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Bacteriophage-based synthetic biology for the study of infectious diseases

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Since their discovery, bacteriophages have contributed enormously to our understanding of molecular biology as model systems. Furthermore, bacteriophages have provided many tools that have advanced the fields of genetic engineering and synthetic biology. Here, we discuss bacteriophage-based technologies and their application to the study of infectious diseases. New strategies for engineering genomes have the potential to accelerate the design of novel phages as therapies, diagnostics, and tools. Though almost a century has elapsed since their discovery, bacteriophages continue to have a major impact on modern biological sciences, especially with the growth of multidrug-resistant bacteria and interest in the microbiome.

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Background

With the discovery of bacteriophages generally being credited to Frederick Twort [1] and Felix d'Hérelle [2] in the early 20th century, these virus particles were so named (Greek, 'bacteria eaters') based on their observed ability to lyse bacterial cells. The use of naturally occurring phages as therapeutics for the treatment of bacterial infections was quickly realized by d'Hérelle and others, with interest continuing to flourish until the discovery and production of penicillin [2–4]. Antibiotics heralded a new age of effective small molecule treatments for bacterial infections, with phage therapy falling out of favor in the Western World [5,6]. Though phages have remained an important tool in the study of molecular biology, genetics, and bacteria [7], concerns over the ever-dwindling arsenal of antibiotics for the treatment of multidrug-resistant bacterial pathogens have also resulted in a renaissance in phage studies and in phage-based therapies as a means to develop alternative therapeutics [8–11]. Correspondingly, advances in synthetic biology have refined the ability to design, modify, and synthesize these viruses, which has enabled novel strategies for creating bacteriophage-based tools for the study and treatment of infectious diseases. The goal of this review is to explore the methods and demonstrations by which such tools can be employed to engineer modified phage and phage parts. For more information concerning the history, applications, and challenges of phage therapy using natural, unmodified viruses, the reader is referred to other reviews [12–15].

Synthetic biology aims to rationally engineer new functionalities in living systems by co-opting and modifying biomolecules crafted from millennia of evolution [16–18]. Cells operate as highly complex computational systems able to dynamically interrogate and respond to their environment. For the past decade, synthetic biologists have laid the foundational rules of biological design [19], constructed a catalog of standardized genetic parts, and assembled simple circuits, such as oscillators [20], switches [21], and Boolean logic gates [22••,23•]. Rational engineering has yielded cellular devices able to produce potent antimalarial compounds [24], to detect and kill pathogenic bacteria [25] and cancer cells [26], to reprogram cell fate [27], and to treat metabolic syndrome [28]. Finally, advancements in DNA synthesis and assembly have enabled the rapid development of higher-order genetic circuits [29–31] of medical [32] and industrial [33,34] relevance. This field has been accelerated by phage-derived technologies, while concomitantly enabling new approaches to engineering phages themselves.

Phage-enabled technologies

Phage display

Described by Smith in 1985 [35], phage display is a methodology employed extensively in both the study of infectious diseases and the development of novel therapeutics. Libraries comprising synthetic random peptides or natural peptides derived from pathogen genomic or cDNA are fused with a coat protein of a bacteriophage, often M13, Fd, or λ, such that the peptide is displayed on the phage surface. Iterative selection steps are employed

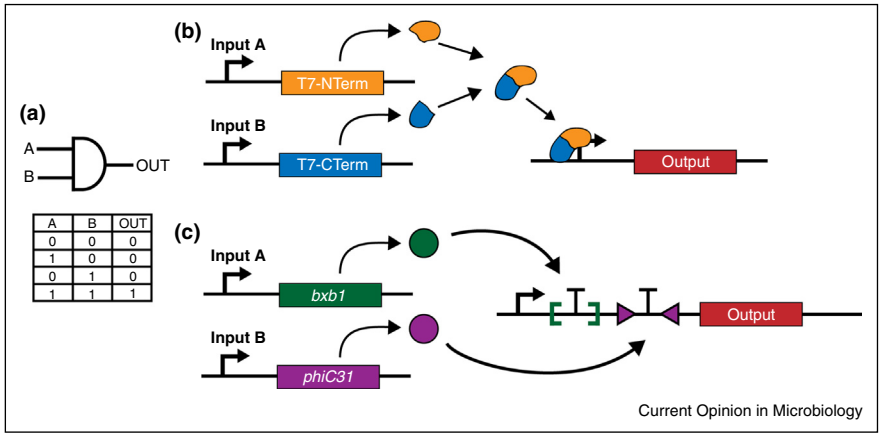
to enrich for phage particles that bind with high affinity to an immobilized target molecule of interest, which are then eluted and propagated in *Escherichia coli*. Since the identity of the displayed peptide is genetically encoded in the phage genome, protein–ligand interactions can be screened in high-throughput to identify molecules with novel biological functions. Phage display has enabled the discovery and characterization of bacterial adhesins [36,37], which bind to receptors on host cells or extracellular matrix and are implicated in establishing infection, as well as antigens used for vaccine development [38]. Moreover, bioactive peptides that block anthrax toxin binding [39] or inhibit cell wall biosynthesis enzymes in *Pseudomonas aeruginosa* [40] were isolated from phage display libraries. Development of antibody-based therapeutics has also greatly benefited from the technology, which can be implemented to rapidly screen random libraries of antigen-binding domains [41]. In a demonstration of direct therapeutic application, phage particles selected for high affinity interaction to *Staphylococcus aureus* were conjugated to chloramphenicol prodrugs to deliver localized, lethal payloads [42]. The applications of phage display are vast and the reader is referred to other literature [43–48] for a more thorough discussion of additional examples.

Bacteriophage-derived parts for synthetic biology

Bacteriophages have formed the backbone of molecular biology, having championed the demonstration of DNA as genetic material [49], the proof of Darwinian natural selection [50], and the ubiquitous use of phage-derived enzymes for common laboratory protocols [51]. Similarly, bacteriophage components constitute a core set of parts in the toolbox of a synthetic biologist. The DNA-dependent RNA polymerase of T7 can specifically drive high-level transcription from the T7 promoter (P_{T7}) both *in vitro* and *in vivo*. The polymerase has been used to reconstitute *in vitro* genetic circuits [52], such as switches [53] and oscillators [54], which permit precise mathematical modeling of biological reactions to inform future predictive design. Moreover, libraries of orthogonal T7 and P_{T7} variants, which exhibit lower toxicity [55] or are split into parts to function as AND gates [56•] (Figure 1a and b), have been constructed to permit higher-order construction of artificial genetic circuits. For example, an AND gate that only outputs a Boolean TRUE value when both inputs are TRUE can be implemented by having an output gene that is only expressed when both parts of a split T7 RNA polymerase are expressed. When coupled with orthogonal ribosomes that do not translate host mRNAs, a fully insulated transcription-translation network was constructed in *E. coli* for protein expression [57].

