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Infectious CTXΦ and the Vibrio Pathogenicity Island Prophage in *Vibrio mimicus*: Evidence for Recent Horizontal Transfer between *V. mimicus* and *V. cholerae*

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Vibrio mimicus* differs from *Vibrio cholerae* in a number of genotypic and phenotypic traits but like *V. cholerae* can give rise to diarrheal disease. We examined clinical isolates of *V. mimicus* for the presence of CTXΦ, the lysogenic filamentous bacteriophage that carries the cholera toxin genes in epidemic *V. cholerae* strains. Four *V. mimicus* isolates were found to contain complete copies of CTXΦ. Southern blot analyses revealed that *V. mimicus* strain PT5 contains two CTX prophages integrated at different sites within the *V. mimicus* genome whereas *V. mimicus* strains PT48, 523-80, and 9583 each contain tandemly arranged copies of CTXΦ. We detected the replicative form of CTXΦ, pCTX, in all four of these *V. mimicus* isolates. The CTX prophage in strain PT5 was found to produce infectious CTXΦ particles. The nucleotide sequences of CTXΦ genes *orfU* and *zot* from *V. mimicus* strain PT5 and *V. cholerae* strain N16961 were identical, indicating contemporary horizontal transfer of CTXΦ between these two species. The receptor for CTXΦ, the toxin-coregulated pilus, which is encoded by another lysogenic filamentous bacteriophage, VPIΦ, was also present in the CTXΦ-positive *V. mimicus* isolates. The nucleotide sequences of VPIΦ genes *aldA* and *toxT* from *V. mimicus* strain PT5 and *V. cholerae* N16961 were identical, suggesting recent horizontal transfer of this phage between *V. mimicus* and *V. cholerae*. In *V. mimicus*, the vibrio pathogenicity island prophage was integrated in the same chromosomal attachment site as in *V. cholerae*. These results suggest that *V. mimicus* may be a significant reservoir for both CTXΦ and VPIΦ and may play an important role in the emergence of new toxigenic *V. cholerae* isolates.

Cholera toxin (CT) is encoded by the *ctxAB* operon, which resides in the genome of CTXΦ, a filamentous bacteriophage that specifically infects *Vibrio cholerae* (40). CTXΦ is found in all epidemic *V. cholerae* isolates but is rarely recovered from non-O1/non-O139 *V. cholerae* environmental isolates (12). The CTXΦ genome can integrate into the *V. cholerae* genome to form a stable prophage or it can replicate as a plasmid in isolates lacking an appropriate integration site. Of the nearly 200 recognized serogroups of *V. cholerae* only the O1 and O139 serogroups are associated with epidemics of cholera (2). In classical biotype strains of *V. cholerae* serogroup O1 there is a CTX prophage on each of the two *V. cholerae* chromosomes (24, 38), whereas in El Tor biotype strains of *V. cholerae* serogroup O1 the CTX prophages are tandemly arranged on the larger of the two chromosomes (26, 30). CTXΦ has a 6.9-kb genome made up of two functionally diverse regions: the core and RS2 regions. The core region encodes CT and contains the genes involved in phage morphogenesis, including genes that are thought to encode the major and minor phage coat proteins and a protein required for CTXΦ assembly (40). The RS2 region contains genes required for replication, integration, and regulation of CTXΦ (20, 43). In El Tor *V. cholerae* isolates, the CTX prophage genome is often flanked by an additional 2.7-kb region, designated RS1, that is similar to RS2 but that contains an additional gene (15, 26, 43).

Uptake of CTXΦ into *V. cholerae* is dependent upon the toxin-coregulated pilus (TCP), a bundle-forming pilus that is also an essential intestinal colonization factor (36). Initially, it was shown that the genes encoding TCP resided on a pathogenicity island known as the TCP island or vibrio pathogenicity island (VPI), which is integrated near the *ssrA* gene (18, 21). However, TCP has recently been proposed to be encoded on a novel filamentous phage named VPIΦ (19). Thus, the filamentous phage VPIΦ encodes TCP, an important virulence factor, and is the receptor for another temperate filamentous phage, CTXΦ, which itself encodes a virulence factor, CT.

The species *Vibrio mimicus* was first proposed to encompass biochemically atypical non-O1 *V. cholerae* isolates. *V. mimicus* is phenotypically and genotypically distinct from *V. cholerae* in several respects, and it can be readily differentiated from *V. cholerae* on the basis of a number of biochemical reactions (10). Thus, unlike *V. cholerae*, *V. mimicus* is negative in sucrose, Voges-Proskauer, corn oil, and Jordan tartrate reactions (10). Sequence analysis of the *mdh* (malate dehydrogenase) gene from a *V. mimicus* strain revealed that the average pairwise divergence between typical *V. cholerae* isolates and *V. mimicus* was 10.5% (6). The natural habitat of *V. mimicus*, like that of *V. cholerae*, is the aquatic ecosystem, where it has been found both as a free-living bacterium and in association with phytoplankton and crustaceans (1, 7). Consumption of *V. mimicus*-contaminated shellfish has been linked to the development of gastroenteritis (1). However, unlike *V. cholerae*, *V. mimicus* has not been associated with epidemics of diarrhea. The virulence determinants of *V. mimicus* have not been well characterized. The ability of *V. mimicus* strains to produce various toxins has been studied (7, 8, 31, 33–35). Several *V. mimicus* strains isolated from clinical and environmental sources were shown to produce multiple toxins, including a

