Filamentous phages linked to virulence of *Vibrio cholerae*

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The pathogenicity of *Vibrio cholerae* depends upon its production of two key virulence factors: the toxin co-regulated pilus (TCP), a colonization factor, and cholera toxin, an exotoxin. Genes encoding both virulence factors were introduced into *V. cholerae* by horizontal gene transfer. The toxin genes are contained within the genome of CTXϕ, an integrated filamentous phage identified in 1996. In the past few years, it has been shown that CTXϕ relies on novel processes for phage DNA integration, replication and secretion. In addition, expression of CTXϕ genes — including the toxin genes — and transmission of CTXϕ were recently found to be promoted by the antirepressor RstC, which is encoded within RS1, a newly described satellite phage of CTXϕ. The genetic island that encodes TCP has also been described as a filamentous phage; however, these sequences are unlike the genome of any previously characterized filamentous phage.

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Abbreviations

Ace	accessory cholera enterotoxin
ACF	accessory colonization factor
CT	cholera toxin
TCP	toxin co-regulated pilus
VPI	vibrio pathogenicity island
Zot	zonula occludens toxin

Introduction

The severe diarrheal disease cholera arises from the colonization of the small intestine by pathogenic isolates of *Vibrio cholerae*. In general, only *V. cholerae* of the O1 serogroup and — for the past decade — the O139 serogroup have been linked to disease. Their ability to colonize a mammalian host is dependent on production of a type IV bundle-forming pilus, known as the toxin co-regulated pilus (TCP) [1]. Pilin production and the resulting colonization induce virulent *V. cholerae* to secrete a potent exotoxin, cholera toxin (CT), which is the primary diarrhea-inducing agent [2]. Uptake of cholera toxin by the intestinal epithelium results in activation of adenylate cyclase, increased Cl^- secretion, and, ultimately, loss of as much as 20 liters of fluid ('rice water stool') per day.

The genes that enable production of CT and TCP are not found in all *V. cholerae* O1 isolates; they appear to have been acquired by a subset of strains via horizontal gene transfer [3–5]. The CT-encoding genes (*ctxAB*) are found within a ~7 kb element initially called the CTX element, but renamed CTXϕ when it was found to be the genome of a filamentous phage [6]. Contemporary toxigenic (*ctxAB*$^+$) *V. cholerae* (both O1 El Tor biotype and O139) typically contain multiple CTX prophages inserted site-specifically near the terminus of the larger of the two *Vibrio* chromosomes (ChrI) [7], whereas O1 classical biotype isolates had prophages near the termini of both ChrI and ChrII [8]. Contemporary toxigenic *V. cholerae* also contain the CTXϕ-related element RS1 inserted adjacent to their CTX prophages. We have recently shown that RS1 is a filamentous phage, although not an autonomously transmissible one [9••]. Instead, RS1 is a 'satellite' phage of CTXϕ; it depends on CTXϕ coat and secretion proteins for packaging of its genome.