Furthermore, bacteriophage recombinases have been used in the construction of genetic circuits that record memory of past reactions [58] (Figure 1c). Recombinases manipulate DNA by recognizing specific sequences and catalyzing the excision, integration, or inversion of DNA segments depending on the location and orientation of the recognition sites. Thus, recombinase expression is coupled with a physical change in genetic material of the cell that can be sequenced to assay exposure to past events. The Cre, Bxb1, and PhiC31 recombinases have been used in the construction of a variety of synthetic circuits including counters [59], a rewritable memory module [60], Boolean logic gates [22••,23•], and digital-to-analog

Figure 1



Bacteriophage-derived parts can perform molecular computation in microbial cells. **(a)** Truth table for AND gate function. An AND gate has a TRUE output only when both inputs are TRUE. **(b)** Split T7 RNA polymerase can act as a transcriptional AND gate. Inputs A and B, which can be exogenous signals such as small-molecule inducers or endogenous signals, control expression of the N-terminal and C-terminal halves of T7 RNA polymerase, respectively, such that simultaneous expression leads to a reconstituted polymerase that can drive production of the output gene [56•]. **(c)** Bacteriophage-derived recombinases Bxb1 and PhiC31 implementing AND gate functionality with integrated memory. Recombinase activity leads to inversion of the intervening terminator sequence flanked by recognition sites. Activation of Bxb1 and PhiC31 leads to inversion of two unidirectional terminators, such that RNA polymerase can drive expression of the output gene [22••].

converters [22••]. Since these recombinases do not require additional host factors, genetic circuits founded on their activity could theoretically function in a wide range of infectious hosts to permit recording of gene expression in various environments.

Genome engineering

In addition to tools for classical molecular biology, phage-derived enzymes and technologies have led to genome-scale engineering techniques critical to the tailoring of strains for specific applications. Recombineering is a powerful technique that uses homologous recombination to introduce highly targeted modifications, insertions, or deletions to loci within cells. This technique has been enabled through the transformation of DNA products bearing flanking homology to target sequences in conjunction with the highly active Red recombination system from phage λ. It has been applied toward modifications in a variety of Gram-negative bacteria [61], including *E. coli* [62], *Salmonella enterica* [63], *Shigella flexneri* [64], *Vibrio cholerae* [65], *Yersinia pestis* [66], and *P. aeruginosa* [67].

A system with the potential for high-efficiency modifications in a large range of bacteria, including both Gram-positive and Gram-negative examples, has recently been described by combining broad-host mobile group II introns, or 'targetrons,' with the widely used Cre/*lox* recombination system from phage P1 [68••]. This technique, known as Genome Editing via Targetrons and Recombinases (GETR), involves first targeting introns containing *loxP*-derived sites to a specific location in a bacterial genome and subsequently using Cre recombinase to catalyze recombination between *loxP* sites on the chromosome and on a targeted construct. This technique can be used to achieve insertions, deletions, inversions, or even relocation of a chromosomal locus, depending on the design of the sites and constructs. Mutations in the wild-type *loxP* sequence allow for control of directionality of recombination as well as the generation of orthogonal sites permitting GETR to be used at multiple loci without crosstalk [68••,69].

Accelerating evolution

An important extension of genome-editing techniques has been the development of multiplex automated genome engineering (MAGE), a technique for generating genetic diversity through the iterative process of λ-Red protein β-mediated recombineering with a pool of short, ssDNA oligonucleotides targeting a single or multiple genomic loci. In the initial study published by Wang *et al.* [70], MAGE was used to generate a mutant strain of *E. coli* with increased production of lycopene using oligonucleotides designed to simultaneously target 24 genetic components. Automating the growth, transformation, and recovery phases of the procedure has the potential to enable hands-free rapid evolution of a population. Along with specialized genome assembly techniques, MAGE was used to recode all UAG stop codons from a strain of *E. coli* in order to generate a free, customizable codon (UAG), thus demonstrating the potential capacity to edit the genetic code in engineered organisms [71,72••]. MAGE presents additional opportunities as a tool for infectious disease research by enabling the rapid optimization of antimicrobial gene circuits or as a means for introducing diversity into organisms and mapping out their evolutionary trajectories. Similarly, MAGE could be applied toward evolving improved or even novel functions in bacteriophages by diversifying key phage proteins, such as host recognition elements.

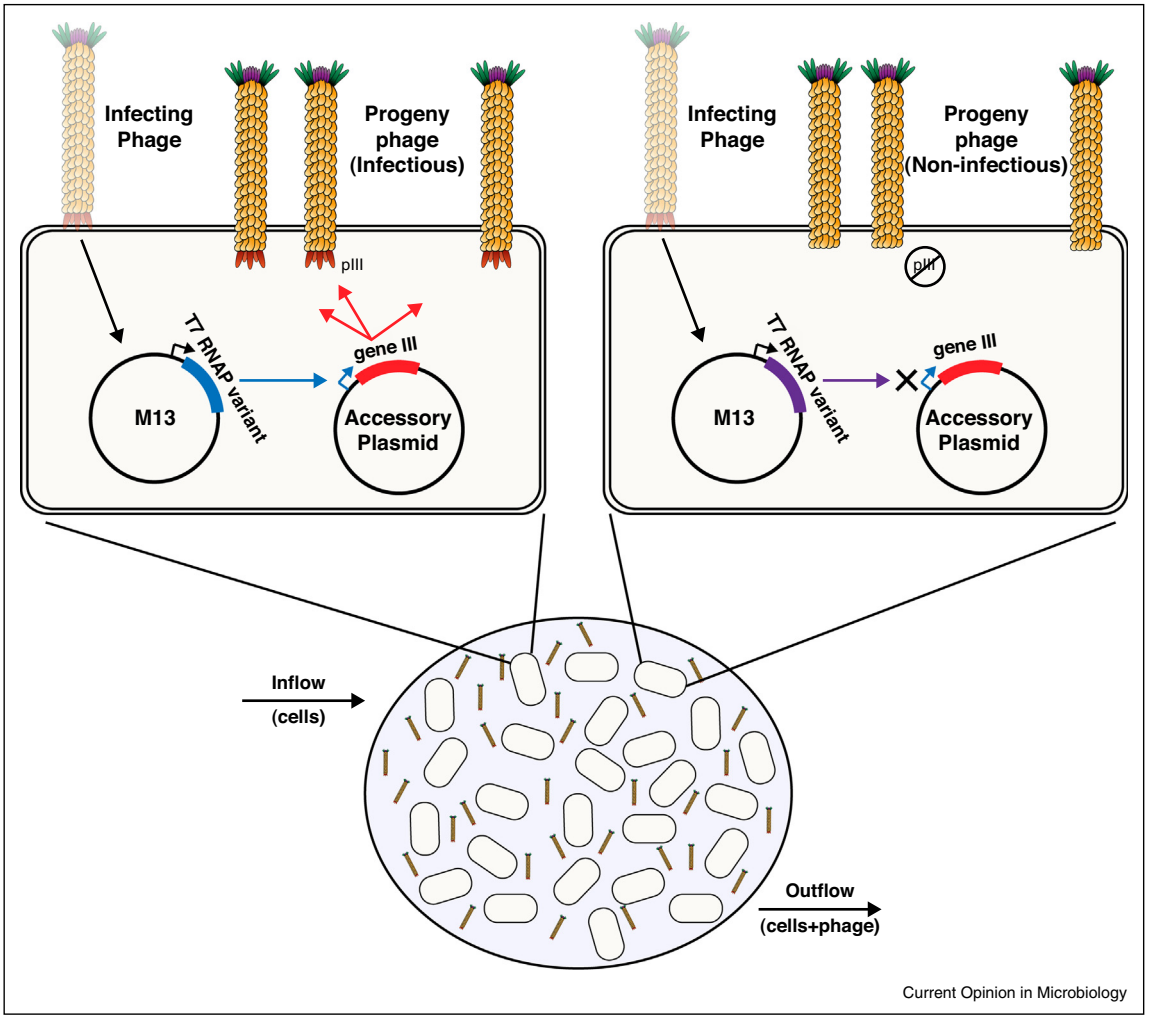
Phage-assisted continuous evolution (PACE) as introduced by Esvelt *et al.* [73] is another stride in accelerated evolution enabled by phage-based technology. In PACE, the life cycle of the filamentous phage M13 is linked with an activity of interest to be evolved, which is used to drive production of pIII, the minor coat protein required for adsorption of infectious phage particles to the cognate receptor on recipient cells (Figure 2). This technique was first used to evolve T7 RNA polymerase variants with new properties, such as the ability to recognize novel promoter sequences, by replacing the gene encoding pIII on the phage genome with the gene for T7 RNA polymerase and moving pIII expression under the control of a target promoter on a heterologous plasmid. In this way, a mutant T7 RNA polymerase with an improved ability to initiate transcription from the target promoter results in increased pIII production and a higher titer of infectious phages. As with MAGE, the PACE platform enables automated evolution, this time by maintaining productive phage in continuous culture within a lagoon with a constant inflow of fresh bacterial cells and an outflow ensuring removal of non-propagative phage variants. PACE has been used to explore how different parameters affect the pathways of genotypic and phenotypic divergence and convergence, contributing toward understanding how evolution acts on single genes and potentially improving the optimization of future engineering efforts [74•,75].

