LAMBDOID PHAGES AND SHIGA TOXIN

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"Sir, I am lysing." Andre Lwoff describing how his technician informed him that UV exposure results in quantitative prophage induction (113).

Observations that many bacterial virulence genes are located in the genomes of prophages have raised the question over the years of whether phages serve merely as vectors for the transfer of virulence genes or additionally contribute to the expression of these genes (6). This question arose anew with the finding in Shiga toxin-producing *Escherichia coli* (STEC) (90) that the genes encoding Shiga toxin, *stx*, are located in genomes of phages of the lambdoid family, downstream of the phage late promoter, and can be expressed by transcription initiating at that promoter (93, 138, 157). The *stx* genes are thus positioned so that the regulatory cascade leading to lytic phage growth can contribute to their expression. The observation that both types of *stx* genes have associated promoters that may be responsible for Stx expression in the absence of transcription from the phage late promoter put a damper on this idea (41, 195). However, as discussed here, there is persuasive evidence that phage functions can contribute significantly to Stx production and/or release from a population of lysogens containing *stx*-encoding prophages. Although this chapter summarizes what is known about *stx*-encoding phages, it is not all-inclusive. The reader is referred to other reviews that may include information which is not discussed here (90, 145, 176, 214, 215). We begin with a brief discussion of STEC, present general information on lambdoid phages, and finally segue into a discussion of the main subject, *stx*-encoding phages.

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STEC AND DISEASE

STEC strains are recognized as emerging foodborne pathogens and are considered a major public health threat in industrialized countries (90). Although there is a diverse range of STEC strains that can cause human disease, with nearly 200 identified serotypes, the most highly reported sources of human infection in North America, the United Kingdom, and Japan are STEC strains that belong to the serogroup

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O157:H7 (89, 197). The STEC strains in the O157 serogroup and their close relatives are collectively referred to as enterohemorrhagic *E. coli* (EHEC). First identified as a unique class of pathogens in 1983 (167), EHEC strains are distinguished from other types of diarrheagenic *E. coli* by their ability to both produce a potent group of related cytotoxins known as Shiga toxins and express functions mediating intimate attachment to and effacement of intestinal cells. In nearly all EHEC strains, the genes encoding Shiga toxin are carried in the complete or partial genomes of resident prophages belonging to the λ family (39, 140, 143, 184, 208).

Patients with EHEC infections suffer from watery diarrhea that, particularly in patients above the age of 65, can progress to hemorrhagic colitis (92). Areas of mucosal hemorrhage and edema, erosions, and ulcerations characterize the histology of colonic tissues isolated from patients with hemorrhagic colitis (129). EHEC infections can also lead to hemolytic uremic syndrome (HUS), which is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure (34, 91). Moreover, HUS is the leading cause of renal failure in childhood, predominantly afflicting children under 4 years of age (91). While the recurrence of HUS is rare, some survivors of the disease suffer residual organ damage manifested as chronic renal insufficiency or end-stage renal disease, hypertension, diabetes mellitus, and neurological disorders (92). In 5 to 10% of cases, HUS results in death (34).

Numerous outbreaks of EHEC infections in North America, Western Europe, Australia, and Asia have drawn attention to the growing danger that EHEC strains pose to public health (32). The largest outbreak recorded to date took place in Japan in 1996 and affected >8,000 individuals, 3 of whom died (92). An outbreak in Scotland in 1996 resulted in the deaths of 19 individuals (37). As was the case for these two outbreaks, the majority of EHEC infections have been linked to the consumption of tainted food, particularly contaminated beef (136). The prevalence of beef as a source of EHEC reflects the fact that although EHEC strains have been isolated from the fecal floras of a variety of animals, cattle are the most common animal reservoir associated with EHEC infections (92). Other EHEC outbreaks, such as one in Ontario, Canada, in 2000, in which an estimated 2,300 people were affected, have resulted from contamination of a central water source (121). EHEC infections associated with person-to-person contact and zoonotic transmission have also been reported and reflect the very low infectious dose for EHEC, which is estimated to be as low as 100 organisms (5, 136).

EHEC VIRULENCE DETERMINANTS

As shown by the use of animal models, EHEC infections, like enteropathogenic *E. coli* (EPEC) infections, produce an intestinal attaching and effacing (A/E) histopathology, which is characterized by localized destruction of the brush border microvilli, intimate adherence of the bacteria to the underlying epithelial cells, and the formation of actin-rich pedestals beneath the adherent bacteria (reviewed in reference 52). These A/E lesions are elicited by proteins which are usually encoded by genes present in a large pathogenicity island referred to as the locus of enterocyte effacement (LEE) (118). The LEE genes encode the components of a type III secretion apparatus and its associated secreted effector proteins (85). LEE also carries the *eae* gene, encoding the surface protein intimin (86), which facilitates the intimate attachment of the bacterial cell to the host epithelial cell. This attachment is mediated by the intimin receptor Tir, a protein that is also LEE encoded, that is translocated from the bacterium into the eukaryotic cell (44, 96). Recent studies have led to the identification of additional effector proteins that are delivered into eukaryotic cells by the LEE-encoded type III secretion system. Some of these proteins which have been shown to be important for EHEC virulence in animal model studies are encoded by genes in putative pathogenicity islands outside of LEE (65, 132). Moreover, in addition to Tir and intimin, the translocated effector protein $EspF_U$, encoded by the cryptic prophage CP-933U, is critical for EHEC-directed pedestal formation in cell culture (28). Although *eae* is indispensable for

EHEC intimate attachment in cell culture and for virulence in an animal model (207), it should be noted that there are several LEE-negative STEC strains that facilitate intimate adherence to epithelial cells by the use of other factors (151, 182).

Additional putative virulence determinants include an enterohemolysin gene (*ehxA*) (10), a catalase-peroxidase gene (*katP*) (15), and a serine protease gene (*espP*) (16). The *ehxA*, *kat*, and *espP* genes are located on the EHEC 60-MDa virulence plasmid (pO157). While not all STEC strains possess the same virulence genes, epidemiological studies indicate that there is a correlation between the presence of certain virulence factors in serogroups and the frequency with which they cause disease in humans (11).

SHIGA TOXINS

Shiga toxin (Stx) was first identified as a virulence factor of *Shigella dysenteriae* type 1 (see the preface to reference 90 for a summary of the history of Stx). There have been extensive characterizations of Shiga toxin, and the reader is directed to other reviews for further details (4, 144). Factors expressed by EHEC in addition to Shiga toxins have been proposed to contribute to the sequelae associated with EHEC infections (11). These include the hemolysin encoded by the virulence plasmid (177) and the factors involved in attachment and effacement encoded by LEE (119). With that said, Shiga toxins have been implicated in mediating the most serious of these sequelae, including hemorrhagic colitis and HUS (94). Shiga toxins are members of a family of bacterial toxins known as AB_5 toxins, which are composed of one A subunit associated with five B subunits (4, 141). The A subunit of the toxin has the catalytic activity (*N*-glycosylase), while the B subunit binds the eukaryotic cell through the glycolipid receptor Gb3 (81, 163). The holotoxin is endocytosed in clathrin-coated pits, translocated from endosomes to the Golgi apparatus, and shuttled to the endoplasmic reticulum via retrograde transport (reviewed in references 173 and 201). In the endoplasmic reticulum, the A subunit is separated from the B pentamer and released into the cytosol, where it rapidly inhibits protein synthesis by cleaving a specific adenine residue in the 28S rRNA of the 60S ribosomal subunit (163). It is this cytotoxigenic effect of Shiga toxin on the intestinal endothelium that is thought to directly mediate the progression of EHEC infection to hemorrhagic colitis. This effect was demonstrated in studies with a natural rabbit EPEC strain that causes the attaching and effacing histopathology but does not express the toxin. A derivative of this strain that was lysogenized with a bacteriophage encoding Stx caused increased vascular changes, edema, and more severe inflammation in the intestine of an infected rabbit compared to that in the intestine of a rabbit infected with the parental nonlysogenized pathogen (188).

Epidemiological studies demonstrate a correlation between EHEC infections and an increased infiltration of monocytes and polymorphonuclear leukocytes (PMNs) into the intestinal lumen (95). In vitro studies suggest that, once it is in the lumen, Stx prevents the apoptosis of PMNs and induces superoxide production by these cells, which can lead to increased endothelial damage (98). Moreover, as discussed below, there is evidence suggesting that superoxide can lead to prophage induction and increased Stx expression (211).

EHEC strains are unable to invade the intestinal epithilium; thus, systemic manifestations of EHEC infections, including HUS, presumably require the translocation of Shiga toxin across the intestinal epithilium (2). Following this translocation, the toxin is thought to enter the bloodstream and target tissues rich in Gb3 receptors (87). The isolation of Stx-bound PMNs from blood taken from patients diagnosed with the early phase of HUS suggests that Stx circulates from the intestine to target organs as a ligand on PMNs (200). The distribution of Gb3 in targeted organs varies significantly among different species, tissue types, and age groups. For example, the relatively high incidence of HUS in young children is thought to reflect the fact that, unlike those of adults, their renal glomeruli express high levels of Gb3 (108). Moreover, the ability of adult cattle to asymptomatically tolerate persistent EHEC infections has been attributed to the absence of Gb3 receptors in

their intestinal epithilia (158). Humans do not express a functional form of the Forssman synthetase, an enzyme that modifies Gb3 to a form that does not bind Stx (47). In contrast, nonprimate cells such as murine and canine cells, which are not affected by Stx, express an active form of Forssman synthetase, resulting in a modified Gb3 receptor that no longer serves as a receptor for Stx.

In addition to halting translation, Shiga toxins have also been shown to upregulate the expression of proinflammatory chemokines and cytokines and to promote apoptosis in endothelial and epithelial cells (reviewed in reference 71). The activation of host immune mechanisms by Stx may play a central role in pathogenesis, particularly in mediating the thrombocytopenia and microangiopathic hemolytic anemia associated with HUS. The stimulation of inflammatory cytokines by Stx has also been implicated in rendering human brain microvascular endothelial cells, which are normally resistant to Shiga toxin-induced cytotoxicity, sensitive to Shiga toxin by upregulating their expression of Gb3 (194).

Two major immunologically distinct toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), have been found associated with different STEC infections (152). Stx1 and Stx2 share similar structures and biological functions, and with the toxin produced by *S. dysenteriae* and several Stx2 variants, have been collectively classified as the Shiga toxin family. For the sake of simplicity, the Stx nomenclature will be used to refer to the Shiga toxins collectively. The genes encoding Stx1 (stx_{1A} and stx_{1B}), which are nearly identical to those encoding both the *S. dysenteriae* Shiga toxin and the plant toxin ricin (20, 75, 104), share only 56% sequence homology with those encoding Stx2 (stx_{2A} and stx_{2B}) (82). Furthermore, the promoters associated with the stx_1 and stx_2 genes differ significantly both in sequence and in regulation. The expression of Stx1, like the expression of Stx in *S. dysenteriae*, is regulated in part by iron, owing to a 21-bp dyad repeat called the Fur box located between the −35 and −10 regions of its promoter, P_{stx1} (21, 41). The Fur box region serves as a binding site for the Fur protein which, when complexed with iron, acts to repress transcription (48). In contrast, the stx_{2AB}-associated promoter P_{stx2} does not contain a Fur binding site, and thus the expression of Stx2 is not regulated by iron (195).

