TIMELINE

A century of the phage: past, present and future

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Abstract | Viruses that infect bacteria (bacteriophages; also known as phages) were discovered 100 years ago. Since then, phage research has transformed fundamental and translational biosciences. For example, phages were crucial in establishing the central dogma of molecular biology — information is sequentially passed from DNA to RNA to proteins — and they have been shown to have major roles in ecosystems, and help drive bacterial evolution and virulence. Furthermore, phage research has provided many techniques and reagents that underpin modern biology — from sequencing and genome engineering to the recent discovery and exploitation of CRISPR–Cas phage resistance systems. In this Timeline, we discuss a century of phage research and its impact on basic and applied biology.

Bacteriophages were discovered independently in 1915 by Frederick Twort, a British pathologist[1], and in 1917 by Félix d'Hérelle, a French–Canadian microbiologist[2] (FIG. 1). Twort described the "glassy transformation" of micrococci colonies, whereas d'Hérelle isolated an "anti-microbe" of *Shigella* and devised the term 'bacteriophage' — literally meaning bacteria-eater.

Phages are obligate intracellular parasites of bacteria and have diverse life cycles (BOX 1). The ability of phages to infect bacteria led d'Hérelle to examine their therapeutic potential against bacterial infection. Even in his first paper, he noted that the presence of phages correlated with disease clearance in patients with dysentery, and he carried out a rabbit study, in which phages provided protection from infection with *Shigella*. Most early phage research conducted in the 1920s and 1930s focused on the development of phage therapy for the treatment of bacterial infections, and companies began to market phage preparations[3]. However, in the late 1930s, the Council on Pharmacy and Chemistry of the American Medical Association concluded that the efficacy of phage therapy was ambiguous and that further research was required[3]. These concerns, and the success of emerging antibacterials, led to a decline in interest in phage therapy, although research continued in the former Soviet Union and other Eastern European countries. During this period, insights into fundamental phage biology were limited, and up until the 1940s the viral nature of phages was still disputed. Visualization of phages by electron microscopy in the early 1940s proved their particulate nature[4].

Since their discovery, phages have had an immense and unforeseen impact on our understanding of the wider biological world. Their 'simplicity' enabled our understanding of core biological processes that are relevant to all biology. Phages provided tractable model systems that gave rise to molecular biology and provided many biotechnologically useful reagents, including restriction enzymes, *en route*. In addition, their influence on nutrient cycles, pathogenicity and bacterial evolution further underlines their central role in global ecology and evolution. Furthermore, the inexorable rise of antibiotic resistance has provided added impetus for 'back to the future' phage-based solutions to bacterial infection. We are also currently witnessing incredible advances in the biotechnological exploitation of CRISPR–Cas phage defence systems, which are revolutionizing both prokaryotic and eukaryotic molecular biology research. In this Timeline, we highlight the impact of phages in the first 100 years since their discovery in terms of the origins of molecular biology, our knowledge of ecology and evolution, and their biotechnological exploitation. We encourage readers to try to imagine what the modern world would look like if phages did not exist; we are clearly indebted to the most abundant biological entities on Earth.

The origins of molecular biology

Key questions in biology addressed. In the early twentieth century, the nature of the gene was a central biological question. Physicists, including Leo Szilard, Salvador Luria and Max Delbrück as well as other researchers (the 'phage group'), began tackling this and other fundamental biological questions by working with phages as biological models[5]. Delbrück urged researchers to use select 'authorized phages', the T-phages, in their studies to facilitate comparability of results between laboratories. The T-phages were able to infect *Escherichia coli*, which was rapidly becoming the model Gram-negative bacterium. In 1939, Emory Ellis and Delbrück characterized phage growth in the 'one-step growth experiment', which revealed key phage-related concepts, such as adsorption, the latent period and viral burst[6]. A few years later, Luria and Delbrück demonstrated that mutations pre-existed in the absence of selection — a prediction of Darwinian theory[7]. This 'fluctuation test' involved growing independent *E. coli* cultures without selection and then plating the bacteria to determine both the total and phage T1-resistant cell numbers. Consistent with a model of pre-existing mutations, the number of mutants varied markedly — a factor influenced by when the mutant arose in each culture. Retrospectively, we now interpret these experiments in terms of mutations in DNA, but the nature of the gene was not known at that time.

By using phages, evidence that genes were composed of DNA was provided by Alfred Hershey and Martha Chase in their 'Waring blender experiment' (REF. 8). Phages provided an ideal model system, as they are composed of a protein coat and internal DNA — the two leading genetic contenders at that

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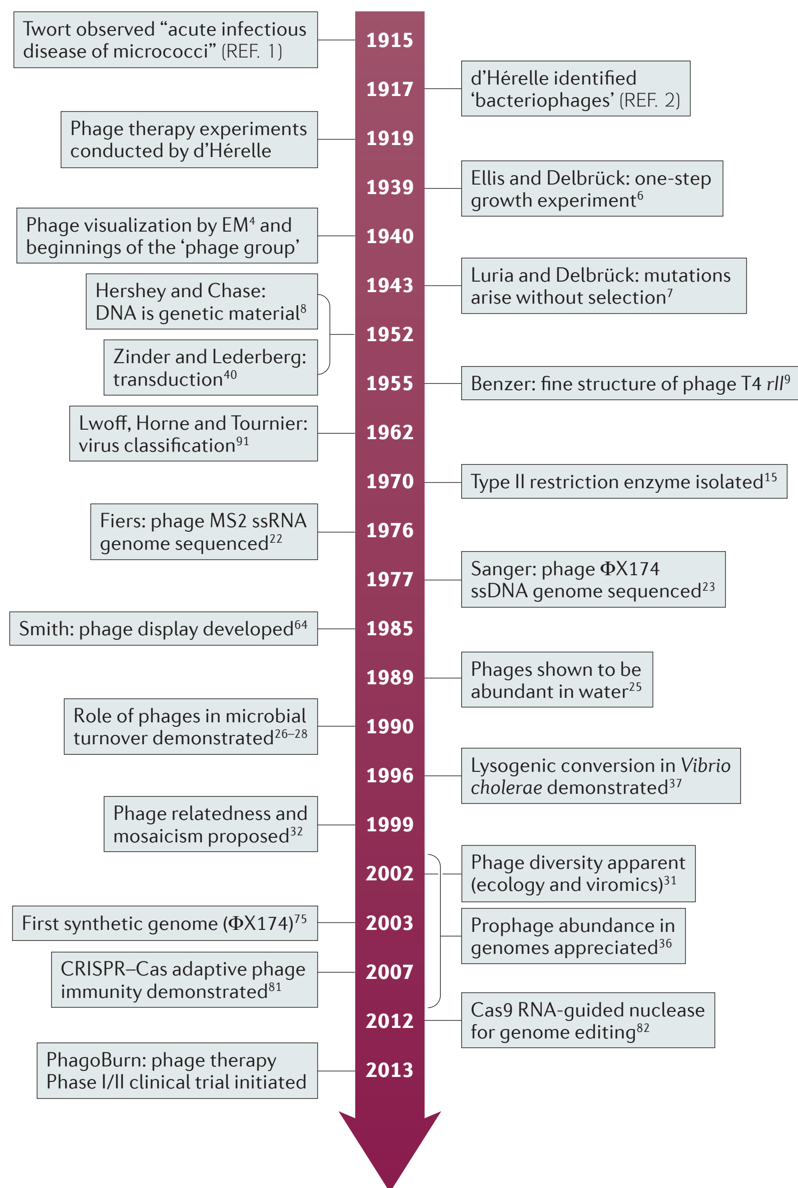

