Title

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Journal

Cell, 186(1)

ISSN

0092-8674

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Publication Date

2023

DOI

10.1016/j.cell.2022.11.017

Cell
Leading Edge

CellPress
OPEN ACCESS

Review

Phage therapy: From biological mechanisms to future directions

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https://doi.org/10.1016/j.cell.2022.11.017

SUMMARY

Increasing antimicrobial resistance rates have revitalized bacteriophage (phage) research, the natural predators of bacteria discovered over 100 years ago. In order to use phages therapeutically, they should (1) preferably be lytic, (2) kill the bacterial host efficiently, and (3) be fully characterized to exclude side effects. Developing therapeutic phages takes a coordinated effort of multiple stakeholders. Herein, we review the state of the art in phage therapy, covering biological mechanisms, clinical applications, remaining challenges, and future directions involving naturally occurring and genetically modified or synthetic phages.

INTRODUCTION

In 2022, the first comprehensive assessment on the global health impact of antimicrobial resistance (AMR) estimated that 4.95 million deaths in 2019 were associated with AMR to which 1.2 million were directly attributable.[1] The majority of these deaths occurred in lower- and middle-income countries, and three-quarters were caused by six bacterial species that had previously been identified as priority pathogens by the World Health Organization (WHO).[2] Although this report confirmed that the magnitude of AMR on morbidity, mortality, and disability is at least as great as that of the human immunodeficiency virus and malaria, the incidence of AMR has significantly worsened during the COVID-19 pandemic.[3,4]

The reasons for the rise of AMR include mis-use and over-use of antibiotics in the food industry, animal husbandry, and medicine, as well as the dwindling antibiotic pipeline as pharmaceutical companies have increasingly opted out of antibiotic discovery and development.[5,6] Moreover, some pathogens are intrinsically antibiotic resistant and challenging to treat with currently available agents. Without a major shift in current trends, it is estimated that at least 10 million people will die from AMR by 2050, at a cost of one trillion dollars per year, primarily due to lost productivity.[7]

The growing AMR crisis has revitalized research into alternatives, with one of the most promising avenues being bacteriophage therapy. Bacteriophages are natural predators of bacteria that have co-evolved with bacteria for nearly 4 billion years. With an estimated 10^{31} bacteriophage particles in the biosphere, they are believed to be the oldest and most abundant organisms on the planet.[8] Over 30 billion phage particles are thought to move in and out of human tissues every day,[9] serving as the stewards of various microbiomes. Although numerous researchers have witnessed what appears to have been the bacteriolytic activity of phages as far back as 1896,[10] it was not until 1917 that self-taught microbiologist Felix d'Herelle deduced that the culprit must be a parasite of bacteria, which he named bacteriophage (derived from the Greek, meaning "bacteria eater").[11]

After d'Herelle successfully used phage preparations to treat children suffering from bacterial dysentery in 1919, phage therapy was used extensively to treat bacterial infections in humans and animals in the 1930s, well before penicillin was first brought to market.[12] The first phage therapy program opened in what is now Tbilisi, Georgia, followed by another in Wroclaw, Poland; both programs still exist to this day. However, after WWII ushered in penicillin to market in the early 1940s, phage therapy fell out of favor in the West. The broad-spectrum activity of penicillin and future antibiotics against bacterial infections was considered an advantage over phages, which require that bacteria express specific surface molecules to which the phage can bind and that lack intracellular defenses capable of inactivating the phage following entry. Moreover, antagonism between the United States and the Soviet Union in the post-war period fueled both distrust of science coming from the former Soviet Bloc and pervasive suspicions about the therapeutic use of phages for decades to come.[12]

In the last 5 years, phage therapy has undergone a revitalization, due to the growing problem of AMR with few new antibiotics in the pipeline and a growing number of high-profile reports where phage therapy was used to successfully treat life-threatening multidrug-resistant bacterial infections.[13–19] Previous limitations that had thwarted the field are now being overcome with advances in high-throughput sequencing, metagenomics, genetic engineering, and synthetic biology. This has encouraged funding agencies to support clinical trials as well as new

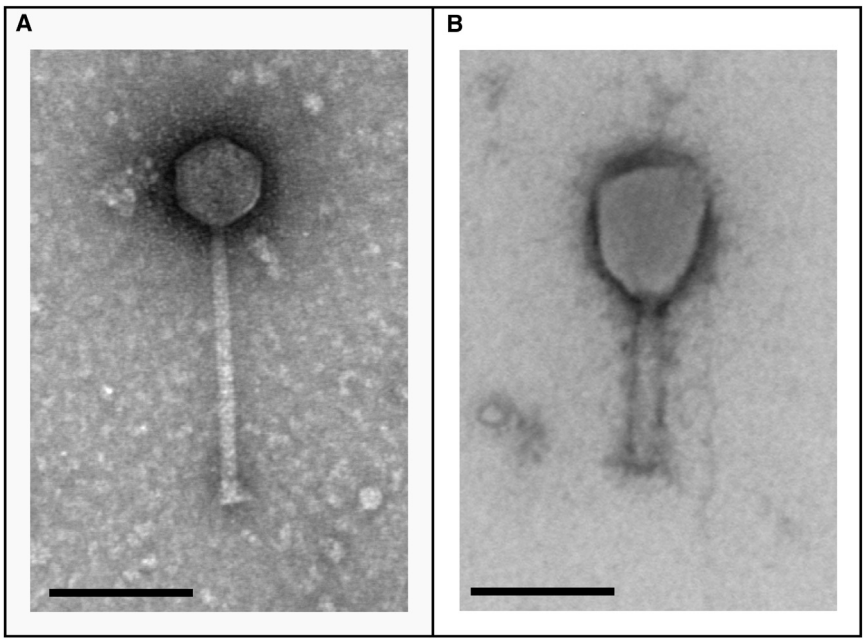

Figure 1. Examples of therapeutically useful phages

(A and B) Bacteriophages Muddy (A) and Maestro (B) have been used to treat *M. abscessus* and *A. baumannii* infections, respectively. Muddy has a siphoviral morphotype with an icsohedral capsid containing the dsDNA genome and a flexible non-contractile tail; Maestro has a myoviral morphology with a contractile tail. Structures at the tail tips of these phages recognize specific receptors on the bacterial cell surface. Scale bars, 100 nm. Images courtesy of Graham Hatfull and Adriana Carolina Hernandez.

investments from biotech start-ups and pharmaceutical companies. Here, we review the state of the art in phage therapy, covering biological mechanisms, clinical applications, remaining challenges, and future directions.

PHAGE BIOLOGY

Phages are viruses and have all the common viral properties: they do not replicate outside of their host, they have relatively small genomes, they make extensive use of host machinery for their replication, and they exhibit tight host cell specificity. There are many different types of virion morphologies, but the most common is the double-stranded DNA (dsDNA) tailed phages, in which the DNA is encapsulated within a capsid (head) that is attached to a tail (Figure 1). Infection is initiated by attachment of the tail tip to the bacterial cell wall and injection of the genome from the capsid, through the cell membrane, and into the cytoplasm. The proteinaceous capsid and tail remain outside of the cell.

