Genetic organization of pre-CTX and CTX prophages in the genome of an environmental *Vibrio cholerae* non-O1, non-O139 strain

Diganta Maiti,¹ Bhabatosh Das,¹ Arjun Saha,^{1†} Ranjan K. Nandy,² G. Balakrish Nair³ and Rupak K. Bhadra¹

Correspondence
Rupak K. Bhadra
rupakbhadra@iicb.res.in

¹Infectious Diseases Group, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700 032, India

²National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata 700 010, India

³International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka 1212, Bangladesh

The cholera toxin (CT) is a critical determinant of the virulence of epidemic *Vibrio cholerae* strains. The *ctxAB* operon encoding CT is part of the genome of a filamentous bacteriophage CTXΦ, which may integrate as a single copy or as multiple copies in the genome of *V. cholerae*. The CTXΦ genome is composed of RS2 (2.4 kb) and core (4.5 kb) regions. In the present study extensive genetic mapping analyses indicated that two copies of tandemly arrayed CTX prophages are integrated in the small chromosome of an environmental *V. cholerae* strain, VCE232, belonging to serogroup O4. Further mapping revealed that the integration of prophages has occurred in the same genetic locus of the small chromosome of VCE232 as that of *V. cholerae* O1 biotype El Tor strains. Interestingly, a new type of RS2-like element 3.5 kb in size was found in the CTX prophage genome in the small chromosome of VCE232. Cloning followed by sequencing of the new RS2-like element of VCE232 revealed the presence of three ORFs, which probably encode highly divergent types of phage regulatory proteins. Furthermore, the strain VCE232 also harbours two copies of a tandemly arranged CTX prophage devoid of the *ctxAB* genes, called pre-CTX prophage, in its large chromosome. The presence of multiple copies of diverse CTX prophages in both the chromosomes of VCE232 suggests that toxigenic environmental *V. cholerae* non-O1, non-O139 strains could play a role in the emergence of new epidemic clones.

Received 14 July 2006
Revised 30 August 2006
Accepted 1 September 2006

INTRODUCTION

The aquatic environment is the natural habitat of *Vibrio cholerae*, which includes both pathogenic and nonpathogenic strains. However, of the more than 206 recognized 'O' serogroups of *V. cholerae* (Li *et al.*, 2002), only the strains belonging to serogroups O1 and O139 are responsible for epidemic and pandemic cholera of humans. The other serogroups of *V. cholerae*, collectively called non-O1, non-O139, have not been associated with epidemics, but can cause sporadic diarrhoea and are ubiquitously distributed in the aquatic environment. The strains of O1 serogroup are further differentiated into two biotypes, classical and El Tor, on the basis of several phenotypic tests (Kaper *et al.*, 1995).

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, which is still continuing (Kaper *et al.*, 1995). In 1992, a newly recognized serogroup, O139, emerged, and resulted in massive cholera epidemics in the Indian subcontinent (Ramamurthy *et al.*, 1993). Several lines of evidence have suggested that strain O139 arose from an El Tor biotype by acquiring the O139 biosynthesis gene cluster from a non-O1 strain (Bhadra *et al.*, 1995; Bik *et al.*, 1995; Waldor & Mekalanos, 1994). Thus, O139 combines the virulent properties of O1 strains and the presence of a capsule of a non-O1 strain.

The strains belonging to O1 and O139 produce a potent enterotoxin, called cholera toxin (CT), encoded by *ctxAB* genes, which is influenced and regulated by environmental cues, and is central to the disease process. An important discovery in cholera research is that the *ctxAB* locus is a part of the genome of a bacteriophage, called CTXΦ (Waldor & Mekalanos, 1996), and thus it is highly probable that this virulence locus may be transferred among diverse *V. cholerae*

†Present address: Department of Developmental Genetics, NICHD, National Institutes of Health, Bethesda, MD 20892, USA.

Abbreviations: CT, cholera toxin; NCBI, National Center for Biotechnology Information.

The GenBank/EMBL/DDBJ accession number for the RS2-like sequence of *V. cholerae* strain VCE232 reported in this paper is DQ288668.