Phage-enabled therapies and diagnostics

Antimicrobial phages

Rather than entirely redesigning or repurposing isolated phages, some engineering efforts in synthetic biology have been made toward adding functions or improving existing phages. For example, Lu and Collins [76] incorporated the gene encoding DspB, an enzyme that degrades a polysaccharide adhesin implicated in biofilm formation, into an engineered T7 phage. This modified phage effectively cleared *E. coli* biofilms through cycles of infection, phage-mediated lysis, and release of the recombinant dispersin enzyme to enzymatically degrade the biofilm material itself and expose protected cells. Additionally, the phage was modified to carry a gene from phage T3 in order to expand its host range and permit infection of the biofilm-forming strain used in the

Figure 2



Phage-assisted continuous evolution. In phage-assisted continuous evolution (PACE), the protein activity of interest is linked to expression of the M13 coat protein pIII, which is required for binding and initiation of the phage infection cycle [73]. Within infected cells (top), the protein of interest is expressed from the injected M13 genome and successful target activity drives expression of gene III on the accessory plasmid, permitting the assembly of infectious phage progeny that can be further amplified and selected through subsequent rounds of infection (left). In the example shown here, the protein of interest is T7 RNA polymerase (T7 RNAP), which can successfully drive production of gene III only if it recognizes the promoter that controls gene III expression, thus enabling the evolution of T7 RNAP variants that can target new promoter sequences. If activity is insufficient to drive expression of the coat protein, progeny will be non-infectious and fail to amplify. The target protein is evolved in a continuous fashion in the 'lagoon' (bottom), where the encoding infectious phages continually amplify via the input of fresh cells, while non-infectious particles fail to infect new cells and are removed in the outflow.

study. However, despite the promise of phage therapeutics, bacteria can display resistance toward phages through innate means, such as restriction-modification systems [77,78], as well as adaptive means, typified by clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) systems [79]. Moreover, mechanisms may emerge in a bacterial population during the course of selective pressure by phages, including phenotypic [80] and genotypic [81] causes of decreased phage adsorption, among others [82]. These hurdles may be tackled through the use of phage cocktails [83], high-throughput phage evolution, or perhaps, given predictable evolutionary pathways, through the rational engineering of phages [10].

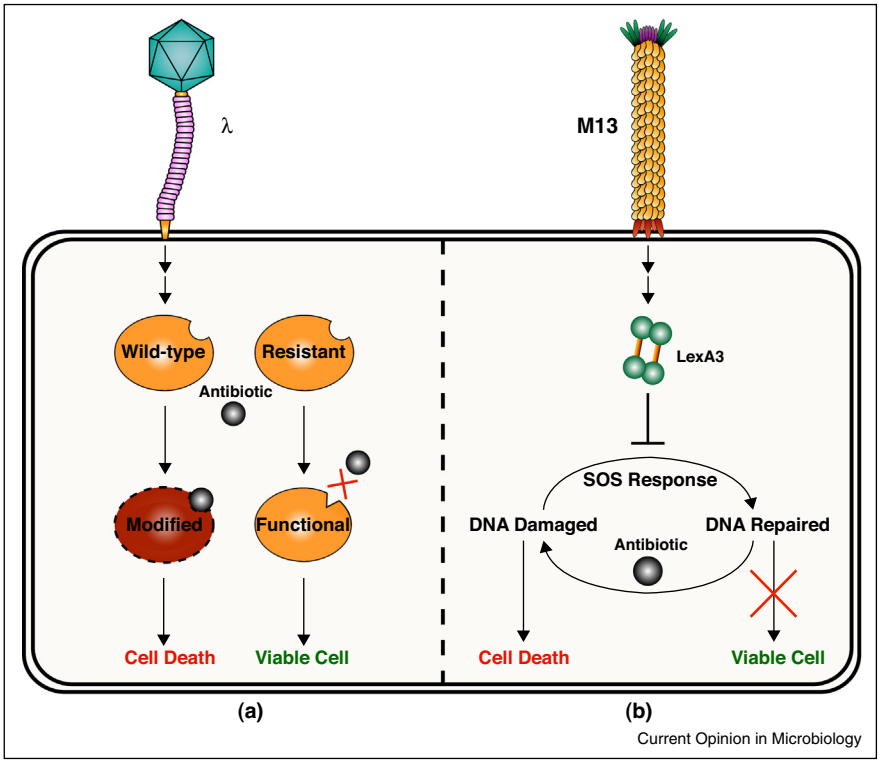
In contrast to taking advantage of a phage's natural ability to lyse a target cell, some studies have focused on using virus particles for their capacity to deliver nucleic acids to target cells. Such an approach was taken by Westwater *et al.* [84], in which the group utilized the non-lytic, filamentous phage M13 to deliver specialized phagemid DNA in place of the phage genome to target cells. The engineered phagemids (plasmids carrying signals to enable packaging into phage particles) were designed

to encode the addiction toxins Gef and ChpBK to elicit destruction of target cells. Hagens and Bläsi [85] also applied this toxic payload concept using M13 to deliver genes encoding the restriction enzyme BglII or the λ *S* holin to kill target *E. coli* by the introduction of double-stranded breaks in the chromosome or the creation of cytoplasmic membrane lesions, respectively. Subsequently, delivery of BglII was used to rescue mice infected with *P. aeruginosa* by adapting the system with an engineered derivative of the *P. aeruginosa* filamentous phage Pf3 [86]. These methods also resulted in a marked decrease in release of endotoxin, one of the major concerns with lytic phage therapy [15], as compared to killing via lysis by a lytic phage [85,86]. More recently, M13-derived particles were used to express a lethal mutant of catabolite activator protein in *E. coli* O157:H7, a food-borne pathogen that causes outbreaks of hemorrhagic colitis [87]. Biotechnology companies have also begun to make use of recombinant phage methods, such as virus-like particles that deliver genes encoding small, acid-soluble proteins to cause toxicity to target cells through non-specific binding to DNA [88].