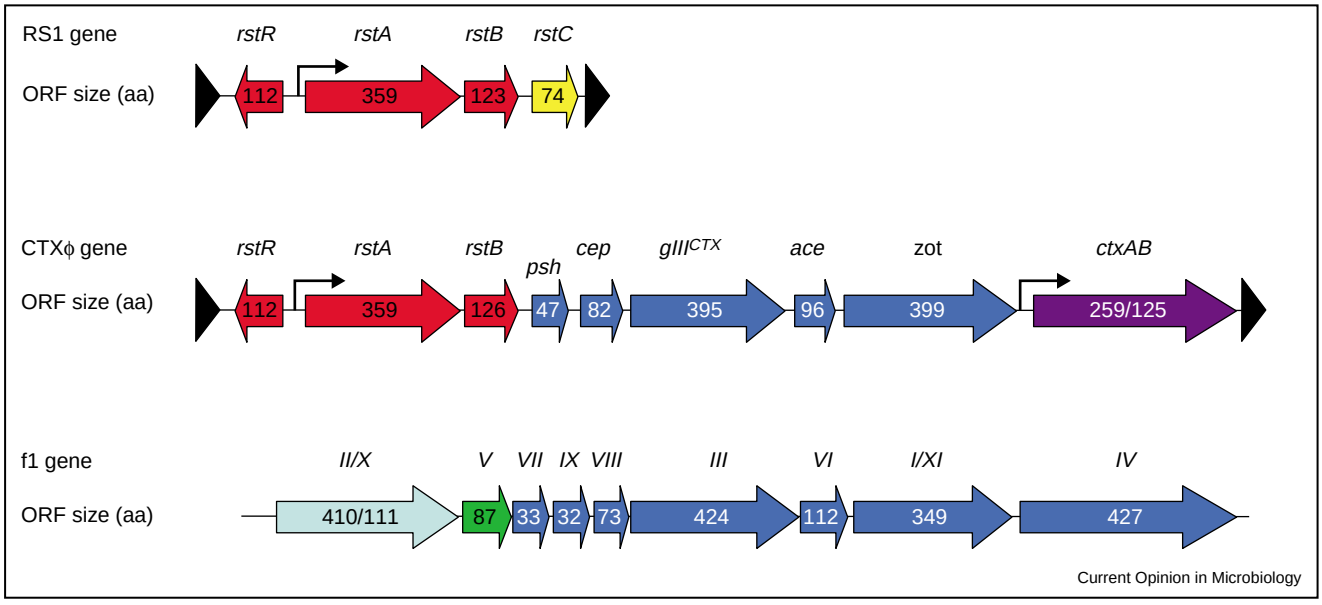
The TCP-encoding genes are clustered within a region known as both the TCP-ACF island [5] and the vibrio pathogenicity island (VPI) [4]. This ~41 kb sequence, which, when present, is found on ChrI, differs significantly in GC content from the remainder of the *V. cholerae* chromosome, suggesting that it originated in a different species. Unexpectedly, it was reported recently that the VPI also corresponds to a filamentous phage genome [10].

In this review, we will present an overview of reports from the past few years concerning the filamentous phages of *V. cholerae*, focusing on their novel features and on the path by which they gave rise to this pathogenic bacterium.

Genomes of CTXϕ and RS1

The sequences at the 5′ ends of integrated CTXϕ and RS1 are essentially identical. They encode proteins used by both elements for replication (RstA), integration (RstB), and regulation of gene expression (RstR; Figure 1) [11]. The remaining genes of CTXϕ encode proteins needed for phage packaging and secretion (Psh, Cep, OrfU, Ace and Zot), as well as CT, which does not contribute to virion formation. RS1 lacks these additional genes; however, since RS1 transmission is mediated by CTXϕ-encoded proteins, it is believed that the two phages have essentially identical packaging, secretion, and infection

Figure 1



Comparison of the genetic structures of the filamentous phages RS1, CTXϕ, and f1. Arrows indicate the direction of transcription. Black triangles represent the repeated sequences that flank the integrated phage DNA in *V. cholerae*. Three of the four genes in RS1 are almost identical to genes of CTXϕ (red arrows). Bent arrows above the CTXϕ genome represent the *rstA* and *ctxAB* promoters. The genes colored blue in CTXϕ and f1 are thought to be involved in packaging and secretion of phage DNA. Genes shown in yellow (*rstC*) and purple (*ctxAB*) have no homologs in the other elements shown; they encode an antirepressor and a toxin, respectively. Gene II/X (aqua) encodes replication proteins analogous in function to RstA, and gene V (green) encodes a single stranded DNA binding protein. Although f1 is represented as a linear element, its genome is actually circular. aa, amino acid.

processes [9••,12]. Instead of the phage morphogenesis proteins, RS1 encodes a novel protein, RstC, that was recently shown to be an antirepressor [9••].

CTXϕ compared with other filamentous phages

In some respects, CTXϕ is quite similar to the prototypical Ff filamentous coliphages: f1, fd and M13. In particular, the genes that encode coat and secretion proteins for all these phages are similar in size and arrangement within their respective genomes (Figure 1), although in most cases they have limited sequence homology [6]. Furthermore, the processes by which virions infect and are released from bacteria appear to be similar. However, coliphage titers typically exceed CTXϕ titers by a dramatic margin (maximum of 10^{11} versus 10^{7}). This is probably a consequence of the fact that Ff are maintained as relatively high-copy plasmids and their genes are continually expressed, whereas CTXϕ is maintained within the chromosome and expression of its genes is restricted by the phage repressor RstR [13]. Because of this repression, most cells in a population of *V. cholerae* do not give rise to CTXϕ. CTXϕ is also more dependent on host-encoded proteins than are the coliphages. Recent studies have revealed that chromosomal gene products mediate both chromosomal integration and secretion of CTXϕ (discussed below) [14,15•]. Interestingly, genomic analyses of filamentous phages in several other bacteria suggest that in this respect CTXϕ, rather than the coliphages, probably represent the norm.

