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Molecular Analyses of a Putative CTXϕ Precursor and Evidence for Independent Acquisition of Distinct CTXϕs by Toxigenic *Vibrio cholerae*

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The genes encoding cholera toxin (*ctxA* and *ctxB*) are encoded in the genome of CTXϕ, a filamentous phage that infects *Vibrio cholerae*. To study the evolutionary history of CTXϕ, we examined genome diversity in CTXϕs derived from a variety of epidemic and nonepidemic *Vibrio* sp. natural isolates. Among these were three *V. cholerae* strains that contained CTX prophage sequences but not the *ctxA* and *ctxB* genes. These prophages each gave rise to a plasmid form whose genomic organization was very similar to that of the CTXϕ replicative form, with the exception of missing *ctxAB*. Sequence analysis of these three plasmids revealed that they lacked the upstream control region normally found 5′ of *ctxA*, as well as the *ctxAB* promoter region and coding sequences. These findings are consistent with the hypothesis that a CTXϕ precursor that lacked *ctxAB* simultaneously acquired the toxin genes and their regulatory sequences. To assess the evolutionary relationships among additional CTXϕs, two CTXϕ-encoded genes, *orfU* and *zot*, were sequenced from 13 *V. cholerae* and 4 *V. mimicus* isolates. Comparative nucleotide sequence analyses revealed that the CTXϕs derived from classical and El Tor *V. cholerae* isolates comprise two distinct lineages within otherwise nearly identical chromosomal backgrounds (based on *mdh* sequences). These findings suggest that nontoxigenic precursors of the two *V. cholerae* O1 biotypes independently acquired distinct CTXϕs.

Vibrio cholerae is the etiologic agent of the diarrheal disease cholera. Humans become infected with *V. cholerae* after ingestion of contaminated food or water. Of the nearly 200 recognized serogroups of *V. cholerae*, only the O1 and O139 serogroups are associated with epidemics of cholera (27). The *V. cholerae* O1 serogroup is further divided into the classical and El Tor biotypes on the basis of several phenotypic differences. Since 1817, seven cholera pandemics have been described. The classical biotype is believed to have given rise to the first six cholera pandemics (2). The ongoing seventh pandemic of cholera, which began in 1961, is caused by the El Tor biotype. In 1992, a newly recognized serogroup, O139, emerged and resulted in cholera epidemics in Southeast Asia (14). The emergence of this novel *V. cholerae* serogroup, along with the re-emergence in 1991 of cholera in South America after a nearly 100-year absence, has renewed interest in the origins and evolution of this pathogen.

Pathogenic *V. cholerae* isolates colonize the small intestine and secrete cholera toxin (CT), an A-B-type toxin, to cause the profuse secretory diarrhea characteristic of cholera (44). CT is encoded by *ctxA* and *ctxB*, which are not integral components of the *V. cholerae* genome but, instead, reside in the genome of CTXϕ, a filamentous bacteriophage that infects *V. cholerae*, as well as its close relative *V. mimicus* (8, 18, 48). CTXϕ utilizes the *V. cholerae* type IV pilus TCP, an essential intestinal colonization factor (46), as its receptor (48). In contrast to the well-characterized filamentous bacteriophages derived from *Escherichia coli*, such as f1, the CTXϕ genome integrates into the genome of *V. cholerae* to form a prophage (48). Integration of CTXϕ is site specific (39, 48). However, following infection of classical strains or El Tor strains lacking a CTXϕ integration site, the El Tor-derived CTXϕ remains extrachromosomal, replicating as a plasmid (48, 49). This plasmid form of CTXϕ was designated the phage replicative form (RF), since cells harboring this plasmid produce relatively large amounts of viral particles (48, 49).

The 6.9-kb CTXϕ genome has a modular structure composed of two functionally distinct domains, the core and RS2 regions (Fig. 1) (48). The core region encodes CT and the genes involved in phage morphogenesis, including genes that are thought to encode the major and minor phage coat proteins (Psh, Cep, OrfU, and Ace) and a protein required for CTXϕ assembly (Zot) (48). The RS2 region encodes genes required for replication (*rstA*), integration (*rstB*), and regulation (*rstR*) of CTXϕ (49). RS2 also contains two intergenic regions, ig-1 and ig-2 (Fig. 1). The RS2 region genes only show sequence similarity to two recently described *Vibrio* sp.-derived filamentous phages (13, 23). In contrast, the genes of the core region (with the exceptions of *ctxA* and *ctxB*) show sequence similarity to the morphogenesis genes of filamentous phages from a range of bacterial species (48). Interestingly, the percent GC contents of the *ctxA* and *ctxB* genes, 38 and 33%, respectively (Fig. 1), are significantly different from those of the rest of the core region genes. The distinct GC content of *ctxAB*, compared to the remainder of the CTXϕ genome, suggests that these genes evolved separately from the remainder of the phage genome and that they were acquired after the emergence of a precursor form of CTXϕ that lacked *ctxAB*. In fact, there have been a number of reports in the literature of *zot*$^+$ *V. cholerae* isolates lacking *ctxAB* (15, 31).

The diversity among CTXϕ genomes has only been examined with reference to the RS2 region genes. DNA sequence analysis has revealed that *rstA* and *rstB* from CTX^{ET}ϕ

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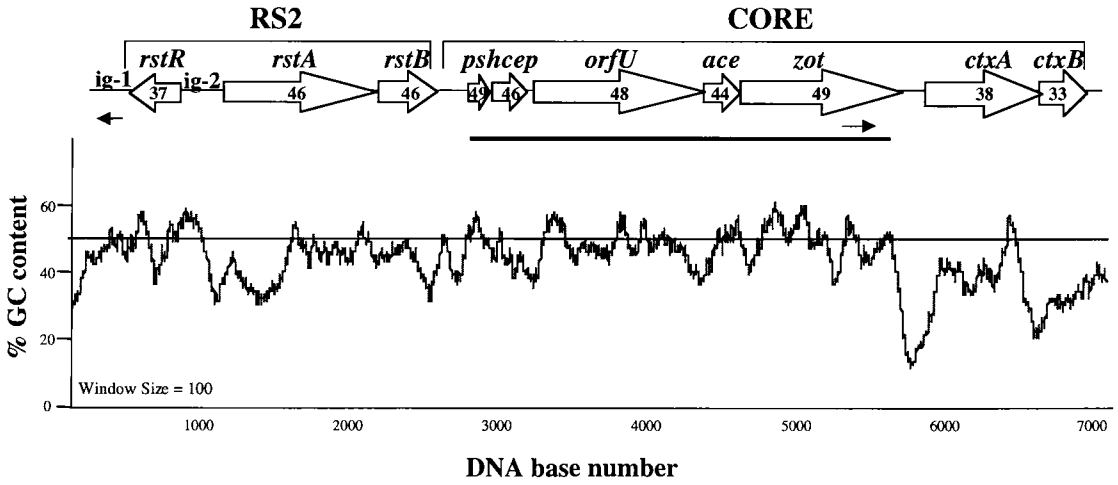