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TABLE 1. Bacterial strains used in this study

Strain	Serogroup (biotype)	Isolation		Reference
		Country	Yr	
V. mimicus				
PT5	O115	Bangladesh	1985	33
PT48	O115	Bangladesh	1985	33
523-80	O115	United States	1980	33
9583	O115	United States	1980	33
9581	O41	India	1990	This study
9582	Rough	India	1990	This study
92-81	O8	United States	1980	This study
531-90	O41	Japan	1990	This study
546-80	Untypeable	United States	1980	This study
V. cholerae				
N16961	O1 (El Tor)	Bangladesh	1975	17
SM115	O1 (El Tor)	Bahrain	1978	15
O395	O1 (classical)	India	1964	26
LAC-1	O1 (classical)	India	1964	39

hemolysin, zonula occludens toxin, and a heat-stable enterotoxin, as well as a CT-like toxin (7, 8, 31, 33–35). To date, the presence of CTXΦ in *V. mimicus* has not been reported.

The distribution of CTXΦ and VPIΦ outside of *V. cholerae* is unknown, as is the extent to which *V. mimicus* and *V. cholerae* share pools of bacteriophages. We therefore examined clinical isolates of *V. mimicus* to determine if this species contains the genes composing CTXΦ and/or VPIΦ and consequently whether *V. mimicus* can serve as a reservoir for these important bacteriophages. We found *V. mimicus* isolates that contained both the CTX and VPI prophages. Nucleotide sequence identity of genes from both these prophages with the respective *V. cholerae* prophages suggests contemporary horizontal transfer of bacteriophages between these two *Vibrio* species.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are shown in Table 1. Bacterial strains were stored at −70°C in Luria-Bertani (LB) broth containing 20% glycerol. The antibiotic streptomycin was used at 200 μg/ml.

Southern hybridization analyses. *V. mimicus* DNA was extracted and purified using the G-nome DNA isolation kit from BIO101 (Vista, Calif.). DNA was digested with several restriction enzymes (New England Biolabs, Beverley, Mass.), and the fragments were separated by electrophoresis in 0.6% agarose. The fragments were then transferred to nylon membranes for hybridization. Three nonradioactive DNA probes to detect the presence of CTXΦ were produced by PCR amplification using *V. cholerae* strain SM115 (15) as a template (Table 2 and Fig. 1). DNA probes were labeled with fluorescein-conjugated nucleotides and, after hybridization, were detected by the ECL system according to the manufacturer's protocol (Amersham; Arlington Heights, Ill.). The rstA probe (5) assays for the presence of the RS2 region, whereas the core (5) and ctx probes assay for the presence of the core region of the CTXΦ genome. Southern blot analyses to detect the presence of DNA sequences that flank the CTX prophage in El Tor *V. cholerae*, the TLC element and an RTX cluster, in *V. mimicus* were performed using the DNA probes tlc (5) and rtx (5).

To determine the distribution of VPIΦ among *V. mimicus* isolates, four DNA probes which span the genome of VPIΦ, ald, tcpA, toxT, and acf (Fig. 1 and Table 2), were utilized. The ald probe includes the *aldA* gene found in the 5′ region of the VPI prophage. The tcpA probe encompasses the *tcpA* gene in the 5′ region of the *tcp* operon, whereas the toxT probe hybridizes to the 3′ region of this operon. The acf probe is limited to the *acfB* gene of the *acf* gene cluster in the 3′ region of the VPI prophage. All probes were made by PCR amplification from *V. cholerae* strain SM115 and purified using the Qiaquick PCR purification kit (Qiagen, Valencia, Calif.).

PCR analyses. To determine whether the chromosomal DNA sequences flanking the 5′ and 3′ ends of the CTX prophage in *V. mimicus* were identical to those of El Tor *V. cholerae*, we carried out a PCR assay with primer pairs rig1 and tlc3 and ctxB3 and rtxA2 (Table 2; Fig. 1A), which are located within the CTX prophage and the known flanking sequences of *V. cholerae* El Tor strain N16961.

