Pseudomonas predators: understanding and exploiting phage–host interactions

Jeroen De Smet, Hanne Hendrix, Bob G. Blasdel, Katarzyna Danis-Wlodarczyk and Rob Lavigne

Abstract | Species in the genus *Pseudomonas* thrive in a diverse set of ecological niches and include crucial pathogens, such as the human pathogen *Pseudomonas aeruginosa* and the plant pathogen *Pseudomonas syringae*. The bacteriophages that infect *Pseudomonas* spp. mirror the widespread and diverse nature of their hosts. Therefore, *Pseudomonas* spp. and their phages are an ideal system to study the molecular mechanisms that govern virus–host interactions. Furthermore, phages are principal catalysts of host evolution and diversity, which directly affects the ecological roles of environmental and pathogenic *Pseudomonas* spp. Understanding these interactions not only provides novel insights into phage biology but also advances the development of phage therapy, phage-derived antimicrobial strategies and innovative biotechnological tools that may be derived from phage–bacteria interactions.

Laboratory of Gene Technology, Department of Biosystems, KU Leuven, Kasteelpark Arenberg 21, Box 2462, 3001 Heverlee, Belgium.

Correspondence to R.L.
Rob.Lavigne@kuleuven.be

doi:10.1038/nrmicro.2017.61
Published online 26 Jun 2017

The *Pseudomonas* genus is one of the most ecologically important groups of bacteria and includes species that are plant commensals (*Pseudomonas stutzeri* and *Pseudomonas fluorescens*) and pathogens (*Pseudomonas syringae*), species that can be used in bioremediation (*Pseudomonas putida*), and species that are pathogens of insects (*Pseudomonas entomophila*), animals and humans (*Pseudomonas aeruginosa*)[1]. The huge metabolic diversity of this genus and the remarkable capacity of its species to adapt to environmental stresses have resulted in a ubiquitous ecological distribution, including in fresh water and soil. This broad distribution and resistance to stress are the result of the high genomic complexity and plasticity of these bacteria, and their unusually large genomic complexity, which also translates to the transcriptional and translational and/or post-translational levels[2]. For example, a comparison of 389 strains of *P. aeruginosa* revealed that collectively only 17.5% of their genomes are shared; this is the 'core' genome. This leaves a large 'accessory' genome, which is capable of rapid modulations during niche-based adaptation in both environmental and pathogenic isolates[3,4]. This genomic versatility also enables *P. aeruginosa* to encode and produce a wide range of cell-associated and extracellular virulence factors, making it well adapted to infect patients who are immunocompromised (for example, patients with burn wounds or cystic fibrosis), and forms the basis of its high level of antibiotic resistance[5]. The diversity and widespread nature of *Pseudomonas* spp. are also reflected in the distribution of their bacterial viruses (phages). Indeed, phages that infect *Pseudomonas* spp. can be readily isolated from almost any rich organic soil or water sample[6], and can be found in human tissues during infection with *P. aeruginosa* (for example, the lungs of patients with cystic fibrosis)[7].

The rapid increase in determined genome sequences of phages that infect *Pseudomonas* species (spp)., from 47 genomes available through the United States National Center for Biotechnology Information (NCBI) in 2010 to 150 at the time of writing (May 2017), has revealed enormous diversity that mirrors the diversity observed in their hosts and that requires a genome-driven classification (BOX 1). Not only do phages that infect *Pseudomonas* spp. differ in genome size (ranging from 3.59 kb for phage PP7 to 316.67 kb for phage 201Φ2-1) but also in genetic content, as sequence comparisons between *Pseudomonas* phages of distinct phage genera revealed little to no shared similarity (apart from within some distinct clades)[8]. Most of this genomic diversity is found in numerous functionally unknown open reading frames (ORFans) that are present in hypervariable regions, which are largely involved in adaptation to host-specific conditions and the formation of tail fibres[9]; the latter are important for host recognition and attachment. These ORFans form the so-called 'viral dark matter', which many presume to represent a goldmine of molecular tools for antimicrobial or biotechnological

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Box 1 | **Phage classification — shifting criteria in the post-genomic era**

It is important to apply an evolution-driven classification system to assess diversity in a viral community[14]. Before the availability of fast genome sequencing technologies, virus classification was based on virion morphology (tail type, polyhedral, filamentous and pleomorphic) and nucleic acid composition[162]. Most *Pseudomonas* phages (>97%) belong to the *Caudovirales* order of tailed double-stranded DNA (dsDNA) phages. The rest, known as PFP phages, have a polyhedral, filamentous or pleomorphic phenotype[14]. Currently, a more molecular-based and genomic-based approach is used for classification to integrate both the phage replication strategy and genome structure[163,164]. This integration has led to an improved classification of phages into subfamilies and genera, driven by vertical evolution but affected by horizontal transfer events[69], and may lead to the classification of metagenome-based virus isolates in the future[165].

applications[10–12]. Surprisingly, although isolates of *Pseudomonas* phages show a high local diversity[13], most *Pseudomonas* phage species seem to be widespread globally, which suggests a finite total diversity[14–16]; this could be the result of a limited number of globally successful core phage genomes. For phages of *P. aeruginosa*, this success might be correlated to the well-studied *P. aeruginosa* population structure. This structure is characterized by the existence of global and local generalists on the one hand and niche specialists on the other hand[17]. Phage genomes that retain the ability to infect global generalist strains, including *P. aeruginosa* str. C and *P. aeruginosa* str. PA14, may have a selective advantage, as these hosts are readily available. The ecological roles of phages are as diverse as their genomes, ranging from predation and the maintenance of local population diversity[18] to catalysing bacterial speciation[19]. The diversity of the *Pseudomonas* genus and its phages enables the investigation of the roles that phages have in microbial physiology and ecology in various physical environments. Therefore, *Pseudomonas* species–phage systems are often used to research the effect of phages on host evolution and fitness, as well as their therapeutic potential against pathogenic *Pseudomonas* spp.

In this Review, we discuss recent findings that relate to the occurrence and effect of phage–host interactions in the *Pseudomonas* genus, both at the molecular and ‘single-cell’ levels during infection, and at the population level. As this genus contains ecologically important bacterial strains, as well as key pathogens, special emphasis is placed on the potential biotechnological or clinical applications that these interactions have or could have in the future.

Infection at the single-cell level

After phages attach to a host cell and pilot their genome through the bacterial membrane, three principal modes of infection cycle can ensue: the lytic or lysogenic cycles, or they may enter a phage carrier state. During the lytic cycle, phages subvert host cellular processes to produce phage progeny, which are rapidly released during host cell lysis. The release of phage progeny in the lytic cycle is primarily associated with host cell lysis; however, some phages (for example, *Escherichia coli* phage M13) leave the host cell intact and are associated with chronic virion release. Temperate phages have the ability to undergo either lytic or lysogenic cycles. During lysogeny, the phage genome either exists as a plasmid or is integrated into the bacterial chromosome as a prophage, which will be stably replicated with the host chromosome[20]. Recent work has found evidence of a third mode of infection cycle, the phage carrier state, in which the phage chromosome exists as a distinct episome without integrating into the host chromosome or triggering cell lysis. In contrast to the lysogenic cycle, this episome is not replicated in a stable manner and can asymmetrically segregate between daughter cells, thereby imposing a distinct expression profile in the resulting host cells[21,22].

Phage infection is often portrayed as a process in which the host cell is helpless against the invading virus; however, in reality, bacteria are equipped with an arsenal of defence systems against phages. In response, phages have evolved a range of methods to circumvent these antiviral mechanisms, which indicates the importance of the struggle between the phage and host for dominance over the cell and its resources (FIG. 1).

Evasion of host defence systems. Before phages can replicate, they must circumvent the defence systems of their host[23]. In this section, we focus on specific examples of how *Pseudomonas* phages cope with host defence systems.

Phage adsorption is the initial process by which phages recognize and bind to the bacterial cell surface. The most common bacterial resistance mechanisms prevent phage adsorption, which phages counteract by adapting their receptor-binding proteins through mutation. Indeed, host recognition genes (which encode tail fibres and structural proteins) are the most rapidly evolving phage genes under selective pressure, and a single point mutation can alter the host range of a phage[24–26].

To gain access to the bacterial cell surface, phages must often overcome a range of extracellular polysaccharides and other biofilm-forming components that are produced by *Pseudomonas* spp. Several virion-associated polysaccharide depolymerases have been identified in *Pseudomonas* phages to achieve this, such as the tail spikes in *P. putida* phages φ15 and AF[27,28], or those of *P. aeruginosa* phage PT-6, which are able to degrade four structurally distinct alginate polymers. Phage PT-6 also encodes a soluble alginase to degrade the capsules of neighbouring cells[5,29].

After infection, phages are able to prevent superinfection with other phages by modulating the expression of cellular receptors. For example, the temperate *P. aeruginosa* phage D3112 encodes a protein (Tip) that binds to PilB, a type IV pilus (T4P) assembly and extension ATPase; this prevents the formation of pili, thus making the bacterium resistant to T4P-dependent phages[30] (FIG. 1). Other prophages in *P. aeruginosa* were shown to prevent superinfection through additional mechanisms, some of which alter the receptor (T4P or O-antigen), whereas others function inside of the cell[31].

After adsorption, phages inject their DNA into the host cell. However, many bacteria encode restriction endonucleases to cut foreign DNA and prevent phage

Phage carrier state
The phage genome is carried as an unstable episome by the host that can segregate asymmetrically between daughter cells. This segregation results in the transient resistance of a subpopulation and ensures the long-term availability of sensitive cells for infection.

Temperate phages
Phages that can undergo either the lytic cycle or a lysogenic cycle, in which they exist as stable plasmids or integrate their genomes into bacterial chromosomes.

Prophage
During lysogeny, the phage genome is either integrated into the bacterial chromosome or maintained as an extrachromosomal plasmid, and this stably present genome is called the prophage.

Type IV pilus
Surface-exposed hair-like filaments that mediate various functions in bacteria, including motility, DNA uptake, protein secretion and adherence to host cells.

O-antigen
The hydrophilic, immunodominant and outermost domain of bacterial lipopolysaccharide that consists of repeated monosaccharide units that are glycosidically linked. The O-antigen forms the basis for the serological classification of Gram-negative bacteria.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

