Molecular Basis of Lysis–Lysogeny Decisions in Gram-Positive Phages

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Abstract

Temperate bacteriophages (phages) are viruses of bacteria. Upon infection of a susceptible host, a temperate phage can establish either a lytic cycle that kills the host or a lysogenic cycle as a stable prophage. The life cycle pursued by an infecting temperate phage can have a significant impact not only on the individual host bacterium at a cellular level but also on bacterial communities and evolution in the ecosystem. Thus, understanding the decision processes of temperate phages is crucial. This review delves into the molecular mechanisms behind lysis–lysogeny decision-making in gram-positive phages. We discuss a variety of molecular mechanisms and the genetic organization of these well-understood systems. By elucidating the strategies used by phages to make lysis–lysogeny decisions, we can improve our understanding of phage–host interactions, which is crucial for a variety of studies including bacterial evolution, community and ecosystem diversification, and phage therapeutics.

INTRODUCTION

Bacteriophages, or phages, are viruses that infect and replicate within bacteria. These bacterial predators can be found in every explored ecological system, ranging from the human intestine to the ocean (45). Phages are highly diverse and differ widely in genomic content, morphology of the virion particle, and lifestyle. Like all viruses, phages are obligatory parasites that hijack their hosts' machinery to ensure their own multiplication and survival. To do so, phages follow a lytic cycle in which the phage genome replicates, taking control of the host machinery and then manufacturing all the structural components required to reproduce. Following programmed cell lysis, these new virions are released into the environment, continuing their life cycle upon infection of a new host. Importantly, some phages, referred to as temperate, can establish an alternative relationship with their host to survive in nature. Unlike virulent phages, which undergo a strictly lytic life cycle, temperate phages can follow two different cycles after infection of sensitive bacteria: the lytic cycle or the lysogenic cycle. During the lysogenic cycle, temperate phages exist in a dormant state, replicating passively with the host. This quiescent replication state usually occurs when the phage genome integrates into the host chromosome (prophage state), although some prophages may exist extrachromosomally, in a plasmid-like state. Reactivation of the lysogen (i.e., the bacterium carrying a prophage) to the lytic cycle usually occurs following scenarios that trigger the host SOS response (Figure 1).

Figure 1 General model of phage lytic and lysogenic life cycles. Following binding to specific host cell receptors, temperate phages inject their DNA (*green*) into the bacterial cell. Once injected, the phage genome can undergo either a lytic or a lysogenic cycle. During the lytic cycle, the phage genome replicates using host cell machinery, synthesizing phage proteins to produce mature virus progeny. Finally, lysis of the bacterial cell releases new virions that then infect other bacterial cells. Conversely, during lysogeny, the phage genome is integrated into the host chromosome (prophage) and replicates passively during host cell division. Prophage induction can occur following certain environmental stresses, where the phage genome is excised from the host chromosome and enters into the lytic cycle.

The SOS response is a conserved pathway in bacteria, composed of more than 40 genes ([66](#)), that is activated under certain stress conditions that compromise the viability of the cell. It is involved in restarting stalled and collapsed replication forks, regulation of cell division, and DNA

repair ([22](#), [87](#)). The inducible pathway is regulated by two proteins, LexA and RecA. LexA is an autoregulatory repressor protein composed of two domains, an N-terminal DNA-binding domain (DBD) and a C-terminal catalytic domain (CTD), separated by a short flexible linker ([62](#)). During normal cellular growth, and in the absence of cellular damage, LexA dimers bind to two palindromic repeat sequences, the SOS boxes, in the promoter region of genes involved in the SOS response. Binding of LexA interferes with RNA polymerase association, thereby repressing transcription ([13](#), [57](#)). Note that, depending on the affinity of LexA for particular SOS boxes, some SOS genes are expressed to a degree even in the repressed state ([66](#)). Full activation of the SOS genes occurs following DNA damage and the activation of RecA, a recombinase that acts as a coprotease for the repressor LexA. RecA detects cellular damage because it binds to single-stranded DNA, creating a nucleoprotein filament that activates RecA. Then, this activated form stimulates the self-cleavage of LexA at a specific alanine–glycine bond, alleviating LexA repression and promoting the expression of the genes contained in the SOS regulon ([58](#)). Proteolysed (cleaved) LexA can no longer bind efficiently to the SOS boxes, leading to reduced levels of LexA in the cell and derepression of SOS genes ([59](#), [66](#)). Overall, this response causes the expression of genes required to repair bacterial DNA damage until normal cellular replication can begin again.

Since resident prophages are dependent on the survival of their host, these sophisticated viruses have adapted to be in tune with, and responsive to, host cell signals such as the SOS response. Following detection of the hosts' DNA damage signaling pathway, and when the damage is irreversible, most prophages rapidly switch to a lytic cycle. This conversion from stable latency to active replication and host lysis involves an efficient transcriptional cascade of phage regulatory components. Ultimately, this process allows temperate phages to ensure their optimal survival and escape potentially doomed host cells.

