Genesis of variants of *Vibrio cholerae* O1 biotype El Tor: role of the CTXφ array and its position in the genome

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The gene encoding cholera toxin, the principal virulence factor of *Vibrio cholerae*, is encoded by a filamentous, lysogenic bacteriophage known as CTXφ. The genome of *V. cholerae*, the host for CTXφ, consists of two chromosomes, one large and one small. Here, it is shown that localization and array of CTX prophage DNA in either the large or small chromosome of *V. cholerae* is likely to be one of the reasons for the emergence of O1 biotype El Tor variants isolated just before and after the *V. cholerae* O139 cholera outbreak in 1992. Analyses of the organization of the CTX region of the genome of pre-O139 El Tor strains revealed that these strains carry two distinct CTX prophages integrated in the small chromosome in tandem: CTX^ET^, the prophage having a conserved NotI site in its repeat sequence segment which seems to be specific for the El Tor strains so far examined, followed by CTX^calc-^like genome, the prophage found in recent O139 clinical isolates from Calcutta. In sharp contrast, in post-O139 El Tor strains only one copy of the CTX^ET^ prophage was found to be integrated in the large chromosome. To the authors’ knowledge, the presence of CTX prophage in the small chromosome of O1 El Tor strains has not been reported previously. It is also shown that the difference in the CTX copy number and the position of the bacteriophage on the genomes of pre- and post-O139 El Tor strains have an effect on cholera toxin production. While a pre-O139 strain produced maximum cholera toxin in yeast extract/peptone medium at 30 °C, a post-O139 El Tor strain showed maximal yield at 37 °C, indicating differential regulation of cholera toxin between the strains. It appears from this study that the variation in the integration site of the CTX prophage, its copy number and the presence of diverse phage genomes in *V. cholerae* O1 biotype El Tor may be strategically important for generating variants with subtle phenotypic modulations of virulence factor production in this longest-ruling seventh pandemic strain.

INTRODUCTION

*Vibrio cholerae*, a non-invasive Gram-negative bacterium, is the causative agent of the diarrhoeal disease cholera. *V. cholerae* strains causing cholera epidemics have until recently been confined to the cholera toxin (CT)-producing serogroup O1, which consists of two biotypes, classical and El Tor. The classical biotype, the sixth pandemic strain, was responsible for cholera epidemics until 1961, when the El Tor biotype displaced it and started the seventh pandemic (Kaper *et al.*, 1995). However, in late 1992, CT-producing *V. cholerae* O139 Bengal emerged as the first non-O1 strain to cause an explosive cholera epidemic in the Indian subcontinent by replacing the seventh pandemic strains of the *V. cholerae* O1 El Tor biotype (Albert *et al.*, 1993; Ramamurthy *et al.*, 1993). *V. cholerae* O139 strains isolated from different parts of India and Bangladesh during the epidemic were found to be of clonal origin, and several lines of evidence have suggested that strain O139 arose from an El Tor biotype (Bhadra *et al.*, 1994, 1995; Bik *et al.*, 1995; Waldor & Mekalanos, 1994). Interestingly, within about one year, O1 El Tor strains reappeared in the same area as the dominant serogroup, replacing the O139 Bengal clone (Mukhopadhyay *et al.*, 1995, 1996a); molecular characterization of El Tor strains isolated prior to and after the O139 epidemic revealed that they were genotypically different from each other (Sharma *et al.*, 1997). The precise molecular mechanism behind the complex epidemiology of El Tor vibrios and the rapid genesis of their variants in this geographical region is currently unknown.