Stx VARIANTS

The Stx2 group of toxins other than Stx2 includes the related cytotoxins Stx2c, Stx2d, Stxe, and Stx2f (62, 156, 181, 183). STEC strains that produce the Stx2c or Stx2d variant have been isolated from symptomatic humans, but STEC strains that produce Stx2d are typically found in asymptomatic patients or those who exhibit less serious forms of disease (58, 156). In contrast, STEC strains that produce Stx2e and Stx2f are commonly isolated from animals. STEC strains that produce Stx2e typically cause edema disease in pigs (217). However, Stx2e-producing STEC strains have been isolated from humans suffering from hemorrhagic colitis and HUS (155, 202). STEC strains that produce Stx2f are generally found in feral pigeons (181) but were also isolated from a patient with diarrhea (62).

The primary sequence of the toxin A subunit is relatively conserved among the Stx2 family members. However, the B subunits of the Stx2 variants contain sequence variations (183, 217) that distinguish the variant toxins from Stx2 both antigenically and by their biological activities. These amino acid variations in the B subunit result in alterations in toxin activation (102, 122) and in receptor affinities (107, 172). As a result, the Stx2 variants differ in the ability to elicit cytotoxicity on cultured eukaryotic cells (122, 147) and virulence in mice (172). For a more detailed discussion of the biological activities of the Stx2 variant toxins, see the review of the Shiga toxin family by Melton-Celsa and O'Brien (123). Although they were originally believed to be bacterial (79, 217), the genes encoding the Stx2 variants have recently been identified in the genomes of prophages (134, 199), similar to the case for Stx1 and Stx2.

Three variants of stx_1 were identified by Paton et al. (149). Although two of these variants have only minimal codon differences from the

stx_{1A} and stx_{1B} genes, one of the variants, stx_{10X3} (also called stx_{1c}), has a number of codon differences. The stx_{1c} gene has been identified in several STEC strains isolated from sheep and humans (100, 227). In humans, these strains were not associated with severe infections, and many were found in asymptomatic individuals. A phage carrying stx_{1c} was isolated and, surprisingly, has significant homology to 933W (100).

PHAGE-MEDIATED HORIZONTAL TRANSFER OF TOXIN GENES

Smith and Linggood (191) showed that the toxigenicity of a clinically isolated diarrheagenic *E. coli* strain can be efficiently transmitted to a nonpathogenic strain of *E. coli* simply by coculturing the two strains. Their finding provided the first evidence that the genes encoding Stx in STEC are encoded in the genomes of temperate phages; i.e., infection of a bacterium with phage can lead to stable lysogens that convert a bacterium from a non-toxin-expressing to a toxin-expressing organism. This was confirmed by the isolation of phages carrying *stx* genes from two different clinical isolates of pathogenic *E. coli*. Smith et al. (184, 190) isolated two different converting phages, H-19A and H-19B, from an O26:H11 clinical isolate. O'Brien et al. (143) identified phages 933J and 933W as two converting phages present in an O157:H7 strain. Upon further analysis, the phage identified initially as 933J did not match the prophage in the O157:H7 genome (142) (see below for further discussion). Sequence analyses and Southern blot studies of the H-19B and 933W genomes showed that these phages encode Stx1 and Stx2, respectively, and that both are members of the lambdoid family of phages (41, 76, 137, 157).

ACQUISITION OF Stx GENES

Phylogenetic analyses of pathogenic *E. coli* suggested that the O157:H7 clones are derived from an EPEC O55:H7 clone. For this conversion, the EPEC clone, encoding the factors required for colonizing the intestine and eliciting A/E lesions, is postulated to have acquired the *stx* genes and the pO157 virulence plasmid by lateral transfer (220). Although most EHEC strains express similar virulence factors, including Stx, enterohemolysin, and catalase, the diversity of the mobile elements associated with genes required for virulence indicates that the acquisition of these secondary virulence determinants occurred independently (162). However, based on phylogenetic analyses, it has been proposed that different lineages of pathogenic *E. coli* have acquired their collections of virulence genes, including *stx*, in the same temporal order (162). Comparisons of the genomes of identified EHEC strains with genomes of nonpathogenic strains of *E. coli* indicated that much of the DNA that is specific to the pathogenic strains consists of phage-related elements (67, 146, 186). Therefore, it is predicted that the genomic diversity of STEC strains can be principally attributed to the phage-mediated horizontal transfer of DNA.

The emergence of new serotypes and species of Stx-producing bacteria associated with disease in humans suggests the promiscuity of the *stx*-encoding phages in nature. The *stx*-encoding phages can infect and lysogenize a broad range of enteric bacteria in vitro, including clinically isolated pathogenic strains of *E. coli*, such as EPEC, enteroaggregative *E. coli* (EAEC), and enteroinvasive *E. coli* (84, 178). An Stx2-producing EAEC strain containing an inducible stx_2-encoding phage was isolated from a patient diagnosed with bloody diarrhea and HUS (80). In another report, serotype O111:H2 Stx2-producing strains, isolated from patients diagnosed with HUS, exhibited enteroaggregative properties and were shown to contain the genetic markers of EAEC (128). It was suggested that the stx_2 genes may be located in a λ-like cryptic prophage in these EAEC isolates. However, it has not been ruled out that an inducible phage is associated with the toxin genes. Derivatives of both *Enterobacter cloacae* and *Citrobacter freundii* that produce Stx2 cytotoxins have been associated with outbreaks of HUS and diarrhea in humans (150, 179). Although inducible Stx-converting phages were not isolated from these Stx-producing strains, the stx_{2A}

and stx_{2B} genes present in these clinical isolates are very similar at the nucleotide level to stx_{2A} and stx_{2B} of STEC. Therefore, it is possible that these species acquired the toxin genes from a common origin with *E. coli*, presumably by lysogenization with an *stx*-encoding phage, possibly from *Shigella* (9), that has since lost essential genes.

In vivo experiments have provided suggestive evidence that phages are involved in the lateral transfer of *stx* genes. Acheson et al. (3) demonstrated that STEC lysogens produce infectious virions in the murine gastrointestinal tract which are able to lysogenize susceptible bacteria that are also present within the gut. In addition, it has been demonstrated that murine intraintestinal transmission of Stx-converting phages is significantly enhanced by the treatment of STEC-infected mice with antibiotics that are known to induce prophages (228). The transduction of susceptible enterobacteria by *stx*-converting phages is most likely a common event in the rumina of cattle and other domestic ruminants, which are the main reservoir of STEC (92, 99). The treatment of STEC lysogens with antibiotics used for animal husbandry purposes in cattle resulted in increased phage titers in vitro (101). Therefore, it is predicted that the supplementation of animal feed with sublethal levels of antibiotics causes prophage induction in the bovine gut and increases the number of free phage that can infect susceptible enteric bacteria that are also present in the gut. Thus, antibiotic supplementation of animal feed, in addition to contributing to increasing levels of antibiotic resistance, may contribute to the phage-mediated lateral spread of genes encoding virulence determinants. It is unlikely that all of the newly infected bacteria encode virulence factors (168). However, some may encode virulence factors that by themselves are not sufficient to cause disease but are able to do so when Stx is also expressed. Thus, phage-mediated lateral transfer in vivo may lead to the conversion of a nonpathogenic bacterium to one that is pathogenic or of an already pathogenic bacterium to one that is more virulent.

PHAGES AND THE BACTERIAL GENOME

Differences in resident λ-like prophages, as observed by Southern analyses, have been used to compare and distinguish EHEC strains from the same and different outbreaks of infection (171). The complete sequencing of two O157:H7 strains has highlighted the significance of phage contributions to the bacterial genome. Perna et al. (154) identified 18 phage-like sequences when they sequenced the EDL933 genome. 933W, with its stx_2 genes, was the only one of the identified phages that was known to form infectious phage particles. A second set of phage genes was found associated with the stx_1 genes. Hayashi et al. (67) also identified 18 phage-like sequences when they sequenced the Sakai (RIMD 0509952) strain. Of these sequences, 13 were related to λ and resembled each other. These included phages carrying stx_1 and stx_2 genes. All 13 λ-like phage sequences were reported to have deletions and/or insertions in regions encoding what were said to likely be essential phage functions and thus are expected to be defective (145) (see below for further discussion). Even though the VT1-Sakai and VT2-Sakai prophages, which carry the stx_1 and stx_2 genes, respectively, have genomes large enough to encode viable phages, they were reported to be defective because plaques could not be isolated on *E. coli* C600. The defectiveness in both cases was thought to result from genes disrupted by an insertion element (IS). Although VT1-Sakai has an insertion in what would be expected to be an essential gene (114, 225), VT2-Sakai has an insertion in a gene with an unknown function (114). Thus, it is possible, at least in the case of VT2-Sakai and possibly that of VT1-Sakai, that the phage is viable but, like phage φ80, is unable to infect C600 (116). If another *E. coli* K-12 strain had been used to test for plaques, it is possible that plaques would have been observed (see below for further discussion).

When the bacterial genome is considered, nearly one-half of the strain-specific sequences of the Sakai genome are of a phage origin, and adding the sequences of what appear to be

prophage-like elements raises the fraction of strain-specific sequences that are of a phage origin to about two-thirds (146). Moreover, significant differences were observed when the sequences of prophages found in Sakai were compared with the genomes of prophages found in other O157 strains. In fact, these differences contribute significantly to the high genomic variation observed among the different O157 isolates.

RELEVANT FEATURES OF LAMBDOID PHAGES

The identified Stx-encoding phages are all members of the lambdoid family. However, like all members of that family, they can differ in their genetic makeup, exhibiting variations in their sequences, genomic restriction patterns, integration site specificities, phage morphologies, and host range specificities (134, 146, 157, 160, 210). Nearly all lambdoid phages share a common genome arrangement that conserves the relative positions of both genes encoding products with similar activities and their associated regulatory signals (24). Such functionally related genes may differ substantially in sequence. Moreover, different lambdoid phages can share sets of genes, e.g., two phages may share the same repressor gene but have different genes encoding the adjacent N transcription regulation protein. However, those two phages may also share their *N* genes with other lambdoid phages. This ability to acquire different sets of genes provides lambdoid phages the capacity to generate recombinants that may have a selective advantage. Hence, lambdoid phages, including those carrying *stx* genes (88), can be thought of as sharing genes from a common genetic pool (69, 196).