PCR assays were also used to determine whether the chromosomal regions flanking the 5′ and 3′ ends of the VPI prophage in *V. mimicus* were identical to those in *V. cholerae*. Primer pairs VPI5 and VPI8 and VPI9 and VPI10, designed from the 5′ and 3′ junction sequences of the VPI prophage from *V. cholerae* strain N16961, were used for these assays. Primer VPI5 is derived from a sequence in the 5′ chromosomal flanking region of the VPI prophage integration site in *V. cholerae*, and primer VPI8 is located within the 5′ region of the VPI prophage genome. To amplify the VPI prophage 3′ chromosomal junction, primer VPI9, which lies within the putative integrase gene of VPIΦ, and primer VPI10, which is located within the 3′ flanking chromosomal DNA of *V. cholerae* strain N16961, were used (Table 2, Fig. 1B).

Detection of the replicative form of CTXΦ. To determine whether the *V. mimicus* strains that were found to contain the CTX prophage also harbored the replicative form of CTXΦ, Qiagen plasmid spin kits were used to isolate plasmid DNA from 5-ml overnight cultures. These plasmid DNA preparations were tested for the presence of the ~7-kb pCTX plasmid band by Southern blot analysis using the CTXΦ core region DNA probe (Table 2, Fig. 1A).

Recovery of infectious CTXΦ particles. We assayed filter-sterilized supernatants from each of the CTXΦ-positive *V. mimicus* strains for the presence of infectious CTXΦ particles as previously described (9, 22). Briefly, 1.5 ml of sterile supernatant from 2-ml overnight cultures was mixed with 1 μl of agglutinated classical strain O395 for 20 min. After addition of 1.5 ml of LB broth, the cultures were incubated overnight at 30°C. Qiagen plasmid spin kits were then used to isolate plasmid DNA from these overnight cultures. These plasmid DNA preparations were digested with *Sph*I, which linearizes the CTXΦ genome, separated on agarose gels, and assayed for the presence of pCTX DNA by Southern analysis.

Nucleotide sequencing and analysis. The *orfU* and *zot* genes from the CTXΦ in *V. mimicus* strain PT5 were sequenced directly from the products of PCR. PCR primers were designed from the published sequences of these genes in *V. cholerae* (14, 37) (Table 2). The *aldA* and *toxT* genes of VPIΦ from PT5 were also sequenced directly from PCR products with primers designed from the published *V. cholerae* sequences (18, 21) (Table 2 and Fig. 1). The *mdh* gene sequence was also determined for *V. mimicus* strain PT5 using primers derived from a *V. cholerae* El Tor strain N16961 sequence (http://www.tigr.org/tigr_home/tdb/mdb/mdb.html). Sequencing was performed with an Applied Biosystems model 373A automated DNA sequencing system at the Tufts University School of Medicine Core Facility using a DyeDeoxy terminator cycle sequencing kit. The sequences were determined in both orientations with additional internal primers, and the overlapping sequences were assembled and edited with the MacVector program. Comparisons of the *V. mimicus mdh*, *orfU*, *aldA*, and *toxT* gene sequences with the respective *V. cholerae* sequences were carried out using MacVector.

Mouse colonization assay. A competition assay between either O395 or a *lacZ* deletion mutant of O395, LAC-1 (42), and spontaneous Sm^r derivatives of three of the four VPIΦ-positive *V. mimicus* strains was done essentially as described previously (42). PT5 is *lacZ* positive, whereas 9583 and 523-80 are *lacZ* negative. The LAC-1 strain has been shown to colonize the suckling mouse intestine as well as O395 does. In this assay LAC-1 and O395 served as standards for comparative analysis of the intestinal colonization properties of the different test strains. Three- to five-day-old suckling mice (Charles River) were inoculated orally with a 1:1 mixture of *V. cholerae* O395 or LAC-1 and a *V. mimicus* test strain and then sacrificed 24 h later. Viable-cell counts were obtained by plating dilutions of the intestinal homogenates on LB agar containing streptomycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml). Three animals were used per group. The relative colonization efficiencies of the two

TABLE 2. Primers and probes used in this study

Phage and primer	Forward or reverse primer sequence (5′–3′)	PCR product size (bp)	Probe	Reference
CTXΦ				
rstA1	ACTCGATACAAACGCTTCTC	1,009	rstA	43
rstA2	AGAATCTGGAAGGTTGAGTG			
orfU1	CGTCACACCAGTTACTTTTCG	2,536	core	37
zot2	AACCCCGTTTCACTTCTAC			14
ctxA1	AGTCAGGTGGTCTTATGCC	1,037	ctx	WS[b]
ctxB2	TTGCCATACTAATTGCGG			
tlc3	GGGAATGTTGAGTTCTCAGTG	1,548	tlc	32
tlc4	GTTGCGAAGTGGATTTTGTG			
rtxA1	CACTCATTCCGATAACCAC	1,366	rtxA	23
rtxA2	GCGATTCTCAAAGAGATGC			
rig1	CACGCTACGTCGCTTATGT	NA[a]	NA	30
ctxB3	CCGCAATTAGTATGGCAA	NA	NA	WS
VPIΦ				
ald1	ATTCTTCTGAGGATTGCTGAT	884	ald	29
ald2	TTTTCTTGATTGTTAGGATGC			
tcpH1	AGCCGCCTAGATAGTCTGTG	1,200	tcpA	28
tcpA4	TCGCCTCCAATAATCCGAC			
toxT1	AGGAGATGGAAGTGGTGTG	1,055	toxT	16
toxT2	CTTGGTGCTACATTCATGG			
acfB1	GATGAAAGAACAGGAGAGA	1,180	acf	21
acfB2	CAGCAACCACAGCAAAACC			
VPI5	GTGAATCTTGATGAGACGC	529	VPIL	18
VPI8	GCCATTGGGTAAGTAGC			
VPI9	CCAATCCTTTGTGACGTTC	705	VPIR	18
VPI10	GGAAATCAGGAAGGTCAAAC			

