

# Importance of prophages to evolution and virulence of bacterial pathogens

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Bacteriophages, or simply phages, are viruses infecting bacteria. With an estimated  $10^{31}$  particles in the biosphere, phages outnumber bacteria by a factor of at least 10 and not surprisingly, they influence the evolution of most bacterial species, sometimes in unexpected ways. “Temperate” phages have the ability to integrate into the chromosome of their host upon infection, where they can reside as “quiescent” prophages until conditions favor their reactivation. Lysogenic conversion resulting from the integration of prophages encoding powerful toxins is probably the most determinant contribution of prophages to the evolution of pathogenic bacteria. We currently grasp only a small fraction of the total phage diversity. Phage biologists keep unraveling novel mechanisms developed by phages to parasitize their host. The purpose of this review is to give an overview of some of the various ways by which prophages change the lifestyle and boost virulence of some of the most dangerous bacterial pathogens.

## Introduction

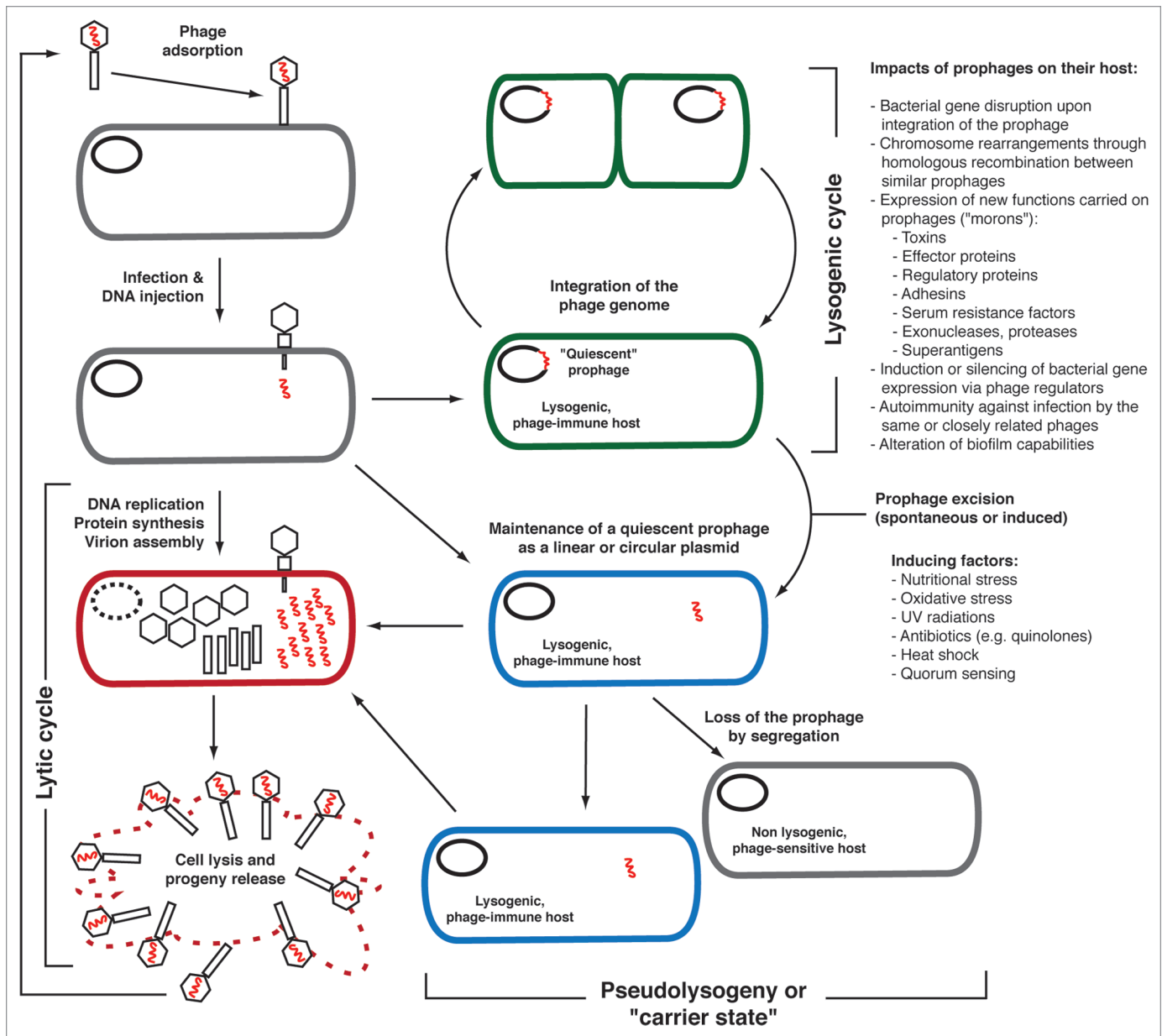
Viruses that infect bacteria are called bacteriophages, or simply phages. They represent the most abundant biological form in the biosphere with an estimated  $10^{31}$  phages, and they outnumber bacteria by a factor of about 10 to 1.<sup>1</sup> Oceans cover about 70% of the Earth's surface and represent the main viral reservoir: each milliliter of sea water contains  $\sim 10^7$ – $10^8$  phages and  $\sim 10^{23}$  phage infections occur every second.<sup>2,3</sup> The mammalian gut is also densely populated with  $10^{13}$ – $10^{14}$  bacteria per gram of fecal matter, and thus represents an incredible playground for phages. The human gut can contain up to  $10^8$  viral particles per gram of feces and most of these viruses are bacteriophages.<sup>4,5</sup> *Escherichia coli* phages dominate, with  $10^3$ – $10^4$  phages per gram of feces in humans and up to  $10^7$  in cows and pigs,<sup>6,7</sup> but phages infecting other important commensal bacteria like *Bacteroides fragilis* and *Salmonella* spp. have been isolated as well.<sup>8</sup> Not surprisingly, phages constitute major players shaping bacterial communities in most if not all ecosystems and they are intimately associated with

virulence and evolution of several important bacterial pathogens.<sup>9,10</sup> The purpose of this review is to give an overview of some of the different ways by which prophages can modulate virulence of their host.