Phage-based delivery of antibiotic-sensitizing cassettes

Rather than encoding killing functions directly within phage particles, Edgar *et al.* [89•] used phage λ as a chassis to generate antibiotic-resensitizing particles through the delivery of dominant wild-type copies of *rpsL* and *gyrA* (Figure 3a). The transduction of these genes into target cells resistant to streptomycin and fluoroquinolones, conferred by mutations in *rpsL* and *gyrA*, respectively, resulted in the production of wild-type enzymes susceptible to the formerly ineffective drugs. In another demonstration, Lu and Collins [90] engineered M13 to carry genes encoding transcription factors that modify the native regulation of bacterial gene networks (Figure 3b). Constructs encoding the LexA3 repressor or SoxR regulator were used to disable the SOS response and DNA repair or to modulate the response to oxidative stress in target cells, respectively, thus potentiating the toxic effects of antibiotic treatment and even resensitizing a resistant bacterial strain. A dual-function phage was also created and validated by using M13 harboring the global regulator *csrA*, to inhibit biofilm formation and the associated increase in antibiotic resistance, and the porin

Figure 3



Bacteriophage can modulate target cells by delivering genetic payloads. Bacteriophage such as the temperate phage λ **(a)** and the filamentous phage M13 **(b)** have been used to deliver non-native DNA to target cells. (a) Delivery of dominant sensitive genes encoding wild-type enzymes such as *gyrA* and *rpsL* results in the resensitization of a cell to antibiotics to which resistance had previously been conferred by mutations in these genes [89•]. (b) Delivery of transcription factors can modulate regulatory networks in bacteria, such as the SOS response normally induced in order to respond to cellular stress and repair damaged DNA [90]. The dominant LexA3 variant can inhibit the SOS response and resensitize cells to some antibiotics as well as reduce the number of emerging resistant cells.

ompF, to improve drug penetration. Examples such as these demonstrate the capacity for bacteriophages to be engineered as gene delivery devices in order to perturb genetic networks in bacteria for both research and therapeutic applications. With this approach, one can alter a gene network at a particular node and observe the qualitative and quantitative effects in order to better characterize native regulatory systems. As models of the interactions in complex regulatory webs of pathogens grow increasingly robust, the ability to know which strands to tug to elicit desired effects may enable rationally designed novel therapeutics based on predictable behaviors.

Genome mining for therapeutics

The development and improvement of next-generation sequencing technology has enabled genomic and metagenomic analyses of phage populations [91,92]. For example, sequencing of gut viral metagenomes has implicated phages as reservoirs of antibiotic-resistance genes [93••] and their role in influencing the intestinal microbiota has been of recent interest [94]. Since bacteriophages must encode mechanisms to control their host cells in order to infect, divert cellular resources to propagate, build progeny phages, and, in many cases, lyse their hosts to release new particles, phage genomes constitute a vast library of parts that can be used to manipulate bacteria for study or treatment. On the basis of this concept, Liu and colleagues [95] developed a method for mining such tools to generate novel therapeutics against *S. aureus*. Predicted phage open reading frames were cloned with inducible expression into the target strain and screened for growth-inhibitory properties. Identified phage proteins were used to pull bacterial targets out of cell lysates and a library of small molecules was screened to identify inhibitors of the protein–protein interaction, with the hypothesis that these molecules might demonstrate similar modulatory action on the host target. In this way, the authors identified novel compounds capable of inhibiting the initiation of bacterial DNA replication in analogy with the phage proteins. Since currently available drugs that target replication only act on topoisomerases, this work demonstrates that mining phage proteins long evolved to inhibit bacterial processes has the potential to expand the antibiotic repertoire by leading us to discover drugs against previously unused targets [95,96].

In addition to random-discovery screens, phage lysins have been specifically investigated in recent years as potential antimicrobials. These enzymes are employed by bacteriophages to degrade the bacterial cell wall and permit the release of progeny phages [97]. In another functional metagenomic study, phage DNA was isolated from a mixture of feces from nine animal species, cloned into a shotgun library for inducible expression in *E. coli*, and used in primary and secondary screens to detect lysins from the phage DNA pool [98]. As a discovery tool, a specific lysin from a phage of *Bacillus anthracis* was used to develop a novel antimicrobial by identifying an enzyme involved in the production of the lysin target and designing a cognate chemical inhibitor [99]. Though lysins are considered useful antimicrobials for Gram-positive pathogens, Gram-negative bacteria possess an outer membrane that prevents access of these extracellular enzymes to the cell wall [100]. To overcome this barrier, a chimeric protein composed of the translocation domain of the *Yersinia pestis* bacteriocin, pesticin, and the enzymatic domain of lysozyme from the *E. coli* phage T4 was engineered. The hybrid bacteriocin was shown to be active against *E. coli* and *Y. pestis* strains, including those expressing the cognate immunity protein conferring resistance to unmodified pesticin [101•,102].

Detection of pathogens

Bacteriophages have also been used to implement real-world applications of biosensing [103–107]. In areas from healthcare and hospital surfaces to food preparation and other industrial processes, methods for the rapid detection of pathogenic organisms are paramount in preventing disease and avoiding the public relations and financial burdens of recalling contaminated products. The amount of time necessary for many conventional detection methods is long due to the requirement for bacterial enrichment before detection of the few bacteria present in complex samples in order to achieve sufficient assay sensitivity and specificity [108]. Engineered bacteriophage-based detectors have the advantage of rapid readouts, high sensitivity and specificity, and detection of live cells [109]. A common design strategy is the creation of reporter-based constructs packaged within phage or phage-like particles that infect target cells and ultimately result in the production of fluorescent, colorimetric, or luminescent signals. Furthermore, sensor designs can include genetically engineered phage that express a product causing ice nucleation [110] or that incorporate tags for linking to detectable elements such as quantum dots [111]. Though most of these examples of specifically modified phages have been enabled by advancements in engineering and synthetic biology to achieve real-world applicability, the concept of using natural phage as sensing tools is not a new one. Phage typing and other techniques have made use of the narrow host range of phage to identify species or strains of bacteria based on a target bacteria's ability to bind, propagate, or be lysed by non-engineered viruses [109].