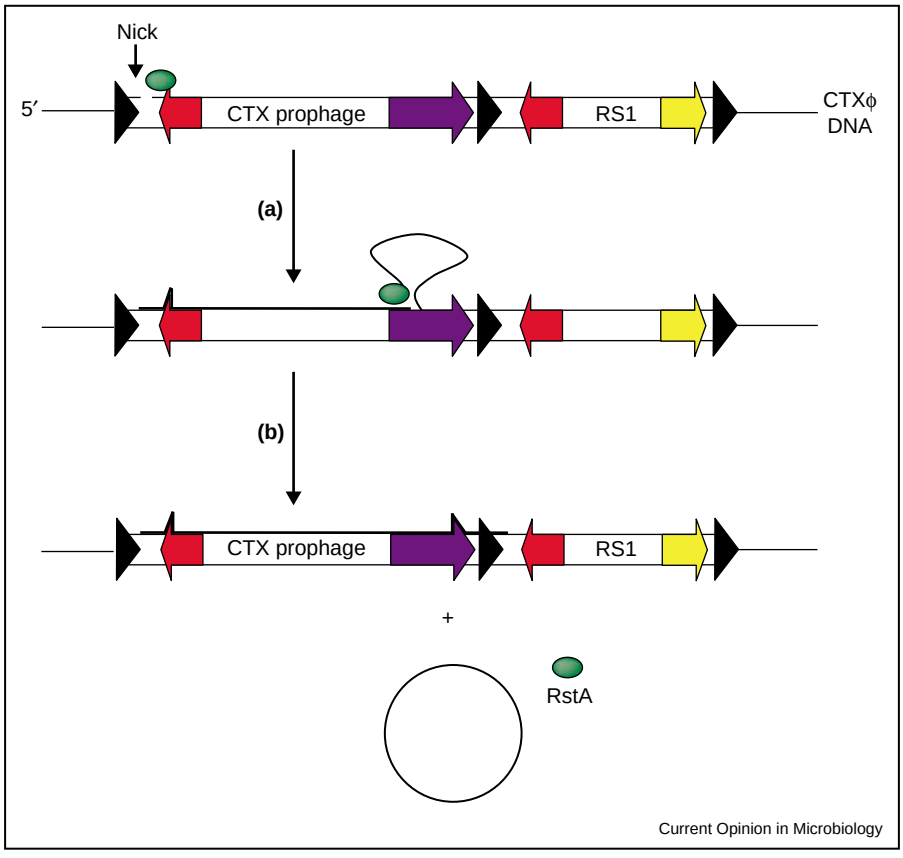
Integration of CTXϕ

Although CTXϕ integrates site-specifically into the *V. cholerae* chromosome, its genome does not include a sequence homologous to a known integrase. Furthermore, although mutational analyses revealed that the phage-encoded protein RstB contributes to integration, it is believed to be an accessory factor, not the primary recombinase [11]. Recently, Huber and Waldor [15•] demonstrated that the chromosome-encoded recombinases XerC and XerD are required for CTXϕ integration. The primary function of these recombinases is to convert chromosome dimers, often generated during replication, into monomers, by catalyzing recombination between sequences near the chromosomal termini. Interestingly, these sequences, which are known as *dif* sites, appear to overlap the CTXϕ integration sites, and disruption of *dif* prevents integration of CTXϕ. However, integration of CTXϕ does not impair conversion of chromosome dimers into monomers.