[a] NA, not applicable.
[b] WS, http://www.tigr.org/tigr_home/tdb/mdb/mdb.html

strains were calculated by comparing the ratios of blue (*lacZ*$^+$) colonies to white (Δ*lacZ*) colonies in the intestinal homogenates and the inocula.

Nucleotide sequence accession numbers. The nucleotide sequences obtained during this study have been deposited in GenBank under accession no. AF207856 to AF207858.

RESULTS AND DISCUSSION

Presence of CTXΦ genes in *V. mimicus*. A recent report by Shi et al. (33) demonstrated the presence of *ctxA*-related sequences in four *V. mimicus* clinical isolates. Since we previously found that the *ctxAB* genes are part of the genome of CTXΦ, we investigated whether these four strains contained a CTX prophage(s). We also tested five additional *V. mimicus* clinical isolates that were previously found not to contain the *ctxA* gene for CTXΦ-related sequences. We found by PCR and DNA hybridization analyses that the four *V. mimicus* isolates previously reported to contain *ctxA*-related sequences, PT5, PT48, 523-80, and 9583, each contained a complete CTXΦ (Table 3). Remarkably, although these four *V. mimicus* strains all belonged to the same serogroup (O115), they were recovered at different times and from different continents (Table 1). The five additional *V. mimicus* isolates examined did not show homology to any of the CTXΦ probes, indicating the absence of CTXΦ in these isolates.

We further analyzed the CTXΦ genes in the four strains containing *ctxAB* by Southern blotting, to determine if the CTXΦ genome was integrated into the *V. mimicus* chromosome (as is observed in *V. cholerae*) and to assess the copy number and arrangement of the CTX prophages. Southern hybridization analyses were carried out on chromosomal DNA prepared from each *V. mimicus* strain digested with restriction enzymes that cut either at no sites (*Eco*RI) or at one site (*Sph*I, *Bgl*II) in the CTX prophage genomes in classical and El Tor *V. cholerae* strains (30). Similar to analyses of classical strains of *V. cholerae*, these analyses of strain PT5 indicate the presence of two CTX prophages at different locations in the genome (Fig. 2). Thus, when strain PT5 chromosomal DNA was digested with *Eco*RI and hybridized with either the core region or rstA probes, two bands were observed (Fig. 3A and B). The presence of two unlinked CTX prophages in PT5 was confirmed in blots of *Sph*I- and *Bgl*II-digested DNA. In these digests, with either the core or rstA probes, two bands were also observed (Fig. 3).