FIG. 1. (Top) Schematic representation of the organization of the El Tor-derived CTXφ genome. Open arrows represent CTXφ ORFs and the direction of transcription of each gene. Numbers within the arrows indicate the genes' percent GC contents. The horizontal bar below the CTXφ map indicates the position of the core probe used in DNA hybridization analysis. The two arrows below the CTXφ map indicate the positions of the PCR primers used to amplify the region between *zot* and ig-1 from pre-CTXφ RF DNA. (Bottom) Regional variation in the mean proportion of GC content based on a sliding window of 100 nucleotides.

$CTX^{class}\phi$, and $CTX^{calc}\phi$ are highly similar (99% amino acid identity) (16, 29). In contrast, comparisons of *rstR* sequences among $CTX^{ET}\phi$, $CTX^{class}\phi$, and $CTX^{calc}\phi$ revealed that they were extremely diverse, with less than 30% amino acid sequence similarity (16, 29). Furthermore, the three known *rstR* genes have a percent GC content (34 to 37%) that is distinct from those of most of the other CTXφ genes (Fig. 1). The hypervariability of *rstR* and its distinct GC content suggest that CTXφ variants obtained diverse *rstR* cassettes via horizontal gene transfer, followed by recombination. A similar mechanism is believed to account for the diversity of repressors in lambdoid phages (12). Since recombination, rather than mutation, probably accounts for the distinct CTXφ *rstR* genes, the diversity of *rstR* sequences does not provide an insight into the relatedness of distinct CTXφs.

We set out to study the evolution and relatedness of CTXφ variants and to examine whether distinct CTXφ genotypes are associated with each of the *V. cholerae* O1 biotypes, with different *V. cholerae* serogroups, or with *V. mimicus*. During the course of this work, three strains harboring CTX-like prophages that lacked the *ctxA* and *ctxB* genes were identified. We hypothesize that these CTX-like prophages lacking *ctxAB* represent derivatives of the ancestral precursor of CTXφ. Comparative nucleotide sequence analyses of two CTXφ core region genes, *orfU* and *zot*, from 13 *V. cholerae* strains revealed that there are distinct phage lineages in classical and El Tor *V. cholerae* isolates. These analyses suggest that acquisition of CTXφ by *Vibrio* spp. has occurred multiple times and has involved several CTXφ genotypes.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used were cultured in Luria-Bertani (34) broth at 37°C. These strains included 13 *V. cholerae* and 4 *V. mimicus* isolates that encompassed seven serogroups (Table 1). These *Vibrio* sp. isolates were derived from both human and environmental sources with a wide geographic distribution between 1930 and 1993 (Table 1).

Molecular analyses. *V. cholerae* DNA was extracted and purified using the G-nome DNA isolation kit from Bio 101, Inc., Vista, Calif. To assay for the presence of the CTXφ genome among *Vibrio* isolates, two pairs of primers were used for PCR assays, orfU1 plus zot2 and ctxA1 plus ctxB2, which were designed from published DNA sequences as previously described (8). For Southern hybridization analyses, a CTXφ core region DNA probe spanning the region between *psh* and *zot* was used (Fig. 1). Southern hybridization was carried out using horseradish peroxidase-labeled DNA probes, which were prepared and hybridized using the ECL direct labeling and detection system (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England) in accordance with the manufacturer's instructions.

To test for the presence of extrachromosomal DNA corresponding to a precursor CTXφ (pre-CTXφ) lacking *ctxAB* in strains 151, 208, and C325, Qiaprep Spin kits (Qiagen, Valencia, Calif.) were used to first isolate plasmid DNA from mid-log-phase cultures. Then, Southern blot analysis with a CTXφ core region probe was used to detect CTXφ-related sequences in these plasmid preparations. O395, which contains a defective CTX prophage, and Bah-2 (pCTX-Kn) were used as negative and positive controls, respectively. To test for transduction of the pre-CTXφs, filtered cell-free supernatants from the same mid-log cultures were mixed with agglutinated (TCP^+) O395 and grown overnight at 30°C. Then, plasmid DNA was prepared from these cells and the presence of CTXφ-related sequences was assayed for by Southern hybridization as described above.

Nucleotide sequencing. Sequencing was performed with an Applied Biosystems 373A automated DNA sequencing system using the DyeDeoxy terminator cycle sequencing kit at the Tufts Medical School sequencing facility. For all of the genes analyzed, both DNA strands were sequenced. The BLAST programs (1) were used to compare sequences to those in the GenBank databases. A 715-bp region of *orfU* and a 708-bp segment of *zot* were amplified by PCR and sequenced. PCR primers to amplify the chromosomal *mdh* locus were designed from the *mdh* sequence of *V. cholerae* strain N16961. With the forward (5′ atgaaagtcgctgttatt 3′) and reverse (5′ gtatctaacatgccatcc 3′) primers, an 892-bp

TABLE 1. Strains used for comparative sequence analyses

Strain	Serogroup (biotype)	Source	Yr	Locality	Reference
V. cholerae					
C1	O1 (classical)	Unknown	1955	Unknown	5
CA401	O1 (classical)	Clinical	1953	India	20
569B	O1 (classical)	Clinical	1948	India	33
N16961	O1 (El Tor)	Clinical	1975	Bangladesh	26
E7946	O1 (El Tor)	Clinical	1978	Bahrain	33
C5	O1 (El Tor)	Unknown	1957	Indonesia	This study
RV79	O1 (El Tor)	Clinical	1930	Vietnam	33
SG20	O139	Clinical	1993	India	This study
CO130	O37	Environmental	1993	India	This study
V52	O37	Clinical	1968	Sudan	50
151	O37	Environmental	1993	Mexico	38
V46	O141	Clinical	1978	United States	This study
208	O11	Clinical	1993	Thailand	38
C325	O1	Clinical	1995	India	31
V. mimicus					
PT5	O115	Clinical	1985	Bangladesh	45
PT48	O115	Clinical	1985	Bangladesh	45
523-80	O115	Clinical	1980	United States	45
9583	O115	Clinical	1980	United States	45