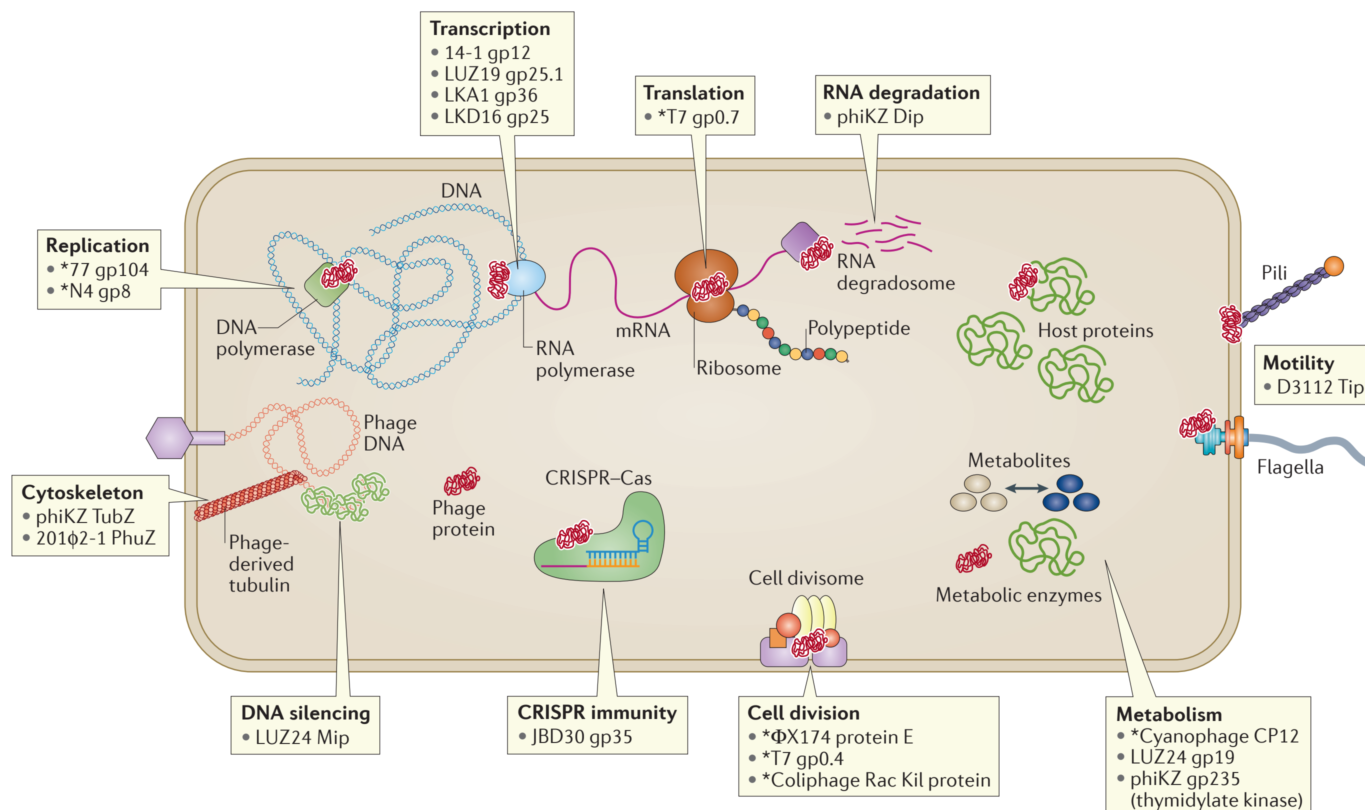

Figure 1 | **Phages influence key processes in host cells.** A schematic representation of a *Pseudomonas* spp. cell, with the different cellular processes that are influenced by phages shown. Following infection, phages will produce proteins (red) that interfere with key host processes to halt or redirect their function. Phages that infect *Pseudomonas* spp. have been shown to influence host cell transcription, RNA degradation, cellular motility, metabolism, CRISPR-mediated immunity to phages and phage DNA silencing. In addition, some phages produce their own cytoskeletal proteins, which optimize phage production by centring phage DNA in the cell. For each process, at least one phage protein that interferes with it is listed as an example. Interestingly, no *Pseudomonas* phage proteins have been reported to interfere with DNA replication, cell division or translation, whereas such proteins have been found in related phages. Examples of phage proteins that do not originate from *Pseudomonas* phages but that also interfere with these processes are marked with an asterisk.

Non-canonical nucleotides
Commonly incorporated into phage genomes, these are nucleotides that have been substituted for, or covalently modified from, the standard adenine, guanine, cytosine andthymine.

CRISPR–Cas system
An RNA-guided prokaryotic adaptive immune system that involves the acquisition of a sequence of the invading DNA (the protospacer) and its insertion into the CRISPR array as a spacer (adaptation). This is transcribed and processed to generate CRISPR RNAs (crRNAs; expression), which direct the cleavage of foreign nucleic acids by Cas proteins at sites that are complementary to the spacer sequence (interference). Type I systems are characterized by the use of a complex of Cas proteins, including Cas3, and are further divided on the basis of their combination of Cas genes and operon organization.

infection. In turn, phages have evolved anti-restriction strategies to defend their DNA against cleavage by restriction endonucleases. One mechanism that is observed in many phages is the incorporation of one or more non-canonical nucleotides during DNA replication[32]. For example, a deoxyuridine monophosphate (dUMP) hydroxymethylase is predicted to be encoded by *P. aeruginosa* phages YuA (gp17), JL001 (gp24) and M6 (gp58)[33].

A different antiviral mechanism that protects the host through the degradation of foreign DNA is the CRISPR–Cas system, which acts as a bacterial adaptive immune system. In CRISPR–Cas systems, genetic information that has been obtained during previous encounters with foreign DNA is stored to trigger sequence-specific DNA degradation during future encounters[34,35]. Large-scale genomic analysis of *P. aeruginosa* isolates revealed that the subtypes I-F, I-E and I-C of type I CRISPR–Cas systems were found in 30%, 12% and 3% of tested isolates, respectively[36]. In response, phages have evolved ways to disable CRISPR–Cas systems[37]. In total, 10 different anti-CRISPR (Acr) protein families have been identified in temperate *Pseudomonas* phages, which interact with components of the subtype I-F CRISPR–Cas interference machinery, AcrF1–F10 (REFS 37,38). Moreover, an in-depth study of one representative from each of the first three families revealed divergent inhibition mechanisms; AcrF1 and AcrF2 bind to the Csy3 and Csy1–Csy2 subunits, respectively, of the CRISPR RNA (crRNA)–effector complex (known as the Csy complex) to block target DNA binding. By contrast, AcrF3 binds to the nuclease Cas3 to prevent the degradation of target DNA[39]. Furthermore, an additional group of phage-borne anti-CRISPR proteins that comprises four distinct families that inhibit the *P. aeruginosa* subtype I-E CRISPR–Cas system has been discovered (ACR88a-32, ACR3-30, ACR3112-31 and ACR5-34)[40]. Interestingly, anti-CRISPR homologues were also found in non-prophage-related genomic regions, which suggests that these genes may protect other mobile DNA elements, besides phages, from degradation by CRISPR–Cas systems and thus may contribute to lateral gene transfer[38,40]. In addition, phages seem capable of evading CRISPR interference through specific mutations. Indeed, several

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

escape mutants of an engineered phage, DMS3, have been found that have a single base mutation within the CRISPR-targeted region[41].

A different host strategy for maintaining cellular control against virus infection involves the silencing of phage DNA through proteins that specifically bind to xenogeneic DNA, which has a lower GC content than the host genome[42]. *P. aeruginosa* str. PAO1 encodes two such proteins, MvaT and MvaU[43,44]. In *P. aeruginosa* phage LUZ24, a protein that inhibits MvaT was identified that blocks it from binding to DNA[45] (FIG. 1).

Subversion of host functions. In addition to circumventing host defences, phages subvert host cellular processes to optimize the intracellular environment for the production of phage progeny. This is achieved by specific, often toxic, protein–protein interactions that occur early during phage infection[45–48].

Bacteriophages have evolved a wide range of transcriptional control strategies that range from full dependence on host transcriptional machinery to near-complete independence. Indeed, infection with *P. aeruginosa* phage phiKZ proceeds normally even in the presence of high concentrations of rifampicin, which is a powerful inhibitor of host transcription[49]. To enable this independence of the host transcriptional machinery, phiKZ encodes two RNA polymerases (RNAP). One of these polymerases is packaged in the virion to be co-injected with phiKZ DNA for early transcription, whereas the other is encoded by transcripts that are expressed during the middle stage of infection and powers late transcription[50].

Most other phages that depend on the transcription machinery of the host for a stage in their infection cycle temporarily inactivate host-mediated transcription, which enables transcription by the phage RNAP. For example, coliphage T7 encodes gp2 and members of the genus Phikmvvirus, including the *P. aeruginosa* phage LUZ19 (REF. 51), encode another protein that interacts with the β′ subunit of host RNAP, resulting in the inactivation of the transcription complex[52] (FIG. 1). These proteins are essential for a productive infection with these phages and are involved in the shutdown of early phage transcription during the later stages of infection (thus helping the transition to late viral transcription), in addition to inhibiting host transcription[53,54].

Last, some phages are completely dependent on the transcriptional machinery of the host and have evolved mechanisms to redirect its function to viral replication. For example, in *P. aeruginosa* phage 14–1, gp12 inhibits *in vitro* transcription from host cell promoters by interacting with the α-subunit of host RNAP, which presumably alters its promoter specificity and directs it towards phage-specific promoters[48] (FIG. 1).

In addition to blocking host transcription, phage infection has been found to modulate the transcription of the *Pseudomonas* host genome. For example, infection of *P. aeruginosa* str. PAO1 with phiKZ results in the upregulated expression of a filamentous prophage, Pf1, which is assumed to attempt to escape the infected cell[49]. Furthermore, in *P. aeruginosa*, infection with the single-stranded RNA (ssRNA) phage PRR1 affects various systems, including transport and export, ribosomal proteins, energy production and conversion, and transcriptional regulators[55]. In addition, the interaction between the LUZ24 phage protein Mip and the foreign DNA-silencing protein MvaT probably prevents the binding of MvaT to its target (AT-rich DNA), which includes the LUZ24 genome, and thus the shutdown of transcription of these genomic regions[45] (FIG. 1).

In addition to controlling transcription, phages can regulate transcript turnover. A novel degradosome-interacting protein (Dip) was discovered in phage phiKZ that influences the degradation of RNA in *P. aeruginosa* to prevent viral RNA degradation in infected cells[56,57] (FIG. 1). RNA turnover was also altered following infection with the phage PAK-P3, which resulted in a decrease in host transcripts. Thus far, the mechanism responsible for PAK-P3-mediated RNA degradation remains unknown[58]. As viruses depend on the metabolism of the host, many viral genomes acquire host-derived metabolic genes, termed 'auxiliary metabolic genes' (AMGs); these genes are hypothesized to overcome bottlenecks in host metabolism to favour viral replication[59]. AMGs have been found to interfere with phosphate[60] and nitrogen metabolism[61], photosynthesis[62,63], the pentose phosphate pathway[64], nucleic acid synthesis[65] and other metabolic pathways[66]. Interestingly, AMG-mediated metabolic reprogramming of bacteria infected with phages can be linked to fluctuations in global nutrient cycles[67].

Unfortunately, there is little experimental data on the net effect of phage infection on host metabolism. In *P. aeruginosa*, infection with the phage Pf1 has been reported to increase the level of agmatine, which is involved in the formation of biofilms[68]. Furthermore, a recent study applied 'high-coverage' metabolomics to analyse the effect of six distinct phages on metabolism in *P. aeruginosa* str. PAO1. Phage-specific alterations were observed, which ranged from the complete depletion of metabolites produced by the host to an active modulation of host metabolism. For example, the phage YuA caused an increase in pyrimidine nucleotides early during infection, possibly owing to degradation of the host genome, whereas phiKZ actively modulated the pyrimidine biosynthesis pathway, resulting in the stimulation of *de novo* synthesis[69]. Although the effect on host metabolism in this study seems to correlate with the AMG content of the infecting phage, known AMG features do not explain all metabolic alterations, which suggests a role for unknown phage-encoded peptides in metabolism takeover.

Apart from interfering directly in metabolic pathways, phages can also conserve energy for infection by shutting off 'non-essential' host processes, such as host replication and cell division[70,71]. For example, the temperate coliphage Rac encodes the Kil protein, which interacts with the tubulin homologue FtsZ to impair cell division in *E. coli*[72]. Thus far, interactions between phage proteins and the replisome or divisome have not been reported for *Pseudomonas* phages. However, an alternative strategy to maximize the use of resources in the host has been reported in the *Pseudomonas*

Degradosome
A multiprotein complex in gammaproteobacteria that is built around the endoribonuclease RNaseE, which is responsible for the decay of mRNA.

Metabolomics
A collective term for the methods used to determine, on a large-scale, the metabolite levels in biological extracts, including both gas and liquid chromatography-based mass spectrometers.

Replisome
The machinery that is used by the cell to replicate DNA by separating the double helix and then synthesizing a complementary sequence on each strand to form two double-stranded DNA polymers that are faithful copies of the original.

Divisome
A transmembrane multiprotein apparatus that forms mid-cell after replication and segregation of the chromosome, and facilitates cell division.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

phiKZ-related phages. These phages have evolved their own tubulin cytoskeletal protein (TubZ) to centre their DNA in the cell for the optimal production of phage progeny[73–75] (FIG. 1). These examples show the diverse strategies that phages have evolved through co-evolution to modulate their host, and, undoubtedly, numerous other mechanisms remain to be discovered.

Phage effects at the population level

In addition to influencing intracellular molecular interactions during infection, phages affect their host at the population level. Indeed, phage infection substantially affects both the evolution and the ecology of the bacterial host, mainly through phage–host co-evolution, lysogenic conversion and transduction[76].

Phage–host antagonistic co-evolution. The continuous adaptation and/or counter-adaptation that occurs between interacting species shape genotypic, phenotypic and community-level diversity[76,77]. Bacteria and phages are increasingly being used to study host–parasite co-evolution (reviewed in REF. 76) owing to their fast generation time and tractability[78]. Below, we focus on the dynamics between *P. fluorescens* str. SBW25 and the *Autographivirinae* family member ϕ2, which is a highly investigated model for antagonistic co-evolution studies[79]. *In vitro*, this model system has an 'arms race' dynamic, which after 200–250 generations switches to a series of oscillations in bacterial and phage genotype populations, referred to as fluctuating selection dynamics[80]. By contrast, in natural environments (such as soil microcosms) the initial 'arms race' is not observed, as bacteria are more resistant to their contemporary phages than to ancestor phages and co-evolution follows fluctuating selection dynamics[79]. This discrepancy may be due to the additional abiotic and biotic selection pressures that are found outside of controlled laboratory conditions, which increase the fitness cost of resistance[81]. For example, if the risk of phage infection is high, constitutive mechanisms (for example, cell wall modification) are preferred over inducible resistance mechanisms (for example, CRISPR), owing to the fixed fitness costs that are associated with constitutive mechanisms[82,83]. Furthermore, the general diversity and the extensive number of genes that are potentially involved in the interplay between phages and bacteria in natural environments should also be taken into account; this may result in continuously changing dominant phage and bacterial isolates in natural environments.