Replication of CTXϕ

Since CTXϕ DNA is maintained as an integrated prophage, production of virions requires an additional step

Figure 2



Model for production of extrachromosomal CTXϕ DNA from a CTX prophage-RS1 array. Extrachromosomal DNA is generated by a replicative process that requires tandem elements as a template. DNA synthesis initiates ~400 bp from the 5′ end of the prophage, near the 3′ end of *rstR* (red arrow), probably at a nick introduced by the CTXϕ encoded protein RstA (dark green oval). (a) Synthesis proceeds through the prophage, displacing one strand of DNA and replacing it with a new strand. Synthesis does not end after the final genes within the prophage (*ctxAB*; purple arrow); instead, (b) it proceeds through the repeat sequence that marks the boundary of the prophage (black triangle) and continues until it encounters the origin of replication within the downstream element (in this case RS1). The downstream element can either be the satellite phage RS1 (shown here, with yellow arrows representing rstC) or it can be a second CTX prophage. Once synthesis is complete, the displaced strand (a complete phage genome) is then circularized into a template for second-strand synthesis. The chromosomal template is left intact.

beyond that required for Ff, namely production of extrachromosomal phage DNA. Other integrated phages (e.g. lambda and P2) excise from the chromosome, using phage-encoded Int and Xis; however, recent studies have shown that extrachromosomal CTXϕ is not formed by prophage excision. Instead, it is generated by a replicative process that depends upon the presence of tandem elements (CTXϕ–CTXϕ or CTXϕ–RS1) within the chromosome (Figure 2; [16]). Prophages in O1 classical biotype *V. cholerae* are not arranged in tandem, and consequently these strains do not produce CTXϕ [8]. The apparent advantage of this replicative process is that phage DNA (and subsequently virions) can be produced without loss of the CTX prophage from the chromosome. Thus, horizontal and vertical transmission of CTXϕ are not mutually exclusive processes; instead, CTXϕ can be transmitted to a new host and simultaneously retained within the genome of its old host.

Secretion of CTXϕ

The simultaneous vertical and horizontal transmission of CTXϕ discussed above is only possible because filamentous phages are secreted by their hosts, and not released through host lysis. Coliphage gene IV (see Figure 1) encodes an outer membrane channel, known as a secretin, through which the phage particle is extruded [17,18]. In contrast, CTXϕ relies upon a chromosome-encoded secretin, EpsD, which is a component of the extracellular protein secretion (Eps) type II secretion system [14]. Interestingly, the Eps system also mediates release of cholera toxin, a CTXϕ-encoded virulence factor, and of a protease and a chitinase [19,20]. Although it is possible that these factors compete with CTXϕ for access to EpsD, *V. cholerae* produces such low titers of CTXϕ that it seems unlikely that phage production significantly impairs protein release. The Eps secretion apparatus, comprising around 15 proteins, is typically localized to

the old cell pole, and this may also be the site of phage release [21•]. However, since only EpsD is required for CTXϕ release, it is also possible that untargeted complexes containing just EpsD and phage secretion proteins (presumably Zot) might form and enable phage secretion from other regions of the cell.

Infection by CTXϕ

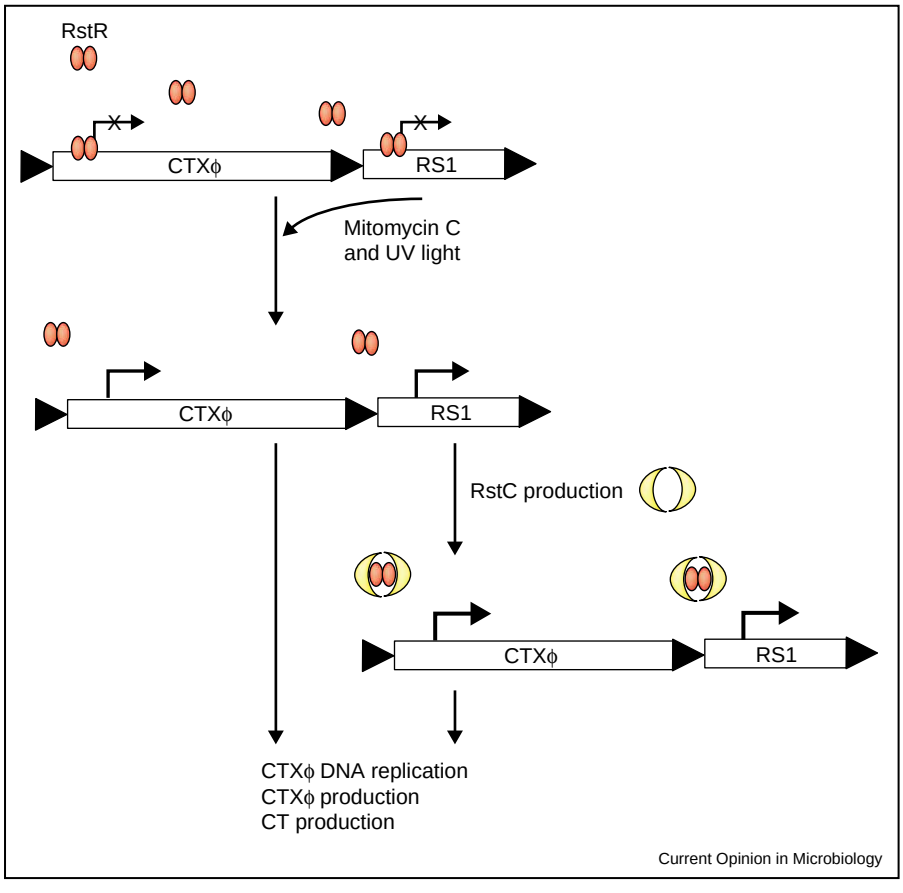
Infection of *V. cholerae* by CTXϕ appears to be similar to infection of *Escherichia coli* by Ff. Both phages utilize a pilus as a receptor (TCP for CTXϕ; F for Ff), and both also require the new host to express TolQ, TolR, and TolA [22]. Each phage also encodes a single infection protein that appears to mediate interaction with these receptor proteins. The CTXϕ 'infection protein' was formerly known as OrfU; however, it was recently renamed $pIII^{CTX}$, as it functions analogously to Ff pIII, which mediates Ff infection of *E. coli*. (D Heilpern and MK Waldor, in press).

