Bacteriophage resistance mechanisms

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Abstract | Phages are now acknowledged as the most abundant microorganisms on the planet and are also possibly the most diversified. This diversity is mostly driven by their dynamic adaptation when facing selective pressure such as phage resistance mechanisms, which are widespread in bacterial hosts. When infecting bacterial cells, phages face a range of antiviral mechanisms, and they have evolved multiple tactics to avoid, circumvent or subvert these mechanisms in order to thrive in most environments. In this Review, we highlight the most important antiviral mechanisms of bacteria as well as the counter-attacks used by phages to evade these systems.

Phase variation
A genetically programmed biological phenomenon that occurs in bacteria that need to adapt to different environments. These bacteria can modify their cellular components according to environmental conditions through the regulation of a complex gene expression network.

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Bacteriophages (or phages) are now widely recognized to outnumber bacteria by an estimated tenfold[1]. The remarkable diversity of phages is best illustrated by the frequency of novel genes that are found in newly characterized phage genomes[2]. Such natural variation is a reflection of the array of bacterial hosts that are available to phages and the high evolution rate of phages when facing selective pressure created by antiphage barriers[2–5] (FIG. 1). In most environments, a large pool of phages and hosts are involved in continuous cycles of co-evolution, in which emerging phage-insensitive hosts help to preserve bacterial lineages, whereas counter-resistant phages threaten such new bacterial strains. Phages and phage resistance mechanisms therefore have key roles in regulating bacterial populations in most, if not all, habitats.

Some bacterial species have been domesticated and used extensively for fermentation processes[2–5]. Because high numbers of bacterial cells are cultivated each day in large vats, it is not surprising that most fermentation industries have experienced, at varying frequencies, problems with phage contamination. Phage–host dynamics may also be important in the re-emerging field of phage therapy. Because the phenomenon of antibiotic resistance has grown into a global public health concern, phage therapy is now being re-evaluated as a means to treat or prevent bacterial infections worldwide. The advantages and disadvantages of phage therapy have already been extensively documented[6–10]. One of the notable disadvantages is the risk of encountering phage-resistant bacterial pathogens or favouring the emergence of phage-insensitive bacterial strains.

As such, phage resistance is a crucial survival phenotype in a range of ecological niches, both natural and man-made. Here, we describe phage resistance mechanisms in bacteria and the known strategies used by phages to subvert these processes.

Preventing phage adsorption

Adsorption of phages to host receptors is the initial step of infection and, perhaps, one of the most intricate events, as phages must recognize a particular host-specific cell component. Phages are faced with an astonishing diversity in the composition of host membranes and cell walls. Futhermore, bacteria have evolved a range of barriers to prevent phage adsorption (FIG. 2). These adsorption-blocking mechanisms can be divided into at least three categories: the blocking of phage receptors, the production of extracellular matrix and the production of competitive inhibitors.

Blocking of phage receptors. To limit phage propagation, bacteria can adapt the structure of their cell surface receptors or their three-dimensional conformation. For example, *Staphylococcus aureus* produces a cell-wall-anchored virulence factor, immunoglobulin G-binding protein A, which binds to the Fc fragment of immunoglobulin G[11] (FIG. 2a). It has been shown that phage adsorption improves when the bacteria produce less protein A, indicating that the phage receptor is masked by this protein[12]. Phage T5, which infects *Escherichia coli*, produces a lipoprotein (Llp) that blocks its own receptor, ferrichrome-iron receptor (FhuA). Llp is expressed at the beginning of the infection, thereby preventing superinfection. This protein also protects newly synthesized phage T5 virions from inactivation by binding free receptors that are released from lysed cells[13]. Host cells also use lipoproteins to inhibit phages, as seen in *E. coli* F^+ strains. The outer-membrane protein TraT, encoded by the F plasmid, masks or modifies the conformation of outer-membrane protein A (OmpA), which is a receptor for many T-even-like *E. coli* phages[14].

Bordetella spp. use phase variation to alter their cell surface[15], which is necessary for the colonization and

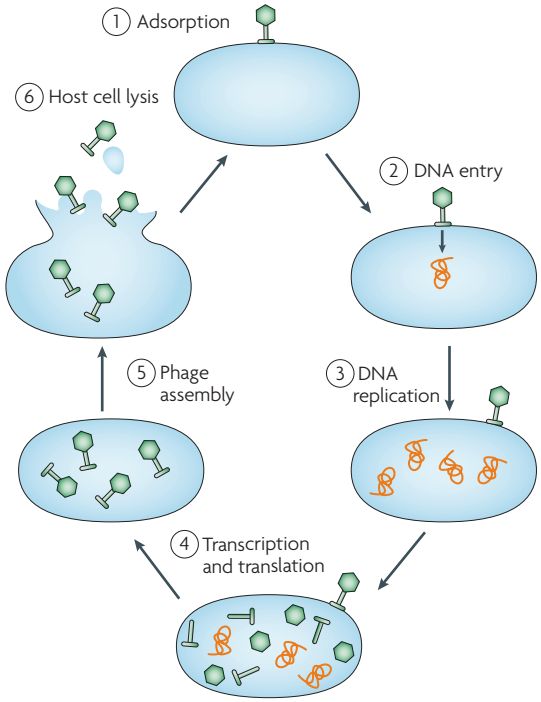

Figure 1 | **The phage replication cycle.** Each step of the cycle can be targeted by antiphage mechanisms.

survival of the bacteria. The production of many adhesins and toxins is under the control of the BvgAS two-component regulatory system. Bvg$^+$ *Bordetella* spp. cells express colonization and virulence factors, including adhesins, toxins and a type III secretion system, that are not expressed in the Bvg$^-$ phase[16]. The phage receptor, pertactin autotransporter (Prn), is expressed only in the Bvg$^+$ phase, thus the efficiency of infection of the *Bordetella* phage BPP-1 is 1 million-fold higher for Bvg$^+$ cells than for Bvg$^-$ cells[17]. Interestingly, although this receptor is absent from Bvg$^-$ cells, the phage BPP-1 is still able to infect them, albeit at a much lower rate, indicating that this phage has evolved a strategy to overcome the absence of its primary receptor.