Fig. 1. Schematic representation of the canonical genetic organization of an RS1 element and a CTX prophage comprising an RS2 segment and a core region (not drawn to scale). Solid triangles represent end repeats. Block arrows represent the indicated genes present in the CTX element and their direction of transcription.

strains in the environment. CTX Φ is a single-stranded filamentous bacteriophage with a small genome of ~6.9 kb (Waldor & Mekalanos, 1996), and is composed of two parts, phage regulatory genetic element RS2, of ~2.4 kb, and a core region of ~4.5 kb (Fig. 1). The RS2 element contains three genes 5'-*rstR*-*rstA*-*rstB*-3' (Waldor *et al.*, 1997), while the core region harbours six genes, including the *ctxAB* operon. The genes of the RS2 element encode functions required for regulation (*rstR* gene product), replication (*rstA* gene product) and integration (*rstB* gene product) of CTX Φ in the genome of *V. cholerae* (Waldor *et al.*, 1997). Several distinct CTX Φ variants have been described, namely CTX^{ET} Φ derived from *V. cholerae* O1 El Tor, CTX^{class} Φ derived from classical *V. cholerae*, CTX^{Calc} Φ derived from O139 *V. cholerae* (Davis *et al.*, 1999), CTX^{Env} Φ derived from non-O1, non-O139 *V. cholerae* (Mukhopadhyay *et al.*, 2001), and CTX^{var} Φ present in pre-O139 *V. cholerae* El Tor isolates (Nandi *et al.*, 2003). Apart from these genes, some RS2 elements may contain an additional gene, *rstC* (Fig. 1); in this instance, the element is termed RS1 and is 2.7 kb in size (Waldor *et al.*, 1997). *RstC* is an antirepressor that controls CTX Φ lysogeny (Davis *et al.*, 2002). RS1 generally flanks 5' and/or 3' of the CTX prophage genome in toxigenic *V. cholerae* O1 El Tor biotype and O139 strains (Davis & Waldor, 2000), but is absent from the classical strains (Davis *et al.*, 2000). *V. cholerae* possesses two circular chromosomes, one small and one large (Heidelberg *et al.*, 2000; Trucksis *et al.*, 1998). In *V. cholerae* El Tor/O139 genomes the CTX prophage may be present either as a single copy or as multiple copies arranged in tandem (Bhadra *et al.*, 1995; Mekalanos, 1983; Nandi *et al.*, 2003). In sharp contrast, in *V. cholerae* classical strains, the prophage is usually present in two copies, one copy in each chromosome (Nandi *et al.*, 2003; Trucksis *et al.*, 1998). The whole-genome sequencing of the *V. cholerae* El Tor strain N16961 has

revealed the integration of only one copy of the CTX prophage flanked by the RS1 element in the large chromosome (Heidelberg *et al.*, 2000). Recently, the integration of the tandem copies of CTX Φ in the small chromosomes of pre-O139 *V. cholerae* El Tor strains has also been demonstrated (Nandi *et al.*, 2003).

Unlike epidemic *V. cholerae* strains, no information is currently available about the organization of the CTX prophage and its chromosomal location in the genomes of CT-positive non-O1, non-O139 strains of *V. cholerae*. The results of this study show for the first time the presence of tandemly arrayed copies of CTX prophages in the small chromosome of a *V. cholerae* non-O1, non-O139 strain, VCE232 (serogroup O4), which was isolated from a year-long environmental study conducted in Calcutta in the 1980s (Nair *et al.*, 1988). Furthermore, we also mapped the large chromosomal tandem integration of two CTX prophages, both devoid of the *ctxAB* genes. Interestingly, the small chromosomal CTX prophage appears to be unique, since its genome contains a novel RS2-like element of 3.5 kb, which was cloned and sequenced.

METHODS

Bacterial strains and growth conditions. Details of *V. cholerae* strains used in this study are described in Table 1. For cloning purposes, *Escherichia coli* DH5 α (Saha *et al.*, 2004) was used. Bacterial strains were routinely grown either in Luria broth (LB) with shaking at 37 °C or in LB containing 1.5 % agar (LA) in plates, as described previously (Nandi *et al.*, 2003). The antibiotic ampicillin was used at 100 μ g ml⁻¹ where needed. Bacterial strains were maintained at -70 °C in LB containing 15 % (v/v) glycerol (Nandi *et al.*, 2003).

