

# Phage-bacterial interactions in the evolution of toxigenic *Vibrio cholerae*

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Understanding the genetic and ecological factors which support the emergence of new clones of pathogenic bacteria is vital to develop preventive measures. *Vibrio cholerae* the causative agent of cholera epidemics represents a paradigm for this process in that this organism evolved from environmental non-pathogenic strains by acquisition of virulence genes. The major virulence factors of *V. cholerae*, cholera toxin (CT) and toxin coregulated pilus (TCP) are encoded by a lysogenic bacteriophage (CTX $\phi$ ) and a pathogenicity island, respectively. Additional phages which cooperate with the CTX $\phi$  in horizontal transfer of genes in *V. cholerae* have been characterized, and the potential exists for discovering yet new phages or genetic elements which support the transfer of genes for environmental fitness and virulence leading to the emergence of new epidemic strains. Phages have also been shown to play a crucial role in modulating seasonal cholera epidemics. Thus, the complex array of natural phenomena driving the evolution of pathogenic *V. cholerae* includes among other factors, phages that either participate in horizontal gene transfer or in a bactericidal selection process favoring the emergence of new clones of *V. cholerae*.

## Introduction

Bacterial evolution is believed to have been significantly driven by their interactions with bacteriophages. Phages contribute to the evolution of bacteria by mediating horizontal transfer of clusters of genes and genomic rearrangements, as well as by bactericidal selection.<sup>1-3</sup> In this latter process, bacterial strains that are able to resist phage predation have an advantage over the susceptible strains in their competition for survival. Toxigenic *Vibrio cholerae*, the causative agent of the epidemic diarrheal disease cholera, interacts with diverse phages, and the interaction can promote genetic diversity and/or cause selective enrichment of particular bacterial clones.<sup>3</sup> *V. cholerae* represents a group of bacteria which is autochthonous to riverine, coastal and estuarine ecosystems, but at the same time, pathogenic for humans.<sup>4,5</sup> In recent times,

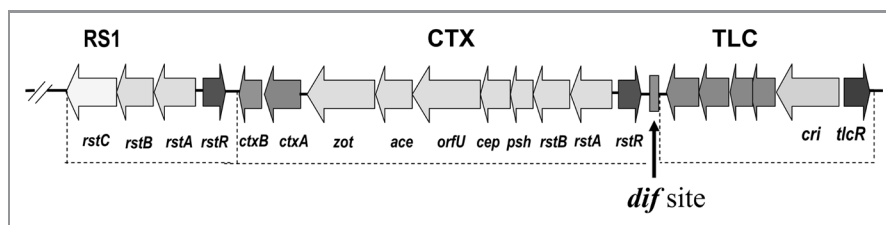
dramatic progress has been made in understanding the genetic determinants that account for virulence of this organism and how virulent strains emerge in nature.

Cholera is an ancient disease, and there has been seven recorded pandemics of cholera since the first pandemic began in 1817.<sup>4,5</sup> However, the disease still affects millions of people worldwide, and causes an estimated 120,000 deaths and manifold more cases each year. The current seventh pandemic of cholera, which originated in Indonesia in 1961, is the most extensive in geographic spread and duration, and the causative agent is *V. cholerae* O1 of the El Tor biotype. The sixth pandemic and presumably the earlier pandemics were caused by the classical biotype, which is now extinct.<sup>6</sup> The two biotypes of *V. cholerae* differ in certain phenotypic and genetic characteristics.<sup>4</sup> The factors associated with the replacement of the classical biotype as the predominant epidemic strain by the El Tor biotype, and eventual disappearance of the classical strains have not been adequately explained. The displacement of the classical strains by the El Tor might have been driven by undefined environmental forces to which the El Tor strains adapted more efficiently. However, possible contribution of particular groups of phages have not been ruled out. Presumably, these phages selectively eliminated the classical biotype strains, while enriching the El Tor biotype.<sup>7</sup>

The best known example of a phage contributing to the emergence of toxigenic strains of *V. cholerae* is the CTX $\phi$  which carries the genes encoding cholera toxin in its genome.<sup>2</sup> There is also a high probability for discovering new phages which are able to transfer other virulence associated genes, pathogenicity islands or genes that promote environmental fitness. This is because the pandemic *V. cholerae* strains carry a number of gene clusters which have structural features consistent with horizontally transferable elements,<sup>8</sup> and evolutionary intermediate strains carrying incomplete sets of these gene clusters, and thus with a lower virulence potential than the pandemic strains have been shown to exist in nature.

The recent recognition that phage predation may play a role in the natural control of cholera epidemics<sup>3,9,10</sup> reinforces prediction that changes in this pathogen may have been to a large extent driven by phages. The emergence of certain strains are likely to be enhanced by phages through the bactericidal mechanism in which phage sensitive strains are killed while providing a selective advantage to phage resistant strains. Therefore, the ability to evade

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**Figure 1.** Chromosomal arrangement of RS1, CTX prophage and TLC gene cluster in *V. cholerae*. Open reading frames are shown as arrows, and the rectangle between CTX and TLC represents the *dif* site.

phage predation constitutes important development in attaining evolutionary fitness. On the other hand, to achieve phage resistance there may be a fitness cost for the pathogen, due to altered expression of surface appendages such as flagella or pili or O-antigens which may often act as receptors for different phages. Therefore the evolutionary success of *V. cholerae* has presumably been dependent on a balance between these various factors. In this review we have summarized available information on the interactions of phage and *V. cholerae*, and the effect of these interactions on the genetics, epidemiology, and evolution of the pathogen.