Some phages that infect *Bordetella* spp. use a newly discovered family of genetic elements known as diversity-generating retroelements to promote genetic variability. These phages switch hosts through a template-dependent, reverse-transcriptase-mediated process that introduces nucleotide substitutions in the variable region of the phage gene *mtd*, which encodes major tropism determinant protein, the protein that is responsible for host recognition[17,18]. Comparative genome analyses have revealed putative diversity-generating retroelement systems in other phages[19], including in those that infect *Bifidobacterium* spp.[20].

Production of extracellular matrix. The production of structured extracellular polymers can promote bacterial survival in various ecological niches by protecting the bacteria against harsh environmental conditions and, in some cases, providing a physical barrier between phages and their receptors (FIG. 2b,c). Some phages have also evolved to specifically recognize these extracellular polymers (FIG. 2c) and even to degrade them (FIG. 2b)[21–25]. Polysaccharide-degrading enzymes can be classified into two groups: the hydrolases (also known as the polysaccharases) and the lyases. The lyases cleave the linkage between the monosaccharide and the C4 of uronic acid and introduce a double bond between the C4 and C5 of uronic acid[23]. The hydrolases break the glycosyl–oxygen bond in the glycosidic linkage[25]. These viral enzymes are found either bound to the phage structure (connected to the receptor-binding complex) or as free soluble enzymes from lysed bacterial cells[22].

Alginates are exopolysaccharides that are mainly produced by *Pseudomonas* spp., *Azotobacter* spp. and some marine algae[22]. An increased phage resistance was observed for alginate-producing *Azotobacter* spp. cells[26,27]. However, phage F116, which targets *Pseudomonas* spp., produces an alginate lyase, facilitating its dispersion in the alginate matrix as well as reducing the viscosity of this matrix[27]. It was proposed that alginate is involved in the adsorption of phage 2 and φPLS-I, which also target *Pseudomonas* spp., as an alginate-deficient mutant was phage resistant[28–30].

Hyaluronan (also known as hyaluronic acid) is composed of alternating *N*-acetylglucosamine and glucuronic-acid residues and is produced by pathogenic streptococci as a constituent of their capsule. This virulence factor helps bacterial cells to escape the immune system by interfering with defence mechanisms that are mediated by antibodies, complement and phagocytes[31–33]. Interestingly, genes encoding hyaluronan-degrading enzymes (known as hyaluronidases) are often found in the prophages that are inserted into the genomes of pathogenic bacterial strains. Not only are these prophage-encoded enzymes able to destroy the bacterial hyaluronan, but they also degrade human hyaluronan, helping the bacteria to spread through connective tissues[34]. Both virulent and temperate streptococcal phages possess hyaluronidase, but the quantity of enzyme produced by temperate phages is several orders of magnitude higher than the quantity produced by virulent phages, therefore enabling the temperate phages to cross the hyaluronan barrier[35,36].

Cell surface glycoconjugates of *E. coli* strains and *Salmonella* spp. serovars are extremely diverse. At least two serotype-specific surface sugars are produced by *E. coli* isolates: the lipopolysaccharide O antigen and the capsular polysaccharide K antigen. Phages have co-evolved with that diversity, and some are specific to these antigens. Capsular-negative mutants are insensitive to K antigen-specific phages[37]. A similar observation was made with *Salmonella* phage P22, which recognized the O antigen[38] (FIG. 2c). Furthermore, the P22 tail spike also possesses an endoglycosidase activity, enabling the phage to cross the 100 nm O antigen layer. Phage ΦV10, which specifically binds to the O antigen of *E. coli* O157:H7, possesses an *O*-acetyltransferase that modifies the O157 antigen to block adsorption of ΦV10 and similar phages[39].

Haemophilus influenzae-specific phages are also confronted with programmed phase variation of the host cell

Receptor-binding complex
A phage-encoded structural protein complex that is essential for the adsorption of the phage to the bacterial cell. In tailed phages, this complex is located at the extremity of the tail.

O antigen
The outer-most part of the lipopolysaccharide on the bacterial outer membrane, containing a repetitive glycan polymer. A great diversity is observed in the structure of *E. coli* O antigens, and they are good targets for serotyping methods and phages.

K antigen
Polysaccharide in the bacterial capsule.

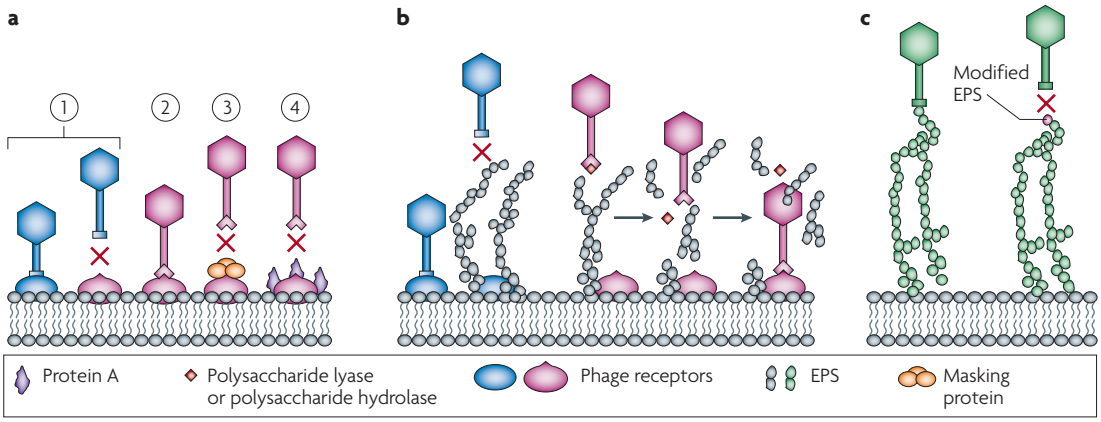

Figure 2 | **Different strategies used by bacteria to block phage adsorption. a** | Escaping phage infection at the adsorption step. Phage adsorption to the bacterial cell surface occurs through recognition of a phage receptor on the surface. Bacteria can become resistant to phages by modifying these cell surface receptors (step 1); phages can adapt to recognize these new receptors (step 2). Bacteria can also produce proteins that mask the phage receptor (step 3). *Staphylococcus aureus* produces protein A, which reduces phage adsorption (step 4). **b** | Phage adsorption can also be blocked by the production of exopolysaccharide (EPS), but phages overcome the EPS layer by producing a polysaccharide lyase or a polysaccharide hydrolase to cleave EPS. **c** | Phages have also evolved to specifically recognize polysaccharides such as O antigens and K antigens.

surface. A modification in the *lic2A* gene, which encodes a protein that is involved in the lipo-oligosaccharide synthesis pathway, is responsible for *H. influenzae* phase variation and preventing the adsorption of *Haemophilus* phage HP1c1 (REF. 40).

