OPINION

A new perspective on lysogeny: prophages as active regulatory switches of bacteria

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Abstract | Unlike lytic phages, temperate phages that enter lysogeny maintain a long-term association with their bacterial host. In this context, mutually beneficial interactions can evolve that support efficient reproduction of both phages and bacteria. Temperate phages are integrated into the bacterial chromosome as large DNA insertions that can disrupt gene expression, and they may pose a fitness burden on the cell. However, they have also been shown to benefit their bacterial hosts by providing new functions in a bacterium–phage symbiotic interaction termed lysogenic conversion. In this Opinion article, we discuss another type of bacterium–phage interaction, active lysogeny, in which phages or phage-like elements are integrated into the bacterial chromosome within critical genes or operons and serve as switches that regulate bacterial genes via genome excision.

Bacterial viruses, or phages, were first discovered by Fredrick Twort and Felix D'Herelle in 1915 and 1917, respectively[1,2]. Soon after, it became clear that phages are pivotal to many aspects of bacterial evolution. Subsequent studies illustrated the never-ending conflict between phages and bacteria, which is evident from the plentiful 'scars' of phage remnants in bacterial genomes and the variety of defence mechanisms acquired by each adversary[3–6].

Bacteria and phages are two of the most abundant and genetically diverse entities known to exist in biology, with phages exceeding bacteria in number by tenfold (the number of phage particles is estimated to be in the order of 10^{31})[7,8]. Phages are obligate parasites that can typically sustain two distinct life cycles — lytic and lysogenic — as defined by their genetics and interaction with the bacterial host[9].

Upon infection, lytic phages immediately enter a productive cycle, in which the phage genome is replicated and packaged into progeny phage particles that are then released through bacterial lysis (FIG. 1a). By contrast, temperate phages can enter a lysogenic cycle, during which the phage genome is integrated into the bacterial chromosome to become a prophage, and persist in what is considered a latent or dormant state that does not promote cell death or the production of phage particles (FIG. 1b). Of note, some prophages persist as low copy number plasmids and do not integrate into the bacterial chromosome (for example, P1 and N15 phages)[10,11]. Prophages are replicated together with the bacterial host chromosome, and this lysogenic state is maintained by the repression of the phage lytic genes. A switch to lytic production is initiated when stressful conditions (that is, DNA damage)[12] induce the excision of the phage genome, which is followed by the expression of lytic genes that promote DNA replication, phage particle assembly, DNA packaging and bacterial lysis. It is important to view temperate phages as heterogeneous populations as not all temperate phages enter the lysogenic cycle upon infection. Even for those phages that do enter the lysogenic cycle, spontaneous induction of lytic production can still occur in the absence of an obvious stressor[13,14].

Phage genome excision and integration are crucial steps in the onset of the lytic and lysogenic cycles, respectively. These events are mediated by phage-encoded DNA recombinases, such as integrases and excisionases, and take place at a specific attachment site in the bacterial genome (*attB*), which is identical to an attachment site (*attP*) in the phage genome[15]. Although these sequences determine the phage specificity to the bacterial genome, secondary sites can be used if the original *attB* site is lost, as was shown with *Escherichia coli* phage λ[16]. Moreover, some phages integrate randomly within their host genome, such as phage Mu, and thus increase variation and possible mutations within the bacterial population[17].

Another documented, but less common, phage life cycle is pseudolysogeny, which represents an unstable situation in which the phage genome fails to replicate as in lytic production or to become established as a prophage[18,19]. This occurs most frequently under nutrient-deprived conditions, when bacterial cells cannot support DNA replication or protein synthesis. The phage genome remains as a non-integrated and non-replicating preprophage, which resembles an episome, until the nutritional status is restored, at which point the phage enters either a lysogenic or a lytic life cycle[20] (FIG. 1c).

Phages are natural predators that exploit bacterial cells for growth. This phenomenon generates a predation pressure that enhances natural selection, as the acquisition of a defence mechanism by the bacteria could potentially lead to near extinction of the phages, and, conversely, an increase in phage virulence risks the extinction of the bacterial population. Evolution of bacteria and phages is thus driven by co-adaptation that supports the reproduction of both[6]. This bacterium–phage co-evolution is extremely rapid, owing to the high turnover rates of phage infections (for example, an estimated 10^{24} productive phage infections per second in the oceans), as well as the short generation times and high mutation rates of bacteria and phages[6,21,22]. The extent of bacterium–phage