Most phages can be classified as being lytic or temperate. Upon infection, lytic phages pursue a single developmental program involving early phage gene expression, genome replication, late lytic expression of the virion structure and assembly genes, assembly of fully packaged particles, and finally bacterial lysis. Temperate phages can also follow this pattern of lytic growth, but a "decision" is made early during infection on whether to undergo lytic growth or to establish lysogeny; lysogenic frequencies vary enormously from a few percent to most infections, depending on the phage, the host, and the conditions.[20,21] In lysogeny, the genes needed for lytic growth are switched off, and the phage genome is established as a prophage, commonly by integration into the host chromosome, although some phages use extrachromosomal (plasmidial) autonomous replication.[22] Lytic phages kill a very high proportion of bacterial cells they infect and therefore are suitable for therapeutic consideration. By contrast, a high proportion of cells survive (as lysogens) following temperate phage infection, and therefore temperate phages are poor choices for therapy. However, these can be engineered to be obligatorily lytic (see below), converting them into therapeutic candidates.[15]

The variety of bacteria productively infected by phages is defined as the host range. Each phage has a specific host range and can be "broad"—infecting many species within a bacterial genus and sometimes in different genera—or "narrow"—infecting only one or a small number of isolates within a bacterial species. These restricted host preferences result from perhaps 3 billion years of microbial warfare in which bacteria evolve to survive lytic phage infection, and phages must co-evolve to have access to susceptible hosts.[23] These dynamics have strongly influenced microbial evolution with a multitude of viral defense systems in bacteria (e.g., restriction-modification [RM] and CRISPR-Cas) and counter-defense systems (e.g., anti-RM and anti-CRISPR) in phages.[24,25] Phage host range is therefore determined by both pre-DNA injection processes at the cell surface (such as receptor recognition) and post-DNA infection defenses.

The long evolutionary span of phages is reflected in several key genomic features. First, phages are genomically highly diverse. This is illustrated by 2,000 sequenced genomes of phages infecting a single strain of *Mycobacterium smegmatis*, encompassing over 30 different genomic types that share few genes with one another and with great variation within each type.[26] Phage genomes are typically tightly packed with overlapping protein-coding and sometimes RNA-encoding genes and are replete with relatively small genes of unknown function (UKF); consequently, the average phage gene size is only about two-thirds that of bacterial genes. The UKF genes are usually not required for lytic growth, but many may influence the efficient production of phage particles and phage-host dynamics. Finally, phage genomes are pervasively mosaic, with single genes (or small subsets of genes) found in different genomic contexts in otherwise unrelated phages.[27] This is likely the result of illegitimate (sequence-independent) recombination events over a long evolutionary time span.[28]

NATURALLY OCCURRING PHAGES

Naturally occurring (or "environmental") phages can be found in virtually any niche on the planet where bacteria are found, including oceans, lakes, soil, plants, and animals. Phages are often abundant in environments where their bacterial hosts are also present.[29] Thus, hunts for environmental phages can be conducted in locations where a plethora of bacteria of the species of interest are found, such as sewage treatment plants and waste downstream of animal or human communities. Typically, water or soil samples from locations of interest are passed through 0.22 micron filters to remove bacteria, fungi, and other entities larger than viruses. Filtrates are layered onto plates seeded with lawns of bacteria for which lytic phages are sought, or bacteria may be incubated with the host to promote enrichment of desirable phages. Plates are then observed for the appearance of plaques (zones of clearance) in the agar, indicating that bacteria in that location have been lysed. Phages can be plaque-purified by serial passage on bacteria supporting their growth and amplified; such hunts can be undertaken at relatively modest costs with little sophisticated equipment.[30] Although there appears generally not to be strong correlations between geography and phage genotype, further investigations are warranted that may facilitate phage discovery for at least some pathogens.

There are likely many naturally occurring phages that cannot be readily isolated because either the host is not available or cannot be readily cultured in the laboratory. Phage genomes have been identified in metagenomic samples and can be very large, and they use alternative genetic codes.[31,32] Although likely hosts can be predicted for these, experimental validation of these prediction and lytic propagation has not been demonstrated.[31,33,34] Phage searches can also be designed to identify phages with specific desirable properties. One example using this approach led to the discovery of *Pseudomonas* OMKO1 that uses the outer membrane porin M (OprM) of the multidrug efflux systems MexAB and MexXY as a receptor-binding site.[35] This characteristic of *Pseudomonas* OMKO1 is of particular therapeutic interest, because it presents an evolutionary trade-off such that resistance to the phage resulting from changes in the efflux pump simultaneously increases antibiotic sensitivity.[36]

Phages intended for clinical use require extensive characterization.[37] This characterization generally includes a definition of host range, whole-genome sequencing for phage speciation and to search for genetic elements encoding AMR, and toxin-encoding genes or genetic elements suggesting the capacity for lysogeny. Prior to clinical use, phages must be propagated on a suitable, well-characterized host and purified to remove/reduce endotoxin levels or other potentially deleterious materials. Phages that are used clinically under non-emergency circumstances should be prepared under Good Clinical Practice (GCP) conditions or conditions approximating this level of rigor. The propagating strain should be prophage-free to avoid contamination of the lysate with spontaneously released prophage-derived particles.[38]

Even though the number of therapeutic successes reported in the literature has been rising, the process of identifying lytic phages remains limiting. More than a century of phage hunting has resulted in the collection of lytic phages with host ranges for most (but not all) clinically important bacterial species. Increasingly accessible databases have made it possible to search for phages of interest that are held in academic and commercial phage banks.[39] A number of recent studies highlighted the importance of systematically characterizing phage receptors and the genetic basis of phage resistance profiles.[40–43] Such efforts can enable rational designing of phage cocktails[44,45] and exploit the evolution of phage resistance toward beneficial clinical phenotypes and outcomes.[46–50] Additional approaches such as directed evolution to promote phage steering and evolutionary traps to exploit phage resistance provide additional opportunities to expand and improve the clinical utility of environmentally sourced phages.[51]

Well-characterized phage banks can serve as libraries from which therapeutic phages can be sourced for clinical use, serving as the starting material for genetically modified phages or as the intellectual framework for the construction of fully synthetic phages (see below). Although well-characterized phage collections are always desired for rational formulations, a growing number of bacterium-specific phage banks have been sufficiently characterized to allow for highly focused phage searches for use in individual patients or in clinical trials. The size of the phage bank required to cover most clinical isolates from a given strain of bacteria is highly dependent on phage biology and target pathogen genetics and interaction determinants. In the case of *Staphylococcus aureus* (*S. aureus*), phages with a relatively broad host range can be identified, and a suitable phage library might consist of only 20 or 30 phages.[52] Bacterial species such as *Acinetobacter baumanni* in which phage host range is narrower might require a phage library of more than 300 phages to provide similar coverage. Phage host range within bacterial species is a critical determinant of whether it is feasible to develop one or more "fixed cocktails" of phages directed at a bacterial species. Such cocktails have been proposed for treatment of bacterial species for which broader host range phages are available (e.g., *S. aureus* and *P. aeruginosa*).[53,54] However, such cocktails are not currently feasible for the treatment of bacterial species with primarily narrow-range phages such as *A. baumannii* or *Mycobacterium abscessus*. These organisms will require either customized phage cocktails or phage engineering focused on the development of broader-host-range phages. Fixed phage cocktails offer the advantage of simpler production and deployment but may lack multivalency, while custom cocktails often provide multivalency but pose more complex challenges for both clinical development and clinical use.