Production of competitive inhibitors. Molecules that are naturally present in the bacterial environment can bind specifically to the phage receptors, rendering these receptors unavailable for phages (FIG. 2a). For example, *E. coli* FhuA is an iron transporter and also the port of entry for coliphages such as T1, T5 and Φ80. The antimicrobial molecule microcin J25 also uses FhuA as a receptor and can outcompete phage T5 for binding to FhuA[41]. Microcin J25 is produced under conditions of nutrient depletion and has a role in microbial competition by inhibiting the growth of phylogenetically related strains and allowing the bacterium to avoid phage infection.

Preventing phage DNA entry

Superinfection exclusion (Sie) systems are proteins that block the entry of phage DNA into host cells, thereby conferring immunity against specific phages. These proteins are predicted to be membrane anchored or associated with membrane components. The genes encoding these proteins are often found in prophages, suggesting that in many cases Sie systems are important for phage–phage interactions rather than phage–host interactions. Many different Sie systems have been identified, although only a few have been characterized.

Sie systems in Gram-negative bacteria. Coliphage T4, as well-characterized virulent phage, has two Sie systems encoded by *imm* and *sp*. These systems cause rapid inhibition of DNA injection into cells, preventing subsequent infection by other T-even-like phages (FIG. 3). The Imm and Sp systems act separately and have different mechanisms of action. Imm prevents the transfer of phage DNA into the bacterial cytoplasm by changing the conformation of the injection site. Imm has two non-conventional transmembrane domains and is predicted to be localized to the membrane, but Imm alone does not confer complete phage immunity and must be associated with another membrane protein to exert its function and achieve complete exclusion[42]. The membrane protein Sp inhibits the activity of the T4 lysozyme (which is encoded by *gp5*), thereby presumably preventing the degradation of peptidoglycan and the subsequent entry of phage DNA. The T4 lysozyme is found at the extremity of the tail and creates holes in the host cell wall, facilitating the injection of phage DNA into the cell[43,44].

The Sim and SieA systems are associated with the prophages that are found in several Enterobacteriaceae species and have been well characterized, although the molecular mechanisms of their blocking activities are not yet fully understood. To exert its activity, Sim must be processed at its amino terminus in a SecA-dependent manner[45]. The resulting 24 kDa Sim protein confers resistance against coliphages P1, c1, c4 and *vir* mutants[46]. The only evidence that has led to the proposal that Sim blocks DNA entry is that phage adsorption is not affected by the presence of this protein and a bacterium can be successfully transformed with the phage genome[44].

Finally, SieA is found in the inner membrane of *Salmonella enterica* subsp. *enterica* serovar Typhimurium carrying lysogenic phage P22 and prevents the infection of phages L, MG178 and MG40 (REF. 47). Notably, it was initially believed that SieB was also involved in superinfection exclusion, but it was later shown to cause phage-abortive infection[47].

Sie systems in Gram-positive bacteria. To date, only a few examples of mechanisms that inhibit phage DNA injection have been identified in Gram-positive

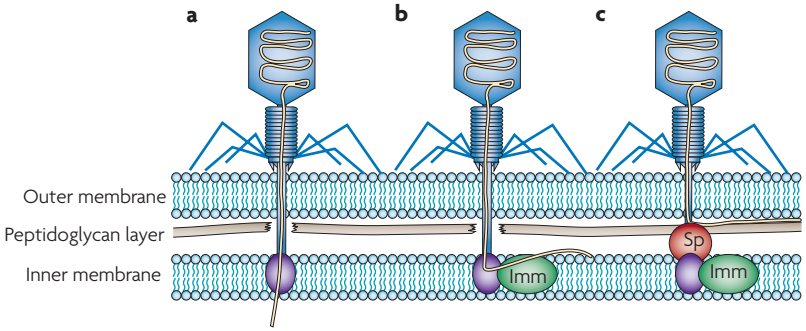

Figure 3 | **Blocking phage DNA entry into the bacterial cell. a** | Normal phage T4 infection of an *Escherichia coli* cell. The peptidogycan layer is degraded, and an inner-membrane protein is involved in the translocation of the DNA into the cytoplasm. **b** | Phage T4 encodes the protein Imm , which blocks the translocation of phage DNA into the cytoplasm, thus preventing infection by other T-even-like phages. **c** | The protein Sp, also encoded by phage T4, blocks degradation of the peptidoglycan, trapping the DNA between the peptidoglycan layer and the outer membrane.

bacteria. Most were identified in *Lactococcus lactis*, a species used in industrial milk fermentation processes[48,49]. The best characterized system is Sie_{2009}, which was identified in the genome of the temperate phage lactococcal phage Tuc2009 and then subsequently found in other prophages in the genomes of several *L. lactis* strains. Most lactococcal prophages (including Tuc2009) belong to the P335 lactococcal phage group, and Sie_{2009} from these phages confers resistance to a genetically distinct group of lactococcal phages (the 936 group). The 936 group is the predominant group of *L. lactis*-specific phages found in the dairy industry. Lactococcal Sie systems are predicted to be localized to the membrane[50,51], and they provide resistance by inhibiting the transfer of phage DNA into host cells[51].