In *V. mimicus* strain PT48, a clinical isolate from Bang-

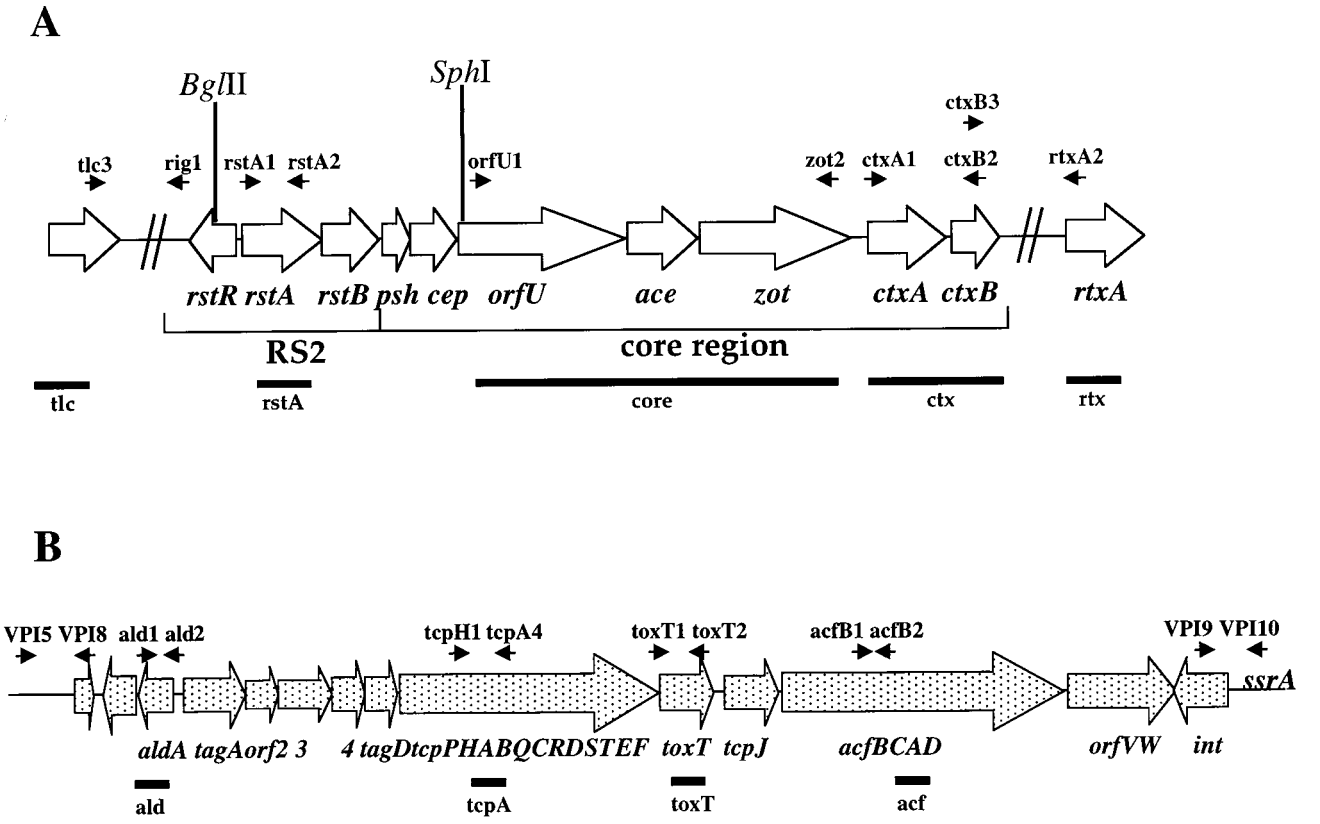

FIG. 1. (A) Schematic representation of the CTX prophage (~7 kb) and surrounding sequences in *V. cholerae*. The open reading frames (ORFs) are shown as arrows. Black arrowheads, locations of the PCR primers listed in Table 2; vertical lines, restriction enzyme sites; horizontal bars, positions of the three DNA probes used for hybridization. (B) Schematic representation of the VPI prophage (~40 kb) in *V. cholerae*. The ORFs and gene clusters are shown as dotted arrows. Black arrowheads, locations of the PCR primers listed in Table 2; horizontal bars, locations of the four DNA probes used for hybridization.

ladesh, Southern blot analyses demonstrated the presence of tandemly arranged CTX prophages, similar to the arrangement present in El Tor *V. cholerae* strains (Fig. 2). Southern hybridization of *Eco*RI-digested PT48 DNA probed with the core or rstA probes revealed only a single hybridizing band >12 kb (Fig. 3A and B). However, PT48 DNA digested with *Sph*I and hybridized with the core probe revealed two hybridization species: a 5-kb band and a 7-kb band (Fig. 3A). The 5-kb band represents the right junction fragment of CTXΦ in the chromosome, and the 7-kb band corresponds to the tandemly integrated copies of CTXΦ. Hybridization with the rstA probe also identified two bands, a 7-kb tandem band and an 8-kb left junction fragment (Fig. 3B). The fact that the size of the tandem band in the *Sph*I digests of PT48 probed with the core and rstA probes is 7 kb and not ~10 kb, as seen with the El Tor strain SM115, suggests that there is no intervening RS1 sequence between the CTX prophages in PT48. *Bgl*II digests of PT48 DNA hybridized with the core and rstA probes revealed two bands, confirming the tandem arrangement of CTX prophages in PT48.

V. mimicus clinical isolates 523-80 and 9583, which were recovered from patients in the United States, also contained tandemly arranged CTX prophages (Fig. 2). The strains gave identical banding patterns when digested with *Eco*RI, *Sph*I, or

TABLE 3. Distribution of CTXΦ and VPIΦ genes in *V. mimicus* strains

Strain	Presence of indicated gene or DNA segment of:										
	CTXΦ					VPIΦ					
	TLC	*rstA*	*orfU-zot*	*ctxAB*	RTX	5′ junction	*ald*	*tcpA*	*toxT*	*acfB*	3′ junction
PT5	−	+	+	+	−	+	+	+	+	+	+
PT48	−	+	+	+	−	+	+	+	+	+	+
523-80	−	+	+	+	−	+	+	+	+	+	+
9583	−	+	+	+	−	+	+	+	+	+	+
9581	−	−	−	−	−	−	−	−	−	−	−
9582	−	−	−	−	−	−	−	−	−	−	−
92-81	−	−	−	−	−	−	−	−	−	−	−
531-90	−	−	−	−	−	−	−	−	−	−	−
546-80	−	−	−	−	−	−	−	−	−	−	−