## Lysogeny is Common in the Bacterial World

Generally speaking, phages can be classified into two main categories based on their lifecycle: strictly lytic phages (or virulent) and temperate phages, although some phages also adopt a pseudotemperate lifestyle, i.e., they generally do not integrate the chromosome of their host and remain as linear or circular plasmids (Fig. 1).<sup>11</sup> Virulent phages can only replicate through a lytic cycle, which is characterized by the lysis of the infected bacterial host at the end of the phage replication cycle. RNA phages such as the *E. coli* phages MS2 and QB are part of this group, but most of the work on lytic phages has involved tailed, double-stranded DNA phages, which are by far the most frequently isolated phages ( $\sim 96\%$  of the phages described in the literature).<sup>12</sup> Typically, the lytic cycle of DNA phages is characterized by the adsorption of the phage to a specific bacterial cell surface receptor, followed by the injection of the phage DNA into the bacterial cytoplasm, transcription of phage genes, and replication of the phage genome. Viral proteins are then synthesized, assembled into capsids (heads) and tails (in the case of tailed phages) and the viral genome is packaged into the capsids to form virions. At the end of the lytic cycle the phage holin, a small protein that forms oligomers, creates pores within the cytoplasmic membrane.<sup>13</sup> This enables the phage-encoded endolysin (often called “lysin”) to gain access to the peptidoglycan layer that it hydrolyzes. This results in cell lysis and the release of hundreds to thousands of new infectious particles into the surrounding environment that will be able to re-infect susceptible bacteria nearby and the cycle will start over again. Temperate phages have the same lytic capacity as virulent phages but in addition, they can opt for a non-lytic lifestyle.<sup>11</sup> Within minutes after phage DNA injection into the bacterial cytoplasm, and depending on the metabolic state of the bacterial cell, the phage can “choose” to initiate a lytic cycle or to integrate its DNA into the bacterial chromosome of its host to become a prophage. If the latter option is chosen, a specific phage repressor (CI in phage  $\lambda$ ) inhibits transcription of most of the phage genes, including those required for the lytic cycle, and the prophage becomes quiescent. Its DNA is replicated

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**Figure 1.** Different lifestyles adopted by phages. True virulent phages will only follow the lytic cycle for their replication and will lead to the lysis of the host cell at the end of the cycle. Temperate phages have the choice to replicate through the lytic cycle like virulent phages, or they can opt for the lysogenic cycle. In most cases of lysogeny, the phage integrates its genome into the host bacterial chromosome and remains quiescent. The prophage DNA is replicated along with the bacterial chromosome and is transmitted to daughter cells. Lysogeny can sometimes have significant impacts on the host (lysogenic conversion).<sup>9</sup> Under certain conditions, including various stresses causing DNA damages, the prophage is excised and initiates a lytic cycle. Some phages also adopt a pseudotemperate lifestyle, i.e., they generally do not integrate the chromosome of their host and replicate as linear or circular plasmids within the cytoplasm. Sometimes, the phage genome is lost during cell division and segregation, which is characterized by instability of the lysogens carrying these phages. Note that chronic infections by non-lytic phages (e.g., filamentous phages such as *V. cholerae* CTX $\phi$ ) are not represented on this figure.<sup>147</sup>

concomitantly with the bacterial chromosome during cell division, and this cycle can last for an infinite period of time. A bacterial cell carrying one or more prophages is called a lysogen and generally, it is "immune" toward lytic or lysogenic infection by other phages of the same group.<sup>11</sup>

The capacity of temperate phages to establish a stable relationship with their host (i.e., lysogens) can have profound repercussions

on the lifestyle, fitness and virulence of the lysogens.<sup>9,10,14,15</sup> With next generation sequencing technologies, an increasing number of complete and draft bacterial genome sequences are now becoming available in public databases. As a direct consequence of that, genome mining and comparative genomics have revealed the pervasiveness of prophages in most bacterial genomes. Prophages constitute one of the main sources of genetic diversity and strain

variation associated with the virulence of many bacterial pathogens including *E. coli*,<sup>16,17</sup> *Streptococcus pyogenes*,<sup>15,18,19</sup> *Salmonella enterica*,<sup>20-23</sup> and *Staphylococcus aureus*.<sup>24-26</sup> Many phages associated with virulent strains encode powerful extracellular toxins, effector proteins participating in invasion, various enzymes such as superoxide dismutase, staphylokinase, phospholipase, DNase, proteins affecting resistance to serum and altering antigenicity, superantigens, adhesion factors, proteinases, and mitogenic factors (for reviews describing these factors, see refs. 9 and 27). The phenomenon of lysogenic conversion, whereby a phage converts a non-virulent bacterial strain into a virulent one, has been linked with the emergence of new virulent and epidemic clones such as the *E. coli* O157:H7 strain that acquired two Shiga toxin-encoding prophages (Sp5 and Sp15)<sup>16,28</sup> and *Vibrio cholerae* that acquired the filamentous phage CTX $\phi$  encoding the cholera toxin.<sup>29,30</sup> Furthermore, prophage induction and mobility, which is often increased by the action of antibiotics, can shape bacterial communities,<sup>31,32</sup> and favor the dissemination of antibiotic resistance genes<sup>33</sup> and other mobile genetic elements such as pathogenicity islands in *S. aureus*,<sup>34,35</sup> thereby promoting bacterial evolution.<sup>9,10</sup> With the massive amount of genomic data available in public repositories, one of the great challenges in phage biology will be to link the presence of prophages in bacterial chromosomes to evident bacterial phenotypes. This task is more complex when prophages do not encode known virulence factors based on bioinformatics analyses.

Several prophages integrate into transfer RNA genes (tRNA), and in most cases the prophage carries a duplication of a portion of the disrupted gene, thereby reconstituting the coding sequence upon integration.<sup>36</sup> A number of prophages also encode their own tRNA genes that can complement the disrupted tRNA sequence. For example, the *Lactobacillus johnsonii* phage Lj965 encodes 4 tRNA genes.<sup>37</sup> It is important to mention however that prophage integration can sometimes lead to a loss of function and a negative lysogenic conversion phenotype. Good examples of that are the L54a and phi13 prophages that integrate into the chromosome of *S. aureus* and cause the inactivation of a lipase and a  $\beta$  toxin gene, respectively.<sup>38,39</sup> Other examples of gene inactivation upon prophage integration can be found in the literature, although in many cases the functional consequences on the host have not been investigated. One such example is the integration of the  $\phi$ C2 prophage into the *C. difficile* chromosome, which disrupts a transcriptional regulator of the GntR family. A possible consequence of this inactivation event could be deregulation of a mannose-specific phosphotransferase system, although experimental evidence of that is not available.<sup>40</sup>