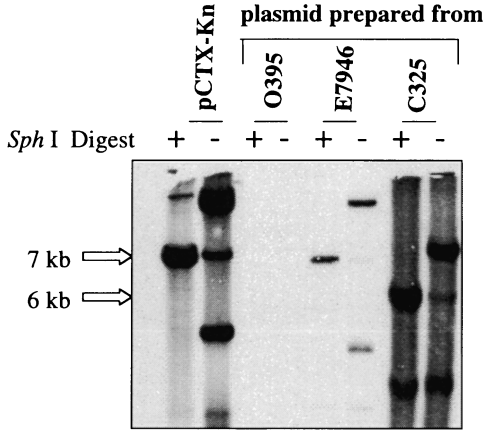

FIG. 2. Detection of an extrachromosomal form of the pre-CTX prophage from *ctxAB* strain C325. Plasmid DNA was prepared from C325, classical strain O395 (a negative control), El Tor strain E7946 (a positive control), and Bah-2(pCTX-Kn). Southern hybridization with a core probe was then used to detect either *Sph*I-digested or undigested plasmid DNA.

region of the 939-bp *mdh* sequence was amplified. PCR products were purified using the Qiaquick (Qiagen) PCR purification kit and subsequently sequenced on both strands. To generate sequencing templates from the three strains that were found to contain CTX-like prophage genomes that lacked *ctxAB*, the DNA sequences between *zot* and ig-1 in the RF were amplified with primers zot5 and rig1 (Fig. 1) using plasmid DNA derived from these strains as templates. The forward primer zot5 (5′ gcagtagcctttgactgag 3′) lies within the *zot* gene, and the reverse primer rig1 (5′ cacgctacgtcgcttatgt 3′) is located within the conserved part of the first intergenic region of CTXϕ. The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.), and the resulting plasmids were subsequently used as templates for sequencing.

Phylogenetic analyses. Regional variation in GC content over the entire length of the CTXϕ genome was calculated using a sliding window of 100 nucleotides. The GC content of each CTXϕ gene was also calculated using the MacVector program. DNA sequence data were assembled and edited with Eyeball Sequence Editor (11). Gene trees were constructed with MEGA (30). Rates (per site) of synonymous (k_S) and nonsynonymous (k_N) substitutions were calculated by the methods of Nei and Gojobori (36) and Nei and Jin (37). The proportions of synonymous (p_S) and nonsynonymous (p_N) substitutions in the *orfU* and *zot* genes between pairs of strains were tabulated in a sliding-window analysis of 30 codons along each gene by the program PSWIN (T. S. Whittam, Pennsylvania State University).

Nucleotide sequence accession numbers. The nucleotide sequences obtained during this study for *orfU*, *zot*, and *mdh* have been deposited in GenBank under accession numbers AF238329 to AF238373.

RESULTS

Identification and analysis of potential CTXϕ precursors. To begin to assess the diversity of CTXϕ, we examined our laboratory collection of natural *V. cholerae* isolates for the presence of CTXϕ-related sequences using PCR tests for the presence of *orfU*, *ace*, *zot*, and *ctxAB*. We identified two isolates, 151 (38) and 208 (38), that contained the CTXϕ sequences *orfU*, *ace*, and *zot* but did not encode *ctxAB*. In addition to these two isolates, we obtained a third *V. cholerae* strain, C325, that was previously reported to contain CTXϕ core region sequences without *ctxAB* (31). *V. cholerae* strains 151, 208, and C325 belonged to three serogroups, O11, O37, and O1, respectively, and were isolated in Mexico, Thailand, and India, respectively. Southern blots revealed that the phage structural genes were present within the chromosomal DNAs of these strains (data not shown). We hypothesized that the phage genes might be components of a prophage that could represent a derivative of a precursor of CTXϕ (pre-CTXϕ) that lacked *ctxAB*. The distinct GC content of *ctxAB* relative to the remainder of the CTXϕ genome strongly suggests that the toxin genes were acquired subsequent to the development of a

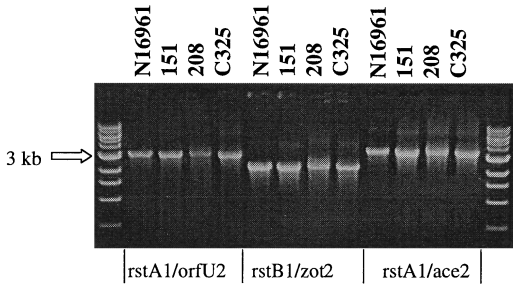

FIG. 3. PCR analyses of the genomic organizations of the plasmid form of CTXϕ from *V. cholerae* strain N16961 (El Tor) or from putative pre-CTXϕs from *V. cholerae* strains 151, 208, and C325. The primer pairs used are indicated below the lanes. The left- and rightmost lanes contained molecular size markers.

pre-CTXϕ. To test whether the phage genes are part of functional prophages in these strains, we first examined whether these three strains contained extrachromosomal DNA that corresponded to the RF of the pre-CTXϕ DNA. Southern blot analyses of plasmid DNAs prepared from these strains showed the presence of extrachromosomal DNA that hybridized with a core region probe in all three cases (Fig. 2 and data not shown). For C325 and 151, this plasmid was ~6.0 kb, a size consistent with a CTXϕ-like RF that is missing *ctxAB*. In strain 208, this plasmid was ~7.0 kb. Furthermore, PCR analyses strongly suggested that the gene content and gene order of core region and RS2 region genes in these three plasmids are identical to those of CTXϕ, with the exception of the missing *ctxAB* genes (Fig. 3). Finally, supernatants derived from all three strains could transmit these pre-CTXϕ genomes to a classical recipient strain (Fig. 4 and data not shown). These results indicate that these putative pre-CTXϕ prophages give rise to infectious particles. These experiments demonstrate that these three CTX-like prophages lacking *ctxAB* are functional and are consistent with the hypothesis that CTXϕ evolved from a pre-CTXϕ lacking *ctxAB*.