As with other predator–prey relationships found in nature, bacteria and phages are part of an ongoing arms race that has resulted in various mechanisms employed by the host and infecting phage to try to ensure the survival of their respective population (for a recent review, see [44](#)). In this sense, lysogeny can be seen as a truce, resulting in complex interactions and elaborate strategies that provide mutual benefits for the phage and the host bacterium ([4](#), [33a](#), [46](#)). Temperate phages can contribute to the pathogenesis of a host bacterium through the expression of the virulence factors they encode. This phenomenon, known as lysogenic conversion, comprises any

beneficial trait that helps increase the fitness of the host bacterium (14, 26). The integrated prophages can also contribute to the survival of the host by additional mechanisms, including but not limited to (a) expression of superinfection exclusion proteins that protect the host from attack by other phages (52, 83, 93), (b) phages that can also regulate host behavior by controlling bacterial gene expression (33a), (c) phages that can serve as a point of bacterial genomic rearrangement (14), and (d) phages that promote gene transfer between bacteria through different transduction mechanisms (20, 21) that, in turn, can be beneficial for the temperate transducing phage (34).

Therefore, when temperate phages infect a cell, phages will “decide” to follow either the lytic or the lysogenic state, a choice that often occurs in response to the signal they receive from their environment. In this review, we focus on the molecular mechanisms behind gram-positive phage lysis–lysogeny decisions and provide an overview of the current literature. Studies on lysis–lysogeny decisions in gram-positive phages have focused on phages that infect economically relevant bacteria, such as the lactic acid bacteria (LAB) used in the dairy industry (*Lactobacillus*, *Lactococcus lactis*, or *Streptococcus thermophilus*) or on phages present in clinically relevant bacteria (*Staphylococcus aureus* or *Streptococcus pneumoniae*), investigating their role in pathogenesis and evolution. Since most of the current knowledge of the mechanisms involved in lysis–lysogeny decision-making in phages has been derived from studies using a handful of *Escherichia coli* phages, we first introduce some basic molecular concepts based on these studies, and then extrapolate or compare these findings with the mechanisms used by phages infecting gram-positive bacteria.

THE λ GENETIC SWITCH AND λ -LIKE LYSOGENY MODULES IN GRAM-POSITIVE PHAGES

The *E. coli* phage λ is the paradigm for the study of regulatory networks in temperate phages. Moreover, phage λ has become a model system for studying cell-fate developmental decision-making through the genetic switch concept (1, 39, 85, 88). The regulatory switch controlling λ lytic or lysogenic development is remarkably intricate, with several layers of control involving different phage-encoded regulators and antiterminator proteins (for detailed reviews, see 18 and 71). However, the essence of the system relies on the balance between the two main regulators, CI and Cro, which act as a bistable switch and are responsible for controlling transcription of most of the phage genes (Figure 2a). Whereas the regulation and maintenance of lysogeny are mainly

under the control of the master repressor CI, Cro indirectly brings about the lytic course by lowering levels of CII, the regulator responsible for activation of *cI* ([71](#), [81](#)). During a stable lysogenic state, the repressor CI binds cooperatively to the *oL* and *oR* operators present in the so-called switch region of the phage genome. Both operators have three binding sites for CI: *oL1*, *oL2*, and *oL3* for *pL* and *oR1*, *oR2*, and *oR3* for *pR*. CI monomers homodimerize, and the helix–turn–helix motif (HTH) in the N-terminal DBD binds to the operator regions. This binding is cooperative and is enhanced when dimers of CI bind to *oL1/oL2* and to *oR1/oR2*, blocking transcription from the *pL* and *pR* early promoters but still allowing transcription from the *pRM* promoter controlling expression of CI. With high concentrations of CI, binding to *oL3* and *oR3* operators promotes formation of CI tetramers that then interact, leading to the formation of a CI octamer and a DNA loop that blocks expression from the *pL*, *pR*, and *pRM* promoters ([71](#), [80](#)). A loss of transcription from the early promoters results in a lack of early gene products, including expression of *cro* and, thus, a lack of late lytic gene products as well. Although CI turns off expression of most of the phage genes, it activates its own transcription, thereby blocking the lytic cycle and allowing vertical transmission of the prophage within the replicating host chromosome. When the lytic cycle starts, Cro dimers binds to the *oL* and *oR* operators, acting as a weak repressor of the *pL* and *pR* promoters and also binding to the *oR3* operator, thereby transcription from *pRM* and blocking expression of *cI* ([92](#)). Another important element in the activation of the lytic cycle is the N protein, an antiterminator that associates with the RNA polymerase subunit and forms a complex with the host Nus proteins, bypassing critical terminator sequences to allow transcription of the late early transcripts ([67](#)). The return to the lysogenic cycle involves the activity of CII, a tetramer with an HTH DNA-binding motif that binds to the promoters *pI*, *pRE*, and *paQ*, resulting in enhanced expression of Integrase and the repressor CI, along with repression of the Q antiterminator protein that activates expression of the morphogenetic genes ([64](#)).