Figure 1 | **Some major events in the 100 years of phage research.** EM, electron microscopy; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

time. They specifically radiolabelled either phage proteins (with ^{35}S) or DNA (with ^{32}P). Unlabelled cells were separately infected with the two differentially labelled phage T2 samples, a blender was then used to remove the attached phage particles and the bacterial cells were collected by centrifugation. This elegant experiment demonstrated that DNA was associated with the bacterial cells and that the progeny phage contained ^{32}P, but not ^{35}S, identifying DNA as the genetic material. In recognition of this early phage work, the Nobel Prize in Physiology or Medicine was shared in 1969 by Delbrück, Hershey and Luria for discovering "the replication mechanism and the genetic structure of viruses".

Shortly after the work of Hershey and Chase, the fine structure of genes was analysed by Seymour Benzer by investigating the *rII* region of phage T4 (REF. 9). He calculated recombination frequencies by co-infecting *E. coli* with multiple phage T4 *rII* mutants to generate a high-resolution genetic map of the *rII* region. The same system was subsequently used by Francis Crick to support the triplet nature of the genetic code[10].

In the 1950s and 1960s, when the central dogma of molecular biology was emerging, it was unknown whether, or how, genes were regulated. François Jacob and Jacques Monod addressed the phenomenon of 'enzyme induction', which described the apparent activation of enzymes only in the presence of the substrate[11]. Using the *lac* system in *E. coli*, they demonstrated co-transcription of genes that were regulated by a common repressor. Although these experiments exploited some phage-based constructions, Jacob had also been working on λ prophage induction, which shared similar principles. Further studies on the genetic circuits of phage λ provided numerous paradigms for gene regulation, including the identification of DNA-binding repressor and activator proteins, and transcriptional antiterminators. The Nobel Prize in Physiology or Medicine in 1965 was shared by Jacob, Monod and André Lwoff "for their discoveries concerning genetic control of enzyme and virus synthesis". These pioneering phage studies defined what we now call 'genetic engineering' by combining analytical power with simple and elegant *in vivo* experiments.

The 'birth' of molecular biology. An unexpected translational benefit of this early research was the discovery of molecular biology reagents, which was facilitated by the increased molecular understanding of phages. Restriction–modification (R–M) was first observed in the early 1950s as a non-heritable variation following phage passage through particular *E. coli* hosts[12,13]. However, it was in the late 1960s and early 1970s that R–M systems were shown to modify DNA (often by methylation) to protect it from cleavage by the partner restriction endonuclease[14]. The sequence-specific nature of a class of restriction enzymes (type II) encouraged their biotechnological development for cutting discrete DNA fragments[15], and phage T4 DNA ligase could be used to join the molecules together[16]. The ability to use these new reagents to clone genes was a major step in the development of molecular biology and biotechnology industries[14]. For their work on "the discovery of restriction enzymes and their application to problems of molecular genetics", Werner Arber, Daniel Nathans and Hamilton Smith were awarded the Nobel Prize in Physiology or Medicine in 1978. To manipulate and amplify DNA fragments, cloning vectors were required. In addition to plasmids, phages provided important cloning solutions. Phage λ was developed as a useful cloning vector, and studies on the packaging of phage λ enabled the development of cosmids that allowed the cloning of large DNA inserts, *in vitro* packaging into phage particles and efficient delivery into *E. coli*[17]. Phage P1-derived artificial chromosomes were also developed for cloning large DNA fragments. In addition, M13

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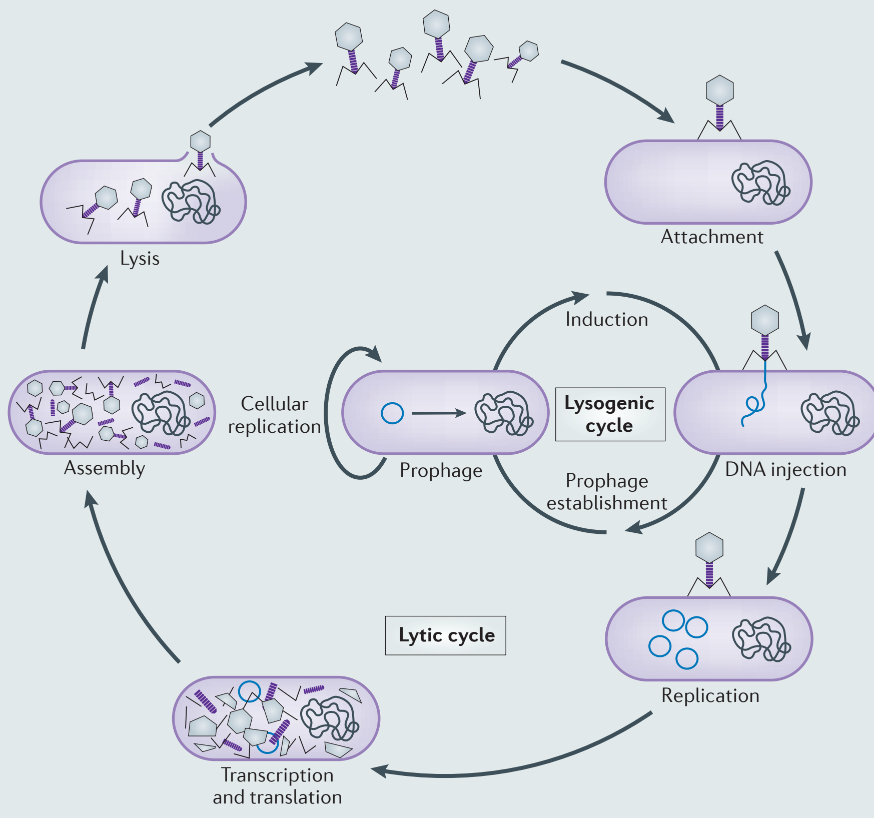
Box 1 | **Phage life cycles and taxonomy**

