VIRULENCE-LINKED BACTERIOPHAGES OF PATHOGENIC VIBRIOS

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Vibrios are gram-negative bacteria that live in aquatic environments and are endemic to many regions of the world. They are particularly prevalent in warmer waters (>17 to 20°C) and can often be isolated from shellfish and other seafood (71). Most *Vibrio* species are rarely, if ever, a cause of human illness, but a few frequently give rise to severe or even fatal diseases. In particular, *Vibrio cholerae* is a cause of epidemic diarrheal disease (3). In recent years, it has also been noted that *V. parahaemolyticus*, another cause of diarrheal disease, may have epidemic potential, due to the emergence of new, highly virulent variants (67). *V. vulnificus* can cause severe soft tissue infections and septicemia but is not associated with epidemic disease.

Vibrios, like most other bacteria, are associated with a multitude of bacteriophages (1, 14, 15, 20, 28, 48, 70, 75, 81, 93). As is true for the bacteria themselves, some of these phages have strong ties to virulence and disease, while most are generally innocuous, both to the bacterial host and to the organisms that the bacteria may colonize. The majority of these phages have not been well characterized, but a few have been the subject of extensive studies. In this chapter, we will focus on the bacteriophages of pathogenic vibrios, particularly those that have been found to contribute directly to bacterial virulence. These phages primarily target *V. cholerae*; however, a virulence-associated phage found in *V. parahaemolyticus* will also be discussed briefly. We will not discuss *V. vulnificus* or *Vibrio* species that are only rarely linked to virulence (e.g., *V. mimicus* and *V. fluvialis*), as virulence-associated phages have not been reported for these organisms.

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V. CHOLERAE AND CHOLERA

V. cholerae is the cause of the severe diarrheal disease cholera. This disease has afflicted humans for centuries, often incapacitating large percentages of affected populations due to its capacity for epidemic spread. In the last two centuries alone, there have been at least seven cholera pandemics. All seven are thought to have been caused by a single serogroup, *V. cholerae* O1, although to date more than 150 serogroups have been identified. Strains of the O1 serogroup can be further classified according to their biotype, based on a variety of phenotypic and

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genetic assays. O1 classical biotype strains are thought to have been responsible for the first six recorded cholera pandemics, while O1 El Tor biotype strains gave rise to the ongoing seventh pandemic, which began in 1961 (90). Classical biotype strains have now largely disappeared from the regions where they used to be endemic (64). They have been replaced both by O1 El Tor biotype strains and, since 1992, by a new virulent serogroup, *V. cholerae* O139. This new serogroup temporarily replaced *V. cholerae* O1 as the primary cause of disease in India and Bangladesh in 1992 (16), and it remains a significant cause of disease to this day (33). O139 strains are the first known non-O1 *V. cholerae* strains to give rise to significant cholera epidemics. *V. cholerae* O139 appears to have evolved from an O1 El Tor strain that acquired a new cassette of genes for O antigen production (7). Whether the emergence of O139 as a widespread etiologic agent of cholera marks the beginning of a new, eighth cholera pandemic is being debated (90).

The hallmark of cholera is the production of copious watery diarrhea (rice water stool), which, if not promptly treated, results in severe, life-threatening dehydration. This diarrhea is a consequence of colonization of the small intestine by *V. cholerae* and secretion by the bacterium of a potent exotoxin, cholera toxin (53). Bacterial colonization requires a relatively large infectious dose, an appropriate O antigen, as described above, and the production of a type four pilus, the toxin-coregulated pilus (TCP). Strains that cannot produce TCP fail to colonize and consequently are even less virulent than strains that lack the ability to produce cholera toxin (43). The production of both TCP and cholera toxin is dependent upon *V. cholerae*'s master regulator of virulence, the transcription factor ToxR, and on intermediate regulators of the virulence cascade, ToxT and TcpP (39, 86).

PHAGES OF *V. CHOLERAE*

V. cholerae is an excellent example of a pathogen whose virulence is largely dependent upon infection by a bacteriophage. A major virulence factor of this bacterium, cholera toxin, is encoded within the genome of CTXϕ, a temperate (and nonlytic) filamentous phage, and consequently only strains of bacteria infected by this phage are capable of causing epidemic disease (93). CTXϕ has also been identified within environmental isolates of *V. cholerae*, although it is much less prevalent among them (18, 62). Both disease-linked and environmentally derived strains that carry CTXϕ usually contain the cluster of genes encoding TCP as well, as this pilus serves as the primary receptor for CTXϕ. Infected strains are usually stable lysogens of CTXϕ, and most produce infectious CTXϕ, although at relatively low frequencies. Phage production is not required for virulence and does not appear to contribute directly to it (22). However, several studies have suggested that the ongoing transmission of CTXϕ may contribute to the evolution of new pathogenic isolates of *V. cholerae*.

Structure and Evolution of CTXϕ

CTXϕ has a single-stranded DNA genome of ~6.9 kb (Fig. 1, top panel). For historical reasons, this genome is typically divided into two parts, known as the RS and core regions (79). The core region encompasses ~4.4 kb from the 3′ end of the prophage genome and encodes several proteins that are utilized for packaging and secretion of new infectious CTXϕ virions. These include Cep (CTXϕ's major coat protein), Zot (a homolog of the f1 phage pI, which mediates phage secretion but is not itself a part of the virion), and Psh, Ace, and $pIII^{CTX}$ (formerly known as OrfU) (42, 93). These last three proteins are thought to be minor coat proteins, with only a few copies present per virion, based on their sizes, positions within the phage genome, and in some cases, homology. The core region also includes the toxin genes, *ctxAB*, which do not contribute to phage production. With the exception of the toxin genes, the organization of the core region is similar to that found in several other filamentous phage genomes (e.g., filamentous phages that infect *Escherichia coli* and additional filamentous phages of *V. cholerae*), although in some cases there is limited sequence similarity.