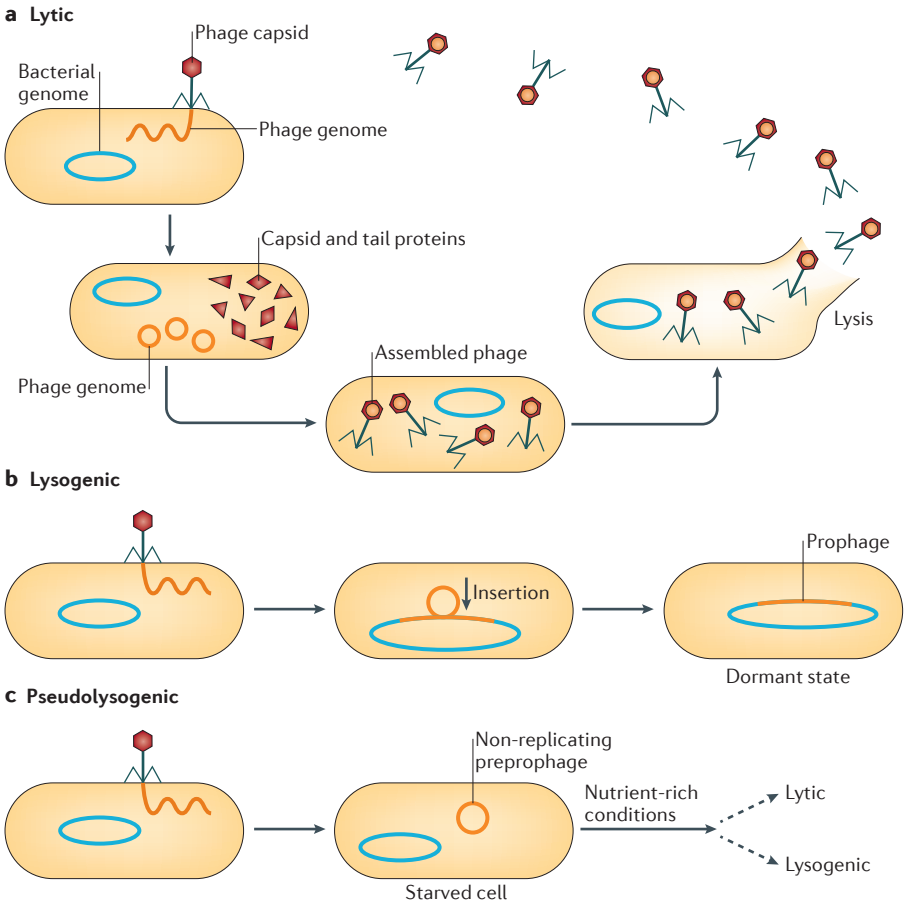


Figure 1 | **The phage replication cycles.** Schematic of lytic, lysogenic and pseudolysogenic cycles. **a** | Lytic phages immediately enter a productive cycle, in which the phage genome is replicated and phage capsid and tail proteins are synthesized using bacterial cell machineries; the phage genome is then packaged into progeny phage particles, which are liberated via bacterial lysis. **b** | Temperate phages enter a lysogenic cycle, in which the phage genome is integrated into the bacterial chromosome (becoming a prophage) and persists in what is considered a phage latent or dormant state that does not promote cell death or production of phage particles. Prophages are replicated together with the bacterial host chromosome during host cell replication and switch into lytic production upon exposure to DNA damage (not shown). **c** | Pseudolysogeny is an unstable situation in which the phage genome fails to replicate (as in lytic production) or become established as a prophage (as in lysogeny). Pseudolysogeny occurs most frequently under nutrient-deprived conditions, when bacterial cells cannot support DNA replication or protein synthesis. In this situation, the phage genome remains for an extended period of time as a non-integrated preprophage, which resembles an episome, until the nutritional status is restored, at which point the phage enters either a lysogenic or a lytic life cycle. Importantly, the pseudolysogenic preprophage does not replicate and so is only inherited by one of the daughter cells following cell division (not shown).

co-evolution is best demonstrated by the remarkable number of phage resistance mechanisms discovered in bacteria and of novel genes identified in phage genomes[5,6].

Lysogeny has a unique role within the bacterium–phage arms race in that it favours the development of symbiotic interactions because the fusion of phage and bacterial genomes, even if temporary, provides an ecological window for the evolution of mutually beneficial functions. As phages depend on their bacterial hosts for survival and proliferation, it is perhaps not surprising that, despite the bacterium–phage evolutionary conflict, phages profit from promoting the survival and proliferation of their hosts. With that in mind, bacterium–phage symbiotic relationships can arise, and there are several known instances of symbiotic interactions between them. For example, some phages encode proteins that enhance the fitness of their bacterial host in a phenomenon known as lysogenic conversion (see below)[23]. A second class of interaction between bacteria and temperate phages that leads to an unusual and fascinating long-term bacterium–phage co-existence is beginning to emerge, which we term 'active lysogeny'. In this case, temperate phages are integrated within bacterial functional genes, and thus need to cooperate with their hosts to regulate the proper and timely expression of the disrupted genes. Active lysogeny results in a highly controlled rearrangement of phage genomes that is distinct from spontaneously occurring phage genome rearrangements.

In this Opinion article, we focus on this newly described bacterium–phage interaction and propose that the integration and excision of prophages in active lysogeny can be viewed as a molecular switch that regulates bacterial genes. This phage regulatory switch (phage-RS) mechanism represents a unique example of bacterium–phage co-evolution that is specific to lysogeny, but distinct from classic (that is, latent) lysogeny, and one in which both parties benefit: the bacteria acquire enhanced fitness and the phages ensure their own survival. It is important to note that some examples discussed here are not bona fide phages but are instead cryptic or defective phages that contain viral elements, such as phage integrase or recombinase genes, but are not competent for infection.

Lysogenic conversion and active lysogeny

Lysogenic conversion is the best-described example of a process that provides a mutually beneficial symbiotic interaction between bacteria and phages. In lysogenic conversion, a phage encodes factors that increase the fitness and survival of the bacterial host[24,25] and that, in most cases, have no apparent value for the phage itself. Although the most common outcome of lysogenic conversion is protection from infection by other phages, lysogenic conversion events have been shown to influence almost every facet of bacterial life[25,26].

The first example of lysogenic conversion was documented in 1927 by Frobisher and Brown[27], who showed that non-toxigenic streptococci can acquire scarlatinal toxin when mixed with filtered supernatants of toxigenic streptococcal cultures. Within these supernatants were free phage particles harbouring the gene for scarlatinal toxin, which was transferred to the genome of non-toxigenic bacteria upon infection. This ability of temperate phages to convert non-pathogenic bacteria into pathogenic bacteria was the basis for the term lysogenic conversion.

Since then, many hallmark examples of lysogenic conversion have been described, with phages encoding various

virulence factors that enhance bacterial invasion into mammalian cells and that inhibit host cellular processes. Among them are potent bacterial toxins and effectors, including: diphtheria toxin of *Corynebacterium diphtheriae*[28]; botulinum toxin of *Clostridium botulinum*[29,30]; shiga toxins of *E. coli* O157:H7 (REF. 31); cholera toxin of *Vibrio cholerae*[32,33]; SpoE effector protein of *Salmonella enterica* subsp. *enterica* serovar Typhimurium[34]; several toxins of *Staphylococcus aureus* that block mammalian host processes and enhance bacterial virulence[35], as well as factors that promote adhesion and colonization, immune system evasion and serum resistance, and even transcription factors that regulate bacterial genes[24,25]. Some of these virulence factors are induced together with late lytic genes upon switching to the lytic pathway and are thus expressed and released upon lytic production and bacterial lysis, as in the case of shiga toxin in *E. coli* O157:H7 (REFS 31,36–38). Alternatively, some virulence factors are expressed during lysogeny, as in the case of cholera and diphtheria toxins[39].

Interestingly, expression of phage-encoded virulence factors during lysogeny can be regulated by bacterial transcription factors, as has been shown for cholera and diphtheria toxins, as well as phage-encoded toxins in *S. aureus*. In *V. cholerae*, the bacterial transcriptional regulators ToxR, ToxT and TcpP respond to environmental stimuli and co-regulate cholera toxin genes together with other bacterial genes that encode virulence factors[39]. Similarly, the production of diphtheria toxin by *C. diphtheriae* is regulated by the bacterial iron-dependent global regulator, DtxR, which also controls the expression of over 40 bacterial genes[25]. In *S. aureus*, phage-encoded toxins are regulated by the bacterial accessory gene regulator (agr) system, which responds to cell density in a process known as quorum sensing[40,41].