### Bacteriophages Acting on *V. cholerae* (Vibriophages)

As far back as the 19th century it was recognized that certain elements (most likely phages) in the waters of the Ganges and Jumna rivers in India had marked antibacterial activity against *V. cholerae*, and could protect against cholera.<sup>11-13</sup> The role of phages in combating cholera, as well as other bacterial infections were eventually ignored due mainly to the discovery and availability of antibiotics. Two remarkable discoveries in recent times have renewed our interest in phages, in particular, phages that grow on *V. cholerae*, and generally known as vibriophages. The discovery that cholera toxin (CT) the major virulence factor of toxigenic *V. cholerae*, is encoded by a lysogenic filamentous phage (CTX $\phi$ ), led to the exploration of other filamentous vibriophages. Accordingly, a number of other phages and satellite phages have been identified in *V. cholerae* O1 and O139 serogroup strains and the possible roles of these phages in horizontal gene transfer have been studied.<sup>11,14</sup>

In contrast to filamentous phages, which do not usually kill the host bacterial cells, the lytic phages can kill the host cells and thus contribute a strong selective force in nature, for the emergence of bacterial clones which are resistant to certain phages. The recent recognition that phage blooms coincide with the decline of *V. cholerae* in water in cholera-endemic areas<sup>3,9,10</sup> has also renewed interest in lytic phages, due to their potential application in developing control measures against cholera epidemics. A considerable number of lytic and temperate vibriophages have now been found, of which the JSF series of environmental phages isolated in Bangladesh are of particular interest because of their role in epidemiology and ecology of *V. cholerae*. At least 16 distinct phages (JSF1 through JSF 16) have been isolated in Bangladesh,<sup>7,15</sup> and efforts are under way to isolate and characterize more cholera phages.

### The Cholera Toxin Converting Phage CTX $\phi$

Bacteriophages can convert their bacterial host from a nonpathogenic strain to a pathogenic strain through a process called phage conversion, by providing the host with phage-encoded virulence genes. Toxigenic *V. cholerae* isolates carry the *ctxAB* genes encoded by a lysogenic filamentous phage CTX $\phi$ .<sup>2</sup> Only those strains that carry CTX $\phi$  cause epidemic and pandemic cholera. The phage is similar in size, structure, and gene order to filamentous coliphages such as M13 and f1 from *E. coli*.<sup>11</sup> The *ctxAB* genes which encode the A-B type enterotoxin CT reside in the CTX $\phi$  genome, which exists as a prophage, the integrated form of the phage genome in the host bacterial chromosome.

CTX $\phi$  has a 6.9-kb genome organized into two functionally distinct modules (Fig. 1). These are RS2 (repeat sequence 2), which comprises three genes, *rstR*, *rstA* and *rstB*, and a core region encoding *psh*, *cep*, *orfU* (*gIII*), *ace*, *zot* and *ctxAB*. The RstA protein is required for CTX $\phi$  DNA replication, which in *E. coli* Ff filamentous coliphage is performed by pII, the gene for which is located at the same genomic region as the *rstA* gene in CTX $\phi$ .<sup>16</sup> The *rstR* gene encodes a repressor protein whereas the *rstB* gene encodes a protein required for integration of the CTX $\phi$  genome into the bacterial chromosome. The RstB protein contains a region similar to single-stranded (ss) DNA-binding proteins, which in other filamentous phages stabilize phage ssDNA.<sup>17</sup> The Psh, Cep, OrfU and Ace proteins are phage structural proteins whereas *Zot* is a protein required for phage assembly.<sup>2</sup> The genes *cep*, *orfU*, *ace* and *zot* correspond to genes gVIII, gIII, gVI and gI respectively of Ff coliphage. The *ctxAB* genes are unique to CTX $\phi$ , but are not essential for phage production, and are located at the same site as gIV in *E. coli* Ff phage. CTX $\phi$  does not encode a pIV protein homolog, which in Ff phage is required for secretion from the bacterial cell. Another important difference between CTX $\phi$  and typical *E. coli* Ff filamentous coliphages is that CTX $\phi$  integrates into the *V. cholerae* genome to form stable lysogens. However, the CTX $\phi$  genome does not encode an integrase gene which should perform this function in lysogenic phages.<sup>2</sup> Instead, CTX $\phi$  uses host encoded integrases XerC and XerD for its integration into the bacterial chromosome. The XerCD proteins are conserved among eubacteria, as they serve to resolve chromosome dimers during cell division.<sup>18</sup> Besides CT, a type IV pilus, the toxin co-regulated pilus (TCP) is the most important virulence factor of *V. cholerae*, and is essential for colonizing the intestine of the host. TCP consists of TcpA

subunits encoded by the *tcpA* gene located in the TCP pathogenicity island. The TCP island is a 40 kb chromosomal region flanked on both sides by a 20-bp *att*-like attachment sequence, and comprised of *tcp* and *acf* gene clusters as well as genes encoding a putative integrase and a transposase.<sup>8</sup>

Interestingly TCP is also the receptor for CTX $\phi$  on the *V. cholerae* cell surface, whereas the periplasmic and inner membrane proteins TolQRA facilitate infection of the bacteria by CTX $\phi$ .<sup>2,19</sup> The OrfU protein of CTX $\phi$ , which has been renamed pIII<sup>ctx</sup>, has been hypothesized to bind to the major pilin protein TcpA of TCP, similar to the binding of F $\phi$  coliphages to the F pilus mediated by the minor coat protein pIII. After CTX $\phi$  is adsorbed to the *V. cholerae* cell wall, the ssDNA is injected to the cell cytoplasm. Upon entry into the cell cytoplasm, the linear ssDNA circularizes, forming pCTX, which can remain as an extra chromosomal element or integrate into the *V. cholerae* genome at specific attachment sites.<sup>2,18</sup> However, in contrast to the classical model of phage integration in which phage ssDNA is converted to dsDNA prior to integration, Val and colleagues<sup>20</sup> proposed that Xer recombinases promote the direct insertion of the ssDNA genome into the *difI* site of *V. cholerae*. The wide distribution of XerCD recombinase system among bacteria and the prevalence of *dif* sites among filamentous phages suggest that this system may be widely involved in filamentous phage integration into the bacterial genome.