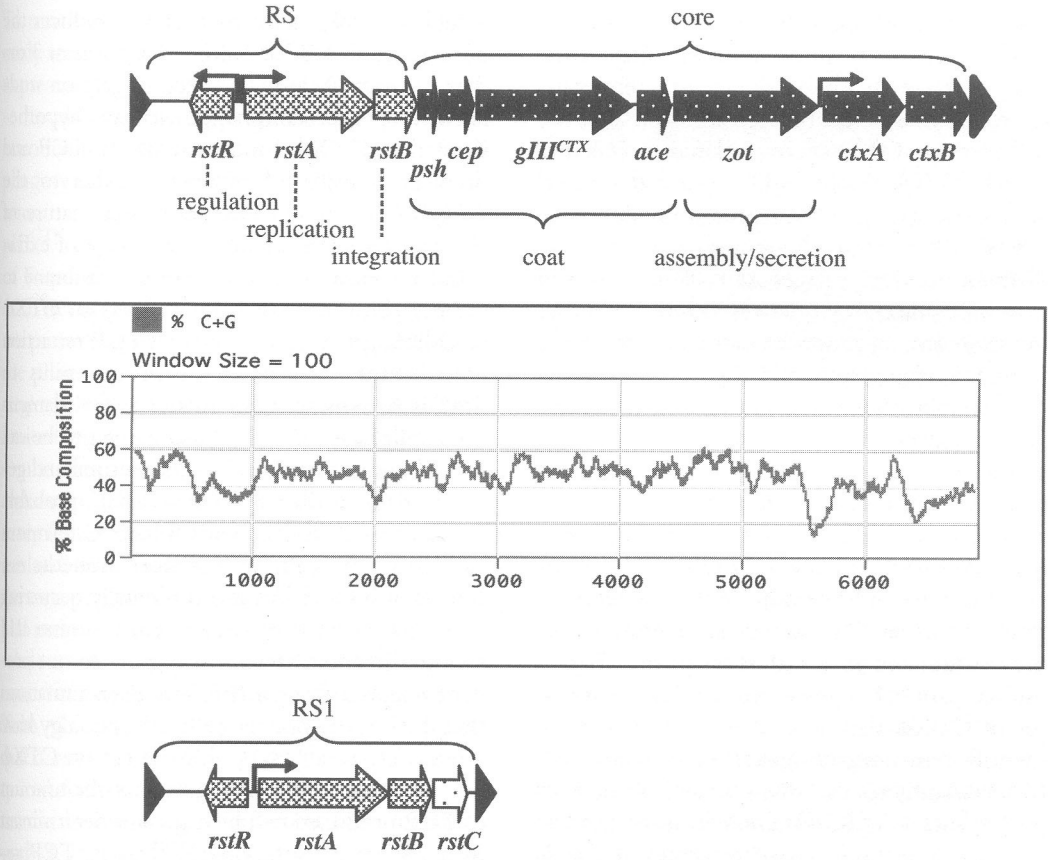

FIGURE 1. (Top) Schematic diagram of the CTXϕ genome, drawn roughly to scale. Arrows show genes and the direction of transcription. Bent arrows represent the *rstR*, *rstA*, and *ctxAB* promoters. Solid triangles represent the 15-bp repeats found at each end of the prophage. Protein functions are indicated underneath the genes. The overall organization of the CTXϕ genome is similar to those of other filamentous phages from *Vibrio* species and of filamentous phages from *E. coli*. (Middle) Graph of the G+C content of the CTXϕ genome, calculated by use of a 100-bp sliding window. The G+C contents of *ctxAB* and *rstR* differ the most dramatically from the average %G+C. The G+C contents of *rstR* and the *rstA* promoter were calculated for $rstR^{ET}$. (Bottom) Schematic diagram of RS1, a satellite phage related to CTXϕ.

The 5′ end of the prophage (~2.4 kb) is known as the RS region. It encodes proteins needed for phage gene regulation (RstR), phage replication (RstA), and phage DNA integration (RstB) (79, 94). RstB may also function as a single-stranded DNA binding protein (14; Mariam Quinones and M. Waldor, unpublished observations). The genes of this region are not homologous to genes in filamentous coliphages, but homologs have been identified in other phages, particularly other vibrio phages, such as the *V. cholerae* phage VSK and the *V. parahaemolyticus* phages Vf33 and VfO3K6. It is possible that the CTXϕ genome is essentially a hybrid element composed of packaging and secretion genes from one ancestral phage and replication and control genes from another source.

Several variants of CTXϕ have been identified. They can be distinguished largely by the sequences of their respective *rstR* genes and the operators to which the variant RstRs bind, which lie within the noncoding region between *rstR* and *rstA* (Fig. 1). The best-studied form of

RstR is that produced by most El Tor biotype strains ($RstR^{ET}$); however, some functional analyses have also been done with repressors identified in classical biotype strains ($RstR^{class}$) and in some O139 strains isolated in Calcutta ($RstR^{calc}$) (19, 57, 94). Additional *rstR* variants have been sequenced but not studied further (18, 62, 73). Overall, the repressor genes in the filamentous vibriophages are far less conserved than their adjacent sequences. It is thought that the different repressor-operator cassettes have been introduced into CTXϕ by recombination, enabling the rapid emergence of diverse CTXϕ variants (8). Repressor diversity in other phages (e.g., lambdoid phages) is thought to have developed via a similar process (12).

The toxin genes, *ctxAB*, do not appear to have coevolved with the remainder of the phage genome. They differ significantly in GC content from the other CTXϕ genes, suggesting that they originated in a different species (Fig. 1, middle panel). Furthermore, a potential ancestor of CTXϕ that lacks toxin genes has been identified in nonpathogenic *Vibrio* strains (8). More distantly related *Vibrio*-derived filamentous phages, such as VSK, VGJ, and Vf1, also lack toxin genes. Interestingly, however, genes at the 3′ ends of these phages are often variable, perhaps because new genes are occasionally acquired due to imprecise excision of integrated phages from a host chromosome (14). It has been speculated that such a process accounts for the presence of *ctxAB* in the CTXϕ genome (8).

Infection by CTXϕ

Genetic studies indicate that at least two protein complexes are used by CTXϕ to infect *V. cholerae*. TCP, a homopolymer of TcpA, is thought to be CTXϕ's primary receptor (93). Infection of strains lacking TCP is reduced by >3 orders of magnitude under standard laboratory assay conditions and is ordinarily undetectable (41). However, under altered assay conditions, a limited amount of TCP-independent infection can be observed. This is dependent upon the TolQRA proteins, which are thought to interact in the inner membrane or periplasm and to function as a secondary receptor. The deletion of *tolQ*, *tolR*, or *tolA* also reduces the occurrence of CTXϕ infection by at least 3 orders of magnitude (41). Based largely on studies of F-specific coliphages, we have hypothesized that CTXϕ binds initially to TCP and then is transferred from the pilus to the TolQRA complex. The molecular nature of the processes that enable the passage of extracellular virions across the outer membrane to the periplasm has not been defined for CTXϕ or coliphages. It is possible that TCP retraction contributes to transfer from the pilus to TolQRA, as is thought to occur for filamentous coliphages (50), but this has yet to be explored experimentally. Both structural and genetic data indicate that CTXϕ probably interacts with amino acids in the C terminus of TcpA (the pilin monomer); nonetheless, studies of both induced and naturally occurring *tcpA* variants have shown that many amino acid changes in the C-terminal region do not prevent phage infection (11, 58). Even mutations that disrupt the normal pilus morphology have a relatively small (<10-fold) effect on CTXϕ infection. Some data suggest that the amount of TCP produced can be a greater determinant of a recipient's infectibility than its TCP sequence (58).