Regulation of Gene Expression

Primarily based on more than 40 years of studies with phage λ, the details of the common regulatory scheme of lambdoid phages are well understood (55, 70). The regulatory circuitry operates through a cascade of gene expression controlled by both negative and positive regulators (Fig. 1). Although the regulatory schemes for these phages are nearly identical, the sequences of the regulatory proteins and their cognate sites can vary significantly. However, as indicated above, a lambdoid phage often will share some genes and sites with another lambdoid phage, and yet other genes and sites will be shared with a different set of lambdoid phages. For example, phages 933W and H-19B share the same N transcription regulatory protein and its site of action, NUT, but each has a different repressor-operator system (138, 139, 157).

We look briefly at the nature of the repressor binding sites, the operators of lambdoid phages, focusing on the λ paradigm. For an enlightening and in-depth presentation of this subject, see the book written by Mark Ptashne (159). The two λ operator regions, O_L and O_R, are located on either side of the *cI* gene (Fig. 1). Each of the operator regions is composed of three similar, but not identical, sequences that are composed of imperfect inverted repeats. Each inverted repeat binds a repressor dimer, but with different efficiencies; e.g., the strength of binding in the O_R region has the order $O_R1 > O_R2 > O_R3$ (Fig. 2). The operators were first identified by mutations in λ that result in the virulent phenotype (74, 83). λ virulent mutants are able to grow in λ lysogens because they have operator defects that reduce repressor binding. The repressor maintains lysogeny in two ways. First, pairs of repressor dimers bind at O_L and O_R, and these interactions are further stabilized by a higher order interaction of the operator-bound tetramers at O_L interacting with those at O_R, resulting in bending of the DNA between the two operator regions (45) and repression of the early promoters (see below) (164). Second, cooperative binding of repressor dimers at O_R1 and O_R2 maintains repressor synthesis during lysogeny by stimulating transcription from the P_{RM} promoter, which is discussed in more detail below.

Transcription of the λ genome initiates at two early promoters, P_L and P_R, whose sequences overlap with their respective operators, O_L and O_R (56). Following infection, initiation at these promoters begins the transcription

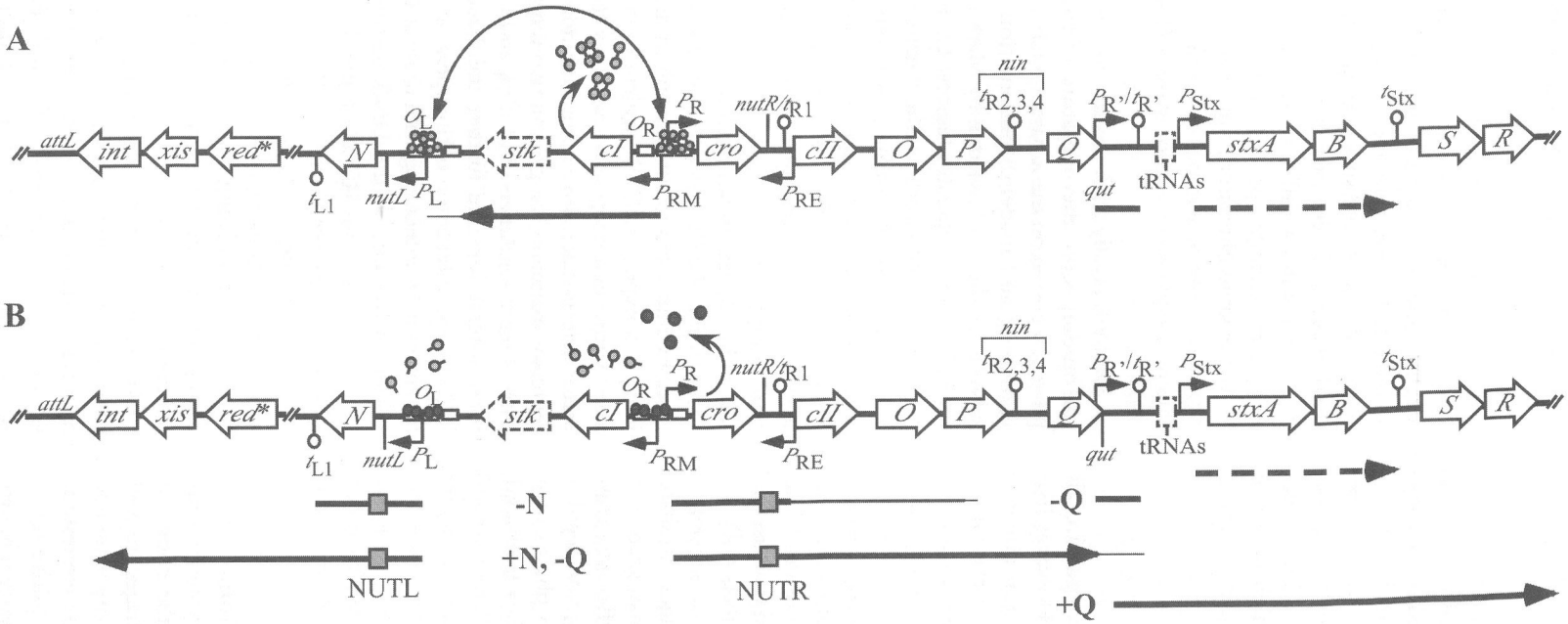

FIGURE 1 Composite genetic map of lambdoid phages showing the regulatory region and associated genes, including *stx* (see the text for details; not drawn to scale). ORFs identified by sequence analyses are shown as block arrows. Genes identified in only some Stx-encoding phages, including *stk* and the tRNA genes, are shown as block arrows with a dotted line border. Arrows are oriented in the direction of transcription of the indicated gene, based primarily on transcription studies with λ and with other phages where appropriate. Promoters are represented as line arrows, and transcription terminators are represented as lollipop structures. Transcription patterns during lysogeny (A) and during prophage induction leading to lytic growth and following the action of the N and Q antitermination proteins (B) are shown by lines below the genome maps. The mRNA NUT sequences are included as gray squares. The curved arrow spanning the *cI* region indicates the interaction between repressor proteins bound at O_L and O_R that further stabilizes repression. Transcription initiating at P_{stx}, which has been observed for phages encoding Stx1 under both prophage-repressing and -inducing conditions, is indicated as a dotted line. For simplicity, the genes encoding the recombination proteins Exo, Bet, and Gam are included in the drawing as a single ORF designated "*red**." In addition to repressing *cI* transcription, Cro binding at O_L and O_R later during lytic growth turns down transcription from P_L and P_R (46, 161).

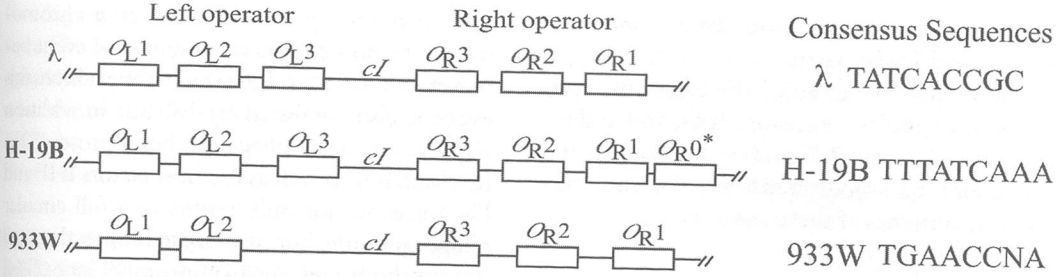

FIGURE 2 (Left) Comparison of the operator regions of λ, H-19B, and 933W depicting relative positions and spacing of the repressor binding repeats (not drawn to scale). (Right) Consensus sequences of operator repeats for each of the phages.

cascades that lead to either lysogeny or lytic growth. The *cI* gene is in the P_R operon, and its product determines the direction taken by the infecting phage. The CII protein (72, 222), whose activity is modulated by a number of host functions, acts to turn on the transcription of two genes whose products are essential for lysogeny, namely, the *cI* gene, encoding the repressor, and the *int* gene, encoding the integration protein (Int). Additionally, CII activates transcription that interferes with lytic gene expression (73). The Int protein, in conjunction with host factors, catalyzes the insertion of the circular phage genome into the bacterial chromosome (105). The integrated prophage is stably maintained in a quiescent state by a repressor that is expressed in the absence of CII (66). As discussed above, the maintenance of CI expression is autogenously regulated. Initial transcription of the *cI* gene is directed by CII when it activates transcription from the P_{RE} promoter (Fig. 1). Later, when P_R transcription and thus *cII* expression are turned off, the repressor is expressed when CI itself stimulates transcription from the P_{RM} promoter (Fig. 1) (159).

The N protein, which is required for the expression of most phage functions (38, 53, 54, 56, 165), is expressed immediately following infection or induction. N acts at sites, called NUTL and NUTR, in the RNA that are located downstream of the early promoters P_L and P_R, respectively (Fig. 1). Note that the following convention will be followed: names of sites in the RNA are given in uppercase letters (e.g., NUT) and those in the DNA are given in lowercase italics (e.g., *nut*). N binding to NUT RNA in concert with a collection of *E. coli* proteins, referred to as Nus proteins, acts to modify RNA Pol to a form that overrides transcription termination signals, i.e., it acts as a transcription antitermination factor. With the exception of N, all phage-encoded functions required for lytic growth ultimately derive from transcription initiation at P_R. The *cro* gene, located immediately downstream of P_R, encodes an antirepressor that serves to ensure that repressor expression is turned off during lytic growth by also binding to the operators (66, 159). Downstream of *cro* is the *nutR* site, which is followed by the *tR1* transcription terminator, the *cII* gene, and the *O* and *P* replication genes. In the absence of N, about 40% of the transcription complexes transcend *tR1* (35), pass through *O* and *P*, and stop at terminators in the *nin* region (30). When N is expressed, transcription complexes initiating at P_R and subsequently modified by N through interactions with Nus proteins and NUTR RNA efficiently transcend the *tR1* and *nin* terminators. N-mediated transcription propelled through the *nin* region extends through Q. Like N, Q is a transcription antitermination protein (170). However, unlike N, Q binds to a DNA site, *qut*, that overlaps in part with the lytic P_R' promoter (Fig. 1). Transcription from P_R' is constitutive, but in the absence of Q, it terminates ~200 nucleotides beyond P_R' at the t_R' terminator. When mediated by Q, transcription initiating at P_R' extends through t_R' and genes encoding lysis functions into regions encoding proteins involved in morphogenesis; in λ, this antiterminated transcript is ~26 kb long.

Replication, Integration, Excision, Lysis, and Induction

We will only briefly cover the topics of replication, integration, excision, lysis, and induction. See Chapters 2, 3, and 4 of this book and the other sources indicated below for more rigorous treatments of these subjects.

REPLICATION (60, 198)

The genes encoding replication functions, named *O* and *P* in λ, are located downstream of *d*II (Fig. 1). Consistent with the cassette structure of lambdoid phage genomes, all of the λ-encoded replication functions are located in this region, with the origin of replication being located within the *O* gene. The O and P proteins are required for the initiation of DNA synthesis, with the host DNA polymerase carrying out the replication process. The replication genes of the *stx*-encoding phages H-19B and 933W vary only slightly from the *O* and *P* genes of λ (137, 157).