Similar to other filamentous phages, CTX $\phi$  is secreted from the bacterial cell without lysis of the cell. However, the genome of CTX $\phi$  differs from that of filamentous phages from *E. coli* in that it lacks the gene for a putative bacteriophage export or secretin protein (a homolog of Ff gIV). Instead, CTX $\phi$  uses the EspD secretin, part of a type II extracellular protein secretion system. The EspD protein, an outer membrane component, appears to be the only component of the type II secretion system required for secretion of CTX $\phi$ .<sup>21</sup> The type II secretion system is the pathway used for export of CT, hemagglutinin-protease, chitinase and other proteins out of the *V. cholerae* cell.<sup>21,22</sup>

**Origin of CTX $\phi$ .** A number of features of the CTX $\phi$  genome suggest that the *ctxAB* genes were acquired by a CTX $\phi$  progenitor (preCTX $\phi$ ) from an unknown source.<sup>2,23</sup> The percent GC content of the *ctxAB* genes (38%), is different from that of the rest of the CTX $\phi$  genome (40%). The production of CT is promoted by two promoters: the P<sub>*rstA*</sub> promoter, which is ~5 kb upstream of *ctxA* is required for CTX $\phi$  virion synthesis but can extend into *ctxAB*, and P<sub>*ctxAB*</sub>, the promoter found immediately upstream of *ctxAB*. Furthermore, *V. cholerae* strains carrying CTX prophage sequences that do not encode the *ctxAB* genes have been identified.<sup>23</sup> These precursor CTX (preCTX) prophages also produce the replicative form (RF) of their genomes, which are very similar to the RF of CTX $\phi$  genome except that they do not carry the *ctxAB* genes. Molecular analysis of these preCTX plasmids revealed that they also lacked the upstream control region normally found 5' of *ctxA*, as well as the *ctxAB* promoter region and coding sequences. These observations suggest that a preCTX simultaneously acquired the *ctxAB* genes and their regulatory sequences. It has been proposed that in the event that led to the acquisition of *ctxAB* genes by a preCTX $\phi$ , integration of

the preCTX $\phi$  genome occurred next to the *ctxAB* genes carried by an unknown organism, and because CTX virion production occurs by rolling circle replication from the chromosome, *ctxAB* genes and the regulatory sequences were acquired from the unknown source by imprecise prophage replication.<sup>11,23</sup>

The acquisition of *ctxAB* genes by the pre-CTX $\phi$  has increased its fitness since CTX $\phi$  confers a selective advantage to its *V. cholerae* host by producing CT; the action of the enterotoxin results in profuse diarrhea, promoting dissemination of the bacterium and hence the phage. CTX $\phi$  forms stable lysogens in *V. cholerae*, and replication of the phage does not cause *V. cholerae* cell lysis; therefore, CTX $\phi$  genome can be both vertically and horizontally transferred.<sup>2,11</sup>

**Diversity of CTX $\phi$ .** The sequence of *rstR* gene of CTX $\phi$  can vary considerably among El Tor, classical and O139 isolates, although *rstA* and *rstB* show a high level of sequence conservation among different *V. cholerae* isolates. The *rstR* sequence variation has been used to classify CTX $\phi$ . For example, CTX $\phi$  from classical isolates carry *rstR*<sup>class</sup> and are designated CTX<sup>class</sup> $\phi$ ; whereas that from El Tor isolates usually carry *rstR*<sup>ET</sup>, and are designated CTX<sup>ET</sup> $\phi$ . *V. cholerae* O139 carry either CTX<sup>ET</sup> $\phi$  or a CTX $\phi$  with a distinct *rstR* designated *rstR*<sup>Calc</sup> and the phage is designated CTX<sup>Calc</sup> $\phi$ .<sup>24,25</sup> *V. cholerae* O139 strains may also carry both CTX<sup>ET</sup> $\phi$  or CTX<sup>Calc</sup> $\phi$  prophages simultaneously.<sup>26</sup> In addition several novel *rstR* sequences have been identified in *V. cholerae* non-O1/non-O139 isolates, and hence there may be diverse CTX $\phi$  carried by these environmental *V. cholerae* strains.<sup>11</sup> Molecular evolutionary analysis of CTX $\phi$ s derived from a variety of *V. cholerae* natural isolates have been done based on sequence analysis of the *rstR* and the gIII<sup>CTX</sup>. These data taken together indicate distinct lineages among CTX $\phi$ , independent acquisition of CTX $\phi$  by different *V. cholerae* isolates, and acquisition of novel CTX $\phi$ <sup>23,27</sup> by classical, El Tor and O139 strains.

**Chromosomal organization of CTX prophages.** The arrangement of CTX prophage on the genome of *V. cholerae* varies depending on (1) whether it is integrated at a single site or two sites, and (2) the biotype or serogroup of the strain. Among El Tor strains, the CTX prophage is located on the large chromosome (chromosome I) ~842 bp downstream from a tandemly duplicated DNA sequence referred to as toxin linked cryptic (TLC) (Fig. 1), which was later shown to be a satellite phage genome.<sup>14,28</sup> CTX prophage in the chromosome of El Tor strains is also flanked by a 2.7 kb repetitive sequence originally referred to as RS1 element, which was also later found to be the genome of a satellite phage, RS1 $\phi$ .<sup>29,30</sup> Many different CTX and RS1 arrangements have been observed among *V. cholerae* O1 El Tor biotype strains.<sup>28</sup> For example, El Tor strain E7946 isolated in Bahrain in 1978 was shown to contain an RS1-CTX<sup>ET</sup>-RS1-CTX<sup>ET</sup>-RS1 arrangement, whereas strain C6709 isolated in Peru in 1991, contained a CTX<sup>ET</sup>-RS1 arrangement.<sup>28,31</sup> The production of infectious CTX $\phi$  particles requires the presence of either a CTX-CTX array comprising two CTX prophages or a CTX-RS1 array, and the DNA in CTX $\phi$  is a hybrid sequence that is derived from these elements.<sup>32</sup> In *V. cholerae* classical biotype strains, CTX prophage is located on both chromosomes and without an RS1 element.<sup>33</sup> On chromosome I, CTX is present in the intergenic