Since TCP is the primary phage receptor, CTXϕ infection of new hosts should occur only under conditions that induce the expression of TCP. The expression of this colonization factor is induced in vivo; thus, it is not surprising that transmission of the phage from one host to another has been found to occur in vivo in animal models (57). In fact, CTXϕ transmission has been used in animal models as an assay for TCP expression (39, 61). CTXϕ infection has not been shown to occur within human hosts, but this possibility seems likely. However, to date, most evidence suggests that CTXϕ production is not induced above basal levels in vivo, so transmission in vivo may be fairly limited (95; Quinones and Waldor, personal communication). CTXϕ production has been found to be induced by DNA-damaging agents such as mitomycin C and UV light, and phage have been isolated from environmental sources (24, 32).

To our knowledge, conditions that simultaneously induce both CTXϕ production and TCP expression have not been identified.

In addition to requiring the two host-cell-encoded receptors, infection also depends on a phage-encoded protein which was initially known as OrfU but subsequently renamed $pIII^{CTX}$ due to its functional similarity to pIII of F-specific filamentous coliphages (e.g., Fd) (42). In coliphages, a few copies of this protein are present at one end of the phage and allow the phage to interact with its receptors in the target cell (83). The CTXϕ and Fd pIIIs have limited sequence similarity; however, they have similar sizes and appear to have similar domains and secondary structures (42, 44). Since the primary Fd receptor is the F pilus rather than TCP, it is not surprising that at least some regions of the two pIIIs differ. A central domain of the Fd pIII protein is thought to interact with the pilin, while an N-terminal sequence revealed by a conformational shift is thought to interact with TolA (25, 65, 82). Genetic evidence suggests that a central region of $pIII^{CTX}$ is similarly required for interaction with TCP and that N-terminal sequences are sufficient for interaction with *V. cholerae* TolQRA. A hybrid Fd phage that displays the N-terminal and central domains of $pIII^{CTX}$ on its surface was able to infect *V. cholerae*, suggesting that $pIII^{CTX}$ is the only CTXϕ protein that is needed for normal recognition and infection of its host (42).

It is not known how phage DNA passes through the bacterial inner membrane, but one possibility is that the phage assembly process is somehow reversed. According to this model, coat proteins are gradually removed from phage DNA and deposited in the inner membrane as the phage DNA enters the cytoplasm. Such coat protein deposition has been observed for F-specific phages (17), but comparable experiments have yet to be performed for CTXϕ.

Integration

Since CTXϕ virions contain single-stranded DNA, the phage DNA that reaches the cytoplasm of a newly infected bacterium is a single strand. It is thought that the next step of infection must therefore be the production of a second, complementary strand. To date, there is no evidence that this process requires any phage-encoded proteins; it may rely entirely on host-encoded replication proteins. The resulting double-stranded phage DNA can then be replicated as a circular plasmid (pCTX). This replication is dependent on, and probably initiated by, the phage-encoded replication protein RstA (94). pCTX can be maintained at a low copy number in strains lacking a functional phage integration site (e.g., most classical biotype strains), although it is fairly unstable in the absence of selective pressure. However, in most *V. cholerae* strains, it appears that double-stranded phage DNA integrates into the chromosome. Integration is not dependent upon a phage-encoded integrase; instead, it relies upon the chromosome-encoded recombinases XerC and XerD (46). These proteins also function to resolve chromosome dimers that are generated by homologous recombination during chromosome replication. XerC and XerD mediate site-specific recombination between short (15 bp) homologous sequences on pCTX and chromosome I, the larger of the two *V. cholerae* chromosomes (46, 80). These short sequences comprise the cores of the phage and chromosome attachment sites, known as *attP* and *attB*, respectively. Interestingly, *attB* either overlaps or is equivalent to the chromosome I *dif* site, which is the target for XerCD's resolution of chromosome dimers. Strains lacking *dif* (e.g., due to deletion) form filaments, presumably due to inadequate chromosome segregation resulting from defective chromosome dimer resolution. *dif*-deficient strains also maintain CTXϕ DNA in its plasmid (replicative) form. However, the integration of phage DNA at the *dif* site does not interfere with *dif* function, nor does it prevent further phage integration events.

Currently, it is not clear how the phage-encoded protein RstB contributes to phage integration, although genetic studies have shown that it is required (94). RstB is not homologous to any known integrases; it may instead function as an accessory factor during integration, perhaps promoting directionality in the recombination reaction.

The sequences of the phage-chromosome junctions, *attL* and *attR*, are consistent with a conservative mechanism of recombination between the core regions of *attB* and *attP*; however, the sites in *attB* and *attP* that are targeted in vitro by XerD are not consistent with such a mechanism (68). XerD cuts outside of the *attB* and *attP* cores, and the numbers of nucleotides between the top (XerC-dependent)- and bottom (XerD-dependent)-strand cleavage sites differ for *attB* and *attP* (68). Since these XerD cleavage sites could not mediate formation of the phage-chromosome junctions detected in vivo, we hypothesize that XerD does not play a catalytic role in CTXϕ integration, but instead is required for synapsis between CTXϕ and the chromosomal DNA. However, if CTXϕ integration is not catalyzed by XerD, then another, as yet unidentified host process must act to resolve the recombination intermediates generated by XerC. Either a host resolvase, such as RecG, or passage of the replication fork may function in this fashion (68).

The integration reaction, in the form that is simplest to envision, yields a strain with a single prophage integrated at the *dif* site, flanked by *attL* and *attR*, each of which contains a short direct repeat (Fig. 2). However, such reactions appear to be relatively uncommon, both in the laboratory and in the natural environment. In the laboratory, the integration of phage DNA often yields strains with two or more prophage integrated in tandem. These two prophage are separated by the core repeat of *attR*, and this sequence, as well as the sequence downstream of the final phage in the array, can serve as an integration site during subsequent phage infections (19, 23). At this point, the mechanistic basis for the formation of tandem prophages is unknown.