### Role of Prophages in the Pathogenesis of Bacterial Infections

Bacteria have to adapt to multiple growth environments and conditions in order to survive. Some bacterial pathogens can transit between very diverse ecological niches throughout their life cycle. For example, aquatic ecosystems are the natural reservoir for *V. cholerae*, but this bacterium can cause severe and lethal diarrhea in humans as well.<sup>41</sup> *Bacillus anthracis* is able to survive

as dormant spores in soil, but can also replicate as vegetative cells in earthworms, and can cause the deadly anthrax disease in animals and humans.<sup>42</sup> To be able to thrive under these very diverse conditions, bacteria have to express different sets of genes and modulate the expression of their genes in a coordinated manner depending on the environmental conditions. The pathogenesis of bacterial infections is often complex and generally involves several stages where bacteria need to express specific genes in a timely fashion. For example one of the very first steps in many bacterial infections is attachment and adhesion to the appropriate cell target, which is often promoted by biofilm formation.<sup>43</sup> This close contact is necessary for several gram-negative bacteria like *S. enterica* and *Pseudomonas aeruginosa* to inject effector proteins through type II and III secretion systems (TTSS) and manipulate the host cell.<sup>44,45</sup> The expression of extracellular toxins is also a hallmark of several pathogens.<sup>46</sup> Some strictly anaerobic intestinal pathogens like *Clostridium perfringens* and *Clostridium difficile* are unable to survive for prolonged periods of time in the presence of oxygen. Bacteria that can reside in soil for years like *B. anthracis* must also be ready to face very unfavorable growth conditions. Therefore, spore formation by these pathogens is intrinsically associated with their survival and dissemination in the environment and in host-to-host transmission. For example, highly resistant spores of *C. difficile* can contaminate hospital settings, leading to nosocomial transmission of highly virulent strains that cause outbreaks.<sup>47</sup> Moreover, pathogens that colonize the mammalian gut must compete for nutrients with trillions of bacteria, in addition to all associated phages that can reach several orders of magnitude.<sup>48,49</sup> In such hostile environments, any metabolic advantage or resistance mechanism provided by prophages has its importance and can make the bacterial host cell more competitive.

### “Morons”, Phage Evolution and Lysogenic Conversion

In most instances, virulence genes carried within prophages are organized as discrete autonomous genetic elements called “morons”, which are flanked on one side by a  $\sigma^{70}$ -like promoter region and on the opposite side by a factor-independent transcriptional terminator. This type of organization minimizes interference with adjacent prophage genes, while allowing optimal expression of the virulence gene(s) during the lysogenic cycle where the prophage is latent and most of its genes are repressed.<sup>50,51</sup> This structure also favors the easy transfer of morons as a functional cassette from one phage to another through genetic recombination, without problems linked to regulation of gene expression. Comparative phage genomics generally enables the quick identification of morons: an extra gene or a group of genes generally interrupts a DNA region that is contiguous in other closely related phage genomes.<sup>50</sup> In addition, morons often have a significantly different G + C content, which is a hallmark of a recent acquisition through horizontal gene transfer.<sup>52</sup> Phage evolution is in part driven by horizontal exchange of functional modules between more or less related phages, thus explaining the extensive genome mosaicism among

related phages.<sup>53,54</sup> Many bacteria harbor more than one prophage in their chromosome, and the higher the degree of DNA homology between these prophages, the higher the probability that a genetic recombination event will occur. These events may lead to large chromosome rearrangements such as inversions and deletions.<sup>9,10</sup> Homologous recombination is not restricted to endogenous prophages. In fact, virulent phages can also recombine with integrated prophages. These events occur at a low frequency, but under strong selective pressure, such as in the presence of phage resistance mechanisms, they can be observed relatively easily.<sup>55-58</sup> A very interesting case was described by Labrie and Moineau in which mutants of the virulent phage  $\lambda$ 36 were successively isolated upon infection of two derivatives of the same *Lactococcus lactis* strain carrying two different abortive phage infection (Abi) systems, AbiK and AbiT.<sup>57</sup> Abi systems are potent antiphage mechanisms expressed by several bacterial species including *E. coli*, *Bacillus subtilis*, *Bacillus licheniformis*, *Shigella dysenteriae*, *S. pyogenes*, *V. cholerae*, and *L. lactis* (for a review see ref. 59). Upon infection by a sensitive phage, the Abi mechanism is activated, leading to abortion of the phage infection process and death of the infected cell, thereby reducing the viral progeny and limiting the propagation of a phage in a given bacterial population.<sup>59</sup> In their study, Labrie and Moineau showed that infection of a *L. lactis* strain expressing the AbiK system with the virulent phage  $\lambda$ 36 led to the isolation of AbiK-resistant phage mutants ( $\lambda$ 36.k1) at a frequency of  $10^{-7}$ . One of these mutants,  $\lambda$ 36.k1, was then used to infect the same *L. lactis* strain, but expressing a different Abi system, AbiT. Again, phage mutants were obtained at a frequency of  $10^{-8}$  and these double phage mutants were fully resistant to both Abi mechanisms. Upon sequencing of the mutant phage genomes, the  $\lambda$ 36.k1t1 mutant was found to have exchanged 79% of its genome compared with the initial  $\lambda$ 36 genome.<sup>57</sup> Unexpectedly, it was found that the genomic sequences were exchanged with endogenous prophages present in the *L. lactis* host used in these experiments.

Virulent phages have received increasing attention over the last decade because they represent a promising therapeutic alternative (“phage therapy”) to fight multidrug-resistant bacterial pathogens.<sup>60-63</sup> For such applications, virulent phages are regarded as safe “professional killers” and are preferred over temperate phages because the only thing they can do upon infection of a susceptible host is to kill it by lysis.<sup>64</sup> However, the elegant experiments described by Labrie and Moineau underline the great capacity of phages to interact with endogenous prophages in order to rapidly adapt to different hosts. Similar recombination events, although they would probably occur at low frequencies, could possibly occur as well if virulent phages are to be used on a large scale for therapeutic applications against pathogenic bacteria. This could eventually lead to the acquisition of virulence factors by homologous recombination between a virulent therapeutic phage and endogenous prophages from the targeted host, or the conversion of a virulent phage into a temperate phage upon acquisition of a lysogeny module or spontaneous mutation. The probability of these events occurring is unknown but it is possible that they could be subjected to selective pressures similar to those seen with antibiotics.<sup>65</sup>