**Table 1.** Phages associated with horizontal gene transfer among *V. cholerae*

Designation	Genome size (bp)	Receptor	Description and function
CTX $\phi$	6,900	TCP	Filamentous bacteriophage that encodes cholera toxin. CTX phage can integrate in the chromosome of <i>V. cholerae</i> forming stable lysogens.
RS1 $\phi$	3,000	TCP/MSHA	A satellite phage that uses CTX $\phi$ -encoded proteins to form RS1 phage particles. RS1 carries the gene for an anti-repressor protein RstC that influences CTX $\phi$ replication and transmission.
KSF-1 $\phi$	7,107	MSHA	A filamentous phage capable of packaging RS1 and possibly other heterologous DNA of <i>V. cholerae</i> .
VGJ $\phi$	7,542	MSHA	VGJ $\phi$ is able to integrate its genome into the same chromosomal <i>attB</i> site as CTX $\phi$ , entering into a lysogenic state.
fs-2 $\phi$	8,651	MSHA	Filamentous phage fs-2 $\phi$ forms turbid plaques on <i>V. cholerae</i> O139 and O1 El Tor biotype strains. A 715 nucleotide fragment located in the large intergenic region of fs-2 was highly homologous to a part of region RS2 of CTX $\phi$ . This phage acts as a helper to produce satellite phage TLC $\phi$ particles.
TLC $\phi$	5,364	MSHA	The TLC satellite phage genome carries a sequence similar to the <i>dif</i> recombination sequence which functions in chromosome dimer resolution during cell division. Lysogeny by TLC phage generates a functional <i>dif</i> site in <i>dif</i> defective strains. The <i>dif</i> site also overlaps with the <i>attB</i> site, which is essential for stable integration of CTX $\phi$ genome.
CP-T1	~43,500	O-antigen	A generalized transducing phage of <i>V. cholerae</i> , which can laterally transfer chromosomal segments among <i>V. cholerae</i> strains.

region between TLC and repeat in toxin (RTX) gene clusters, often designated as the El Tor site, and on chromosome II in the intergenic region between the genes designated VCA0569 and VCA0570, defined as the classical site.<sup>33</sup> Within certain classical strains (O395, GP12 and C33), CTX<sup>class</sup> is present as an array of truncated, fused prophages at the El Tor integration site on chromosome I whereas at the classical integration site on chromosome II, a solitary CTX<sup>class</sup> prophage is present.<sup>33</sup> In several other classical strains (CA401, C1, C14, C21 and C34), a single CTX<sup>class</sup> prophage is present in the El Tor site whereas at the classical site on Chromosome II, a truncated CTX<sup>class</sup> array is found. The classical prophage arrangements cannot generate pCTX<sup>class</sup> and CTX<sup>class</sup> $\phi$ .<sup>33</sup>

A number of CTX prophage arrangements have been described within *V. cholerae* O139 serogroup strains.<sup>24,25,32-35</sup> Whereas strain SG24 contains a CTX<sup>ET</sup>-RS1-CTX<sup>ET</sup> array, strains AS197 and AS209 contain a RS1-CTX<sup>ET</sup>-CTX<sup>Calc</sup>-CTX<sup>Calc</sup> array. CTX prophage arrangement appears to be dynamic and continually giving rise to new variant strains. For example, genomic analysis of a *V. cholerae* strain B-33 isolated in Mozambique in 2004 revealed the presence of CTX<sup>class</sup> with a CTX<sup>class</sup>-CTX<sup>class</sup> array at the classical insertion site on chromosome II.<sup>36</sup>

**Distribution of CTX $\phi$ .** Although most toxigenic *V. cholerae* strains belonging to the O1 or O139 serogroups carry CTX prophages, the distribution of CTX is sporadic among non-O1/non-O139 serogroup strains.<sup>5,37-39</sup> The limited distribution of CTX $\phi$  among non-O1/non-O139 strains is possibly due to the limited distribution of TCP, which is the host receptor for CTX $\phi$ . TCP is encoded on the TCP pathogenicity island, and the bacterium must acquire this island before it can uptake CTX $\phi$ .<sup>2</sup> A range of *V. mimicus* isolates are also known to carry CTX $\phi$  and most of these isolates encode TCP, indicating a similar method of

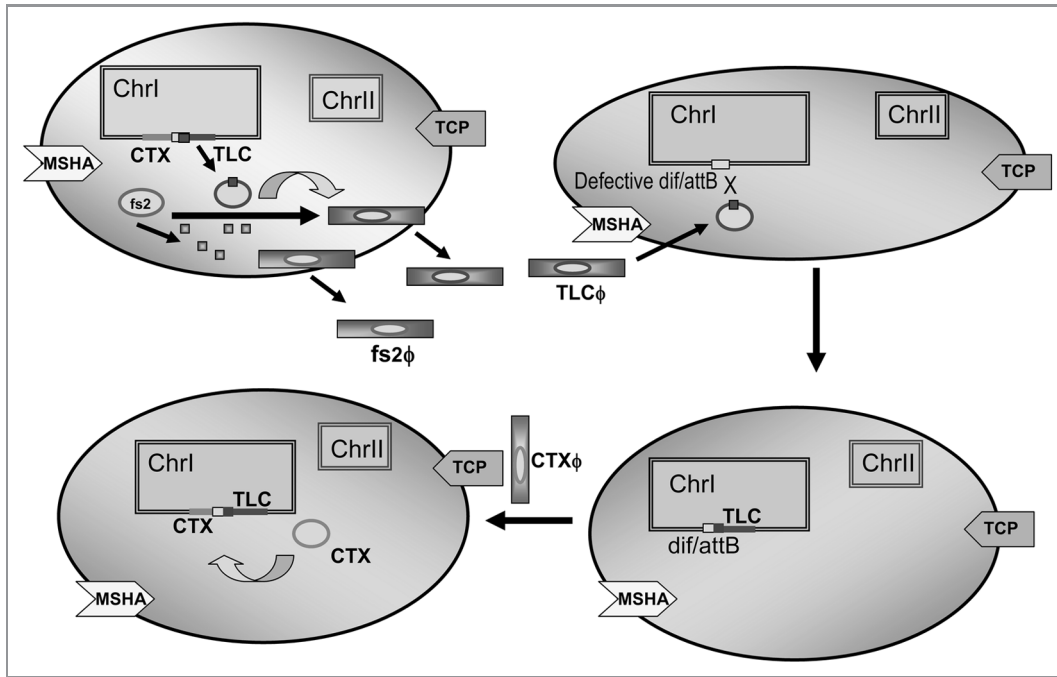
phage infection. There have been preliminary reports on the presence of homologs of CTX prophage as well as the CTX $\phi$  receptor TCP in the genomic sequence of *V. fischeri*. However, it is not known whether the TCP sequence is functional in this species. The CTX prophage from *V. fischeri* has been found to lack the *ctxAB* genes.<sup>11</sup> Nevertheless, the presence of CTX $\phi$  and TCP island or their homologs in *Vibrio* species other than *V. cholerae* may represent additional reservoirs of potential *V. cholerae* virulence genes.<sup>11,40</sup>

### Other Filamentous Phages of *V. cholerae*

Besides CTX $\phi$ , at least nine other filamentous phages or satellite filamentous phages that lack genes required for phage morphogenesis have been described. These include the RS1 and TLC satellite phages, and KSF-1, VGJ, VSK, VSKK, 493, fs1 and fs2 phages. Some of these phages are also involved in horizontal gene transfer (Table 1). Filamentous phages such as KSF-1 $\phi$  and VGJ $\phi$  can supply capsid, assembly and packaging proteins for RS1 and possible other satellite phage genomes and thus facilitate the horizontal transfer of the satellite phage genomes. With increasing numbers of different *V. cholerae* genomes being sequenced, the number of filamentous phage genome sequences and identification of novel prophages are likely to increase dramatically.