New phage engineering strategies

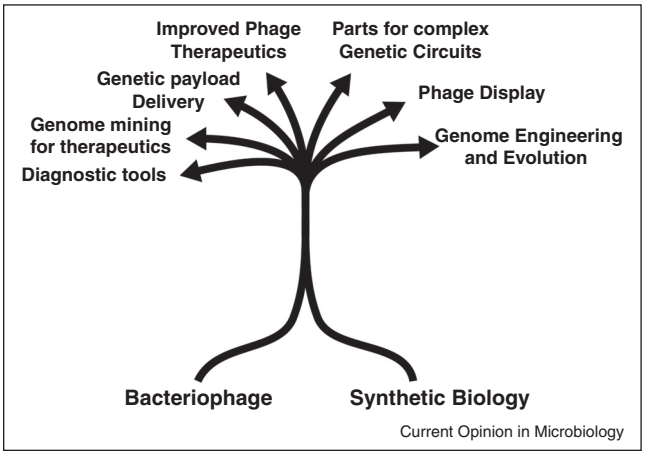
Historically, modifications to bacteriophage relied on random mutagenesis or homologous recombination, both of which are inefficient and necessitate intensive screening to identify mutants of interest. The relatively large size of most bacteriophage genomes and their inherent toxicity to bacterial hosts has confounded the use of

conventional molecular biology techniques for engineering. However, recent synthetic biology tools have revitalized the ability to make rational additions or modifications to phage genomes. Among such improvements, the phage defense function encoded by CRISPR-Cas systems, previously adapted for genome editing [112–117] and reviewed in [118], has been described for improving recombineering in bacteriophages by counter-selecting unmodified phages with wild-type target sequences [119]. *In vitro* assembly of large constructs has also been made possible with techniques such as Gibson assembly [120], which enzymatically stitches together DNA fragments with overlapping homology, thus allowing for insertions of heterologous DNA and site-directed mutagenesis using PCR. Moreover, transformation of overlapping fragments into yeast in conjunction with a compatible yeast artificial chromosome leads to *in vivo* recombination-based assembly of large constructs [121,122]. Genomes can be assembled with modifications or be modified post-assembly in yeast, where they are non-toxic to the host, and then purified and rebooted in bacteria to produce engineered phage progeny. Current DNA synthesis technology, in concert with *in vivo* and *in vitro* recombination, also permits *de novo* chemical synthesis of bacteriophage genomes. Smith *et al.* [123] utilized this approach to synthesize, clone, and produce infectious particles of the 5386 bp phage ΦX174, and a similar scaled-up approach has created the first bacterial cell with a synthetic genome of 1.1 Mb [124]. By rendering bacteriophages genetically accessible, synthetic biology can permit more precise studies of their underlying biology and inspire creation of novel therapeutic agents.

Refactoring and genome stability

Despite advances in rational engineering of bacteriophages, tampering with systems finely tuned by evolution can lead to fitness defects [125]. For example, roughly 30% of the genome of the bacteriophage T7 was 'refactored,' a process whereby genes and their respective regulatory elements were separated into distinct modules to permit systematic analysis and control [126]. The refactored genome produced viable bacteriophage, albeit with significantly reduced fitness. Multiple rounds of *in vitro* evolution restored wild-type viability at the expense of some of the design elements, implying that rational design can be coupled with evolution to ensure the creation of robust biological systems [127]. Similarly, the evolutionary stability of a T7 phage engineered to infect encapsulated *E. coli* by producing a capsule-degrading endosialidase as an exoenzyme was investigated *in vitro* [128]. While the engineered phage permitted replication in the encapsulated strain, the benefit conferred by endosialidase production was shared by wild-type, non-producing 'cheater' phages, which could quickly outcompete the engineered viruses due to their higher fitness. Although these studies point to the fragility of current synthetic biology efforts, bacteriophage-based systems can serve as an excellent platform to understand the constraints placed on synthetic genetic circuits by evolution and inform future designs.

Figure 4



Synergy between synthetic biology and bacteriophage. New tools and techniques from synthetic biology have enabled phage research and the development of novel therapies. Likewise, bacteriophage components have fueled innovation in synthetic biology.