To further characterize these potential pre-CTXϕ derivatives and to determine if these three genomes contain another toxin-encoding gene in place of *ctxAB*, we sequenced the re-

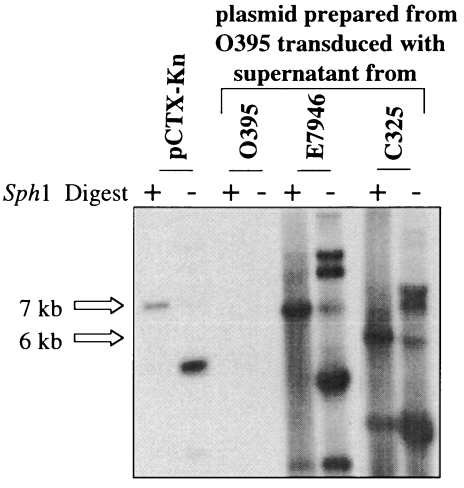

FIG. 4. Detection of an infectious form of pre-CTXϕ derived from strain C325. Cell-free supernatants from C325, O395 (a negative control), and E7946 (a positive control) were mixed with agglutinated O395. Twenty-four hours later, plasmid DNA was prepared from these three cultures and the presence of CTXϕ core genes was determined by Southern blot analysis.

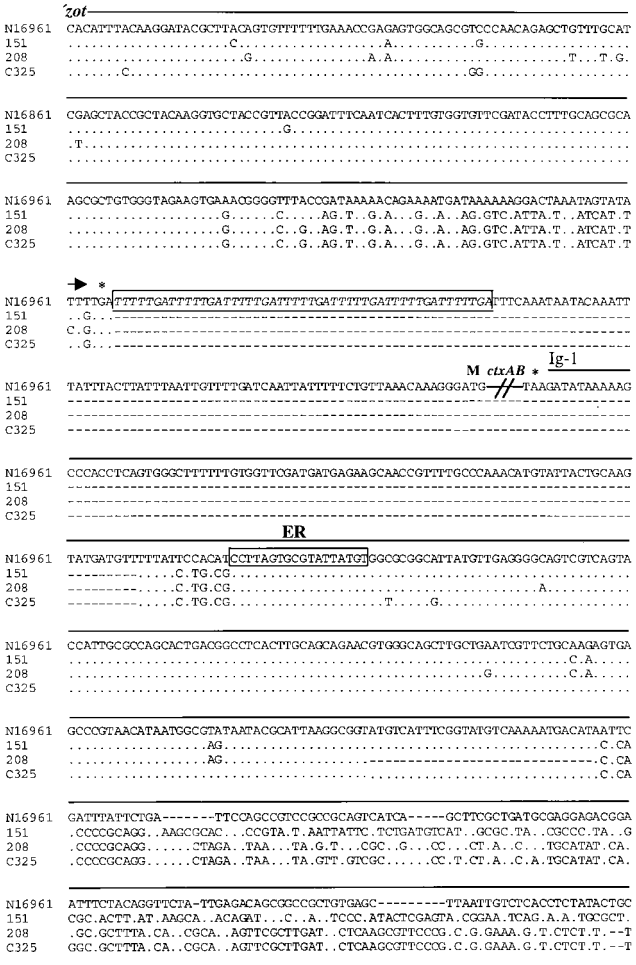

FIG. 5. Alignment of the nucleotide sequences of *zot* to ig-1 from *V. cholerae* strain N16961 (El Tor) and from *V. cholerae* strains 151, 208, and C325. The latter three sequences were determined from PCR-amplified pre-CTXφ RF DNAs derived from these strains. The arrow above the sequences depicts the 3′ end of the *zot* ORFs. Dots indicate nucleotide identity, and dashes represent gaps introduced to allow alignment of the CTXφ sequences with the pre-CTXφ sequences. Boxed and italicized nucleotides indicate the repeat sequences that play a role in ToxR binding. The boxed nucleotides designated ER represent the end repeat sequence thought to constitute the core of the CTXφ attachment site. The *ctxAB* genes of strain N16961 are not shown. M represents the *ctxA* start codon, and the asterisk designates the stop codons of *ctxB* and *zot*. The line above the N16961 sequence beginning after the *ctxB* stop codon represents the El Tor ig-1 sequence.

gion 3′ of *zot* in these plasmids. To accomplish this, PCR primers within the 3′ end of *zot* and a conserved region of ig-1 (Fig. 1) were used to amplify this region from these three plasmids. The three nucleotide sequences of this region were highly similar. A comparison of these sequences with each other and with the corresponding sequence from El Tor strain N16961 is shown in Fig. 5. There is almost complete nucleotide sequence identity at the 5′ end of these four sequences; however, the terminal 60 bp of N16961 *zot* was highly divergent from the new sequences (28 polymorphic sites).

The sequences downstream of *zot* in N16961 also were dissimilar to the new sequences. The heptad direct repeat sequences that facilitate regulation of *ctxAB* expression by the transcriptional activator ToxR (32, 35, 40) were absent from the 151-, 208-, and C325-derived plasmids, as were the *ctxAB* promoter and coding sequences (Fig. 5). No open reading frames replaced *ctxAB* in these plasmids; rather, 3′ of the presumed *zot* stop codon in these three sequences was a sequence that aligned nearly perfectly with a part of ig-1 from strain N16961. This region of identity began near the 18-bp end repeat sequence that is thought to constitute part of the CTXφ attachment site and extended for 215 bp (49). 3′ of this conserved region, which probably constitutes a critical part of CTXφ *attP*, these three sequences diverged from the N16961 sequence. However, the N16961 sequence for this region of ig-1 also differs from the ig-1 of classical CTX prophages (29). These data are consistent with a model of CTXφ evolution in which a pre-CTXφ simultaneously obtained *ctxAB*, *cis*-acting sequences required for activation of toxin gene expression, and a new carboxyl terminus for Zot. The presence of the 18-bp end repeat sequence that likely corresponds to the core region of the CTXφ attachment site, as well as other parts of the CTXφ *attP* sequence in the putative pre-CTXφ genomes, suggests that these phages have integration sites and integration mechanisms similar to those of CTXφ.