In general, expressed phage-encoded virulence factors are either actively secreted during lysogeny by bacterial secretion systems (FIG. 2a), such as the type II secretion system that secretes cholera toxin, or released by diffusion during bacterial lysis in the lytic cycle (FIG. 2b), as occurs with shiga toxin. Interestingly, during lysogenic conversion by bacterial lysis, lysis is thought to occur only in a subset of the bacterial population, which may either be owing to bacterial altruism (when a proportion of the bacterial community sacrifices itself for the common good) or to a phage mechanism that ensures the survival of

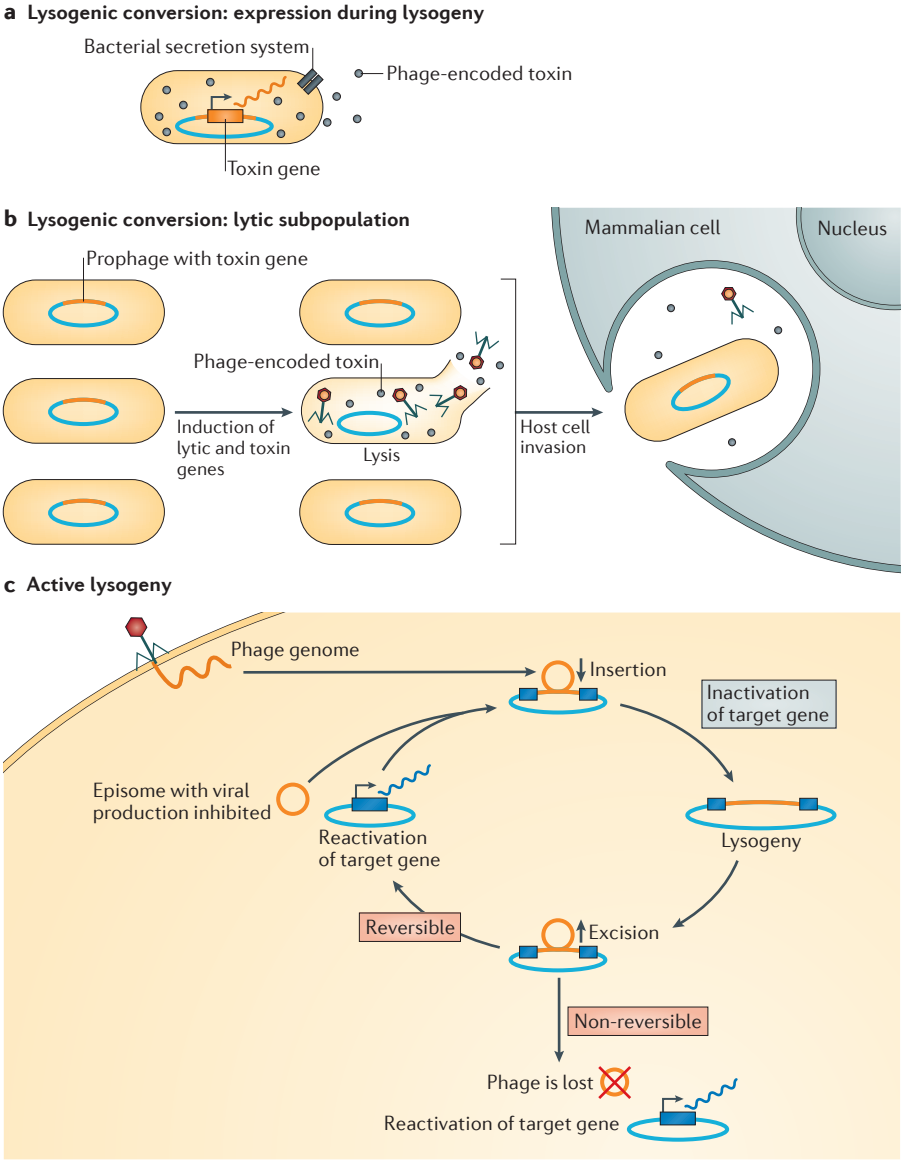


Figure 2 | **Bacterium–phage lysogenic interactions.** Lysogenic conversion and active lysogeny are two lysogenic processes whereby bacteria and phages cooperate. Lysogenic conversion is an interaction in which expression of phage-encoded proteins contributes to bacterial fitness with no apparent value to the phage. Illustrated in parts **a** and **b** are two examples of phage-encoded virulence factors that promote bacterial invasion into mammalian cells. **a** | Virulence factors are expressed from lysogenic prophages during bacterial infection of mammalian cells and secreted by bacterial secretion systems. **b** | Alternatively, phage-encoded virulence factors are expressed only in those cells in a subpopulation that switch to the lytic life cycle. These phage-encoded virulence factors are released by diffusion following bacterial cell lysis; although the requirement for lysis means that part of the bacterial community dies, the remainder invade mammalian cells and propagate within them. The sacrifice of the lytic subpopulation thus provides a benefit to other bacteria in the population. **c** | Active lysogeny is a newly described type of bacterium–phage interaction in which an integrated prophage serves as a regulatory switch that controls the expression of bacterial genes, which we term a phage regulatory switch (phage-RS). The prophage is integrated within the open reading frame (or adjacent regulatory region) of a bacterial gene with a crucial function, thereby deactivating the expression of the gene. A precise excision of the prophage, which restores the disrupted gene, is induced under conditions that require the gene's expression. Reversible active lysogeny is a complete on–off mechanism of gene regulation that occurs when the phage excision and reintegration events are reversible; that is, the excised phage is maintained as an episome that can be reintegrated into the target gene under conditions that once again permit the inactivation of the gene. For the phage-RS to be reversible, the phage cannot undergo lytic production, even when excised from the bacterial genome. Alternatively, in non-reversible active lysogeny, the excision event is followed by phage loss.

future hosts[24] (FIG. 2b). The overall outcome of lysogenic conversion in this context is that converted bacteria, which carry the phage-encoded virulence genes, are more virulent and thus more efficient at infecting mammalian cells. Notably, although the advantages of bacterium–phage interactions are clear in cases of lysogenic conversion, as bacterial host populations acquire genes that promote survival in an existing niche or invasion of a new one (reviewed in REFS 24,25,42,43), the evolutionary benefits are not always obvious in other bacterium–phage interactions and can seem to be highly complicated.

A second type of cooperative behaviour between bacteria and phages occurs when the insertion of temperate phages into the bacterial chromosome disrupts bacterial genes or regulatory regions. In many cases, phage insertions into functionally important genes or regulatory regions lead to deleterious effects for the host. One mechanism to overcome this problem is to restore the disrupted gene (or regulatory region) by providing a viral copy of that gene, or part of it that can be fused to the bacterial remainder of the gene. This restores the coding sequence of the gene (or regulatory region), as shown to occur with phages integrated within tRNA genes[44,45]. In other cases, integration of phages into functional genes or regulatory regions can be tolerated if the affected genes are non-essential or required only under certain conditions (for example, virulence factors that are expressed only during mammalian infection). Notably, for an insertion into conditionally expressed genes to be sustainable, the inserted prophage must respond to the same cues that induce expression of the target gene and permit its timely expression. Theoretically, such a bacterium–phage interaction could involve a controlled and precise excision of the prophage that results in a functional target gene but does not trigger lytic production and bacterial lysis (as normally occurs upon phage excision); that is, a phage-RS (FIG. 2c). Remarkably, several such cases have been documented, with phages inserted in crucial but conditional genes. In some instances, a mutually beneficial interaction has evolved, whereas in others a complete transformation of the prophage into a non-infective phage-RS has occurred. Whether infectious or not, phage-RSs are in all cases specific to lysogeny. We therefore suggest naming this phenomenon active lysogeny, to refer specifically to the active genome rearrangements of prophages as a form of bacterial gene regulation.