**RS1 satellite phage.** The satellite filamentous phage RS1 $\phi$  encodes four open reading frames (ORF) namely *rstR*, *rstA*, *rstB* and *rstC* (Fig. 1). The first three of these are homologous to the corresponding genes of the RS2 region of CTX $\phi$ , whereas the additional ORF *rstC* encodes an antirepressor protein RstC which causes the RstR repressor to aggregate and prevent binding to the P<sub>*rstA*</sub> promoter.<sup>29,30</sup> The RstC protein promotes transcription of CTX $\phi$  genes required for phage production, supporting the





**Figure 2.** Role of TLC $\phi$  in CTX $\phi$  mediated toxigenic conversion of *V. cholerae*. Infectious TLC $\phi$  particles are produced from TLC satellite phage genome by using morphogenesis proteins of the helper phage fs2 $\phi$ . The TLC $\phi$  infects recipient strains using MSHA pilus as the receptor. Upon chromosomal integration of the TLC $\phi$  genome, a functional *dif/attB* sequence is generated in TLC-negative strains that apparently exhibit defective *dif/XerC/XerD*-mediated chromosome dimer resolution. Lysogeny by such TLC phages also generates a chromosomal *attB* site which is essential for stable integration of CTX $\phi$  and conversion of *V. cholerae* to a toxigenic form. The CTX $\phi$  uses TCP as its receptor on the bacterial cell surface.

transmission of both RS1 $\phi$  and CTX $\phi$ .<sup>30</sup> On the other hand, RS1 being a satellite filamentous phage lacks genes required for phage morphogenesis and depends on CTX $\phi$  for the capsid proteins, packaging and transmission.<sup>29</sup> Upon infection of recipient cells the RS1 $\phi$  genome, similar to CTX $\phi$ , integrates site-specifically into the *V. cholerae* chromosome.<sup>29</sup> The interactions and cooperation of RS1 and CTX prophages thus appear to promote the propagation of both these phage genomes.

**The TLC satellite phage.** In toxigenic *V. cholerae*, the CTX prophage is associated with a tandemly duplicated DNA sequence originally referred to as toxin linked cryptic (TLC) which is located upstream of the CTX prophage (Fig. 1). In most strains, TLC-related sequences also exist extrachromosomally as plasmids (pTLC). Each copy of chromosomally integrated TLC element was found to comprise of five ORFs. For example, in strain AL 33457, the two copies of TLC comprised of ORFs, VC1466 to VC1470 and VC1472 to VC1476 respectively, with VC1471 located in between the two copies of the element.<sup>14</sup> The largest ORF in TLC encodes a protein named Cri replicase which is similar to the replication initiation protein (pII) of *E. coli* F-specific filamentous phages. The TLC also encodes TlcR, a protein that displays sequence similarity to RstR, the repressor controlling lysogeny of the filamentous CTX $\phi$ . It has been recently confirmed that the TLC is the genome of a satellite filamentous phage which uses morphogenesis proteins of another filamentous phage known as fs2 $\phi$  to form infectious TLC $\phi$  particles.<sup>14</sup>

Interactions of *V. cholerae* with TLC $\phi$  appears to be important in the evolutionary biology of *V. cholerae*. The TLC phage

genome carries a sequence similar to the *dif* recombination sequence which functions in chromosome dimer resolution during cell division. *Dif* sequences are required for XerC/XerD-mediated resolution of chromosome dimers in *E. coli* and *V. cholerae* but are also exploited by various filamentous phages for integration of their genomes into the host chromosome.<sup>41-46</sup> The *dif* site (Fig. 1) overlaps with the *attB* sequence which is utilized by CTX $\phi$  and RS1 $\phi$  for their chromosomal integration though XerC/XerD-mediated recombination with the corresponding *dif/attP* site formed by annealing of ssDNA derived from the phage genomes.<sup>45,46</sup>

Bacterial cells defective in the dimer resolution often show a pathology of cell filamentation.<sup>41,42</sup> It has been shown that TLC $\phi$ -related transducing phages can, upon chromosomal integration of their genome generate a functional *dif* sequence and correct aberrant filamentous morphology in cells of naturally occurring, TLC-negative strains that apparently exhibit defective *dif/XerC/XerD*-mediated chromosome dimer resolution. Lysogeny by such TLC phages also generates a chromosomal *attB* site which is essential for stable integration of CTX $\phi$  and conversion of *V. cholerae* to a toxigenic form (Fig. 2). Thus, TLC $\phi$  plays a critical role in improving not only the chromosome replication efficiency of *V. cholerae* but also its emergence as a human pathogen.

**KSF-1 $\phi$ .** Besides CTX $\phi$ , the first filamentous phage found to act as a helper for the RS1 satellite phage was KSF-1 $\phi$ , which supports the production of infectious RS1 $\phi$  particles independently of CTX $\phi$  by providing the gene products required for capsid, assembly and packaging of RS1 $\phi$  particles.<sup>47</sup> KSF-1 $\phi$  is a viable infectious phage

that integrates into the host genome and contains an 18-bp *att*-like sequence. The phage particles have a length of 1.2  $\mu\text{m}$ , and a width of 7 nm, and the particles contain a single-stranded DNA genome. The genome was found to comprise 7,107-bp with a GC content of 44% encoding 14 ORFs.<sup>48</sup> The host cell receptor for KSF-1 $\phi$  is the mannose sensitive hemagglutinin (MSHA) pilus. Since the receptor for KSF-1 $\phi$  is the MSHA pilus, RS1 $\phi$  particles produced by this phage can infect MSHA positive strains, and is independent of TCP for infecting recipient bacterial hosts. Thus, KSF-1 $\phi$  is capable of facilitating the transfer of RS1 to strains that do not express toxin coregulated pilus. Given that RS1 can enhance coproduction of CTX $\phi$  particles, KSF-1 $\phi$ -mediated dissemination of RS1 may indirectly promote the spread of toxin genes among *V. cholerae* strains.<sup>47</sup>