PFGE. Intact genomic DNA of *V. cholerae* was prepared and digested with the rare-cutter restriction enzyme *NotI* (New England Biolabs), essentially as described by Nandi *et al.* (2003). For the

Table 1. *V. cholerae* strains used in this study

Strain	Serotype (biotype)	Origin	Chromosomal location of CTX prophage (copy no.)	Reference
O395	O1 (classical)	Clinical	Large (1), small (1)	Trucksis <i>et al.</i> (1998)
N16961	O1 (El Tor)	Clinical	Large (1)	Heidelberg <i>et al.</i> (2000)
VC44	O1 (El Tor)	Clinical	Small (2)	Nandi <i>et al.</i> (2003)
CO471	O1 (El Tor)	Clinical	Large (1)	Nandi <i>et al.</i> (2003)
VCE232	O4	Environmental	Large (2)*, small (2)	This study

*CTX prophages are devoid of *ctxAB* genes.

separation of the two chromosomes of *V. cholerae*, undigested washed agarose slices were used directly (Nandi *et al.*, 2003; Trucksis *et al.*, 1998). PFGE was carried out in a Pulsaphor Plus system with a hexagonal electrode array (Amersham Pharmacia Biotech) in 0.5 × TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.3), essentially as described previously (Nandi *et al.*, 2003). After electrophoresis, gels were stained with ethidium bromide (0.5 µg ml⁻¹) and DNA was visualized.

PCR and other molecular genetic techniques. Standard molecular biological methods were followed throughout the study (Sambrook *et al.*, 1989), unless otherwise mentioned. Primers used in PCR experiments are described in Table 2. For hybridization experiments, an ~1.0 kb fragment of the *ctxAB* operon or 2.3 kb of the RS segment were PCR-amplified using the primers CtxAF/CtxBR or Ig1F/RSR (Table 2), respectively, and genomic DNA of the *V. cholerae* El Tor strain N16961 (Table 1) as a template. An ~0.8 kb *PstI*-*NruI* internal fragment of the PCR amplicon obtained by the primer pair OrfU-F/CtxAR (Table 2), using the genomic DNA of the *V. cholerae* O1 El Tor strain N16961 (Heidelberg *et al.*, 2000; Table 1) as template, was used as a *gIII*^{CTX} gene (Heilpern & Waldor, 2003) probe. Similarly, an ~1.5 kb *AvaI*-*BglII* internal fragment of the 3.7 kb insert DNA present in the recombinant plasmid pCTCP1 (this study) was used as a probe (called URS probe) for the detection of a new type of RS2-like element identified in this study. The PCR amplification conditions used in this study were as follows: a single step of denaturation of template DNA at 94 °C for 1 min, followed by amplification of 30 cycles, each of

which consisted of three steps, denaturation at 94 °C for 30 s, primer annealing at 55–60 °C (depending upon the primers used) for 30 s, and primer extension at 72 °C for a time period based on the expected size of the amplicon (usually 1 min per kb of DNA fragment), followed by a single step of final extension at 72 °C for 7 min. The PCR assay was carried out in a GeneAmp PCR system (model 9700, Applied Biosystems). The PCR-amplified DNA fragment was always purified by the electroelution method (Sambrook *et al.*, 1989) and was used for various purposes. For labelling of DNA, ~50 ng DNA was labelled with [α -³²P]dCTP (Amersham Biosciences) by the random-priming method, using the NEBlot kit (New England Biolabs). For Southern blot hybridization, restriction-enzyme-digested bacterial genomic DNA was separated by electrophoresis, transferred to Hybond-N+ membranes (Amersham Biosciences) and hybridized with a labelled DNA probe at 60 °C in a hybridization oven (Amersham Biosciences), essentially as described previously (Bhadra *et al.*, 1995; Nandi *et al.*, 2003). The membranes were washed under stringent conditions (Bhadra *et al.*, 1995), dried, and exposed to Kodak X-OMAT AR5 film.

Cloning and DNA sequencing. Initially, a PCR experiment was done using the primers CtxBF (*ctxB* gene-specific sequences) and CepR (*cep* gene-specific sequences) (Table 2) to amplify the 4.4 kb RS2-like element of the small chromosomal CTX prophage of VCE232. Sequencing of the 3' and 5' regions of this PCR product using the same primers helped us further in designing two primers, CtxBF2 and CepR2 (Table 2), for PCR amplification of a ~3.7 kb DNA segment that contained the entire RS2-like element (data not shown). A plasmid named pCTCP1 carrying a 3.7 kb insert was selected for sequencing. Sequencing was done in an automated DNA sequencer (Genetic Analyser 3130, Applied Biosystems) using company-supplied kit reagents and protocols. For initial sequencing, M13 forward and reverse universal primers (New England Biolabs) were used, since the pCR4TOPO cloning vector (Invitrogen) contains M13 forward and reverse primer binding sites. The sequence obtained was subjected to National Center for Biotechnology Information (NCBI) BLASTN version 2.2.10 (<http://www.ncbi.nlm.nih.gov/blast/>) to check the authenticity and homology of the sequence, followed by the design of several forward and reverse primers (see Table 2) for sequencing the DNA of both strands of the entire 3.7 kb insert.