Regulation of CTXϕ production

As mentioned above, *V. cholerae* produce relatively few CTXϕ, probably because transcription of the prophage genes is usually inhibited by the phage repressor RstR. This repressor binds to the *rstA* promoter and thereby prevents expression of all the downstream phage morphogenesis genes, which appear to be co-transcribed (Figure 3) [9••,13]. As predicted by this model, we have found that when RstR levels are reduced (e.g. in response to the DNA damaging agent mitomycin C) more CTXϕ are produced (M Quinones and BM Davis, unpublished data). Sunlight, probably its DNA-damaging UV component, is also a phage-inducing agent, although its effect on RstR levels has not been assessed [23].

CTXϕ titers also increase in response to the RS1-encoded antirepressor RstC (Figure 3) [9••]. RstC binds to and sequesters RstR, thereby preventing it from binding to its operators in the *rstA* promoter. When both proteins are

Figure 3



Regulation of CTXϕ production. Most phage genes are generally not expressed by CTXϕ lysogens grown in Luria broth, as the repressor RstR (red ovals) blocks transcription from the *rstA* promoter (bent arrow). Stimuli such as mitomycin C and UV light are thought to reduce RstR levels, thereby relieving repression. These stimuli also induce expression of *rstC* in cells carrying RS1. RstC (yellow cresents) binds to RstR, blocking its activity and further augmenting transcription from the *rstA* promoter. Mitomycin C, UV light, and RstC all induce phage DNA replication, CTXϕ production and CT production.

overexpressed, RstC induces the formation of large, stable aggregates of RstR that are usually found at one cell pole. Similarly, purified RstC induces the formation of insoluble aggregates of RstR *in vitro*. The subcellular distribution and solubility of endogenous RstR and RstC have not yet been assessed; however, RstC expressed from its natural promoter can induce CTXϕ production as much as several thousand fold. Interestingly, RstC appears to be transcribed from the *rstA* promoter, along with *rstA* and *rstB*. Consequently, a stimulus that impairs RstR probably initiates production of RstC, which then acts to inhibit the re-establishment of repression and prolongs the cycle of virion production.

RstC promotes production of cholera toxin

Expression of RstC also results in increased production of CT, as at least some of the transcripts that initiate at the *rstA* promoter extend through *ctxAB* (Figure 3) [9••]. Thus, it is possible to augment CT production even in the absence of ToxT, the transcriptional activator generally thought to regulate toxin synthesis [24]. ToxT-dependent synthesis typically occurs following the colonization of the intestine by *V. cholerae*, and ToxT is clearly the primary regulator of toxin synthesis [2,25•]. However, it is possible that RstC might allow bacteria within alternate intestinal niches (e.g. cells in which *toxT* is not turned on) to contribute to cholera pathogenesis.