Finally, a Sie-like system was recently found in the prophage of *Streptococcus thermophilus*, another bacterial species used in industrial milk fermentation processes[48,49]. Prophage TP-J34 encodes a signal-peptide-bearing 142-amino-acid lipoprotein (LTP) that blocks the injection of phage DNA into the cell. Surprisingly, this system confers resistance to some lactococcal phages when transformed into *L. lactis*[52].

Cutting phage nucleic acids

Restriction–modification systems. Many, if not all, bacterial genera possess restriction–modification (R–M) systems. Their activities are due to several heterogeneous proteins that have been classified into at least four groups (type I–type IV). The principal function of the R–M system is thought to be protecting the cell against invading DNA, including viruses. For mechanistic information on R–M systems, see REFS 53,54.

When unmethylated phage DNA enters a cell harbouring a R–M system, it will be either recognized by the restriction enzyme and rapidly degraded or, to a lesser extent, methylated by a bacterial methylase to avoid restriction, therefore leading to the initiation of the phage's lytic cycle. The fate of phage DNA is determined mainly by the processing rates of these two enzymes. As the restriction enzyme is often more active than the methylase, the incoming phage DNA is usually cleaved, although the host DNA is always protected by the methylase activity. Moreover, methylases are usually more specific for hemimethylated DNA (that is, DNA containing methyl groups on only one of the two DNA strands)[55]. When the phage DNA is methylated, the new virions become insensitive to the cognate restriction enzyme and readily infect neighbouring cells containing the same R–M system. The phage will remain insensitive until it infects a bacterium that does not encode the same methylase gene, in which case the new virions will become unmethylated again and will therefore be sensitive once again to the R–M system of the original bacterium.

To cope with these R–M systems, phages have evolved several antirestriction strategies. One of these strategies is the absence of endonuclease recognition sites in their genomes through the accumulation of point mutations. For example, the polyvalent *Staphylococcus* phage K has no *Sau*3A sites (which have a 5′-GATC-3′ recognition sequence) in its double-stranded-DNA genome[56,57]. The antiviral efficiency of an R–M system is directly proportional to the number of recognition sites in a viral double-stranded-DNA genome[58]. Furthermore, some phages have overcome R–M systems through the acquisition of the cognate methylase gene in their genomes[59].

Perhaps, the most striking example of an antirestriction system is found in phage T4 (FIG. 4). The genome of this virulent phage contains the unusual base hydroxymethylcytosine (HMC) instead of the cytosine that is found in the host DNA. This modification allows phage T4 DNA to be impervious to R–M systems that recognize specific sequences containing a cytosine. In the co-evolutionary arm race, some bacteria have acquired the ability to attack modified phage DNA. In contrast to classical R–M systems, modification-dependent systems (MDSs) are specific for either methylated or hydroxymethylated DNA[60]. Only a few MDS enzymes have been thoroughly characterized, such as DpnI from *Streptococcus pneumoniae*[61] and McrA, McrBC and Mrr from *E. coli*[62]. Interestingly, phage T4 is also resistant to MDS enzymes, because its HMC residues are glucosylated.

In yet another twist, *E. coli* CT596 is able to attack glucosylated DNA, as it possesses a two-component system consisting of glucose-modified restriction S (GmrS) and GmrD proteins encoded by a prophage[63,64]. This system specifically recognizes and cleaves DNA containing glucosylated HMC but has no effect on unglucosylated DNA[64]. Some T4-like phages have a gene encoding internal protein I (IPI) , which is specifically designed to disable the GmrS–GmrD system. During infection, mature IPI (IPI*) is injected into the host cell along with the phage genome. According to its structure, IPI* may interact with the GmrS–GmrD complex to inactivate its restriction activity[65]. Interestingly, some bacterial strains have found ways to bypass IPI* by using a single, fused polypeptide[65].

Coliphage λ also possesses a restriction alleviation mechanism encoded by *ral*[66]. It was shown that the

Restriction enzyme
An endonuclease protects the bacterial cell against infection by cleaving foreign DNA at specific sites. These enzymes are generally coupled with a cognate DNA methylase, which modifies and protects the host DNA.

Two-component system
A biological mechanism necessitating the presence of two enzymes to be functional.