INTEGRATION AND EXCISION (105, 135, 218)

The linear infecting λ genome circularizes via its complementary single-stranded ends and integrates (recombines) into the bacterial genome (26). Two functions, the Int protein and the CI repressor, must be expressed for these genomes to become stable prophage (discussed above). The Int protein, with the bacterial IHF protein, catalyzes the site-specific recombination that results in integration. This activity is said to be site specific because recombination occurs at unique sites, called attachment, or *att*, sites, on the phage genome (*attP*) and the host chromosome (*attB*). As a general rule, lambdoid phages have different attachment sites (*attB*) in the bacterial chromosome. However, some do share sites, e.g., H-19B shares the same *attB* site as HK022 (109). Integration generates hybrid sites, *attL* and *attR*, at the junctions between the phage genome and the bacterial chromosome that differ from *attP* and *attB*. All four sites share a common core within which site-specific recombination occurs.

The prophage exits the bacterial chromosome by a site-specific recombination event between *attL* and *attR*. Referred to as excision, this event is also catalyzed by Int but in addition requires a second phage-encoded protein, Xis (excisionase), as well as the host factors IHF and Fis. Excision not only results in a full circular phage genome but also reestablishes the *attB* site on the bacterial chromosome. The excised phage genome enters the lytic cycle and is ready to be transcribed and replicated, leading to the packaging of progeny DNA into phage particles that are ultimately released by phage-directed lysis of the bacterial host.

LYSIS (see Chapter 3 and references therein for a detailed discussion)

The set of genes involved in lysis (*S*, *R*, *Rz*, and *Rz1*) is located immediately downstream of the *stx* genes in phages that carry *stx* genes and, as in λ (216), are expressed from P_R' (138, 157) (Fig. 1). In λ, the *R* gene encodes an endolysin that degrades the peptidoglycan of the bacterial cell wall. R does not have a signal sequence and thus requires the action of the S protein to transit the cell membrane. S, a transmembrane protein, is a member of the holin family that disrupts the cytoplasmic membrane, allowing R into the periplasm at the proper time during infection.

PROPHAGE INDUCTION (this topic is covered in detail in Chapter 4 and references therein)

Although the prophage state maintained by the CI repressor is quite stable, the growing population of lysogens always has a small fraction that loses repression and becomes induced. The induced fraction can be greatly increased if lysogens are treated with DNA-damaging agents that activate the expression of the SOS system. Specifically, the bacterial RecA protein activated by bound single-stranded DNA binds and facilitates autocleavage of the phage repressor, causing prophage induction. DNA-damaging agents (e.g., mitomycin C or one of the quinolone antibiotics) lead to activation of the

RecA protein and thus cause prophage induction. The low-level release of phage from lysogens without the addition of an inducing agent was originally called spontaneous phage release (112), but we will refer to it as spontaneous induction.

INDUCING AGENTS AND Stx PRODUCTION

An additional role for the *stx*-encoding phages beyond serving as vectors for the transfer of *stx* genes was first indicated by studies showing that agents that induce prophages also increase the expression of Stx. For example, Head et al. (68) showed that treatment of STEC with mitomycin C increases Stx expression 100-fold. The fact that prophage induction can influence the course of infection was noted by Acheson and Donohue-Rolfe (1). They pointed out that the association of HUS with the use of mitomycin C in cancer treatment (106) may have resulted from an induction of prophage in an infecting STEC strain, which in turn resulted in increased Stx expression. In another study (224), mitomycin C treatment of a STEC strain led to both phage production and increased Stx expression. The *stx* genes could not be identified in the DNA isolated from the phage particles, leading the authors to conclude that the *stx* genes were not carried on the genome of a prophage (224). However, the possibility that the *stx* genes were located within the genome of a defective prophage was not ruled out.

Succeeding studies showed that several antibiotics that are commonly used to treat gastrointestinal infections induce both prophages and Stx expression. In particular, the quinolone antibiotics (e.g., ciprofloxacin) that are commonly used to treat infections causing diarrhea have been shown to be potent inducers of both phage and Stx production (101, 117, 228). In one study, antibiotics affecting the cell wall (e.g., amoxicillin) also induced the expression of higher levels of Stx (97). However, whether concomitant prophage induction occurred was not determined. Results from other studies yielded data supporting the opposite conclusion. Zhang et al. (228) showed with both in vitro and in vivo studies that treatment with fosfomycin, an inhibitor of peptidoglycan synthesis, did not increase either Stx expression or phage production, while treatment with ciprofloxacin, a gyrase inhibitor, induced both. Teel et al. (199) similarly found that while ciprofloxacin induced Stx production, fosfomycin failed to induce Stx production from the stx_{2d1}-encoding prophage ϕB2F1 in the STEC strain B2F1. They also found that an in vivo treatment of mice infected with B2F1 with ciprofloxacin increased Stx2d1 levels. A study from the Cohen lab (124) sheds light on the question raised by the results of treatment with amoxicillin. That study reported that β-lactam antibiotics, by inducing the expression of DpiA through an interaction with the DpiBA two-component system, can induce the SOS response in *E. coli*.

Clinically, the antibiotic treatment of STEC infections has been correlated with a significantly higher incidence of secondary sequelae such as HUS (18, 29, 153, 221). For example, in one prospective cohort study (221) of 71 children, 9 received antibiotic treatment. Ten developed HUS, and half of these were treated with antibiotics. Of the five who received antibiotic treatment, two were treated with trimethoprim, which affects DNA metabolism and would be expected to activate the SOS response. Three were treated with cephalosporin which, like amoxicillin, is a β-lactam and thus, as discussed above, can induce an SOS response (124).

In contrast to the work discussed above, STEC strains have been isolated in which Stx expression is not significantly elevated after mitomycin C treatment (168, 199). This subject will be covered in more detail below.

RELEVANT BIOLOGY OF *stx*-ENCODING PHAGES

We focus our discussion on the *stx*-encoding phages that have been best characterized biologically: H-19B and 933W. These phages have different operator-repressor systems but share the same *N*-NUT and *Q*-QUT genes and sites. Although H-19B encodes stx_1 and 933W

encodes stx_2, both sets of *stx* genes are located at the same relative position in their genomes, downstream of the lytic P_R' promoter.

In addition to H-19B and 933W, other *stx*-encoding phages have been partially or completely sequenced. Table 1 lists some relevant information about those phages as well as references to the papers reporting the sequences. All of these phages are members of the lambda family, sharing at least some of the same genomic elements. Moreover, some share the same regulatory genes and sites, e.g., *cI*-operators, Q-P_R', lysis genes, and attachment sites.

Studies by Mühldorfer et al. (131) suggested that functions expressed from 933W and H-19B activate the expression of Stx. They used a plasmid containing a translation fusion between the proximal portion of the stx_{2A} gene, including the promoter and upstream sequences, and a *phoA* reporter gene. In lysogens with either a 933W or H-19B prophage and the plasmid, mitomycin C induction of the prophage led to significantly increased levels of Stx expression from the plasmid, as measured by PhoA activity. A subsequent study showed that the *trans*-acting factor is the Q protein (138). Consistent with this finding, sequence analyses showed that H-19B and 933W share the same Q protein and P_R' (138, 157). The *stx* genes are located downstream of P_R' and upstream of the phage lysis genes, and thus both sets of *stx* genes are under the control of Q. Because Stx is secreted into the periplasm without any identifiable mechanism for release from that space, there has been a question of how Stx is released from the bacterium (141). It has been proposed that phage-mediated lysis may be the answer to this question (138). Hence, effective Stx production and/or release may depend on transcription from P_R' and ultimately on lysis of those bacteria in which the *stx*-encoding prophage is induced. In the absence of an inducing agent, such as an antibiotic, only a small portion of the population is spontaneously induced (110) to produce and/or release Stx.

Studies with the stx_2-encoding lambdoid phage ϕ361, a relative of 933W, provided compelling evidence that *stx* expression can be primarily dependent on transcription from the phage P_R' promoter (213). The ramifications of this idea will be discussed below. ϕ361, ob-

TABLE 1 Relevant information from sequenced and partially sequenced Stx-encoding phage genomes[a]

Phage	*stx* gene	*attB* site	*N*	*cI*	*Q*	Holin cassette	tRNAs	Reference
H-19B	stx_1	K-12 min 22.7[b]	H-19B	+	H-19B	+	−	137
933W	stx_2	*wrbA*	H-19B	+	H-19B	933W	933W	157
VT2-Sakai	stx_2	*wrbA*	+	+	H-19B	+	933W	114
VT2-Sa	stx_2	*wrbA*	+	+	H-19B	VT2-Sa	933W	126
Stx2ϕ-I	stx_2	*wrbA*	H-19B	933W	H-19B	933W	933W	174
STX2ϕ-II	stx_2	*wrbA*	VT2-Sakai	VT2-Sakai	H-19B	933W	933W	175
Stx1ϕ	stx_1	*wrbA*	VT2-Sakai	VT2-Sakai	H-19B	933W	−	175
VT1-Sakai	stx_1	*yehV*	H-19B	+	H-19B	933W	933W	225
CP-933V	stx_1	*yehV*	+	+	H-19B	VT2-Sa	−	154
ϕP27	stx_{2e}	*yecE*[c]	ND	+	+	+	933W[d]	160
ϕB2F1	stx_{2d1}	ND	ND	+	−	+	933W	199
ϕSC370	stx_2	ND	ND	+	H-19B	ND	933W	133
ϕLC159	stx_2	ND	ND	+	H-19B	ND	933W	133
ϕ7888	*stx*	ND	ND	ND	+	+	−	193

[a]Data include the nature of the *stx* gene, the site of insertion in the bacterial genome (*attB*), the presence or absence of selected phage genes and, if known, their homology with a gene(s) in other lambdoid phages, and whether the phage carries putative tRNA genes. +, present and not related to a gene(s) in other known phages; −, absent; ND, not done.

[b]This *att* site is also used by phage HKO22 (109).

[c]The Stx2-encoding phage ϕ297 also integrates at *yecE* (41a).

[d]ϕP27 contains *ileZ* and *argO* but not *argN*.

tained from the clinically isolated O157:H7 EHEC strain 1:361, shares the *Q*, P_R', and stx_2 genes of 933W. For an assessment of the roles of P_R' and *Q* in Stx production and release, a derivative of a ϕ361 prophage with a deletion of the *Q*-P_R' region was constructed. The deleted prophage, ϕ361Δ*Q*-P_R', still retains the *stx* genes as well as the *stx*-associated promoter. A lysogen carrying a wild-type ϕ361 prophage produced measurable quantities of Stx, but a lysogen carrying ϕ361Δ*Q*-P_R' failed to produce measurable levels of Stx under identical conditions. In accord with these results, Northern blotting showed that an *stx* message was produced in the former lysogen but not in the latter, even though the P_{stx2} promoter was retained in the ϕ361Δ*Q*-P_R' prophage. The role of P_R' in Stx expression in vivo was assessed by use of a mouse model in which streptomycin was used to eliminate the normal flora. Mice were orally inoculated with either the 1:361 EHEC strain or an isogenic derivative with the Δ*Q*-P_R' deletion in the ϕ361 prophage. Stools from mice challenged with the original 1:361 strain had ~30 times the levels of Stx as did those from mice challenged with equal amounts of 1:361-Δ*Q*-P_R', even though the stools from both groups of animals had approximately equal numbers of 1:361 or 1:361-Δ*Q*-P_R' bacteria.