Single CTX prophages have rarely been observed in clinical isolates of *V. cholerae* (Fig. 3). O1 serogroup, El Tor biotype strains typically have one or more CTX prophages flanked by a related element, the satellite phage RS1 (Fig. 1, bottom panel) (69; discussed below). Most O139 serogroup strains also have multiple CTX prophages flanked by RS1 (4, 56). However, the pattern is somewhat different for O1 serogroup, classical biotype strains (now rarely seen), which is consistent with the hypothesis that the classical and El Tor biotypes of *V. cholerae* independently acquired CTXϕ (8). Classical biotype strains typically have CTX prophage DNA integrated at the *dif* sites in both of the *V. cholerae* chromosomes, whereas the prophage arrays of El Tor biotype strains are typically found in chromosome I (chrI). In some classical biotype isolates, chrI contains a solitary prophage and chrII contains the remnants of two prophages that have been truncated and fused, while other isolates contain the reverse arrangement (22). To date, classical biotype strains have not been found to contain RS1. As mentioned above, classical biotype strains also seem to lack functional CTXϕ integration sites, although the strains appear to have functional *dif* sites for chromosome dimer resolution. The integration site deficiency appears to be a consequence of

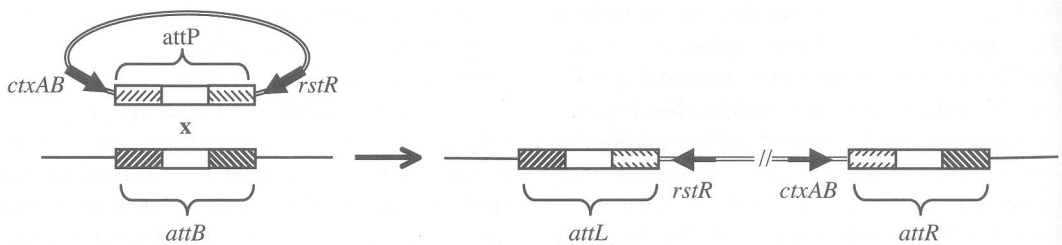

FIGURE 2. Schematic representation of CTXϕ integration. The chromosomal integration site (*attB*) recombines with the integration site in pCTX (*attP*) through short regions of homology (white rectangles; equivalent to the solid triangles seen in Fig. 1). This process yields a single integrated prophage.

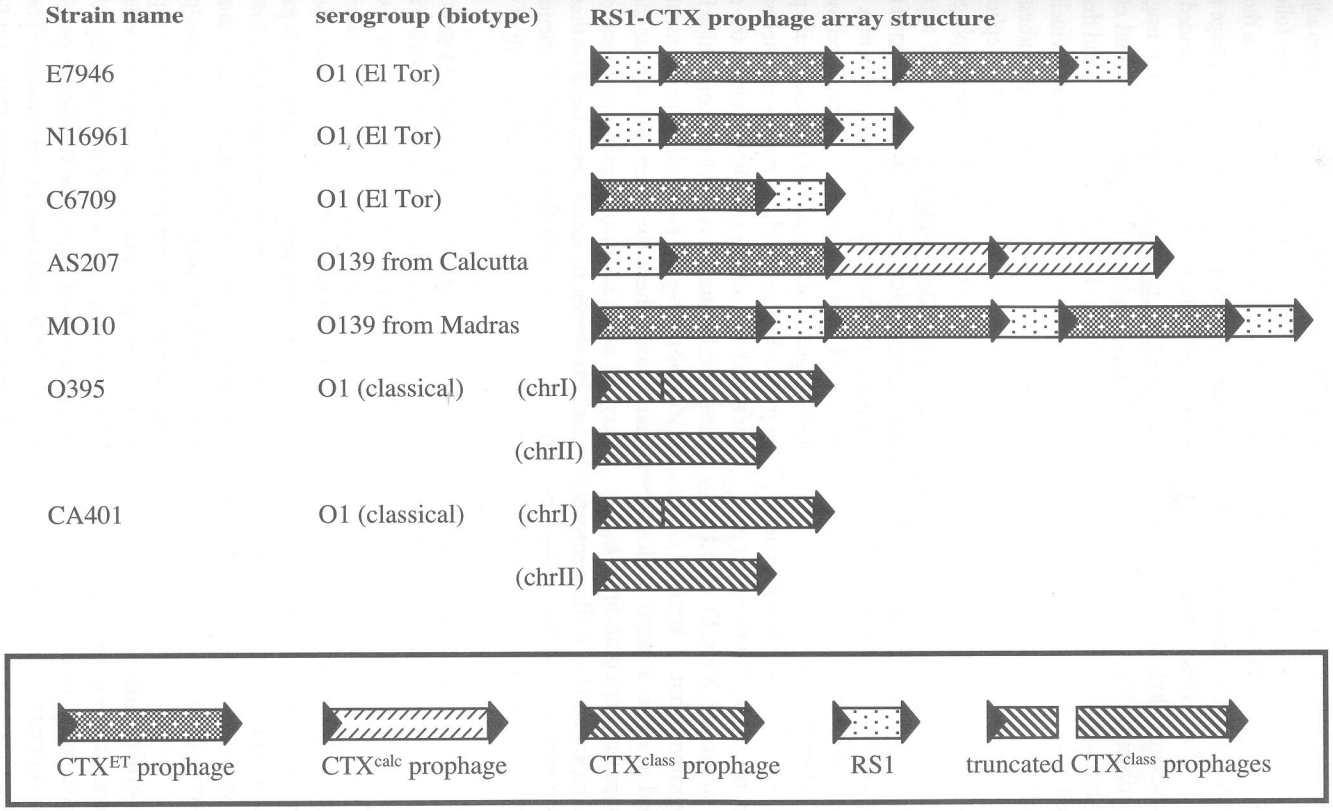

FIGURE 3. Arrangement of CTX prophage and RS1 DNA integrated in a variety of clinical isolates of *V. cholerae*. Most O1 El Tor biotype strains and O139 strains (all of those shown here) have CTXϕ DNA integrated into the large chromosome (chrI). Phage DNA is typically part of an array of tandemly integrated elements. We have found that *V. cholerae* strain N16961 has two copies of RS1 that flank a prophage rather than a single RS1 element downstream of the prophage, as was reported previously (40). O1 classical biotype strains have CTXϕ DNA integrated into both chromosomes. Each chromosome has either a single prophage or an array of two truncated, fused prophages.

a few nucleotides that differ between the *dif* sites of El Tor biotype and classical biotype strains (22, 45). It is not clear how CTXϕ initially integrated into chrII of classical strains. Interestingly, Nandi et al. recently reported that a subset of El Tor biotype strains from India also contain CTXϕ integrated into the small chromosome (chrII) (74). These strains do not contain CTXϕ DNA on chrI, and one of the phages on chrII differs from those typically found in El Tor strains. These atypical El Tor strains were isolated later than were most previously characterized strains isolated in India, and hence may be of a different origin. Still, it is not apparent why CTXϕ has a different integration pattern in these strains.