Nucleotide sequence and other computer-based analysis. DNA sequence data were compiled and analysed by the DNASIS program (Hitachi). BLASTN version 2.2.10 was used to search for homologous sequences in the database. The NCBI ORF finder tool (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to identify potential ORFs within the sequenced fragment. The ORFs were subsequently subjected to a database search using BLASTP version 2.2.10 (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

RESULTS AND DISCUSSION

Two copies of the CTX prophage in tandem are located in the small chromosome of VCE232

We have reported recently that the non-O1, non-O139 strain VCE232 harbours the *ctxAB* genes in its small chromosome (Bhattacharya *et al.*, 2006). In this study, fine mapping with the restriction enzymes *AvaI*, *BglI*, *BglII*, *PstI* and *XbaI*, which have single sites either in the RS2 or in the core of the phage genome (Bhadra *et al.*, 1995; Mekalanos, 1983; Nandi *et al.*, 2003; Pearson *et al.*, 1993), and using the *ctxAB* region as a probe in hybridization experiments, helped us in deducing the genetic organization of the CTX

Table 2. Sequences of primers used in this study

Primer	Primer sequence (5'–3')	Use
CtxAF	CTCAGACGGGATTTGTTAGGCACG	PCR
CtxAR	TCTATCTCTGTAGCCCCTATTACG	PCR
CtxBF	GATACACATAATAGAATTAAGGAT	PCR, sequencing
CtxBR	GGTTGCTTCTCATCATCGAACCAC	PCR
Ig1F	GAGCCTGTGACACTCACCTTGAT	PCR
RstAR	GCATATGGAACCGACCAAGCAAGAT	PCR
RSR	GCTCAGTCAATGCCTTGAGTTG	PCR, sequencing
RstBF	GTGGTGTGTTTCCTTGTTTGAC	PCR
OrfUF	GGTGTTATTTGATGGCTGCATG	PCR
CepR	AGACCCAGCCACAACCTCAAG	PCR, sequencing
CIIF	CTCACGCTGAACAGCAAGTC	PCR
CIIR	TTGCTTGAATCGAAAGGACA	PCR
RtxAR	GCTGATAAGCTTCGTCAAGCCAA	PCR
TlcF	GATTGTGCGTCTTGCAATTTAGG	PCR
TlcR	GTGAATAAATCAGGTGTAATGTCTG	PCR
CtxBF2	GCCGCAATTAGTATGGCAAA	PCR, sequencing
CepR2	CGCCTCGTTCCAGACATAAT	PCR, sequencing
Ig1aF	GTGAGAACATAAGCGACGTA	Sequencing
UR2	ACCTTCGAGCTTCATATTTTCATTC	Sequencing
UR3	CGTAACCGCCATCTGTCATT	Sequencing
UR5	GCTCGTGATTTGGAATTGGT	Sequencing
UR6	GTACCGCTTTAAGGTTGAGCAT	Sequencing

prophage in the small chromosome of VCE232. When the *ctxAB* probe was used, two fragments of equal intensity were observed in the autoradiogram, indicating the presence of two copies of the CTX prophage in VCE232 (Fig. 2a, b). Further detailed analyses of the autoradiograms and a comparison of the data with the similar information available for the *V. cholerae* O1 biotype El Tor strain VC44 (Table 1) reported previously (Nandi *et al.*, 2003) indicated that the small chromosomal CTX prophages are arranged in tandem in strain VCE232, and that the two cores of the phages are connected by an unusual 3.5 kb DNA segment, instead of the canonical 2.4 kb RS2 element found in epidemic *V. cholerae* strains (Waldor & Mekalanos, 1996). Our analysis indicates that the genome size of the CTX prophage present in VCE232 should be ~ 8.0 kb (3.5 kb of RS2-like element + 4.5 kb of core = 8.0 kb), instead of the 6.9 kb (2.4 kb of RS2 + 4.5 kb of core = 6.9 kb) found in epidemic *V. cholerae* strains (Mekalanos, 1983; Pearson *et al.*, 1993; Waldor & Mekalanos, 1996; Waldor *et al.*, 1997). Hybridization of PFGE-separated *NotI* fragments of VCE232 with the *ctxAB* probe produced only one signal in the 130 kb region (data not shown), which is a similar result to that of *V. cholerae* El Tor strains containing tandemly arranged CTX prophages in the small chromosome (Nandi *et al.*, 2003). All this evidence indicated that the two copies of the CTX prophage in the VCE232 genome are

likely to be arranged in tandem. It also appears that the CTX prophage identified in VCE232 has a novel structure not reported before.