Prophage-mediated lysogenic conversion and host genome diversification. Next-generation sequencing has revealed the ubiquity of prophages in the genomes of *Pseudomonas* spp. At least 60 distinct temperate phages have been characterized in *P. aeruginosa* alone and most strains contain at least one prophage[3]. Notably, the genome of *P. putida* str. KT2440 contains four prophages that account for 2.6% of the total genome[84]. Under specific conditions, prophages can escape the genome and complete a lytic cycle; when a prophage loses this capacity it becomes cryptic[85]. The presence of a prophage can influence the phenotype of a lysogen, which is known as lysogenic conversion. For example, a prophage can transform a non-virulent bacterial strain to a virulent strain (lysogenic conversion), as was the case with *E. coli* O157:H7 after it acquired two Shiga toxin-encoding prophages (Sp5 and Sp15)[86]. Furthermore, the presence of temperate phages was shown to accelerate genome diversification and adaptive evolution in pathogens; for example, through the insertional inactivation of clinically important traits, such as motility and quorum sensing[87]. Interestingly, a form of phage carrier state can also arise following infection by lytic phages in *P. aeruginosa* str. PAO1, which aids in the accumulation of mutations that result in host resistance against multiple phages. This higher-than-expected mutation frequency is most likely the result of the constitutive production of new phage particles in these colonies, which exerts a permanent selective pressure on the bacterium[88]. Last, transducing phages, such as *P. aeruginosa* phage DMS3 (REF. 89) and *P. aeruginosa* phage φPA3 (REF. 90), can incorporate random bacterial DNA fragments during genome packaging and transfer these fragments to other bacteria through a process known as transduction. This phage-mediated horizontal gene transfer has a substantial role in shaping the accessory genome of *Pseudomonas* spp.[3], which encompasses those genes that are found in some strains of *P. aeruginosa* but not others.

The effect of phages on* Pseudomonas *spp. in the environment. *Pseudomonas* phages can affect bacterial ecosystems in the natural environment through several mechanisms (FIG. 2). First, we consider well-defined communities that comprise a single species of bacteria and phage under laboratory conditions to limit additional biotic and abiotic factors from influencing co-evolution. Comparing *in vitro* cell densities of communities of either a motile or a non-motile strain of *P. aeruginosa*, in the presence of the phage phiKZ, revealed that motility provides a fitness advantage to the bacterium. This is probably due to decreased adherence of phages to motile bacteria. In addition, motile bacteria can form clusters that result in a 'safety in numbers' effect, as the risk for an individual cell of having to defend against phage infection decreases[91] (FIG. 2). By contrast, when *P. fluorescens* is grown in a homogeneous spatial structure, co-evolution selects for the mucoid phenotype, which prevents phage adhesion but decreases bacterial motility[92] (FIG. 2). These examples illustrate the unpredictability of host–phage co-evolution in more complex environments due to the influence of additional abiotic and biotic parameters[92]. Indeed, under laboratory conditions, phages can increase rates of mutation in *P. fluorescens*[93], whereas in soil this increase is not observed, owing to the spatial heterogeneity and increased costs that are associated with phage resistance mechanisms found in this natural environment[94]. Moreover, variation in the natural environment influences the roles that phages can have in *Pseudomonas* genome diversification. This is due to dispersal barriers that exist between communities in spatially structured niches of the environment that

Lysogenic conversion
Following insertion into the genome, specific prophage elements can induce changes in the phenotype of the infected bacterium.

'Arms race' dynamic
In this model, selective pressure between a host (*Pseudomonas*) and its parasite (phage) leads to an increasingly more resistant host population and virulent parasite population, as each species has to constantly evolve to maintain the same level of fitness. Consequently, each generation is better adapted than its ancestor generations in both species.

Fluctuating selection dynamics
A mode of co-evolution that is characterized by fluctuating selection pressures in variable environments. In this instance it leads to phages evolving to infect common bacterial genotypes, providing a benefit to rare host resistance alleles that then become dominant; at which point phages start targeting the new dominant bacteria.

Lysogen
A bacterial cell in which a mobilizing prophage exists in a dormant state, with its host-lethal genes suppressed by a transcriptional repressor.

Mucoid phenotype
Pseudomonas strains that cause persistent infections as they produce excessive amounts of the extracellular polysaccharide alginate that render them resistant to phagocytosis and to antibiotics.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

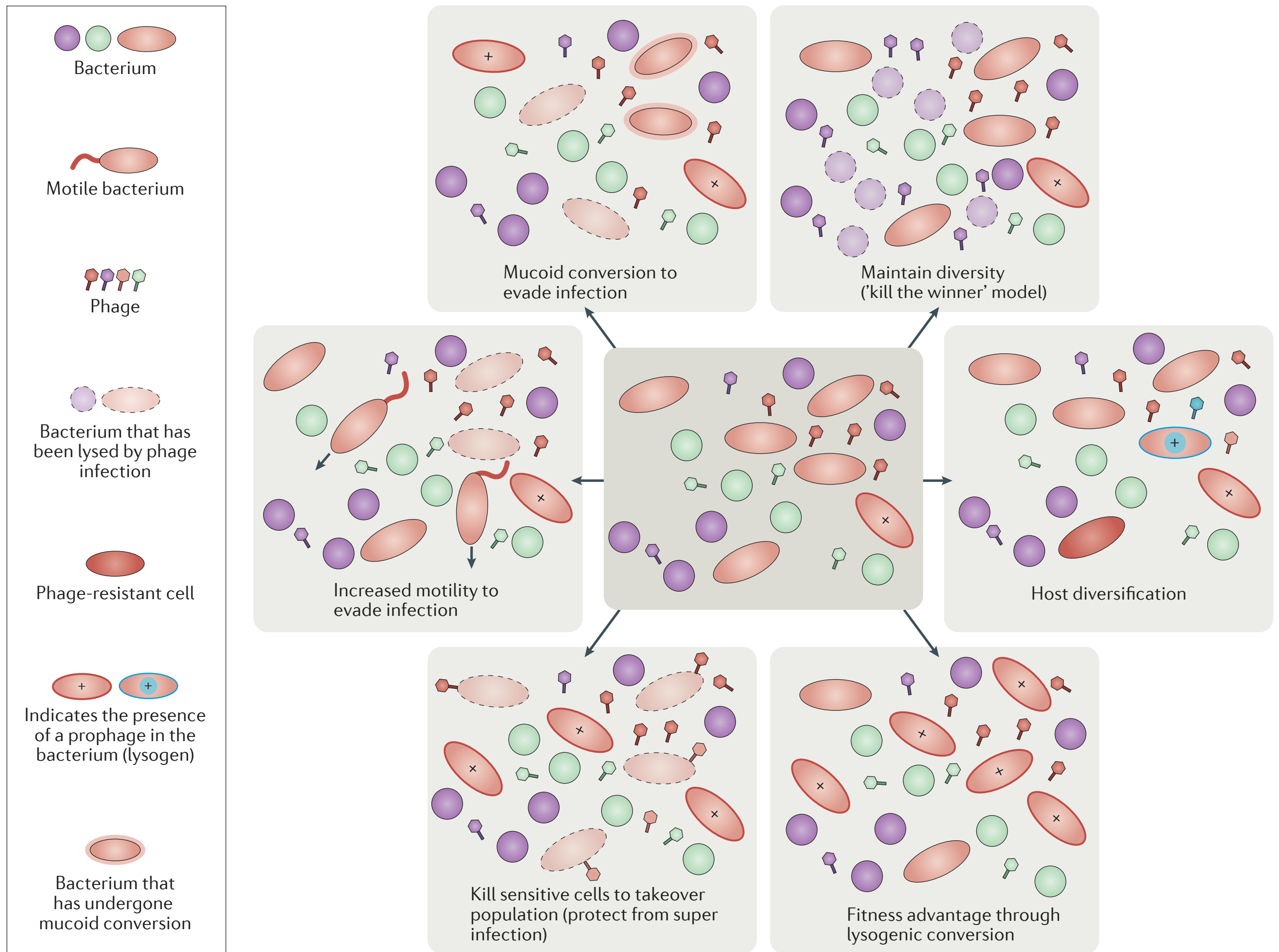

Figure 2 | **The effect of *Pseudomonas* phages on complex bacterial communities.** This figure provides an overview of the already described roles that *Pseudomonas* phages have in the environment. The central square depicts a normal bacterial community. Such communities comprise different bacterial species and even in these species heterogeneity is present, as some bacteria are lysogens that contain a prophage. The presence of a diverse set of phage species in such environments can trigger several ecological effects. Phages can catalyse phenotypic changes in the host population; for example, by triggering selection for motile bacteria that are able to escape from phage-rich niches (middle left) or by triggering mucoid conversion in the bacterial population to evade phage infection (top left). Furthermore, phages have a role in maintaining bacterial diversity in the environment. The 'kill the winner' model states that the likelihood of phage infection for a bacterial species increases when its abundance in the population increases. This ultimately leads to a decrease in the population of this species; these dynamics prevent the takeover of the environment by one species (top right). Apart from conserving diversity, phages can increase the bacterial diversity in an environment by selecting for resistant bacterial genotypes (dark red cell), which can be the result of diverse mechanisms (middle right). Last, phages can also negatively affect bacterial diversity. By providing a selective advantage to their host due to lysogenic conversion, temperate phages can increase host fitness (and potentially abundance) in the environment (bottom right). By killing sensitive cells, temperate phages help their lysogens to consolidate and takeover a niche (bottom left).

prevent locally arising phage-resistant genotypes from widely dispersing through the environment, which provides phage-resistant genotypes with a lower fitness the opportunity to arise[95].

Phage–host co-evolution also occurs in more complex communities, which can comprise more than just one species of bacteria and phage[96]. In such communities, conflicting interactions between different organisms affect co-evolution, which can trigger a diversification of defence strategies. As an example of this, we consider the effect of co-evolution on the pathogenesis of *P. aeruginosa* in a wax moth larvae model. After *P. aeruginosa* had been cultured with a predatory protozoan (*Tetrahymena thermophila*) for one month, it was used to infect wax moth larvae. This revealed that the protozoan had caused an attenuation of bacterial virulence by targeting *P. aeruginosa*. The addition of the PMN phage into this model was shown to limit the protozoa-driven attenuation of bacterial virulence, presumably owing to competition and conflicting defence mechanisms between

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

both predators of the bacterium[97]. Phages can also shape the community composition of coexisting bacteria, as was shown using *in vitro* cultures of *P. aeruginosa* and *P. fluorescens*[18]. In this study, phages enabled the two species to coexist by infecting the superior competing *Pseudomonas* species more frequently, which gave the weaker competitor a chance to secure its own niche. This observation is an example of how phages can maintain diversity by killing the winner in a specific niche (FIG. 2).

In addition, prophages can have detrimental or beneficial effects on the physiology of their *Pseudomonas* spp. hosts, affecting their fitness in the environment. For example, the removal of all four prophage elements from the genome of *P. putida* str. KT2440 resulted in an increase in tolerance to environmental stresses[84]. By contrast, prophages can protect their host cell from superinfection with other phages, which can increase its competiveness compared with phage-sensitive bacteria[31] (FIG. 2). Furthermore, some phages, such as phage Pf4, help *P. aeruginosa* to combat its competitors. The virions of phage Pf4 can chelate iron, which is a crucial component for the formation of biofilms; this leads to the destabilization of preformed biofilms from *Aspergillus fumigatus*[98], which is a competitor microorganism. Thus, the release of Pf4 virions from *P. aeruginosa* populations provides a competitive advantage. Last, prophage-derived genes can increase the fitness of the host in specific environments, which is known as lysogenic conversion (FIG. 2). For example, the prophage-derived transcriptional regulator LscR in *P. syringae* pv. *Glycinia* PG4180 mediates the expression of levansucrase, which results in a fitness advantage for the bacterium in the high concentrations of sucrose in plant tissue[99].