Reversible active lysogeny

Active lysogeny can have two outcomes for the phage: following phage insertion and subsequent excision, the excised phage can either persist until it is reintegrated into the host genome or be lost from the cell. We term these two forms of active lysogeny reversible active lysogeny and non-reversible active lysogeny, respectively (FIG. 2c). In both cases, the initial prophage genome excision event allows host gene transcription, but unique to reversible active lysogeny, which is discussed below, is a controlled reintegration of the phage that once again terminates host gene transcription.

Regulation of competence genes during phagosomal escape. The ability of bacteria to undergo natural DNA transformation is a regulated physiological state referred to as 'competence'. The canonical function of the competence (Com) system is the facilitation of exogenous DNA uptake across bacterial membranes by DNA transformation[46]. In Gram-positive bacteria, the Com system has been extensively examined using *Bacillus subtilis* as a model, and has been shown to be regulated by quorum sensing[47]. In this context, the small peptide ComX is exported outside of the bacterium, where it is sensed at high concentrations by the surface receptor kinase ComP. This kinase, in turn, activates a series of events that ultimately stabilize ComK, the master transcriptional activator of the late *com* genes required for competence. Transcription of these genes results in the assembly of the competence apparatus — comprising a cell wall-crossing pseudopilus, a DNA translocation channel, a DNA receptor and a helicase — that facilitates DNA uptake[48].

Intriguingly, the expression of the Com system has been associated with reversible active lysogeny in *Listeria monocytogenes*, a bacterium that cannot naturally take up DNA and is therefore not considered to be competent. The *L. monocytogenes* genome contains homologues for almost all of the structural genes of the competence apparatus, including a *comK*-like gene [49]. However, it lacks homologues for the quorum-sensing genes that encode the proteins that regulate competence in *B. subtilis* (for example, ComX, ComP and the downstream regulatory proteins), and functional orthologues have not been identified. The only remnant of the Com regulatory machinery is the *comK*-like gene, which, however, is inactivated in some strains by the insertion of a ~40-kb *L. monocytogenes*-specific prophage[50] (the A118-like prophage, which belongs to the Siphoviridae family of double-stranded DNA viruses that can reproduce via both lysogenic and lytic cycles[51,52]). Production of lytic virions is induced in response to nutritional stress, during *L. monocytogenes* stationary growth, or in response to mutagenic stress upon ultraviolet irradiation and is accompanied by bacterial lysis mediated by the combined action of phage-encoded holin and endolysin[53]. Although various aspects of this phage's biology have been studied, its impact on *L. monocytogenes* general fitness and virulence had been unclear.

Recently, it was shown that the *L. monocytogenes comK*-like gene and the genes encoding competence system apparatus, particularly the pseudopilus and the DNA channel, are highly transcribed during mammalian cell infection and are required to facilitate efficient bacterial escape from the phagosomes of the cell[54]. Escaping the phagosome is a crucial step in *L. monocytogenes* infection, as this bacterium is adapted to grow within the cytosol of host cells and to spread from cell to cell by recruiting the host actin polymerization machinery[55]. Bacteria that fail to escape the phagosome do not grow and are eventually killed within the phagosomes by host antibacterial mechanisms (for example, generation of free radicals, low pH and degradative enzymes). This unexpected function of the *com* genes was shown to be independent of Com components that involve DNA binding (that is, the DNA receptor and helicase) and thus indicated additional roles for the Com machinery in *L. monocytogenes*[54].

Remarkably, the expression of the *com* genes during *L. monocytogenes* infection of mammalian cells was found to require the formation of a functional *comK* gene via a precise excision of the prophage. Prophage excision was strongly induced within phagosomes, but, unlike classic prophage excision, did not lead to the production of progeny virions and bacterial lysis. Furthermore, although phage genes encoding capsid and tail proteins were induced during *L. monocytogenes* infection of mammalian cells, genes responsible for bacterial lysis (for example, the genes encoding holin and lysin) and virion formation (for example, the gene encoding terminase) were effectively repressed. These observations led to a model of reversible active lysogeny for the A118-like prophage, in which the prophage is stably integrated into the *L. monocytogenes* genome except for during mammalian infection, when the prophage turns into a phage-RS that regulates the expression of *comK* via genomic rearrangement. Under