The principal virulence factor of *V. cholerae* is CT. Previously, it has been shown that the genes encoding CT, ctxAB, along with other virulence-related genes reside on a 4·5 kb DNA segment called the core region (Baudry *et al.*, 1992; Pearson *et al.*, 1993; Trukcik *et al.*, 1993). The core
region is flanked by one or multiple copies of direct repeat sequences (RSs) that vary in length from 2-4 to 2-7 kb, and this approximately 7 kb DNA segment (RS + core) is called the CTX genetic element (Pearson et al., 1993). However, it has been discovered (Waldor & Mekalanos, 1996) that the CTX genetic element of V. cholerae corresponds to the genome of a filamentous bacteriophage designated CTXφ (Fig. 1). The RS region present just upstream of the core of CTXφ, named RS2 (2-4 kb in size), encodes functions required for regulation (rstR gene product), replication (rstA gene product) and integration (rstB gene product) of CTXφ into the V. cholerae genome (Fig. 1) (Waldor et al., 1997). Apart from these genes, some RS2 elements may contain an additional ORF, termed rstC (Fig. 1), and are called RS1 (2-7 kb in size). This element, when present, always flanks (5’ and/or 3’) the CTX prophage genome (Davis et al., 2000; Waldor et al., 1997) (Fig. 1). Although the role of the rstC gene of RS1 is not clear, it has been predicted that this region could form a stem-loop structure that might act as a transcriptional terminator (Waldor et al., 1997). CTXφ gains entry into the V. cholerae cell through the toxin co-regulated pilus, another important virulence factor of V. cholerae, and integrates its genome into the V. cholerae chromosome by a RecA-independent site-specific process to form a stable lysogen (Pearson et al., 1993; Waldor & Mekalanos, 1996). Interestingly, it has been shown that the sixth pandemic classical biotype vibrio strains are unable to generate infectious CTXφ particles while the El Tor biotype and O139 serogroup strains can give rise to such particles (Davis et al., 2000; Kimsey & Waldor, 1998). Apart from this difference, the organization of the CTX prophage genome in V. cholerae can be used as one of the reliable molecular methods for the differentiation of classical and El Tor biotypes. In El Tor genomes, the CTX prophage may be present either as a single copy or as multiple copies arranged in tandem (Mekalanos, 1983). In sharp contrast, in classical vibrios, the CTX prophage is present in two copies and these are widely separated on the chromosome (Mekalanos, 1983). It has been shown that V. cholerae contains two unique chromosomes, one large and one small (Trucksis et al., 1998). Genetic mapping revealed that in the classical biotype strain O395 the two copies of the CTX prophage are present in one copy on each chromosome (Trucksis et al., 1998). However, the whole-genome sequencing of an El Tor strain, N16961, revealed the integration of only one copy of the CTX prophage flanked by the RS1 element in its large chromosome (Heidelberg et al., 2000). Thus, no reports were available to show that CTXφ can integrate in the small chromosome of El Tor O1 strains.

The results of this study show, for the first time, that multiple copies of CTX prophage in tandem integrate in the small chromosome of certain V. cholerae O1 El Tor strains. Surprisingly, such El Tor strains were prevalent just before the O139 outbreak. In sharp contrast, El Tor strains isolated just after the O139 outbreak carried a single copy of the CTX prophage in their large chromosome. Moreover, from restriction mapping and hybridizations with region-speciﬁc gene probes, it was found that pre-O139 El Tor strains were infected with two distinct types of CTXφ. The difference in CTX prophage array and the location of integration sites observed between the El Tor strains isolated prior to and after the O139 outbreak also affected the regulation of production of CT.

**METHODS**

**Strains.** V. cholerae strains used in this study are listed in Table 1. All strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. V. cholerae strains were maintained at −70 °C in Luria broth (LB) containing 15% (v/v) glycerol as described previously (Nandi et al., 1997).