Figure 2 The lysis–lysogeny modules of (a) phage λ and (b) phi 11. (a) The lysis–lysogeny decision in phage λ is mainly under the control of the regulators CI and Cro. During lysogeny, CI represses the *oL* and *oR* operators by binding to *oR1/oR2* and *oL1/oL2*. Cooperative CI binding induces a DNA loop, which enhances the stability of the lysogenic state by blocking expression of phage early genes, including *cro*. The switch to the lytic cycle is achieved by RecA-induced CI autocleavage following activation of the host SOS response. Reduced levels of CI leads to expression of the early lytic genes, including *cro*. Cro binds preferentially to the *oR3* operator, blocking CI expression and allowing a switch to the lytic cycle. The thickness of the blunt end lines shows the affinity for each of the binding sites by CI and Cro. (b) In phi 11, the lysogenic state is maintained through binding of the CI repressor to the *O1* operator followed by *O2* and *P2*, thus repressing the expression of *cro* and *gp07*. Lysogeny is further maintained by binding of the host LexA to the *gp07* operator (*L1*). Following activation of the SOS response and activation of RecA, the autoproteolytic activity of LexA and CI repressor is enhanced, ultimately reducing concentrations of LexA and CI. Cleaved LexA removes the repression of *P2*, while cleaved CI

removes the repression of *cro*. These events cause expression of Cro, the lytic repressor. Moreover, derepression of *L1* causes expression of Gp07, enhancing the activity of Cro. Binding of Cro to its cognate operator, *O3*, results in repression of *cI*.

In λ , the switch to the lytic cycle occurs following activation of the host SOS response and the presence of activated RecA. As mentioned above, activated RecA stimulates the autoproteolysis of LexA, the bacterial SOS response master repressor. Importantly, and through a similar process, activated RecA also induces cleavage of the CI repressor (86). Like LexA, the CI repressor contains two domains joined by a flexible hinge, an HTH N-terminal DBD and a CTD involved in the oligomerization containing an identical alanine–glycine cleavage site (72, 86). Following RecA-stimulated inactivation by autocleavage, CI is unable to bind to its operator sites, launching activation of the λ lytic cycle, which is favored by the expression of Cro. Phage repressors with LexA-like domains are widespread in nature, and numerous studies have identified phage repressors undergoing self-cleavage upon binding to active RecA, including the repressor of the archetypical enterobacterial P22 and phi80 phages (27, 31).

The λ -like system is not limited to gram-negative phages and can be found in various other SOS-inducible phages, including those infecting gram-positive bacteria. Temperate *Siphoviridae* phages that infect low-GC-content gram-positive bacteria share a common genetic organization in the lysogeny module analogous to the CI–Cro system observed in lambdoid phages (28, 61, 95). For example, bacteriophages of *S. aureus*, including phi11, phi12, phi13, L45a, phi42, and phiSa3, possess the *cI* and *cro* genes in divergent orientation in their genome, analogous to λ (Figure 2b). Interestingly phi11 encodes an additional protein, Gp07, involved in the lysis–lysogeny decision system (25a). Phage phi11 is a 43.6-kb double-stranded DNA temperate *Siphoviridae* phage that has been used as a model for studying lysogeny in phages infecting gram-positive bacteria. As with the λ CI, phi11 encodes in its immunity region a λ CI-like repressor protein consisting of an N-terminal HTH DBD characteristic of XRE family-like proteins (2) and a C-terminal domain involved in oligomerization and RecA-dependent cleavage. As in λ , this repressor recognizes direct repetitions in the intergenic region that contains two divergently encoded promoters controlling expression of the early lytic (P_{e}) and lysogenic genes (P_{L}). Unlike in λ , there are three operator sites in the intergenic region between CI and Cro (*O1*, *O2*, and *O3*) and an additional operator region situated downstream of Cro (*P2*) (9, 25a). When CI is expressed, it binds initially

to operator *O1* following cooperative binding to operators *O2* and *P2* and blocking transcription of the early lytic genes. Conversely, Cro acts as an antirepressor of the system by binding to *O3* to inhibit expression of CI and promote lysis. Interestingly, the system adds another layer of control by incorporating a LexA-binding site, the operator *L1* located upstream from *gp07*. Thus, during lysogeny LexA binds to *L1*, which helps maintain the lysogenic state by repressing expression of Gp07 (25a). Following activation of the SOS response and activation of RecA, the repressor CI and LexA undergo autoproteolysis, releasing repression of the early gene promoter (P_R). Phi11 does not encode a CII homolog, but instead encodes Gp07, an antirepressor protein with two domains: an N-terminal Bro-like domain and a C-terminal KilA domain. The role of Gp07 is to bind to Cro, enhancing its binding to operator *O3* and promoting repression of CI expression, thus switching the phage into the lytic state (24–25a).

There are other examples of phages with antirepressor proteins required to switch between lysogenic and lytic cycles. The *Lactobacillus casei* phage A2 is a 43.4-kb double-stranded DNA temperate phage with cohesive ends that belongs to the *Siphoviridae* family. The genetic switch is composed of two nonoverlapping, divergent promoters: the P_L promoter, which controls the expression of the lysogenic genes such as *CI*, and the P_R promoter, which controls the expression of the lytic genes such as *Cro*. This regulatory region contains three similar, but not identical, 20-bp inverted DNA repeats (operators O_1 , O_2 , and O_3) that are recognized by CI and Cro (40, 53). The CI repressor binds cooperatively to the *cI–cro* intergenic DNA region. At low concentrations, CI binds to operator O_1 (situated downstream of the transcription start site of the *cro* gene) and operator O_2 (which overlaps P_R), resulting in displacement of the σ RNA polymerase, thus blocking expression of the lytic genes from the P_R promoter. At higher concentrations, CI binds to operator O_3 , which overlaps with the P_L promoter, inhibiting expression of the lysogenic genes. Conversely, Cro binding is not cooperative and is maximal to O_3 , resulting in repression of P_L transcription, while with high levels of Cro expression, binding to O_1 and O_2 likely induces a DNA looping that provokes the displacement of the σ RNA polymerase from the genetic switch (53, 54). The system requires another element that modulates the switch between lysis and lysogeny: the Gp25 protein, which functions as an antirepressor that binds to the *cro* mRNA. The *gp25* gene is situated downstream of *cro* and is partially homologous to other antirepressor proteins present in phages infecting gram-positive bacteria, but it is one-third shorter and encodes only for the putative Bro motif (residues 12–99), hypothesized to be involved in DNA/RNA interactions (100). In contrast