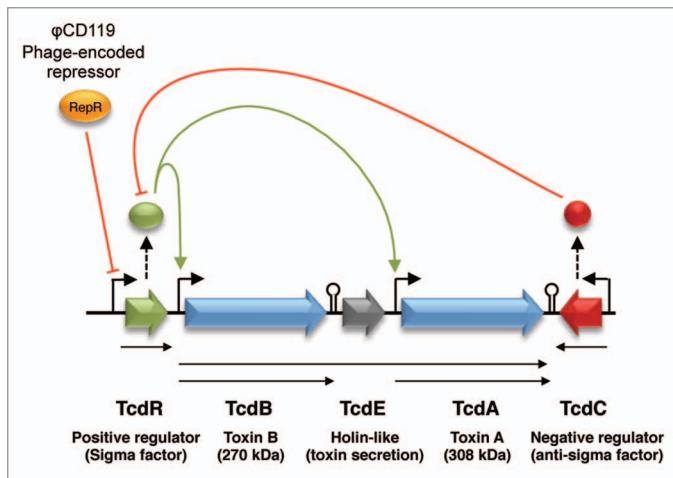
## The Role of Antibiotics in Phage Mobility, Horizontal Gene Transfer, and Evolution

The maintenance of lysogeny depends on a fine balance between phage and host factors. Typically, a phage repressor (CI in phage  $\lambda$ ) binds to phage operator sequences and represses early promoters controlling the expression of genes involved in the lytic cycle. Prophage induction generally requires proteolytic cleavage and displacement of the phage repressor, which most of the time occurs upon activation of the RecA-dependent SOS response following DNA damage.<sup>66</sup> Prophage repression is not totally efficient and depending on the phage and the host, some level of spontaneous prophage induction can occur. DNA-damaging agents such as UV light, mitomycin C, and antibiotics, in particular quinolones, are often used to force quiescent prophages to switch to a lytic cycle in laboratory conditions (Fig. 1).<sup>67</sup> However, in “real life” situations, antibiotic-induced prophage activation can have multiple consequences. For example, the expression and secretion of Shiga toxins rely on prophage induction and ciprofloxacin treatment of mice infected by Shiga-encoding *E. coli* O157:H7 can aggravate the infection.<sup>68,69</sup> Similarly, the temperate phage  $\phi$ sc1 from *Streptococcus canis* was found to encode a gene called *scm* for *S. canis* mitogen factor. In vitro experiments demonstrated that *scm* was able to elicit an inflammatory response similar to the one induced by superantigens, although to a lesser extent. Fluoroquinolones were found to induce transcription of the *scm* gene by 58-fold and it was concluded that the higher incidence of canine streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) observed after the introduction in canine veterinary medicine of enrofloxacin (a fluoroquinolone antibiotic) might be caused by prophage induction.<sup>70</sup> Interestingly, the *scm* gene is encoded in the middle of a putative tail minor structural gene, in a different coding frame, thus underlining the importance of scrutinizing phage genomic sequences to find potential virulence genes.<sup>70</sup> Prophage induction can also lead to transduction, i.e., the phage-mediated transfer of genetic material between bacterial cells. For example, the staphylococcal pathogenicity islands SaPII and SaPIBov1 are excised and circularized, encapsidated in phage-like particles and transferred at high frequency upon activation of the SOS response and induction of prophages such as 80 $\alpha$ ,  $\phi$ 11,  $\phi$ 13, and  $\phi$ 147.<sup>35,71,72</sup> Because SaPIs from *S. aureus* encode various virulence factors, transduction of SaPIs represents an important mechanism driving bacterial evolution in this species.<sup>34,73</sup>

## Prophages Altering Production and Secretion of Toxins

The phage-mediated transfer of extracellular toxin genes is the classical type of lysogenic conversion. For example, phage CE $\beta$  encodes one of the most powerful toxins ever described, the botulinum neurotoxin type C1.<sup>74</sup> Some bacterial toxins rely on a specialized secretion apparatus to be exported to the extracellular milieu. For instance, the release of cholera toxin and the enterotoxin A produced by *V. cholerae* and *P. aeruginosa*, respectively, rely on a type II secretion system.<sup>75</sup> Other toxins however, rely





**Figure 2.** Organization of the pathogenicity locus (PaLoc) in *C. difficile*. Arrowheads indicate a positive effect on the target and blunt ended lines indicate repression of the target. Phage  $\phi$ CD119 expresses a repressor that binds to the promoter region of TcdR, thereby repressing transcription of *tcdR* and hence, transcription of the toxin genes.<sup>103</sup>

on bacterial cell lysis to be released, like all clostridial neurotoxins produced by *Clostridium botulinum* and *Clostridium tetani*.<sup>76</sup> The Shiga toxins encoded by *E. coli* prophages also require cell lysis.<sup>77,78</sup> The Shiga toxins carried on the *E. coli* phages H-19B and 933W are expressed from a late phage promoter ( $P_R$ ) located downstream of the antiterminator protein Q and upstream of the lysis cassette of this lambdoid phage.<sup>79</sup> Upon prophage induction and initiation of the lytic cycle, the  $P_R$  promoter initiates transcription of the late genes, including the lysis cassette and the Shiga toxins. Subsequent phage-induced cell lysis enables the release of toxins into the extracellular environment.<sup>80</sup> Transcription of the *stx2* gene is mainly initiated at the  $P_R$  phage promoter and thus, Stx2 production is greatly dependent and influenced by prophage induction.<sup>68,81</sup> Lysogenic conversion involving other phage-encoded toxins has been well described in other pathogens, including *Corynebacterium diphtheriae* producing the diphtheria toxin, which is encoded on phage  $\beta$ ,<sup>82</sup> *S. pyogenes* that produces several phage-encoded toxins (A, A1, A3, C, I, H, M, L, K),<sup>83-86</sup> and *S. aureus* that encodes enterotoxins (A and P), exfoliative toxin A, and Panton-Valentine leukocidin, all carried on prophages.<sup>87-90</sup>

In many cases however, phages do not code for toxin genes and hence, they are incapable of directly converting their host into toxin producers. Nevertheless, recent studies in *C. difficile* have shown that prophages can indirectly modulate toxin production in an unexpected way. *C. difficile* is an emerging gram-positive nosocomial pathogen causing opportunistic intestinal infections in humans and animals. The toxigenic potential of *C. difficile* is mainly due to the expression of two large exotoxins, TcdA and TcdB.<sup>91</sup> These toxins are encoded on a ~19.6 kb pathogenicity locus called the PaLoc and are under the positive regulation of the alternative sigma factor TcdR (Fig. 2).<sup>92</sup> TcdC is an anti-sigma factor also encoded on the PaLoc and which interferes with the RNA polymerase/TcdR holoenzyme, thereby