It is now recognized that CTXϕ is not the only phage to integrate at a *dif* site, and it is suspected that XerC and XerD mediate the integration of these other phages as well. Huber and Waldor reported that the chromosomal junctions of phages in *Xyella*, *Xanthomonas*, and *Yersinia* resemble targets of XerCD (46), and Gonzalez et al. reported that a *Yersinia pestis* prophage integrated at *dif* in *Y. pestis* is closely related to a prophage integrated at *dif* in *E. coli* K1 (38). Iida et al. reported that a filamentous phage in *V. parahaemolyticus* is also integrated at a *dif*-like site near the chromosome terminus (47), and Campos et al. have recently reported that another phage of *V. cholerae*, VGJϕ, integrates into the same chromosomal locus as CTXϕ (14). Thus, it seems that many integrating filamentous phages utilize a host-encoded recombinase rather than a phage-encoded integrase. It may be that many of these phages evolved from a common ancestor that, through an unknown process, incorporated a *dif*-like sequence into its chromosome.

CTXϕ Phage Replication

Unlike most characterized integrating phages, CTXϕ does not have to excise from the chromosome in order to generate extrachromosomal phage DNA that can be packaged into virions. Instead, CTXϕ generates extrachromosomal phage DNA by an unusual replicative process in which the prophage DNA acts as a template for the synthesis of viral DNA independent of chromosomal DNA replication (Fig. 4). This process is dependent upon the presence of tandem chromosomal elements, either two adjacent phage genomes or a phage genome followed by an RS1 element (23). Replication is thought to initiate after production by the phage replication protein, RstA, of a single-stranded nick in the CTX prophage at the phage origin of replication, which lies near the 3′ end of *rstR* (72). Nicking should generate a 3′ end that can be extended by DNA polymerase, which displaces the old phage DNA as it synthesizes a new plus strand of phage DNA. Replication does not end at the 3′ end of the prophage; instead, synthesis continues into the adjacent downstream element until a new origin of replication is reached. At this point, a new nick is introduced into the displaced strand, releasing a full-length, single-stranded equivalent of a phage genome. However, this strand is not necessarily identical to a single prophage within the chromosome, because it is a hybrid containing DNA corresponding to two adjacent elements. In many cases, these two elements are identical at their 5′ ends, so a novel element is not generated. However, in some cases, because there are differences between the tandem elements, this process does allow for the reassortment of some phage sequences.

This replicative process is advantageous for CTXϕ in that it allows for both horizontal and vertical transmission of CTXϕ from a single host. Thus, unlike the case for phages that excise, phage transmission is not necessarily coupled to loss from the host. Of course, such parallel transmission processes are only feasible for nonlytic phages such as filamentous phages, since the host of a lytic phage does not survive phage production, and preservation of the prophage within the genome is therefore not valuable. However, there can also be a disadvantage to this replicative process, namely, solitary prophages are unable to produce phage progeny. The fact that solitary prophages are not replication competent is one factor underlying the failure of classical biotype strains of *V. cholerae* to produce new CTXϕ (22). To date, it has not been re-

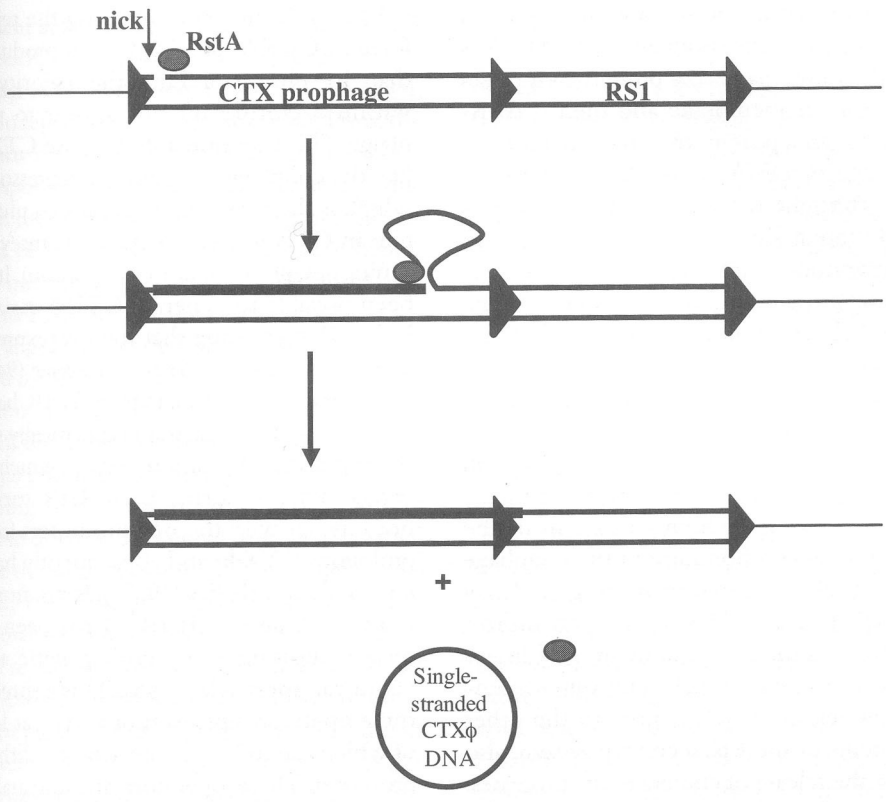

FIGURE 4. Production of extrachromosomal CTXϕ DNA from a prophage array relies on a replicative process mediated by RstA. Phage DNA excision does not occur. Replication initiates at the origin of replication, which lies near the 3′ end of *rstR*. As the replication complex moves along the phage DNA template, it displaces a single strand of the phage genome and replaces it with a newly synthesized strand. Replication continues past the end of the prophage, into the adjacent element downstream (in this case, RS1). Replication terminates at the origin of replication of the downstream element. A single-stranded hybrid phage genome, containing DNA from both the upstream and downstream elements in the array, is then released.

ported whether other integrating filamentous phages rely on a similar replicative process.

Once an extrachromosomal copy of the phage genome has been generated, it is probably converted from a single-stranded to a double-stranded form, as occurs following phage infection. The double-stranded genome can then be replicated by basically the same process as that used for a chromosomal template, except that with a plasmid template, the start and end sites of the replicon do lie within the genome of a single phage (72). This process was shown by Moyer et al. (72) to have a rolling-circle mechanism, as has also been found for filamentous coliphages. Presumably, this process is also used to produce single-stranded phage DNA that can be packaged within new virions. It has not been proven that the plasmid form of the genome, pCTX, must be generated prior to phage production; however, the differences in phage gene expression from the prophage and pCTX suggest that this is probably the case.

Secretion

CTXϕ, like other filamentous phages, is secreted by its host cell rather than released following host cell lysis. Fully assembled virions are apparently never present within the host cell

cytoplasm. Instead, phage assembly and secretion are thought to occur simultaneously: DNA is packaged within phage coat proteins as it passes across the inner membrane and then is transmitted through a pore in the outer membrane. The passage of DNA across the inner membrane is thought to depend on the phage-encoded protein Zot, which is homologous to pI of filamentous coliphages (59, 93). This inner membrane protein contains an NTP-binding motif, probably for binding to ATP, and is likely to energize phage production (59). Coat proteins are also probably present in the inner membrane prior to incorporation within virions.