to Gp07 in phi11, when Gp25 accumulates after activation of the lytic cycle, it binds to the newly synthesized early lytic mRNA transcript (*cro* mRNA), abolishing translation of the early lytic genes. This inhibition of *cro* mRNA translation, together with a low level of expression of CI, switches the phage into the lysogenic cycle and explains the high stability and lysogenization rates of *L. casei* A2 ([17](#)).

LYSOGENY SYSTEMS IN PHAGES INFECTING LACTIC ACID BACTERIA

During the late 1990s, there was a great effort to study the genetic switches controlling lysogeny for temperate phages that infect LAB (*L. lactis* or *S. thermophilus*), which are essential for the production of fermented foods such as cheeses and yogurts ([41](#)). Spontaneous phage induction in lysogenic LAB during fermentation can lead to production failure; thus, understanding the genetic switch for lysis–lysogeny has been important for the dairy industry. Most of the LAB phages studied since then have described a conserved genetic organization reminiscent of the one present in the λ phage, with two divergently encoded regulatory proteins, CI and Cro, flanking an intergenic region carrying the promoters that control expression of the lytic and lysogenic genes ([28](#)). These studies have shown singularities between the lactococcal genetic switches of the different LAB phages studied, especially in the variation in the number of direct repeats recognized by CI: two in lactococcal phages r1t, phiLC3, and TP901-1 ([10](#), [48](#), [50](#)); three for the *L. casei* phage A2 and *L. lactis* phage Tuc2009 ([40](#), [50](#)); and up to five binding sites for the *Lactobacillus plantarum* phage ϕ gle ([49](#)). Interestingly, the CI repressors of the LAB phages studied can be divided into two groups according to their size and structural characteristics ([50](#), [61](#), [79](#)). Phages Tuc2009, phiLC3, r1t, and BK5-T as well as lactococcal phage A2 (see the preceding section) encode repressors that are similar in size to the λ -like ones and possess the two classical distinctive domains: an N-terminal DBD involved in the recognition of the binding sites in the promoter region and a CTD involved in the oligomerization of the protein with the characteristic RecA cleavage site ([10](#), [15](#), [40](#), [50](#), [69](#)). In contrast, lactococcal phage TP901-1, *S. thermophilus* phage Sf21, and *Lactobacillus gasseri* phage ϕ adh encode repressors that are shorter and do not possess the C-terminal oligomerization and RecA-mediated autocleavage domain, although these phages are SOS inducible ([16](#), [32](#), [63](#)). Brüssow and colleagues ([61](#)) proposed the lysogenic module present in streptococcal phage Sf21 as the model of a new lysogenic organization family. The

module for this family contains genes with well-conserved functions including an integrase gene, a superinfection exclusion gene (not always present), a metalloproteinase motif-containing gene and a CI-like repressor ([61](#)). The best-characterized models are those present in phages $\phi 105$ ([11](#), [61](#); see the next section) and TP901-1.

The lactococcal phage TP901-1 bistable genetic switch comprises an immunity region with two regulatory proteins: the CI repressor (ORF4) and the MOR antirepressor (ORF5), divergently oriented flanking an intergenic region with two divergently transcribed promoters (P_L and P_R) ([63](#)). This intergenic region contains three palindromic operator sites: O_R , located between the two divergent promoters P_L and P_R ; O_L , which overlaps the transcriptional start site for the lytic promoter and P_L ; and O_b , located downstream of MOR ([48](#)). Studies have shown that these operator regions are recognized by the CI repressor, which binds cooperatively to all three operator sites in its hexameric form, generating a CI–DNA loop structure that prevents transcription by the RNA polymerase subunit ([74](#)). The role of the antirepressor MOR is to directly interact with the repressor to generate a CI:MOR complex to block the activity of the repressor, leading to the lytic cycle ([3](#), [73](#)).

THE PROTEASE-ENCODED LYSOGENY MODULE

Other temperate phages from the *Siphoviridae* family encode an alternative lysogeny module with distinctive differences from the λ -like genetic switch described above. First characterized at a molecular level by the Grossman lab in 2008 ([11](#)), this system presents a regulatory module composed of a transcriptional repressor (ImmR) and a site-specific protease (ImmA). Remarkably, this regulatory control system is also found in other mobile genetic elements, such as ICEBs1, the integrative and conjugative element (ICE) in *Bacillus subtilis*, where it has been extensively characterized ([5](#)). The regulatory module of the ICEBs1 element comprises a repressor, ImmR, which possesses an N-terminal HTH DBD that binds to the lysogenic promoter, thus repressing the system. In contrast to the λ -like repressors, ImmR does not have the LexA-like C-terminal domain with the conserved alanine–glycine motif present in LexA and other phage repressors; thus, it does not undergo direct autocleavage following activation of RecA during the SOS response ([59](#)). In contrast, the ImmR cleavage is mediated by ImmA, encoded by the *immA* gene, which localizes 3' to *immR* (**Figure 3**). ImmA is a metalloproteinase containing a catalytic