**Culture conditions and assay of CT.** V. cholerae cells were routinely grown in a gyratory shaker at 37 °C in LB. For detection of CT produced by various V. cholerae strains, yeast extract/peptone (YPE) medium [1-5% bactopeptone (Difco), 0-4% yeast extract (Difco), 0-5% NaCl, pH 7.0–7.5] was used as described by Mukhopadhyay et al. (1996b). V. cholerae culture was inoculated into YEP medium and grown at two different temperatures,
Table 1. *V. cholerae* O1 strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source, place of isolation</th>
<th>Year of isolation</th>
<th>Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC20</td>
<td>Clinical, India</td>
<td>1992</td>
<td>El Tor</td>
</tr>
<tr>
<td>VC44</td>
<td>Clinical, India</td>
<td>1992</td>
<td>El Tor</td>
</tr>
<tr>
<td>CO457</td>
<td>Clinical, India</td>
<td>1994</td>
<td>El Tor</td>
</tr>
<tr>
<td>CO471</td>
<td>Clinical, India</td>
<td>1994</td>
<td>El Tor</td>
</tr>
<tr>
<td>CO473</td>
<td>Clinical, India</td>
<td>1994</td>
<td>El Tor</td>
</tr>
<tr>
<td>C6709</td>
<td>Clinical, Peru</td>
<td>1991</td>
<td>El Tor</td>
</tr>
<tr>
<td>N16961</td>
<td>Clinical, Bangladesh</td>
<td>1975</td>
<td>El Tor</td>
</tr>
<tr>
<td>569B</td>
<td>Clinical, India</td>
<td>1948</td>
<td>Classical</td>
</tr>
<tr>
<td>O395</td>
<td>Clinical, India</td>
<td>1964</td>
<td>Classical</td>
</tr>
</tbody>
</table>

All strains possessed the CT gene.

RESULTS AND DISCUSSION

Restriction fragment length polymorphism (RFLP) analysis of pre- and post-O139 El Tor strains

To get a deeper molecular insight into the rapid emergence of El Tor variants in the mid-1990s (Mukhopadhyay *et al*., 1995; Sharma *et al*., 1997), we have done PFGE assays on *NotI*-digested genomes of representative O1 El Tor strains (Table 1) isolated in Calcutta, India, before and after the outbreak caused by a novel non-O1 strain of *V. cholerae* named O139 Bengal (Ramamurthy *et al*., 1993). In this study, we have also included the well-studied El Tor strain C6709, which was responsible for the Peru epidemic (Levine, 1991). When the *NotI* digestion profiles of the El Tor genomes were compared, extensive RFLPs were observed among the strains, indicating that they were variants (Fig. 2a, arrowheads). This result supports our earlier findings using I-*CeuI*, a group I intron-encoded restriction endonuclease that only cuts in the 23S rRNA gene sequences of prokaryotic *rrn* operons (Nandi *et al*., 1997). The RFLPs observed among the genomes of pre- and post-O139 El Tor strains as well as in the genome of strain C6709 were further confirmed by Southern blot hybridization of *NotI*-digested genomic DNA using *ctxA* as a probe. The *ctxA* gene hybridized with a single *NotI* fragment from each of the genomes of the El Tor strains (Figs 2b and 4e), with the sizes of the *NotI* fragments being 130, 78 and 7 kb, respectively (Figs 2b and 4e). It has been shown previously that there is a *NotI* site in the *rrs* of El Tor strains but not in the *rrs* of classical vibrios (Pearson *et al*., 1993). Thus, the hybridization of *ctxA* with only one *NotI* fragment, of greater than 7 kb in size, of the genome of each El Tor strain indicated that each strain has only one copy of the CTX prophage and no downstream *rs* or there are multiple copies of the CTX prophage in tandem from which the *NotI* site in the *rrs* connecting the core is lost. From the above experiment, it was confirmed that the 7 kb *NotI* fragment from the genome of the Peru strain C6709 can accommodate only one copy of the CTX prophage genome, which
is also 7 kb in size, and its 3’ region contains an RS1 with a NotI site in it. Waldor & Mekalanos (1994), using other restriction enzymes and region-specific DNA probes, reported a similar organization for the CTX element in the Peru strain. Thus, the strain responsible for the epidemic in Peru contained a typical El Tor-specific CTX prophage since it had a conserved NotI site in its RS region (see Fig. 5). Analysis of the DNA sequences of RS elements of CTX of El Tor origin reveals that there is a conserved NotI site in the intergenic region \( \text{ig-1} \), which is physically linked to the \( \text{rstR} \) gene of the RS (Waldor et al., 1997). Davis et al. (1999) designated such El Tor-specific phages as CTXET\( \phi \). The correlation that there is a conserved NotI site in the \( \text{ig-1} \) region of the RSs of El Tor strains is further supported by the whole-genome sequence of El Tor strain N16961 (Heidelberg et al., 2000).