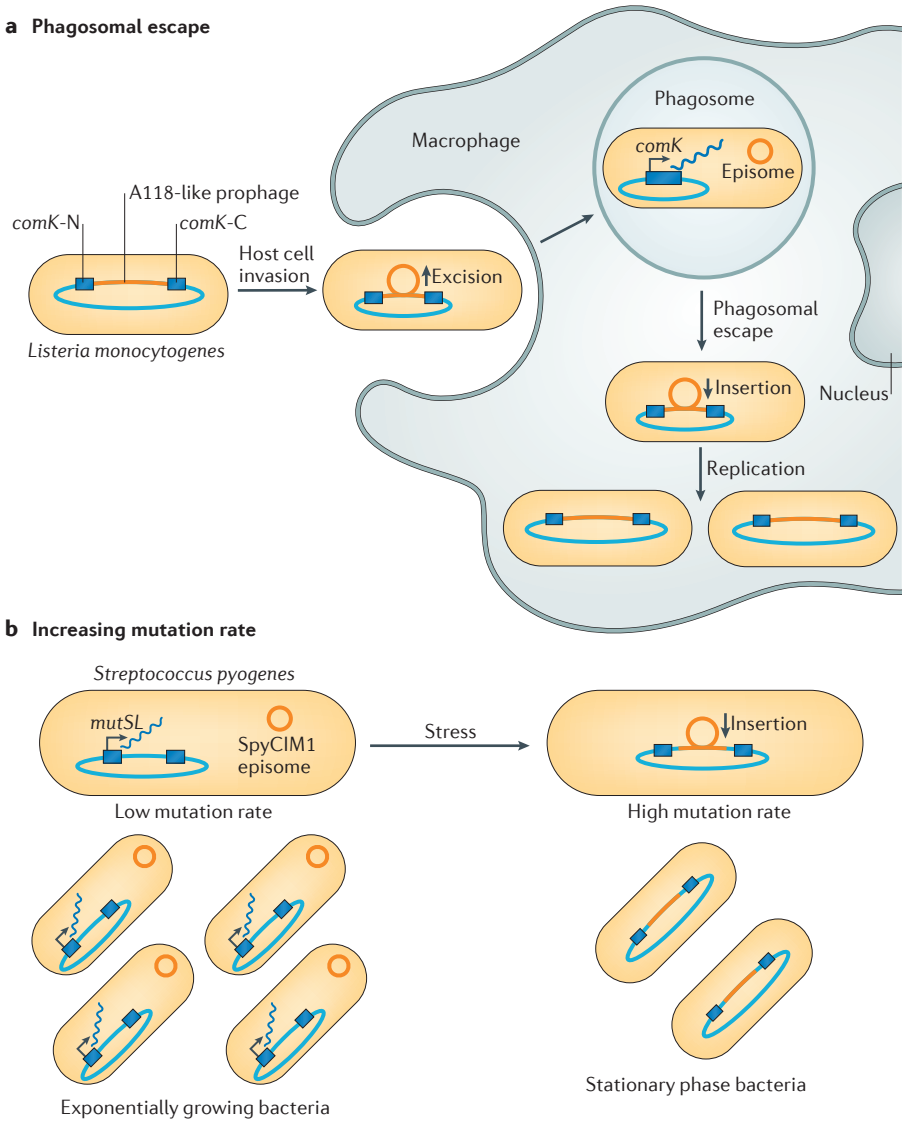


Figure 3 | **Reversible active lysogeny regulates critical processes in bacteria. a** | Regulation of *Listeria monocytogenes* escape from mammalian cell phagosomes. Upon *L. monocytogenes* invasion into mammalian cells, active lysogeny ensures that the bacteria rapidly escape the phagosome to enable replication within the host cell cytosol. An infective prophage (A118-like) inserted within the *comK* gene, which encodes the competence system master regulator, is excised in the phagosomal environment. This excision event reactivates *comK*, providing a temporal regulation of gene expression. The competence system of *L. monocytogenes* is necessary to promote efficient phagosomal escape, which is promoted by this phage regulatory switch (phage-RS). After the bacterium has escaped into the cytosol, the phage reintegrates into *comK* and blocks the expression of the competence system. **b** | Regulation of a mutator phenotype in *Streptococcus pyogenes*. In *S. pyogenes*, the *mutS* and *mutL* genes encode the mismatch repair (MMR) system, which is responsible for detection and removal of randomly occurring mutations in the bacterial genome. During exponential growth, the *mutSL* operon is expressed, but a phage-RS, SpyCIM1, is maintained, in an 'off' position, as an episome. Under conditions of stress, when bacteria enter stationary phase, the phage-RS is inserted within the *mutSL* operon and renders the MMR system non-functional, leading to an increased mutation rate, which is favourable during this growth phase. When returning to exponential growth, the phage-RS excises (not shown) and replicates once again as an episome, restoring the function of the MMR system. C, C terminus; N, N terminus.

these conditions, phage excision results in an intact *comK* gene that produces a functional ComK protein, which in turn activates the expression of the competence system to allow efficient phagosomal escape through an unknown mechanism (FIG. 3a). Notably, throughout this process the lytic pathway is blocked, preventing bacterial lysis, and the phage genome eventually re-integrates into the *comK* gene during bacterial growth in the cytosol of the host cell.

This unique bacterium–phage interaction exemplifies a reversible mode of active lysogeny and demonstrates an intriguing adaptation of the prophage to the intracellular lifestyle of its host. Indeed, switching to lytic production during mammalian infection would be detrimental for both the bacterium and the phage, as phages cannot reproduce in mammalian cells and are unlikely to find a new bacterial host in the inner tissues where *L. monocytogenes* propagates because these sites are normally sterile. That *L. monocytogenes* and the A118-like prophage both require repression of lysis for survival in the intracellular environment is an unusual example of shared interest between a bacterium and a phage that probably underlies the evolution of this symbiotic interaction and its specificity to the mammalian niche. The stable integration of this phage within the *L. monocytogenes* genome supports the idea that the A118-like prophage provides a fitness advantage for the bacterium, and it is possible that this advantage applies even outside the mammalian niche. It is also possible that the phage may have acquired a resistance to bacterial defence mechanisms, such as the restriction-modification or CRISPR systems (both encoded in the *L. monocytogenes* genome), which has allowed its persistence.

Regulation of mutator genes. Many bacteria exhibit increases in mutation rates, especially during times of nutrient deprivation or environmental stress[56]. For example, under such conditions the genes encoding components of the DNA mismatch repair system (MMR), which is responsible for the detection and removal of randomly occurring mutations, often acquire loss-of-function mutations, ultimately causing a hypermutator phenotype. Such a phenotype diversifies the population and increases the chance of mutations arising that could facilitate bacterial survival[57,58].

The bacterial MMR system comprises two proteins, MutS and MutL, which are encoded in a single operon[59]. Interestingly, in certain strains of the human pathogen *Streptococcus pyogenes*, a 13.5 kb non-infective prophage remnant (named SF370.4 or chromosomal island M1 (SpyCIM1))[60] paralyses the MMR system when inserted between *mutS* and *mutL* by truncating the *mutSL* operon[59,61]. Remarkably, in such strains the prophage adopts two alternative modes of replication in response to

changes in bacterial growth conditions, thus regulating the expression of *mutS* and *mutL*. During bacterial exponential growth, SpyCIM1 is excised and replicates as an episome, leaving an intact and functional MMR system, resulting in a low mutation rate. By contrast, under stress conditions, such as during the stationary growth phase, SpyCIM1 reintegrates into the *mutSL* operon, increasing the mutation rate up to 160-fold and thereby enhancing the probability of bacterial survival[59] (FIG. 3b).This conditional suppression of the MMR system exemplifies how temperate phages can be co-opted to regulate important bacterial processes as DNA regulatory switches.

Notably, although SpyCIM1 is capable of excision, replication and reintegration into its host genome, it has lost the ability to produce infectious phage particles as it lacks most of the genes needed for lytic growth[62]. So, unlike the previous example of the prophage in *L. monocytogenes* that retained infectivity (an infective phage-RS), in this bacterium–phage interaction, the prophage has been neutralized to a harmless non-infective phage-RS.