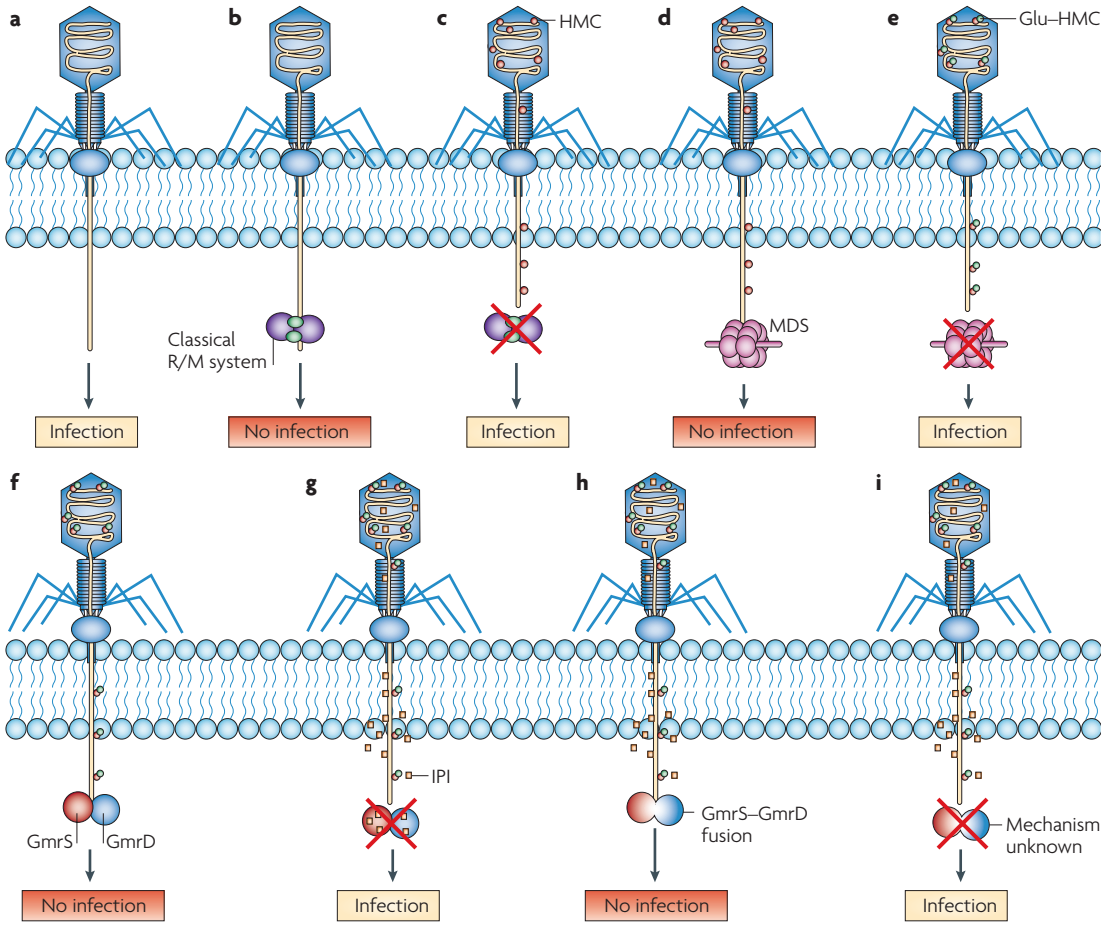

Figure 4 | **The arms race between bacteria and phages.** Using phage T4 as a model whenever possible, the different strategies of phages for escaping the restriction–modification (R–M) systems and modification-dependent systems (MDSs) that are encoded by *Escherichia coli* are shown. **a** | Phage T4 infecting a phage-sensitive host. **b** | Phage T4 infecting a phage-resistant *E. coli* cell containing a R–M system. The phage genome is cut at specific sites by the restriction enzyme. **c** | The genome of phage T4 contains hydroxymethylcytosine (HMC) and can also be methylated, thereby avoiding specific endonucleases. **d** | Some bacteria have acquired modification-dependent systems that can exclusively cleave HMC-containing DNA, thus preventing infection by HMC-containing phages. **e** | Phage T4 acquired a resistance to MDSs through the glucosylation of HMC residues (glu–HMC). **f** | A few *E. coli* strains have acquired a glucose-modified restriction (Gmr) system that targets and cleaves glu–HMC-modified phage T4 genomes, thereby blocking phage infection. This system is composed of two subunits (GmrS and GmrD) that specifically cut glu–HMC DNA but have no effect on unglucosylated DNA. **g** | Some T4-like phages have a gene encoding internal protein I (IPI), a protein that hinders the Gmr system and allows these phages to successfully infect *E. coli* strains containing this system. **h** | Some *E. coli* strains harbour a modified Gmr system in which a translational fusion of GmrS and GmrD is produced, rendering IPI ineffective. **i** | Finally, phage T4 mutants can bypass the GmrS–GmrD fusion by a unknown mechanism, leading to a successful infection.

proportion of methylated phage λ progeny was increased in the presence of Ral, indicating that Ral enhances the methylase activity of type I R–M systems. A similar system named Lar, encoded by a phage λ derivative (namely, phage λ reverse, which is a recombinant of the prophages λ and Rac of *E. coli* str. K12 (REF. 67)), also enhances methylase activity and alleviates the restriction activity of type I R–M systems[68,69].

Coliphage T7 possesses the *0.3* gene encoding Ocr (overcomes classical restriction activity), which mimics the conformation of the phage double-stranded B-fold DNA to sequester the R–M enzymes and prevent restriction of the phage genome[70]. Ocr is one of the first proteins to be expressed by phage T7 immediately after the entry of its genome into the cell[71]. Ocr specifically binds and inhibits type I R–M enzymes[72–74]. The genome of coliphage T3 encodes a protein that is similar to phage T7 Ocr. This Ocr-like protein is not only efficient against type I R–M systems but also inhibits type III R–M systems[60]. It can also hydrolyse *S*-adenosyl methionine[75], thereby subverting those endonucleases that require *S*-adenosyl methionine for their activity (reviewed elsewhere[76]).

Finally, phage P1, which targets *E. coli*, possesses proteins that are injected with its DNA genome, similarly to IPI* of phage T4. The proteins defence against restriction A (DarA) (which is 69 kDa) and DarB (which is 251 kDa) are apparently ejected through the tail together with the phage genome and temporarily occlude restriction sites to protect the phage P1 genome against type I R–M systems[77].

The CRISPR–Cas system. The effects of clustered regularly interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated (*cas*) genes on phage multiplication were recently described[78] and have been reviewed elsewhere[79,80–83]. CRISPR–*cas* loci were first described in 1987 (REF. 84), but comparative analysis has revealed that they are present in an increasing number of complete bacterial and archaeal genomes[85–89]. These loci are generally composed of 21–48 bp direct repeats interspaced by non-repetitive spacers (26–72 bp) of similar length and, usually, flanked by a varying number of *cas* genes (ranging between 4 and 20 in number, if present) (FIG. 5). Many hypotheses have been proposed to explain the biological role of CRISPR–*cas* loci, but it was only recently found that they are an immunity system that targets foreign nucleic acids, including phage genomes and plasmids[88,90–94]. It was also reported that the CRISPR–Cas system is involved in the lysogeny-dependent inhibition of biofilm formation and swarming motility in *Pseudomonas aeruginosa*[95].

When a phage-sensitive strain of *S. thermophilus* is challenged with a virulent phage, natural phage-insensitive host mutants will eventually emerge. Analyses of these mutants indicate that they have acquired at least one new repeat-spacer unit at the 5′ end of the repeat-spacer region of a CRISPR locus. The newly added spacer (which is usually 30 bp in *S. thermophilus*) is 100% identical to a sequence named the proto-spacer found in the genome of the infecting virulent phage. The molecular mechanism behind the acquisition of a repeat-spacer unit is still unknown. Interestingly, different phage-insensitive host mutants can acquire distinct spacers from the same phage genome. The only common feature is the presence of a short conserved nucleotide motif (for example, NNAGAAW) known as the proto-spacer-associated motif (PAM) that flanks the proto-spacer in the phage genome. It should be noted that the sequence and the location (that is, whether it is upstream of downstream of the proto-spacer) of the PAM varies between CRISPR–Cas systems[96].