### Mapping of the locations of CTX prophage in the El Tor genomes

The distinct RFLP patterns recorded for the genomes of El Tor strains isolated before and after the O139 outbreak prompted us to map the \( \text{ctx} \) loci in these strains more precisely. We also wanted to know in which chromosome, large or small, of El Tor vibrios the CTX prophage was located. The whole-genome sequence of the \( \text{V. cholerae} \) O1 El Tor strain N16961 revealed a single copy of the CTX prophage flanked by an RS1 element located on the large chromosome (Heidelberg et al., 2000). However, it was not known whether CTX\( \phi \) always integrates in the large chromosome of El Tor strains or if it was also present in the small chromosome. To determine the integration site of CTX\( \phi \) in the El Tor strains, undigested intact chromosomal DNAs from different \( \text{V. cholerae} \) strains were subjected to PFGE to separate their two chromosomes (Trucksis et al., 1998), stained with ethidium bromide and visualized under a long wavelength UV transilluminator (Fig. 3). For comparison with the El Tor strains, we also included a classical strain, O395, which carries two copies of the CTX

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**Fig. 2.** RFLP analysis of the pre- and post-O139 El Tor O1 strains VC44 (lane 1) and CO457 (lane 2), respectively, and the Peru epidemic strain C6709 (lane 3). (a) NotI-digested genomic DNA of \( \text{V. cholerae} \) was subjected to PFGE with pulse times interpolated between 5 and 25 s for 22 h at 10 V cm\(^{-1}\) at 3 °C; after PFGE, the gel was stained with ethidium bromide. Arrowheads indicate RFLPs among the El Tor strains. Numbers to the right of the image represent the molecular size markers. (b) RFLP analysis of the genomes of El Tor strains VC44, CO457 and C6709 with respect to the \( \text{ctx} \) locus. NotI-digested genomic DNAs of the El Tor strains were subjected to PFGE followed by hybridization with the \( \text{ctxA} \) gene as a probe. Numbers to the right of the image indicate molecular size markers, λ ladder and λ DNA digested with HindIII. Numbers to the left of the image indicate the sizes of the NotI fragments of the genomes of each El Tor strain containing the CTX prophage.