PHAGE THERAPY

As noted above, a number of high-profile and well-described clinical case reports, coupled with a more widely available technology for phage identification and production, have led to more widespread use of phages in clinical medicine over the past several years. In the United States, the Food and Drug Administration (FDA)'s Emergency Investigational New Drug process has provided the regulatory framework under which most of these

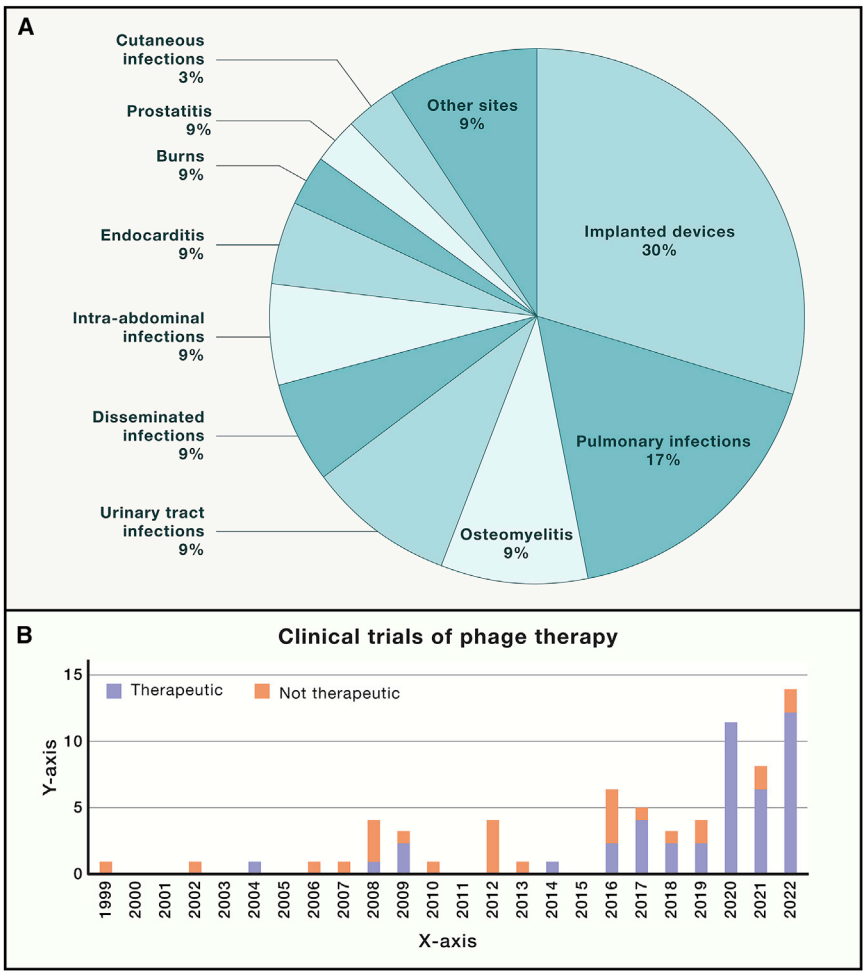

Figure 2. Phage therapy reports and phage studies by year listed

(A) Case reports of phage therapy since 2000. A PubMed search was performed on September 22, 2022, using the search terms "(bacteriophage) AND (therapy) AND (case report)." Sites of infection in each of the 70 cases reported in 53 manuscripts are depicted.

(B) Clinical trials of phage therapy reported to ClinicalTrials.gov since 1999. The registry was queried using the key word "phage" on September 9, 2022.

experiences have been undertaken.[55] Although the regulatory framework in Europe is a bit more heterogeneous, it has recently begun to be more centralized and systematic.[56] Established phage therapy programs now exist in the United States, Belgium, France, and Sweden, in addition to long-standing programs in the Republic of Georgia and in Poland. In Europe and Australia, collaborative initiatives were successful in developing standardized phage therapy protocols to facilitate therapeutic applications.[57,58] Most recently, the United Kingdom announced that it will begin to consider compassionate-use phage therapy requests through the National Health Service.

Regulation of clinical development of phages in the United States is overseen by the FDA's Office of Vaccines Research and Review (OVRR) in the Center for Biologics Evaluation and Research (CBER). The OVRR is well equipped to provide regulatory oversight regarding the safety, purity, potency, and consistency of phage manufacturing. As in the case of all drugs under regulatory oversight by the FDA, licensure of phage products also requires that they have been demonstrated to treat, prevent, cure, or mitigate a disease in humans. In their most straightforward clinical application as antimicrobial agents, regulatory decisions regarding clinical efficacy will likely initially be based on considerations analogous to those used to determine efficacy of "traditional" antibiotics.

Earlier case series describe the treatment of infections with phage alone or in combination with antibiotics in a wide variety of anatomic sites.[59] Although treatment success was reported in a subset of patients, two features stand in contrast to more recent experience with phage therapy. First, phage production technology during this early era required that most treatment courses be administered by oral, topical, intravesicular, intrarectal, or inhaled routes of delivery. Second, phages were often administered as incompletely characterized mixtures of phages that had not undergone *in vitro* assessments for their activity against the specific organism under treatment. Although substantial progress in the area of phage susceptibility testing has occurred over recent years, assay methodologies are not yet fully systematized, and their predictive value for clinical activity requires substantial additional evaluation.[53]

Over the past 10–15 years, an increasing number of more detailed case reports have arisen (Figure 2A), reflected by the amount of literature on PubMed.gov. With allowances for the selection bias associated with case reports, pulmonary infections and those of implanted vascular and orthopedic devices account for over half of the described cases. Most of the more modern experiences have reported the addition of phages to optimized background antibiotic therapy. With the development of technologies that have enabled the preparation of near-GCP-grade therapeutic phages, phage therapy is increasingly being delivered intravenously. A recent comprehensive review of the safety of phage administration to humans and animals concluded that phage administration, regardless of route, is generally well tolerated.[60] Non-parenteral routes of delivery allow for the use of phages that have been less rigorously prepared, but their efficient delivery to sites of infection may be compromised by a number of factors. For example, phage treatment of enteric infections requires formulations that ensure delivery of phages past the stomach where gastric acid may destroy viability.