**VGJ $\phi$ .** The filamentous phage VGJ $\phi$  infects O1 and O139 strains, and integrate at the same chromosomal site as CTX $\phi$ .<sup>49</sup> The genome of VGJ $\phi$  is 7,542 bp with a GC content of 43% encoding 10 ORFs and is known to use the type IV pilus mannose-sensitive hemagglutinin (MSHA) as a receptor for infecting host cells.<sup>50</sup> The genome sequence of VGJ $\phi$  revealed the presence of two sites homologous to functional *att* sequences from other filamentous phages. These include *V. cholerae* phages CTX $\phi$  and VSK, and *V. parahaemolyticus* phage f237. VGJ $\phi$  integrates through its own *attP* sequence into the same chromosomal *attB* site in *V. cholerae* where CTX $\phi$  integrates, and the integration is likely mediated by the XerCD recombinases of the host bacterium.<sup>49</sup>

The VGJ $\phi$  also provides functions required for production and transmission of both CTX $\phi$  and RS1 $\phi$ .<sup>50</sup> In a novel type of specialized transduction, the site-specific co-integration of VGJ $\phi$  and CTX $\phi$  (or RS1 $\phi$ ) genome results in single hybrid molecules that generate ssDNA hybrid genomes that are packaged into hybrid phages (VGJ $\phi$ /CTX $\phi$  hybrid or VGJ $\phi$ /RS1 $\phi$  hybrid).<sup>50</sup> Since the host cell receptor for VGJ $\phi$  is the MSHA pilus, VGJ $\phi$  is capable of transferring CTX $\phi$  to *V. cholerae* cells by a TCP-independent mechanism. Given that MSHA is present in a wide variety of *V. cholerae* strains and is presumed to express in the environment, diverse filamentous phages using this receptor are likely to contribute significantly to *V. cholerae* evolution.

**Other MSHA dependent filamentous vibriophages.** MSHA is used as the host cell receptor by at least five other filamentous phages namely, VSK, 493, VSKK, fs1 and fs2.<sup>51-55</sup> Phages fs1 and fs2 were initially isolated from certain *V. cholerae* O139 strains. These phages were later found to infect both *V. cholerae* O1 and O139 strains and the phage genome could also integrate into the host bacterial genome. Phage fs1 was also isolated from a number of clinical *V. cholerae* O1 isolates.<sup>56</sup> This phage has a 6,340-bp ssDNA genome that encodes 15 ORFs, whereas the fs2 $\phi$  genome size is 8,651 bp, containing nine ORFs and a large stem loop region.<sup>52,53</sup> Almost all *V. cholerae* O139 and O1 El Tor biotype strains tested were sensitive to fs2 $\phi$ , whereas most O1 classical biotype strains and non O1/nonO139 strains were found to be resistant.<sup>53</sup> A 715 nucleotide fragment located in the large intergenic region of fs-2 $\phi$  was highly homologous to a part of region RS2 of CTX $\phi$ . Recently, a phage similar to fs2 $\phi$  has been shown to act as a helper for the TLC satellite phage by encoding

functions leading to the morphogenesis of infectious TLC $\phi$  particles.<sup>14</sup>

The VSK $\phi$  was also isolated from a *V. cholerae* O139 strain. This phage has a genome of 6,880-bp with a GC content of 43%, and the phage genome integrates into the genome of the host bacterium.<sup>51</sup> VSK $\phi$  genome has high sequence similarity to VGJ $\phi$  (83%) and fs1 $\phi$  (87%).<sup>51</sup> Phage 493 was isolated from a 1994 isolate of *V. cholerae* O139, and was shown to inhibit pre-O139 El Tor biotype *V. cholerae* strains but not post-O139 El Tor strains in plaque assays.<sup>54</sup> The emergence and decline of *V. cholerae* O139 in India and Bangladesh were suggested to have been influenced by the susceptibility and resistance of El Tor strains to 493 $\phi$ .<sup>55</sup> The 493 $\phi$  has an approximate genome size of 9,300 bp with a 2,300-bp stem-loop structure, which may represent a transposable element.

In summary, most of the known filamentous phages of *V. cholerae* have genomes of a similar size (range 6 kb to 9.3 kb) and encode a similar number of ORFs (range 7 to 15). Distinct replication and morphogenesis modules are present in most of these phages. Most of the filamentous vibriophages studied integrate into the host bacterial genome, and probably use the XerD and XerC mechanism of integration since most of these phages contain *att*-like sites similar to that of CTX $\phi$ . The TCP pathogenicity island which has a significantly larger size (~41 kb), and distinct from the known *V. cholerae* filamentous phage genomes was also reported to be the genome of a filamentous phage called VPI $\phi$ .<sup>57</sup> However, a lack of reasonable similarity in sequence of the TCP island (alternatively known as VPI<sup>58</sup> for *Vibrio cholerae* pathogenicity island) to the morphogenesis genes of canonical filamentous phages was one of the reasons for skepticism regarding the existence of the VPI $\phi$ . Subsequently it was shown that the initial study was incorrect and that VPI is not itself a phage genome.<sup>59</sup> However, the TCP island, which includes putative integrase and transposase genes and is flanked by *att*-like sequences, clearly resembles a horizontally acquired element.<sup>60</sup>

**Possible role of transducing phages in horizontal gene transfer.** Besides the TCP pathogenicity island, the *V. cholerae* genome is known to carry a number of other gene clusters with structural features, that are consistent with the ability of these genomic regions to move horizontally between strains.<sup>8</sup> These islands include, VPI-2, encoding genes for neuraminidase (nanH) and amino sugar metabolism, and the *Vibrio* seventh pandemic islands VSP-1 and VSP-2.<sup>11,61</sup> The VPI-2 is a 57 kb region that is required for growth of *V. cholerae* on sialic acid as a sole carbon source. In the mucus-rich environment of the gut, where sialic acid availability is extensive, the capacity to utilize sialic acid as a carbon and energy source by *V. cholerae* is believed to confer an advantage. *V. cholerae* non-O1/O139 pathogenic strains also contain VPI-2, which in addition to sialic acid catabolism genes also encodes a type 3 secretion system in these strains. *Vibrio* seventh pandemic islands VSP-1 and VSP-2 are unique to seventh pandemic El Tor and O139 isolates.<sup>61</sup> VSP-1 spans a 16 kb region with a GC content of 40 %, in contrast to 47 % for the entire *V. cholerae* genome. The VSP-2 encompasses a 7.5 kb region with a GC content of 41%, and was found to show homology to a 43.4 kb genomic island in *V. vulnificus*.<sup>62</sup> the

precise functions of all genes in VSP-1 and VSP-2 have not been defined, but the presence of these islands in pandemic strains suggest their role in evolutionary fitness and spread of the seventh pandemic clone.