repressing toxin gene transcription.<sup>93,94</sup> Under normal laboratory conditions, TcdC represses transcription of the toxin genes during logarithmic growth. Upon entry into stationary phase, the expression of TcdC decreases, thus relieving the inhibition of TcdR. Transcription of *tcdA* and *tcdB* is then allowed and toxins are produced. Various signals have been identified that participate in the control of toxin gene expression, like the presence of carbon sources, various amino acids and temperature,<sup>95-99</sup> and the master regulator CodY plays a central role in this regulation.<sup>100</sup> In 2005, Goh et al. reported an increased toxin production in lysogens of *C. difficile* carrying the temperate phages  $\phi$ C2,  $\phi$ C6, and  $\phi$ C8.<sup>101</sup> Likewise, the temperate phage  $\phi$ CD38-2 was shown to stimulate the production of TcdA and TcdB toxins about 2-fold once introduced as a prophage into epidemic strains of *C. difficile*.<sup>102</sup> In the latter case, the level of mRNA from all 5 PaLoc genes was 2- to 3-fold higher in the lysogens carrying the prophage and the level of expression was even greater in some lysogens.<sup>102</sup> The molecular mechanism by which these phages affect toxin gene expression remains unknown, but in a similar study, Govind et al. demonstrated that RepR, a transcriptional regulator encoded by the temperate phage  $\phi$ CD119, could directly repress toxin gene transcription in *C. difficile*.<sup>103</sup> In lysogens carrying  $\phi$ CD119, the *repR* gene is transcribed and the gene product binds to its own promoter region. However, RepR also binds DNA regions downstream of the *tcdR* promoter thereby blocking TcdR expression. In the absence of TcdR, transcription of *tcdA* and *tcdB* is not possible (Fig. 2).<sup>103</sup>

The similarity between PaLoc genes in *C. difficile* and phage genes, as well as the possibility for a phage protein like RepR to bind regulatory regions from the PaLoc underlines the existence of some level of crosstalk between prophages and bacterial hosts for millions of years. As a consequence of this proximity, some prophages have been seamlessly integrated within the regulatory network of their host. A good example of this is the phage-encoded SopE effector protein for which secretion is dependent on the invasion-associated type III secretion system (T3SS-1) of the *Salmonella* pathogenicity island (SPI-1).<sup>104</sup> SopE is a potent inducer of intestinal inflammation and activates several innate immune signaling pathways.<sup>105</sup> *Salmonella* cells lysogenized with phage SopE $\phi$  are more invasive because they produce the membrane-associated effector protein SopE, which functions as a guanine nucleotide exchange factor activating Rac1 and Cdc42 causing membrane ruffling and promoting *Salmonella* invasion.<sup>106,107</sup> The interesting feature of SopE is that its expression and secretion are controlled in *trans* by the T3SS-encoded factors InvB, InvF, and SicA. The InvF/SicA complex binds the *sopE* promoter region within the SopE $\phi$  prophage to allow its transcription, and the InvB chaperone enables secretion of SopE.<sup>104,108</sup> This example shows that regulatory circuits of prophages within their hosts can be complex and exquisitely interconnected.

### “Phage-Like” Genes Involved in Toxin Secretion

The fact that a phage protein can bind regulatory regions of the *C. difficile* PaLoc supports the hypothesis that the PaLoc evolved

from an ancient prophage. Indeed, one very interesting characteristic of the PaLoc is the functional similarity of one of its gene products, TcdE, with phage holins.<sup>109</sup> Because of its potential cell-permeabilizing activity, TcdE was proposed to participate in toxin secretion.<sup>109</sup> This hypothesis was later confirmed experimentally in the study by Govind et al. who observed a dramatic reduction in extracellular toxin accumulation in a mutant *C. difficile* strain 630 carrying an inactivated *tcdE* gene. In addition, the *C. difficile* *tcdE* gene was able to complement an *E. coli*  $\lambda$  lysogen encoding a non-functional holin gene.<sup>109,110</sup> Although there is yet no proof that phage holins participate in toxin secretion in *C. difficile*, this possibility cannot be ruled out considering the functional similarities between holins and TcdE.<sup>109</sup> Lysis-associated genes are normally expressed at the end of the phage lytic cycle, thus suggesting that prophage induction could be linked to toxin release in *C. difficile*, as for Shiga toxins in *E. coli*. Spontaneous prophage induction of phage  $\phi$ CD38-2 did not contribute significantly to toxin release in vitro,<sup>102</sup> but the situation might be different with other phages, or during a real infection, especially if antibiotics that stimulate prophage induction are present.<sup>67</sup>

Recently, *Salmonella enterica* serovar Typhi was found to encode a homolog of phage muramidases that control secretion of the typhoid toxin. *S. Typhi* is an exclusive human pathogen causing the life-threatening typhoid fever, due to the expression of the cytolethal distending toxin (CDT). This toxin is composed of three subunits encoded by the *cdtA*, *cdtB*, and *cdtC* genes. CdtA and CdtC form the heterologous protein subunit B necessary for the delivery into mammalian cells of subunit A, the active part of the toxin composed of CdtB.<sup>111</sup> Several bacterial species produce the CDT toxin, including *E. coli*, *Shigella dysenteriae*, and *S. Typhi*, but in the latter pathogen, orthologs of the *cdtA* and *cdtC* genes are absent. The consequence is that *S. Typhi* must be internalized within mammalian cells in order to deliver the CDT toxin.<sup>111</sup> A gene encoding an *N*-acetyl- $\beta$ -D-muramidase similar to a phage endolysin has been identified within the islet encoding the *cdtB* gene in *S. Typhi*. Deletion of this gene prevented delivery of the toxin upon infection of epithelial cells, so the gene product was named TtsA for typhoid toxin secretion A.<sup>112</sup> Although TtsA is not encoded within a phage genome in *S. Typhi*, a number of other muramidases of the same family were identified in the context of complete or incomplete prophages. As observed with many phage lysis cassettes, a gene encoding a holin is often located next to the muramidase, but not in *S. Typhi*. This has led to the suggestion that a holin encoded elsewhere in the genome of *S. Typhi* probably fulfills the task of permeabilizing the membrane in order for TtsA to reach the peptidoglycan layer. Thus, TtsA might represent the recent adaptation of a phage-associated enzyme carrying out a different function, which might also be the case for TcdE encoded in the PaLoc of *C. difficile*.<sup>110,112</sup>

### The Contribution of Prophages to Biofilm Formation

The importance for microorganisms to build complex, surface-attached microbial communities termed biofilms is now well established.<sup>113,114</sup> Usually characterized by a complex environment composed of exopolysaccharides (EPS), proteins, and DNA,

biofilms have been tightly linked to bacterial persistence and dissemination in particular niches.<sup>115</sup> The mechanisms and ecological consequences of biofilm formation and dispersal have been studied in detail (for a recent review see ref. 116) and temperate phages have been shown to drastically influence the dynamics of biofilm formation and dispersal by a number of important pathogens such as *P. aeruginosa*,<sup>117</sup> *Streptococcus pneumoniae*,<sup>118</sup> *B. anthracis*,<sup>42</sup> and *E. coli*.<sup>119</sup>