The evolutionary origin of this non-infective phage-RS from phage genomes is discussed in detail elsewhere[60,62]. Briefly, SpyCIM1 is classified as a phage-related chromosomal island (PRCI) because it contains a specific set of phage-associated genes and features that enable excision, integration and replication, as well as genes responsible for lysogeny regulation (for example, *cI* repressor and *cro* regulator)[62]. Indeed, it has now been shown that PRCIs are a class of bacterial mobile genetic element that specifically evolved from prophages[60]. Other examples of non-infective prophages integrated within the *mutSL* operon have been identified in related *Streptococcus* species, with genomes ranging from 13–20 kb. As with SpyCIM1, these examples all have integrase and replication genes but no identifiable genes encoding capsid proteins[63]. Moreover, a bioinformatics search of *Streptococcus* spp.genome sequences using the phage integrase gene sequence as a query revealed additional PRCIs integrated within other functional genes. These include: *rpsD*, which encodes the 30S ribosomal protein S4; *manA*, which encodes α-1,2-mannosidase; and *metE*, which encodes methionine synthase[63]. The influence of these non-infective prophages on their bacterial hosts has not yet been investigated but it is conceivable that some may affect cell physiology and behaviour in a similar manner to the SpyCIM1 phage-RS.

Non-reversible active lysogeny

Whereas the examples described above are defined by the reversible excision and reintegration of the prophage from and into the target gene, there are other scenarios in which the phage is not reintegrated. In those cases, the prophage serves as a controlled single mode switch, like those that regulate developmental processes.

Regulation of mother cell genes during sporulation. In *B. subtilis*, the *skin* (*sigK*-intervening DNA element) phage-RS, which is a 48 kb remnant of an ancestral phage[64], is inserted within the open reading frame of *sigK*, separating it into two parts[65–67]. This phage-RS encodes a range of proteins, including arsenate- and arsenite-resistance genes[68], a quorum-sensing system, a peptidoglycan hydrolase, an essential Cro–like regulator[64], the putative immunity repressor SknR, a terminase gene, a cell wall lytic autolysin enzyme[67] and a toxin–antitoxin system[69] that is thought to be maintained in the host genome by an addiction mechanism[70,71]. Notably, the *skin* phage-RS offers a mechanism to activate mother cell genes during sporulation.

Specifically, although the *skin* phage-RS cannot produce infective viral particles, and thus does not function as an active phage, it has retained its ability to excise itself from the bacterial genome in a highly controlled manner[67]. Interestingly, during *B. subtilis* sporulation, the mother cell undergoes a specific recombination event between 5 bp repeats flanking the *skin* phage-RS. This results in excision of *skin* and rejoining of the two parts of *sigK*, leading to an intact and functional gene that can express the σ^K transcription factor. In turn, σ^K regulates many genes that are required in the final stages of mother cell differentiation, such as those responsible for spore polysaccharide biosynthesis, mother cell metabolism, germination and mother cell lysis[72]. The *skin* excision event relies on a recombinase encoded by the *skin* element itself, termed CisA (also known as SpoIVCA)[73]; however, some reports have shown that the bacterial RecA protein can also fulfil this function in the absence of CisA[74]. The excised *skin* element is eventually lost in the mother cell, which dies late during sporulation, whereas the forespore, in which *skin* is not excised, gives rise to an endospore that contains *skin* within its *sigK* gene[73]. Thus, through the use of a non-infective phage-RS, the bacterium has gained a mechanism that specifically activates mother cell genes in the course of sporulation (FIG. 4a).

skin-like elements are also integrated within *sigK* genes in other Gram-positive sporulating bacteria, including several *Clostridium* species, although it is not yet known whether these function as phage-RS elements[75]. More recently, examples of phage-RS insertions into mother cell-specific genes of spore-forming bacteria have been identified in *B. subtilis, Bacillus amyloliquefaciens, Bacillus weihenstephanensis* KBAB4 and *Geobacillus thermoglucosidasius*[76,77]. In *B. weihenstephanensis,* a 42 kb non-infective phage-RS, which corresponds to the *vfbin* locus, is inserted in the gene for dipicolinic acid synthase β-subunit (*spoVFB*). As with *sigK* and *skin* in *B. subtilis,* expression of *spoVFB* required a precise excision of the *vfbin* phage-RS in the mother cell, facilitating spore dormancy[76]. In the case of *B. subtilis* and *B. amyloliquefaciens,* a temperate infective phage, SPβ, is integrated within yet another sporulation-related gene, *spsM*, which is associated with polysaccharide synthesis[77]. Excision of SPβ during sporulation results in transcription of the intact and functional *spsM* specifically in mother cells, thereby promoting the addition of polysaccharides to the spore envelope. The prophage excision depends on two phage-encoded proteins, SprA recombinase and SprB accessory protein, but does not lead to lytic production during the sporulation process. Interestingly, although SPβ is a non-infective phage-RS in *B. amyloliquefaciens*, it is fully functional in *B. subtilis* and stands out as the only lytic phage among sporulation-related phage-RS elements known so far[77].

Regulation of nitrogen fixation genes. When the cyanobacteria *Anabaena* spp. and *Nostoc* spp. are exposed to nitrogen-limiting conditions, approximately one out of 10–20 cells differentiates into a nitrogen-fixing cell called a heterocyst. These heterocysts are separated from one another by vegetative cells, which use the nitrogen fixed by heterocysts to carry out photosynthesis[78].

Three different genomic rearrangements are thought to be required for heterocyst differentiation in these cyanobacteria. These DNA rearrangements result in the expression of three genes involved in the nitrogen fixation process, which encode an α-subunit of nitrogenase (*nifD*), a heterocyst-specific ferredoxin (*fdxN*) and the large subunit of an uptake hydrogenase (*hupL*)[79]. All three genes are interrupted by non-infective phage-RS elements (*nifD* 11 kb long, *fdxN* 59.4 kb long and *hupL* 10.5 kb long; named after the genes they interrupt) that render them non-functional.

In the late stages of heterocyst differentiation, the phage-RS elements are precisely excised from their respective genes by a recombinase encoded by each phage-RS element. These recombinases perform site-specific recombination between two direct repeat sequences flanking each element. Both DNA and protein sequence analysis suggest that these phage-RS elements are remnants of temperate phages[80]. This is supported by the observation that the self-encoded recombinase is located near the 5′ end of each element and belongs to the tyrosine family of recombinases, which resemble site-specific phage integrases. In addition, each phage-RS element is inserted in the same location within its cognate gene for all examined strains, suggesting a shared ancestral phage-specific integration event[80]. In all three cases, the excision process results in intact and functional genes that are expressed solely in the heterocyst cell[81,82] (FIG. 4b).

This example represents yet another case of a non-reversible regulatory switch that does not undergo phage-RS reintegration. For these phage-RS elements, reintegration is not required because the heterocyst cells are fully differentiated and do not replicate.