Diversity of CTX core region sequences. Since our understanding of the diversity of CTXφs was limited and rested entirely on RS2 region sequences, we compared the sequences of core region genes from a set of *Vibrio* sp. isolates that we found to harbor CTXφ. This set included 13 *V. cholerae* strains and 4 *V. mimicus* strains (Table 1). These strains were chosen to represent the diversity of CTXφs in our laboratory collection based on the dates and sites of their isolation. The sample of *V. cholerae* included seven O1 serogroup strains (three classical and four El Tor biotype strains). Although classical and El Tor strains are thought to be essentially clonal (3, 10, 28), the similarity of the CTXφs in these strains has not been assessed. The six non-O1 serogroup strains included four serotypes (O139, O37, O141, and O11) and two strains that contained the putative pre-CTXφ derivatives (151 and 208).

To enable comparisons of the diversity of CTXφ sequences with the diversity of a *V. cholerae* chromosomal sequence, we also performed sequence analyses of *mdh*, which encodes the metabolic enzyme malate dehydrogenase. We selected *mdh* because this locus was used previously in several evolutionary studies of a number of enteric bacteria, including *V. cholerae*, *E. coli*, and *Salmonella enterica* (6, 7, 10, 41). These studies demonstrated that phylogenetic trees based on *mdh* are congruent with analyses of evolutionary relatedness based on other methods and that such trees give a reliable estimate of genotypic divergence. For this study, we analyzed 693 bp of the 936-bp coding region of *mdh*. Among the 13 *V. cholerae* and 4 *V. mimicus* isolates we compared, there were 82 polymorphic sites resulting in nine amino acid replacement substitutions among the *V. cholerae* and *V. mimicus* isolates examined (Table 2). The *mdh* sequence from *V. cholerae* isolates in pairwise comparisons revealed an average difference of four polymorphic nucleotide sites. Consistent with a recent report (10), the *mdh* sequences in epidemic *V. cholerae* isolates were essentially identical. Among *V. mimicus* strains, the *mdh* sequence differed, on average, by two polymorphic nucleotide sites. Most of the genetic variation observed at the *mdh* locus was between the two *Vibrio* species; thus, pairwise comparisons between *V. cholerae* and *V. mimicus* strains yielded an average difference of 68 polymorphic nucleotide sites.

The nucleotide sequences of 715 bp (codons 46 to 396) of the 1,188-bp coding region of *orfU* for the 13 *V. cholerae* strains and 4 *V. mimicus* strains were similarly compared. In total, there were 72 polymorphic sites within the *orfU* sequences, which resulted in 27 sites of amino acid replacements (Table 2). Among the 17 *orfU* sequences determined, there were 8 variant sequences. Interestingly, the *orfU* sequences were highly variable between the classical and El Tor biotypes but

TABLE 2. Nucleotide sequence variation in the *orfU* and *zot* genes of CTXϕs derived from *V. cholerae* and *V. mimicus* strains

Gene	No. of sites				k_S	k_N
	Total	Total poly[a]	Silent poly[b]	AA poly[c]		
CTXϕ						
orfU (*V. cholerae* only)	715	71	44	27	0.0898 ± 0.0159[d]	0.0272 ± 0.0047
orfU (*V. mimicus* only)	715	58	37	21	0.1040 ± 0.0199	0.0279 ± 0.0052
zot (*V. cholerae* only)	708	32	23	9	0.0407 ± 0.0096	0.0051 ± 0.0018
zot (*V. mimicus* only)	708	12	7	5	0.0244 ± 0.0093	0.0052 ± 0.0023
V. cholerae vs *V. mimicus*						
orfU (all strains)	715	72	45	27	0.0993 ± 0.0176	0.0289 ± 0.0051
zot (all strains)	708	32	23	9	0.0385 ± 0.0088	0.0053 ± 0.0018
Chromosomal						
mdh (*V. cholerae*)	693	13	4	7	0.0229 ± 0.0072	0.0013 ± 0.0008
mdh (*V. mimicus*)	693	4	2	2	0.0071 ± 0.005	0.0027 ± 0.0019
V. cholerae vs *V. mimicus*						
mdh (all strains)	693	82	73	9	0.1618 ± 0.0216	0.0054 ± 0.0018

[a] Total poly, total number of polymorphic sites.
[b] Silent poly, silent polymorphic sites.
[c] AA poly, amino acid polymorphic sites.
[d] k_S and k_N values are means ± standard deviations.

identical within biotypes, with the exception of El Tor strain RV79, a 1930 clinical isolate from Vietnam. The O139 *orfU* sequence (strain SG20) was identical to the sequences derived from El Tor strains N16961, C5, and E7946. *V. mimicus* strain PT5 shared *orfU* sequence identity with these three El Tor strains as well. Comparison of the *orfU* nucleotide sequences between classical and El Tor *V. cholerae* isolates revealed 61 polymorphic nucleotide sites resulting in 23 amino acid replacements. The *orfU* sequences from nonepidemic *V. cholerae* isolates CO130, V52, V46, 151, and 208 were more similar to the *orfU* sequences derived from the El Tor isolates, whereas *V. mimicus* isolates PT48, 523-80, and 9583 shared sequence similarity with *orfU* from classical *V. cholerae* isolates.

The nucleotide sequences of a 708-bp segment of *zot* (codons 54 to 290) of the 1,200-bp coding region of *zot* were also compared. From the 17 strains examined, there were eight variant *zot* sequences. Among the eight variant *zot* sequences, there were a total of 32 polymorphic sites of which 9 resulted in an amino acid replacement (Table 2). Similar to the findings for the *orfU* gene, the classical biotype *V. cholerae* strains all had the same *zot* sequence that was distinct from the El Tor strains' *zot* sequence. Also, again with the exception of RV79, the El Tor sequences and the O139 sequences were identical. The level of sequence divergence observed at the *zot* locus was less than that observed at the *orfU* locus. There were a total of 18 polymorphic sites between the classical and El Tor biotype strains.