*B. anthracis* causes anthrax, which is a deadly zoonotic disease. It is generally assumed that once *B. anthracis* has killed its host, the bacterium remains in the carcass and the surrounding environment in the form of highly resistant and inert spores until another host ingests the spores and the infection cycle starts again.<sup>42</sup> An elegant series of experiments by Schuch and Fischetti<sup>42</sup> demonstrated the importance of temperate phages in altering multiple phenotypes in *B. anthracis*, including biofilm formation, thus readdressing the “spore dogma” around *B. anthracis*. Temperate phages infecting *B. anthracis* were isolated from various environmental samples (e.g., phage Wip1, Wip2, Frp1, Slp1, and Bcp1) or induced from bacteria from the soil, fern rhizosphere, or the gut of the earthworm *Eisenia fetida* (e.g., phage W $\beta$ , Wip4, Wip5, and Frp2). The role of these phages on *B. anthracis* lifestyle has been assessed in the prophage-free  $\Delta$ Sterne strain into which either of these phages was introduced as a prophage. Among multiple phenotypes, biofilm formation was deeply influenced by lysogenization. As demonstrated in laboratory conditions, the  $\Delta$ Sterne strain did not produce biofilm after 3 mo. However, lysogenization of  $\Delta$ Sterne with phages Bcp1, Wip1, Wip4, and Frp2 resulted in formation of a robust, complex and viable biofilm as witnessed by microscopy observations and bacterial counts. A  $\Delta$ Sterne/Wip2 lysogen was isolated from the 3-mo-old biofilm and the Wip2 prophage was cured from the strain. The cured  $\Delta$ Sterne strain had completely lost the ability to form viable biofilms but reintroduction of Wip2 reversed the phenotype. Using expression libraries of Bcp1 and Wip4, a phage-encoded RNA polymerase sigma factor was identified in each phage genome and when cloned and expressed on a plasmid, the genes induced EPS production and biofilm production similar to that observed with  $\Delta$ Sterne lysogens carrying the entire prophages. Insertional inactivation of the sigma factor in a  $\Delta$ Sterne/Wip4 lysogen completely abolished biofilm formation. This study clearly demonstrated that phage-encoded RNA polymerase sigma factors can act in *trans* to specifically induce the expression of bacterial genes required for biofilm formation by *B. anthracis*.<sup>42</sup>

*P. aeruginosa* is well known for its capacity to produce biofilm and to colonize water lines and various abiotic surfaces. This important gram-negative pathogen often colonizes the lungs of cystic fibrosis (CF) patients. *P. aeruginosa* is also capable of forming small colony variants (SCVs) and since these variants grow slowly, they are generally resistant to drug therapies, which makes CF patients more vulnerable to chronic infection by *P. aeruginosa*.<sup>120</sup> Interestingly, the most highly activated genes in *P. aeruginosa* PAO1 biofilms are those within a filamentous prophage, Pf4,<sup>121</sup> and accordingly, free phages have been isolated from biofilm effluents from strain PAO1 and other clinical isolates. Rice

et al. showed that complete deletion of the filamentous phage Pf4 from *P. aeruginosa* PAO1 prevented the typical lysis and death of bacteria at the late stages of biofilm development.<sup>117</sup> The occurrence of SCVs at late stages of biofilm development generally correlates with the conversion of Pf4 into a superinfective form, but SCVs were not detected in biofilm effluents of the PAO1 strain cured of Pf4. Using a nasal aspiration mouse model of acute pneumonia, Rice et al. demonstrated that mice infected with *P. aeruginosa* PAO1 lacking the Pf4 prophage survived significantly longer than mice infected with the wild-type strain. Therefore, their study confirmed the important role of filamentous prophages in modulating biofilm dispersal, in the formation of drug-resistant SCVs, and in the virulence of *P. aeruginosa*.<sup>117</sup>

Extracellular DNA (eDNA) appears to be an important constituent of robust biofilms in many bacterial species including *Staphylococcus epidermidis*,<sup>122</sup> *V. cholerae*,<sup>123</sup> *Bordetella* sp.,<sup>124</sup> and *S. pneumoniae*.<sup>118</sup> In the latter study, Carrolo et al. demonstrated that the presence of eDNA in *S. pneumoniae* biofilms was the result of bacterial lysis caused by the synergic action of the host-encoded autolysin LytA and a phage-encoded lysin Sv1 encoded on the temperate phage SV1. Total biofilm production was compared between *S. pneumoniae* R36A and the R36AP lysogen carrying phage SV1, and strain R36AP produced denser and more robust biofilm than its non-lysogenic counterpart. This difference seemed to rely on the activity of the phage lysin Sv1, since inactivation of the corresponding gene resulted in lower biofilm production. Inactivation of the bacterial autolysin LytA had a similar effect, but the capacity of *S. pneumoniae* to produce biofilm was completely abolished in a double mutant lacking both LytA and Sv1.<sup>118</sup> The expression of the phage lysin Sv1 is expected to occur at the end of the lytic cycle, which suggested that the Sv1-mediated cell lysis and release of eDNA could in fact be the result of spontaneous induction of the SV1 prophage. Accordingly, free phage particles were detected during biofilm formation and the highest titers were observed at the peak of biofilm formation. Taken together, these results strongly suggest that eDNA is essential for proper biofilm formation by *S. pneumoniae*, which is mainly resulting from cell lysis of a fraction of the bacterial population either by autolysis (mediated by LytA) or upon spontaneous prophage induction.