HEXXH zinc-binding motif that is required for its proteolytic activity (11). The metalloproteinase IrrE from *Deinococcus deserti*, which mediates the radiation response cleaving the repressor DdrO, is the best-characterized member of this family (29). The structure of IrrE shows that the HEXXH motif coordinates a single zinc that catalyzes the cleavage of the target repressor (98). A sequence homology analysis has indicated that ImmA can share a common folding and catalytic mechanism to cleave ImmR. This assumption is supported by the null phenotype of an ImmA mutant in the H75 of the HEXXH zinc-binding motif (11). Therefore, ImmA acts as an antirepressor, producing site-specific cleavage that causes degradation of ImmR and derepression of the system (6, 11). Interestingly, in the ICEBs1 element, activation of the system can occur by two independent mechanisms: activation by the RecA-mediated DNA damage response (SOS response) or activation by the RapI–PhrI quorum-sensing system. RapI plays a crucial role in inducing the ICEBs1 element by enhancing ImmA proteolytic activity and ImmR repressor degradation. RapI belongs to the RRNPP family of quorum-sensing regulators and possesses the characteristic C-terminal regulatory domain composed of seven tetratricopeptide (TPR) repeats. The activity of RapI is regulated by a small peptide encoded downstream, PhrI, that is secreted as a propeptide through the host secretion machinery, processed into a five-amino-acid mature peptide, and incorporated into the cell through the host oligopeptide permease transporter. PhrI peptide accumulates when the surrounding cells encode for the ICEBs1 element and the binding of PhrI to the TPR domain of RapI inhibits its activity (5). In the ICEBs1 element, the derepression mediated by activation of the RecA-dependent SOS response and RapI–PhrI cell density system may be caused by an increase in the proteolytic activity of the ImmA protease (12). This dual regulation for this mobile genetic element (MGE) allows for passive propagation in the host cell until either the potential mating partners lacking the element or the host cell is in distress (7).

Figure 3 General model of the protease-encoded lysogeny module. In $\phi 105$ and other phages carrying similar protease-encoded lysogeny modules, the phage gene *immR* encodes a repressor that binds to the intergenic region controlling expression of P_m and P_r promoters. This repression is lifted after proteolysis mediated by the phage-encoded metalloproteinase ImmA. Regulation of ImmA is proposed to be under the control of the host RecA and Rap–Phr system.

As mentioned above, this regulatory module is present in many different phages that infect gram-positive bacteria and has been partially characterized in the *B. subtilis* phage $\phi 105$ (11) (**Figure 3**). Phages harboring this lysogenic module require activation of the SOS response to enter into the lytic cycle. In this regulatory system, however, cleavage of ImmR depends on ImmA, although activation of RecA is also required to activate the SOS response. It is not yet understood how the SOS response activates ImmA function, although the ImmA C-terminal region might be important for this regulation (12). Interestingly, multiple gram-positive phages that carry ImmR–ImmA systems such as $\phi 105$, skin, PBSX and $\lambda Ba04$, also encode a quorum-sensing Rap–Phr system, similar to the one encoded in the ICEBs1 element (RapI–PhrI) and multiple others present in the *Bacillus* genome (12). The fact that other phages such as $\phi 105$ carry homologs of RapI and PhrI, and encode for ImmR and ImmA, suggests a conserved mechanism for inactivating repressor proteins and performing the switch from lysogeny to lysis depending on population cell density. However, this possibility has not yet been experimentally tested.

In ICEBs1, ImmR regulates the transcription of the *xis* promoter. *xis* encodes for a small charged protein that acts as an excisionase. Overexpression of *xis* is sufficient to induce excision of ICEBs1 but depends on the presence of the tyrosine recombinase *int*, which acts as an integrase during the excision and integration of ICEBs1 (56). These results suggest that transcriptional repression of *xis* promoter controls excision in this system. Phages like $\phi 105$ with an ImmR–ImmA system also encode an integrase in the same position as in ICEBs1. Furthermore, immediately upstream and in the opposite direction of ImmR, phage $\phi 105$ encodes a small charged protein that could act as *xis* despite a lack of homology to the ICEBs1 excisionase. Importantly, this regulatory module is not exclusive to phages or ICEs but is also present in a new family of mobile pathogenicity islands, the phage-inducible chromosomal islands (PICIs) (65, 75). Interestingly, PICIs carrying this module are not SOS induced but require induction by a second, coresident PICI, which must first be induced by a phage (A. F. Haag, et al, manuscript in revision). This induction cascade thus represents a delightful intracellular *ménage à trois* involved in the transfer

of MGEs.