The mechanistic details of the antiphage or antiplasmid activity of the CRISPR–Cas system are also still unclear, but several hypotheses have been put forward. It was first suggested that this system targets RNA and acts as a bacterial RNA interference mechanism[88]. It was recently proposed, however, that the CRISPR target might be the DNA[94,97]. Several research groups have now purified Cas proteins and shown that some of these proteins cleave CRISPR mRNA in the repeat region into smaller RNAs ready to target incoming phage or plasmid RNA or DNA[97–99].

Although it is undoubtedly fascinating, the CRISPR–Cas system is still not infallible. *S. thermophilus*-specific phages can bypass the resistance provided by the newly acquired spacers by acquiring a simple point mutation (or deletion) in the targeted proto-spacer. Of note, a mutation in the conserved PAM in the phage genome is also sufficient to dodge CRISPR activity[100]. Because the PAM is found only in the phage genome and not in the repeat next to the host-acquired spacer, it provides a way to differentiate between host DNA and the phage target. Interestingly, iterative addition of new spacers targeting the emerging phage mutants is still possible. In fact, the CRISPR–Cas system and the phage response perfectly illustrate the dynamic co-evolution between both groups of microorganisms, and it is now acknowledged that CRISPR–Cas systems play a substantial part in the structure of microbial communities[101].

Abortive infection systems

Bacteria carry a wide range of heterologous proteins that provide resistance through the abortion of phage infection. These abortive infection (Abi) systems also lead to the death of the infected cell, which is not the case for the antiphage systems described above. Typically, these Abi systems target a crucial step of phage multiplication such as replication, transcription or translation. Studies on Abi systems began over 50 years ago and, even now, their modes of action are still not completely understood, partly because they are complex and partly because of knowledge gaps in phage biology.

The Rex system found in phage λ-lysogenic *E. coli* strains is not only the best characterized Abi system to date (see REFS 102,103) but has also provided fundamental knowledge about molecular genetics and about phage T4 biology. This two-component system requires both RexA and RexB proteins for protection against phages (FIG. 6). When phage infection occurs, a phage protein–DNA complex is produced as a replication or recombination intermediate, and this activates RexA[103,104]. RexA is an intracellular sensor that activates the membrane-anchored RexB. At least two RexA proteins are needed to activate one RexB protein, indicating that the protein ratio is important for Abi activity[105]. RexB is an ion channel that reduces membrane potential, leading to a drop in the cellular ATP level, thereby decreasing the synthesis of macromolecules and stopping cell multiplication[103,104]. During the process, phage infection will also abort, because it needs either ATP or ATP-dependent cellular components. Phage T4 encodes two proteins (RIIA and RIIB) that help the phage to circumvent this Abi mechanism; however, this phenomenon is only partial, as overproduction of the Rex proteins confers phage resistance[103]. The Rex system was studied using the phage T4rII mutant as a model, as this phage contains mutations in *rIIA* and *rIIB* and is therefore susceptible to Rex exclusion. However, a mutation in *motA* renders the phage T4rII mutant insensitive to the Rex system[102]. MotA is a transcription factor that activates the middle gene promoters during the switch of transcription from early to middle genes[106].

Two other *E. coli* Abi systems, Lit and the anticodon nuclease PrrC, abort phage infection by inhibiting the translation machinery (for more information see REFS 102,103,107). The *lit* gene is found in a defective prophage (prophage e14) that is integrated into the genome of *E. coli* K12. Late during phage infection, the 29-amino-acid Gol peptide that is present in the major capsid protein of phage T4 activates Lit[108]. Activated Lit, which possesses a zinc metalloproteinase domain, cleaves elongation factor-Tu between Gly59 and Ile60, stopping protein synthesis and leading to bacterial death and abortion of the infection[103].