The effect of phages on the pathogenicity of* Pseudomonas *spp. Interestingly, the presence of phages in communities of pathogenic *Pseudomonas* spp. can affect the virulence, capacity for biofilm formation or antibiotic resistance of a pathogen, both positively and negatively (FIG. 3). Bacterial resistance against pili-dependent phages, such as the *P. aeruginosa*-infecting phage D3112, (achieved by decreasing pili synthesis) leads to decreased surface adherence of *P. aeruginosa*, which, in turn, decreases biofilm formation while also increasing antibiotic susceptibility[100]. In *P. aeruginosa*, the lipopolysaccharide (LPS)-specific phage E79 selects for alterations in LPS that lead to decreased swarming and increased biofilm formation[100]. Furthermore, the prophage Pf4 also has a crucial role in the formation of biofilms in *P. aeruginosa*[101]. The production of long negatively charged Pf4 filaments, which are released from infected cells, structures the biofilm as a liquid crystal, increasing its tolerance to desiccation and antibiotics[102] (FIG. 3). Furthermore, oxidative stress can induce phage Pf4 to convert to a super-infective phage (capable of lysing the existing lysogens), which can target and kill sensitive cells and generate more phenotype diversity inside the biofilm, making the biofilm communities more resilient[103]. Remarkably, *P. aeruginosa* has evolved a mechanism to control the onset of this lysis through the host two-component regulator BfmR-mediated control of the expression of PhdA, which is an antitoxin homologue[104]. In addition to triggering phage Pf4 to become lytic, *P. aeruginosa* can use an endolysin of a cryptic prophage to mediate explosive cell lysis to release extracellular DNA (eDNA), which is an essential component of the biofilm matrix[105,106].

Shifts in virulence can result from alterations in the bacterial membrane that emerge as phage resistance mechanisms. For example, *P. aeruginosa* PA1 strains that are resistant to phage PAP-1 exhibit decreased virulence due to the loss of the O-antigen[107] (FIG. 3). In addition, the presence of phages can augment the pathogenicity of the host bacterium. In *P. fluorescens,* co-evolution with phage ϕ2 selects for the overproduction of alginate, which protects *P. fluorescens* from phage adhesion and also increases its virulence by forming a protective barrier against phagocytes and oxidative radicals[92] (FIG. 3). Furthermore, *in vitro* work has shown that phage-resistant *P. aeruginos*a PAO1 variants are more virulent than wild-type bacteria[108].

Temperate phage-mediated lysogenic conversion can affect virulence through the transfer and expression of virulence factors. A well-known example is the cholera toxin that is encoded by the *Vibrio cholerae* phage CTXΦ[109], and a similar pore-forming toxin is encoded by the temperate *Pseudomonas* phage ΦCTX, which substantially increases the virulence of *P. aeruginosa*[110] (FIG. 3). Another example of a prophage gene that can interfere with bacterial virulence is the phage-encoded peptide inhibitor of α-polymerase protein (Iap) of phage D3, which mimics Wzz, a protein that regulates the chain length of the O-antigen side chain of LPS. This triggers a serotype conversion in *P. aeruginosa* to avoid superinfection by phages that use these LPS as receptors, which, at the same time, increases adherence and evasion of the host immune response[111]. In addition, *P. aeruginosa* str. LESB58 has four prophages that enhance pathogenicity; this is probably through the modulation of host gene expression, although the exact mechanisms are unknown[112,113].

Notably, phage-induced alterations in the bacterial host membrane can revert antibiotic resistance in multidrug-resistant *P. aeruginosa*. For example, this reversion is mediated by the lytic bacteriophage OMKO1, which uses the outer membrane porin M as a receptor binding site. As porin M is a key part of multidrug efflux systems (which pump antibiotics out of bacterial cells), phage resistance that is mediated through the decreased expression of porin M is linked to a decrease in antibiotic resistance[114] (FIG. 3). Furthermore, phages have been shown to decrease the development of antibiotic resistance in a kanamycin and phage combination treatment in *P. fluorescens*, as this combinatorial use limits the evolution of bacterial resistance[115].

Biotechnological applications of phages

Inappropriate therapy and the overuse of antibiotics have led to the emergence of acquired resistance among pathogenic bacteria in the past decades, forcing us to search novel drugs or risk being left with no treatment options. Bacterial viruses act as inspiration for complementary

Endolysin
A type of peptidoglycan hydrolase that is encoded by many bacteriophages to weaken the peptidoglycan layer of the host and release phage progeny at the end of the lytic cycle.

Cryptic prophage
An ancestral prophage that no longer has the ability to produce infectious phage progeny following induction.

Serotype conversion
A subset of cells in a species of bacteria that shares the same exposed cell surface antigens. Modifications to these antigens can lead to the conversion to another serotype.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

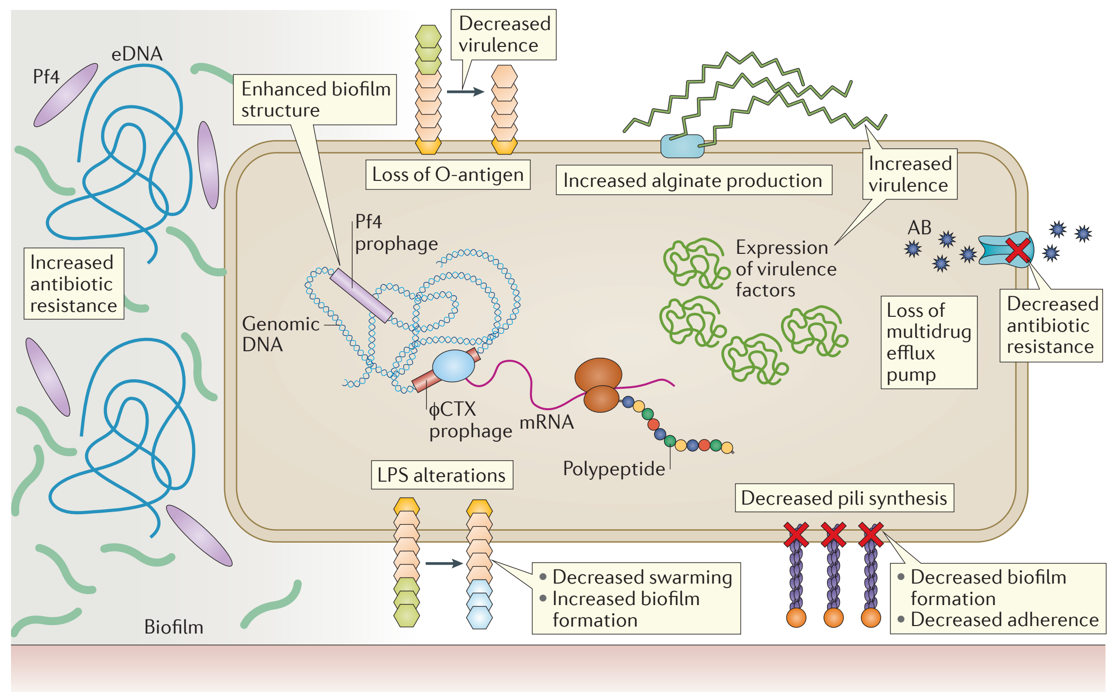

Figure 3 | **Conflicting effect of phages on *Pseudomonas* pathogenicity.** *Pseudomonas* phages are known to affect three key aspects of *Pseudomonas* pathogenicity: the formation of biofilms, virulence and antibiotic resistance. Examples of the mechanisms responsible for this interference, which can have a beneficial or a detrimental outcome on host pathogenicity, are depicted in this figure. In regard to biofilm formation, the temperate phage Pf4 is reported to have a major role in the release of extracellular DNA (eDNA), which stabilizes the biofilm matrix and makes it less penetrable to antibiotics. Another phage, D3112, was found to block the formation of pili, which resulted in decreased adherence and, consequently, decreased biofilm formation. Conversely, phage-resistant mutants often arise owing to alterations or truncations in the lipopolysaccharide (LPS) of *Pseudomonas* spp., which have been linked to increased biofilm formation and reduced virulence, respectively. However, phages can also increase virulence through the production of specific virulence factors, such as exotoxins (for example, cholera toxin) and polysaccharides (for example, alginate). Last, the sensitivity of *Pseudomonas* spp. to antibiotics is also influenced by phages. The stabilized biofilm matrix, as a result of the released eDNA, decreases this sensitivity, whereas the loss of specific efflux pumps that are used by phages as receptors in phage-resistant mutants renders them more sensitive to treatment with antibiotics. All of these examples show the complex roles that phages can have in shaping the pathogenicity of environmental *Pseudomonas* spp.

or even alternative antibacterial therapy, and a broad research field is emerging that aims to develop phages and phage-derived products for antibacterial therapy, food decontamination, disinfection, for bacterial detection, as nanomedicine and for drug delivery.

Phage therapy. The idea of using phages to target and kill pathogenic bacteria, and thus treat bacterial infections, in human medicine predates the discovery of antibiotics[116] and is undergoing a revival[117,118]. Proponents of phage therapy extoll the specificity and self-dosing effect (as they replicate and produce phage progeny at the infection site) of phage preparations, as well as their potential synergy with antibiotic treatments[119]. However, key hurdles for implementing human phage therapy include the development of resistance and immunogenicity to administered phages, as well as legislative and patenting challenges[117]. Progress has been made for the implementation of phage therapy in food protection, and preclinical, production, delivery and clinical phase trials are ongoing to bring phage therapy to the clinic and are outlined below[120].

A first hurdle for phage therapy is that published phage research often focuses exclusively on the isolation, genome sequencing and basic microbiological characterization of phages, with no clear follow up towards (more expensive) preclinical experiments to show efficacy. Nevertheless, a wide range of *in vitro* and *in vivo* assays have been developed to assess phage efficacy, including the *in vitro* gentamicin exclusion assay in the airway surface liquid (ASL) infection model, which can mimic the normal or cystic fibrosis lung environments depending on the human cell line used; for example, NuLi-1 cells derived from normal human bronchial epithelium and CuFi-1 cells derived from the bronchial epithelium of patients with cystic fibrosis. The opportunistic pathogen *P. aeruginosa* is often correlated with lung pathogenesis in individuals with cystic fibrosis and infection is almost impossible to eradicate with the therapies that are currently available. This assay enables the assessment of the prophylactic and curative potential of phages against bacterial lung infection and internalization into the epithelial cells of the lungs[121]. A second assay that has worked towards using phages to treat lung infections,

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

assessed the delivery efficiency and efficacy of nebulized phage against *P. aeruginosa* strains isolated from patients with cystic fibrosis[122,123].

For *in vivo* evaluation, the *Galleria mellonella* insect model is a straightforward and inexpensive method, although data must be carefully interpreted[124,125]. This model was used to assess the virulence of six *P. aeruginosa* (cystic fibrosis and non-cystic fibrosis) strains and the efficacy of phage treatment, both using single phages and phage cocktails[125]. Phage treatment significantly reduced mortality, and the most potent phage, KT28, rescued approximately 20% of larvae two days after the application of a lethal dose of non-cystic fibrosis strains. However, results were highly strain and phage dependent.

An excellent *in vivo* model of a mouse lung infection has been optimized to also assess infections with *P. aeruginosa*, in which the efficacy of phage administration is combined with real-time imaging of a bioluminescent strain of *P. aeruginosa*[126]. Phages that are isolated and then propagated on the desired target bacterial strain are the most efficient *in vivo*, which supports a personalized approach for an optimal treatment[126]. In a mouse model of *P. aeruginosa* keratitis, the administration of a single phage or a phage cocktail suppressed bacterial infection and prevented keratitis[127]. In contrast to conventional methods, such as antibiotic eye ointment, which only retained sufficient drug concentration for 30 min, phages exhibited a self-dosing effect at the site of infection. Moreover, when phages were combined in phage cocktails they worked in synergy, had an extended host range and decreased the emergence of phage-resistant bacteria, thus significantly improving disease outcome[127]. This was confirmed in a mouse burn wound model, in which no resistance against a three-phage cocktail was observed after 72 hours in more than 100 isolates[128]. However, *in vivo* models are insufficiently used to study the development of resistance following phage therapy in *Pseudomonas* spp., and this should be addressed in future research.

Aside from proving the efficacy of phage therapy in *in vitro* and *in vivo* models, the pharmacokinetics and immunological responses of the human host present a unique challenge to phage therapy, which is difficult to model (owing to the self-replicating nature of phages) and introduces additional variability for phage efficacy in individual patients. A limited number of studies show that phages can pass the stomach–duodenal pH barrier and can be delivered to the systemic circulation through the gut epithelium, but this depends on the type of phage and the health status of the patient[129]. Phage clearance is mediated by the liver, kidneys or spleen, and depends on the route of administration and the type of phage[130].