THE ARBITRIUM QUORUM-SENSING SYSTEM REGULATING LYSOGENY

Recent studies have shown that phages not only tune into the viability of the bacterial host through the SOS response but also utilize bacterial quorum-sensing systems ([55](#), [89](#)) or directly encode their own quorum-sensing systems to be able to sense the bacterial population and regulate their lysis–lysogeny decisions accordingly. The arbitrium system, a phage-encoded quorum-sensing system, was first identified in the *B. subtilis* SP β group of phages using phi3T phage as a model ([33](#)). In this system, phages communicate during infection by using a short peptide, AimP, as a signal. As its name suggests, arbitrium AimP peptide concentration acts as an arbitrator, tipping the scales to either the lytic or lysogenic cycle. High AimP levels guide the phage into a lysogenic cycle, while low levels favor the lytic cycle. In the prototypical phi3T phage, the arbitrium system is composed of three genes: *aimP*, which encodes the arbitrium peptide; *aimR*, which encodes a transcriptional activator; and *aimX*, whose transcription is enhanced by AimR to produce a small noncoding RNA that exerts a negative regulatory effect on lysogeny, thus inducing lysis by a mechanism that is poorly understood ([33](#), [90](#)).

As in Rap–Phr quorum-sensing systems ([76](#), [78](#)), the active AimP is a short peptide [six amino acids long in the case of phi3T ([33](#))] but is initially synthesized as a longer (43-amino-acid) inactive propeptide that is secreted into the growth medium, where it is proteolytically cleaved to generate the mature AimP. The peptide accumulates in the extracellular medium and is imported into the cell through the bacterial oligopeptide permease transporter ([77](#)). Once inside the bacterial cytoplasm, AimP binds to the arbitrium peptide receptor AimR, which controls its DNA regulatory activity ([33](#)). AimR is a transcriptional activator that promotes *aimX* expression. At the initial stages of infection, when the number of active phages is low, the AimP peptide is absent and AimR binds to its DNA operator region, thus activating *aimX* expression and keeping the lytic cycle active. During phage replication, AimP accumulates in the medium and is internalized by nearby bacteria, increasing the intracellular concentration of mature AimP peptide. After reaching the threshold level, AimP binds to its cognate AimR receptor, inducing conformational changes in AimR that prevent its binding to DNA and, consequently, inhibits *aimX* expression (**Figure 4**). For some phages, such as phage Wbeta, which infects *Bacillus cereus*, and Waukesha92-like, which infects *Bacillus thuringiensis* serovar Kurstaki HD-1, a gene encoding a putative CI-like

repressor has been identified. This gene is located in the opposite orientation as *aimX*, suggesting that the AimX noncoding RNA could control lysogeny through direct RNA antisense interactions (33, 90). This simple, direct, and elegant communication system allows phages to decide between lytic and lysogenic life cycles according to the progeny population, preventing the killing of the entire bacterial host population by the phage.

Figure 4 Model for the arbitrium-mediated lysis-lysogeny decision. During initial stages of phage infection within a bacterial population, when the concentration of the mature arbitrium peptide is low, the regulator AimR activates transcription of *aimX*. AimX functions to silence expression of the phage *cI* repressor encoded downstream. Following successive rounds of phage infection and increased production of the mature arbitrium peptide, the concentration of AimP reaches a threshold that allows AimR to bind the arbitrium peptide, thus preventing the expression of *aimX*. The absence of AimX allows expression of the CI phage repressor, leading to suppression of lytic genes and promotion of lysogeny.

Crystal structures of the AimR receptors from phi3T and SPbeta phages, two representatives of the SPβ phage family, have recently been solved in their apo and AimP peptide-bound forms by different groups (30, 38, 99), allowing the proposition of, paradoxically, two alternative molecular mechanisms of peptide inhibition. These crystal structures revealed that the AimRs are members of the RRNPP family of quorum-sensing regulators (38), which are widespread in gram-positive bacteria. As with other members of this family, each AimR monomer has an N-terminal effector and a C-terminal regulatory domain. The short N-terminal domain adopts a characteristic HTH DNA-binding fold, while the C-terminal domain is composed of nine highly degenerated TPR repeats. The HTH domain accounts for the AimR DNA operator recognition, and binding activity was recently confirmed by the crystal structure of the SPbeta AimR–DNA complex (38).

The AimR operator in SPbeta phage is located downstream of *aimP* and presents two perfect inverted repeats, separated by an unusually long (25-bp) spacer. A DNA-binding site with similar organization was also identified in phi3T phage (38), suggesting that this operator architecture is common in the AimR family. The structure of the SPbeta AimR–DNA complex showed that AimR dimers recognize this unusual operator by weak direct DNA readout that involves only three AimR residues interacting exclusively with three of the six inverted repeat basis and by indirect readout of the long spacer with several contacts between the DNA backbone and polar residues of AimR. This fact, together with the high AT content of the spacer that allows DNA bending, suggests that AimR could recognize sequences with shorter or longer spacer and/or degenerated recognition sequences. Evidence of AimR promiscuity has been confirmed in vitro for SPbeta phage, showing that its AimR recognizes alternative phage operators with 23–30-bp spacer sequences and degeneracy in the palindromic sequences (38). These alternative operators were identified not only in the phage genome but also in the genome of the *B. subtilis* host, leading the researchers[**AU: Ref. 38?**) to propose that AimR controls multiple phage and/or bacterial genes in addition to *aimX* and, consequently, regulates processes beyond the lysis–lysogeny decision.