It has been suggested that the TCP island or VPI can excise from the chromosome and form circular DNA,<sup>63</sup> and thus provides support to the possibility of horizontal transfer of this region. Similarly, VPI-2 and the two VSP islands were also shown to excise to form extrachromosomal products.<sup>64</sup> Each of the pathogenicity islands VPI-2 and VSP-2 encodes a P4-like integrase adjacent to a tRNA locus, and the P4-like integrase was required for their excision.<sup>64,65</sup> It seems possible that these pathogenicity islands are transmitted by phage transduction, and the excision from the chromosome is likely a first step in its horizontal transfer from cell to cell. For example, inter strain transfer of VPI mediated by a generalized transducing phage CP-T1 has been documented.<sup>66</sup> Further studies directed toward identifying possible phages involved in the transfer of these pathogenicity islands will provide more insights into the contribution of phages in *V. cholerae* evolution.

### Phage Predation and Selection of *V. cholerae* Strains

A distinctive epidemiological feature of cholera is its appearance with seasonal regularity in endemic areas, such as the Ganges Delta region of Bangladesh and India. These cholera epidemics are also known to be self-limiting, since the epidemics subside after reaching a peak, even without any active intervention. Among other factors, phages that can kill *V. cholerae* (cholera phages) have been demonstrated to play a significant role in modulating the course of epidemics.<sup>3,9,10</sup> The dynamics of the interactions between *V. cholerae* and lytic phages was studied during cholera epidemics in Dhaka, Bangladesh between 2001 and 2004. These studies showed that the number of cholera patients increased whenever the number of lytic vibriophages in water decreased. Similarly, cholera epidemics tended to end concurrent with large increases in the concentration of these viruses in the water.

The incidence of phage excretion in stools of cholera patients during the entire course of an epidemic was also monitored. The buildup to the phage peak in the environment coincided with increasing excretion of the same phage in the stools of cholera patients.<sup>9</sup> Further studies using animal models showed that the infectious dose (ID<sub>50</sub>) of *V. cholerae* cells in stools of cholera patients as well as that of laboratory-grown cells was higher in the presence of a phage, and ~10-fold-more cells of *V. cholerae* would be required to cause a productive infection under the conditions in which cholera patients excrete *V. cholerae* together with phages in their stools.<sup>67</sup> The increase of the required infectious dose due to the deleterious effect of co-ingesting phage with *V. cholerae* would therefore lead to a reduced number of patients. Thus phages antagonize the transmissibility of cholera during the late stage of a cholera epidemic,<sup>9,68</sup> eventually leading to the collapse of the epidemic. Therefore, cholera phages in the environment, and their amplification in cholera patients remarkably influence the epidemiology of cholera, due mainly to phage mediated elimination of the causative epidemic strain. However, since the

same *V. cholerae* strain often reemerges and causes a subsequent epidemic during the next cholera season, it is obvious that a proportion of the epidemic *V. cholerae* cells survive the phage attack. Therefore, factors or genetic changes which enhance the ability of the bacteria to resist phage predation may also be a strong driving force in the evolution of *V. cholerae*.

The resistance to phage may be manifested by the metabolic status of the *V. cholerae* cells, inadequate expression of phage receptors or other mutations that affect phage bacterial interactions. Under laboratory conditions, co-culture of a phage and *V. cholerae* or dilutions of phage-positive cholera stools has been shown to cause emergence of phage-resistant derivatives of the bacteria.<sup>67</sup> Frequently these resistant derivatives were found to have lost their O1 antigen which often acts as the receptor for these phages. However, challenge studies in mice with a mixture of *V. cholerae* and phage suggested that the intestinal environment did not favor the emergence of phage-resistant derivatives that lost the O1 antigen.<sup>67</sup> These results suggest that the outcome of phage bacterial interactions are more complex in nature and involves a multitude of factors including environmental factors, the lytic phages, and the role of the human host.<sup>69</sup>

**Changing prevalence of vibriophages in the environment.** The environmental surveillance system to monitor phages and *V. cholerae* in the aquatic environment in Bangladesh yielded various phage isolates and data on phage prevalence. Different phage strains<sup>3,7,15</sup> (JSF1 through JSF16) which interact with *V. cholerae* have been identified and partially characterized (Table 2). A lytic vibriophage ICP1 was consistently prevalent in stools of cholera patients in Bangladesh.<sup>70,71</sup> Genomic sequence analysis indicated that ICP1 is in fact identical to JSF4 and is closely related to JSF11 (W. Robins, S. Faruque and J. Mekalanos, unpublished results). Studies were also conducted to identify bacterial factors which mediate resistance to phage susceptibility, and thus provide a mechanism to survive predation by phages. As mentioned above, often phage resistant strains were found to have lost their O-antigen. Incorporation of mutations in the *cyaA* or *crp* genes encoding adenylate cyclase or cyclic AMP (cAMP) receptor protein (CRP) respectively, into *V. cholerae* O1 strains was also found to cause alterations in their phage susceptibility patterns, and the susceptibility correlated with the ability of the bacteria to adsorb these phages. These results suggested that cAMP-CRP-mediated downregulation of phage adsorption may contribute to a mechanism for the *V. cholerae* strains to survive predation by phages in the environment.<sup>15</sup>

Fluctuation in the prevalence of the different predatory phages have also been observed (unpublished data), with a temporal change in the most prevalent phage type (Table 3) which is a factor in the collapse of epidemics by phage predation.<sup>3</sup> The significance of this change is not clear. Nevertheless, occasional change in the predominant phage appears to maintain susceptibility of the epidemic strain to the most prevalent phage responsible for ending epidemics in a self-limiting manner.