From an immunological perspective, phages trigger the production of phage-specific antibodies[131] and stimulate both the innate and adaptive immune systems[132]. Nevertheless, studies on animal models and clinical case studies on patients in Georgia and Poland have indicated that phage therapy has low toxicity and induces minimal side effects[133]. In addition, the investigation of interactions between phages and mammalian cells has indicated that phages may exert immunomodulatory or antitumor effects[134].

The final step towards the clinical implementation of phage therapy is the analysis of its efficacy in clinical trials. However, to date, only a few clinical trials and human case studies for the treatment of *P. aeruginosa* infections with phages are available, all of which rely heavily on the quality of production standards, phage production methods, optimized delivery methods and patient health status (BOX 2). Currently, one large multi-centre phase II clinical trial that is funded by the European Commission (PhagoBurn)[135], is testing phage cocktails against *E. coli* or *P. aeruginosa* for the topical treatment of infected burn wounds[136]. A previous phage therapy study in a patient who had a skin graft rejection due to colonization with *P. aeruginosa* resulted in eradication of the infection, which enabled successful skin grafts[137]. Another clinical trial showed the efficacy and safety of a cocktail that contained six phages for the treatment of chronic otitis media caused by antibiotic-resistant isolates of *P. aeruginosa*; phage-treatment led to significantly lower bacterial counts and increased clinical indicators[138].

Box 2 | **Phage production, processing and delivery methods**

Strategies that ensure consistent toxin-free and stable phage preparations for the commercial use of phage therapy have been previously described in a limited manner[166], but this information is often kept confidential by commercial companies. However, it is speculated that the propagation of phages to high titres is carried out using non-pathogenic bacterial host strains, which are cultured in disposable fermentation vessels to limit contaminants that may trigger unwanted immune responses. The concentration and purification of phages could be achieved by scalable centrifugation and chromatography-based techniques[167]. Next, quality control protocols (stability and pyrogenicity) can be more easily integrated into contract research organization-driven standardized and validated assays to pass ethical and legislative requirements in product development[168].

Specific protocols have also been described for small-scale preparations for non-commercial individual use, which has enabled the compassionate use of phage cocktails in patients[168]. A recent breakthrough for the non-commercial application of phage therapy was the approval by Belgian authorities to enable quality-controlled phage preparations to be included in magistral preparations. This is de facto enabling individual patient treatment on the authority of a medical doctor and a pharmacist, outside of the ‘compassionate use’ framework that is based on article 37 of the declaration of Helsinki concerning ‘unproven’ therapies (J.-P. Pirnay, personal communication).

Various phage delivery approaches have been assessed in animal models[169]. However, application emphasis has been placed on topical and gastrointestinal use, thus avoiding the hurdles of immunogenicity to systemically injected phages[132]. Non-commercial initiatives also focus on inhalation therapy for the direct delivery of phages to the lungs. Nebulization of high titres of *Pseudomonas aeruginosa* bacteriophages to the lower respiratory tract caused lysis in 66–98% of phenotypically diverse isolates of *P. aeruginosa* associated with cystic fibrosis[123]. However the effectiveness was dependent on the class of nebulizer that was used[122]. Several studies have addressed novel ways of phage processing (such as spray freeze drying or spray drying) for the production of inhalable phage dry powder formulations that are suitable for pulmonary delivery in a form of aerosols or inhalers[170,171]. These had variable success rates, depending on the selected phages and the additional stabilizing excipients, and showed limited stability during long-term storage[170,172]. A promising option for *in vivo* systemic delivery is the use of cationic liposomes as phage carriers, which increased the phage retention times in mice (up to 14 days in the spleen) compared with those of free-phage administration (undetectable after 48 h in all organs)[173].

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Apart from clinical trials, phage therapy centres in Georgia and Poland have a long tradition of treating multidrug-resistant purulent septic infections, bronchopneumonia, and urinary tract and digestive system infections with custom-made phage cocktails[139,140]. In Georgia, physicians treat topical infections with PhageBioDerm, which is a polymer that releases antibiotics and lytic bacteriophages against *P. aeruginosa*, *E. coli*, *Staphylococcus aureus*, *Streptococcus* spp. and *Proteus* spp. Indeed, this bioactive composite was used on 107 patients who had wounds infected by various pathogens, which resulted in complete recovery in 70% of the cases[139].

Pseudomonas phages could potentially be particularly attractive for the treatment of patients with cystic fibrosis, as phages can infect multidrug-resistant strains and encode alginate lyases that actively degrade the bacterial extracellular matrix[29,141]. An *in vivo* study in a mouse model showed that phage treatment of lung infection, caused by a *P. aeruginosa* strain isolated from a patient with cystic fibrosis, can have curative and preventive properties[142]. Moreover, several patient case studies have been reported that show the successful application of phage therapy after several antibiotic regimens had proven ineffective[143]. At the National Center of Cystic Fibrosis in Tbilisi, Georgia, eight patients with cystic fibrosis that received phage preparations through nebulization had a longer infection-free period and an overall improved health status compared with patients who were treated with antibiotics[144].

Several studies in veterinary medicine and agriculture illustrate other potential applications of phage therapy against *Pseudomonas*. In dogs, chronic otitis due to *P. aeruginosa* can be treated by applying a topical bacteriophage cocktail, with no evidence of toxicity or allergy[145]. In aquaculture, skin lesions infected with *P. aeruginosa* in catfish could be successfully treated[146], and a phage cocktail that was administered in the food of Ayu fish protected them from infection with *Pseudomonas plecoglossicida*[147]. In agriculture, a phage cocktail against *P. syringae* pv. *porri* was developed to protect leek production, although field trials resulted in mixed success[148].

Enzyme-based antimicrobials — harvesting the enzymatic potential of phages. As phage therapy still faces many legislative hurdles, research is also focused on investigating the option to directly use phage enzymes as enzyme-based antimicrobials. Although medicine has relied on small molecules as antibiotics, in recent years enzymes have been introduced as antimicrobials. A successful example is dornase alfa (also known as Pulmozyme), which is a DNase used for reducing biofilm viscosity in patients with cystic fibrosis by degrading eDNA. The most promising class of phage enzymes being investigated to treat infections caused by antibiotic-resistant bacteria are phage-encoded peptidoglycan hydrolases, for which recent studies have clearly demonstrated their potential[149]. During the phage infection cycle, the expression of such enzymes in the cell (endolysins) is responsible for the degradation of the peptidoglycan layer and the consequent lysis and escape of phage progeny. We can also use this enzymatic function by applying such enzymes extracellularly to Gram-positive bacteria, which would result in the breakdown of their peptidoglycan layer and cell lysis. Only a few enzymes that are naturally produced by phages manage to exert limited antibacterial activity against Gram-negative species by partially destabilizing the negatively charged LPS[150]. By contrast, most endolysins fail to breach the Gram-negative outer membrane barrier to reach the peptidoglycan layer. However, through the use of protein-engineering approaches, peptides that physically disrupt the outer membrane were fused to endolysins to enable them to reach the peptidoglycan layer, which enabled the potent killing of Gram-negative bacteria and reduced their minimum inhibitory concentrations (MICs). This technology is known as 'Artilysin' and is non-cytotoxic and thus far seems to be impervious to the development of resistance. Artilysins have been described for the treatment of multidrug-resistant strains of *P. aeruginosa*[150] and persistent *Acinetobacter baumannii*[151], primarily for topical applications. As a proteinaceous antimicrobial agent, systemic applications may present similar immunological questions to the use of phage therapy. However, natural biodegradability also marks such proteins as a sustainable approach, as they will exert a reduced resistance selection pressure in the environment; this should limit the ecological burden compared with antibiotics[152,153].

Conclusions and outlook

The widely distributed *Pseudomonas* genus has been clearly established as an ecologically important group of bacteria. However, the ecological role of phages that infect *Pseudomonas* spp. has only just come into focus. In this regard, phage–host co-evolution experiments have uncovered some initial governing principles in strictly controlled environments. However, the intricacies and population complexities (of both phage isolates and strains of *Pseudomonas*) in natural environments represent an almost infinite number of potential interaction events and ecological consequences. These intricate interactions go well beyond the predator–prey role, as both viral and bacterial communities are mutual catalysts of evolution.

In addition to being used to study evolution and ecology, the current antibiotic resistance crisis has accelerated the applied use of *Pseudomonas* phages as an alternative antibacterial treatment (FIG. 4). Following the evolutions of the field, one may distil three principal approaches on how phage therapy may be implemented. First, to date, research has focused on the development of a stable and fixed product, such as a standardized phage cocktail for a specific infection. This approach has the benefit of sustaining potentially cost-efficient large-scale production, but does not encompass the principal asset of phage therapy: continuous adaptability towards the specific infecting pathogen strain. Second, to address this, a tailored approach has been proposed that involves the selection of phages that efficiently target the specific

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

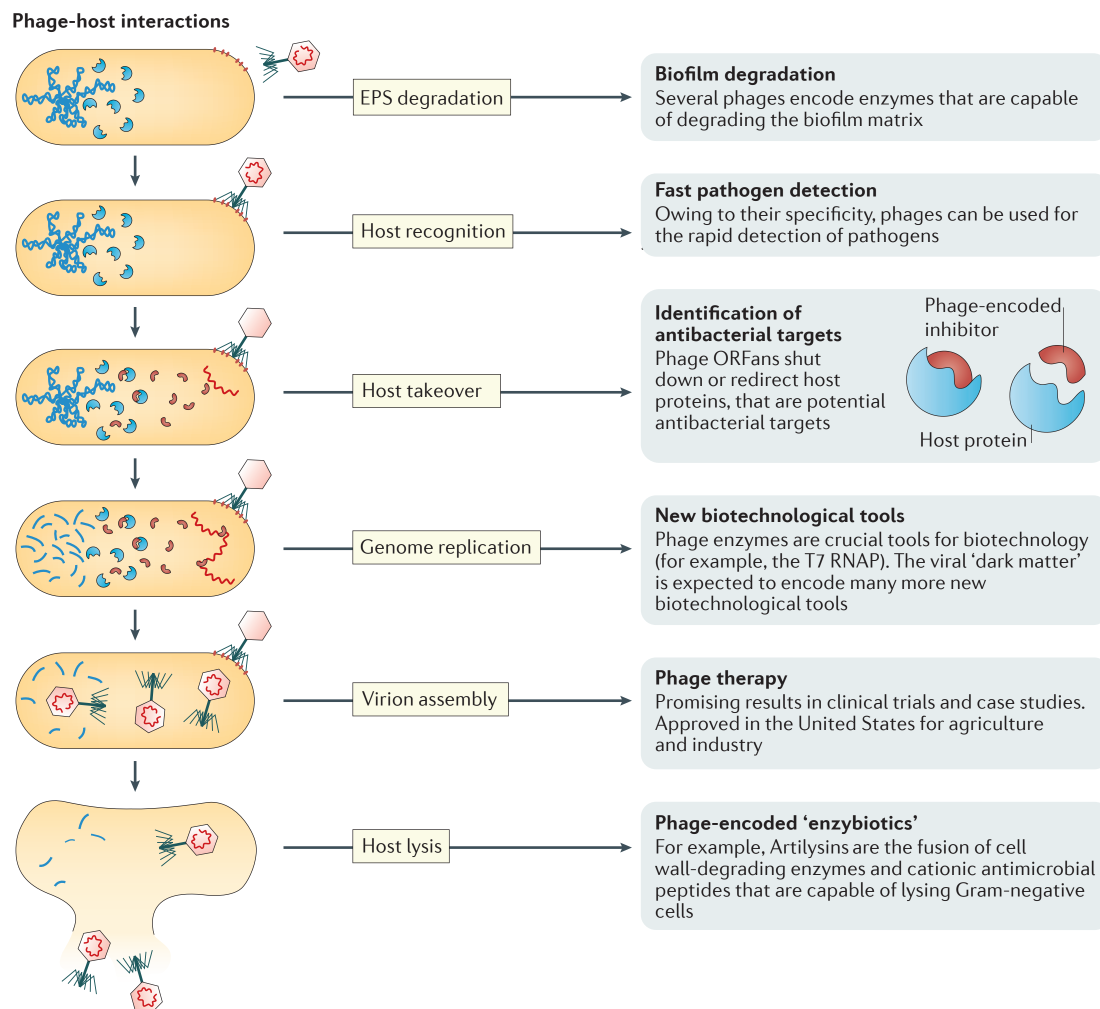

Figure 4 | **'Step-by-step' potential of phage applications.** Apart from the use of whole phages as antimicrobials to control bacterial infections, the past decades of research into the interactions between phages and *Pseudomonas* spp. has illustrated the potential of these biological entities for biotechnology. In fact, phage proteins in each phase of the infection cycle have potential biotechnological applications. Phage enzymes that enable the phage to reach its host by degrading extracellular polysaccharides (EPS) can be used for the degradation of biofilms. The specificity of the virion-associated host receptors makes them ideal tools for rapid pathogen identification. Specific interactions between small phage proteins and host enzymes to ensure the takeover of the host cell early during infection could yield novel antibacterial targets or strategies to selectively modulate host pathways. Several phage transcription and/or replication enzymes and genetic elements have already become commonly used tools in molecular laboratories (for example, T7 RNA polymerase (RNAP)), but a wide range of biotechnological tools remains to be discovered. The enzymes that are involved in the lysis of an infected cell are being investigated and engineered as novel antibiotics. These examples show the need for in-depth molecular investigation of *Pseudomonas* phages and their interactions with their hosts, not only to expand our understanding of phage biology but also to optimally exploit the capacities of these organisms.