There is considerable diversity among phages, and both genomic and morphological information is currently used in their classification[67,90]. As we learn more about the diversity of phages and their genomic mosaicism, their taxonomy and classification require regular updating and have been said to be "as much an art as a science" (REF. 67). Initially, d'Hérelle believed that there was only one phage with many 'races'. Subsequently, electron microscopy from the 1940s[4] enabled phage taxonomy based on morphology. In 1962, the Lwoff, Horne and Tournier (LHT) virus classification system was developed, which included the type of nucleic acid (DNA or RNA), capsid morphology and enveloped nature[91]. In 1966, what is now the International Committee on Taxonomy of Viruses (ICTV) was established to provide a universal viral taxonomy, and in 2011 it released its ninth report[90]. The genetic material of phages consists of double-stranded (ds) or single-stranded (ss) DNA or RNA, and their genome sizes can range from very simple (for example, ~3.5 kb ssRNA genome in phage MS2) to highly complex (for example,~500 kb dsDNA genome in *Bacillus* phage G) and can include modified nucleotides as protection against restriction enzymes. Morphologically, phages can be tailed, polyhedral, filamentous or pleomorphic, and some have lipid or lipoprotein envelopes[67,90]. Recently, the prokaryote virus subcommittee of the ICTV has considerably systematized phage and archaeal virus taxonomy[90]. Details of the classification system can be accessed on the ICTV website. Most characterized phages belong to the *Caudovirales* order (dsDNA genome with a tailed morphology), which is divided into the following families: *Myoviridae* (for example, phage T4), *Siphoviridae* (for example, phage λ) and *Podoviridae* (for example, phage T7)[67].

Phages can have lytic or lysogenic life cycles (see the figure)[80]. To infect a host bacterium, a phage will first interact with receptors on the host cell, adsorb and then inject its genome. The subsequent replication strategy will depend on whether the phage is virulent or temperate. Virulent phages, such as phage T4, are only able to replicate through the lytic cycle, a process involving the production of new viral progeny and their release from the infected cell. Alternatively, temperate phages (for example, phage λ) enter either the lytic cycle or form a stable association with the host, termed lysogeny. During lysogeny, the viral genome is termed a prophage and replicates in concert with the host DNA, either in a free, plasmid-like state (for example, phage P1) or integrated into the bacterial chromosome (for example, phage λ). Under conditions of stress, prophages can exit the lysogenic state and produce more virions that are released from the bacterium. Typically, release of phage progeny results in bacterial death through cell lysis[52,54], although filamentous phages are released by 'secretion' through the outer membrane, thereby avoiding bacterial lysis but causing a chronic infection that slows growth of the host cell[63].



phage-based vectors were developed and phage T7 DNA polymerase has provided a high-fidelity DNA sequencing solution[18].

These advances provided the necessary tools to isolate and study genes. Phage technologies also enabled mutagenesis of bacterial genes for functional studies. For example, phage λ suicide vectors (which, upon injection into the host, are unable to replicate) were used to deliver transposons in random mutagenesis studies. Furthermore, site-directed mutagenesis systems were derived from M13 and fd phages[19,20]. Another example is phage Mu (mutator), a temperate phage that randomly transposes in the *E. coli* genome and generates mutants[21]. These features have been exploited in diverse bacterial species to generate random mutants and, in combination with reporter genes, transcriptional and translational fusions were applied to examine gene expression[21].

Many phage-derived tools were instrumental for DNA sequencing, but phage genomes were also the first to be sequenced. For example, the phage MS2 genome was the first single-stranded RNA (ssRNA) genome to be sequenced in 1976 by the group of Walter Fiers[22]. In 1977, Fred Sanger and his team sequenced the phage ΦX174 genome, which was the first complete single-stranded DNA (ssDNA) genome to be sequenced[23], and in 1982 the first double-stranded DNA (dsDNA) genome to be sequenced was that of phage λ[24]. The phage λ genome was shotgun sequenced using restriction enzymes, T4 DNA ligase and M13 vectors — almost entirely phage-derived products. These sequencing approaches were subsequently applied to many whole-genome projects — from *E. coli* to humans. It is therefore undeniable that the impact of phages on the progress of biology has been immensely far reaching.

Ecology and evolution

Diversity, abundance and ecosystems. The success of fundamental and translational phage research had high impact. Many researchers began to focus on bacteria other than *E. coli* and more complex eukaryotic organisms that became genetically tractable, owing to the new phage-inspired molecular biology 'toolkit'. However, research from the late 1980s to the 2000s also reinvigorated our understanding of basic phage biology. Before 1989, phage numbers in aquatic environments were thought to be low, until up to 2.5×10^8 viruses per ml were detected in natural waters (the typical range is ~10^6–10^7), suggesting that phages are important in the

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turnover of microorganisms and in gene transfer in the environment[25]. Subsequently, it became clear that viruses were abundant and active partners in food webs and in carbon and nitrogen cycling in the oceans[26–28]. Furthermore, some marine phage genomes carry auxiliary metabolic genes, such as those encoding proteins involved in photosynthesis, which are thought to assist infection by 'complementing' rate-limiting steps in host metabolism[29]. Metagenomics of ocean samples then revealed the true extent of phage abundance and diversity[30,31]. The abundance and diversity of phages in almost all natural environments, and in association with plants and animals, are now widely accepted[29].

Improvements in sequencing technologies generated a surge in phage genome data, revealing evolutionary relatedness and genomic mosaicism[32] with concomitant implications for phage classification. However, despite the deluge of phage genomic data, particularly since the emergence of next-generation sequencing methods, bacterial viruses often encode viral proteins with no known homologues[33], and presumably these 'unknowns' define new biological processes. Therefore, there are still enormous gaps in our knowledge of phages and their life cycles (BOX 1).

To understand the ecological and evolutionary role of phages, their co-evolution with their bacterial hosts must be considered. During his research in 1917, d'Hérelle observed increased phage numbers in stool samples taken from patients before their recovery from dysentery — perhaps an early ecological insight into the dynamics of the interaction between phages and bacteria[2]. Co-evolutionary experiments in natural and laboratory settings have begun to show that co-evolution promotes the rate of phage and bacterial evolution, sustains both genetic and phenotypic variation and can alter microbial community structures[34]. Indeed, it is perhaps unsurprising that phages and bacteria constitute the greatest genetic diversity on the planet. The ease and speed with which large bacterial and phage populations can be manipulated in laboratory microcosms has favoured their application towards addressing more general questions in evolutionary theory — an approach termed experimental evolution[35]. The power of rapid next-generation sequencing to elucidate genetic changes underpinning phenotypic and population shifts will further increase the utility of phage–bacterium systems as ecological and experimental evolution models. Finally, the ability to analyse CRISPR–Cas systems (BOX 2) to link host bacteria with phages will undoubtedly improve our understanding of their interactions in complex ecosystems and in an evolutionary context.