Presence of two tandemly arrayed copies of pre-CTX prophages in the large chromosome of VCE232

Apart from the *ctxAB* probe, we also tested another core gene, *gIII^{CTX}* (Fig. 1), in our hybridization experiments, for two reasons: first, it has been reported that *V. cholerae* may possess CTX prophages devoid of *ctxAB* genes, called pre-CTX prophages (Boyd *et al.*, 2000; Jiang *et al.*, 2003; Li *et al.*, 2003); second, we wished to confirm further the presence of the CTX prophage in the small chromosome of VCE232. PFGE of intact chromosomal DNAs of VCE232 along with control *V. cholerae* strains, namely, O395, N16961 and VC44 (Table 1), was performed to separate their two chromosomes (Fig. 3a) followed by transfer to a membrane and hybridization first with the *ctxAB* probe (Fig. 3b) and, after stripping, with the *gIII^{CTX}* probe (Fig. 3c). While the *ctxAB* probe hybridized with the small chromosome of VCE232, surprisingly, the *gIII^{CTX}* gene hybridized with both the chromosomes of VCE232 (Fig. 3c). The O1 strains used as controls, however, gave identical results with both *ctxAB* and *gIII^{CTX}* probes (Fig. 3b, c). Furthermore, the

Fig. 2. Southern hybridizations of enzyme-digested *V. cholerae* genomic DNA with *ctxAB* (a, b), *gIII^{CTX}* (c) RS (d) and URS (e) as probes. (a) Lanes: 1, 2 and 3, *AvaI*, *BglI* and *PstI* digests of VCE232, respectively; 4, 5 and 6, *AvaI*, *BglI* and *PstI* digests of VC44, respectively. (b) *XbaI*-digested genomic DNA of VCE232 (lane 1) and VC44 (lane 2); *BglI*-digested genomic DNA of VCE232 (lane 3) and VC44 (lane 4). (c) *AvaI* digests of VCE232 (lane 1) and VC44 (lane 2); *BglI* digests of VCE232 (lane 3) and VC44 (lane 4); *BglI* digests of VCE232 (lane 5) and VC44 (lane 6); *PstI* digests of VCE232 (lane 7) and VC44 (lane 8); *XbaI* digests of VCE232 (lane 9) and VC44 (lane 10). The 5.5 kb band corresponds to the restriction fragment originating from the pre-CTX prophages present in tandem in the large chromosome of VCE232. (d) Lanes 1–8 as explained in (c). Intensity of the single *BglI* band (lane 3) detected by the RS probe indicates the presence of multiple co-migrating fragments (see Fig. 6). (e) *BglI* digests of VCE232 (lane 1) and VC44 (lane 2); *PstI* digests of VCE232 (lane 3) and VC44 (lane 4). 'M' denotes λ DNA digested with *HindIII* used as a molecular mass marker (from top to bottom the fragment sizes are 23.1, 9.4, 6.5, 4.3, 2.3 and 2.0 kb).

Fig. 3. Chromosomal location of the pre-CTX prophage in *V. cholerae* strain VCE232. (a) Ethidium bromide-stained PFGE profile of undigested intact genomic DNAs of *V. cholerae* strains. (b) Southern hybridization of DNAs shown in panel (a) probed with the *ctxAB* genes. (c) The blot shown in panel (b) was stripped and reprobed with the *gIII^{CTX}* gene. Lanes: 1, O395; 2, N16961; 3, VC44; 4, VCE232. LC and SC indicate large and small chromosomes of *V. cholerae*, respectively. Numbers in the left-hand margin indicate yeast chromosomes as molecular mass markers.