These results provide strong evidence that, at least for this *stx*-encoding phage, the lytic promoter P_R' plays a major role in Stx expression. Transcription of the *stx* genes initiating at P_R' depends on the expression of Q, which, in turn, depends on transcription from the early promoter P_R', which is repressed until the prophage is induced. Like the case with λ, after induction, transcription initiating at the P_R promoter of H-19B is modified by an interaction of the phage-encoded N protein and host Nus factors at NUT sites in the RNA (138). The same is true for 933W, which has the same N-NUT system as H-19B, with both differing from the N protein of λ (138, 157). However, unlike the N protein of λ, whose action requires the *E. coli* proteins NusB and ribosomal protein S10 in addition to NusA (55), the action of the N proteins of H19B and 933W requires NusA but neither NusB nor the ribosomal protein S10 (139). This requirement of fewer proteins for N action may allow for an extended host range relative to that of λ.

The results of two different types of studies in which the induction of *stx*-encoding phages was blocked are consistent with the idea that transcription from P_R is important for Stx expression. For one type of study, the $recA^+$ allele in the host bacterium was replaced with a mutant *recA* allele (59, 117, 134, 199). As discussed above, under physiological conditions, RecA is required for induction resulting from autocleavage of the repressor (see Chapter 4). For the other type of study, the autocleavage activity of the repressor was inactivated, thereby eliminating induction even under $RecA^+$ conditions. Based on studies with λ (189), a point mutation was placed in the 933W prophage *cI* gene (encoding the repressor), rendering the prophage noninducible, presumably because the repressor is uncleavable, hence the name ind^- (206). Neither *recA* mutant nor ind^- lysogens produced measurable levels of Stx. A failure in Stx expression was also observed when either the *recA* or ind^- lysogens were treated with mitomycin C, whereas the Stx levels in the rec^+ ind^+ controls were increased by orders of magnitude with this treatment. The results of the experiments with the ind^- prophage provide definitive evidence that prophage induction is indeed required for Stx expression from the 933W prophage. As a corollary, it follows that the *stx*-associated promoter P_{stx2} is unlikely to be a significant contributor to Stx expression, at least in the case of the stx_2-encoding phage used for these studies.

As discussed above, STEC strains in which Stx2 levels are not significantly elevated following mitomycin C treatment have been isolated. Ritchie et al. (168) reported that STEC strains isolated from patients with HUS showed a large increase in Stx production following mitomycin C treatment, while STEC strains isolated from cattle generally showed only a small increase in Stx2 levels following a similar treatment. Although the *stx* genes in the strains showing low levels of induction have associated

phage genes, the prophages are most likely defective.

The situation is somewhat different for the stx_1-encoding phage H-19B. Although the *stx* genes in this phage are located at essentially the same genomic position as the stx_2 genes in 933W, in addition to significant sequence differences between the stx_1 and stx_2 genes, the associated promoters, P_{stx1} and P_{stx2}, are quite different. In H-19B, the expression of Stx can occur independently of phage induction from the iron-regulated stx_1 promoter. In fact, following mitomycin treatment, there is little increase in Stx1 production by STEC strains isolated from patients with HUS (168). However, it is not known whether or not the stx_1 genes in these isolates are carried by defective prophages that, for any number of reasons, may be uninducible. If such were the case, then mitomycin C treatment would obviously have no effect on Stx expression. What is known is that if P_{stx} is removed from the *stx* genes carried by an intact H-19B prophage, then high levels of Stx are expressed from the lytic promoter P_R' after prophage induction (212). Additionally, induction leads to phage replication, which in this case increases the gene dosage of *stx*. As noted previously, the stx_1-encoding phage H-19B and the stx_2-encoding phages 933W and ϕ361 share the same Q gene and P_R' promoter. Although transcription from P_R' is not required for Stx expression from the H-19B genome, it is required for Stx release. This likely reflects the presence of a putative terminator downstream of *stx* and upstream of the phage genes encoding lysis functions (41, 195). Whereas transcription from P_{stx} would stop at a terminator downstream of the *stx* genes, Q-modified transcription initiating at P_R' would read through the terminator into the downstream genes, which include those encoding lysis functions. The relative contribution of these two promoters to Stx release was assessed in H-19, a clinical strain carrying an H-19B prophage (212). Stx release was measured either after growth in low iron, by monitoring transcription from P_{stx}, or after treatment with mitomycin C, by monitoring transcription from P_R'. After overnight growth, <1% of the Stx produced in the culture grown in low iron was in the supernatant, while >99% of the Stx produced in the mitomycin C-treated culture was in the supernatant. Thus, Stx release for this strain requires phage induction, presumably because it leads to phage-directed lysis.

Studies with the STEC strain B2F1 (199) confirmed the central role that prophage induction can play in Stx2 production but raised a question about the universality of Q control in lambdoid phages. B2F1 encodes two forms of the Stx2 variant Stx2d, Stx2d1 and Stx2d2 (79). Teel et al. (199) found that the genes encoding the two forms of Stx2d are nested within a group of genes which partially resemble those found in lambdoid phages. Derivatives of B2F1 with a knockout of either stx_{2d1} or stx_{2d2} were constructed. The derivative expressing Stx2d2 but not Stx2d1 exhibited the same cytotoxicity to cultured mammalian cells as the original strain expressing both forms of Stx. However, the derivative expressing Stx2d1 but not Stx2d2 exhibited a ninefold decrease in the level of cytotoxicity compared to the original strain. Further experiments revealed an even larger difference in toxicity. Treatment with mitomycin C resulted in large increases in the expression of Stx2d1 yet had no effect on the levels of expression of Stx2d2. Only phage particles carrying stx_{2d1} were identified after mitomycin C treatment of B2F1. Hence, it was concluded that Stx2d1 is encoded in a complete phage, ϕB2F1, and that the expression of the *stx* genes is controlled by the phage regulatory system. Stx2d2, on the other hand, is expressed constitutively, leading to the conclusion that it is encoded by a defective prophage (see below for further discussion).

An examination of sequences ~4 kb upstream of stx_{2d1} failed to identify a sequence with significant homology to any Q genes in the database. However, a 50-bp sequence that was 90% homologous to the 5′-terminal sequence of the 933W Q gene was identified. This short region likely encodes only a truncated nonfunctional fragment of a Q protein. However, the possibility of a gene encoding a hybrid Q, with the amino-terminal end derived from 933W and the rest of the protein de-

rived from an undiscovered Q protein or a Q protein with a sequence that differs in total from those of all known Q proteins, has not been ruled out. The observations of Teel et al. (199) that "the stx_{2d1}-flanking regions resembled other toxin-producing-converting phages in organization" and that Stx production is induced by mitomycin C make it likely that the expression of ϕB2F1 late genes, including *stx*, is under the control of a late gene regulator such as Q. In this regard, note that although in the initial analysis of the region of phage ϕP27 encoding stx_{2e}, a Q gene could not be identified (134), one was identified when sequencing was extended further upstream (160).

The results of these studies make a compelling argument that phage-directed events following induction are critical for Stx release in the case of stx_1-encoding phages and for both production and release in the case of stx_2-encoding phages. The phage activity directly involved is the Q-mediated modification of transcription initiating at P_R'. The expression of Q, however, requires transcription initiating at P_R, which, in turn, requires a release of repression, i.e., prophage induction. This means that, at least for the class of STEC strains with *stx* genes located in complete phages, only the fraction of the population undergoing induction is primarily responsible for Stx production and/or release. This raises the question of what fraction of the lysogen population is spontaneously induced.

For an answer to this question, an engineered lysogen, called SIVET, was constructed that not only allows enumeration of the fraction of lysogens that are induced, but also provides a way to select that population from an orders of magnitude larger population of uninduced lysogens (110). SIVET (for selectable in vivo technology), an extension of recombinase-based in vivo technology (22), allows the selection of a subpopulation of bacteria in a culture in which a particular promoter is turned on. To study the fraction of bacteria in cultures of H-19B and 933W lysogens in which prophages were induced, SIVET lysogens were constructed with the following features (110) (Fig. 3). The prophages were altered so that

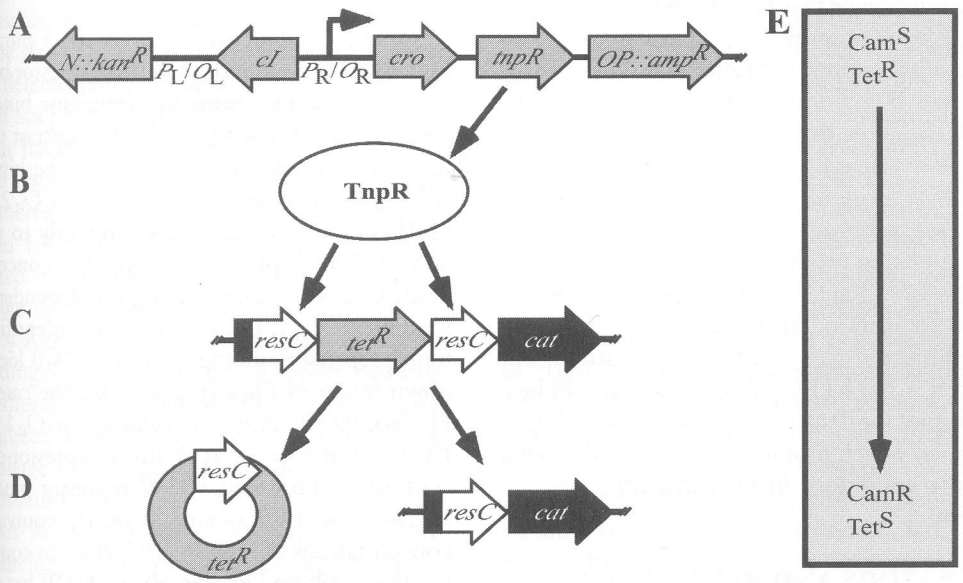

FIGURE 3 SIVET action. (A) Altered prophage carrying *tnpR*. (B) The induced prophage expresses TnpR. (C) Insert of *resC-tet-resC* cassette at a distal site on the bacterial chromosome. A single nucleotide change in each *res* site (resulting in *resC*) was required to allow expression of a functional gene product from the reconstituted *cat* gene. (D) Action of TnpR removes the *tetR* cassette from within the *cat* gene, leaving a functional *cat* gene. The DNA circle with the *tetR* gene does not replicate and is lost by segregation. (E) Action of TnpR makes a scoreable and heritable change, converting the bacterium from Tet^r Cam^s to Cam^r Tet^s.

their induction was not lethal to the host bacterium. The induced lysogens could be selected because, after the loss of repression, the prophage expressed a recombinase, the γδ resolvase, which specifically acts at sites called *res* sites (64). A *cat* gene disrupted by a *tetR* cassette flanked by *resC* sites was located at another site on the bacterial chromosome (see the legend to Fig. 3). With these constructs, resolvase-directed recombination occurring at the *res* sites removes the *tetR* cassette, resulting in the formation of a functional *cat* gene. The change is both irreversible and inheritable. Thus, the induced population can be selected because they and their progeny are resistant to chloramphenicol and sensitive to tetracycline, while the much larger uninduced population remains sensitive to chloramphenicol and retains resistance to tetracycline.