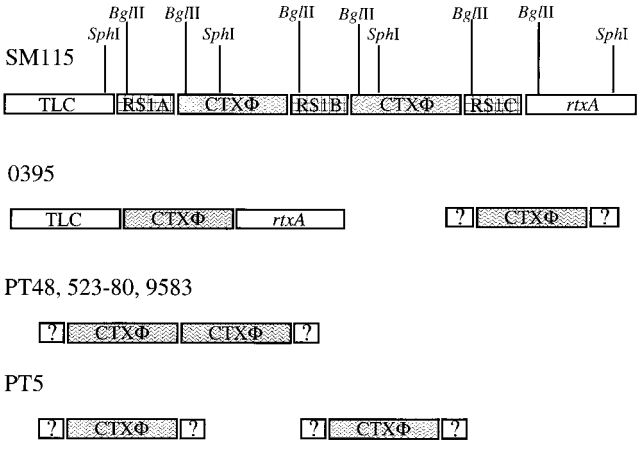

FIG. 2. Chromosomal arrangement of CTX prophages in *V. cholerae* strains SM115 and O395 and in *V. mimicus* strains PT5, PT48, 523-80, and 9583 as determined by Southern blot and PCR analyses. Vertical lines indicate restriction enzyme sites. Boxes with question marks adjacent to the CTX prophages represent those cases where the phage-chromosome junction sequences are unknown.

*Bgl*II, and probed with the core region or the rstA probe. Thus, two fragments of *Eco*RI-digested 523-80 DNA hybridized with the core probe: a 7-kb band and an 8-kb band (Fig. 3A). An identical pattern was found with the rstA probe, suggesting the presence of two CTX prophages at different map positions. However, Southern blots of *Sph*I-digested DNA probed with the core probe gave two bands: a 7-kb band and a 5-kb band; similarly, hybridization with the rstA probe gave a 7-kb band and an 8-kb band, suggesting a tandem arrangement of CTX prophages similar to the pattern seen with strain PT48. Taken together, these results suggest that the CTX prophages in 523-80 and 9583 contain an *Eco*RI site not present in the PT5, PT48, or *V. cholerae* serogroup O1 CTX prophages, explaining the two bands seen with this enzyme (Fig. 3A and B). Digestion

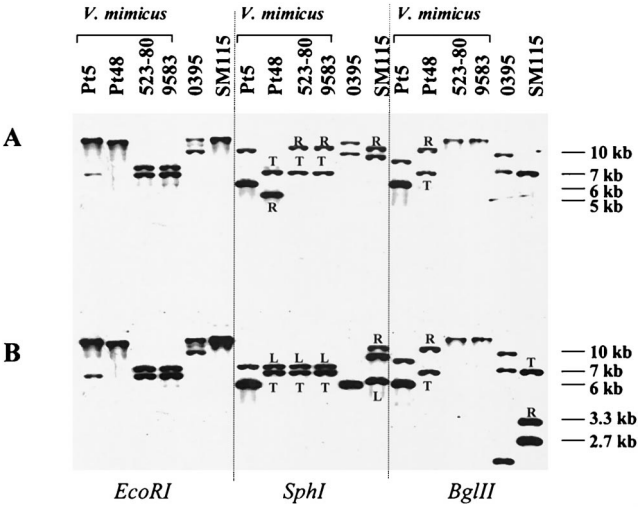

FIG. 3. Southern blot analyses of the integrated copies of CTXΦ in *V. mimicus* strains PT5, PT48, 583-80, and 9583 and *V. cholerae* classical strain O395 and El Tor strain SM115. Equal amounts of chromosomal DNAs from the indicated strains were digested with *Eco*RI, *Sph*I, and *Bgl*II, electrophoresed, transferred to nitrocellulose, and probed with CTXΦ core (A) and rstA (B) probes. R and L, right and left junction fragments, respectively; T, tandem arrangements of integrated CTXΦ.

of 523-80 and 9583 DNA with *Bgl*II and hybridization with core and rstA probes also suggested DNA sequence polymorphisms in the CTX prophages in these strains, as both probes identified a single >12-kb band, indicating the absence of a *Bgl*II site in *rstR*. These analyses of the arrangements of the CTX prophages in these four *V. mimicus* isolates demonstrate that, as for *V. cholerae*, the CTX prophages in *V. mimicus* occur in multiple copies and have alternative arrangements in the genome.