Additional CTXϕ-encoded toxins

Two CTXϕ-encoded proteins — Ace (accessory cholera enterotoxin, a minor coat protein) and Zot (zonula occludens toxin, an Ff pI homolog) — have also been reported to have enterotoxic activity. Both increase short-circuit current across rabbit intestinal tissue in Ussing chambers, although their modes of action differ [26–29]. Although Ace is thought to be a minor coat protein of CTXϕ, it appears that its toxicity is not dependent on incorporation into CTXϕ [27]; however, the process by which Ace (which appears to contain a transmembrane domain) can be independently released from *V. cholerae* has not been characterized. Zot is also predicted to be a membrane protein, and it is essential for CTXϕ production, although it is not thought to be incorporated into the phage particle. Interestingly, Di Pierro *et al.* [30] recently determined that the toxin activity of Zot requires only a carboxy-terminal fragment of the protein, which is not similar in sequence to other pI homologs [31]. As it has been shown indirectly that proteolysis *in vivo* generates such a fragment [31], cleavage of full-length Zot may release the toxin from bacteria. It is not known whether the residual amino-terminal fragment can still contribute to CTXϕ assembly.

Zot and Ace were both initially identified through investigations of the residual diarrhea induced by several *ctxA*$^-$ candidate vaccine strains of *V. cholerae*, and it was hypothesized that they might contribute to the unacceptable 'reactogenicity' of these strains. It is clear that both Ace and Zot (or a Zot fragment) increase short-circuit current in Ussing chambers; however, there is currently little evidence that either protein plays a significant role in the pathogenesis of cholera. Vaccine trials have shown that some strains with intact *ace* and *zot* do not induce diarrhea (e.g. 395-N1 and CVD 103-HgR [1,32]), and that some strains lacking *ace* and *zot*, as well as *ctxA*, can still cause diarrhea, even if they have no effect in Ussing chambers (e.g. CVD110 [33]). Thus, it appears that Ace or Zot, or both, might not contribute to the virulence of *V. cholerae*.

VPI: a filamentous phage?