Cryptic or “defective” prophages are sometimes considered harmless because it is generally assumed that they are quiescent and that most of their genes should be repressed. Moreover, since they have lost their capacity to produce genuine infectious particles, they should not pose any problem to the host. Wang et al.<sup>119</sup> underlined the importance of such cryptic prophages in modulating biofilm formation in *E. coli*. The *E. coli* K-12 strain has 9 cryptic prophages in its chromosome, comprising 3.6% of the total genomic DNA. Precise removal of all nine cryptic prophages yielded a cured *E. coli* strain termed Δ9. When assessed for biofilm formation in minimal medium, the cured strain Δ9 had almost completely lost the ability to form biofilm. Additionally, single-phage mutants within the same *E. coli* K-12 strain have been constructed in order to assess the contribution of each individual phage to the observed phenotype. The phage remnants e14, rac, and CP4-44 were found to have

the greatest impact on biofilm formation since curing *E. coli* K-12 from any of these remnants impaired biofilm production.<sup>119</sup> Furthermore, single gene deletions were created to identify specific genes within e14 and rac that were directly responsible for the observed phenotype. The genes identified were *intE* (integrase) and *ymlD* (putative SAM-dependent methyl transferase) for e14 and *intR* (integrase), *stfR* (putative tail-fiber), *ydaS* and *ydaW* (putative transcriptional regulators), and *ydaF* (unknown function) for rac. The exact mechanism(s) by which these genes affect biofilm formation remains to be elucidated. Interestingly, the susceptibility of *E. coli* toward quinolones and β-lactams was significantly increased in the Δ9 strain cured of its prophage remnants. Single prophage deletions showed that CP4-6, rac, and Qin were responsible for the lower susceptibility to these antibiotics. The authors showed that rac and Qin encoded DicB and KilR respectively, and whose expression correlated with an inhibition of cell division and reduced susceptibility to quinolones and β-lactams. The study by Wang et al. clearly showed that the presence of defective prophages can contribute significantly to important bacterial phenotypes such as biofilm formation and drug-resistance.<sup>119</sup>

### Phages that Modulate Sporulation

A number of bacterial species sporulate in order to survive under nutrient limitation or harsh environmental conditions. The endospore is a metabolically dormant and inert form of the bacterial cell, which can withstand extremes of temperature, ionizing radiation, and desiccation. Spores are also resistant to many chemical agents, including antibiotics and several disinfectants.<sup>47,125</sup> Estimates of the longevity of bacterial spores range from thousands to millions of years.<sup>125</sup> Sporulation is a multistage process involving a cascade of gene activation and repression that has been studied extensively in *B. subtilis*, which is to date one of the best models for studying the molecular mechanism of sporulation.<sup>126,127</sup> In practical terms, bacterial spores formed by pathogenic bacteria can be of serious concern in food safety and human health. Important human and animal pathogens produce spores that cause food poisoning (*Bacillus cereus* and *C. botulinum*), wound infections (*C. perfringens* and *C. tetani*) and intestinal diarrhea and colitis (*C. difficile*).<sup>128,129</sup> Spores from highly pathogenic bacteria such as *B. anthracis* that cause anthrax also represent potential biological weapons.<sup>130</sup> In hospitals and long-term care facilities, spores of *C. difficile* are frequently responsible for outbreaks due to the nosocomial transmission of spores that are difficult to eradicate from surfaces and equipments and that can be transmitted via the hands of healthcare workers and visitors.<sup>47</sup>

Several spore-converting phages have been isolated in *B. subtilis*,<sup>131,132</sup> *Bacillus pumilus*,<sup>133,134</sup> *Bacillus thuringiensis*,<sup>135</sup> and *B. cereus*.<sup>136</sup> These phages were isolated based on their capacity to enhance the rate or extent of sporulation of wild-type cells or their ability to complement different mutant strains defective in sporulation. For example, lysogenization of *B. subtilis* with phages PMB12 and SP10 convert sporulation-negative strains into sporulating cells.<sup>131-133,137</sup> PMB12 was found to suppress the stage 0 sporulation mutations *spoCM-1*, *spo0J93*, and *spoOK141*,



suggesting that this phage influenced the sporulation process at an early stage,<sup>132,137</sup> and at least 3 genes from PMB12 (*scn* genes) seemed to be required to induce sporulation.<sup>137</sup> A subsequent study showed that lysogens of *B. subtilis* strain 3-13 carrying either of PMB12 or SP10 were able to sporulate in the presence of enough glucose to inhibit sporulation and the expression of  $\alpha$ -amylase in the wild-type parental strain.<sup>132</sup> Therefore, PMB12 and SP10 relieve the catabolite repression-associated inhibition of sporulation and  $\alpha$ -amylase expression in *B. subtilis*.

A few studies have addressed the role of temperate phages in sporulation of *C. perfringens*. In 1977, Stewart and Johnson reported that curing *C. perfringens* strain S9 from its integrated prophage  $\phi$ S9 resulted in a 1-log reduction in the maximum number of spores. The time that was necessary to reach the maximum number of spores was also increased to 9–10 h in the cured strain S9CR instead of 3 h in the S9 lysogen.<sup>138</sup> Reintroduction of  $\phi$ S9 into the cured strain restored sporulation to levels close to the parental lysogen. These results suggested that  $\phi$ S9 was able to stimulate sporulation in *C. perfringens*. However, the genome sequence of  $\phi$ S9 was published recently and the capacity of the phage to influence sporulation of *C. perfringens* was reassessed.<sup>138,139</sup> The sporulation-promoting phenotype initially reported by Stewart and Johnson could not be reproduced and the sporulation kinetics and maximum number of spores observed in the lysogen and the cured strain were similar, although similar experimental conditions were used.<sup>139</sup> In 2002, the genome sequence of  $\phi$ 3626, another temperate phage infecting *C. perfringens*, was published.<sup>140</sup> The presence of a sporulation-associated sigma factor (ORF32) in the genome of  $\phi$ 3626 suggested that reprogramming of the RNA polymerase toward sporulation genes could possibly enable the prophage to promote sporulation of its host as reported for  $\phi$ S9, but the experimental demonstration of this has not yet been reported.<sup>138,140</sup>

The alternative sigma factor  $\sigma^K$  is the last sigma factor to be activated during the sporulation cascade.<sup>127</sup> In *B. subtilis*, the full-length *sigK* is encoded by two genes, *spoIVCB* and *spoIIIC*, interrupted by a 48-kb intervening region.<sup>141</sup> This DNA fragment, called *skin<sup>Bs</sup>* in *B. subtilis* for *sigK* intervening element, is precisely excised from the chromosome of the mother cell compartment of sporulating cells by a recombination event between two 5-bp repeated sequences, thereby enabling expression of *sigK* in this specific compartment.<sup>142</sup> A 14-kb *skin<sup>Cd</sup>* element interrupting the *sigK* gene was also identified in the genome of *C. difficile* strain 630.<sup>143</sup> Creation of a merodiploid CD196 strain carrying both interrupted and uninterrupted copies of *sigK* led to a defective sporulation phenotype as opposed to wild-type CD196 cells that sporulated efficiently. This result led to the conclusion that interruption of *sigK* and timely excision of the *skin<sup>Cd</sup>* element were required for full sporulation of *C. difficile*.<sup>144</sup> The observation that strains CD37 and ATCC9689, two natural isolates of *C. difficile* lacking a *skin<sup>Cd</sup>*, were defective in sporulation further supported the above conclusion.<sup>144</sup> The *skin<sup>Bs</sup>* and *skin<sup>Cd</sup>* elements contain phage-related genes, which suggests that the *skin* elements might represent cryptic remnants of ancient prophages.<sup>141,143</sup> In agreement with that hypothesis, the temperate phage  $\phi$ S63 was found to integrate within  $\sigma^K$  in *C. perfringens*, thus representing the

first example of a *skin* element in this species, and also the first example of a functional prophage interrupting  $\sigma^K$ .<sup>139</sup> However, contrary to *C. difficile* where interruption of  $\sigma^K$  is required for efficient sporulation, interruption of  $\sigma^K$  in *B. subtilis*, and probably *C. perfringens* as well, is not essential because in the two latter species,  $\sigma^K$  is expressed as an inactive pro- $\sigma^K$  that must be processed by a membrane-associated metalloprotease to release the active  $\sigma^K$ .<sup>126,139</sup>