Topical administration of phages requires attention to stability of phages in the vehicles used for their delivery and to the anatomy of the infection under treatment. Aerosolized administration of phages in the treatment of pulmonary infections has been widely used, but attention to the stability of each phage in the specific nebulizer being used for that phage is critical. The development of efficient methodologies for the purification of phages has enabled intravenous administration and, in principle, the delivery of phages to anatomic sites that are not reliably accessible by topical or oral routes of administration. These include, in particular, systemic infections and those on implanted prosthetic devices. Intravenously administered phages are generally cleared from the bloodstream over the course of 1–3 h, but as they circulate, they reach sites of infection and can then propagated on the pathogen under treatment. Phage preparations are well tolerated by this route and can be given in both inpatient and outpatient settings. A concern regarding intravenous administration, especially in patients who are immunocompetent and requiring longer courses of therapy, relates to the potential that adaptive immune responses to the administered phage(s) may compromise therapeutic efficacy.[61] Although phage-specific immune responses have been reported in some patients receiving phage therapy, the impact on treatment outcomes has been variable.[62,63] Rigorous pharmacokinetic (PK) and pharmacodynamic (PD) studies are required for the development of a systematic understanding of optimal routes of administration. Quantifying phages at externally accessible sites of infection is possible using molecular or culture-based approaches. PD studies of infections at less easily accessible sites such as joint prostheses and pacemaker wires may require more novel approaches. For example, the use of -fluorescently labeled peptides that specifically bind to a therapeutic phage could enable real-time monitoring of phage populations at deeper sites.[64] Finally, the potential impact of phage-specific immunity on therapeutic outcomes must also be carefully evaluated in phage development programs.

CLINICAL TRIALS OF PHAGE THERAPY

Further development of phage therapeutics requires an investment in rigorous clinical trials of the same design and scope as those that would be applied to the development of small-molecule antibiotics.[65] These studies must be based on strong preclinical studies and be conducted in an orderly fashion in phases that are analogous to antimicrobial studies. Failure to fully appreciate key pre-clinical and pharmacologic principles has led to a number of high-profile failures in early clinical trials of phage therapeutics.[66,67]

Over the past 7 years, the number of clinical trials registered in ClinicalTrials.gov that use phages has increased. Of the 44 clinical trials with therapeutic intent, 29 have been posted since the beginning of 2020 (Figure 2B). Although most phage trials propose to use environmental phages, three trials propose the use of CRISPR-enhanced phage products. Most of the registered trials seek to exploit the bacteriocidal activity of lytic phages, although an increasing number are focused on the ability of phages to disrupt biofilms that challenge sterilization of infected implanted biomedical devices.[68] As these trials progress, we will need to learn which applications are most amenable to phage therapy, how to more accurately select lytic phages for clinical use, and how to optimize the use of phages in combination with antibiotics. We will need to define PKs and PDs of phage administration in order to determine whether pre-existing or induced adaptive immune responses compromise phage activity and how to monitor patients for the emergence of bacteria with reduced phage susceptibility.[69,70] Although the body of knowledge required to optimally utilize phages as therapeutic agents is substantial, a framework for how to develop this body of knowledge has been developed over 80 years of experience with antibiotics and should be thoughtfully applied to the development of phage therapeutics moving forward.

The development of rigorous and reproducible laboratory techniques that predict clinical activity of phages is still in its infancy and must also be prioritized as clinical investigations proceed. Classical agar "spot tests" as well as assays undertaken in multiwell plates are most frequently used for the selection of phages for clinical use and in clinical trials.[51,71] The "spot" test has the advantages of simplicity and the absence of the need for sophisticated laboratory equipment, and it provides quantitative efficiencies of plaquing (EOPs); it also offers opportunities to identify, recover, and characterize host-range mutants for expansion of the therapeutic phage repertoire. However, spot tests have the disadvantage of requiring visual endpoint assessments that are more subjective and less quantitative than can be obtained from microwell-based liquid assays. Neither approach has been fully standardized, and choices of media, temperature, and inoculum density can affect the interpretation of either type of test. Further development of rigorous, reproducible laboratory assays to detext synergy and antagonism of phages with one another and with antibiotics is also sorely needed.[72,73] Clinical trials provide an important opportunity to address critical remaining issues in the area of clinical laboratory testing for phage susceptibility.

DESIGNING NEW PHAGES

There are three potential impediments to using naturally occurring phages therapeutically: (1) the only available phages with desired tropisms are temperate, (2) phages that infect the target bacterial host do not kill it efficiently, and (3) any of the many phages that code for dozens of proteins of UKF could be potentially harmful. It thus may be necessary to engineer phages with enhanced therapeutic properties, safety features, and host range. For example, phages can be engineered to carry payloads that modulate host responses or reduce the potential for horizontal gene transfer of antibiotic resistance genes by rapidly degrading the bacterial genome.[74] Such engineering approaches may also allow for the functional and programmed arrangement of phage particles that increased penetrance of biofilms, target intracellular pathogens, or have enhanced PK and PD properties. Construction of such recombinants with increased genome length may encounter packaging constraints of the phage capsids, warranting identification and removal of non-essential genes to increase cloning capacity, similar to strategies used in development of the first phage cloning vectors.[75,76]

Fundamentally, there are two alternative strategies available for engineering phages for desired functionality. The first is genome modification to imbue a known phage with altered properties; the second is build-by-design, using synthetic genomics to construct phages designed from the known rules of phage biology.[74] Phage synthetic genomics is still in its infancy but holds enormous promise as it is unencumbered by constraints of naturally occurring phages. The methods for the first approach—phage genome modification—are further advanced, and engineered phages have been used therapeutically[15,77]; we will discuss this first.

Phage genome engineering

Phage genome engineering involves two main steps: to "build" and to "select or recover" (Figure 3). There have been a number of methods developed over the years to build engineered phages and recover desired progeny from a pool of parental strain. The build component is typically mediated by host- or phage-derived recombination systems, and phage-encoded recombinases can confer high levels of homologous recombination in "recombineering" strains.[78] These are readily available for many Gram-negative bacteria based on the phage lambda Red system, but host-specific systems can be developed by harnessing the recombination systems from phages of those hosts. This approach is the basis of the bacteriophage recombineering of electroporated DNA (BRED) developed for engineering phages of mycobacteria,[79] but which has been adapted for other bacterial hosts. Because the recombination process is efficient, desired progeny can be identified by simple PCR analysis of a few (12–18) plaques.[79] However, the efficiency of recovery varies for different types of mutations; for example, simple deletions are recovered at higher frequencies than gene insertions or replacements. BRED was used to construct obligatorily lytic variants of temperate mycobacteriophages through precise deletion of the phage repressor, and these were used therapeutically.[15,77] These approaches can be applied to other genetically tractable bacteria, but they are less useful for understudied pathogens.