NotI-digested PFGE-separated genome of VCE232 also hybridized with the *gIII^{CTX}* probe, apart from a 130 kb band originating from the small chromosome (the same band was detectable by the *ctxAB* probe), an additional *NotI* fragment of ~97 kb was observed in the autoradiogram (data not shown). The detection of signals in both the chromosomes of VCE232 with the *gIII^{CTX}* probe (Fig. 3c), but only in the small chromosome with the *ctxAB* probe (Fig. 3b), indicated the probable presence of a pre-CTX prophage in the large chromosome. This observation prompted us to determine the genetic organization of the pre-CTX prophage in VCE232. Extensive Southern hybridization analyses of *AvaI*-, *BglI*-, *BglII*-, *PstI*- and *XbaI*-digested genomic DNA of VCE232 using the *gIII^{CTX}* probe (Fig. 2c), and comparison of the data with that of the similarly digested, but probed with the *ctxAB* genes, genomic DNA of VCE232, as described in the previous section, indicated that the large chromosome of VCE232 also harbours two copies of the pre-CTX prophage arranged in tandem (see below). Interestingly, unlike the RS2 element of the small chromosomal CTX prophage, the RS2 segment of the pre-CTX prophage of the large chromosome appears to be similar to that of RS2 of *V. cholerae* O1 strains, which is supported by the fact that an RS2 probe generated from the *V. cholerae* O1 El Tor strain N16961 hybridized only with those restriction fragments which originated from the pre-CTX prophage region of the large chromosome (Fig. 2d). To our knowledge, this type of structural organization of pre-CTX and CTX prophages in *V. cholerae* per se has not been reported previously, and the information obtained from this study raises the possibility that non-O1, non-O139 *V. cholerae* may play a role in the evolution of diverse CTX prophages, which in turn may influence the emergence of new pathogenic clones of *V. cholerae*.

Site of integration of the pre-CTX and CTX prophages in VCE232

In the large chromosome of epidemic strains, the CTX prophage is integrated between the 'toxin-linked cryptic element' or *tlc* and the 'repeats in toxin' or *rtx* loci (Davis *et al.*, 2000; Heidelberg *et al.*, 2000; Lin *et al.*, 1999; Rubin *et al.*, 1998). In the small chromosome of *V. cholerae* classical

strains (Davis *et al.*, 2000) and in certain clinical *V. cholerae* El Tor strains (Nandi *et al.*, 2003), it is integrated between the *traF* and *yciH* genetic loci (Davis *et al.*, 2000). In both cases the integration site (*attB*) is a 17 bp sequence, called the end repeat, which is almost identical to an 18 bp repeat sequence present in the phage genome (Davis & Waldor, 2000). To determine the integration sites of pre-CTX and CTX prophages in the large and small chromosomes of VCE232, respectively, site-specific PCR assays were performed. As controls, strains N16961 (CTX prophage present in the large chromosome) and VC44 (CTX prophage present in the small chromosome) belonging to the *V. cholerae* El Tor biotype were employed. Two primers were designed, called CIIF (forward) and CIIR (reverse) (Table 2), using the small chromosomal sequences (Heidelberg *et al.*, 2000) which flank the predicted CTX prophage integration site, i.e. the *traF* and *yciH* gene regions. Using the primers CtxAF and CIIR (Table 2), and VCE232 or VC44 genomic DNA as template, an amplicon of ~1.4 kb was obtained and, as expected, no amplicon was obtained in the case of N16961 (Fig. 4). The result strongly suggests that the CTX prophage is integrated in the same locus of the small chromosome of VCE232 as in epidemic *V. cholerae* strains (Nandi *et al.*, 2003). Sequencing of the 1.4 kb PCR product obtained from strains VCE232 and VC44 confirmed this conclusion (data not shown). Furthermore, while the *V. cholerae* El Tor strains N16961 and CO471 (Table 1), which carry the CTX prophage in the large chromosome only (Heidelberg *et al.*, 2000; Nandi *et al.*, 2003), gave the expected PCR amplicon of 0.8 kb with primers CIIF and CIIR due to the absence of the CTX prophage between the *traF* and *yciH* genes, called the 'empty site' (Davis *et al.*, 2000), *V. cholerae* strains VCE232 and VC44 (as control strains) both failed to give any PCR product using the same primer pair (Fig. 4). The results also support the integration of the CTX prophage in the *traF*–*yciH* region of the small chromosome of VCE232, as in epidemic strains. Similarly, to determine whether the pre-CTX prophage in the large chromosome of VCE232 integrates between the *tlc* and *rtx* loci, OrfUF and RtxAR primers (Table 2) were used for PCR amplification. While in the *V. cholerae* El Tor strain CO471, which has an intact