The C-terminal regulatory TPR domain of AimR adopts a superhelical fold with a concave inner groove where the regulatory peptide is accommodated, as observed in the crystal structures of phi3T and SPbeta AimRs in complex with their respective AimPs (30, 38, 99). This TPR domain also mediates AimR dimerization, which is required for recognition of the palindromic operator. The AimP hexapeptides encoded by phages from the SPβ family present a highly conserved C-terminal RGA signature, while the first three amino acids are more variable and have been proposed to account for the AimR–AimP specificity. AimR from SPbeta phage showed a high plasticity in its apo state, but AimP binding induces a constricted conformation of the TPR. In the case of phi3T, changes in the TPR domain modify the dimer interface and induce dimer dissociation, hindering AimR from recognizing its palindromic operator. However, changes induced in the dimer interface of the AimR of SPbeta phage by its cognate AimP are not as drastic as in phi3T, conserving the protein in its dimeric state. However, the high conformational plasticity observed in its apo form is blocked by the AimP peptide, inducing an AimR-locked conformation wherein the HTH domains approach one another, preventing the regulator from reaching the distant palindromes of its DNA operator. Remarkably, in the locked conformation induced by the peptide, the AimR recognition helices remain exposed in a competent DNA-binding conformation,

opening the possibility that the inhibited AimR–AimP complex could recognize alternative operators with shorter spacers (38). In this way, although the peptide shows a similar molecular mechanism of action for blocking the arbitrium operator recognition in both AimRs, the results are different (monomerization versus dimer locked). These alternative regulatory responses await further analysis.

Interestingly, recent homology searches in the bacterial, archaeal, and phage genomes have revealed the existence of more than 1,000 arbitrium-like systems, ~95% of which show the characteristic *aimR–aimP* organization (33, 90). A phylogenetic analysis clustered these arbitrium-like systems into 10 different clades, corresponding to the original arbitrium system found in SPβ phages in clade 2. Furthermore, these arbitrium-like systems are not restricted to phages but are also present in other mobile genetic elements (~16% of the systems), such as conjugative plasmids, or reside in the bacterial host genome (~15%). It is intriguing that ~30% of these systems, whose function is to control the lysis–lysogeny balance, belong to elements that do not perform these life cycles and do not have to make this decision. This observation suggests that arbitrium systems have additional functions and/or that the regulation of lysis–lysogeny by AimR goes beyond the control of *aimX* expression (90).

PSEUDOLYSOGENY IN BACTERIOPHAGES INFECTING GRAM-POSITIVE BACTERIA

The phenomenon of pseudolysogeny has been described as an intermediate state in the phage life cycle, where the phage is neither integrated into the bacterial chromosome (lysogenized) nor actively replicating and entering the lytic cycle. This phenomenon is greatly influenced by the growth conditions of the host cell and represents an adaptation of the phage for survival in adverse conditions, especially in natural environments (60). Although pseudolysogeny has been known since the early 1960s, the difficulty of studying this phenomenon under appropriate conditions of suboptimal bacterial growth or starvation means that it is still poorly understood. The classical examples of phages able to enter this pseudolysogenic state are mainly in gram-negative bacteria; for example, phage T4, a typical virulent phage, can enter pseudolysogeny after infecting cells growing in starving conditions (51). Nonetheless, examples of pseudolysogeny in phages infecting gram-positive bacteria can be found. For instance, the *Clostridium botulinum* phage c-st (84) is a large temperate phage of 185 kb that carries a botulinum neurotoxin (BoNTX/C1) and exoenzyme

C3. First described as a temperate toxin–encoding lysogenic converting phage, it can exist as a circular plasmid prophage, which accounts for its unstable lysogenic state. Another example can be found in *S. aureus*, where temperate phages ϕ s80b and ϕ s84b can integrate into the genome of some hosts but remain in an extrachromosomal state in others (42). The importance of pseudolysogeny may have been underestimated with regard to its role in pathogenicity, as revealed by a 2014 study by Utter et al. (94). These authors performed a massive sequencing analysis of selectively enriched extrachromosomal DNA samples from natural *S. aureus* strains, which showed a widespread distribution of multiple extrachromosomal prophages carrying virulence factors (94). One of these circular extrachromosomal prophages was completely sequenced and annotated as phage phiBU01, a 43.8-kb prophage carrying the immune evasion cluster isolated from a vancomycin-intermediate *S. aureus* strain. This extrachromosomal prophage is inducible by the SOS response, and its genomic content is similar to other classical *S. aureus* temperate phages. In particular, high homology is observed between phiBU01 and ϕ NM3, apart from the immunity cluster region where less homology is observed (94). The precise molecular mechanisms behind this phenomenon must be further explored, which may bring about an understanding of its impact on the biology of both phage and host.

Some possible advantages of pseudolysogeny have been proposed. As mentioned above, pseudolysogeny can be regarded as an adaptative condition to increase phage survival rates when encountered by unfavorable growth conditions for the host and can have a major impact on host–phage interactions in natural environments (68), but it can also be beneficial for the host, as it will increase the gene dosage of the beneficial traits for the host encoded by the phage or act as genomic switches (94). Nonetheless, there are potential disadvantages for the phage when it enters pseudolysogeny; phages can be harder to maintain as this pseudolysogenic state has an associated metabolic cost for the host. Also, phages entering into pseudolysogeny will lose the potential benefits of promoting gene transfer, such as engaging in lateral transduction. These downsides cannot overshadow the advantages of lysogeny, though they might explain why pseudolysogeny is employed only in limited scenarios and has not been adopted as a conventional lifestyle for most of the phages studied.