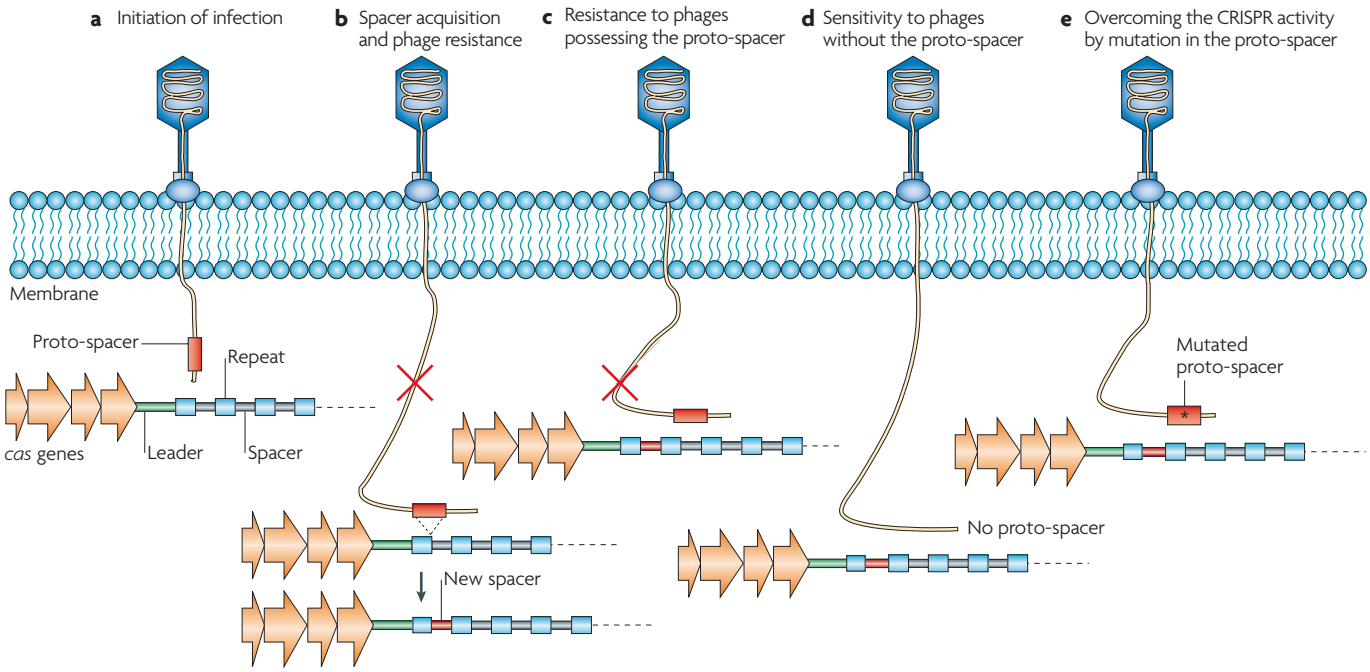

Figure 5 | **The CRISPR (clustered regularly interspaced short palindromic repeat) mode of action. a** | Phage DNA enters the bacterial cell and the lytic cycle of the phage initiates. Most bacterial cells will undergo lysis on completion of the phage lytic cycle. **b** | A small portion of phage-infected cells (approximately 1×10^{-6}) will survive the infection. The CRISPR (clustered regularly interspaced short palindromic repeat) locus of such a phage-insensitive mutant contains an additional repeat (duplicated from the CRISPR locus) and a new spacer (which is 30 nucleotides long in *Streptococcus thermophilus*) that has been acquired from the infecting phage genome. **c** | The newly acquired repeat-spacer unit is responsible for the phage resistance of these phage-insensitive mutants. Any incoming phage genome carrying a proto-spacer with 100% nucleotide identity to the new spacer-repeat unit will be inactivated and the phage infection process will be blocked. **d** | These so-called phage-insensitive mutants are still sensitive to phages that do not possess this specific proto-spacer in their genomes. **e** | Phage mutants carrying a single point mutation or a deletion in their proto-spacer (or in the motif next to the proto-spacer) can bypass the CRISPR activity and successfully complete their lytic cycles. *cas* genes, CRISPR-associated genes.

The *prrC* gene is located in a cryptic genetic element on the chromosome of *E. coli* CT196. This gene is part of a cassette containing three other genes: *prrA*, *prrB* and *prrD*. This cassette also encodes a type IC R–M system that probably acts as a first line of defence against phages[103]. Activated PrrC cleaves $tRNA^{Lys}$ in the anticodon loop and, in phage T4 that are defective in polynucleotide kinase or RNA ligase activities, this cannot be repaired, causing protein synthesis to stop[103]. PrrC activity is neutralized by the associated *prrD*-encoded restriction enzyme, *Eco*prrI. The phage T4 peptide Stp (suppressor of the three-prime phosphatase) alters the interaction of *Eco*prrI and PrrC, releasing activated PrrC protein and causing phage abortion[103].

Some Abi systems are more complex and involve several resistance-induced physiological changes. PifA is a membrane-associated protein that confers resistance to virulent phages T3 and T7 expressing gene *1.2* and to other related phages[109]. In phage-infected bacteria carrying the PifA system, early phage gene transcription occurs normally, but anomalies rapidly arise. For example, macromolecule synthesis is severely reduced and only half of the phage genome is injected into the cell, thereby limiting late transcription[110]. Little phage DNA replication occurs and the bacterial chromosome is degraded. In addition, bacterial membrane permeability is altered, causing leakage of molecules such as ATP[111]. It is not known which of these physiological changes occur first, but the result is that the bacterium dies and the phage remains trapped, limiting its propagation. To bypass the PifA resistance mechanism, phage T7 requires a mutation in gene *1.2* or a double mutation in gene *10* (REF. 102). The protein encoded by gene *1.2*, gp1.2, is an inhibitor of deoxyguanosine triphosphate (dGTP) triphosphohydrolase and is necessary for phage propagation in host strains that overexpress this dGTPase, whereas gp10 is the major capsid protein[110].

The Abi mechanisms described above are active in *E. coli*, but many systems have also been discovered in Gram-positive bacteria, especially in *L. lactis*. To date, at least 23 distinct lactococcal Abi mechanisms have been reported[112]. The Abi phenotype is usually mediated by a single host gene, although in a few cases the system consists of two to four genes. The different lactococcal Abi proteins are thought to affect different steps of the phage multiplication cycle. For example, AbiA, AbiF, AbiK, AbiP and AbiT were shown to interfere with DNA replication[113–117], whereas AbiB, AbiG and AbiU affected RNA transcription[118–120]. AbiC was shown to limit production of the major capsid protein[121], and AbiE, AbiI and AbiQ affected the packaging of phage DNA[122] (FIG. 1). AbiD1 was found to interfere with a phage-encoded

Cryptic genetic element
An incomplete or defective prophage that is unable to excise from the host genome and multiply as a result of host genome evolution. Such prophages provide a pool of phage genes that can be tapped into by an incoming virulent phage.