**Fig. 3.** Differences in the localization and arrangement of the CTX prophage in the chromosomes of pre- and post-O139 El Tor variant strains. Left panel, undigested intact genomic DNAs of \( \text{V. cholerae} \) O1 strains were subjected to PFGE (pulse times interpolated between 50 and 100 s for 24 h at 10 V cm\(^{-1}\) at 4 °C); after PFGE, the gel was stained with ethidium bromide. Lanes: M, yeast chromosomes as molecular mass markers; 1, VC20 (pre-O139, El Tor); 2, C6709 (Peru outbreak, El Tor); 3, CO473 (post-O139, El Tor); 4, VC44 (pre-O139, El Tor); 5, O395 (classical). Right panel, the same gel shown in the left panel was subjected to Southern transfer and hybridized with the \( \text{ctxA} \) gene. LC and SC, large and small chromosomes of \( \text{V. cholerae} \), respectively. In the pre-O139 El Tor strains VC20 (lane 1) and VC44 (lane 4) the \( \text{ctxA} \) gene showed hybridization with the small chromosome, but in the Peru epidemic strain C6709 (lane 2) and the post-O139 El Tor strain CO473 (lane 3) the same probe hybridized with the large chromosome of the pathogen; in classical strain O395 (lane 5) both chromosomes showed hybridization signals.
element, with one copy present in each chromosome (Trucksis et al., 1998). We found a distinct difference in the migration of the small chromosomes of the El Tor and classical strains (Fig. 3). This difference was expected, as it has been reported by Trucksis et al. (1998) that the size of the small chromosome of the classical strain O395 is about 1600 kb as against 1070 kb, the size of the small chromosome of the El Tor strain N16961 (Heidelberg et al., 2000). We also found that the small chromosomes of all the El Tor strains examined in this study migrated in the 1100 kb region (Fig. 3). The gel was processed to transfer the DNA to a nylon membrane, followed by hybridization with the ctxA gene and autoradiography. To our surprise, the ctxA gene hybridized only with the small chromosome of the pre-O139 El Tor strains VC20 and VC44 (Fig. 3). However, in the post-O139 El Tor strain CO473, as well as in the Peru strain C6709, the large chromosome contained the CTX prophage (Fig. 3). Like CO473, the post-O139 El Tor strains CO457 and CO471 also contained CTX prophage in the large chromosome (data not shown). As expected, the ctxA probe hybridized with both chromosomes of strain O395 of the classical biotype (Fig. 3), confirming the result of Trucksis et al. (1998). Our result indicates, for the first time, that CTXφ can integrate in the small chromosome of El Tor vibrios, and this is probably one of the reasons for the genome rearrangements leading to the genesis of variants. The result also suggests the presence of a functional attB-like sequence, needed for the integration of CTX prophage (Davis et al., 1999; Pearson et al., 1993), in the small chromosome of the El Tor strains examined in this study. Taken together, our results confirm that the pre- and post-O139 strains of V. cholerae O1 El Tor are variants and probably evolved from two independent clones.

The different integration sites of the CTX prophage in pre- and post-O139 El Tor strains prompted us to map the ctx locus precisely to determine whether this locus is present in a single copy or in tandemly repeated multiple copies. Besides, fine mapping of the region can also provide important information about the genome structure of CTXφ, as a recent study has shown that there are strain-specific CTXφ present in various isolates. For example, CTX\textsuperscript{class}φ is found in classical strains, CTX\textsuperscript{res}φ is present in El Tor and O139 strains and CTX\textsuperscript{calc}φ is found in resurgent O139 strains (Davis et al., 1999, 2000; Kimsey & Waldor, 1998; Kimsey et al., 1998). The diversity of CTXφ among biotypes is mainly due to the extensive variations in the RS element, particularly in the rstr gene region (Davis et al., 1999; Kimsey et al., 1998). For fine mapping of the ctx region of the genomes of El Tor strains, various restriction endonucleases such as AvaI, PstI and BglII, which have a single digest site either in the RS or in the core of the phage genome, were utilized. As discussed above, the rare cutter NotI, which has a conserved site in the RSs of El Tor strains, also helped to map the cxt region. The enzyme-digested V. cholerae genomic DNA was hybridized with the ctxA or RS probe (Fig. 4) and the blots were analysed extensively as described previously (Bhadra et al., 1995; Khetawat et al., 1999). The hybridization results are summarized in Table 2. Analyses of the hybridization results (Fig. 4) indicated that there were two copies of tandemly arranged CTX prophage; thus, the two cores in the genomes of pre-O139 El Tor strains are connected by a single RS2 element (Fig. 5). In contrast, the genomes of post-O139 El Tor strains contained only a single copy of the CTX prophage (Fig. 5). Restriction mapping of the CTX region of pre- and post-O139 El Tor strains further revealed that the upstream RS copy flanking the 5’ of the CTX prophage is likely to be RS1 according to Waldor et al. (1997) (Fig. 5). It should be noted that in both categories of