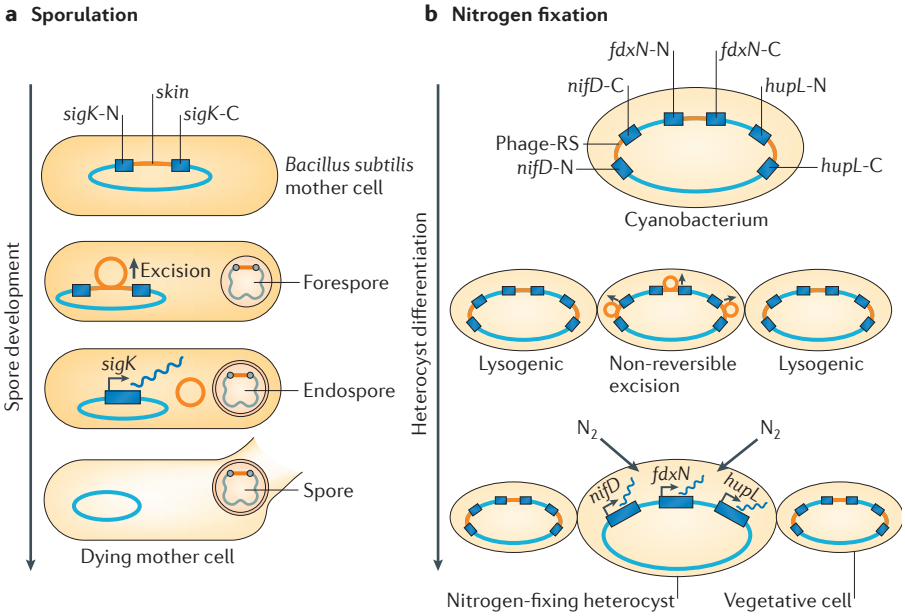


Figure 4 | **Non-reversible active lysogeny regulates developmental processes in bacteria.** **a** | Regulation of mother cell-specific genes during sporulation in *Bacillus subtilis*. A phage regulatory switch (phage-RS), named *skin*, is inserted within the *sigK* gene, which encodes σ^K, which regulates the expression of late-stage sporulation genes in the mother cell. During sporulation, *skin* excises itself, leaving an intact *sigK* gene that produces a functional σ^K protein, which in turn activates the mother cell's late-stage sporulation genes. Following excision, the excised *skin* element is eventually lost in the mother cell, which dies late during sporulation. By contrast, the forespore, which did not undergo element excision, gives rise to an endospore that still encodes the *skin* element within its *sigK* gene. **b** | Regulation of heterocyst differentiation in the cyanobacteria *Anabaena* spp. and *Nostoc* spp. Under nitrogen-limiting conditions, a subset of cyanobacterial cells differentiate into nitrogen-fixing cells, named heterocysts. Three different DNA rearrangements have been described that lead to the expression of three genes involved in the nitrogen fixation process, *nifD*, *fdxN* and *hupL*. All three genes are interrupted by non-infective phage-RS elements that render them non-functional. During the late stages of heterocyst differentiation, each of the three phage-RS elements is precisely excised from its cognate gene by the action of a specific recombinase encoded by the phage-RS. Although the differentiated cells eventually die, the neighbouring vegetative cells, which did not undergo DNA rearrangements, still contain the phage-RS elements and further propagate. C, C terminus; N, N terminus.

Concluding remarks

Functional and evolutionary considerations suggest that temperate phages in most cases will not persist in the genome of the host bacterium when integrated into functionally important genes[83]. However, the examples presented here show that such a phenomenon is more common than expected and represents a unique bacterium–phage interaction. Moreover, phages that do persist when integrated into functionally important genes form part of a newly described phage-mediated regulatory mechanism, the phage-RS. Thus, in contrast to the lytic life cycle, lysogeny provides a platform for the co-evolution of bacteria and phages that is different from the classic antagonistic co-evolution of two adversaries. In this regard, lysogeny could be considered as a mechanism that expands the repertoire of bacterium–phage interactions, especially those that are mutually beneficial and support co-reproduction.

When considering fitness of the phage, lysogeny is commonly regarded as a beneficial state, as it promotes propagation of the prophage together with its host chromosome as a mechanism to survive hostile environments[84]. From the perspective of the bacterium, the question of fitness benefit is more complex, although as early as 1961 Campbell proposed that lysogeny must confer a selective advantage to bacteria (because otherwise the prophage would not be tolerated) and that "One therefore must look for possible means by which the phage might impart a selective advantage to its host" (REF. 85). Indeed, cases of improved fitness were later demonstrated[10,86,87], and mechanisms such as lysogenic conversion and active lysogeny further support Campbell's original premise.

Remarkably, in the case of active lysogeny, evolution of temperate phages inserted within crucial bacterial genes has yielded a new phage-mediated mechanism that regulates bacterial genes and processes, which may have further contributed to improving bacterial fitness. Whereas lysogenic conversion occurs mostly via lateral gene transfer by phages, transferring genes that improve the host's fitness[24], active lysogeny seems to be a more complex phenomenon that probably evolves through alternating bacterium–phage adaptations driven by the need to support efficient lysogenic growth. These may result in an optimized molecular switch that regulates the expression of its target gene(s). In this scenario, the initial integration of a phage into a critical gene is expected to result in a decrease in bacterial fitness, which is then gradually restored by reciprocal adaptations and counter-adaptations between the phage and the bacterium. This process can lead to a mutually beneficial outcome, as in the case of phagosomal escape by *L. monocytogenes* and its A118-like prophage, or only to the enhancement of the bacterial host's fitness, as in the cases of *S. pyogenes* SpyCIM1 and *B. subtilis skin* phage-RS elements, which only benefit by replicating with the host genome. The persistence of the prophage in its host genome suggests a fitness advantage for the

bacterium or alternatively the existence of a phage addiction mechanism, such as in the case of the toxin–antitoxin system encoded by the *skin* element.