Methods for enriching desired phage progeny in the recovery stage are confounded by the inability to use antibiotic resistance as commonly used for bacterial genetics. Some specialized phage/host genes essential for phage growth have been developed as selectable markers, but their use is very limited[80]; phenotypic differences (e.g., plaque type/size) may be useful characteristics, and recombinants also can be detected by plaque hybridization.[81] However, CRISPR-Cas-based technologies provide a simple and powerful method to enrich for mutant progeny through counterselection against the parent phage.[82–84] CRISPR-mediated antagonism of phage infection is often very efficient (reducing phage titers by more than four orders of magnitude), and PAM (protospacer adjacent motif) site selection for discrimination between parent and mutant progeny may be the chief limitation. This requires construction of a recombinant host strain expressing an active Cas protein (e.g., RNA-guided DNA nucleases such as Cas9 and Cas12 or RNA-guided RNA nuclease such as Cas13) and a guide RNA targeting the parental phage. Cas9-mediated interference has also been coupled with the BRED strategy (CRISPY-BRED) to enrich for less efficiently produced recombinants including fluorescent reporter phages.[84]

Recently, two studies reported the use of RNA-targeting CRISPR-Cas13a for phage genome engineering.[85,86] When combined with homologous recombination, these enabled engineering of a broad diversity of phages, including single codon deletions, and introduction of fluorescent tags into nucleus-forming 200–500 kbp genome-sized jumbo phages.[87] We and others speculate that in a couple of years, a suite of CRISPR-based toolboxes will facilitate creating smaller edits or whole-phage genome engineering to meet any design specifications defined by diverse therapeutic applications.[88] This could include integrated tools to simultaneously detect the presence of temperate markers, AMR genes, and virulence genes.[89] It may be possible to use a combination of CRISPR-based programmable base editors, nucleases, transposases/recombinases, and prime editors for creating defined genome-scale changes in diverse phages.[90,91]

Synthetic phage genomics

CRISPR-Cas systems allow for marker-free engineering of diverse phages but are limited by bacterial hosts that have genetic toolboxes available for expressing CRISPR-Cas systems and maintaining editing templates. Furthermore, engineering of complex genetic traits that require multiple modifications around the genome needs sequential cloning and counterselection, making it a time-consuming exercise. Although phage editing and engineering projects are much easier than just a couple of years ago, researchers are still in need for faster and broadly applicable technologies that are not limited by the genetic toolboxes and pathogen bacteria for manufacturing large volumes of phages.

Synthetic genome construction offers powerful new strategies for building genomes by design (Figure 4). Methods are now available for synthesis of phage-sized genomes (~50 kbp), which can be propagated by rebooting in a permissive bacterial host or by cell-free transcription-translation (TXTL) systems.[74,92–94] This approach is especially applicable when natural phages are not available or if there is a defined need for specifically designed genetic additions. Synthesis strategies have either used synthetic oligonucleotides to assemble the entire phage genome or have created chimeric phages by partially replacing a section of an available genome scaffold with a modified/synthetic one.[74] The *in vitro* assembled phage genomes are either electroporated into bacterial cells directly or subcloned within *S. cerevisiae*-bacterial shuttle vector in *S. cerevisiae* before moving the constructs into bacterial cells to induce phage production (for "booting up" phages).

Assembling phage genomes *in vitro* or in yeast and moving them into bacterial cells can become inefficient for building larger phage genomes requiring highly efficient transformations. Some of these limitations have been overcome by assembling large synthetic phages (<150 kbp) using synthetic or PCR amplified DNA fragments and transforming them into cell-wall-deprived (called L-form) *Listeria monocytogenes* cells.[95] Functional phages produced by this process are then used to infect and propagate in the target host bacteria. This L-form process was also shown to be broadly efficient in cross-genus rebooting *Bacillus* and *Staphylococcus* synthetic phages.[96] Although a single infection of a sensitive host may be sufficient for rebooting, this dependency on using living cells may become limiting in some therapies. Notably, phage propagation on a single