Fig. 4. Site of integration of the CTX prophage in the small chromosome of VCE232. PCR amplification was done with ClIF/ClIR primers (lanes 1–4) and CtxAF/ClIR primers (lanes 5–8) using genomic DNA of *V. cholerae* as template. Lanes: 1 and 5, N16961; 2 and 6, CO471; 3 and 7, VC44; 4 and 8, VCE232. Numbers in the right-hand margin indicate the sizes of the PCR amplicons (kb). M, ϕ X174 DNA digested with *Hae*III and used as molecular size markers.

core and no downstream RS1 element (Nandi *et al.*, 2003), an expected amplicon size of ~ 6.6 kb was obtained, the VCE232 genomic DNA when used as template also yielded an amplicon, but its size was 1.1 kb less than that of CO471 (data not shown), which was due to the presence of tandemly arranged pre-CTX prophages in the same locus, but devoid of *ctxAB* genes. On the other hand, both by hybridization and PCR analysis using primers TlcF and TlcR

(Table 2), it was confirmed that strain VCE232 lacks the *tlc* element (data not shown). This is not surprising, because it has been reported elsewhere that in non-O1, non-O139 strains the *tlc* element is absent (Dalsgaard *et al.*, 2001). The 5' region of the pre-CTX prophage integration in the large chromosome of VCE232 is still being determined.

Cloning and sequence analyses of the unique RS2-like element of VCE232

The RS2-like element was cloned as a 3.7 kb fragment in plasmid pCTCP1 and sequenced. Analysis revealed three complete ORFs within the 3.7 kb region (Fig. 5), none of which at the nucleotide level strongly matched the genes of the RS element of CTX prophages reported for *V. cholerae*. However, there was no doubt that the RS2-like sequence of VCE232 was of bacteriophage origin. A 114 amino acid-coding ORF (nucleotide positions 704–1048), lying immediately adjacent to *igl* (nucleotide positions 149–703), showed about 36 % identity at the amino acid level with the RstR^{class} of the RS2 region present in *V. cholerae* strain 569B (accession no. AAC24223; Kimsey & Waldor, 1998). Further analysis of this ORF also revealed homology with helix–turn–helix DNA-binding proteins, a characteristic feature of many bacteriophage repressors (Aggarwal *et al.*, 1988), including RstR^{ET}, RstR^{class} and RstR^{Calc} (Davis *et al.*, 1999; Kimsey & Waldor, 2004; Waldor *et al.*, 1997). Based on its position, orientation and identity to the RstR of the classical CTX phage, this particular ORF may encode an RstR-like repressor protein, and thus the ORF may be provisionally designated *rstR*-232 according to Mukhopadhyay *et al.* (2001). Adjacent to *rstR*-232, a 709 amino acid-coding ORF (nucleotide positions 1172–3301) was observed. The product of this ORF showed 49 % identity at the amino acid level with the 716 amino acid replication protein-coding ORF of the fs-2 phage of *V. cholerae* O139 strain

Fig. 5. Comparison of the genetic structures of different RS2 elements found in *V. cholerae*. The triangle represents an end repeat (ER). Arrows indicate genes present in the RS2 element with their direction of transcription, and the number within each arrow indicates the number of amino acids encoded by a gene, except for the *rstB* gene of the classical strain 569B, for which only a partial sequence is available. The thin lines indicate intergenic DNA segments. Parentheses indicate the serotype/biotype of *V. cholerae*. GenBank accession numbers of each of the RS2 elements are as follows: 569B, AF055890 (Kimsey & Waldor, 1998); N16961, AE004224 (Heidelberg *et al.*, 2000); AS207, AF110029 (Davis *et al.*, 1999); VCE232, DQ288668 (this study).

Fig. 6. Schematic representation of the genetic organization of pre-CTX and CTX prophages in the large (L) and small (S) chromosomes of strain VCE232, respectively. Restriction maps (not drawn to scale) of the genomic regions are also shown. Various restriction fragment sizes (in kb) obtained by hybridization experiments (see Fig. 2) are indicated below each representation of the genetic organization of the prophages. Thin lines represent chromosomal DNA. A rectangular box represents a truncated core devoid of *ctxAB* genes, a filled box indicates *ctxAB* genes, and a solid bar indicates the location of the URS probe. Diverse RS elements present in strain VCE232 are shown. A, *Ava*; BI, *Bgl*I; BII, *Bgl*II; N, *Not*I; P, *Pst*I; X, *Xba*I.