Bacterial pathogenicity and evolution. The impact of phages on the evolution of bacteria (FIG. 2) is underscored by the estimation that, globally, $\sim 2 \times 10^{16}$ phage-mediated gene transfer events occur every second[36]. Furthermore, in 1996, the discovery that the *Vibrio cholerae* toxin — a key virulence factor — is encoded in the genome of the transferrable filamentous phage CTXΦ highlighted the importance of phages in the evolution of bacterial pathogenicity[37]. This process, termed lysogenic conversion (or phage conversion), was first observed in the 1950s[38], and it describes a situation in which a prophage provides additional genes that benefit the lysogen (FIG. 2). The importance of phages for pathogenicity in bacteria was further demonstrated in the 1990s, when genome sequencing revealed the abundance of prophages and established that they account for the main genetic variability between closely related bacterial strains (for example, pathogens versus non-pathogens)[36]. For example, in *Streptococcus pyogenes*, ~10% of the genome consists of prophages, which encode multiple virulence factors, and in *E. coli* O157:H7 str. Sakai, 18 prophages constitute 16% of its genome[36]. In some cases, the prophages define core aspects of bacterial pathogenesis. For example, in Shiga toxin-producing *E. coli*, prophage induction upregulates the toxin genes, and cell lysis is important for toxin release[36,39]. Finally, prophages that exhibit regions of homology can drive evolutionary changes through inversions or deletions and other chromosomal rearrangements[36] (FIG. 2). For example, an *S. pyogenes* M3 strain isolated in Japan differs from an isolate from the United States owing to a chromosomal inversion between two different prophages, which encourages reshuffling of the prophage virulence genes[36]. Inversions and deletions can also modulate fitness through selection events that drive rapid evolutionary changes.

Phages are also responsible for horizontal gene transfer (HGT) between bacteria. For example, by inducing bacterial lysis, phages promote release of bacterial DNA, which can then be acquired by neighbouring competent cells (FIG. 2). Furthermore, much of the phage-derived HGT occurs by generalized transduction, where bacterial DNA is accidentally packaged during phage replication and then delivered into neighbouring cells (FIG. 2). This phenomenon was discovered in 1952 by Norton Zinder and Joshua Lederberg[40], which contributed to Lederberg's shared 1958 Nobel Prize in Physiology or Medicine "for his discoveries concerning genetic recombination and the organization of the genetic material of bacteria". Specialized transduction is the transfer of DNA located adjacent to the integrated prophage after imprecise excision (FIG. 2). Transduction facilitates the mobilization of antibiotic resistance and virulence genes, and antibiotic exposure can promote these processes[41,42]. A form of 'constitutive generalized transduction' is promoted by gene transfer agents (GTAs), which have a significant role in HGT in bacteria[43]. GTAs are prophage-like elements encoded in bacterial genomes that package random host DNA but cannot package enough to enable the transmission of their own genes[44] (FIG. 2). GTAs might have evolved from mutant prophages that became defective and subsequently decayed. Phage-inducible chromosomal islands (PICIs) can hijack phages to assist in their transfer, giving rise to high-efficiency transduction, where the islands are transferred to neighbouring bacteria[45] (FIG. 2). For example, the *Staphylococcus aureus* pathogenicity islands (SaPIs) encode superantigens and 'parasitize' phages for high-frequency transduction[45]. As identification of PICIs and GTAs is challenging, their general contribution to gene transfer is probably underestimated[44,45].

In addition to providing virulence genes to bacteria, phages themselves may have been co-opted by bacteria during the evolution of R-type pyocins and type VI secretion systems[46] (FIG. 2). Type VI secretion systems use a phage tail-like cell-puncturing mechanism to deliver effector proteins into both eukaryotic and prokaryotic cells. R-type pyocins are phage tail-like structures encoded in bacterial genomes that are released during cell lysis and bind to and kill other bacteria. Notably, some marine bacteria release arrays of tail-like structures, which can induce metamorphosis in marine tubeworms, suggesting that phage-like structures can be used by bacteria to interact with eukaryotic organisms[47]. Collectively, these studies demonstrate that there are many ways in which phages contribute to bacterial virulence and host interactions.

Biotechnological exploitation

Phages as alternative antimicrobials. Recently, increasing antibiotic resistance, coupled with a paucity of new antibiotics,

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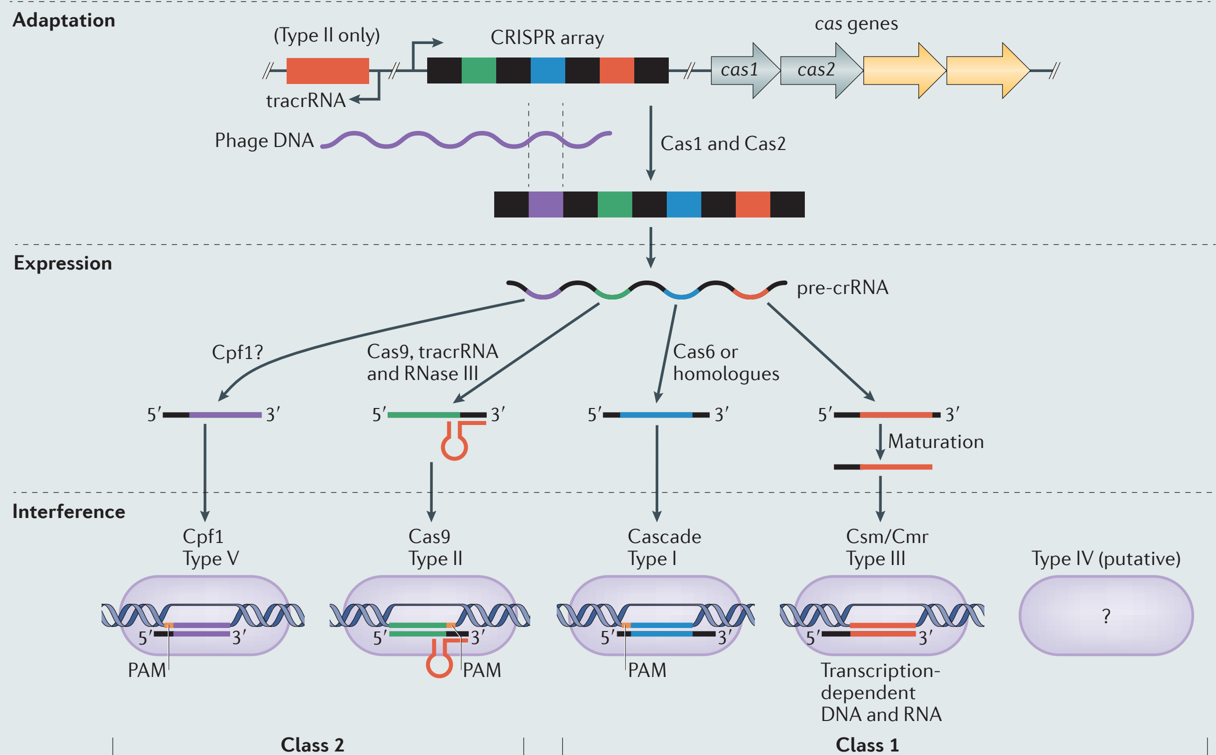
Box 2 | CRISPR–Cas adaptive immune systems

CRISPR–Cas systems provide adaptive immunity to bacteria and archaea against foreign invaders, such as plasmids and phages[83]. However, they can also be involved in diverse cellular processes[92].