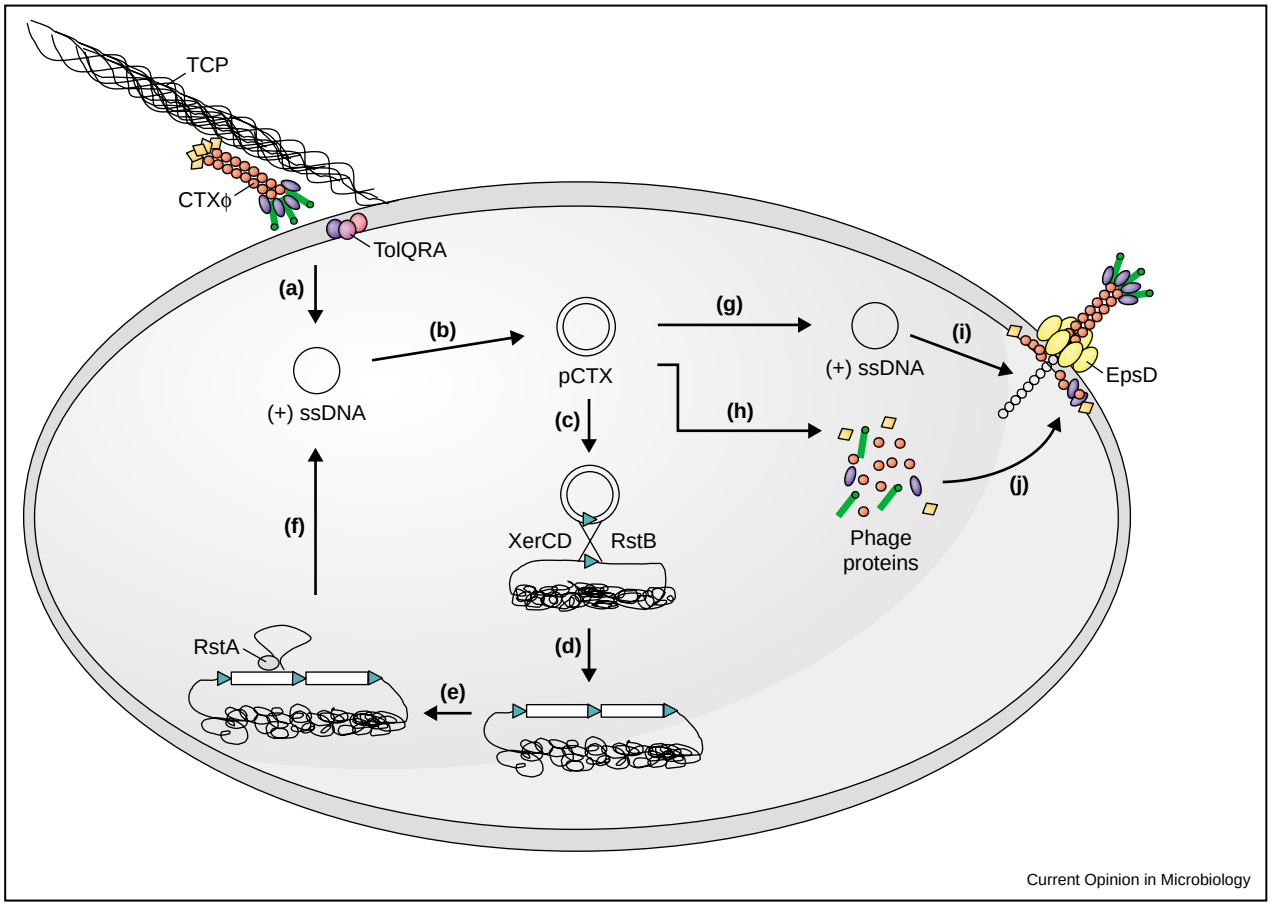
In 1999, it was proposed that TcpA, the major pilin subunit of TCP, also has an unexpected biological role. Karaolis *et al.* [10] reported that TcpA is a coat protein for a newly identified filamentous phage, VPIϕ, whose genome consists of the VPI. They detected VPI genes, but not chromosomal genes, in cell-free, nuclease-treated, concentrated *V. cholerae* culture supernatants, and found that these genes could be transmitted at high frequency to a new host. Release of VPI genes into culture supernatants depended on production of TcpA by the donor cell, and VPI genes could be co-immunoprecipitated from supernatants with anti-TcpA antisera. From these results, the authors concluded that TCP is not only a pilin subunit but also a coat protein for a novel filamentous phage [10]. The authors did not conclusively determine if TCP and VPI are a single entity; however, they speculated that this is the case [34].

The data presented by Karaolis *et al.* [10] are interesting and provocative; however, at this point they raise many more questions than they answer. On the basis of its morphology, VPIϕ was classified as a filamentous phage, yet it is unlike any previously characterized. Its genome (although never measured directly) is thought to be ~41 kb, much larger than the average ~7 kb filamentous phage genome, and it does not encode any proteins with significant homology to the conserved gene products of filamentous phages [7,10]. Although the prophage was reported to give rise to an extrachromosomal replicative form (RF), no replication genes were identified, either by homology or by functional analyses, and no evidence for replication was presented. Also unlike other filamentous phages, VPIϕ appears to be resistant to chloroform extraction, as this was one step of phage purification. It is puzzling that the phage purification protocol reliably yielded CTXϕ as well as VPIϕ, as CTXϕ is disrupted by chloroform [6]. Finally, the use of CTXϕ as a control was surprising in that Karaolis *et al.* [10] isolated CTXϕ DNA from supernatants of strains (e.g. classical biotype strains O395 [also known as 395] and TCP2) that we believe are incapable of producing CTXϕ.

There is also a conflict between the report [10] that TcpA is a coat protein for VPIϕ — presumably the major coat

protein, if TCP is in fact VPIϕ — and the consensus of reports concerning *tcpA* expression *in vitro* [2,35,36]. Karaolis *et al.* [10] found that an El Tor biotype strain of *V. cholerae* yielded an average of nine VPIϕ per cell when cultures were grown overnight at 37°C in Luria broth. The much smaller filamentous phages of *E. coli* are estimated to contain ~2800 copies of their major coat protein per phage [37]; thus, a conservative estimate would be that each VPI-producing *Vibrio* would have to produce ~25,000 molecules of TcpA. However, it is generally recognized that El Tor biotype strains express very little *tcpA* or other genes of the ToxT regulon *in vitro* when grown in Luria broth [2,35,36]. It is hard to comprehend how production of the quantities of TcpA needed for VPIϕ assembly could have previously escaped detection. It is, of course, possible that TCP/VPIϕ could represent a completely new class of phage; phage with filamentous structures could theoretically be formed by numerous and diverse pathways. Furthermore, several recent studies have demonstrated that secretion pathways previously thought to be distinct are, in fact, overlapping (e.g. [38,39]); the production of VPIϕ could be one more example of this paradigm. However, we think that alternate explanations for the results of Karaolis *et al.* should also be considered. It is clear that many aspects of VPIϕ biology are in need of further study, as the report by Karaolis *et al.* [10] has prompted many questions concerning *Vibrio* evolution, pathogenicity, and phage biology (see for example Lee [40]) that remain unanswered.