In experiments using either MC1000 H-19B or 933W SIVET lysogens grown to stationary phase, ~0.004% of the H-19B and 0.01% of the 933W SIVET lysogens per generation became chloramphenicol resistant, i.e., were spontaneously induced. These studies also provide evidence that H-19B and 933W exhibit higher levels of spontaneous induction than do three other lambdoid phages that do not carry *stx* genes. Based on an admittedly small sample, it was suggested that the repressor-operator systems of *stx*-encoding phages might have evolved to induce more readily than those of non-*stx*-encoding prophages. In this way, the induced subpopulation produces Stx and contributes to the growth advantage of the surviving members of the population. The SIVET system has yet to be transferred to a clinical STEC strain, but when it is, the STEC SIVET strain should be a useful tool for determining both the fraction of STEC in which prophages are induced in vivo and the conditions in vivo that influence induction.

OPERATORS AND REPRESSORS

The preceding discussion indicates that, at least for those *stx*-encoding phages that we have discussed, the nature of the interactions of repressors with operators is likely to play an important role in determining Stx expression and/or release. To this end, the operator regions of H-19B and 933W (Fig. 2) and their interactions with their cognate repressors have been characterized (103, 187, 206). Sequence analyses identified the two putative operator regions of H-19B (137) and 933W (49, 157). Isolation and sequencing of H-19B (187) and 933W (206) virulent mutants provided genetic evidence that the putative sites were indeed the O_R and O_L regions of these phages. As discussed above, the operator regions are composed of a series of imperfect inverted repeats that bind repressor dimers. The classical λ virulent mutant has mutations in the imperfect inverted repeats O_R1, O_R2, and O_L1 (74, 83, 159). However, it has since been determined that either of two mutations in O_R and one mutation in O_L or one mutation in O_R and two mutations in O_L are necessary and sufficient for virulence in λ (51). The only isolated virulent mutants of phage 933W contain three mutations in O_R and one mutation in O_L (206). In contrast to the case for λ, virulent mutants of phage H-19B only require one mutation in O_R and one mutation in O_L (187).

DNase I protection assays with a purified H-19B CI protein indicated that repressor binding at O_R differs from the repressor binding patterns seen for λ O_R (187). In contrast to λ, in which the repressor binds with the highest affinity to O_R1 and cooperatively to O_R2, the H-19B repressor binds independently to O_R1 and O_R2 and appears to bind O_R2 concomitantly with O_R3. This binding is independent of binding to O_R1. These studies also identified a fourth repressor binding site in O_R, O_R0, located downstream of O_R1 (Fig. 2). Like the case for H-19B, 933W repressor binding to O_R1 and O_R2 does not occur at identical repressor concentrations. However, 933W repressor binding to O_R2 and O_R3 occurs at nearly equivalent concentrations of protein (103, 206). In contrast to other lambdoid phages, phage 933W has only two identifiable inverted repeats in the left operator region (Fig. 2), and this was confirmed by in vitro binding studies with the purified CI protein (103, 206).

POSSIBLE IN VIVO INDUCING AGENT

The compelling evidence, both in vitro and in vivo, that the induction of lysogens with *stx*-encoding prophages leads to significant increases in Stx production and release suggests that the severity of a STEC infection may reflect the presence of inducing agents during the infection. Based on these considerations, Wagner et al. (211) postulated that H_2O_2 was a possible candidate for such an in vivo inducing agent. H_2O_2 is known to be an inducer of the SOS response (78) and is produced in vivo by scavenger cells such as neutrophils (33). Their study provided suggestive, if far from conclusive, evidence that exposure to H_2O_2 may play such a role. Treatment of the STEC strain 1:361 (carrying the ϕ361 stx_2-encoding prophage) with H_2O_2 led to a maximum, approximately threefold, increase in Stx production. Coculture of the 1:361 strain with human neutrophils led to an approximately fivefold increase in Stx production. The increase in Stx production observed in the presence of neutrophils was shown to result from induction of the *stx*-encoding prophage. A control strain in which the phage P_R' promoter, the major contributor to transcription of the *stx* genes in this strain, as discussed above, was deleted failed to show the same high levels of Stx production as those observed with the strain having an intact prophage when both were cocultured with neutrophils. Moreover, no increase in Stx production was observed when 1:361 was cocultured with neutrophils treated with diphenyleneiodonium, an agent known to specifically block the production of H_2O_2. Although the results of this study are intriguing, it still remains an open question whether there is sufficient H_2O_2 or other agents produced in infected individuals to lead to prophage induction resulting in enhanced Stx production.

An in vitro study (192) with the Stx2-producing EHEC strain 86-24 suggested that quorum sensing (223) may influence prophage induction, which, as we have seen, can lead to Stx expression and release. Gene array technology was used to characterize the RNAs obtained from 86-24 and a mutant derivative with a deletion of *luxS* which is thus unable to synthesize the autoinducer molecule that is active in this mode of cell-to-cell communication. Transcripts encoding gene products involved in the SOS response were found at an approximately 20- to 25-fold higher concentration in the $luxS^+$ strain than in the isogenic *luxS* mutant strain. However, an analysis of *stx* expression with an *stx*::*lacZ* transcription fusion indicated that the expression of the *stx* genes was approximately threefold higher in the the $luxS^+$ strain than in the isogenic *luxS* mutant strain. A corresponding difference was observed in the Stx levels, as measured by a Western blot that showed a sixfold higher level of Stx2 produced by the $luxS^+$ strain than by the *luxS* mutant. Hence, it was concluded that quorum sensing induces the bacterial SOS response and, as a result, prophage induction, resulting in an increase in expression of the *stx* genes.

stx GENES AND DEFECTIVE (CRYPTIC) PROPHAGES

Several *stx* genes have been reported to be encoded by defective prophages (67, 120, 127, 145, 154, 168). The term "cryptic" is used to describe defective prophages that have lost the ability to produce a repressor and thus do not express immunity. However, Campbell concluded that "the term has had limited utility," and he uses "the terms as synonyms" (27).

In some cases, prophages carrying *stx* genes have been designated as defective based on the identification of IS elements in their genomes or a failure to identify PFU corresponding to the prophages (67, 127). We offer a cautionary note in considering whether or not a prophage is defective. First, IS elements are assumed to inactivate the phage because they are often polar (61) and thus, even when located in an intergenic region, can interfere with the expression of downstream genes. However, lambdoid phages have highly effective transcription antitermination systems and thus can suppress the effect of IS polarity, allowing the expression of essential genes located downstream of the IS element (12). Hence, the presence of an IS insertion in a lambdoid phage, provided it does not

inactivate an essential gene, does not necessarily mean that the phage is defective. Second, the failure to observe the release of PFU from a STEC strain with a prophage carrying *stx* genes does not necessarily mean that the prophage is defective. The choice of *E. coli* strain used for the lawn may determine whether plaques are observed. As previously discussed, C600, a K-12 strain that is frequently used to look for plaques, has a mutation in the *tonB* gene that makes it resistant to infection from the lambdoid phage ϕ80 and hybrid phages carrying the *h* gene of ϕ80 (116).

The stx_1-encoding prophages CP-933V and Sp15 (VT1-Saki), found in the O157:H7 strains EDL933 and Sakai, respectively, are reported to be defective (67, 142, 145, 154) either because they fail to produce observable phage particles or because they have IS sequences. These prophages may not be able to produce viable phage particles, yet they appear to have genome sizes sufficient to encode all of the functions necessary for viability (145, 154). Although plaque-forming phage were not identifiable with VT1-Sakai, phage particles that carry stx_1 genes, as assessed by PCR analysis, were identified (225).

The release of a viable phage carrying *stx* genes from a STEC strain would appear to be rather conclusive evidence that the prophage with those *stx* genes in the STEC strain encodes a viable phage. However, as early studies with *stx*-encoding phages have shown, such a conclusion may not always be warranted. EHEC strain EDL933 was the subject of very early studies examining the question of whether the genome of the stx_1-encoding prophage encodes a viable phage. As discussed above, 933J was first identified as the product of induction of the stx_1-encoding prophage in EDL933 (143). Although two later studies concluded that 933J was not derived from EDL933, they differed as to whether the stx_1-encoding prophage served as a template for the production of a viable phage. One group identified a phage that was not related to 933J but was released from EDL933 and carried the stx_1 genes (166), while the other group reported that a phage carrying stx_1 could not be isolated from the same strain (142). Another prophage in EDL933, 933W, which carries stx_2, was reported by both groups to encode viable phage production (143). Two things appear peculiar about the finding of a viable stx_1-encoding phage by Rietra et al. (166). First, following UV induction of the EDL933 strain, only 0.2% of the phage particles had the stx_1 genes, while the rest had stx_2 genes. Second, the two phages were indistinguishable morphologically, had the same size of genome, and had a high level of DNA homology. These findings suggested to O'Brien et al. (142) that the viable stx_1-encoding phage was a product of recombination between a defective stx_1-encoding prophage and 933W. This explanation is supported by information gained from the recent sequencing of EDL933 (154) showing that although the prophage carrying the stx_1 genes, now called CP-933V, shares some sequences with 933W, it also has extensive differences. Therefore, it is unlikely that the isolated stx_1-encoding phage originated from CP-933V, and it seems likely that the viable stx_1-encoding phage was a product of recombination in the *stx* regions of CP-933V and 933W. Accordingly, this recombination generated a phage that was primarily derived from 933W but that had stx_1 genes in place of stx_2 genes. The observation that the two phages share homologies on either side of the *stx* genes is consistent with this idea. This example also serves to show that the production of a viable phage carrying an appropriate genetic marker does not necessarily mean that the prophage with that marker encodes a viable phage.