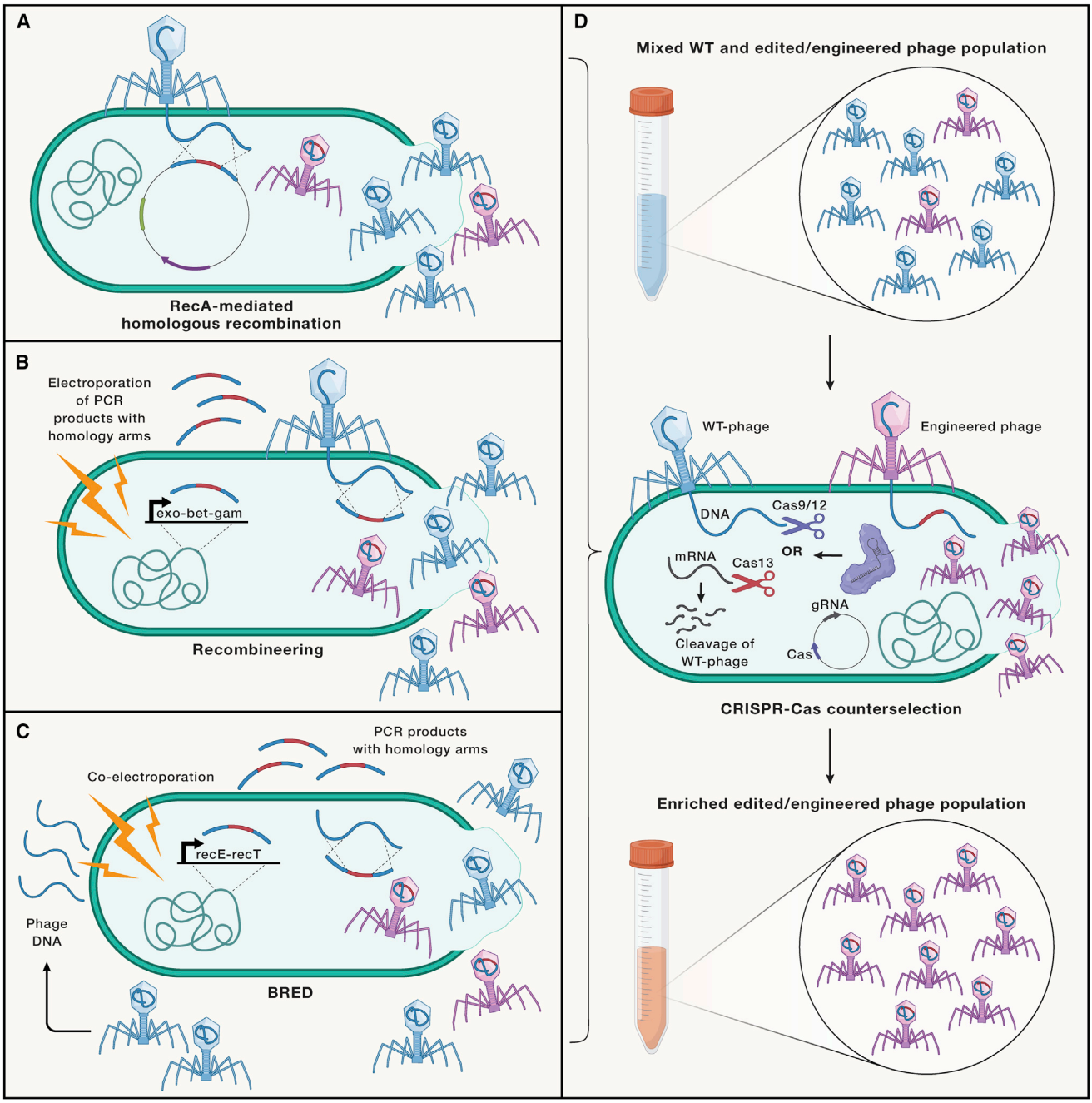

Figure 3. Methods used in phage engineering

Commonly used *in vivo* homologous recombination methods in combination with CRISPR-Cas system-based counterselection strategy.

(A) Rec-A-mediated homologous recombination method involves phage DNA recombination with the homology region (shown in blue-red loci) present on plasmid DNA to yield recombinant phages.

(B) *In vivo* recombineering method involves recombination between phage genome and electroporated PCR products with homology arms (shown in blue-red fragments).

(C and D) (C) BRED method involves recombination between co-electroporated phage DNA (blue fragments) and PCR products with homology arms (shown in blue-red fragments). Because of different recombination efficiencies, each of these methods produces phage progenies made up of recombinant and wild-type phages (D). RNA-guided DNA nucleases such as Cas9 and Cas12 or RNA-guided RNA nuclease such as Cas13 counterselection is then applied to selectively remove unedited phages to enrich edited/engineered phages.

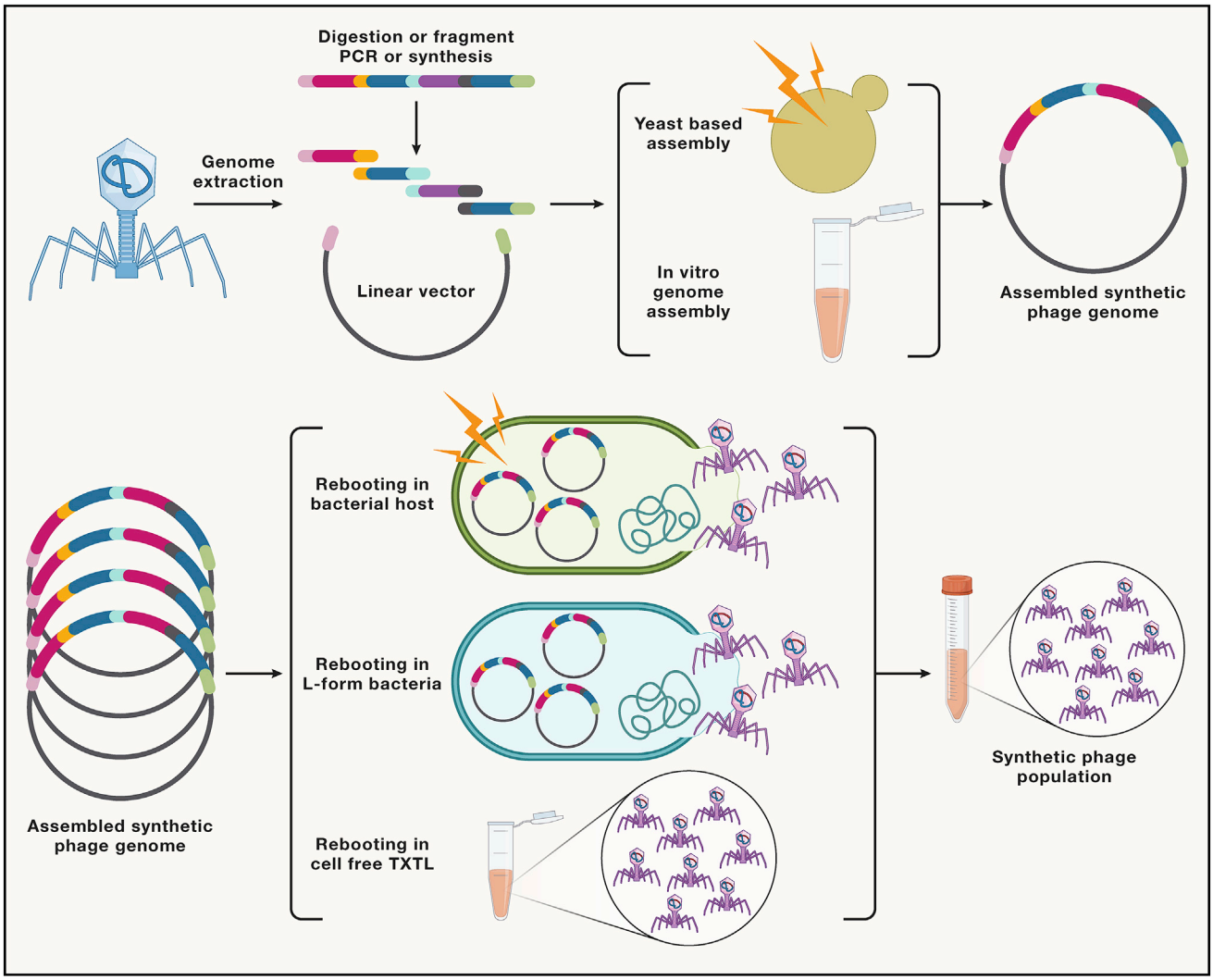

Figure 4. Building synthetic phage genomes

Using combination of phage genome fragments amplified via PCR and/or built using synthetic oligonucleotides; synthetic phage genomes are assembled into a vector using yeast-based assembly or *in vitro* assembly methods. Thus assembled genomes are then "rebooted" using suitable permissive bacterial host, cell-wall-deprived (L-form) bacterial hosts, or by using cell-free transcription-translation (TXTL) systems.

bacterial strain may change the host range of phages and may limit its applicability in therapy.

Alternatively, the toxicity and inefficient cellular transformation steps associated with rebooting of synthetic phage genomes in bacterial cells can be overcome by using TXTL systems.[93,94] In this technology, completely synthetic versions are assembled in the test tube using PCR DNA fragments of a phage template or synthetic oligonucleotides and *Escherichia coli* (*E. coli*) cytoplasmic extracts amended with additional host-specific factors as needed. The TXTL technology has been used successfully in assembling and rebooting synthetic phages from diverse groups albeit with lower efficiency. Recently, TXTL was used to reboot clinically relevant phages using genomic DNA isolated from purified phage stocks.[97] As it has become possible to build >500 kb genomes, thanks to improvements in DNA synthesis and assembly methods, we anticipate this trend of producing completely *de novo* phage genomes to continue. These improvements may complement other technologies mentioned above to produce phages on demand, addressing evolved phage resistance during therapies.