strain from the patient. This approach is more consistent with the traditional application of phage therapy in Georgia, Russia and Poland, in which phage cocktails are gradually updated over time[154]. Although this approach definitely has benefits for a patient-specific treatment, the commercial potential is limited. In addition, both approaches have patentability issues. Third, the engineering and biotechnological design of bacteriophages represent an alternative avenue, which deliberately moves away from the natural interplay between phages and their hosts[155–157]. Although this biotechnological design potentially overcomes several hurdles, including patentability and the development of resistance, it would still be difficult to register and implement as a genetically modified organism (GMO)-based approach. Phage-derived approaches that focus on endolysins or engineered endolysins as enzyme-based antimicrobials do not face these challenges and may therefore be easier to commercialize[158] (FIG. 4).

Last, the study of molecular phage–host interactions provides opportunities for synthetic biology, as many of its essential tools are phage-derived parts; for example, the T7 RNAP, λ Red recombinase or phage integrases[159]. The characterization of the unknown ORFans could

Synthetic biology
The application of design and engineering principles to biological systems to artificially manipulate them for useful purposes.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

yield new tools, not only for synthetic biologists but also metabolic engineers (FIG. 4). Metabolic engineering is the effort to understand, perturb and design metabolic networks for the production of pharmaceuticals, food and speciality chemicals[160]. In this emerging field there is a continuous quest for tools to dynamically alter metabolic pathways. Specific phage genes that are involved in host takeover[64] might form a new set of tools for the construction of controllable metabolism in bacteria[161].

Despite the breakthroughs in *Pseudomonas* phage co-evolution studies, a large knowledge gap exists between the governing mechanisms at the ecological and molecular levels in the interaction between phage and host. Thus, the integration of evolution studies and omics approaches, and the functional elucidation of unknown viral genes, remain important challenges and require a framed system biology approach. This level of understanding of the interactions between phages and *Pseudomonas* spp. will ensure a better understanding of these species as pathogens. In addition, understanding how phages affect these organisms could lead to novel insights to optimize current phage-based antibacterial strategies or identify totally new avenues to control *Pseudomonas* spp.

1. Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B. & Jackson, R. W. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* **35**, 652–680 (2011).
2. Stover, C. K. *et al.* Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964 (2000).
3. Kung, V. L., Ozer, E. A. & Hauser, A. R. The accessory genome of *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* **74**, 621–641 (2010).
4. Freschi, L. *et al.* Clinical utilization of genomics data produced by the International *Pseudomonas aeruginosa* Consortium. *Front. Microbiol.* **6**, 1036 (2015).
5. Breidenstein, E. B. M., de la Fuente-Núñez, C. & Hancock, R. E. W. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol.* **19**, 419–426 (2011).
6. Ceyssens, P.-J. & Lavigne, R. Bacteriophages of *Pseudomonas*. *Future Microbiol.* **5**, 1041–1055 (2010).
7. James, C. E. *et al.* Lytic activity by temperate phages of *Pseudomonas aeruginosa* in long-term cystic fibrosis chronic lung infections. *ISME J.* **9**, 1391–1398 (2015).
8. Hatfull, G. F. & Hendrix, R. W. Bacteriophages and their genomes. *Curr. Opin. Virol.* **1**, 298–303 (2011).
9. Cazares, A. *et al.* Core and accessory genome architecture in a group of *Pseudomonas aeruginosa* Mu-like phages. *BMC Genomics* **15**, 1146 (2014).
10. Rohwer, F. & Youle, M. Consider something viral in your research. *Nat. Rev. Microbiol.* **9**, 308–309 (2011).
11. Yin, Y. & Fischer, D. Identification and investigation of ORFans in the viral world. *BMC Genomics* **9**, 24 (2008).
12. Hatfull, G. F. Dark matter of the biosphere: the amazing world of bacteriophage diversity. *J. Virol.* **89**, 8107–8110 (2015).
13. Sepúlveda-Robles, O., Kameyama, L. & Guarneros, G. High diversity and novel species of *Pseudomonas aeruginosa* bacteriophages. *Appl. Environ. Microbiol.* **78**, 4510–4515 (2012).
14. Ceyssens, P.-J. *et al.* Survey of *Pseudomonas aeruginosa* and its phages: *de novo* peptide sequencing as a novel tool to assess the diversity of worldwide collected viruses. *Environ. Microbiol.* **11**, 1303–1313 (2009).
15. Kwan, T., Liu, J., Dubow, M., Gros, P. & Pelletier, J. Comparative genomic analysis of 18 *Pseudomonas aeruginosa* bacteriophages. *J. Bacteriol.* **188**, 1184–1187 (2006).
16. Angly, F. E. *et al.* The marine viromes of four oceanic regions. *PLoS Biol.* **4**, e368 (2006).
17. Tümmler, B., Wiehlmann, L., Klockgether, J. & Cramer, N. Advances in understanding *Pseudomonas*. *F1000Prime Rep.* **6**, 9 (2014).
18. Brockhurst, M. A., Fenton, A., Roulston, B. & Rainey, P. B. The impact of phages on interspecific competition in experimental populations of bacteria. *BMC Ecol.* **6**, 19 (2006).
19. Buckling, A. & Rainey, P. B. The role of parasites in sympatric and allopatric host diversification. *Nature* **420**, 496–499 (2002).
20. Feiner, R. *et al.* A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat. Rev. Microbiol.* **13**, 641–650 (2015).
21. Cenens, W. *et al.* Expression of a novel P22 ORFan gene reveals the phage carrier state in *Salmonella* Typhimurium. *PLoS Genet.* **9**, e1003269 (2013).
22. Cenens, W., Makumi, A., Govers, S. K., Lavigne, R. & Aertsen, A. Viral transmission dynamics at single-cell resolution reveal transiently immune subpopulations caused by a carrier state association. *PLoS Genet.* **11**, e1005770 (2015).
23. Samson, J. E., Magadán, A. H., Sabri, M. & Moineau, S. Revenge of the phages: defeating bacterial defences. *Nat. Rev. Microbiol.* **11**, 675–687 (2013).
This study provides a comprehensive overview of identified phage defence mechanisms against bacterial host resistance.
24. Paterson, S. *et al.* Antagonistic coevolution accelerates molecular evolution. *Nature* **464**, 275–278 (2010).
25. Scanlan, P. D., Hall, A. R., Lopez-Pascua, L. D. C. & Buckling, A. Genetic basis of infectivity evolution in a bacteriophage. *Mol. Ecol.* **20**, 981–989 (2011).
26. Le, S. *et al.* Mapping the tail fiber as the receptor binding protein responsible for differential host specificity of *Pseudomonas aeruginosa* bacteriophages PaP1 and JG004. *PLoS ONE* **8**, e68562 (2013).
27. Cornelissen, A. *et al.* The T7-related *Pseudomonas putida* phage φ15 displays virion-associated biofilm degradation properties. *PLoS ONE* **6**, e18597 (2011).
28. Cornelissen, A. *et al.* Identification of EPS-degrading activity within the tail spikes of the novel *Pseudomonas putida* phage AF. *Virology* **434**, 251–256 (2012).
29. Glonti, T., Chanishvili, N. & Taylor, P. W. Bacteriophage-derived enzyme that depolymerizes the alginic acid capsule associated with cystic fibrosis isolates of *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* **108**, 695–702 (2010).
30. Chung, I.-Y., Jang, H.-J., Bae, H.-W. & Cho, Y.-H. A phage protein that inhibits the bacterial ATPase required for type IV pilus assembly. *Proc. Natl Acad. Sci. USA* **111**, 11503–11508 (2014).
31. Bondy-Denomy, J. *et al.* Prophages mediate defense against phage infection through diverse mechanisms. *ISME J.* **10**, 2854–2866 (2016).
32. Weigele, P. & Raleigh, E. A. Biosynthesis and function of modified bases in bacteria and their viruses. *Chem. Rev.* **116**, 12655–12687 (2016).
33. Ceyssens, P.-J. *et al.* The genome and structural proteome of YuA, a new *Pseudomonas aeruginosa* phage resembling M6. *J. Bacteriol.* **190**, 1429–1435 (2008).
34. Barrangou, R. *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712 (2007).
35. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71 (2010).
36. van Belkum, A. *et al.* Phylogenetic distribution of CRISPR–Cas systems in antibiotic-resistant *Pseudomonas aeruginosa*. *mBio* **6**, e01796-15 (2015).
37. Pawluk, A. *et al.* Inactivation of CRISPR–Cas systems by anti-CRISPR proteins in diverse bacterial species. *Nat. Microbiol.* **1**, 16085 (2016).
38. Bondy-Denomy, J., Pawluk, A., Maxwell, K. L. & Davidson, A. R. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature* **493**, 429–432 (2013).
The study is the first report of the presence of CRISPR inhibitors in phages.
39. Bondy-Denomy, J. *et al.* Multiple mechanisms for CRISPR–Cas inhibition by anti-CRISPR proteins. *Nature* **526**, 136–139 (2015).
40. Pawluk, A., Bondy-Denomy, J., Cheung, V. H. W., Maxwell, K. L. & Davidson, A. R. A New group of phage anti-CRISPR genes inhibits the type I-E CRISPR–Cas system of *Pseudomonas aeruginosa*. *mBio* **5**, e00896-14 (2014).
41. Cady, K. C., Bondy-Denomy, J., Heussler, G. E., Davidson, A. R. & O'Toole, G. A. The CRISPR/Cas adaptive immune system of *Pseudomonas aeruginosa* mediates resistance to naturally occurring and engineered phages. *J. Bacteriol.* **194**, 5728–5738 (2012).
42. Ali, S. S., Xia, B., Liu, J. & Navarre, W. W. Silencing of foreign DNA in bacteria. *Curr. Opin. Microbiol.* **15**, 175–181 (2012).
43. Castang, S., McManus, H. R., Turner, K. H. & Dove, S. L. H-NS family members function coordinately in an opportunistic pathogen. *Proc. Natl Acad. Sci. USA* **105**, 18947–18952 (2008).
44. Castang, S. & Dove, S. L. Basis for the essentiality of H-NS family members in *Pseudomonas aeruginosa*. *J. Bacteriol.* **194**, 5101–5109 (2012).
45. Wagemans, J. *et al.* Antibacterial phage ORFans of *Pseudomonas aeruginosa* phage LUZ24 reveal a novel MvaT inhibiting protein. *Front. Microbiol.* **6**, 1242 (2015).
46. Roucourt, B. & Lavigne, R. The role of interactions between phage and bacterial proteins within the infected cell: a diverse and puzzling interactome. *Environ. Microbiol.* **11**, 2789–2805 (2009).
47. Wagemans, J. *et al.* Functional elucidation of antibacterial phage ORFans targeting *Pseudomonas aeruginosa*. *Cell. Microbiol.* **16**, 1822–1835 (2014).
48. Van den Bossche, A. *et al.* Systematic identification of hypothetical bacteriophage proteins targeting key protein complexes of *Pseudomonas aeruginosa*. *J. Proteome Res.* **13**, 4446–4456 (2014).
This report describes an effective strategy for the identification of interacting unknown phage proteins.
49. Ceyssens, P.-J. *et al.* Development of giant bacteriophage ϕKZ is independent of the host transcription apparatus. *J. Virol.* **88**, 10501–10510 (2014).
50. Yakunina, M. *et al.* A non-canonical multisubunit RNA polymerase encoded by a giant bacteriophage. *Nucleic Acids Res.* **43**, 10411–10420 (2015).
51. Lavigne, R. *et al.* A multifaceted study of *Pseudomonas aeruginosa* shutdown by virulent podovirus LUZ19. *mBio* **4**, e00061-13 (2013).
52. Klimuk, E. *et al.* Host RNA polymerase inhibitors encoded by ϕKMV-like phages of *Pseudomonas*. *Virology* **436**, 67–74 (2013).
53. Qimron, U., Kulczyk, A. W., Hamdan, S. M., Tabor, S. & Richardson, C. C. Inadequate inhibition of host RNA polymerase restricts T7 bacteriophage growth on hosts overexpressing *udk*. *Mol. Microbiol.* **67**, 448–457 (2007).
54. Sheppard, C. *et al.* A non-bacterial transcription factor inhibits bacterial transcription by a multipronged mechanism. *RNA Biol.* **10**, 495–501 (2013).
55. Ravantti, J. J., Ruokoranta, T. M., Alapuranen, A. M. & Bamford, D. H. Global transcriptional responses of *Pseudomonas aeruginosa* to phage PRR1 infection. *J. Virol.* **82**, 2324–2329 (2008).
56. Van den Bossche, A. *et al.* Structural elucidation of a novel mechanism for the bacteriophage-based inhibition of the RNA degradosome. *eLife* **5**, e16413 (2016).
This paper describes a novel mechanism of phage interference with bacterial RNA degradation.
57. Dendooven, T. *et al.* Viral interference of the bacterial RNA metabolism machinery. *RNA Biol.* **14**, 6–10 (2016).