Few of the *stx*-encoding prophages identified as defective (or cryptic) have been characterized regarding the nature of the regulation of expression and the mode of release of the encoded Stx proteins. As discussed above, Teel et al. (199), in their studies of the STEC strain B2F1, identified two Stx-encoding phages. One of these, the Stx2d2-encoding phage CP-B2F1, was considered defective primarily because phage particles encoding Stx2d2 could not be observed. This study was particularly interesting

because of the finding that the CP-B2F1 prophage produced significantly more Stx than the viable Stx2d1-encoding prophage ϕB2F1. Moreover, while a mitomycin C treatment of B2F1 resulted in significantly higher levels of ϕB2F1-encoded Stx2d1, the treatment did not affect the levels of CP-B2F1-encoded Stx2d2. Hence, the *stx* genes carried by the intact prophage appear to be under phage control, while the *stx* genes of the putative defective phage are constitutively expressed and are not under phage control. The limited amount of sequencing of these prophage genomes reveals that, as with other *stx*-encoding phages, both ϕB2F1 and CP-B2F1 carry the homologs of the *S* lysis gene downstream of the *stx* genes. The phage-encoded lysis genes were proposed to be major contributors to the release of Stx when the *stx* genes are carried in the genome of an intact prophage (138). It follows, then, that induction of the prophage leads to the expression of Stx and downstream lysis functions. The high level of constitutive expression of Stx from the CP-B2F1 prophage may lead to expression of the downstream lysis genes. Because terminators can be leaky (31), some readthrough into the lysis genes is likely to occur even if, as has been shown for other *stx* genes (41, 195), the *stx* genes of CP-B2F1 have an associated terminator. This may explain why the *S* gene downstream of the stx_{2d2} genes in that defective prophage has an internal stop codon. Based on these considerations, one can imagine that selection for the constitutive expression of *stx* from the CP-B2F1 prophage genome led, in turn, to the selection of an inactivated lysis gene.

Among the numerous questions raised by these studies that remain to be answered, three that have not been addressed in any detail pique our curiosity. First, are the phage promoters involved in the expression of Stx by defective phages, and if not, what promoters are involved? Second, is Stx expression from defective prophages regulated? Third, if genes encoding an intact phage lysis system are not located in *cis* with the *stx* genes, how are the products of the latter released from the bacterial cell?

DIVERSITY OF Stx-ENCODING PHAGES

Several studies have reported on *stx* genes and their relationship to lambdoid phages. However, it is beyond the scope of this chapter to summarize all of them. Hence, we have selected a few of the studies that we think are most representative for further discussion.

Wagner et al. (210) isolated seven *stx*-encoding phages from clinical STEC strains that were placed in five different restriction fragment length polymorphism (RFLP) groups. No relationship between RFLP patterns and the serotype of the parent STEC was discerned. Comparisons of Stx expression normalized against the total cellular protein yielded some surprising results. Lysogens constructed from the same *E. coli* strain, MC1000, with each carrying one of the seven phages, produced Stx at significantly different levels, with variations of as much as 800-fold in overnight cultures. Significant differences in the levels of Stx were also observed among the clinical strains from which the phages were isolated. There was little correlation between the relative Stx levels in a particular clinical strain and in the MC1000 lysogen carrying the prophage from the same clinical isolate. However, a correlation between Stx production in the MC1000 derivatives and phage production was observed. Because the clinical strains carried several prophages, phage production in those strains was not measured. The correlation between Stx and phage production in MC1000 lysogens is consistent with the idea that the subpopulation in which the prophage is induced is responsible for Stx production, at least in the case of bacteria carrying stx_2-encoding phages. The reasons for the observed differences in phage production between the different strains, however, are not obvious. Wagner et al. (210) suggest that differences in binding by the different phage repressors might explain these differences in phage and Stx production. However, this explanation, as they pointed out, fails to account for the observed differences between MC1000 derivatives and the clinical isolates with the same *stx*-encoding

prophages. One other explanation is the possibility of different numbers of *stx*-encoding phages in the lysogens. Multiple prophage of the same type, of course, would influence the copy number of both the *stx* and *cI* genes. However, it is unlikely that a two- or threefold increase in these genes could explain the large observed differences in Stx production. The differences in Stx expression observed between the clinical and laboratory strains may have resulted from differences in the ability of the hosts to support phage gene expression (57).

Unkmeir and Schmidt (208) examined 40 independently isolated non-O157 STEC strains and showed that in nearly all of these strains, the *stx* genes were surrounded by phage sequences. A PCR assay with an H-19B-specific set of primers showed that all 20 of the Stx1-expressing strains examined had phage-specific sequences in the *stx* region that were similar to those of H-19B. Phage-related sequences in the *stx* region similar to those of 933W were found in 19 of 20 Stx2-expressing strains examined by a PCR assay with a 933W-specific set of primers.

These studies provide overwhelming evidence of the relationship between *stx* and lambdoid phage genes, suggesting that at one time all of the *stx* genes in these strains were part of phage genomes. It also appears that in many STEC strains, prophages have gone through extensive rearrangements that include deletions of large genetic components.

Stx PHAGES IN *SHIGELLA* SPP.

The observation that *S. dysenteriae* type 1 has genes which are highly homologous to those in lambdoid phages both upstream and downstream of the *stx* genes, albeit with intervening IS sequences (120, 208), makes it plausible that the current genomic arrangement around the *S. dysenteriae* type 1 *stx* genes evolved from a prophage. Thus, the *stx* genes may well have been imported into *S. dysenteriae* by a phage whose genome was subsequently partially deleted and invaded by IS elements. Whether the *stx*-encoding phages found in *E. coli* genomes derived from *S. dysenteriae* through phage transfer or from a common progenitor bacterium is an open question.

Additional information was brought to bear on the question of phage and lateral transfer of *stx* genes with the report of a *Shigella sonnei* strain, CB7888, isolated from a stool sample obtained from a patient suffering from diarrhea, that expressed Stx1 from genes encoded in a prophage (9). This was the first report of an *S. sonnei* strain carrying *stx* genes. A comparison of the sequences of the *stx* genes from this strain with those of the *stxA* and *B* genes of *S. dysenteriae* type 1 showed that the *stxA* genes are identical, while the *stxB* genes differ by only a single nucleotide. Because this sequence difference results in a replacement by a synonymous codon, the amino acid sequences of the two *stxB* genes are also identical. A phage, 7888, isolated from this *S. sonnei* strain was shown to transduce *stx* genes to non-*stx*-encoding *S. sonnei* and *E. coli* K-12. The fascinating aspect of this report is that the *stx* genes encoded by the phage resemble those in *S. dysenteriae* type 1 rather than those in *E. coli*, which are commonly identified in resident prophages. Moreover, as the authors suggest, these results imply that the phage may have acquired the *stx* genes from *S. dysenteriae* type 1. Further studies (193) showed that the genome of phage 7888 is related to that of 933W.

ACCESSORY FUNCTIONS OF LAMBDOID PHAGES: PROVEN AND POSSIBLE VIRULENCE FACTORS

The lambdoid family of phages share similarities in genetic organization, but at some identical positions in their genomes, lambdoid phages often contain insertions of genes encoding proteins with unrelated functions (25, 69) (see Chapter 5). Such genes are considered accessory genes in the sense that they appear not to be required for lytic growth or the maintenance of lysogeny under laboratory conditions (reviewed in reference 36). However, some of these accessory genes are expressed and useful in the prophage state because they add to the fitness of the host lysogen (24, 36). The *stx* genes obviously are prime examples of accessory functions car-

ried by lambdoid phages that, although not contributing to successful lytic growth or the formation of a stable prophage, presumably provide fitness to their bacterial hosts. However, there are several examples of other genes, carried by both *stx*-encoding and non-*stx*-encoding lambdoid phages isolated from STEC, that either have been demonstrated to contribute to or look like they might contribute to the pathogenic potency of their bacterial hosts.

Bor and Lom

To begin this discussion, we look at the experimental grande dame of the family, phage λ. Among its accessory genes, two, *lom* and *bor*, encode functions that are apparently nonessential for the phage but have activities capable of contributing to the successful growth of the lysogenized bacterium in a mammalian host (7, 8). Both are expressed by the λ prophage; Lom is implicated in conferring enhanced adhesion to mammalian cells, and Bor is implicated in increased serum resistance (7, 8, 209). Putative *lom* and *bor* homologs have since been identified in the genomes of several Stx-encoding transducing phages and cryptic prophages present in the genomes of STEC strains (114, 145, 157, 175).

$EspF_U$

The cryptic prophage CP-933U, which is found in the genome of the EHEC strain EDL933 (154), encodes a protein, $EspF_U$, that is delivered into eukaryotic cells via the LEE-encoded type III secretion apparatus (28). In the host cell, $EspF_U$ associates with the already present EHEC Tir protein, promoting the recruitment and polymerization of actin. For this reason, $EspF_U$ is critical for pedestal formation in cell culture experiments. However, it is has not yet been determined if $EspF_U$ is essential for virulence in an animal model, nor has it been determined if regulatory functions of the CP-933U prophage influence the expression of *esp*F_U.

NleA

The gene encoding NleA, a protein delivered into eukaryotic cells by the LEE-encoded type III secretion system, was identified in the genome of the EHEC strain EDL933 in a putative pathogenicity island that is associated with the cryptic prophage CP-933P (65). In addition, *nleA* was identified in the genomes of other pathogenic *E. coli* strains in regions adjacent to or within predicted cryptic prophage genomes. In the eukaryotic cell, NleA localizes to the Golgi apparatus. Although the function of NleA has yet to be determined, it is known that it is required for virulence in animal models of infection. Like the case for $EspF_U$, it is unclear if the regulatory functions of the prophage influence NleA production in those pathogenic *E. coli* strains in which the *nleA* gene is in a prophage genome.

Cif

A gene encoding Cif, so named because it inhibits the eukaryotic cell cycle (cycle inhibiting factor), is transported into eukaryotic cells by the LEE-encoded type III secretion system (115). Genes encoding Cif have been identified in both EPEC and EHEC strains. However, the *cif* genes identified in EHEC strains appear to encode nonfunctional proteins, and they are not found in the genomes of either of the two sequenced O157:H7 strains. The *cif* gene is flanked by open reading frames (ORFs) that have been identified in cryptic prophages of EHEC strains; however, as far as we are aware, it has not been determined whether the *cif* gene is carried in a lambdoid phage in EHEC genomes. Although Cif is reported to block the cell cycle transition from G_2 to M in the eukaryotic cell, its contribution to the pathogenesis of EPEC or EHEC strains has not been determined as of this writing. What is known is that Cif does not influence attaching and effacing by either pathogen.