Fig. 4. Southern blot hybridization of (a) AvaI-, (b, d) BglII-, (c) PstI- and (e) NotI-digested genomes of V. cholerae strains with cxtA (a–c and e) or RS (d) as a probe. (e) Shows the blot obtained from a PFGE gel. (a–c) Lanes: 1, VC20; 2, VC44; 3, CO457; 4, CO471; 5, CO473; 6, C6709; 7, 568B; 8, O395. (e) Lanes: 1, VC20; 2, VC44; 3, CO471; 4, CO473. The asterisk in (b) indicates the 6.9 kb BglII fragment containing the genome of CTX prophage of strain C6709 which has an RS1 element downstream of ctxAB (see Fig. 5). Thus, to determine the chromosomal BglII site downstream of ctxAB (3.9 kb in size), an RS probe (see Fig. 1) was used (d). Arrowheads correspond to the sizes of the hybridized restriction fragments described in Table 2. Numbers to the left of the images indicate λ DNA digested with HindIII (a–d) or λ DNA concatemers (e) used as molecular size markers.
El Tor strains are the upstream RS1 and RS2 elements have NotI and BglII restriction sites (Fig. 5). Hybridization of the PFGE-separated NotI fragments of the pre-O139 El Tor strains VC20 and VC44 with the ctxA gene produced only one hybridization signal in the 130 kb region (Figs 2b and 4e), indicating that there is no NotI site in the RS2 connecting the two cores of the CTX prophage (Fig. 5). This result suggests that the tandemly repeated second copy of the CTX prophage present in the pre-O139 El Tor strains VC20 and VC44 is probably similar to the CTXcalc prophage present in the pre-O139 El Tor strains (Davis et al., 2000). Comparison of the presence of Aval, PstI, BglII and NotI sites downstream of the CTX prophages of pre- and post-O139 El Tor strains also indicated that the location of the CTX prophage in the genomes of pre-O139 El Tor strains was completely different from that in the post-O139 El Tor vibrios (Fig. 5). A similar type of analysis done on the genomes of different classical strains revealed specific loci for the integration of CTX prophage in the large and small chromosomes (Davis et al., 2000). In the large chromosome of classical, El Tor and O139 strains, CTXφ is located between the tlc and rtx gene clusters (Davis et al., 2000; Heidelberg et al., 2000; Lin et al., 1999; Rubin et al., 1998), while in the small chromosome of classical strains it is located between the traF and yciH genetic loci. In El Tor vibrios the same region of the small chromosome contains only a 14 bp end repeat sequence (Davis et al., 2000). Davis et al. (2000) termed the traF and yciH region of El Tor an ‘empty’ region. They failed to detect integration of any CTX prophage in the ‘empty’ locus in various El Tor strains and concluded that the site is probably not preferred by CTX prophage. However, the results of the present study indicate that in certain clinical El Tor strains CTXφ may integrate in the small chromosome. An attempt was made to find out whether the ‘empty’ locus was located in the same region as suggested by Davis et al. (2000) by analysing the region downstream of the prophage insertion locus using the restriction enzymes Aval, PstI, BglII and NotI. Our rationale was that if the CTX prophage integrates in between the same traF and yciH loci of the small chromosome of the pre-O139 El Tor strains then the restriction map determined by us for downstream of the CTX prophage should match with the restriction map determined for the same region from the published V. cholerae whole-genome sequences (Heidelberg et al., 2000). When such a comparison was done for the enzymes Aval, PstI, BglII and NotI, an excellent similarity was found between the restriction maps (Fig. 5; Table 2). The restriction analysis indicated that the integration of the CTX prophage had occurred in the same region of the small chromosome of pre-O139 El Tor strains as in the classical strains (Davis et al., 2000). Similar restriction site analysis of the post-O139 El Tor genomes together with other published reports for various El Tor strains also supported the finding that the CTX prophage integrates between the tlc and rtx gene clusters of the large chromosome (Table 2). Taken together, our mapping results indicate that the El Tor strains linked to the cholera outbreak before the emergence of O139 Bengal were novel strains of V. cholerae that carry two different types of CTX