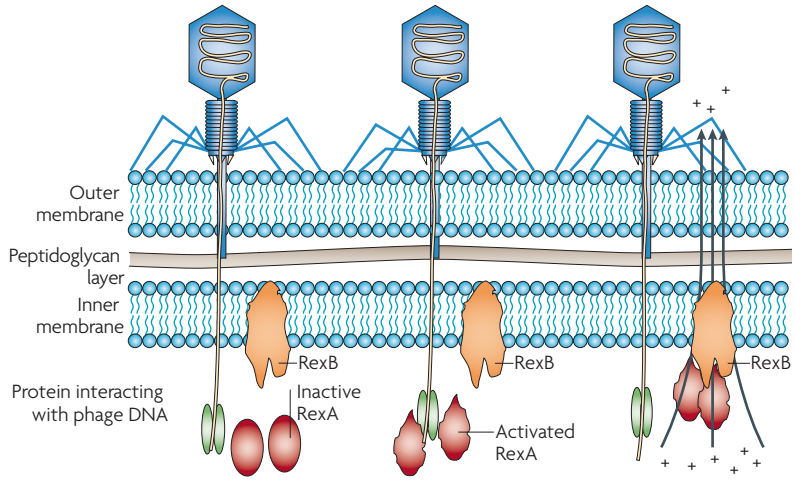

Figure 6 | **The mode of action of the Rex system.** The Rex system is an example of a two-component abortive infection system that operates in *Escherichia coli*. An inactive form of RexA, the sensor protein, is activated by the phage protein–DNA complex that forms as a replication or recombination intermediate on phage infection. Two activated RexA proteins are needed to trigger the membrane-anchored protein RexB, which acts as an ion channel and allows the passage of monovalent cations through the bacterial inner membrane, destroying the membrane potential and killing the cell.

RuvC-like endonuclease[123], and the presence of AbiZ caused premature lysis of the infected cells[124].

More specifically, AbiP inhibits transcription of the middle and late genes of the phage, and this is accompanied by the accumulation of early transcripts and a reduction in phage DNA replication[125–127]. AbiK blocks the phage lytic cycle in an unknown process that involves a phage-encoded single-stranded-DNA-annealing protein of the Rad52 family, named Sak[128]. It has been suggested that AbiK could synthesize a complementary DNA molecule from an unknown RNA template using its putative reverse transcriptase activity. In this model, the RNA molecule is degraded and a single-stranded DNA released. Then, Sak binds to the single-stranded DNA, protecting it from degradation but still allowing it to bind to the complementary phage RNA and block the gene translation that is necessary for phage propagation[129].

Toxin–antitoxin (TA) systems are widespread in many bacteria, and one such system was recently associated with an Abi phenotype[130]. TA systems were first identified on plasmids and found to have a role in plasmid maintenance. Later, they were found to be associated with gene regulation, bacterial population control and programmed cell death[131]. The core of a TA system can involve different regulatory interactions, including protein–protein, RNA–RNA or protein–RNA interactions. In all cases, a toxic molecule is produced by the cell and neutralized by the antitoxin product. The expression of these molecules is tightly controlled and varies from system to system but often involves promoter repression or the use of a specific transcriptional terminator. When the balance between the two regulatory halves is altered, the toxin is released and the bacterium dies.

The ToxIN system from *Pectobacterium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*) consists of a genetic cassette containing a *toxI* gene (which is composed of 5.5 repeats of 36 nucleotides each) encoding an antitoxin followed by a transcription terminator and the *toxN* gene encoding the toxin. The antitoxin is composed of at least one RNA molecule, encoded by the repeats, that neutralizes ToxN. This mechanism has been shown to abort phage infection[130,132]. Other TA systems identified in *E. coli* act as phage defence mechanisms, such as the *hok–shok* system (which is an RNA–RNA system) that prevents phage T4 infection[133] and the MazEF system (which is a two-protein system) that protects against phage P1 (REF. 134).

Conclusions

Phages are now widely recognized as major ecological contributors in various environments. Throughout the 1950s–1970s, phages were pillars of genetic research and molecular biology[135]. For the past decade, phage research has been going through a renaissance, mainly owing to the prospect of their use in phage therapy or the need to combat them in the food and biotechnology industries. It will be increasingly important to better understand the interactions between phages and their bacterial hosts in order to fully exploit their antimicrobial potential and to effectively control their populations in bio-industries. For example, it is imperative to go beyond the classical analysis of phage host range and to try to understand why a phage successfully infects one strain but not another.

One area that requires substantial attention is in the identification of phage receptors. The phage infection process begins with the specific adsorption of the phage to a receptor on the host surface. This interaction, which is often described as a lock (the host receptor) and key (the phage receptor-binding protein) association, is still poorly understood. Indeed, outside *E. coli*, very few phage receptors are known, and the means by which phages have evolved in response to mutations in these receptors remain to be determined.

Many more phage resistance barriers are likely to be uncovered, as these natural antiviral systems reflect the remarkable diversity of bacterial viruses and the role of resistance in maintaining the phage–host balance in either natural or man-made environments. It is now essential to go beyond the initial discovery of a phage resistance system to try to understand the molecular mechanisms behind these antiviral activities. In that regard, progress in phage biology will undoubtedly be needed to fully comprehend these biological systems. Moreover, most antiphage systems were found using double-stranded-DNA phages as targets. Systems targeting phages with single-stranded DNA, single-stranded RNA or double-stranded RNA genomes are almost certainly waiting to be revealed.

Finally, phage resistance mechanisms are often studied in a laboratory-controlled environment, one at a time and using a single phage–host model. However, bacterial strains often contain multiple antiphage barriers. The conjunction of many mechanisms in the same bacterial cell has rarely been assessed, and the effect of such combinations on phage population and evolution is often overlooked. Similarly, the efficacy

of phage resistance mechanisms against genetically diverse phages are not always measured. As phage and bacteria have a long co-evolutionary history, we can assume that phages can effectively raise a counter-resistance, through various means and with limited fitness cost, to cope with such selective forces. One can also imagine that one combination of phage resistance mechanisms may be better than another, favouring specific clonal population structures to persist in phage-containing environments. Clearly, a global approach is needed to fully appreciate the persistence of these survivors.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES

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gp5 | *imm* | *motA* | *mtd* | *ral* | *sp*
Entrez Genome: http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome
Bordetella phage BPP-1 | coliphage λ | coliphage T3 | coliphage T4 | phage T5 | coliphage T7 | lactococcal phage Tuc2009 | *Salmonella* phage P22 | *Staphylococcus* phage K
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Escherichia coli | *Haemophilus influenzae* | *Lactococcus lactis* | *Pectobacterium atrosepticum* | *Pseudomonas aeruginosa* | *Salmonella enterica* subsp. *enterica* serovar Typhimurium | *Staphylococcus aureus* | *Streptococcus pneumoniae* | *Streptococcus thermophilus*
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