Stk

The 933W genome carries an ORF, *stk*, that, based on its amino acid sequence, was originally annotated as encoding a eukaryotic-like serine/threonine protein kinase (PK) (157). However, enzymatic studies showed that *stk* encodes a PK with only tyrosine kinase activity (205). Nine of 30 STEC strains independently isolated from infected patients over a 20-year

period carry the *stk* gene. Located downstream of the *cI* gene (Fig. 1), *stk* is cotranscribed with *cI*. A functional role for Stk has yet to be determined. In vitro studies using a derivative of 933W with a deletion of *stk* failed to show any obvious phenotypic changes, indicating that Stk is unlikely to contribute to either lytic growth of the phage or in vitro growth of a lysogen carrying the phage. Because it resembles a eukaryotic PK rather than a bacterial PK, one plausible, but as yet untested, role for Stk would be contributing to bacterial virulence through some effect of its PK activity in eukaryotic cells. Such an activity has been shown for kinases and phosphatases of other pathogens (43), and like those enzymes, Stk might be transferred to the eukaryotic cell.

tRNA Genes

Three putative tRNA genes are found upstream of most phage-associated stx_2 and stx_2 variant genes (Fig. 1). Based on homologies to known tRNA sequences and the prediction that the encoded RNAs can be folded into the cloverleaf structures of tRNAs, these genes appeared to encode tRNAs and were designated *ileZ*, *argN*, and *argO* (114, 126, 157, 180, 199, 225). What makes these observations relevant to our discussion is the finding that stx_{2A} contains a large number of rare isoleucine and arginine codons that are cognate to the inferred activities of the putative tRNA species encoded in the genomes of these phages (157, 180). Studies with *E. coli* demonstrated a direct correlation between the frequency at which a codon occurs in a genome and the quantity of its cognate tRNA species produced (77). Therefore, a high occurrence of rare codons within an mRNA species can lead to defects in translation due to ribosome stalling, including premature termination, frameshifts, misincorporation, and ribosomal hopping (40). However, increasing the concentration of tRNA species cognate to the rare codons can overcome these translational difficulties (19, 42). Hence, these phage-encoded tRNA species may supplement the host tRNA pool, thus augmenting the efficiency of translation of the stx_{2A} message.

The location of these putative tRNA genes downstream of the P_R' promoter (Fig. 1) means that they, like the *stx* genes, are transcribed as components of the phage late transcript. However, there is a putative promoter and a putative downstream transcription terminator associated with the tRNA genes (157), indicating that these tRNAs could be expressed by the repressed prophage. If expressed by the prophage, these tRNAs could influence the expression of *stk*, which, like stx_{2A}, has an abundance of rare isoleucine and arginine codons (157). The requirement of rare tRNA species for the translation of virulence factors has been observed for other pathogens (169, 203). Moreover, the coliphage T4 encodes several tRNA species which are thought in some cases to complement the host repertoire of tRNA species, accommodating the low G+C content of the phage genome (reviewed in reference 130).

ORFs Encoding Possible Virulence Factors

Sequencing projects have identified other ORFs in lambdoid phages whose putative products may contribute to the virulence of the host bacterium. There was no evidence at the time of this writing that these genes encode functional products or even that products of these genes are expressed by the host bacterium. Therefore, it is important to bear in mind that it is no more than informed speculation to propose that the following putative gene products contribute to the virulence of the host bacterium.

SOD

Two examples of such phage-carried ORFs are the putative [Cu,Zn] superoxide dismutase (SOD) and catalase genes identified in the genomes of lambdoid prophages present in O157:H7 STEC strains (145, 154). In other bacterial pathogens, SOD and catalase proteins neutralize superoxide radicals and H_2O_2, respectively, and through this activity, they enhance intracellular survival. The [Cu,Zn] SOD encoded by the Gifsy-2 prophage of *Salmonella enterica* serovar Typhimurium has been shown to

contribute to the virulence of the host bacterium (50) (for more details, see Chapter 8). Unlike what was found with *Salmonella*, there is no evidence that STEC strains are intracellular at any stage during infection. However, as discussed, low doses of Stx have been demonstrated to induce oxygen free radical production by PMNs in vitro (98). Therefore, these prophages may contain genes to protect the host bacterium from oxygen radicals in the lumen. The possibility that STEC carries phages with genes encoding a product that contributes to the bacterium's ability to inactivate reactive oxygen compounds poses an apparent contradiction. We previously discussed the possibility that reactive oxygen compounds, by their action in inducing prophages, lead to increases in Stx production and release. By reducing the levels of reactive molecules, these enzymes would reduce phage induction and thus Stx production and release, but at the same time they would contribute to the protection of the bacterial population from the untoward effects of the reactive molecules. It is difficult to extrapolate results from the in vitro studies of Wagner et al. (211) to the in vivo situation. Thus, we are left with the question of whether the levels of reactive molecules present during an infection are sufficient to override the bacterial defenses to result in bacterial DNA damage sufficient to induce the prophage and, in turn, Stx expression.

Ehly2

ORFs encoding products with sequence similarity to what had been described as an enterohemolysin, Ehly2, have been identified in the genomes of temperate phages, including those of several *stx*-encoding phages (10, 114, 126, 157, 174, 175, 225). Ehly2 is distinct from the plasmid-encoded enterohemolysin (Ehly1) and the *E. coli* alpha-hemolysin with respect to cell specificity, serology, and epidemiology (10). Although hemolytic activity has been demonstrated for Ehly2, it appears that this activity is not directly due to the action of Ehly2. Rather, the reported hemolysis is due to lysis of the bacterium caused by Ehly2 and the release of cytolysin A, which is responsible for the observed lysis of red blood cells (148). The sequences of the putative Ehly2 genes in STEC also have similarity to the λ ORF encoding Ea22, which plays a role in blocking the initiation of *E. coli* DNA synthesis, causing the Bin phenotype (185).

DNA METHYLASE

Putative DNA adenine methyltransferase genes have been identified in several *stx*-encoding phages (114, 134, 157, 174, 175, 225). Although Dam, which methylates the A in GATC sequences, has been shown to influence the virulence of a number of pathogenic bacteria (111), it is not known whether other DNA adenine methylases that probably do not have the global effects of Dam have any influence on virulence.

Trc

Resident lambdoid prophages of the O157 Sakai strain contain two ORFs that encode products with similarities to the amino acid sequence of the EPEC TrcA protein (67). TrcA serves as a cytoplasmic chaperone that functions to stabilize the precursor of bundle-forming pili (204). In this way, it contributes to the adherence of EPEC to host cells. TrcA was also demonstrated to interact with intimin, a protein which is essential to virulence. However, the physiological relevance of this interaction has not yet been defined. Although EHEC strains do not produce bundle-forming pili, they do express intimin. Therefore, a bacteriophage-encoded TrcA-like protein may function as a chaperone for intimin or other unidentified proteins.

CONCLUDING THOUGHTS

The study of STEC strains has provided new information highlighting the importance of phage genomes as components of the bacterial chromosome. Complete or incomplete phage genomes, particularly those of lambdoid phages, account for a large portion of the strain-specific sequences in O157:H7 strains (17, 67, 145). In addition to *stx*, these lambdoid genomes also encode other known and putative virulence factors. Because they belong to the well-studied λ family, the *stx*-encoding phages have proven to

be an experimental windfall for addressing the question of the contribution of phages to virulence factor expression. By exploiting the encyclopedic collection of information acquired over the years on λ and its relatives (63, 70), it was possible to design experiments that led to uncovering the role of phages in Stx expression (138, 212, 213). This is particularly striking in the case of the stx_2-encoding phages, as experiments with two different phages, ϕ361 and 933W, demonstrated that Stx expression ultimately depends on prophage induction. Hence, it was proposed that, at least in the case of STEC strains with these prophages, stx_2 production is driven primarily from the fraction of the total population of STEC in which the prophage is induced. In this regard, note the interesting findings of Muniesa et al. (133) from a study of STEC strains isolated from patients. As expected, they found a direct correlation between phage and Stx production. What was more striking was the finding that only one isolate, which produced low levels of phage, was found in all of the asymptomatic patients, while all of the STEC strains that produced medium or high levels of phage were isolated from symptomatic patients. These findings, coupled with the finding that treatments with SOS-inducing antibiotics exacerbate STEC infections, make a compelling case supporting the idea that the subpopulation of induced lysogens is responsible for the production and release of Stx2. The situation is slightly different for Stx1, as it appears that under conditions of low iron, significant levels of Stx production can occur independently of phage induction, but Stx release appears to require phage lysis functions.

The idea that the release of virulence factors can be mediated by lysis is not a novel idea (see below). For example, the product of the *lytA* gene of *Streptococcus pneumoniae,* an autolysin, has been implicated in the release of virulence factors (125). Moreover, the mosaicism seen in small regions of *lytA* genes from many *S. pneumoniae* isolates appears to have been generated by recombination with phage genes, such as the amidase genes of phages HB-3 and EJ-1, which encode cell wall lytic enzymes (219).

Although phage lysis genes appear to be associated with *stx* genes, it is not clear whether the expression of phage lysis genes is the only mode of Stx release. However, if the expression of phage genes plays a pivotal role in the expression and/or release of Stx, then the question of what fraction of the infecting bacteria are induced in vivo remains to be answered. Continuing this line of reasoning, we have yet to determine whether there are SOS-inducing agents in the intestines that would add to spontaneous induction of the prophage and thus lead to an increase in the levels of Stx production and/or release. The identification of a factor from pharyngeal cells that induces both phage and the toxin associated with the phage from *Streptococcus pyogenes* (13, 14) offers an attractive precedent for such a scenario. With the development of better animal models, we look forward to the results of experiments addressing these questions.

Finally, we look at the issue of lambdoid phages as repositories of *stx* genes. The lambda family, as postulated by Campbell (23), because its members commonly recombine with each other to generate variants, are like "members of a sexually reproducing species, drawing on a common gene pool." This allows for constant genetic rearrangements, permitting new combinations that are likely to extend the host range of phages and allow for extensive lateral distribution of the phages and their *stx* genes. Because in many cases the recombination that generates phages with reassorted genomes appears to have occurred in regions with little homology, it has been postulated that these rearrangements occur primarily by illegitimate (nonhomologous) recombination (69). However, studies examining recombination by the λ Red system show that homologies of ~20 nucleotides are sufficient to allow this system to promote homologous recombination (226). Hence, provided that there are appropriate regions of short homologies, exchanges of genome regions among lambdoid phages may occur nearly anywhere in the phage genomes by homologous recombination catalyzed by the phage-encoded re-

combination system. In a similar way, nonphage elements, such as the *stx* genes, may be acquired by the phage genome through recombination within short regions of homology. Defective phages carrying *stx* genes may also serve as vehicles for lateral transfer by recombining with resident or infecting intact lambdoid phages. As pointed out by Campbell and Botstein (25), the progenitor lambdoid phage likely evolved by acquiring sets of functional genes from "genetic elements of the host." The *stx* genes may have been similarly acquired. The question that follows is this: what selective pressures are operating to maintain phages that have acquired *stx* genes? This is of particular interest if, as is the case for stx_2-encoding phages, prophage induction is required for Stx production and release. Since there is no evidence that Stx plays a role in fostering either lytic growth or the establishment of the prophage state, selective pressure must therefore be on the lysogen, i.e., Stx provides a selective advantage to the host bacterium. Hence, the fraction of lysogens that spontaneously induce and release Stx with phage provide a selective advantage to the surviving uninduced majority members of that population. Such a model provides another example of the symbiotic relationship between phages and their bacterial hosts.

Coming full circle, we end where we began by quoting Andre Lwoff, this time from his classic 1953 review article (112). We learn that no idea is really new. Lwoff, quoting from a footnote of Félix d'Herelle's 1926 book, *Le Bacteriophage et Son Comportement*, states, "It could be that the toxic character of the diphtheria bacillus is bound to the presence of the bacteriophage which would cause the lysis of bacteria and thus liberate the toxin."

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