### Table 2. Comparison of sizes of restriction fragments (Aval, BglII, PstI and NotI) originating downstream of the ctxAB genes of various V. cholerae strains that hybridized with the ctxA or RS probe, and determination of the chromosomal location of the CTX prophage

<table>
<thead>
<tr>
<th>V. cholerae strain</th>
<th>Chromosomal location of CTXφ</th>
<th>Size (in kb) of restriction fragments originating downstream of ctxAB*</th>
<th>Symbolic CTX prophage array</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC20 Small</td>
<td>6-1</td>
<td>Aval 6-1, BglII 11.5, PstI 9.6, NotI 130.0</td>
<td>RS1-RS2ET-CORE-RS2var-CORE</td>
</tr>
<tr>
<td>VC44 Small</td>
<td>6-1</td>
<td>Aval 6-1, BglII 11.5, PstI 9.6, NotI 130.0</td>
<td>RS1-RS2ET-CORE-RS2var-CORE</td>
</tr>
<tr>
<td>CO457 Large</td>
<td>17-5</td>
<td>Aval 17.5, BglII 7.8, PstI 5.8, NotI 78.0</td>
<td>RS1-RS2ET-CORE</td>
</tr>
<tr>
<td>CO471 Large</td>
<td>17-5</td>
<td>Aval 17.5, BglII 7.8, PstI 5.8, NotI 78.0</td>
<td>RS1-RS2ET-CORE</td>
</tr>
<tr>
<td>CO473 Large</td>
<td>17-5</td>
<td>Aval 17.5, BglII 7.8, PstI 5.8, NotI 78.0</td>
<td>RS1-RS2ET-CORE</td>
</tr>
<tr>
<td>C6709 Large</td>
<td>20-2</td>
<td>Aval 20.2, BglII 3.9, PstI 8.7, NotI 6-8</td>
<td>RS2ET-CORE-RS1</td>
</tr>
<tr>
<td>N16961 Large</td>
<td>20-6</td>
<td>Aval 20.6, BglII 3.9, PstI 8.7, NotI 6-8</td>
<td>RS1-RS2ET-CORE-RS1</td>
</tr>
<tr>
<td>569B Large</td>
<td>13-5</td>
<td>Aval 13.5, BglII 7.8, PstI 5.8, NotI ND</td>
<td>RS2class-CORE</td>
</tr>
<tr>
<td>O395 Small</td>
<td>6-1</td>
<td>Aval 6-1, BglII 11.5, PstI 9.6, NotI ND</td>
<td>RS2class-CORE</td>
</tr>
</tbody>
</table>

ND, Not determined.

*Sizes of restriction fragments as indicated by arrowheads in Fig. 4 except for strain N16961, which were obtained from Heidelberg et al. (2000).
†Determined by using an RS probe (see Fig. 4).
prophage in tandem in their small chromosome, which are most probably located between the traF and yciH region. The presence of diverse CTXφs in the genomes of resurgent V. cholerae O139 strains has been reported by Kimsey et al. (1998). It seems probable that the CTXcalcφ detected and characterized in the genomes of resurgent V. cholerae strains belonging to different serotypes and biotypes are shown. The restriction sites are highly conserved in the core region of the prophage but vary considerably in the RS2 region, leading to evolution of diverse phages. Precise chromosomal locations of CTX prophages were determined by comparing the restriction fragment sizes originating from the downstream chromosomal insertion regions of various V. cholerae strains belonging to different serotypes and biotypes are shown. The restriction maps of the CTX prophages and downstream chromosomal insertion regions of various V. cholerae strains belonging to different serotypes and biotypes are shown. The restriction sites are highly conserved in the core region of the prophage but vary considerably in the RS2 region, leading to evolution of diverse phages. Precise chromosomal locations of CTX prophages were determined by comparing the restriction fragment sizes originating from the downstream chromosomal region of the core (see Table 2); their integration in the large or small chromosome is indicated in parentheses after each strain as L or S, respectively. In the case of classical strain 569B it is not known exactly which one of the two prophage copies is present in the large or small chromosome; thus, they are denoted as copy I and II (in parentheses). However, comparison of the restriction map downstream of the core with that of strain VC44 suggests that copy number II is most likely present in the small chromosome. The presence of a unique NotI site (N) in the RS1 and RS2 regions of CTXφ of El Tor and O139 strains and its absence in classical vibrio is also shown. AvaI, A; BglII, B; HindIII, H; PstI, P.