The CRISPR arrays provide the 'immune memory' and consist of short repeats separated by 'spacer' sequences derived from the invading mobile genetic elements (MGEs). The Cas proteins impart the machinery for defence against the incoming nucleic acids[93]. CRISPR–Cas systems are divided into two major classes, five major types (type I–V) and multiple subtypes[94]. Three types (type I–III) and recently type V[95] have been characterized mechanistically, and they show significant differences, despite conserved overall functional principles.

CRISPR–Cas systems work in three steps, termed adaptation (or acquisition), expression and interference (see the figure). Adaptation involves the endonucleases Cas1 and Cas2, which acquire the new spacer and add this to the CRISPR, with concomitant repeat duplication. During expression, the *cas* genes and CRISPRs are transcribed. The full length CRISPR transcript is termed the pre-CRISPR RNA (pre-crRNA) and is processed into CRISPR RNAs (crRNAs) containing the MGE-derived spacer sequence and portions of the repeats. Generation of crRNAs differs between systems. In class 1 (type I and type III) systems, crRNA production typically involves a Cas6 endoribonuclease. In class 2 (type II and type V) systems: type II systems require Cas9, host RNase III and a non-coding RNA (transactivating crRNA (tracrRNA)) for pre-crRNA processing, whereas in the type V systems Cpf1 generates crRNA independently of a tracrRNA. In class 1 systems, the crRNA forms part of a Cas–ribonucleoprotein complex, whereas in class 2 systems a single protein (Cas9 or Cpf1) forms a complex with crRNA and, for Cas9, also with tracrRNA. During interference, these complexes recognize and degrade the MGE. In type I systems, recognition of complementary DNA (the protospacer) results in the recruitment of Cas3, which degrades the invader DNA. Type III systems target RNA and DNA in a transcription-dependent manner. Finally, type II and type V interference results in DNA degradation by the Cas9–tracrRNA–crRNA or Cpf1–crRNA complexes, respectively (for recent reviews please refer to REFS 83,92,93).

In addition to the applications of CRISPR–Cas, the biology of these systems is equally impressive and is revealing new dimensions of phage–bacterium interactions. For example, when becoming 'immunized' by a MGE, some CRISPR–Cas systems preferentially acquire new spacers from the invader in a replication-dependent manner[96]. This limits problems of 'autoimmunity', and suggests an enhanced response against aggressively replicating MGEs. Type III systems have another trick to target lytic phage reproduction, without self-targeting prophages in the bacterial genome[97]. By targeting both RNA and DNA in a transcription-dependent manner, these systems only interfere with transcriptionally active phages. However, phages can evade CRISPR–Cas through point mutations that disrupt the complementarity during targeting[81,98]. Surprisingly, in a process termed 'priming', CRISPR–Cas systems can detect even heavily mutated invaders and rapidly acquire spacers to generate renewed immunity[98]. Not to be outdone, some phages overcome CRISPR–Cas immunity by expressing anti-CRISPR proteins that inhibit the resistance machinery[99]. In a further twist, a *Vibrio cholerae* phage encodes a CRISPR–Cas system required for replication in strains containing an anti-phage-inducible chromosomal island (PICI)[100]. Evidently, there is still much to learn about CRISPR–Cas systems and their evolutionary relationship with phages and other MGEs. PAM, protospacer adjacent motif.



has encouraged a resurgence in phage-inspired antibacterial approaches in agriculture, medicine and some food industries (FIG. 3). There is also interest in utilizing phages for sensitive and specific detection of bacteria[48]. Multiple detection methods are being developed, including systems based on phage-induced lysis, phage amplification, delivery of reporter genes, cell surface-binding proteins and biosensors[48]. Phage therapy using natural or modified virulent phages is showing encouraging therapeutic results, with controlled clinical trials underway[49,50]. For example, a cocktail of phages used to treat *Pseudomonas aeruginosa* ear

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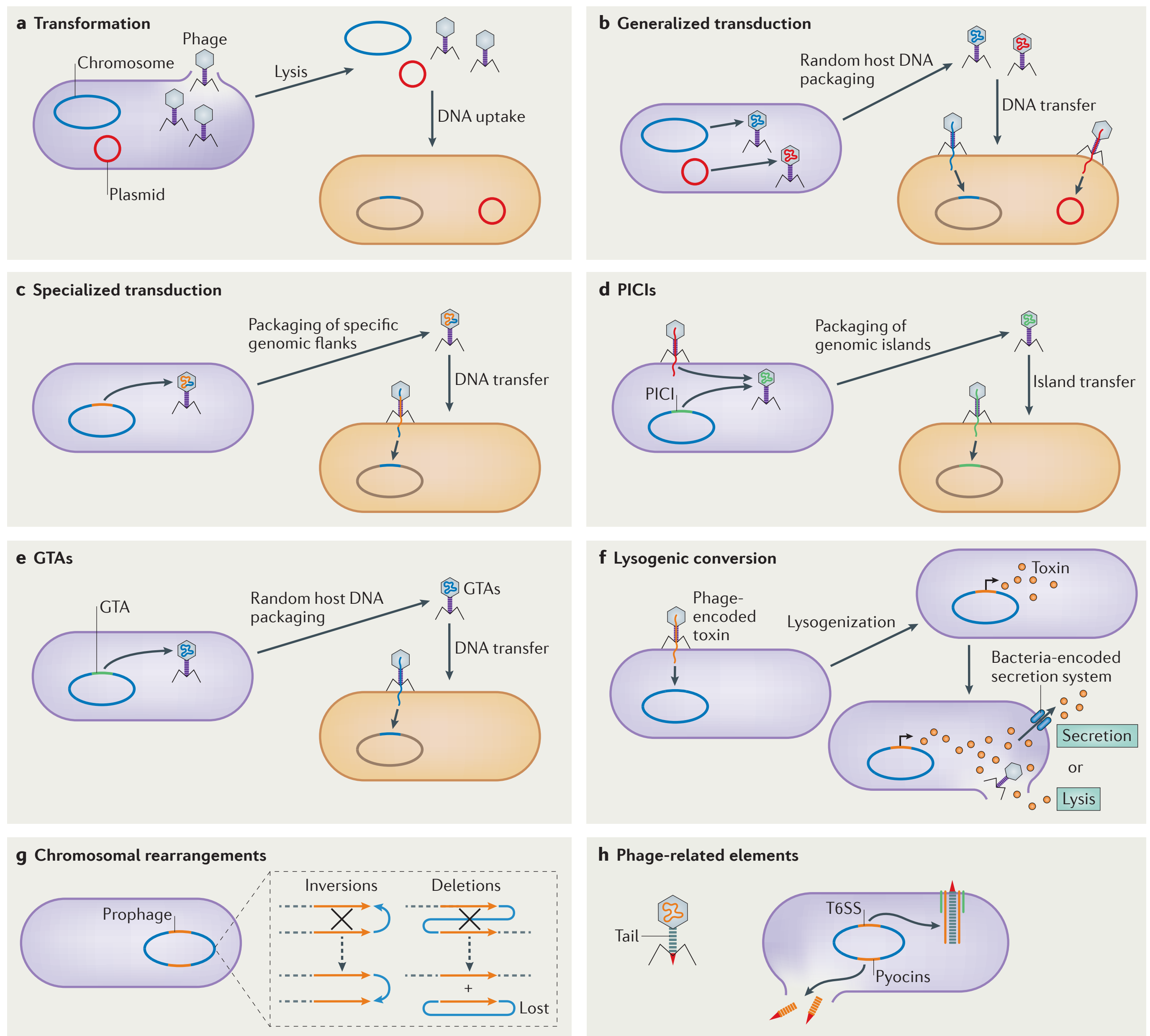