Figure 4



Model of the key steps (infection, integration, replication, assembly and secretion) in the life cycle of CTXϕ. (a) CTXϕ infection of *V. cholerae* requires TCP and TolQ, TolR and TolA. The single stranded DNA of CTXϕ ((+) ssDNA) loses its protein coat and is transported into the bacterial cytoplasm. (b) DNA complementary to the phage genome is synthesized to generate pCTX, the replicative (plasmid) form of CTXϕ. (c) The chromosome-encoded recombinases XerC and XerD, along with the phage-encoded protein RstB, are required for integration of pCTX into the chromosome at or near the *V. cholerae dif* site. (d) Recombination between nearly identical sequences in pCTX and the *V. cholerae* chromosome (green triangles) generates either a single CTX prophage or (as shown) tandem prophages. (e) Tandem prophages can serve as a template for production of extrachromosomal phage DNA. This process is initiated by the phage-encoded protein RstA; (f) it results in the formation of the single-stranded phage genome ((+)ssDNA). (g) pCTX can serve as a template for further replication of the phage genome; (h) its genes can also be transcribed and translated, resulting in synthesis of phage proteins. (i and j) Phage proteins are thought to be inner membrane proteins prior to insertion into phage particles. Phage DNA (chain of circles) is simultaneously packaged into virions and secreted from the cell. Phage secretion depends upon the outer membrane channel EpsD. Ss, single-stranded.

Evolution of pathogenic *V. cholerae*

As mentioned above, two crucial events in the evolution of pathogenic *V. cholerae* were the acquisition of the TCP-encoding gene cluster and the acquisition of *ctxAB*. As TCP is the receptor for CTXϕ, which carries *ctxAB*, it is likely that the ~41 kb region that includes the TCP-encoding genes was acquired first. Some segments of this region differ significantly between O1 El Tor biotype and O1 classical biotype strains; however, sequence analyses suggest that it was acquired by the two lineages around the time that they diverged, so it may have been acquired just once by a common ancestor [41]. Alternatively, two distinct lineages may have acquired a very similar element. In either case, integration of the new DNA into the chromosome was probably mediated by the integrase encoded at one end of the island.

In contrast, CTXϕ was clearly acquired by El Tor and classical biotype strains after they diverged from a common ancestor; thus, toxigenic *V. cholerae* has evolved at least twice [42]. RS1 is also present in most, if not all, toxigenic seventh pandemic El Tor biotype strains, although it has never been detected in classical strains [3,8]. It is not clear why CTXϕ and RS1 are so tightly coupled in the El Tor strains. Interestingly, Vibrios infected with a CTXϕ precursor phage have also been identified [42]. This phage includes all of CTXϕ except for *ctxAB* and some adjacent sequences, including the *ctxAB* regulatory sequences. It appears that *ctxAB* were a late addition to the CTXϕ genome and that imprecise excision of the CTXϕ precursor from the chromosome of another host resulted in fusion of adjacent, formerly chromosomal, toxin genes to the phage genome. It is intriguing — and probably evolutionarily advantageous — that the virulence genes acquired in separate events (e.g. *ctxAB* and the TCP gene cluster) are nonetheless controlled by a single regulator, ToxT.

Conclusions and future studies

In the past few years, a better understanding of key steps of the CTXϕ life cycle has been obtained (Figure 4). As several of these steps exploit chromosome-encoded proteins, subtly adapting their functions to suit the phages' ends, it is increasingly evident that study of CTXϕ — and presumably other phages as well — can also expand our knowledge of cellular processes.

Acknowledgements

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- •• of outstanding interest

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