For the two CTXϕ genes *orfU* and *zot*, we estimated the genetic diversity in all pairwise comparisons using the methods of Nei and Gojobori (36) and Nei and Jin (37). The results are summarized in Table 2 along with those from analysis of the chromosomal gene *mdh* for comparison. The values k_S and k_N are the average numbers of nucleotide differences per synonymous (silent) site and per nonsynonymous (replacement) site, respectively, among all pairwise comparisons. Table 2 shows that the estimates of CTXϕ gene diversity within either *V. cholerae* or *V. mimicus* are significantly greater than those calculated for the chromosomal locus *mdh*. Also, as noted above, *orfU* sequence diversity was greater than *zot* sequence diversity.

Spatial distribution of polymorphic sites. The OrfU protein is thought to be functionally equivalent to pIII of filamentous phages derived from *E. coli* (22, 48). pIII is a phage coat protein that mediates phage attachment to a host cell. We hypothesized that the significant differences in the OrfU sequences between classical strain- and El Tor strain-derived CTXϕs reflect the functional constraints that these two OrfU proteins face in binding to the CTXϕ receptor, TCP. The sequence of TcpA, the major subunit of TCP, is known to vary considerably between the two *V. cholerae* O1 biotypes (24, 42). To begin to address this possibility, we analyzed whether the synonymous and nonsynonymous substitutions within *orfU* are clustered in the central domain of the genes, which encodes the putative TCP-binding domain of OrfU (21). The results of this analysis, shown in Fig. 6, revealed a striking clustering of synonymous and nonsynonymous site variation in the central portion of *orfU*. Two distinct peaks of nonsynonymous site variation were present in the central region. We predict that these two *orfU* hypervariable regions constitute parts of OrfU that are important for OrfU-TcpA interaction. In contrast, similar analysis of the *zot* sequences did not reveal any clustering of the polymorphic sites.

Evolutionary relationships among CTXϕs and their *Vibrio* sp. host strains. To compare the evolutionary relationships between CTXϕs and their *Vibrio* sp. host strains, the 17 *zot*, *orfU*, and *mdh* sequences were used to construct three phylo-

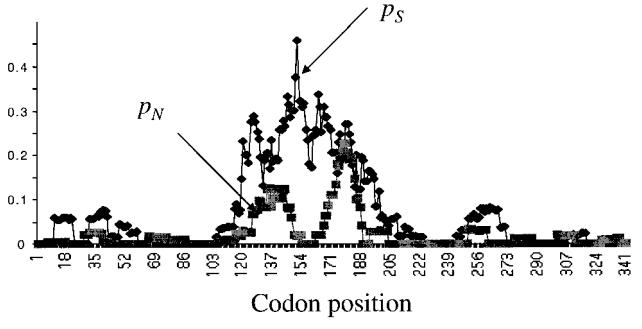

FIG. 6. Regional variation in the mean proportion of synonymous (p_S) differences between pairs of strains and the mean proportion of nonsynonymous (p_N) differences between pairs of strains based on a sliding window of 90 nucleotides in *orfU*. Squares represent p_N values, and diamonds indicate p_S values.