Figure 2 | **The role of phages in bacterial pathogenicity and evolution.** Phages contribute to bacterial pathogenicity and evolution by multiple mechanisms, including by generating genetic diversity through horizontal gene transfer (HGT). **a** | Phage-mediated cell lysis can release naked DNA that is acquired by transformation. **b** | Phages can also directly inject random fragments of host or plasmid DNA into neighbouring bacteria, by generalized transduction. **c** | Temperate phages can move flanking host genes through specialized transduction. **d** | Phage-inducible chromosomal islands (PICIs) can hijack 'helper' phages for high-efficiency transduction of the island. **e** | Gene transfer agents (GTAs) are non-replicative phage-like elements in the bacterial chromosome that package and transduce host DNA for constitutive transduction. **f** | During lysogenization, temperate phages can cause lysogenic conversion by carrying genes encoding diverse proteins, such as toxins, that are either secreted, or released on bacterial lysis. **g** | Prophages can also promote recombination events that lead to prophage and genomic rearrangements, by inversions or deletions. **h** | Elements related to phages (such as R-type pyocins and type VI secretion systems (T6SSs), which are both structurally similar to the phage tail), are used by bacteria as part of their arsenal.

infections showed significant efficacy and safety[51]. Currently, a Phase I/II clinical trial involving three European countries is investigating the safety and efficacy of phages for the treatment of burn wounds infected with *E. coli* and *P. aeruginosa* (known as the PhagoBurn clinical trial: further information can be found on the PhagoBurn trial website). For food safety and agriculture, there has been faster uptake of phage technologies, with several approved products commercially available. Examples include LISTEX P100 (Micreos; the Netherlands) and ListShield (Intralytix; Baltimore, Maryland, USA) for the protection of processed foods from *Listeria monocytogenes*.

Other innovative antimicrobial approaches using phage products or engineered phages are being developed (FIG. 3). Considerable research has focused on endolysins, which

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are peptidoglycan hydrolases involved in cell lysis during phage replication[52]. The loss of structural integrity upon peptidoglycan degradation causes lysis through osmotic imbalance[53]. In Gram-negative bacteria, the outer membrane must also be breached through complexes (known as spanins) that fuse both membranes[54]. Endolysins are typically composed of an enzymatically active domain and a cell wall-binding domain[52]. Extensive endolysin diversity exists, and most endolysins are species specific, although some are more promiscuous. The modularity of endolysins has facilitated 'mixing and matching' of domains to increase activities and alter host ranges[52]. By fusing endolysins to other domains, it is possible to deliver endolysins across the Gram-negative outer membrane or into eukaryotic cells to target intracellular bacteria[52,55]. Phase I and Phase II clinical trials are underway for the topical or intravenous use of endolysins for the control of *S. aureus* infection in humans, and preclinical trials have been carried out in various infection models[52]. The first commercially available endolysin, Staphefekt (Micreos), is available for the treatment of human skin infections caused by *S. aureus*.

A complementary approach to the use of endolysins involves exploiting tailspike or diffusible polysaccharide-depolymerizing enzymes, which can reduce the levels of surface polymers such as exopolysaccharides (EPSs) and lipopolysaccharides (LPSs)[52]. Rather than lysing bacteria, polysaccharide removal can disrupt biofilms, reduce virulence and assist in bacterial clearance by host immune systems[56]. In a variation on this theme, phage T7 was engineered to express an EPS-degrading enzyme during infection of an *E. coli* biofilm. Cell lysis released the EPS-degrading enzyme, which aided phage infection and increased the anti-biofilm and antibacterial effect[57] (FIG. 3). The specificity of phages for their target bacteria is often considered to be a therapeutic advantage as this limits collateral damage to beneficial microorganisms, but altering the host range can be desirable. For the EPS-degrading phage T7, utility was enhanced by introducing gene *1.2* from phage T3 that extended its host range, enabling infection of *E. coli* containing the F plasmid. Evolution can also be exploited to select for altered receptor recognition and host range. For example, *in vitro* evolution of phage λ resulted in recognition of a new porin receptor, outer membrane protein F (OmpF)[58]. In nature, *Bordetella* phage BPP-1 accelerates its tail fibre evolution to alter its