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

58. Chevallereau, A. *et al.* Next-generation '-omics' approaches reveal a massive alteration of host RNA metabolism during bacteriophage infection of *Pseudomonas aeruginosa*. *PLoS Genet.* **12**, e1006134 (2016).
This paper is the first to combine omics-based analyses of both the transcriptome and metabolome to evaluate phage PAK-P3 infection.
59. Breitbart, M., Thompson, L. R., Suttle, C. A. & Sullivan, M. B. Exploring the vast diversity of marine viruses. *Oceanography* **2**, 135–139 (2007).
60. Martiny, A. C., Huang, Y. & Li, W. Occurrence of phosphate acquisition genes in *Prochlorococcus* cells from different ocean regions. *Environ. Microbiol.* **11**, 1340–1347 (2009).
61. Sullivan, M. B. *et al.* Genomic analysis of oceanic cyanobacterial myoviruses compared with T4-like myoviruses from diverse hosts and environments. *Environ. Microbiol.* **12**, 3035–3056 (2010).
62. Lindell, D., Jaffe, J. D., Johnson, Z. I., Church, G. M. & Chisholm, S. W. Photosynthesis genes in marine viruses yield proteins during host infection. *Nature* **438**, 86–89 (2005).
63. Frank, J. A. *et al.* Structure and function of a cyanophage-encoded peptide deformylase. *ISME J.* **7**, 1150–1160 (2013).
64. Thompson, L. R. *et al.* Phage auxiliary metabolic genes and the redirection of cyanobacterial host carbon metabolism. *Proc. Natl Acad. Sci. USA* **108**, E757–E764 (2011).
This report provides a clear example of how phage-encoded AMGs can alter host metabolism to favour viral replication.
65. Miller, E. S. *et al.* Bacteriophage T4 genome. *Microbiol. Mol. Biol. Rev.* **67**, 86–156 (2003).
66. Sharon, I. *et al.* Comparative metagenomics of microbial traits within oceanic viral communities. *ISME J.* **5**, 1178–1190 (2011).
67. Brussaard, C. P. D. *et al.* Global-scale processes with a nanoscale drive: the role of marine viruses. *ISME J.* **2**, 575–578 (2008).
68. Sonkar, K., Purusottam, R. N. & Sinha, N. Metabonomic study of host–phage interaction by nuclear magnetic resonance- and statistical total correlation spectroscopy-based analysis. *Anal. Chem.* **84**, 4063–4070 (2012).
69. De Smet, J. *et al.* High coverage metabolomics analysis reveals phage-specific alterations to *Pseudomonas aeruginosa* physiology during infection. *ISME J.* **10**, 1823–1835 (2016).
70. Liu, J. *et al.* Antimicrobial drug discovery through bacteriophage genomics. *Nat. Biotechnol.* **22**, 185–191 (2004).
71. Yano, S. T. & Rothman-Denes, L. B. A phage-encoded inhibitor of *Escherichia coli* DNA replication targets the DNA polymerase clamp loader. *Mol. Microbiol.* **79**, 1325–1338 (2011).
72. Conter, A., Bouché, J. P. & Dassain, M. Identification of a new inhibitor of essential division gene *ftsZ* as the *kil* gene of defective prophage Rac. *J. Bacteriol.* **178**, 5100–5104 (1996).
73. Kraemer, J. A. *et al.* A phage tubulin assembles dynamic filaments by an atypical mechanism to center viral DNA within the host cell. *Cell* **149**, 1488–1499 (2012).
This study is the first report of a prokaryotic tubulin array that functions similarly to the microtubule-based spindles of eukaryotes.
74. Aylett, C. H. S., Izoré, T., Amos, L. A. & Löwe, J. Structure of the tubulin/FtsZ-like protein TubZ from *Pseudomonas* bacteriophage ΦKZ. *J. Mol. Biol.* **425**, 2164–2173 (2013).
75. Erb, M. L. *et al.* A bacteriophage tubulin harnesses dynamic instability to center DNA in infected cells. *eLife* **3**, e03197 (2014).
76. Koskella, B. & Brockhurst, M. A. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* **38**, 916–931 (2014).
An excellent review that discusses the role of co-evolution in ecology.
77. Scanlan, P. D. *et al.* Coevolution with bacteriophages drives genome-wide host evolution and constrains the acquisition of abiotic-beneficial mutations. *Mol. Biol. Evol.* **32**, 1425–1435 (2015).
78. Bohannan, B. J. M. & Lenski, R. E. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. *Ecol. Lett.* **3**, 362–377 (2000).
79. Gómez, P. & Buckling, A. Bacteria–phage antagonistic coevolution in soil. *Science* **332**, 106–109 (2011).
80. Hall, A. R., Scanlan, P. D., Morgan, A. D. & Buckling, A. Host–parasite coevolutionary arms races give way to fluctuating selection. *Ecol. Lett.* **14**, 635–642 (2011).
81. Gorter, F. A., Scanlan, P. D. & Buckling, A. Adaptation to abiotic conditions drives local adaptation in bacteria and viruses coevolving in heterogeneous environments. *Biol. Lett.* **12**, 20150879 (2016).
82. Westra, E. R. *et al.* Parasite exposure drives selective evolution of constitutive versus inducible defense. *Curr. Biol.* **25**, 1043–1049 (2015).
83. Chabas, H., van Houte, S., Høyland-Kroghsbo, N. M., Buckling, A. & Westra, E. R. Immigration of susceptible hosts triggers the evolution of alternative parasite defence strategies. *Proc. R. Soc. B Biol. Sci.* **283**, 20160721 (2016).
84. Martínez-García, E., Jatsenko, T., Kivisaar, M. & de Lorenzo, V. Freeing *Pseudomonas putida* KT2440 of its proviral load strengthens endurance to environmental stresses. *Environ. Microbiol.* **17**, 76–90 (2015).
85. Wang, X. & Wood, T. K. Cryptic prophages as targets for drug development. *Drug Resist. Updat.* **27**, 30–38 (2016).
86. Fortier, L.-C. & Sekulovic, O. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence* **4**, 354–365 (2013).
87. Davies, E. V. *et al.* Temperate phages both mediate and drive adaptive evolution in pathogen biofilms. *Proc. Natl Acad. Sci. USA* **113**, 8266–8271 (2016).
88. Latino, L., Midoux, C., Hauck, Y., Vergnaud, G. & Pourcel, C. Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa*. *Microbiology* **162**, 748–763 (2016).
89. Budzik, J. M., Rosche, W. A., Rietsch, A. & O'Toole, G. A. Isolation and characterization of a generalized transducing phage for *Pseudomonas aeruginosa* strains PAO1 and PA14. *J. Bacteriol.* **186**, 3270–3273 (2004).
90. Monson, R., Foulds, I., Foweraker, J., Welch, M. & Salmond, G. P. C. The *Pseudomonas aeruginosa* generalized transducing phage PA3 is a new member of the KZ-like group of 'jumbo' phages, and infects model laboratory strains and clinical isolates from cystic fibrosis patients. *Microbiology* **157**, 859–867 (2011).
91. Taylor, T. B. & Buckling, A. Bacterial motility confers fitness advantage in the presence of phages. *J. Evol. Biol.* **26**, 2154–2160 (2013).
92. Scanlan, P. D. & Buckling, A. Co-evolution with lytic phage selects for the mucoid phenotype of *Pseudomonas fluorescens* SBW25. *ISME J.* **6**, 1148–1158 (2012).
93. Pal, C., Maciá, M. D., Oliver, A., Schachar, I. & Buckling, A. Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature* **450**, 1079–1081 (2007).
94. Gómez, P. & Buckling, A. Coevolution with phages does not influence the evolution of bacterial mutation rates in soil. *ISME J.* **7**, 2242–2244 (2013).
95. Vogwill, T., Fenton, A. & Brockhurst, M. A. Coevolving parasites enhance the diversity-decreasing effect of dispersal. *Biol. Lett.* **7**, 578–580 (2011).
96. Friman, V.-P. & Buckling, A. Effects of predation on real-time host–parasite coevolutionary dynamics. *Ecol. Lett.* **16**, 39–46 (2013).
97. Friman, V.-P. & Buckling, A. Phages can constrain protist predation-driven attenuation of *Pseudomonas aeruginosa* virulence in multienemy communities. *ISME J.* **8**, 1820–1830 (2014).
98. Penner, J. C. *et al.* Pf4 bacteriophage produced by *Pseudomonas aeruginosa* inhibits *Aspergillus fumigatus* metabolism via iron sequestration. *Microbiology* **162**, 1583–1594 (2016).
99. Abdallah, K., Hartman, K., Pletzer, D., Zhurina, D. & Ullrich, M. S. The bacteriophage-derived transcriptional regulator, LscR, activates the expression of levansucrase genes in *Pseudomonas syringae*. *Mol. Microbiol.* **102**, 1062–1074 (2016).
100. Hosseinidoust, Z., Tufenkji, N. & van de Ven, T. G. M. Formation of biofilms under phage predation: considerations concerning a biofilm increase. *Biofouling* **29**, 457–468 (2013).
101. Rice, S. A. *et al.* The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J.* **3**, 271–282 (2009).
102. Secor, P. R. *et al.* Filamentous bacteriophage promote biofilm assembly and function. *Cell Host Microbe* **18**, 549–559 (2015).
103. Hui, J. G. K., Mai-Prochnow, A., Kjelleberg, S., McDougald, D. & Rice, S. A. Environmental cues and genes involved in establishment of the superinfective Pf4 phage of *Pseudomonas aeruginosa*. *Front. Microbiol.* **5**, 654 (2014).
104. Petrova, O. E., Schurr, J. R., Schurr, M. J. & Sauer, K. The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. *Mol. Microbiol.* **81**, 767–783 (2011).
105. Gloag, E. S. *et al.* Self-organization of bacterial biofilms is facilitated by extracellular DNA. *Proc. Natl Acad. Sci. USA* **110**, 11541–11546 (2013).
106. Turnbull, L. *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **7**, 11220 (2016).
107. Le, S. *et al.* Chromosomal DNA deletion confers phage resistance to *Pseudomonas aeruginosa*. *Sci. Rep.* **4**, 4738 (2014).
108. Hosseinidoust, Z., van de Ven, T. G. M. & Tufenkji, N. Evolution of *Pseudomonas aeruginosa* virulence as a result of phage predation. *Appl. Environ. Microbiol.* **79**, 6110–6116 (2013).
109. Waldor, M. K. & Mekalanos, J. J. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910–1914 (1996).
110. Nakayama, K., Kanaya, S., Ohnishi, M., Terawaki, Y. & Hayashi, T. The complete nucleotide sequence of ΦCTX, a cytotoxin-converting phage of *Pseudomonas aeruginosa*: implications for phage evolution and horizontal gene transfer via bacteriophages. *Mol. Microbiol.* **31**, 399–419 (1999).
111. Taylor, V. L., Udaskin, M. L., Islam, S. T. & Lam, J. S. The D3 bacteriophage α-polymerase inhibitor (Iap) peptide disrupts O-antigen biosynthesis through mimicry of the chain length regulator Wzz in *Pseudomonas aeruginosa*. *J. Bacteriol.* **195**, 4735–4741 (2013).
112. Davies, E. V. *et al.* Temperate phages enhance pathogen fitness in chronic lung infection. *ISME J.* **10**, 2553–2555 (2016).
113. Lemieux, A.-A. *et al.* Genes required for free phage production are essential for *Pseudomonas aeruginosa* chronic lung infections. *J. Infect. Dis.* **213**, 395–402 (2016).
114. Chan, B. K. *et al.* Phage selection restores antibiotic sensitivity in MDR *Pseudomonas aeruginosa*. *Sci. Rep.* **6**, 26717 (2016).
This study presents an innovative way of using the phage-associated development of resistance to our benefit by selecting against antibiotic resistance.
115. Zhang, Q.-G. & Buckling, A. Phages limit the evolution of bacterial antibiotic resistance in experimental microcosms. *Evol. Appl.* **5**, 575–582 (2012).
116. Summers, W. C. Bacteriophage therapy. *Annu. Rev. Microbiol.* **55**, 437–451 (2001).
117. Górski, A. *et al.* Phage therapy: combating infections with potential for evolving from merely a treatment for complications to targeting diseases. *Front. Microbiol.* **7**, 1515 (2016).
118. Cisek, A. A., Dąbrowska, I., Gregorczyk, K. P. & Wyżewski, Z. Phage therapy in bacterial infections treatment: one hundred years after the discovery of bacteriophages. *Curr. Microbiol.* **74**, 277–283 (2017).
119. Loc-Carrillo, C. & Abedon, S. T. Pros and cons of phage therapy. *Bacteriophage* **1**, 111–114 (2014).
120. Pirnay, J.-P. *et al.* Quality and safety requirements for sustainable phage therapy products. *Pharm. Res.* **32**, 2173–2179 (2015).
121. Danis-Wlodarczyk, K. *et al.* A proposed integrated approach for the preclinical evaluation of phage therapy in *Pseudomonas* infections. *Sci. Rep.* **6**, 28115 (2016).
This paper proposes a standardized approach to the preclinical evaluation of phages.
122. Cooper, C. J., Denyer, S. P. & Maillard, J.-Y. Stability and purity of a bacteriophage cocktail preparation for nebulizer delivery. *Lett. Appl. Microbiol.* **58**, 118–122 (2014).
123. Sahota, J. S. *et al.* Bacteriophage delivery by nebulization and efficacy against phenotypically diverse *Pseudomonas aeruginosa* from cystic fibrosis patients. *J. Aerosol Med. Pulm. Drug Deliv.* **28**, 353–360 (2015).
124. Beeton, M. L., Alves, D. R., Enright, M. C. & Jenkins, A. T. A. Assessing phage therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* infection model. *Int. J. Antimicrob. Agents* **46**, 196–200 (2015).
125. Olszak, T. *et al.* *In vitro* and *in vivo* antibacterial activity of environmental bacteriophages against *Pseudomonas aeruginosa* strains from cystic fibrosis patients. *Appl. Microbiol. Biotechnol.* **99**, 6021–6033 (2015).