CTXϕ differs from filamentous coliphages in that it does not encode its own outer membrane pore through which to exit from the periplasm. It lacks a homolog of the coliphage gene IV, which lies downstream of gene I (*zot* in CTXϕ). Instead, CTXϕ secretion utilizes EpsD, the secretin component of *V. cholerae*'s type II secretion system (21). This outer membrane channel, in conjunction with the other ~15 proteins of the Eps secretory system, also allows for the release of cholera toxin, proteases, and chitinase from the periplasm (84). The multiprotein Eps complex has been shown to be localized to the cell poles, and thus it is possible that CTXϕ secretion also occurs from the cell pole (85). However, CTXϕ secretion is not dependent upon Eps proteins other than EpsD (21). Instead, EpsD likely interacts with Zot to direct phage secretion. Phage secretion does not appear to inhibit the transport of other proteins that pass through EpsD, probably because phage production is a relatively rare event for *V. cholerae* (see below). Analyses of other phage genomes suggest that reliance upon a host-encoded secretin may be a common strategy for filamentous phages (21).

CTXϕ Regulation

Compared to the filamentous coliphages, which can reach titers of 10^{11} phage/ml in culture supernatants, CTXϕ is produced at very low levels. Phage lysogens grown in the lab in Luria broth typically yield maximum titers of ~10^5/ml. Even strains carrying the replicative form of CTXϕ (pCTX) do not produce more than 10^7 phage/ml. Thus, the majority of cells within a culture do not appear to produce phage. This is presumably because CTXϕ, unlike the coliphages, produces a repressor, RstR, which is the most highly expressed phage protein in CTXϕ lysogens (H. H. Kimsey and M. Quinones, personal communication). It has not been possible to generate CTXϕ variants that lack *rstR*, suggesting that the overexpression of phage proteins is toxic to *V. cholerae* (94).

Several different variants of RstR have been characterized, and each one specifically regulates the adjacent *rstA* promoter (P_{rstA}), which also has several different forms. Each RstR variant will not interact with the *rstA* promoters from heterologous CTXϕs and consequently has no effect on their activities (19, 56). RstR from El Tor biotype *V. cholerae* ($RstR^{ET}$) has been characterized thoroughly by both genetic and biochemical approaches (55). This repressor has three operators upstream of $rstA^{ET}$, at least two of which can influence the activity of the $rstA^{ET}$ promoter. These operators are unusually large (~50 bp), and several analyses indicated that a tetramer of $RstR^{ET}$ binds to each site. As is the case for other phage repressors with multiple operator sites (e.g., cI of phage λ), $RstR^{ET}$ has a different affinity for each of its operators, presumably to allow fine-tuning of phage gene expression. Preliminary evidence suggests that $RstR^{ET}$ binding regulates the *rstR* promoter as well as P_{rstA} in both a positive and a negative fashion (H. H. Kimsey, personal communication).

P_{rstA} is also bound by LexA, a chromosome-encoded repressor that controls the expression of numerous DNA repair genes (80a). LexA is a key regulator of the bacterial SOS response, and its binding to P_{rstA} enables the regulation of CTXϕ production in response to DNA damage. Treatment of *V. cholerae* CTXϕ lysogens with DNA-damaging agents, such as UV light or mitomycin C, results in elevated expression of *rstA* and the downstream phage genes and increased production of CTXϕ virions. UV-stimulated CTXϕ production requires RecA-

dependent autocleavage and depletion of LexA (80a). RstR levels also decrease following UV treatment of CTXϕ lysogens, via an unknown, RecA-independent mechanism (80a). Thus, the molecular mechanisms by which the *V. cholerae* SOS response controls CTXϕ production differ from those used by *E. coli* to control the induction of lambdoid prophages (see Chapter 3). It is not yet clear whether RstR and LexA bound to P_{rstA} interact with each other.

P_{rstA} appears to be the only CTXϕ promoter required for CTX prophage development. The repression of P_{rstA} prevents transcription of *rstA* and all of the downstream phage genes required for phage production. Studies of mutant phages containing transcription terminators indicate that these downstream genes are cotranscribed and that the single polycistronic message is subsequently converted into smaller fragments (20). These mature transcripts are likely subject to distinct regulatory processes, as there is not an equal need for proteins encoded by different genes. In particular, phage production requires high levels of CTXϕ's major coat protein, Cep. The termination of some transcripts immediately downstream of *cep* may also allow for elevated synthesis of Cep relative to phage proteins that are encoded downstream.

The maximal activity of P_{rstA} requires sequences that include the LexA binding site, which is centered at bp −48 relative to the start site of *rstA* transcription (80a). We hypothesize that all or part of this LexA binding site may also function as a promoter UP element. UP elements increase promoter strength by binding the carboxy-terminal domain of the alpha subunit of RNAP (30). LexA may prevent the binding of this subunit of RNAP to P_{rstA}. After LexA is cleaved following an SOS stimulus, the alpha subunit of RNAP may bind to the LexA binding site in P_{rstA}, thereby facilitating high-level transcription of the CTXϕ genes required for virion formation.

In addition to the SOS-mediated pathway, the repression of P_{rstA} can also be relieved by an antirepressor, RstC, which is encoded within the satellite phage RS1 (discussed below). RstC interacts directly with RstR to form large complexes, and as a result, seems to prevent binding of RstR to its operators (20). Interestingly, RstC is able to block the activity of a variety of RstR variants, including RstR from El Tor, classical, and Calcutta phages. The expression of RstC causes a dramatic increase in both phage gene expression and phage production. Since *rstC* transcription initiates at the *rstA* promoter, it is unlikely that the production of RstC initiates phage production. Instead, it appears that RstC allows phage production to be prolonged following an initial inducing stimulus.

Toxin Regulation

Unlike most CTXϕ transcripts, the majority of transcripts of *ctxAB*, which encode cholera toxin, do not initiate at the *rstA* promoter. Thus, the regulation of cholera toxin expression differs markedly from the expression of some other phage-encoded virulence factors (e.g., Shiga toxins), whose expression is generally coregulated with that of adjacent phage genes (91, 92) (see Chapter 7). Instead, *ctxAB* are transcribed from a promoter directly upstream of the toxin genes. This promoter is absent from the CTXϕ precursor phage that lacks the toxin genes, and it is thought to have been acquired in conjunction with *ctxAB* (8). The *ctxAB* promoter is one endpoint of a transcriptional cascade controlled by *V. cholerae*'s master regulator of virulence, ToxR. Along with the coactivator TcpPH, ToxR activates transcription of a secondary activator, ToxT, which then binds to the *ctxAB* promoter to induce toxin expression (27, 39). Thus, production of toxin is modulated by a multitude of agents that alter expression of its regulators, including temperature, pH, bile, salt, and amino acids (86). El Tor biotype strains require fairly specialized growth conditions in vitro to induce toxin production (49); however, in vivo (e.g., in the infant mouse model host), the ToxR regulon is turned on in the majority of *V. cholerae* cells (61).