MDO14 (accession no. BAA33476) reported by Ikema & Honma (1998). This ORF was followed by a 106 amino acid-coding ORF (nucleotide positions 3350–3670) that showed 39% identity at the amino acid level with the product of *orf104* (accession no. BAA33477) of an fs-2 phage, which corresponds to gene *V* (single-stranded DNA-binding protein of coliphage) involved in DNA replication (Ikema & Honma, 1998; Mukhopadhyay *et al.*, 2001). These two ORFs were provisionally designated *rstA*-232 and *rstB*-232, respectively, based on their positions, arrangement and direction of transcription in the RS element, and the similarity of their products with that of fs-2 phage replication proteins. The overall similarity in genetic organization, including location and homology, presence of intergenic regions and direction of transcription of the genes, of the RS2-like element of VCE232 with that of a filamentous single-stranded phage such as fs-2 of *V. cholerae* O139, as well as with the RS2 element of the *V. cholerae* classical strain 569B, suggests that the small chromosomal RS2-like element of VCE232 is most likely a part of the filamentous CTX Φ integrated in its genome. A schematic representation of this newly identified 3.5 kb RS2-like element (nucleotide positions 131–3670) of VCE232 and its comparison with the common RS2 elements found in some

of the epidemic *V. cholerae* strains is shown in Fig. 5. Thus, the organization of the VC232 RS2-like element is similar to that of the other *V. cholerae* strains.

Organization of CTX prophages in the genome of *V. cholerae* strain VCE232

Hybridization with a canonical RS probe (Fig. 2d) and with a 1.5 kb internal fragment of the unique RS2-like element of VCE232 obtained by *Ava*I and *Bgl*II double digestion of the 3.7 kb insert DNA of plasmid pCTCP1, known as the URS probe (Fig. 2e), allowed the positions to be determined of two different types of RS2 elements in the large and small chromosomes of VCE232 (Fig. 6). In addition to hybridization experiments, region-specific PCR assays also helped to determine the organization of the CTX genetic elements in VCE232. The presence of an RS1 element in the VCE232 genome was confirmed by amplifying its genomic DNA with the primers Ig1F and RSR (Table 2), for which the *V. cholerae* El Tor strain VC44 served as a control (Nandi *et al.*, 2003). PCR of VCE232 genomic DNA using either the primer pair CtxAF (*ctxA*-specific forward primer) and RSR (*rstC*-specific reverse primer) or OrfUF (*gIII*^{CTX}-specific forward primer) and RSR gave no amplicon (data not

shown), indicating the absence of an RS1 downstream of the prophages present in the large or small chromosome. Similarly, PCR assay using the VCE232 genomic DNA as template and the primers CIIF (Table 2) and RSR (*rstC*-specific reverse primer) also failed to give any amplicon (data not shown), suggesting the absence of an RS1 element immediately upstream of the CTX prophage in the small chromosome of VCE232. To determine the upstream position of the RS1 of the large chromosome, a PCR experiment was done using primers RstBF and RstAR (Table 2). If RS1 is located immediately upstream of RS2 in VCE232, then, according to the whole-genome sequence data (Heidelberg *et al.*, 2000), one would expect an amplicon size of ~1.5 kb using primers RstBF and RstAR. Interestingly, while the control *V. cholerae* strain N16961 (Heidelberg *et al.*, 2000) gave an expected PCR amplicon of 1.5 kb, in the case of VCE232 the same primer pair gave a 2.0 kb amplicon (data not shown). It seems that the upstream RS1 is not arranged exactly in tandem; rather, it is very close to the pre-CTX prophage, and thus it may be considered as a free RS1 element, as shown schematically in Fig. 6.

In conclusion, the results of this study have shown that a non-O1, non-O139 *V. cholerae* strain can be simultaneously infected by diverse CTX Φ , and that the genetic organization of its genome is highly complex. Further studies on the organization and function of the CTX prophages of environmental toxigenic strains of *V. cholerae* would enhance our understanding of how these environmental strains play a role in the emergence of new variants of toxigenic *V. cholerae*.

ACKNOWLEDGEMENTS

We are indebted to Professor Siddhartha Roy for his constant support to this study. We are grateful to Dr T. Ramamurthy, National Institute of Cholera and Enteric Diseases, for generous donation of *V. cholerae* strains. The work was supported by research grant SMM003 from the Council of Scientific and Industrial Research (CSIR), Government of India. D. M. is grateful to the CSIR for a Senior Research Fellowship.

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