### Conclusion

Although there are multiple other factors which drive the evolution of *V. cholerae*, phages have been conclusively shown

**Table 2.** Lytic vibriophages isolated from surface water and cholera patients in Bangladesh<sup>3,7,15</sup>

Phage designation	Primary host strains	Alternative host strains	Plaque type	Isolation of lysogens
JSF-1	<i>V. cholerae</i> O1	Not found	Clear	-
JSF-2	<i>V. cholerae</i> O1	Not found	Turbid	+
JSF-3	<i>V. cholerae</i> O139	Not found	Clear	+
JSF-4	<i>V. cholerae</i> O1	Not found	Clear	+
JSF-5	<i>V. cholerae</i> O1	Not found	Clear	-
JSF-6	<i>V. cholerae</i> O1	<i>V. cholerae</i> non-O1 non-O139	Clear	-
JSF-7	<i>V. cholerae</i> O1	<i>V. cholerae</i> O141 strain V50; <i>V. cholerae</i> O139 strain AI1853	Clear on O1 strain; Clear/turbid on non-O1 strains	+
JSF-8	<i>V. cholerae</i> O1	<i>V. cholerae</i> non-O1 non-O139 strains 3565, 3548; <i>V. mimicus</i> strains 957V1621, 778V1349, and 1016V1721	Clear on O1 strain; Clear/turbid on non-O1 strains	+
JSF-9	<i>V. cholerae</i> O1	<i>V. cholerae</i> O141 strain V50; non-O1 strains 79, 3565, 3548; <i>V. mimicus</i> strains 957V1621, 778V1349, and 1016V1721	Clear	-
JSF10	<i>V. cholerae</i> O1	<i>V. cholerae</i> O139 strain Arg-3 <i>V. cholerae</i> O141 strains V46 and V47	Clear	-
JSF-11	<i>V. cholerae</i> O1	Not found	Clear	-
JSF12	<i>V. cholerae</i> O1	<i>V. cholerae</i> non-O1 strains 79; <i>V. mimicus</i> strains 957V1621, 1016V1721	Clear	-
JSF-13	<i>V. cholerae</i> O1	Not found	Clear	-
JSF-14	<i>V. cholerae</i> O1	Not found	Clear	-
JSF-15	<i>V. cholerae</i> O1	<i>V. cholerae</i> O141 strain V50; non-O1 strain 79, <i>V. mimicus</i> strains 957V1621, and 778V1349	Clear	-
JSF-16	<i>V. cholerae</i> O1	<i>V. cholerae</i> O141 strain V50	Clear/turbid	+

to contribute to horizontal gene transfer among *V. cholerae* strains, and in selection of particular clones. Complete genomic sequencing of a number of *V. cholerae* strains is beginning to reveal changes that the organism has undergone and that have been linked to its emergence as a pathogen. Some of these genetic changes define important virulence altering traits. Pathogenic strains typically carry genes for different virulence factors and the lipopolysaccharide O1 or O139 antigens that are typically absent in environmental strains.<sup>5,8</sup> Thus, while there are over 200 serogroups of *V. cholerae*, only two serogroups (O1 and O139) are

associated with epidemic and endemic disease. The O139 serogroup is a derivative of the highly successful CTX<sup>+</sup> TCP<sup>+</sup> O1 El Tor “7th pandemic clone,”<sup>31</sup> and hence carry the CTX prophage and TCP pathogenicity island, but many O1 environmental strains do not. Both classical and El Tor biotypes of *V. cholerae* O1 carry CTX and TCP; so the explanation for the evolutionary success of the seventh pandemic clone over the preexisting sixth pandemic strain remains largely a mystery. Recent studies are beginning to suggest possible role of phages in this process as well.<sup>7</sup>

Similar to CTX prophage, the CTX $\phi$  receptor TCP is limited to subsets of strains within three species, *V. cholerae*, *V. mimicus* and *V. fischeri*, thus explaining the limited distribution of the CTX prophage. The bacterial cell surface receptor for VSK, VJG, KSF-1 and fs2 phages is MSHA, a type IV pilus that is widely present among *Vibrio* species, indicating the potential broad host range of these filamentous phages and their potential role in horizontal gene transfer across species boundaries. However, emergence of pathogenic strains of *V. cholerae* with endemic and pandemic potential has been exclusively linked to the acquisition of the TCP and CTX genes by only a limited spectrum of *V. cholerae* strains. This fact suggests that the precursors of these pandemic clones probably display traits that are lacking in typical environmentally adapted *V. cholerae* regardless of their serogroup. This is not to say that new pathogenic clones do not emerge from environmental *V. cholerae*. But the evolution of environmental

**Table 3.** Predominant phages isolated from surface water and cholera patients in Bangladesh during 2002–2011

Year	Abundant phages	Predominant phage
2002–2004 <sup>3</sup>	JSF1 through JSF6	JSF4
2005	JSF1 through JSF9	JSF4
2006	JSF1, JSF4, JSF6 through JSF9	JSF4
2007	JSF1 through JSF11	JSF4/JSF11
2008	JSF1, JSF4 through JSF11	JSF11
2009	JSF1, JSF4 through JSF11	JSF11
2010	JSF1, JSF4 through JSF11	JSF11
2011	JSF1, JSF4 through JSF16	JSF11



strains to typical pathogenic strains would require more widespread gene transfer events than shown to occur through the known phages until now. While conjugative plasmids and a single generalized transducing phage for *V. cholerae* namely CPT-1 are known to exist,<sup>70</sup> other studies are beginning to suggest more extensive role of possible additional vibriophages in the horizontal transfer of chromosomal segments as well as in selection of different *V. cholerae* clones. *V. cholerae* has been reported to become naturally competent to uptake exogenous DNA, while growing on a chitin substrate.<sup>71</sup> More recently, it has been suggested that free DNA released in the aquatic environment by phage mediated lysis of *V. cholerae* can be harnessed and

assimilated by surviving *V. cholerae* strains.<sup>72</sup> Thus, phages contribute to bacterial evolution in numerous ways and the full potential of these mechanisms in the evolution of *V. cholerae* as a pathogen is yet to be appreciated.

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