The ToxR-responsive cascade is clearly the dominant pathway for the induction of cholera toxin production by *V. cholerae*; however, *ctxAB* transcription can also initiate at the *rstA*

promoter and thus be rendered independent of ToxR and ToxT (20). Thus, stimuli that activate the *rstA* promoter, such as DNA-damaging agents and RstC, also increase the production of cholera toxin. The replicative form of CTXϕ, which seems less subject to RstR-mediated repression, also yields increased toxin (60). Under conditions in which the ToxR regulon is active, these alternate pathways make a relatively insignificant contribution to toxin levels. However, these pathways do allow for some toxin production under conditions in which the toxin-specific promoter is not active. It has also been noted that the infection of O1 serogroup strains with a non-*ctxAB*-encoding temperate phage from serogroup O139 strains alters the regulation of toxin synthesis, although no explanation for this phenomenon was reported (29).

Alternate Transfer Pathways for CTXϕ

CTXϕ is generally thought of as a self-transmissible agent, dependent only upon a few host-encoded proteins for phage production and a few recipient-encoded proteins that serve as phage receptors. However, several researchers have also noted nonautonomous pathways for CTXϕ transmission. These alternate mechanisms rely on packaging of the CTXϕ genome within the coat proteins of other phages, such as the generalized transducing phage CPT1 and the filamentous phage VGJϕ (10, 13). Interestingly, packaging of the CTXϕ genome by CPT1 appears to be more efficient than packaging of other chromosomal loci (10). VGJϕ-dependent transmission follows the formation of a hybrid (cointegrate) between CTXϕ and VGJϕ, so both phage genomes are transferred to the new host. These alternate pathways allow the dissemination of CTXϕ (and thus cholera toxin genes) to hosts that lack the genes encoding the phage receptor TCP and thus may allow a wider spread of virulence genes among the vibrio population. However, it is questionable whether these mechanisms make a significant contribution to the emergence of new pathogenic strains, as strains lacking TCP cannot colonize the human host and are therefore avirulent, regardless of whether they can produce toxin or not.

Alternate CTXϕ Hosts

CTXϕ is most prevalent among pathogenic strains of *V. cholerae*, as it is required for their virulence. The vast majority of these strains are O1 or O139 serogroup strains that also contain the genes encoding TCP. However, CTXϕ lysogens have also been detected among non-O1/O139 strains and among environmental isolates of *V. cholerae*. For example, Daalsgard et al. identified several pathogenic isolates of *V. cholerae* O141, all of which contained both CTXϕ and TCP genes (18). In contrast, environmental isolates of *V. cholerae* O141 examined in the same study generally lacked CTXϕ genes. Li et al. also found that CTXϕ was present in 7 of the 300 non-O1, non-O139 strains that they analyzed, all of which were also TCP^+ and linked to infection (62). In addition, CTXϕ has been identified in a subset of *V. mimicus* strains (9). As observed above, all natural CTX^+ strains also contained the TCP gene cluster; however, Faruque et al. reported that a subset of TCP^- strains of *V. mimicus* could be infected in the laboratory by CTXϕ at a low frequency (35). The mechanism underlying this infection was not determined. It is possible that *V. mimicus* serves as an additional environmental reservoir for CTXϕ.

Additional CTXϕ-Encoded Toxins

In addition to cholera toxin, two other CTXϕ-encoded proteins, Ace and Zot, have been reported to act as enterotoxins (5, 37, 87, 88). Both Ace (thought to be a minor coat protein of the phage) and Zot (thought to mediate phage secretion) have been found to increase short-circuit current across rabbit intestinal tissue in Ussing chambers; however, their precise mechanisms of action have not been determined. Apparently, Ace toxicity does not require its incorporation into CTXϕ; however, it is unclear how Ace is released from *V. cholerae* when it is not serving as a CTXϕ coat protein. The toxic Zot moiety may not be the full-length protein; it may instead be a carboxy-terminal fragment of the protein that is generated by the proteolysis of full-length Zot (26,

89). It has been proposed that these proteins might contribute to the residual diarrhea induced by some *ctxA*-negative *V. cholerae* strains which are being considered for use as live vaccines. However, to date there has been little evidence that either protein contributes significantly to cholera pathogenesis.

RS1

RS1 is a mobile genetic element that is closely related to CTXϕ and is often found adjacent to the CTX prophage in the bacterial chromosome (Fig. 1, bottom panel). Approximately 2.4 kb of the ~2.7-kb RS1 element is identical to CTXϕ. These sequences include *rstR*, *rstA*, most of *rstB*, and intergenic regions, including the repeat sequence that mediates CTXϕ integration. Consequently, a plasmid variant of RS1 is capable of both autonomous replication and chromosomal integration. RS1 lacks the remaining genes of the CTXϕ genome, which encode phage coat proteins, a phage secretion protein, and cholera toxin. In their place, it encodes the antirepressor, RstC (discussed above). Since RS1 lacks the genes of CTXϕ's core region, it cannot independently package its genome for transmission to a new host. However, infectious RS1 can be generated with contributions from a helper phage such as CTXϕ (20, 31). RS1 replicates by the same mechanism as does CTXϕ, i.e., it requires the presence of tandem elements. Following the production of extrachromosomal RS1 DNA, it can be packaged within coat proteins produced by a helper phage. In most cases, this helper phage is probably CTXϕ, since *rstC* is transcribed from extrachromosomal RS1 and can therefore promote CTXϕ coat protein production. However, an apparently unrelated filamentous phage, KSF-1, can also package RS1 DNA into infectious particles, as can VGJϕ (13, 34). Like the transmission of CTXϕ via alternate transfer pathways, this process allows for RS1 DNA to be introduced into new TCP^- hosts. However, as noted above, it is unclear how likely this process is to contribute to the emergence of new pathogenic vibrios.