Analysis of CTX prophage flanking DNA. *V. cholerae* serogroup O1 strains contain tandemly arranged copies of an integrated toxin-linked cryptic plasmid, pTLC, adjacent to the 5′ end of the CTX prophages (32). Adjacent to the region 3′ of the CTX prophages there is a recently described RTX toxin gene cluster (23). We used PCR analyses to investigate whether the TLC element and the RTX cluster are present in the CTX prophage-positive and -negative *V. mimicus* strains. The primer pairs for these PCRs, rig1 and tlc3 and ctxB3 and rtxA2 (Fig. 1), were designed to amplify TLC sequences 5′ of the CTX prophage and RTX sequences 3′ of the CTX prophage. No PCR products were detected in the nine strains tested using these primer pairs (Table 3). To further investigate whether there are sequences homologous to TLC and RTX in these *V. mimicus* strains, we performed Southern blot analyses with a 1.5-kb tlc probe and a 1.3-kb rtxA probe. No hybridization bands were obtained. Therefore, *V. mimicus*, unlike *V. cholerae*, for which previous studies have demonstrated the co-occurrence of the CTX prophage and the TLC element, has no TLC element.

Detection of CTXΦ RF and infectious virions. To determine whether the four *V. mimicus* strains that harbor the CTX prophage also contained the ~7-kb plasmid replicative form (RF) of CTXΦ, pCTX, plasmid DNA preparations from these strains were probed with a CTXΦ core region probe in a Southern blot. The plasmid preparations from all four strains yielded hybridizing DNA of the appropriate size (data not shown), indicating that these strains harbored the CTXΦ RF in addition to the CTX prophage.

We then tested whether these strains produced infectious CTXΦ particles. To accomplish this, filtered sterile supernatants from overnight cultures of *V. mimicus* strains were used to infect agglutinated (TCP^+) classical strain O395 with CTXΦ (27), according to an established protocol (9, 22, 40). Cell-free supernatant from one strain, PT5, was found to transfer CTXΦ DNA to O395, thus demonstrating the ability of this strain to produce infectious CTX virions (data not shown). This is the first report of the isolation of CTXΦ outside of *V. cholerae*. This finding indicates that CTXΦ has more than one host species and hence that *V. mimicus* may represent an important reservoir of CTXΦ in the natural environment. For reasons which are not yet known, we were unable to detect transfer of CTXΦ DNA from supernatants derived from PT48, 523-80, and 9583. It is possible that our inability to detect transfer of CTXΦ from these three *V. mimicus* strains into O395 is a reflection of phage immunity. We have found that the CTXΦ repressor, RstR, functions as an effector of phage immunity by repressing transcription of *rstA*, a gene required for CTXΦ replication (9, 20). If *rstA* in the CTXΦ derived from *V. mimicus* strains PT48, 523-80, and 9583 is repressed by the $RstR^{classical}$ repressor in O395, then these three *V. mimicus*-derived CTXΦs would not replicate in this strain.

Comparison of *V. mimicus* and *V. cholerae* CTXΦ nucleotide sequences. There is a sporadic distribution of CTXΦ in *V. cholerae* and *V. mimicus* strains. Among *V. cholerae* isolates, CTXΦ is confined to epidemic strains and is rarely recovered from non-O1/non-O139 isolates. This sporadic distribution of CTXΦ in *V. cholerae* and *V. mimicus* populations is most likely

the result of horizontal transfer of CTXΦ between *Vibrio* species. However, it is also possible that the CTX prophage was present in the most recent common ancestor of *V. cholerae* and *V. mimicus*, with subsequent loss of this phage from most isolates of these two species. To distinguish between these two possibilities and to begin to elucidate the evolutionary history of CTXΦ, we sequenced two genes from the core region of the CTXΦ derived from *V. mimicus* strain PT5 and compared these sequences with sequences of the same region from El Tor *V. cholerae* strain N16961 from the TIGR database (http://www.tigr.org/tigr_home/tdb/mdb/mdb.html). We also sequenced the chromosomally encoded malate dehydrogenase gene (*mdh*), which is a basic metabolic housekeeping gene found in all *Vibrio* species and therefore is ancestral to the species. Comparison of the *mdh* sequences from *V. mimicus* and *V. cholerae* provides a measure of the divergence of the two species. Of the 640 bp of *mdh* sequenced from *V. mimicus* strain PT5, there were 64 polymorphic sites, compared with the *V. cholerae mdh* from strain N16961. This indicates that the level of nucleotide sequence divergence between the two species is approximately 10%, similar to values from previous reports (6). This level of nucleotide sequence divergence is similar to the level of divergence between *Escherichia coli* and *Salmonella enterica* (3, 4). In striking contrast, the 992 bp of the *orfU* gene and the 1,036 bp of the *zot* gene derived from *V. mimicus* strain PT5 and *V. cholerae* strain N16961 contained no polymorphic nucleotide sites. This nucleotide sequence identity of the CTXΦ genes within otherwise divergent chromosomal backgrounds strongly argues against the possibility that CTXΦ was inherited from a common ancestor of *V. cholerae* and *V. mimicus*. Rather, the CTXΦ sequence identity between these two species suggests that there was relatively recent horizontal transfer of this phage between these two species. The site of this phage transfer could be their shared ecological niche: the estuarine environment. However, since the *V. mimicus* isolates examined are clinical isolates, we cannot formally rule out the possibility that CTXΦ transfer between these two species may have occurred in the human host.