But what could be the advantage for a phage to promote sporulation, knowing that spores are refractory to phage infection? A possible reason could be that phage genomes trapped inside the spores have a selective advantage over free viral particles, due to the protection provided by the spore under extreme conditions. For typical prophages that integrate into the genome of their host, promoting sporulation might not be a critical feature because every single lysogen carries one copy of the phage genome. However, a common feature of spore-converting phages, including phage SP10 in *B. subtilis*, is their pseudotemperate nature, i.e., prophages do not integrate into the genome of their host but remain as extrachromosomal DNA during lysogeny (Fig. 1).<sup>145</sup> This leads to a characteristic instability of the prophage genome in these lysogens and a fraction of the bacterial population eventually loses the phage during cell division.<sup>145-147</sup> A number of pseudotemperate phages have evolved in such a way that their genome is efficiently partitioned in the growing forespore along with the bacterial chromosome, thus escaping a certain death by remaining trapped inside the endospore. Even the strictly lytic phage  $\phi$ 29 is able to exploit this survival strategy. When sporulation of *B. subtilis* initiates, the lytic cycle of  $\phi$ 29 is repressed, the phage genome is partitioned inside the spore where it remains quiescent until favorable growth conditions are met to allow spore germination.<sup>148</sup> If a pseudolysogenic bacterial species is unable to sporulate efficiently, survival of pseudotemperate phages might be seriously compromised because only a fraction of the spores will contain the trapped phage genome, as opposed to all spores derived from true lysogens. Hence, these pseudotemperate phages manage to increase the frequency of sporulation of their host possibly to increase the chances that a sufficient number of phage genomes will get trapped inside spores in order to sustain the phage population.<sup>131,132</sup>

On the other hand, preventing sporulation could also provide some advantage to the bacterial host, as observed for temperate phages infecting *B. anthracis*.<sup>42</sup> A number of temperate phages active on *B. anthracis* have been isolated from diverse environments and the role of these phages on the host lifestyle of *B. anthracis* has been assessed in lysogens of the  $\Delta$ Sterne strain carrying either of these phages. Findings by Schuch and Fischetti revealed that some of the prophages were able to profoundly alter the sporulating capacity of the bacterium in broth cultures.<sup>42</sup> For example, after 1 d of incubation at 37 °C under good aeration, the  $\Delta$ Sterne strain produced about  $5 \times 10^8$  spores/ml whereas lysogens carrying Wip4, Wip5, or Frp1 prophages did not sporulate (<10 spores/ml). Blocking of *B. anthracis* sporulation by some prophages (e.g., Wip1 and Wip4) also coincided with strong biofilm formation, resulting in lysogens surviving in soil for several months whereas the non-lysogenic strain  $\Delta$ Sterne did not



survive under these conditions.<sup>42</sup> On the other hand, after 5 d of incubation at 24 °C under poor aeration, the ΔSterne strain did not sporulate (<10 spores/ml), but lysogens carrying phages Wβ, Wip1, Wip2, or Frp2 produced between 10<sup>5</sup> and 10<sup>6</sup> spores/ml. Modification of the sporulation phenotype required the expression of an alternative RNA polymerase-associated sigma factor encoded by the phage. The expression of the phage locus encoding this gene (locus *bcp25,26* in phage Bcp1 and *wip48,49* in phage Wip4) gave the same phenotype as the complete prophage.<sup>42</sup> These experiments demonstrated that some prophages efficiently block sporulation of *B. anthracis*, therefore enabling the bacterium to replicate in the form of vegetative cells in an alternative host, for instance the gut of the earthworm *E. fetida* that serves as a natural reservoir for the active amplification of this pathogen. Long-term survival of *B. anthracis* vegetative cells within biofilms in the soil could also favor survival and mobility of this group of phages that seem to have adopted a pseudotemperate lifestyle.<sup>42</sup>

### Concluding Remarks and Future Perspectives

Prophages can modify the lifestyle, fitness, virulence, and evolution of their bacterial host in numerous ways. Lysogenic conversion involving potent toxins is probably the most determinant contribution of prophages known to date, but as we study phage-host interactions in different bacterial species, we find novel ways by which prophages can influence their host. We currently grasp only a small fraction of the total phage diversity, both in terms of genetic content, but also in terms of novel molecular mechanisms enabling modulation of bacterial phenotypes and lifestyles, including virulence. The growing number of whole bacterial genomic sequences released in public databases will provide great opportunities to seek novel prophage sequences. However, all

this sequence data will be of limited use if the underlying phage biology is not understood and this is why it will be important to continue basic phage research in the post-genomic era. There is a current trend toward understanding phage–host interactions and bacterial–host interactions on a global scale. For example, the gut microbiota is now recognized as a key element in human health. Dysbiosis, which occurs when the “normal” microbiota is altered, for example by antibiotic treatments, is associated with opportunistic bacterial pathogens such as *C. difficile*, probably being one of the most striking cases. But dysbiosis of the gut microbiota is also associated with important chronic disorders, such as obesity, type 2 diabetes, and inflammatory bowel diseases (Crohn disease and ulcerative colitis).<sup>149</sup> The human gut contains large amounts of free viral particles, most of them being bacteriophages probably released after spontaneous prophage induction of lysogenic gut bacteria.<sup>5</sup> This is not surprising since phages outnumber bacteria by a factor of at least 10 and the gut is colonized with trillions of bacteria with 60–70% of them being lysogenic.<sup>150</sup> However, although the influence of phages on bacterial populations from aquatic environments and other ecological niches has been studied in more detail,<sup>2</sup> the influence of bacteriophages in shaping the human gut microbiota has been addressed in only a few studies (for a review see ref. 32) and this will certainly represent one of the major challenges over the next era of phage research.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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