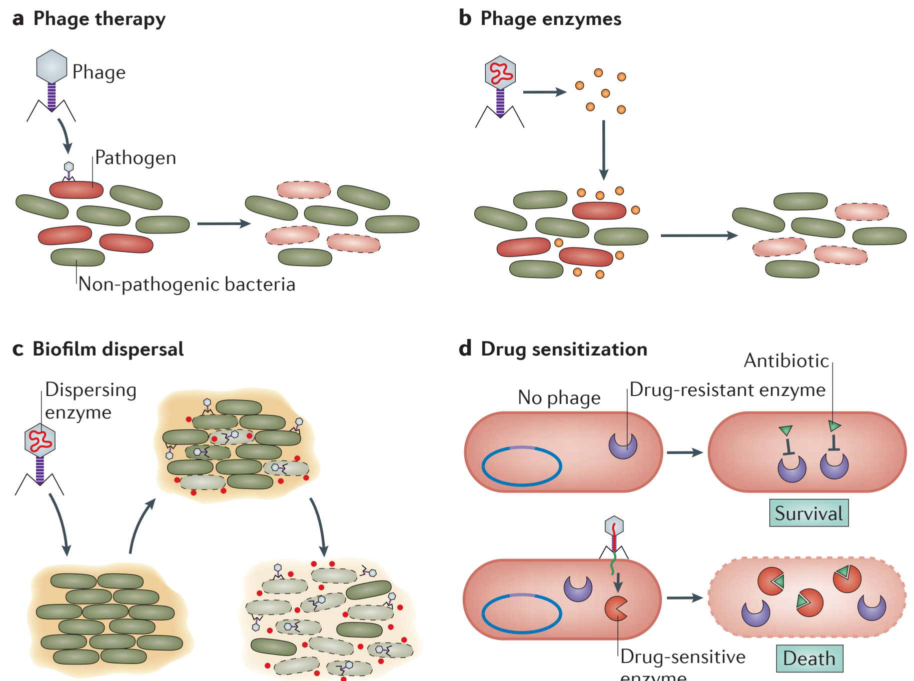

Figure 3 | **Some phage-inspired antimicrobial approaches.** Phages and their products provide routes that could lead to the creation of novel antimicrobial strategies. **a** | The specificity of phages can be explored for phage therapy, by which phages target particular bacterial pathogens. **b** | Phage products, such as enzymes, can be used to target specific bacteria, including pathogens. **c** | Phages can be used to disrupt biofilms, by targeting bacteria embedded in these structures, and can be engineered to release specific enzymes that degrade the biofilm matrix. **d** | Phages can be used to sensitize antibiotic-resistant bacteria. For example, phages can introduce antibiotic-sensitive genes into drug-resistant hosts, and this strategy can be combined with antibiotic treatment.

host range through diversity-generating, site-specific, error-prone reverse transcription[59]. The specificity of phages, and their efficiency of DNA delivery, has been exploited for the injection of bacteria with lethal genes, which encode restriction endonucleases, holins, toxins of toxin–antitoxin systems or proteins that condense DNA (for example, SASPject; Phico Therapeutics, Cambridge, UK).

Phages can also improve antibiotic efficacy (FIG. 3). For example, antibiotics can be conjugated to phages, enabling delivery to specific cells and causing an increase in local drug concentration[60]. Furthermore, antibiotic resistance can be overcome by using phages to inject sensitizing alleles of the mutated genes (for example, *rpsL* and *gyrA*) to restore drug efficacy[61]. Alternatively, delivery of regulatory genes can reprogramme cells in a defined manner. For instance, the introduction of genes that inhibit the stress response (such as *lexA*), improve drug uptake (such as *ompF*) or repress biofilm production (such as *csrA*) can increase the antibiotic sensitivity of *E. coli*[62]. Therefore, phages offer a wide range of potential methods to tackle bacterial infections and antibiotic resistance. However, regulatory issues need to be addressed for engineering approaches.

Synthetic biology and nanotechnology. One group of phages that are having a major impact on biotechnology and nanotechnology are the filamentous phages[63]. These phages (for example, M13 and fd) have small circular ssDNA genomes and are secreted from the bacterium without lysis. They have been exploited in phage display, a technique pioneered by George Smith[64]. This commercially available technology exploits the physical link between genotype and phenotype, and exploits the screening of large phage numbers. Libraries of variable DNA sequences are fused to a coat protein gene and the corresponding encoded proteins are displayed on the virion surface upon viral assembly. Typically, phage display selects proteins that bind to a specific target; phages that bind to the target are isolated and re-amplified, whereas phages that do not bind to the target are washed away. The applications of phage display are diverse. For example, the emerging field of nanomedicine involves using nanostructures for the

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targeted delivery of therapeutic cargo or for molecular detection or diagnostics. By using phage display, peptides or the entire phage can be engineered, for example, to bind to and deliver drugs, vaccines or imaging labels to specific locations, or to identify or target cancer cells or bacterial infections[65]. Similar methods are being applied to engineer phages with useful properties for materials science. For example, high-powered lithium-ion batteries have been built from filamentous phages, and liquid crystal nanostructures can be assembled[63].

As well as being engineered into useful nanostructures, natural phages are intricate molecular self-assembling nanomachines. Since phages were first viewed in the electron microscope, many groups have combined X-ray crystallography and electron microscopy to determine high-resolution virus structures[66]. The majority of phages visualized (~96%) are tailed and belong to the *Caudovirales* order, which is subdivided into three families (*Myoviridae*, *Siphoviridae* and *Podoviridae*)[67]. *Myoviridae* have straight contractile tails, *Siphoviridae* have flexible non-contractile tails, and *Podoviridae* have short tails[67]. The structural proteins of *Caudovirales* have similar folds, suggesting a shared evolutionary history, but considerable morphological variation exists[66]. Most phage capsids are icosahedral, but some are prolate (that is, elongated). Phage heads are capped at one vertex with a portal (or connector), where assembly of the scaffolding and major capsid proteins is typically initiated[68]. The major capsid proteins of *Caudovirales* have a highly conserved HK97 fold[69] and require scaffolding proteins to establish appropriate capsid geometry[66]. The empty capsid (that is, procapsid) is matured by proteases that degrade the scaffolding proteins, and DNA is then translocated in an energy-dependent manner through a packaging complex to tightly package the linear dsDNA genome. Packaging involves small and large terminases — some of the strongest and fastest molecular machines — to cleave genome-length DNA units and drive them into the capsid. Multiple proteins are then added to plug the portal and prepare the head for binding to the phage tails. In contrast to the *Podoviridae*, in which tails assemble directly on the portal vertex, *Myoviridae* and *Siphoviridae* synthesize tails independently and then attach these to the capsid to complete assembly[66]. Tail structures are involved in the recognition of specific receptors, penetration of cell membranes and delivery of the viral genome into the host cell[70]. In recent years, cryo-electron microscopy has revealed remarkable aspects of the viral 'search' for receptors (which in phage T7 occurs in a 'random walk') and has uncovered structural remodelling upon DNA injection, intracellular capsid maturation, tight DNA packaging and the lysis process[68,71–73].

Phages have also affected the development of synthetic biology[74]. Synthetic biology aims to build systems 'from scratch' with predictable properties, and phages provide novel molecular parts[74] and tractable model systems. Indeed, phage ΦX174 was the first genome to be completely artificially synthesized[75]. Phage integrases and recombinases catalyse the site-specific recombination of two sequences (*att* sites), and many, including those from phage Bxb1 and phage ΦC31, have been exploited in synthetic biology and other applications to drive integration, excision and inversion events[76]. These enzymes classically control the integration and excision of temperate phages into, and out of, the bacterial genome[76] (BOX 1). Another widely used system is the Cre–*loxP* site-specific recombination system of phage P1, which can generate defined genetic alterations in eukaryotes or bacteria in a controlled manner[77]. When the expression of recombinases is linked to an input signal, they can elicit DNA sequence changes that can be used for circuit generation and provide re-writable genetic memory[78]. In addition, T7 RNA polymerase can be applied to synthetic circuit design. By splitting T7 RNA polymerase into two parts that can be controlled independently by different inputs, AND logic gates that can be engineered generate output only when both signals are present[79]. Therefore, the future feasibility of biocomputing using phages in batteries, and as the memory, may not be purely in the realms of science fiction.