Presence of VPIΦ genes in *V. mimicus*. The receptor for CTXΦ in *V. cholerae* is TCP. The genes encoding TCP have recently been proposed to be an integral part of the genome of the newly described novel filamentous phage VPIΦ, found in pathogenic isolates of *V. cholerae* (19). Since TCP plays a critical role in the uptake of CTXΦ into *V. cholerae*, we tested whether VPI prophage sequences were present in the four toxigenic and five nontoxigenic clinical isolates of *V. mimicus*. All four CTXΦ$^+$ *V. mimicus* isolates yielded PCR products with four sets of primer pairs that span the VPIΦ genome (Table 3; Fig. 1). In contrast, PCR products were not amplified from the five *V. mimicus* strains that did not harbor a CTX prophage (Table 3). Southern analyses confirmed that these five strains lacked sequence homology to VPIΦ. In *V. cholerae* the VPI prophage is found adjacent to the *ssrA* locus (19). Interestingly, by PCR analyses we found that the VPI prophage was integrated at the identical chromosomal sites in the four VPI prophage-positive *V. mimicus* isolates (Table 3), and we were able to amplify by PCR the chromosomal junction fragments of the VPI prophage integration site of *V. cholerae* from all *V. mimicus* toxigenic isolates.

Since TCP is required for CTXΦ infection of *V. cholerae*, it was suggested that there were two critical sequential steps in the evolution of pathogenic *V. cholerae* (5, 12, 13, 25, 40, 41). First, strains had to acquire TCP (presumably via infection with VPIΦ), and second, having acquired the CTXΦ receptor, these TCP$^+$ strains were then infected with and lysogenized by CTXΦ. Our finding that all four *V. mimicus* isolates that contained CTX prophages also contained VPI prophages supports the notion that TCP is required for acquisition of CTXΦ. The co-occurrence of these two bacteriophages in two different species is striking from an evolutionary perspective, as it would appear that these two unrelated bacteriophages have similar host ranges and that their acquisition and potentially their function are intimately interconnected.

Comparison of *V. mimicus* and *V. cholerae* VPI prophage nucleotide sequences. To begin to address the evolutionary history of VPIΦ in *V. cholerae* and *V. mimicus*, we sequenced two genes carried by VPIΦ, *aldA* and *toxT* from *V. mimicus* strain PT5, and compared these sequences with the *aldA* and *toxT* sequences from *V. cholerae* strain N16961. One of the sequenced genes, *aldA*, codes for an aldehyde dehydrogenase (29), and the other, *toxT*, codes for a transcriptional activator for virulence gene expression (11). The *aldA* and *toxT* sequences from PT5 and N16961 were identical. The identity of these VPI prophage sequences from *V. mimicus* and *V. cholerae* suggests recent horizontal transfer of VPIΦ between *V. mimicus* and *V. cholerae* and lends additional support to the idea that the VPI is a mobile genetic element.

Mouse colonization assay. The identification of *V. mimicus* strains containing the CTX and VPI prophages, whose genomes encode many of the known virulence factors of *V. cholerae*, raised the question of whether these *V. mimicus* isolates are as pathogenic as *V. cholerae* isolates that contain these prophages. To begin to experimentally assess the virulence properties of three of the *V. mimicus* strains containing both prophages (PT5, 523-80, and 9583), we performed mouse colonization assays. Twenty four hours after 1:1 mixtures of O395 with each of these three strains were intragastrically inoculated into suckling mice, the ratio of *V. mimicus* cells to O395 cells in intestinal homogenates was found to be less than 1:1,000 for all three *V. mimicus* strains. Thus, compared to *V. cholerae*, these *V. mimicus* isolates appear to be extremely attenuated for intestinal colonization. The molecular basis for this attenuation is not known. If TCP is properly expressed in vivo by these *V. mimicus* strains containing the VPI prophage, these data indicate that other colonization factors, such as the serogroup antigen, are critical for intestinal colonization.

Conclusions. The extent to which *V. cholerae* and *V. mimicus* share gene pools is unknown, as is the ancestry of bacteriophages in these species. The data presented in this report indicate that *V. cholerae* is not the only host of both CTXΦ and VPIΦ. The finding of nucleotide sequence identity of genes from both CTXΦ and VPIΦ derived from *V. mimicus* and *V. cholerae* strongly suggests that contemporary horizontal transfer of bacteriophages between these species has occurred. However, we cannot conclude from this sequence identity whether these phage genomes were transferred from *V. cholerae* to *V. mimicus* or vice versa. Also, although it seems likely that these two phage genomes were transferred between these *Vibrio* species via transduction, we cannot strictly come to this conclusion from our data. Regardless of this limitation, *V. mimicus* may represent an important environmental reservoir for both bacteriophages and, therefore, may play a significant role in the emergence of new toxigenic *V. cholerae* isolates.

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