PHAGE APPLICATIONS BEYOND ANTIMICROBIAL RESISTANCE IN HUMANS

To tackle the global threat of AMR, the WHO and United Nations Interagency Working Group have endorsed a multipronged approach based on the concept of One Health: the interactions between humans, animals, and the environment.[98] While this framework has traditionally been applied to antibiotic stewardship, it has recently been assessed in the context of phage therapy[72] and can be applied to other phage applications that we discuss below (Figure 5).

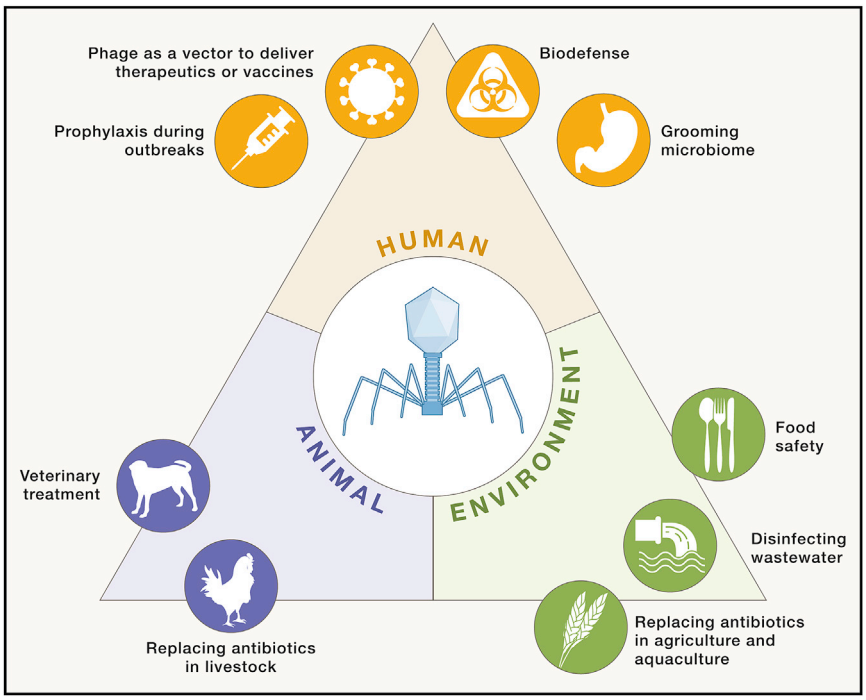

Figure 5. Potential phage therapy applications from the One Health perspective
Depicted are phage applications that could be implemented to address AMR arising from interactions between humans, animals, and the environment.

Other medical applications

Apart from the therapeutic use of phages to treat bacterial infections, phage therapy has the potential for treating chronic diseases where bacteria contribute to pathogenesis. For example, the microbiome gut-liver axis has been implicated in inflammatory responses associated with alcoholic and non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)[99] as well as with irritable bowel syndrome (IBS). Although the precise causal pathways have yet to be elucidated, pre-clinical studies are promising,[100,101] and clinical trials are planned to determine if phage therapy could be used to selectively target *Enterococcus faecalis* and *Klebsiella pneumoniae* in the gut microbiome to reduce progression to liver disease and invasive *E. coli* associated with Crohn disease.[102–104] The recent discovery of a prophage active against *Helicobacter pylori* offers hope that phage therapy could be used to target the etiologic role of this pathogen in gastric ulcer disease and gastric cancer.[105]

During outbreaks, phages could be used as prophylaxis to prevent infection among close contacts of patients acquiring highly transmissible bacterial pathogens such as *Vibrio cholerae*.[106] Phage prophylaxis could also help to interfere with transmissible pulmonary pathogens such as *Mycobacterium tuberculosis*.[107] Furthermore, filamentous phages possess properties that enable them to be manipulated into hydrogels that could be used to prevent biofilms associated with implanted hardware (e.g., prosthetic devices).[108] Another application of phages might be to groom the gut microbiome by activating prophages using medications or dietary additives such as *Stevia rebaudiana* and bee propolis extracts.[109–111] This approach to modulating bacterial composition or function has been differentiated from phage therapy and was recently referred to as "phage rehabilitation."[112]

Phage-based approaches can also be used to improve the diagnosis of elusive bacterial pathogens. Reporter phages have been described for several human pathogens,[113] and quantitative PCR has been developed to target the multicopy terminase large subunit gene encoded by prophages that are only found in *Borrelia burgdorfeii*, the primary pathogen responsible for Lyme disease.[114] Since many pathogenic bacteria harbor prophage-encoded markers, similar approaches could have wider diagnostic applications. Phages also have potential applications in biodefense to detect bacterial pathogens such as *Bacillus anthracis* or *Yersinia pestis*, and they could be used to treat victims of bioterrorist attacks.[115]

Applications in veterinary medicine and animal husbandry

The lytic activity of phages against *Salmonella enterica* (serotype pullorum) was demonstrated *in vitro* as far back as 1926. However, when the same phage was administered orally to chickens to treat salmonellosis, it was unsuccessful, probably because it was destroyed by gastric enzymes or acids. The application of phage therapy in veterinary medicine and animal husbandry was largely ignored afterward until the 1980s, and it has been the subject of several reviews[116,117] covering its use to treat or prevent *Salmonella*, *E. coli*, and *Campylobacter* in poultry and livestock.

In the beginning of the 21st century,[118] phage was administered orally to broiler chickens with antacid protection and was found to successfully reduce the bio-burden of several *Salmonella enterica* serotypes.[119] Subsequent studies using phage as a preventive versus therapeutic treatment for salmonellosis in chickens found the former approach more effective.[120,121] Other studies in chickens have shown the success of phage therapy for treating *Campylobacter jejuni* and colibacillosis, caused by avian pathogenic *E. coli*.[116] Colibacillosis mortality was also reduced when phage preparations were sprayed on the bedding of contaminated chickens,[122] indicating its role as an environmental disinfectant.