Conclusions

Bacteriophages and functional components derived from their genomes have long been powerful tools that have allowed us to understand basic biological processes and that sparked the field of molecular biology. Mounting concerns over the spread of multidrug-resistant bacterial pathogens, as well as the development of enabling technologies from synthetic biology, have resulted in the resurgence of studies involving these highly evolved and specialized viruses. Recent efforts have made strides in engineering phages with modified properties, endowing entirely new functions, and deriving repurposed parts for the study, detection, and treatment of infectious diseases (Figure 4). As our ability to engineer phages through genome synthesis and modification continues to improve, we will be able to further leverage these finely tuned products of evolution that constitute the most numerous biological entities known to man.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Twort FW: **An investigation on the nature of ultra-microscopic viruses**. *Lancet* 1915, **186**:1241-1243.
2. D'Hérelle F: **Sur un microbe invisible antagoniste des bacilles dysentérique**. *C R Acad Sci* 1917, **165**:373-375.
3. Bruynoghe R, Maisin J: **Essais de thérapeutique au moyen du bacteriophage**. *C R Soc Biol* 1921, **85**:1120-1121.
4. Summers WC: **Bacteriophage therapy**. *Annu Rev Microbiol* 2001, **55**:437-451.
5. Fruciano DE, Bourne S: **Phage as an antimicrobial agent: d'Herelle's heretical theories and their role in the decline of phage prophylaxis in the West**. *Can J Infect Dis Med Microbiol* 2007, **18**:19-26.
6. Summers WC: **The strange history of phage therapy**. *Bacteriophage* 2012, **2**:130-133.
7. Henry M, Debarbieux L: **Tools from viruses: bacteriophage successes and beyond**. *Virology* 2012, **434**:151-161.
8. Barrow PA, Soothill JS: **Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential**. *Trends Microbiol* 1997, **5**:268-271.
9. Kutateladze M, Adamia R: **Bacteriophages as potential new therapeutics to replace or supplement antibiotics**. *Trends Biotechnol* 2010, **28**:591-595.
10. Lu TK, Koeris MS: **The next generation of bacteriophage therapy**. *Curr Opin Microbiol* 2011, **14**:524-531.
11. Gravitz L: **Turning a new phage**. *Nat Med* 2012, **18**:1318-1320.
12. Carlton RM: **Phage therapy: past history and future prospects**. *Arch Immunol Ther Exp (Warsz)* 1999, **47**:267-274.
13. Sulakvelidze A, Alavidze Z, Morris JG: **Bacteriophage therapy**. *Antimicrob Agents Chemother* 2001, **45**:649-659.
14. Merril CR, Scholl D, Adhya SL: **The prospect for bacteriophage therapy in Western medicine**. *Nat Rev Drug Discov* 2003, **2**:489-497.
15. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM: **Phage treatment of human infections**. *Bacteriophage* 2011, **1**:66-85.
16. Slusarczyk AL, Lin A, Weiss R: **Foundations for the design and implementation of synthetic genetic circuits**. *Nat Rev Genet* 2012, **13**:406-420.
17. Lu TK, Khalil AS, Collins JJ: **Next-generation synthetic gene networks**. *Nat Biotechnol* 2009, **27**:1139-1150.
18. Cheng AA, Lu TK: **Synthetic biology: an emerging engineering discipline**. *Annu Rev Biomed Eng* 2012, **14**:155-178.
19. Brophy JAN, Voigt CA: **Principles of genetic circuit design**. *Nat Methods* 2014, **11**:508-520.
20. Elowitz MB, Leibler S: **A synthetic oscillatory network of transcriptional regulators**. *Nature* 2000, **403**:335-338.
21. Gardner TS, Cantor CR, Collins JJ: **Construction of a genetic toggle switch in *Escherichia coli***. *Nature* 2000, **403**:339-342.
22. •• Siuti P, Yazbek J, Lu TK: **Synthetic circuits integrating logic and memory in living cells**. *Nat Biotechnol* 2013, **31**:448-452.
This paper was the first application of phage-derived recombinases to construct all two-input logic gates integrated with memory in living cells, as well as digital-to-analog converter circuits.
23. • Bonnet J, Yin P, Ortiz ME, Subsoontorn P, Endy D: **Amplifying genetic logic gates**. *Science* 2013, **340**:599-603.
This work showed the use of phage-derived recombinases to build genetic logic gates, and phages to pass the state of these logic gates between cells.
24. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D *et al.*: **High-level semi-synthetic production of the potent antimalarial artemisinin**. *Nature* 2013, **496**:528-532.
25. Saeidi N, Wong CK, Lo T-M, Nguyen HX, Ling H, Leong SSJ, Poh CL, Chang MW: **Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen**. *Mol Syst Biol* 2011, **7**:521.
26. Xie Z, Wroblewska L, Prochazka L, Weiss R, Benenson Y: **Multi-input RNAi-based logic circuit for identification of specific cancer cells**. *Science* 2011, **333**:1307-1311.
27. Galloway KE, Franco E, Smolke CD: **Dynamically reshaping signaling networks to program cell fate via genetic controllers**. *Science* 2013, **341**:1235005.
28. Ye H, Charpin-El Hamri G, Zwicky K, Christen M, Folcher M, Fussenegger M: **Pharmaceutically controlled designer circuit for the treatment of the metabolic syndrome**. *Proc Natl Acad Sci U S A* 2013, **110**:141-146.
29. Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA: **Genetic programs constructed from layered logic gates in single cells**. *Nature* 2012, **491**:249-253.
30. Ausländer S, Ausländer D, Müller M, Wieland M, Fussenegger M: **Programmable single-cell mammalian biocomputers**. *Nature* 2012, **487**:123-127.
31. Daniel R, Rubens JR, Sarpeshkar R, Lu TK: **Synthetic analog computation in living cells**. *Nature* 2013, **497**:619-623.
32. Ruder WC, Lu T, Collins JJ: **Synthetic biology moving into the clinic**. *Science* 2011, **333**:1248-1252.
33. Keasling JD: **Synthetic biology and the development of tools for metabolic engineering**. *Metab Eng* 2012, **14**:189-195.
34. Roquet N, Lu TK: **Digital and analog gene circuits for biotechnology**. *Biotechnol J* 2014, **9**:597-608.
35. Smith GP: **Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface**. *Science* 1985, **228**:1315-1317.
36. Jacobsson K, Frykberg L: **Phage display shot-gun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones**. *Biotechniques* 1996, **20**:1070-1076 1078, 1080–1081.
37. Jönsson K, Guo BP, Monstein H-J, Mekalanos JJ, Kronvall G: **Molecular cloning and characterization of two *Helicobacter pylori* genes coding for plasminogen-binding proteins**. *Proc Natl Acad Sci U S A* 2004, **101**:1852-1857.
38. Bazan J, Całkosiński I, Gamian A: **Phage display — a powerful technique for immunotherapy: 2. Vaccine delivery**. *Hum Vaccin Immunother* 2012, **8**:1829-1835.
39. Basha S, Rai P, Poon V, Saraph A, Gujraty K, Go MY, Sadacharan S, Frost M, Mogridge J, Kane RS: **Polyvalent inhibitors of anthrax toxin that target host receptors**. *Proc Natl Acad Sci U S A* 2006, **103**:13509-13513.
40. Paradis-Bleau C, Lloyd A, Sanschagrin F, Clarke T, Blewett A, Bugg TDH, Levesque RC: **Phage display-derived inhibitor of the essential cell wall biosynthesis enzyme MurF**. *BMC Biochem* 2008, **9**:33.
41. Frenzel A, Kügler J, Wilke S, Schirrmann T, Hust M: **Construction of human antibody gene libraries and selection of antibodies by phage display**. *Methods Mol Biol* 2014, **1060**:215-243.
42. Yacoby I, Shamis M, Bar H, Shabat D, Benhar I: **Targeting antibacterial agents by using drug-carrying filamentous bacteriophages**. *Antimicrob Agents Chemother* 2006, **50**:2087-2097.
43. Rakonjac J, Bennett NJ, Spagnuolo J, Gagic D, Russel M: **Filamentous bacteriophage: biology, phage display and nanotechnology applications**. *Curr Issues Mol Biol* 2011, **13**:51-76.

44. Nicastro J, Sheldon K, Slavcev RA: **Bacteriophage lambda display systems: developments and applications**. *Appl Microbiol Biotechnol* 2014, **98**:2853-2866.

45. Huang JX, Bishop-Hurley SL, Cooper MA: **Development of anti-infectives using phage display: biological agents against bacteria, viruses, and parasites**. *Antimicrob Agents Chemother* 2012, **56**:4569-4582.

46. Mullen LM, Nair SP, Ward JM, Rycroft AN, Henderson B: **Phage display in the study of infectious diseases**. *Trends Microbiol* 2006, **14**:141-147.

47. Bratkovič T: **Progress in phage display: evolution of the technique and its applications**. *Cell Mol Life Sci* 2009, **67**:749-767.

48. Smith GP, Petrenko VA: **Phage display**. *Chem Rev* 1997, **97**:391-410.

49. Hershey AD, Chase M: **Independent functions of viral protein and nucleic acid in growth of bacteriophage**. *J Gen Physiol* 1952, **36**:39-56.

50. Luria SE, Delbrück M: **Mutations of bacteria from virus sensitivity to virus resistance**. *Genetics* 1943, **28**:491-511.

51. Green MR, Sambrook J: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press; 2012.

52. Noireaux V, Bar-Ziv R, Libchaber A: **Principles of cell-free genetic circuit assembly**. *Proc Natl Acad Sci U S A* 2003, **100**:12672-12677.

53. Kim J, White KS, Winfree E: **Construction of an in vitro bistable circuit from synthetic transcriptional switches**. *Mol Syst Biol* 2006, **2**:68.

54. Kim J, Winfree E: **Synthetic in vitro transcriptional oscillators**. *Mol Syst Biol* 2011, **7**:465.

55. Temme K, Hill R, Segall-Shapiro TH, Moser F, Voigt CA: **Modular control of multiple pathways using engineered orthogonal T7 polymerases**. *Nucleic Acids Res* 2012, **40**:8773-8781.

56. • Shis DL, Bennett MR: **Library of synthetic transcriptional AND gates built with split T7 RNA polymerase mutants**. *Proc Natl Acad Sci U S A* 2013, **110**:5028-5033.

This paper constructed a library of AND gates using split T7 RNA polymerases.

57. An W, Chin JW: **Synthesis of orthogonal transcription–translation networks**. *Proc Natl Acad Sci U S A* 2009, **106**:8477-8482.

58. Purcell O, Lu TK: **Synthetic analog and digital circuits for cellular computation and memory**. *Curr Opin Biotechnol* 2014, **29C**:146-155.

59. Friedland AE, Lu TK, Wang X, Shi D, Church G, Collins JJ: **Synthetic gene networks that count**. *Science* 2009, **324**:1199-1202.