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

126. Henry, M., Lavigne, R. & Debarbieux, L. Predicting *in vivo* efficacy of therapeutic bacteriophages used to treat pulmonary infections. *Antimicrob. Agents Chemother.* **57**, 5961–5968 (2013).
This study describes an excellent *in vivo* model that is available for *Pseudomonas* lung infections in mice.
127. Furusawa, T. *et al.* Phage therapy is effective in a mouse model of bacterial equine keratitis. *Appl. Environ. Microbiol.* **82**, 5332–5339 (2016).
128. McVay, C. S., Velásquez, M. & Fralick, J. A. Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrob. Agents Chemother.* **51**, 1934–1938 (2007).
129. Gorski, A. *et al.* Bacteriophage translocation. *FEMS Immunol. Med. Microbiol.* **46**, 313–319 (2006).
130. Łusiak-Szelachowska, M. *et al.* Phage neutralization by sera of patients receiving phage therapy. *Viral Immunol.* **27**, 295–304 (2014).
131. Górski, A. *et al.* in *Advances in Virus Research* Vol. 83 (eds Lobocka, M. & Szybalski, W.) 41–71 (Elsevier, 2012).
132. Hodyra-Stefaniak, K. *et al.* Mammalian host-versus-phage immune response determines phage fate *in vivo*. *Sci. Rep.* **5**, 14802 (2015).
133. Górski, A. & Weber-Dabrowska, B. The potential role of endogenous bacteriophages in controlling invading pathogens. *Cell. Mol. Life Sci.* **62**, 511–519 (2005).
134. Budynek, P., Dąbrowska, K., Skaradziński, G. & Górski, A. Bacteriophages and cancer. *Arch. Microbiol.* **192**, 315–320 (2010).
135. US National Library of Medicine. *ClinicalTrials.gov* https://clinicaltrials.gov/ct2/show/NCT02116010 (2015).
136. Rose, T. *et al.* Experimental phage therapy of burn wound infection: difficult first steps. *Int. J. Burns Trauma* **4**, 66–73 (2014).
137. Marza, J. A., Soothill, J. S., Boydell, P. & Collyns, T. A. Multiplication of therapeutically administered bacteriophages in *Pseudomonas aeruginosa* infected patients. *Burns* **32**, 644–646 (2006).
138. Wright, A., Hawkins, C. H., Anggård, E. E. & Harper, D. R. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clin. Otolaryngol.* **34**, 349–357 (2009).
139. Markoishvili, K., Tsitlanadze, G., Katsarava, R., Morris, J. G. & Sulakvelidze, A. A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. *Int. J. Dermatol.* **41**, 453–458 (2002).
140. Weber-Dąbrowska, B. *et al.* Bacteriophage procurement for therapeutic purposes. *Front. Microbiol.* **7**, 1177 (2016).
141. Saussereau, E. *et al.* Effectiveness of bacteriophages in the sputum of cystic fibrosis patients. *Clin. Microbiol. Infect.* **20**, O983–O990 (2014).
142. Morello, E. *et al.* Pulmonary bacteriophage therapy on *Pseudomonas aeruginosa* cystic fibrosis strains: first steps towards treatment and prevention. *PLoS ONE* **6**, e16963 (2011).
143. Kutateladze, M. & Adamia, R. Phage therapy experience at the Eliava Institute. *Med. Mal. Infect.* **38**, 426–430 (2008).
144. Kutateladze, M. & Adamia, R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol.* **28**, 591–595 (2010).
145. Hawkins, C., Harper, D., Burch, D., Änggård, E. & Soothill, J. Topical treatment of *Pseudomonas aeruginosa* otitis of dogs with a bacteriophage mixture: a before/after clinical trial. *Vet. Microbiol.* **146**, 309–313 (2010).
146. Khairnar, K. *et al.* Novel bacteriophage therapy for controlling metallo-beta-lactamase producing *Pseudomonas aeruginosa* infection in Catfish. *BMC Vet. Res.* **9**, 264 (2013).
147. Park, S. C. & Nakai, T. Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu *Plecoglossus altivelis*. *Dis. Aquat. Organ.* **53**, 33–39 (2003).
148. Rombouts, S. *et al.* Characterization of novel bacteriophages for biocontrol of bacterial blight in leek caused by *Pseudomonas syringae* pv. *porri*. *Front. Microbiol.* **7**, 279 (2016).
149. Czaplewski, L. *et al.* Alternatives to antibiotics — a pipeline portfolio review. *Lancet Infect. Dis.* **16**, 239–251 (2016).
150. Briers, Y. *et al.* Engineered endolysin-based 'artilysins' to combat multidrug-resistant Gram-negative pathogens. *mBio* **5**, e01379-14 (2014).
The study is the first report of a promising strategy to combat multidrug-resistant pathogens using engineered phage enzymes.
151. Defraine, V. *et al.* Efficacy of artilysin art-175 against resistant and persistent *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **60**, 3480–3488 (2016).
152. Fischetti, V. A. Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. *Int. J. Med. Microbiol.* **300**, 357–362 (2010).
153. Briers, Y. *et al.* Art-175 is a highly efficient antibacterial against multidrug-resistant strains and persisters of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **58**, 3774–3784 (2014).
154. Pirnay, J.-P. *et al.* The phage therapy paradigm: prêt-à-porter or sur-mesure? *Pharm. Res.* **28**, 934–937 (2011).
155. Bikard, D. *et al.* Exploiting CRISPR–Cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.* **32**, 1146–1150 (2014).
156. Citorik, R. J., Mimee, M. & Lu, T. K. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat. Biotechnol.* **32**, 1141–1145 (2014).
157. Pires, D. P., Cleto, S., Sillankorva, S., Azeredo, J. & Lu, T. K. Genetically engineered phages: a review of advances over the last decade. *Microbiol. Mol. Biol. Rev.* **80**, 523–543 (2016).
158. Gerstmans, H., Rodríguez-Rubio, L., Lavigne, R. & Briers, Y. From endolysins to Artilysin®s: novel enzyme-based approaches to kill drug-resistant bacteria. *Biochem. Soc. Trans.* **44**, 123–128 (2016).
159. Citorik, R. J., Mimee, M. & Lu, T. K. Bacteriophage-based synthetic biology for the study of infectious diseases. *Curr. Opin. Microbiol.* **19**, 59–69 (2014).
160. Woolston, B. M., Edgar, S. & Stephanopoulos, G. Metabolic engineering: past and future. *Annu. Rev. Chem. Biomol. Eng.* **4**, 259–288 (2013).
161. Maynard, N. D., Gutschow, M. V., Birch, E. W. & Covert, M. W. The virus as metabolic engineer. *Biotechnol. J.* **5**, 686–694 (2010).
162. Ackermann, H.-W. 5500 phages examined in the electron microscope. *Arch. Virol.* **152**, 227–243 (2007).
163. Lavigne, R., Seto, D., Mahadevan, P., Ackermann, H.-W. & Kropinski, A. M. Unifying classical and molecular taxonomic classification: analysis of the *Podoviridae* using BLASTP-based tools. *Res. Microbiol.* **159**, 406–414 (2008).
164. Lavigne, R. *et al.* Classification of *Myoviridae* bacteriophages using protein sequence similarity. *BMC Microbiol.* **9**, 224 (2009).
165. Simmonds, P. *et al.* Consensus statement: virus taxonomy in the age of metagenomics. *Nat. Rev. Microbiol.* **15**, 161–168 (2017).
166. Kramberger, P., Urbas, L. & Štrancar, A. Downstream processing and chromatography based analytical methods for production of vaccines, gene therapy vectors, and bacteriophages. *Hum. Vaccin. Immunother.* **11**, 1010–1021 (2015).
167. Adriaenssens, E. M. *et al.* CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. *Virology* **434**, 265–270 (2012).
168. Merabishvili, M. *et al.* Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. *PLoS ONE* **4**, e4944 (2009).
169. Ryan, E. M., Gorman, S. P., Donnelly, R. F. & Gilmore, B. F. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. *J. Pharm. Pharmacol.* **63**, 1253–1264 (2011).
170. Vandenheuvel, D. *et al.* Feasibility of spray drying bacteriophages into respirable powders to combat pulmonary bacterial infections. *Eur. J. Pharm. Biopharm.* **84**, 578–582 (2013).
171. Leung, S. S. Y. *et al.* Production of inhalation phage powders using spray freeze drying and spray drying techniques for treatment of respiratory infections. *Pharm. Res.* **33**, 1486–1496 (2016).
172. Vandenheuvel, D., Meeus, J., Lavigne, R. & Van den Mooter, G. Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix. *Int. J. Pharm.* **472**, 202–205 (2014).
173. Singla, S. *et al.* Phospholipid vesicles encapsulated bacteriophage: a novel approach to enhance phage biodistribution. *J. Virol. Methods* **236**, 68–76 (2016).

Acknowledgements
The Laboratory of Gene Technology is supported by a GOA grant 'Phage biosystems' (GOA/15/006) from KU Leuven. J.D.S., K.D-.W. and B.G.B. hold a post-doctoral mandate (PDM) from KU Leuven.

Competing interests statement
The authors declare competing interests: see Web version for details.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

FURTHER INFORMATION
PhagoBurn: http://www.phagoburn.eu/
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.