Additional Filamentous Phages in *V. cholerae*

Several filamentous phages with no known connections to virulence have also been identified in *V. cholerae* (e.g., fs1, fs2, VSKϕ, VGJϕ, and 493) (1, 14, 15, 28, 48, 51). Many of these have large regions of sequence similarity and thus appear to be highly related. For example, VGJϕ and VSKϕ are 83% homologous at the nucleotide level, while VSKϕ and fs1 are 87% homologous (14). In particular, the genes encoding proteins involved in replication and packaging of the phage genomes appear to be conserved, while regulatory proteins (e.g., repressors) appear to be much less conserved, as is the case among CTXϕ variants. Some proteins from CTXϕ also have clear homologs in other phages (e.g., Zot and RstA), but overall, CTXϕ appears to be more distantly related to characterized phages than are several of the other *Vibrio*-derived filamentous phages. The fs2 genome is more closely related to those of phages from other organisms, particularly *E. coli*, than to *Vibrio*-derived phages, and it does not cross-hybridize on Southern blots with DNA from phage fs1 (28). Despite their sequence differences, the genomes of all of these phages appear to be similar in organization, although several of the phages also contain open reading frames that lack obvious homologs in other phages, particularly at their 3′ ends. CTXϕ appears to be the only phage that utilizes TCP as a receptor. At least two of the other phages (493, VGJϕ, and possibly fs1 and fs2) use another type four pilus, the mannose-sensitive hemagglutinin, as their receptor (14, 28, 52).

TCP

In 1999, it was reported that the genes encoding TCP were also part of the genome of a filamentous phage (54). TcpA, the major pilin subunit, was said to be the major coat protein of this phage (VPIϕ), but it was not determined whether pili and phage were distinct entities or the same structure. The purified "phage" resembled pili; however, there was no way to determine if they were in fact phage rather than pili. The genome of the putative VPIϕ did not

resemble those of other filamentous phages, and the strategy used for the purification of VPIϕ was incompatible with the purification of these phages, raising doubts for some that this was in fact a filamentous phage (24). A subsequent thorough analysis failed to detect the production of VPIϕ by numerous *V. cholerae* strains that contained the TCP gene cluster (36). Thus, it seems likely that the TCP gene cluster is not part of the genome of an autonomous filamentous phage and that an alternative transfer mechanism accounts for the horizontal transmission of this region. One possibility is that transmission requires the presence of a helper phage that is only present in a small fraction of *V. cholerae* strains.

K139

One additional phage in *V. cholerae* has also been linked to virulence, namely, phage K139 (81). Unlike the other phages described here, K139 is not a filamentous phage; instead, it is more similar to phage P2. K139 is a temperate phage that was initially isolated from *V. cholerae* O139; however, in the laboratory, it only infects nonlysogenic El Tor strains. It is not able to infect O139 strains, as its receptor is the O1 antigen (77). It is hypothesized that the O1 El Tor strain that gave rise to O139 strains was a K139 lysogen and that this accounts for K139's initial isolation from the O139 background.

In 1995, K139 was found to encode a secreted protein, named Glo, with similarity to eukaryotic Gs(α) proteins. Glo is expressed in lysogens, and disruption of the gene encoding it reduced the virulence of *V. cholerae* in assays of suckling mouse survival (81). Subsequently, Glo was found to be a periplasmic protein that contributes to phage exclusion and thus to K139's inability to infect K139 lysogens (76). At this point, it is not clear whether Glo's role in phage exclusion accounts for its contribution to the virulence of K139 lysogens or if, under some circumstances, Glo is released from the bacterium to interact directly with host tissues and contribute to virulence.

Other Phages of *V. cholerae*

Numerous other phages that infect *V. cholerae* have been described, but little characterization of them has been reported. Although they do not appear to be directly relevant to *V. cholerae* virulence, some have potential utility as tools for the study of *V. cholerae*. For example, the JA1 phage is a lytic phage that uses the capsule of O139 strains as its receptor (1). Thus, this phage can be used to identify *V. cholerae* strains with alterations in the capsule synthesis pathway, as some such strains are resistant to the phage (2). This phage can also facilitate the purification of capsular components, as it encodes an enzyme that depolymerizes the capsule (63). Other phages have been reported to induce biotype switching between the O1 El Tor and O1 classical biotypes (70, 78). Although the true distinction between the two biotypes is not simply a matter of phage infection, these phages may nonetheless be useful for exploring some of the phenotypes associated with each of the biotypes.

V. PARAHAEMOLYTICUS

V. parahaemolyticus, though not as widely recognized as a cause of disease as *V. cholerae*, is nonetheless a significant cause of disease in some parts of the world. For example, this halophilic bacterium is a frequent cause of gastroenteritis and the leading cause of food-borne illness in Japan (71). *V. parahaemolyticus* also occasionally gives rise to wound infections and septicemia. The factors that differentiate pathogenic from nonpathogenic isolates have not been well studied, but one long-known feature of pathogenic strains is their hemolytic activity. The primary hemolysin gene, *tdh*, lies within a pathogenicity island that contains several additional genes that may also contribute to virulence (66). Recently, genome sequencing of *V. parahaemolyticus* revealed that pathogenic isolates also encode a type three secretion system that is absent from nonpathogenic strains (66). It was speculated that this apparatus may account for the inflammation associated with *V. parahaemolyticus* infections.

In the past decade, there has been a notable increase in the frequency of *V. parahaemolyticus* infections in both Japan and the United States. This increase has been linked to the emergence of new serotypes, particularly O3:K6 and, more recently, O4:K68. Initially, it was noted that the O3:K6 strains all contained a filamentous phage, f237, with a new open reading frame that distinguished it from previously identified *V. parahaemolyticus* phages (e.g., Vf33) (75). No connection between this phage and virulence was demonstrated, but it was proposed that the new open reading frame could serve as a marker for the new and highly virulent strains. However, subsequent studies have revealed that f237, while detectable within the majority of O3:K6 and O4:K48 strains, is not 100% accurate as a predictor of virulence for these serogroups (6). Furthermore, several new disease-associated serogroups have been detected recently, and these also lack the new phage and/or the new gene, suggesting that the new gene may have limited utility as an indicator of highly pathogenic strains.

CONCLUSIONS

CTXϕ has played a pivotal role in the evolution of pathogenic *V. cholerae*. Studies of this lysogenic filamentous phage have yielded knowledge of new types of virus-host interactions. CTXϕ has a remarkable dependence on its host: its chromosomal integration requires host recombinases and probably other host factors, its development is controlled by a cellular repressor, and its secretion requires a chromosome-encoded secretin. Despite the requirement for many host factors in the CTXϕ life cycle, this phage does not appear to negatively affect the growth of *V. cholerae*. It seems that CTXϕ and *V. cholerae* have coevolved to the point where their relationship lacks the antagonism that usually characterizes certain aspects of virus-host interactions. We anticipate that future studies of CTXϕ will provide additional examples of how a virus and its host can coevolve in a symbiotic fashion. Furthermore, it seems likely that future studies of other pathogenic vibrios will uncover new bacteriophages that influence pathogenicity.

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