Phage has also been used extensively to treat several *Staphylococcus* species that cause mastitis in bovines, where it has also shown efficacy as a prophylactic.[123] In swine, phage has been used to treat infections caused by *E. coli* and *Salmonella enterica* as well as swine respiratory disease caused by *Bordetella*

bronchiseptica and *Pasteurella multocida*. In a recent study, dried phages delivered prophylactically in pig feed reduced *Salmonella* colonization upon challenge.[124] Taken together, these studies suggest that phage preparations could be used as a substitute or an adjunct to antibiotics pre-slaughter in poultry, cows, and pigs to prevent several types of food-borne bacterial infections from entering the food chain. Another recent study showed the utility of innovative genetic mining techniques to identify Salmophages that have application for the biocontrol of *Salmonella enterica*.[125]

Phage therapy has been less studied among pets; like human studies, the field has had a number of regulatory hurdles and clinical trials are lacking. Since companion animals are a well-known source of zoonotic multidrug-resistant bacterial infections,[126] the application of phage therapy in veterinary medicine is worthy of additional research. In a recent review, Huang et al. summarized 38 veterinary phage products, of which 9 have been approved by the FDA and 3 by the European EFSA.[117] Most research has been primarily conducted among dogs[127] but has also been encouraging for human relevance. In a pilot study, a phage cocktail was successful in reducing morbidity associated with canine otitis media caused by *P. aeruginosa*.[128] Recently, an antimicrobial treatment for animal pyoderma associated with *Staphylococcus intermedius* was developed based on cutaneous permeation of bacteriophage particles impregnated in a hydroxyethylcellulose gel with ionic liquid as a permeation enhancer.[129]

At least 150 bacterial pathogens have been identified in farmed and wild-caught fish,[130] some of which seriously affect the success of aquaculture operations and can also cause disease in humans. Phage has been shown to reduce mortality associated with *Vibrio*, *Pseudomonas*, and *Aeromonas*, most notably among fish and shrimp.[117,131,132] In one of the few rigorous field trials to have evaluated the prophylactic use of phage in aquaculture, fish mortality due to *Pseudomonas plecoglossicida* decreased by 30% after the fishpond was exposed to phage-impregnated feed for several weeks. Further, no evidence of phage-resistant bacteria or neutralizing antibodies were observed in either infected or cured fish.[133] Since most studies lack controls and key parameters such as dosing, Richards has published helpful recommendations for future studies.[132] Such studies should also consider the potential effects of therapeutic phage on marine environments.[134]

Environmental applications

Increasing regulatory restrictions on the use of antibiotics in agriculture has stimulated greater interest in the use of phage to reduce AMR in the food chain, which has been the subject of other reviews.[117,131,135] The first documented use of phage to treat bacterial pathogens in plants occurred in 1924 where it was used to prevent rot in cabbages.[136] Subsequently, phage has been evaluated as a means to prevent soft rot in potatoes, corn wilt,[131] blight, and citrus canker. Several phage products have been commercialized and obtained approval from the U.S. Environmental Protection Agency,[117] including a phage cocktail to prevent Pierce's disease in grapevines, caused by *Xylella fastidiosa* subsp. *fastidiosa*.[137] Despite some successes, results have been highly variable in many studies, in part due to issues related to the consistency of field conditions, variable weather, and the need to determine ideal timing and route for biocontrol delivery,[131] prompting recommendations to standardize protocols and improve outcomes.[138]

Phage preparations that eliminate bacterial pathogens in animal food (i.e., meat and dairy products) and plant food (i.e., fruits and vegetables) were designated as "generally recognized as safe" by the FDA as early as 1958,[117] and they have also been approved in the European Union, Switzerland, Israel, Canada, China, Australia, and New Zealand.[139] Huang and colleagues recently documented 14 phage products used in food processing, 11 of which have been approved by the FDA, which target *E. coli*, *Listeria*, *Salmonella*, *Shigella*, and *Staphylococcus* species.[117] Phages are also being evaluated to decontaminate meat from *Campylobacter jejuni*[140] and to prevent beehive collapse associated with foulbrood, caused by a spore-forming bacteria, *Paenibacillus larvae*.[141,142]

Apart from their applications to food safety, phages could be used to detect multidrug-resistant bacteria in the built environment such as hospital settings, where it could also be used to decontaminate surfaces. The potential for phages to be applied as biocontrol agents in wastewater treatment was recently reviewed by Runa and colleagues,[143] which includes their potential use as effluent quality indicators. A jumbo phage has been identified that attacks *Vibrio coralilliticus*, a widespread pathogen of coral.[144]

Conclusions

Phage therapy has been standard of care in parts of the former Soviet Union for over 80 years. After having been largely abandoned by the West for decades, it has undergone a robust revitalization in the last 7 years, especially in medicine. A growing number of clinical trials are underway in Europe, the UK, and Australia to evaluate the role of various phage preparations to treat multidrug-resistant bacterial infections in different patient populations. Clinical trials of genetically engineered and synthetic phages are now beginning but face greater scrutiny in terms of safety. Even in the absence of efficacy data from clinical trials, an increasing number of countries (e.g., the United States, Belgium, France, Sweden, Australia, and most recently, the United Kingdom) have created a "parallel track" whereby phage therapy can be approved for compassionate use on a case-by-case basis when antibiotic options have failed. Obstacles to scaling-up phage therapy include both logistical and regulatory challenges but are clearly surmountable.

There is also great potential for phage preparations to significantly reduce antibiotic use in agriculture, aquaculture, animal husbandry, and veterinary medicine, but additional empirical data are needed to standardize methods, measures, and outcomes.[72] Given the growing burden of AMR worldwide that has worsened during the COVID-19 pandemic, there is an urgent need for globally coordinated approaches to standardize guidelines and protocols, and to develop shared resources—such as phage libraries and GMP facilities—to optimize manufacturing of clinical grade phage, and to extend these resources to lower- and middle-income countries. Although it is unlikely that phages will ever entirely replace antibiotics, given that the majority of antibiotics are used in agriculture and in livestock, phage-based approaches could significantly improve antibiotic stewardship from the One Health perspective.

ACKNOWLEDGMENTS

S.A.S. and R.T.S. are grateful for support to the Center for Innovative Phage Applications and Therapeutics from the UC San Diego Chancellor, Toffler Foundation, Mallory Smith Legacy Fund, Emily's Entourage, and the Cystic Fibrosis Foundation. R.T.S. receives research support from the NIAID's Antimicrobial Resistance Group (UM1AI104681). This work was supported by awards from NIGMS (R35GM131729) and the Howard Hughes Medical Institute (GT12053) to G.F.H. V.K.M. also acknowledges support by the Laboratory Directed Research and Development Program of Lawrence Berkeley National Laboratory under U.S. Department of Energy contract no. DE-AC02-05CH11231. We thank Elizabeth Lampley and Sharon Trillo-Park for assistance with manuscript preparation and Jason Gill and Adriana Carolina Hernandez for sharing the electron micrograph of the Maestro phage.

AUTHOR CONTRIBUTIONS

S.A.S. conceived of the review content. S.A.S., G.F.H., V.K.M., and R.T.S. all contributed to drafting and editing the manuscript as well as generating the figures.

DECLARATION OF INTERESTS

S.A.S. owns stock in Adaptive Phage Therapeutics and is an unpaid advisor to Felix Biosciences. R.T.S. is a scientific consultant to LyseNTech and GSK. G.F.H. receives support from Janssen Inc through a Collaborative Research Agreement and is a consultant for Janssen, Inc. and Tessera, Inc. V.K.M. is a co-founder of Felix Biotechnology.

WEB RESOURCES

- ClinicalTrials.gov, https://clinicaltrials.gov/
- PubMed.gov, https://pubmed.ncbi.nlm.nih.gov/

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