, a phage of significant size.⁹⁷⁾ If these advances can be escalated to a level able to meet the quantities necessary for different applications, this could prove to be a game changer, especially for phage therapy, since this would also remove the necessity for endotoxin removal, allowing for safer and more straightforward phage production.

9. CONCLUSION

As the reader realizes, there is still much to be researched before many of these exciting applications can find their way into daily use. However, a shift in the public perception of phages seems to be taking place, in part due to the looming threat of antibiotic resistance, but also due to the spread of promising results in the first publicized cases of phage therapy. If this is accompanied by legislative and regulatory efforts by government bodies and organizations, phage therapy and other phage-related applications may become mainstream, contributing, through disease management, clinical diagnostics, food safety and industry, to a higher QOL around the world.

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Conflict of Interest The authors declare no conflict of interest.

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