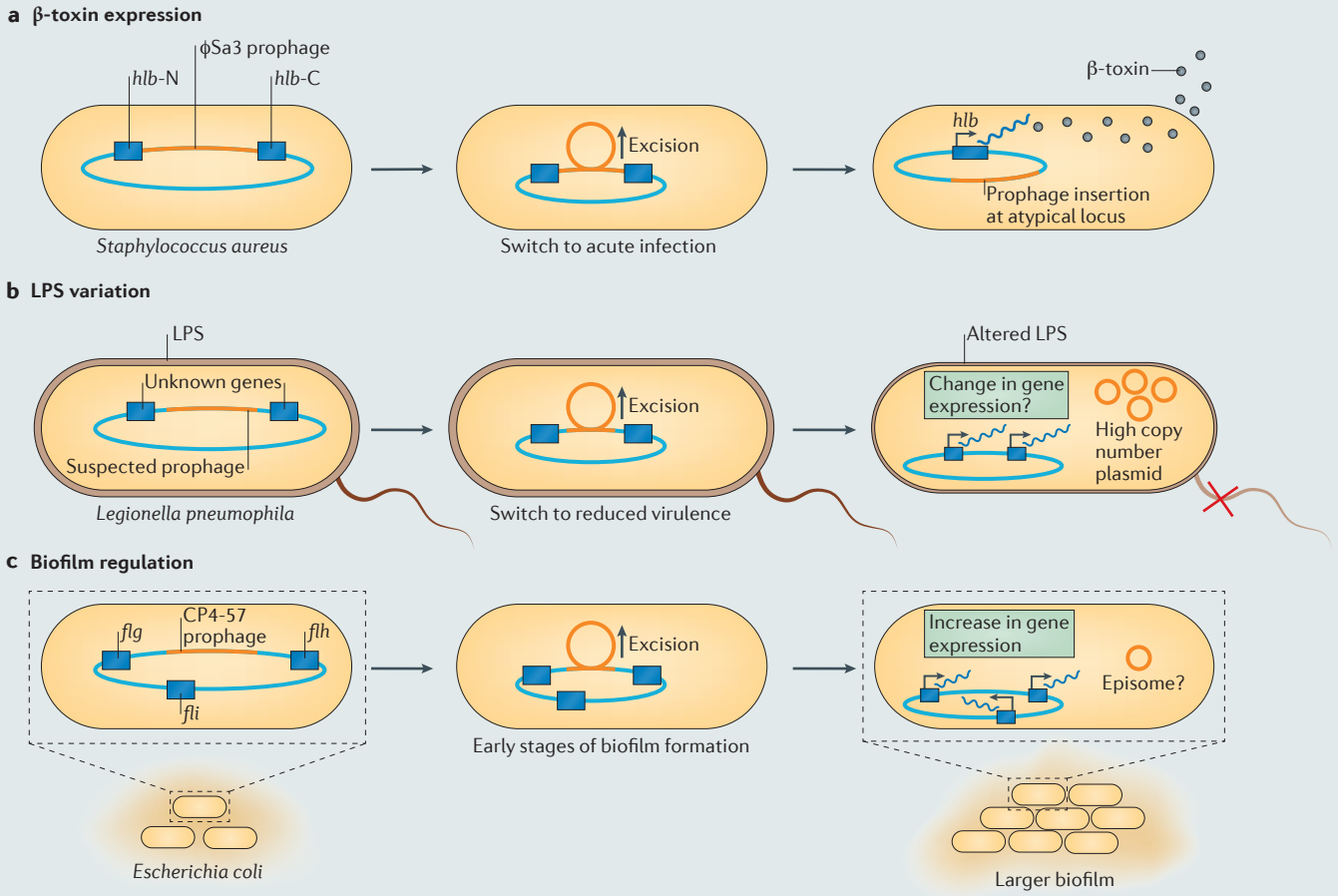
It is perhaps not surprising that further selective pressures — for example, hostile environments that induce lytic production — seem to have promoted the fixation of loss-of-function mutations and gene losses that have caused prophages to lose their lytic capabilities. Indeed, it is an open question whether all phage-RS elements will ultimately become non-lytic, and thus non-infective. Another question is how the newly adopted phage-RS interacts with the original native regulatory system of the target gene (its promoter and associated transcription factors). Do they work in parallel?

Box 1 | **Putative phage regulatory switches**

Although not completely understood, the following three examples may also represent cases in which prophage excision leads to regulation of crucial bacterial processes. The highly successful bacterial pathogen *Staphylococcus aureus* expresses β-toxin, which is a toxic haemolysin and sphingomyelinase that promotes human nasal colonization and acute infections. In most human *S. aureus* isolates, the gene encoding β-toxin, *hlb*, is disrupted by the prophage ϕSa3 (also known as *hlb*-converting phage)[88,89]. Interestingly, during *in vivo* infection (when switching from colonization to acute infection) the prophage excises itself from *hlb*, restoring the contiguity of the gene. With *hlb* now intact, the β-toxin is expressed, thus enhancing bacterial virulence. Although this phage is capable of lytic production, and thus is infectious, it seems that when *S. aureus* infects mammalian cells, some of the phages avoid entering the lytic cycle and are integrated in the bacterial chromosome in atypical loci that do not disrupt *hlb* expression[90–93] (see the figure, part **a**) or are maintained as episomes.

The second example involves the human pathogen *Legionella pneumophila*. This bacterium is known to alternate between two phenotypes exhibiting enhanced or reduced virulence, which are associated with variable synthesis of lipopolysaccharides (LPS) and the flagellum[94,95]. The switch between the two phenotypes occurs upon excision and reintegration of a 30 kb element that is a suspected phage, which is inserted within an intergenic region between two unknown genes, potentially affecting their expression. As the identity of these genes is not yet known, how a change in their expression might affect the LPS and flagellum phenotypes remains to be established. When excised, the phage is maintained as a high copy number plasmid (see the figure, part **b**).

The third example relates to a phage excision event in *Escherichia coli* that was shown to enhance biofilm formation (see the figure, part **c**), a structured type of bacterial growth that promotes resistance to many types of stresses[96]. One important factor in biofilm formation is cell motility, which is required for attachment and dispersal of bacteria during the process[97]. The *E. coli* K-12 genome encompasses a cryptic prophage (CP4-57) that lacks the genes necessary for lytic production but encodes a functional integrase[98]. Although the prophage is not integrated directly into a bacterial open reading frame[98], excision of CP4-57 has been shown to increase the expression of the motility operons *flg*, *flh* and *fli* during early stages of biofilm formation, an increase that leads to the establishment of a larger biofilm community[99]. In each of these cases, the mechanism by which the phage regulates the relevant processes is not clear, and thus it has not been confirmed that a phage-RS switch is responsible, but the possibility that phage excision alters the expression of bacterial genes to affect the change in phenotype warrants further study.



Is there any crosstalk between them? Does the phage-RS take the lead? Although these questions are yet to be answered, it is clear that the phage-RS has to respond to the same conditions and signals that originally triggered the native regulatory network. One possible mechanism would be to regulate the phage-RS with the same factors that control the expression of the target gene. Future studies identifying the environmental cues and the signalling cascades that trigger a controlled phage DNA excision to activate gene expression will reveal exciting insights into active lysogeny.

As detailed here, temperate phages take part in some of the most crucial decisions in bacterial life, such as whether to express virulence genes, to sporulate or to differentiate. Nevertheless, for most lysogenized bacteria, and particularly pathogens, we do not know whether (or how) the presence of a prophage affects bacterial behaviour, particularly the ability to infect mammalian cells. As most pathogenic bacteria contain prophages, sometimes even more than one[61], we anticipate that some of these genetic elements play key parts in the interactions between humans and bacteria. Indeed, *S. aureus*, *Legionella pneumophila* and *E. coli* are all associated with prophages that are putative phage-RS elements (BOX 1). We foresee that future research on bacterium–prophage interactions will result in exciting discoveries and surprises.

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Competing interests statement
The authors declare no competing interests.