LYSIS–LYSOGENY DECISION IN EXTRACHROMOSOMAL TEMPERATE TECTIVIRUSES

Following infection of a susceptible bacterium, some phages reside within the host as autonomous linear plasmids without integrating into the chromosome. Temperate tectiviruses that infect the *B. cereus sensu lato* group have been described as undergoing this alternative lysogenic lifestyle. Furthermore, these phages have developed a sophisticated approach to the lysis–lysogeny decision by directly incorporating the host LexA into the mechanism as the repressor of their genetic switch. Phage GIL01 represents a nice example of these phages. This phage was isolated from *B. thuringiensis* serovar Israelensis, an insect pathogen used in agriculture and mosquito control (97). The GIL01 genome consists of two major functional regions, and transcription is controlled by three promoter regions, P1, P2, and P3 (35). The P1 and P2 promoters control expression of the first domain, containing phage early genes involved in DNA replication and transcription regulation. The second domain contains the structural and lysis genes, which are under the control of the P3 promoter. Intriguingly, these genomic domains are not regulated by a phage-encoded repressor but are directly regulated by the host LexA (35, 37). The canonical LexA-binding site, dinBox1, flanks promoter P1, resulting in repression of genes involved in phage replication. LexA binds to dinBox1 and also an adjacent low-affinity binding site, dinBox1b, in complex with a small phage-encoded accessory protein, gp7 (37). The tight association of gp7 to LexA not only improves LexA binding but also protects LexA from RecA-induced autocleavage (19, 37). A closer examination of the GIL01 lysis–lysogeny switch revealed that the LexA–gp7 complex additionally represses expression from P3, with the aid of another phage-borne factor, gp6 (36). GIL01 gp6 possesses significant homology to the DNA-binding region of LexA and has been observed to bind to P3 at the same time as, but not in complex with, LexA–gp7 (36). Therefore, the promoter that controls expression of phage capsid and lysis genes is controlled by two LexA family members with differing functions, allowing timing-specific expression of these late genes. By expressing gp6 and gp7 and by directly interfering with the host SOS DNA repair pathway via LexA, GIL01 can refrain from premature reactivation and remain a stable lysogen until persistent DNA damage is detected (36).

Figure 5 Temperate tectiviruses utilize host LexA for lysis–lysogeny decisions. During lysogeny in phage GIL01, host LexA forms a complex with the phage-encoded Gp7, repressing the expression of phage genes directed from the P1, P2, and P3 promoters. The switch to the lytic cycle occurs following persistent DNA damage, whereby LexA undergoes autoproteolysis. When the concentration of LexA falls below a threshold level, derepression of P1 and P2 occurs, resulting in high-level expression of the replication and regulatory genes to initiate the lytic cycle.

B. cereus sensu lato infecting *Tectiviridae* are less well known than their gram-negative infecting relatives, such as the lytic phage PRD1 (70), although interest in these phages has recently increased. Temperate phages Bam35 and GIL16, also isolated from *B. thuringiensis* (serovar Alesti and strain B16, respectively), show considerable homology to GIL01 but display slightly different host spectra (91, 96). Similarly to GIL01, these prophages possess gp6 (LexA-like) and gp7; thus, a similar lysis–lysogeny system is presumed for these phages. Berjón-Otero et al. (8) found that P6 (gp6) of Bam35 interacts with other phage-encoded proteins (P24, P29, and P30), although they did not provide a biological explanation. This group also observed that P7 (gp7) is able to interact with itself, hypothesizing that the P7 complexes might increase or decrease binding to LexA. A recent study using whole-genome sequence analysis identified extended *Tectiviridae* hosts, expanding the spectrum beyond *Bacillus* to four additional genera (47). An examination of the array of tectiviruses in this study showed variation in the previously demonstrated LexA-binding site, and the researchers proposed that mutations in this region might be useful for the production of lytic-only phages in certain ecological environments, such as in the presence of a large susceptible host population that might contribute to our understanding of the

host–parasite arms race between these phages and their hosts.

CONCLUDING REMARKS

Phages are the most abundant entities on Earth and because of their predatory role are fundamental regulators of bacterial populations in different ecological niches. It is becoming increasingly clear that the enormous complexity and variety of phages are reflected in the different regulatory networks that control their life cycles. Furthermore, phages have evolved different layers of control to tightly regulate their lysis–lysogeny decisions and have developed different sensing systems to tune into their host and their surroundings. This allows them to make timely decisions to adapt to an ever-changing environment by sensing the vitality of the host through the SOS response. In other systems, such as arbitrium, an additional layer of control is involved, as the phage has the ability to sense its surrounding viral population. It is interesting to observe some differences in host cell dependency for control of the phage life cycle in these systems. While some phages have developed complex networks controlled by multiple self-encoded regulators, others have evolved a simpler process that relies directly on the bacterial SOS response, for example by incorporating the host LexA as a component of the lysogenic switch. Although the latter results in phages being more reliant on the host, it can allow for a swift activation of the lytic cycle. Nevertheless, we cannot discard the possibility that this apparent simplicity is due to the fact that there are still phage regulators to be discovered, as the lysogenic switch in gram-positive phages has not been as intensely studied as their gram-negative counterparts. Understanding the biology of phages and how they regulate their life cycle will be essential for us to understand their role in the intricate ecological networks of bacterial populations.

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