Differential regulation of CT production in pre- and post-O139 El Tor strains

To see whether changes in the location and variations in the copy number of CTX prophage in the genomes of pre- and post-O139 El Tor strains have any effect on CT production, we used YEP medium and two incubation temperatures, 30 or 37 °C, as described by Mukhopadhyay et al. (1996b). These experiments showed that CT production...
by *V. cholerae* El Tor strains grown in YEP medium at 30 °C with shaking was the highly favoured condition compared to 37 °C. When we compared the production of CT by various strains of *V. cholerae* in YEP medium, the pre-O139 El Tor strains VC20 and VC44 showed optimal production at 30 °C (about 710 ng CT ml⁻¹) compared to 37 °C (about 310 ng CT ml⁻¹) with shaking. Surprisingly, strains CO457, CO471 and CO473, isolated just after the O139 outbreak, produced their maximal amount of CT at 37 °C (about 950 ng CT ml⁻¹) and not at 30 °C (about 750 ng CT ml⁻¹). However, the Peru strain C6709, like other El Tor strains reported by Mukhopadhyay *et al.* (1996b), showed optimal production of CT at 30 °C (750 ng CT ml⁻¹) compared to 37 °C (600 ng CT ml⁻¹). The CT values mentioned here are expressed as the mean of three independent experiments with each strain. Although the exact reasons for the differential regulation of CT production in pre- and post-O139 El Tor strains is currently unknown, it appears from this study that the genomic positions of the CTX prophage may play some role in such variations; these variations need further investigation. Thus, it appears that apart from various environmental cues that control the expression of various virulence factors in *V. cholerae*, the genomic positions of virulence-determining genes may also intrinsically fine-regulate their expression. This type of subtle phenotypic modulation of a major virulence factor may be a selective advantage when a pathogen persists in an endemic zone for a long time. However, further work is needed in this direction to come to a definite conclusion.

**Conclusion**

Possible environmental or host factors that determine the emergence and temporal domination of a particular variant of toxigenic *V. cholerae* and the displacement of an existing variant through natural selection are currently unknown. It is now well established that the major virulence genes of *V. cholerae* that have been studied extensively are located on mobile elements (Karaolis *et al.*, 1998, 1999; Waldor & Mekalanos, 1996). Previously, we have shown the presence and expression of two critical virulence genes, *ctxAB* and *tcpA*, in diverse environmental non-O1, non-O139 strains of *V. cholerae*, which appear to constitute an environmental reservoir for virulence genes (Chakraborty *et al.*, 2000). The present study also indicates that the mobile element CTXφ, ferrying the virulence genes *ctxAB*, is also forced to diversify, probably by ‘mixing and matching’ with other CTXφs leading to the genesis of new versions of phages. This type of diversity in the phage genome and variation in the sites of integration is probably needed for survival of the phage within the bacterial host. However, *V. cholerae* probably capitalizes on this property of CTXφ by rearranging its genome, which may lead to phenotypic modulation of expression of virulence factors such as CT, as shown in this study, and most probably modulation of expression of other virulence-related factors. Thus, the variants of *V. cholerae* El Tor generated by diverse CTXφs can maintain their pathogenic and epidemic potentials under various stressful conditions. The striking temporal association of the location of CTX prophages in the small chromosomes of *V. cholerae* O1 El Tor strains isolated just prior to the emergence of strain O139, and the displacement of strain O139 by the O1 El Tor strains carrying a single copy of the prototype CTXφ in their large chromosomes, may be one of the contributory factors for the emergence of El Tor variants.

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