60. Bonnet J, Subsoontorn P, Endy D: **Rewritable digital data storage in live cells via engineered control of recombination directionality**. *Proc Natl Acad Sci U S A* 2012, **109**:8884-8889.

61. Murphy KC: **Phage recombinases and their applications**. *Adv Virus Res* 2012, **83**:367-414.

62. Datsenko KA, Wanner BL: **One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products**. *Proc Natl Acad Sci U S A* 2000, **97**:6640-6645.

63. Husseiny MI, Hensel M: **Rapid method for the construction of *Salmonella enterica* serovar Typhimurium vaccine carrier strains**. *Infect Immun* 2005, **73**:1598-1605.

64. Beloin C, Deighan P, Doyle M, Dorman CJ: ***Shigella flexneri* 2a strain 2457T expresses three members of the H-NS-like protein family: characterization of the Sfh protein**. *Mol Genet Genomics* 2003, **270**:66-77.

65. Yamamoto S, Izumiya H, Morita M, Arakawa E, Watanabe H: **Application of lambda Red recombination system to *Vibrio cholerae* genetics: simple methods for inactivation and modification of chromosomal genes**. *Gene* 2009, **438**:57-64.

66. Derbise A, Lesic B, Dacheux D, Ghigo JM, Carniel E: **A rapid and simple method for inactivating chromosomal genes in *Yersinia***. *FEMS Immunol Med Microbiol* 2003, **38**:113-116.

67. Lesic B, Rahme LG: **Use of the lambda Red recombinase system to rapidly generate mutants in *Pseudomonas aeruginosa***. *BMC Mol Biol* 2008, **9**:20.

68. •• Enyeart PJ, Chirieleison SM, Dao MN, Perutka J, Quandt EM, Yao J, Whitt JT, Keatinge-Clay AT, Lambowitz AM, Ellington AD: **Generalized bacterial genome editing using mobile group II introns and Cre-lox**. *Mol Syst Biol* 2013, **9**:685.

This paper demonstrated the use of mobile group II introns and the Cre-lox system to create a broadly useful bacterial genome editing system.

69. Langer SJ, Ghafoori AP, Byrd M, Leinwand L: **A genetic screen identifies novel non-compatible loxP sites**. *Nucleic Acids Res* 2002, **30**:3067-3077.

70. Wang HH, Isaacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, Church GM: **Programming cells by multiplex genome engineering and accelerated evolution**. *Nature* 2009, **460**:894-898.

71. Isaacs FJ, Carr PA, Wang HH, Lajoie MJ, Sterling B, Kraal L, Tolonen AC, Gianoulis TA, Goodman DB, Reppas NB *et al.*: **Precise manipulation of chromosomes in vivo enables genome-wide codon replacement**. *Science* 2011, **333**:348-353.

72. •• Lajoie MJ, Rovner AJ, Goodman DB, Aerni H-R, Haimovich AD, Kuznetsov G, Mercer JA, Wang HH, Carr PA, Mosberg JA *et al.*: **Genomically recoded organisms expand biological functions**. *Science* 2013, **342**:357-360.

This work used the lambda Red recombineering system to recode all known UAG stop codons in *Escherichia coli*, which rendered the modified strain more resistant to T7 infection.

73. Esvelt KM, Carlson JC, Liu DR: **A system for the continuous directed evolution of biomolecules**. *Nature* 2011, **472**:499-503.

74. • Dickinson BC, Leconte AM, Allen B, Esvelt KM, Liu DR: **Experimental interrogation of the path dependence and stochasticity of protein evolution using phage-assisted continuous evolution**. *Proc Natl Acad Sci U S A* 2013, **110**:9007-9012.

This paper used a phage-based continuous evolution system with high-throughput sequencing to rapidly evolve and track protein evolution.

75. Leconte AM, Dickinson BC, Yang DD, Chen IA, Allen B, Liu DR: **A population-based experimental model for protein evolution: effects of mutation rate and selection stringency on evolutionary outcomes**. *Biochemistry* 2013, **52**:1490-1499.

76. Lu TK, Collins JJ: **Dispersing biofilms with engineered enzymatic bacteriophage**. *Proc Natl Acad Sci U S A* 2007, **104**:11197-11202.

77. Bertani G, Weigle JJ: **Host controlled variation in bacterial viruses**. *J Bacteriol* 1953, **65**:113-121.

78. Tock MR, Dryden DTF: **The biology of restriction and anti-restriction**. *Curr Opin Microbiol* 2005, **8**:466-472.

79. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P: **CRISPR provides acquired resistance against viruses in prokaryotes**. *Science* 2007, **315**:1709-1712.

80. Bull JJ, Vegge CS, Schmerer M, Chaudhry WN, Levin BR: **Phenotypic resistance and the dynamics of bacterial escape from phage control**. *PLoS ONE* 2014, **9**:e94690.

81. Manning PA, Puspurs A, Reeves P: **Outer membrane of *Escherichia coli* K-12: isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3**. *J Bacteriol* 1976, **127**:1080-1084.

82. Labrie SJ, Samson JE, Moineau S: **Bacteriophage resistance mechanisms**. *Nat Rev Microbiol* 2010, **8**:317-327.

83. Levin BR, Bull JJ: **Population and evolutionary dynamics of phage therapy**. *Nat Rev Microbiol* 2004, **2**:166-173.

84. Westwater C, Kasman LM, Schofield DA, Werner PA, Dolan JW, Schmidt MG, Norris JS: **Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy**

for treatment of bacterial infections. *Antimicrob Agents Chemother* 2003, **47**:1301-1307.

85. Hagens S, Bläsi U: **Genetically modified filamentous phage as bactericidal agents: a pilot study**. *Lett Appl Microbiol* 2003, **37**:318-323.

86. Hagens S, Habel A, von Ahsen U, von Gabain A, Bläsi U: **Therapy of experimental pseudomonas infections with a nonreplicating genetically modified phage**. *Antimicrob Agents Chemother* 2004, **48**:3817-3822.

87. Moradpour Z, Sepehrizadeh Z, Rahbarizadeh F, Ghasemian A, Yazdi MT, Shahverdi AR: **Genetically engineered phage harbouring the lethal catabolite gene activator protein gene with an inducer-independent promoter for biocontrol of *Escherichia coli***. *FEMS Microbiol Lett* 2009, **296**:67-71.

88. Fairhead H: **SASP gene delivery: a novel antibacterial approach**. *Drug News Perspect* 2009, **22**:197-203.

89. • Edgar R, Friedman N, Molshanski-Mor S, Qimron U: **Reversing bacterial resistance to antibiotics by phage-mediated delivery of dominant sensitive genes**. *Appl Environ Microbiol* 2012, **78**:744-751.
This paper demonstrated the delivery of dominant genes to antibiotic-resistant bacteria in order to resensitize them to antibiotic therapy.

90. Lu TK, Collins JJ: **Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy**. *Proc Natl Acad Sci U S A* 2009, **106**:4629-4634.

91. Henn MR, Sullivan MB, Stange-Thomann N, Osburne MS, Berlin AM, Kelly L, Yandava C, Kodira C, Zeng Q, Weiand M *et al.*: **Analysis of high-throughput sequencing and annotation strategies for phage genomes**. *PLoS ONE* 2010, **5**:e9083.