CRISPR–Cas resistance revolution. During co-evolution with phages, bacteria have acquired many antiviral strategies, and new types are still being uncovered[80]. Fundamental studies of these resistance systems have produced serendipitous findings that enabled powerful innovation and translational applications. R–M systems are the classical example of these unexpected and unpredictable rewards[14]. Another resistance class providing significant commercial benefit are the abortive infection systems, which give population-level protection through the 'altruistic cell suicide' of phage-infected bacteria[80]. More recently, the discovery and characterization of CRISPR–Cas adaptive immune systems (BOX 2) has again demonstrated that basic phage research often yields sophisticated tools with wide-ranging biotechnological utility. The first demonstration that CRISPR–Cas provides immunity against phages was reported in 2007 (REF. 81), and after only 5 years the possibility of exploiting these systems for genome editing was realized[82].

Since 2012, there has been an explosion in the number of applications based on the CRISPR–Cas system[83,84]. These advances are centred on the type II CRISPR–Cas system (BOX 2), owing to the ability of one protein (Cas9) and an engineered single-guide RNA (sgRNA) to direct double-strand breaks in complementary DNA sequences. By either non-homologous end joining, or homology-directed repair, spontaneous or specific mutations can be generated in diverse organisms, including phages, bacteria, fungi, plants and animals[84]. In addition, a nuclease-deficient Cas9 yields an RNA-guided protein that binds to specific DNA regions to repress gene expression or, when fused to activator domains, enhances transcription[83,84]. Further developments include the generation of large sgRNA libraries, which enable genome-wide screening for gene function to assist drug target identification[84].

CRISPR–Cas9 is readily applicable to both model and non-model organisms, and is driving widespread studies of gene function and the development of modified crops and animals in the agricultural sector and for diverse biomedical applications[84]. For example, cells were engineered to target HIV, and this disrupted both latent viral genomes and protected cells from new viral infection[85]. There is also interest in using CRISPR–Cas9 to modify ecosystems to control pest species or eliminate disease vectors, such as mosquitoes. Indeed, a CRISPR–Cas9 gene drive was able to rapidly spread a mutant allele through *Drosophila* in laboratory experiments[86]. In bacteria, CRISPR–Cas has also been used to generate mutations in several bacterial genomes with potential use for synthetic biology and metabolic pathway engineering[87], and has been explored as a novel antimicrobial strategy. For example, CRISPR–Cas can kill bacteria in a sequence-specific manner to selectively eliminate particular strains, to select for less virulent survivors owing to the loss of pathogenicity islands, and to inhibit antibiotic-resistant bacteria by targeting resistance genes[83,87]. In addition, CRISPR–Cas9 can be used to manipulate phage genomes to study phage biology with greater ease and precision[88]. Although promising, these developments also raise many ethical and regulatory questions[84].

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Conclusions and the future

The biological impact of phage research in one century has been phenomenal, and the commercial impact incalculable. Without phage research, there would be very few, if any, global biotechnology businesses (without, for example, restriction enzymes, phage display and now CRISPR–Cas), and a long list of important phage-based technologies and potential therapeutics would not exist. In addition to the historical role of phages in helping to define core biological principles, it is important to appreciate their superb educational value. This is exemplified by the remarkable 'Citizen Science' (Howard Hughes Medical Institute (HHMI)–Science Education Alliance (SEA)–Phage Hunters Advancing Genomics and Evolutionary Science (PHAGES)) educational engagement programme developed by Graham Hatfull *et al.*, which has introduced thousands of undergraduates and high school students, through practical phage work, to core biological and evolutionary principles but, perhaps more importantly, the exciting and capricious nature of scientific research. This has produced the largest number (>800) of genome sequences for phages infecting a single bacterial strain and highlighted our ignorance about the functions of around 70% of phage gene products[33].

Recent discoveries have also revealed 'jumbo' phages with genomes larger than those of some bacteria; so, at least in terms of size, genomic demarcation zones between bacteria and their viruses may be gradually dissolving into a genetic continuum, raising interesting evolutionary questions about the nature of life on Earth[89]. This serves to highlight an important fact that will make the second century of phage research unpredictably exciting. Given the sheer global abundance of phages and our stark ignorance of the functions of most of their genes, there must be many discoveries still to be derived from basic phage work. New technologies for transcriptomic, proteomic and metabolomic analyses of phage infection will uncover new functional and regulatory complexities of phages. Based on the experience of the past 100 years, some of these future discoveries will undoubtedly undergo powerful translation into medical, agricultural and industrial biotechnologies.

Surely we can also expect much more in the coming years, and not least from the exploitation of phage-based tools that can be repurposed for diverse synthetic biology applications. Focusing on even a single, contemporary phage research 'product' should be enough to convince us of the exciting and impactful future possibilities. CRISPR–Cas-based technology is already revolutionizing eukaryotic biology and is creating collateral ethical issues because of the ability to manipulate genomes with high fidelity— ultimately perhaps for targeted re-engineering (editing) of our own genomes. Man discovered phages and, in only a century, phages have given up a few of their biological and biochemical secrets, with incredible results. So perhaps the ultimate biotechnological translation of phage research may be fundamental genetic manipulation of man himself: from therapeutics to evolution.

What will the next 100 years reveal? If Twort and d'Hérelle had been asked that question a century ago there is no way that they could have imagined what we know today. So, for us too, the full potential of phages is unpredictable. Importantly, applications with the greatest impact that have been derived from phage biology (such as restriction enzymes and CRISPR–Cas) originated from curiosity-driven research on fundamental phage phenomena. There may be no better argument for investment in basic bioscience than this. Inventive innovation cannot be generated solely through policies that preferentially fund low-risk strategic research. So, assuming continued investment in basic phage research, we expect an exciting second century. Surely the fantastic impact of the first century of phage biology must have convinced everyone of that.

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doi:10.1038/nrmicro3564
Published online 9 November 2015

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Acknowledgements

Research in the laboratory of G.P.C.S. is supported by grants from the UK Biotechnology and Biological Sciences Research Council (BBSRC) and the Cambridge Trusts, and through the financial support of the Society for General Microbiology, the Society for Applied Microbiology and the British Society for Plant Pathology. Research in the laboratory of P.C.F. is supported by the Marsden Fund, the Royal Society of New Zealand (RSNZ), a Rutherford Discovery Fellowship (RSNZ) to P.C.F., a University of Otago Research Grant, a Bequest Fund for Research in the Otago School of Medical Sciences and the Bio-Protection Centre of Research Excellence. The authors thank R. Dy for assistance with the preparation of some of the figures and R. Staals for comments on the manuscript. The authors apologize to the many phage researchers whose significant contributions could not be cited owing to space constraints.

Competing interests statement

The authors declare no competing interests.

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