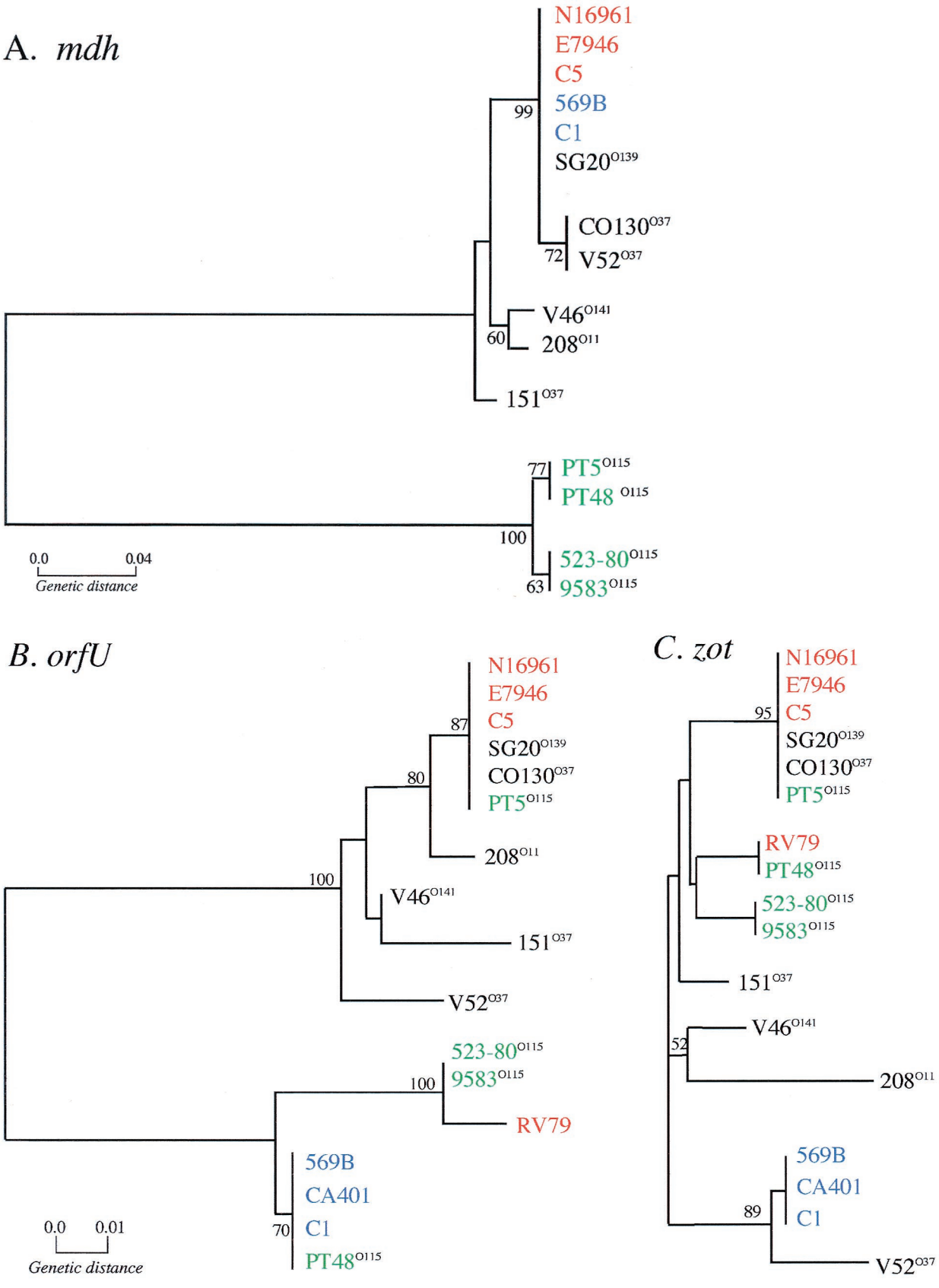

FIG. 7. Evolutionary relationships based on synonymous site variation in the *orfU*, *zot*, and *mdh* genes. The neighbor-joining method was used to construct the trees. The *V. cholerae* El Tor strains are in red, classical strains are in blue, non-O1 serogroup strains are in black with the particular serogroup in the superscript, and *V. mimicus* strains are in green. Bootstrap values based on 1,000 computer-generated trees are indicated at the nodes, and only values greater than 50 are shown.

genetic trees. These three trees, shown in Fig. 7, were constructed by the neighbor-joining method from a matrix of pairwise genetic distances based on all polymorphic nucleotide sites, with correction for multiple substitutions by the Jukes-Cantor method (25, 43). The most notable feature of this analysis is the divergent clustering of CTXϕ genes from classical and El Tor biotype *V. cholerae* isolates (with the exception of RV79). The *orfU* and *zot* sequences derived from strains of the same biotype invariably clustered together (Fig. 7). For the most part, *orfU* sequences derived from nonepidemic *V. cholerae* isolates clustered with El Tor *orfU* sequences. The relationships among the *V. mimicus* strains based on CTXϕ gene trees are very different. *V. mimicus* strain PT5 clusters with the El Tor strains on both the *orfU* and *zot* gene trees. However, *V. mimicus* strains PT48, 523-80, and 9583 grouped with the classical strains in the *orfU* tree but with the El Tor strains in the *zot* gene tree (Fig. 7). These discrepancies are probably due to the low number of polymorphic sites analyzed at the *zot* locus, as indicated by the low bootstrap values obtained for these nodes in the *zot* gene tree. Similarly, the limited number of polymorphic sites among the *zot* sequences probably explains the discrepancy between the *orfU* and *zot* gene trees for non-O1 serogroup strains V52, 208, and V46. Another notable feature of Fig. 7 is the lack of congruence of the phage gene trees with the gene tree derived from *mdh* sequences. The classical and El Tor epidemic *V. cholerae* isolates are all identical based on *mdh* sequence analysis, yet they have very divergent CTXϕ sequences. This lack of similarity between the phage and chromosomal gene trees is indicative of horizontal transmission of CTXϕ, as expected for a mobile genetic element like CTXϕ. The clustering of *V. mimicus* strain PT5-derived CTXϕ sequences with El Tor *V. cholerae* CTXϕ sequences and the *V. mimicus* strain PT48-derived *orfU* sequence with the classical *orfU* sequence (Fig. 7) provides additional evidence for the proposition (8) that horizontal transfer of CTXϕ between *V. cholerae* and *V. mimicus* occurred relatively recently. Similarly, these gene trees suggest that there was a relatively recent transfer of CTXϕ between O1 and non-O1 strains of *V. cholerae*.

DISCUSSION

CTXϕ evolution. A number of toxins and other virulence factors are encoded within the genomes of bacteriophages (4, 47). In most, if not all, cases, phage-encoded virulence genes are not thought to directly influence the biological properties of the phage. Rather, these genes are believed to be accessories to the bacteriophage genome that can affect the properties of the host cell and thereby potentially indirectly influence the viability of the phage genome. Since neither CT nor its structural genes seem to affect CTXϕ functions and since the GC content of *ctxAB* differs from that of most of the other CTXϕ genes, we hypothesized that *ctxAB* was acquired after the evolution of a pre-CTXϕ that lacked these genes.

We identified and analyzed three *V. cholerae* isolates (151, 208, and C325) that contained several CTXϕ core region genes but lacked *ctxAB*, under the assumption that such strains may contain a derivative of a pre-CTXϕ. These three isolates were all found to harbor an extrachromosomal circular DNA molecule whose genomic organizations were very similar to that of the CTXϕ RF, with the exception of missing *ctxAB*, the ToxR binding sites found 5′ of *ctxAB*, and 173 bp of the ig-1 region 3′ of *ctxAB*. Since the GC content of *ctxAB* is distinct from the remainder of the core region, and since the 3′ end of *zot* in these three plasmids is significantly different than the *zot* sequence in CTXϕ, we believe that these three plasmids are more likely to be derivatives of a pre-CTXϕ that never contained *ctxAB* rather than of CTXϕs that have lost *ctxAB*. If these plasmids (or their prophage forms) do represent derivatives of pre-CTXϕs, then it seems probable that the sequences downstream from *zot* and 5′ of *ctxAB* were acquired along with *ctxAB* by a pre-CTXϕ. Since ToxR and ToxT are required for *ctxAB* expression, this suggests that the strain that donated *ctxAB* to a pre-CTXϕ contained these transcriptional activators. The origin of *ctxAB* and the mechanism of its acquisition by a pre-CTXϕ remain matters for speculation. As is the case for several toxin-encoding phages (4), *ctxAB* is adjacent to the CTXϕ attachment site, raising the possibility that an imprecise excision of the pre-CTX prophage generated a new phage that included adjacent chromosomal *ctxAB* sequences.

We determined the nucleotide sequence of large portions of two CTXϕ core region genes, *zot* and *orfU*, from 13 *V. cholerae* and 4 *V. mimicus* strains in order to study the relatedness of different CTXϕs. There were significant differences in these two sequences between the *V. cholerae* O1 biotypes. However, except for El Tor strain RV79, no differences in these sequences were found in strains of the same biotype that were isolated at different times and locations, reflecting the clonality of the sixth (classical)- and seventh (El Tor)-pandemic strains of *V. cholerae*. The similarity of RV79-derived CTXϕ sequences to CTXϕ sequences from classical *V. cholerae* (Fig. 7) most likely reflects the fact that this El Tor strain was isolated 31 years prior to the onset of the current seventh pandemic of cholera, during a period when the classical biotype was predominant.