92. Reyes A, Semenkovich NP, Whiteson K, Rohwer F, Gordon JI: **Going viral: next-generation sequencing applied to phage populations in the human gut**. *Nat Rev Microbiol* 2012, **10**:607-617.

93. •• Modi SR, Lee HH, Spina CS, Collins JJ: **Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome**. *Nature* 2013, **499**:219-222.
This work showed how the phage metagenome is modulated by antibiotic treatment in the mouse gut.

94. Mills S, Shanahan F, Stanton C, Hill C, Coffey A, Ross RP: **Movers and shakers: influence of bacteriophages in shaping the mammalian gut microbiota**. *Gut Microbes* 2013, **4**:4-16.

95. Liu J, Dehbi M, Moeck G, Arhin F, Bauda P, Bergeron D, Callejo M, Ferretti V, Ha N, Kwan T *et al.*: **Antimicrobial drug discovery through bacteriophage genomics**. *Nat Biotechnol* 2004, **22**:185-191.

96. Walsh C: *Antibiotics: Actions, Origins, Resistance*. ASM Press; 2003.

97. Pastagia M, Schuch R, Fischetti VA, Huang DB: **Lysins: the arrival of pathogen-directed anti-infectives**. *J Med Microbiol* 2013, **62**:1506-1516.

98. Schmitz JE, Schuch R, Fischetti VA: **Identifying active phage lysins through functional viral metagenomics**. *Appl Environ Microbiol* 2010, **76**:7181-7187.

99. Schuch R, Pelzek AJ, Raz A, Euler CW, Ryan PA, Winer BY, Farnsworth A, Bhaskaran SS, Stebbins CE, Xu Y *et al.*: **Use of a bacteriophage lysin to identify a novel target for antimicrobial development**. *PLoS ONE* 2013, **8**:e60754.

100. Fischetti VA: **Exploiting what phage have evolved to control Gram-positive pathogens**. *Bacteriophage* 2011, **1**:188-194.

101 • Lukacik P, Barnard TJ, Keller PW, Chaturvedi KS, Seddiki N, Fairman JW, Noinaj N, Kirby TL, Henderson JP, Steven AC *et al.*: **Structural engineering of a phage lysin that targets Gram-negative pathogens**. *Proc Natl Acad Sci U S A* 2012, **109**:9857-9862.
This paper reported the adaptation of phage-derived lysins to target Gram-negative bacteria.

102. Patzer SI, Albrecht R, Braun V, Zeth K: **Structural and mechanistic studies of pesticin, a bacterial homolog of phage lysozymes**. *J Biol Chem* 2012, **287**:23381-23396.

103. Smartt AE, Ripp S: **Bacteriophage reporter technology for sensing and detecting microbial targets**. *Anal Bioanal Chem* 2011, **400**:991-1007.

104. Schofield DA, Sharp NJ, Westwater C: **Phage-based platforms for the clinical detection of human bacterial pathogens**. *Bacteriophage* 2012, **2**:105-283.

105. Lu TK, Bowers J, Koeris MS: **Advancing bacteriophage-based microbial diagnostics with synthetic biology**. *Trends Biotechnol* 2013, **31**:325-327.

106. Singh A, Poshtiban S, Evoy S: **Recent advances in bacteriophage based biosensors for food-borne pathogen detection**. *Sensors (Basel)* 2013, **13**:1763-1786.

107. Tawil N, Sacher E, Mandeville R, Meunier M: **Bacteriophages: biosensing tools for multi-drug resistant pathogens**. *Analyst* 2014, **139**:1224-1236.

108. Velusamy V, Arshak K, Korostynska O, Oliwa K, Adley C: **An overview of foodborne pathogen detection: in the perspective of biosensors**. *Biotechnol Adv* 2010, **28**:232-254.

109. Smartt AE, Xu T, Jegier P, Carswell JJ, Blount SA, Sayler GS, Ripp S: **Pathogen detection using engineered bacteriophages**. *Anal Bioanal Chem* 2012, **402**:3127-3146.

110. Wolber PK, Green RL: **Detection of bacteria by transduction of ice nucleation genes**. *Trends Biotechnol* 1990, **8**:276-279.

111. Edgar R, McKinstry M, Hwang J, Oppenheim AB, Fekete RA, Giulian G, Merril C, Nagashima K, Adhya S: **High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes**. *Proc Natl Acad Sci U S A* 2006, **103**:4841-4845.

112. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**:816-821.

113. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM: **RNA-guided human genome engineering via Cas9**. *Science* 2013, **339**:823-826.

114. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA *et al.*: **Multiplex genome engineering using CRISPR/Cas systems**. *Science* 2013, **339**:819-823.

115. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh J.R., Joung JK: **Efficient genome editing in zebrafish using a CRISPR-Cas system**. *Nat Biotechnol* 2013, **31**:227-229.

116. Cho SW, Kim S, Kim JM, Kim J-S: **Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease**. *Nat Biotechnol* 2013, **31**:230-232.

117. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA: **RNA-guided editing of bacterial genomes using CRISPR-Cas systems**. *Nat Biotechnol* 2013, **31**:233-239.

118. Mali P, Esvelt KM, Church GM: **Cas9 as a versatile tool for engineering biology**. *Nat Methods* 2013, **10**:957-963.

119. Kiro R, Shitrit D, Qimron U: **Efficient engineering of a bacteriophage genome using the type I-E CRISPR-Cas system**. *RNA Biol* 2014, **11**:1-3.

120. Gibson DG, Young L, Chuang R, Venter JC, Hutchison CA, Smith HO: **Enzymatic assembly of DNA molecules up to several hundred kilobases**. *Nat Methods* 2009, **6**:343-345.

121. Lu TK, Koeris MS, Chevalier BS, Holder JW, McKenzie GJ, Brownell DR: *Recombinant phage and methods* 2013. US 13/627,060.

122. Jaschke PR, Lieberman EK, Rodriguez J, Sierra A, Endy D: **A fully decompressed synthetic bacteriophage øX174 genome assembled and archived in yeast**. *Virology* 2012, **434**:278-284.

123. Smith HO, Hutchison CA, Pfannkoch C, Venter JC: **Generating a synthetic genome by whole genome assembly: phiX174**

bacteriophage from synthetic oligonucleotides. *Proc Natl Acad Sci U S A* 2003, **100**:15440-15445.

124. Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang R-Y, Algire MA, Benders GA, Montague MG, Ma L, Moodie MM *et al.*: **Creation of a bacterial cell controlled by a chemically synthesized genome**. *Science* 2010, **329**:52-56.

125. Sleight SC, Bartley BA, Lieviant JA, Sauro HM: **Designing and engineering evolutionary robust genetic circuits**. *J Biol Eng* 2010, **4**:12.

126. Chan LY, Kosuri S, Endy D: **Refactoring bacteriophage T7**. *Mol Syst Biol* 2005, **1**:2005.0018.

127. Springman R, Molineux IJ, Duong C, Bull RJ, Bull JJ: **Evolutionary stability of a refactored phage genome**. *ACS Synth Biol* 2012, **1**:425-430.

128. Gladstone EG, Molineux IJ, Bull JJ: **Evolutionary principles and synthetic biology: avoiding a molecular tragedy of the commons with an engineered phage**. *J Biol Eng* 2012, **6**:13.