The *orfU* sequences were significantly more diverse than the *zot* sequences. Also, the polymorphic sites in *orfU* were clustered in the central region of this gene whereas no clustering was evident in the *zot* polymorphic sites. We suspect that the clustering of the polymorphisms in *orfU* developed in response to a high level of selective pressure upon this domain of OrfU, which is predicted to bind CTXϕ particles to TCP, the CTXϕ receptor on *V. cholerae*. Interestingly, the amino acid sequence of TcpA, the major subunit of TCP, is known to be significantly different in classical and El Tor *V. cholerae* isolates (80% amino acid similarity). We therefore predict that the two hypervariable regions (Fig. 6) within OrfU interact directly with TcpA and that the abundant interbiotype polymorphisms in OrfU reflect selective pressures to change in parallel with TcpA, its ligand.

Diverse CTXϕs in the evolution of toxigenic *Vibrio* spp. Comparative sequence analyses of the chromosomal *mdh* locus in CTXϕ host strains yields an inferred phylogeny that differs significantly from the inferred phylogeny based on either *zot* or *orfU* sequences (compare Fig. 7A with B or C). It was recently reported (10) that epidemic isolates of *V. cholerae* are very closely related, and our comparisons of *mdh* sequences confirm this finding. All of the *mdh* sequences for *V. cholerae* serogroup O1 epidemic isolates that we analyzed were identical and thus cluster together on the *mdh* gene tree. However, this was not the case for the two CTXϕ gene trees. The phage sequences derived from classical and El Tor strains formed two divergent branches on both the *orfU* and *zot* gene trees. This finding of distinct CTXϕ lineages within essentially identical *V. cholerae* chromosomal backgrounds is not consistent with a simple evolutionary scenario of clonal descent, in which a single CTXϕ progenitor infected an ancestral *V. cholerae* isolate and evolved within *V. cholerae* to the present level of diversity. Rather, it suggests the hypothesis that distinct CTXϕs independently infected *ctxAB* progenitors of the classical and El Tor *V. cholerae* pandemic strains (Fig. 8).

We also found that *mdh* sequences were more variable be-

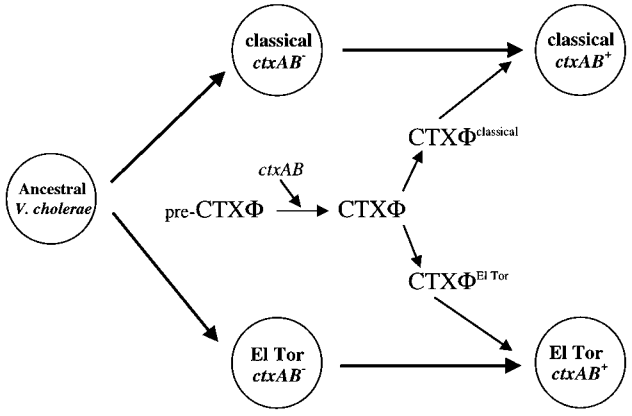

FIG. 8. Model for acquisition of *ctxAB* by the two *V. cholerae* O1 biotypes. An ancestral *V. cholerae* isolate gave rise to the classical and El Tor biotypes, which were subsequently independently infected with divergent CTXφs. CTXφ probably arose from a precursor CTXφ that acquired the CT genes by imprecise excision from a unknown donor strain.

tween *V. mimicus* and *V. cholerae* isolates than either the *orfU* or *zot* sequences derived from these two *Vibrio* species (Table 2). *V. mimicus mdh* sequences and *V. cholerae mdh* sequences are located on distinct branches of the *mdh* gene tree, whereas there is no consistent clustering of *V. mimicus*-derived CTXφ sequences on the *zot* and *orfU* gene trees (Fig. 7). This indicates that CTXφ(s) infected *V. cholerae* and *V. mimicus* after these two *Vibrio* species diverged from their most recent common ancestor. As recently suggested (8), the identity of *orfU* and *zot* sequences in *V. mimicus* strain PT5 with the El Tor sequences suggests recent horizontal transfer of the El Tor CTXφ between these two *Vibrio* species. Likewise, the similarity of the *orfU* sequences from the other three *V. mimicus* isolates we studied (PT48, 523-80, and 9583) to the classical *orfU* sequence suggests that these isolates were infected by a CTXφ closely related to classical CTXφ. Although the classical CTX prophage is thought to be defective in classical *V. cholerae* isolates, the similarity of *orfU* in *V. mimicus* isolates to the classical *orfU* gene suggests that classical CTXφ was not always defective and that at some time in the past, classical CTXφ infected *V. mimicus*. However, since the *zot* sequences from these three *V. mimicus* isolates do not cluster with the classical *zot* sequences, it is possible that a distinct CTXφ, perhaps derived from recombination, infected the *ctxAB* progenitors of these *V. mimicus* strains.

The *orfU* and *zot* sequences analyzed from the six non-O1 *V. cholerae* strains examined were diverse. In O139 strain SG20, these sequences were identical to the El Tor *zot* and *orfU* sequences. Since serogroup O139 *V. cholerae* is believed to be derived from El Tor *V. cholerae* via recombination of the locus encoding the serogroup antigen, this sequence identity likely represents vertical inheritance of this phage genome from the El Tor precursor of this newly emerged epidemic serogroup given the known sequence identity of El Tor and O139 *V. cholerae* at several loci (42). CTXφ sequence identity was also found between serogroup O37 strain CO130 and El Tor isolates. This CTXφ sequence identity probably reflects horizontal transfer of CTXφ between these isolates, given the differences in chromosomal background among these isolates. Since strain CO130 is an environmental rather than a clinical isolate, such horizontal transfer of CTXφ may have occurred outside of human hosts in the aquatic ecosystems that are the natural habitats of *V. cholerae*. Faruque et al. recently proposed that the natural habitats of *V. cholerae* may be an important site for the emergence of new toxigenic strains (17). Although the site where CTXφ-mediated transfer of *ctxAB* occurred is not known, the identity of CTXφ sequences from otherwise diverse O1 and non-O1 *V. cholerae* strains and *V. mimicus* isolates strongly suggests that horizontal transmission of CTXφ has occurred relatively recently and that such transmission is an ongoing process that contributes to the emergence of new toxigenic *Vibrio* species.

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