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Pathogenic Potential of Environmental *Vibrio cholerae* Strains Carrying Genetic Variants of the Toxin-Coregulated Pilus Pathogenicity Island

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The major virulence factors of toxigenic *Vibrio cholerae* are cholera toxin (CT), which is encoded by a lysogenic bacteriophage (CTXΦ), and toxin-coregulated pilus (TCP), an essential colonization factor which is also the receptor for CTXΦ. The genes for the biosynthesis of TCP are part of a larger genetic element known as the TCP pathogenicity island. To assess their pathogenic potential, we analyzed environmental strains of *V. cholerae* carrying genetic variants of the TCP pathogenicity island for colonization of infant mice, susceptibility to CTXΦ, and diarrheagenicity in adult rabbits. Analysis of 14 environmental strains, including 3 strains carrying a new allele of the *tcpA* gene, 9 strains carrying a new allele of the *toxT* gene, and 2 strains carrying conventional *tcpA* and *toxT* genes, showed that all strains colonized infant mice with various efficiencies in competition with a control El Tor biotype strain of *V. cholerae* O1. Five of the 14 strains were susceptible to CTXΦ, and these transductants produced CT and caused diarrhea in adult rabbits. These results suggested that the new alleles of the *tcpA* and *toxT* genes found in environmental strains of *V. cholerae* encode biologically active gene products. Detection of functional homologs of the TCP island genes in environmental strains may have implications for understanding the origin and evolution of virulence genes of *V. cholerae*.

Cholera caused by toxigenic *Vibrio cholerae* is an acute watery diarrhea which can occur as spreading epidemics (15). The pathogenesis of cholera depends on the synergistic effect of a number of factors produced by toxigenic *V. cholerae*. Profuse watery diarrhea is caused by an enterotoxin, cholera toxin (CT), produced by *V. cholerae* when it colonizes the small intestine (15, 31). The *ctxAB* operon, which encodes the A and B subunits of CT, resides in the genome of CTXΦ, a lysogenic filamentous bacteriophage (37). In addition to genes encoding CT, all strains capable of causing cholera carry genes for a colonization factor known as toxin-coregulated pilus (TCP), the expression of which is coordinately regulated with CT (21, 36). Although the major structural subunit of TCP is encoded by the *tcpA* gene, the formation and function of the pilus assembly require the products of a number of other genes located on a larger genetic region referred to as the TCP pathogenicity island (10, 15, 23, 27). Expression of CT and TCP are coregulated by the ToxR regulatory system, which includes the ToxT protein (9). The TCP gene cluster comprises at least 15 open reading frames, including the *tcpA* and *toxT* genes as well as a number of other regulatory genes. It has been suggested that regulators such as TcpI act downstream of the *toxR* and *toxT* genes to fine-tune the expression of TCP throughout the pathogenic cycle of *V. cholerae* (19). Other genes of the TCP island, including *tcpP* and *tcpH*, have also been suggested to have a role in the transcriptional activation of the *toxT* promoter (20). A notable example of evolutionary coadaptation is that the CTXΦ virion uses TCP as its receptor for infecting *V. cholerae* cells (37), whereas the *toxT* gene, which is located in the TCP island, encodes a transcriptional regulator which controls the expression of both TCP and CT genes in response to particular host or environmental conditions (9, 19, 20, 23).

Recently, new variants of the TcpA protein have been found in several *V. cholerae* non-O1, non-O139 strains (3). Although clinical isolates of *V. cholerae* are normally expected to carry virulence-associated genes, recent studies have also identified environmental *V. cholerae* strains which possess virulence genes or their homologs, including genetic variants of the TCP pathogenicity island (4, 30). These environmental strains were found to carry new alleles of the *tcpA*, *toxT*, and *tcpF* genes or variant forms of a regulatory sequence upstream of *toxT* (30). The present study was undertaken to further analyze these *V. cholerae* strains for pathogenic potential by using animal models and for toxigenic conversion by CTXΦ. This has implications for understanding the emergence and evolution of new pathogenic strains of *V. cholerae*.

A total of 14 *V. cholerae* strains initially cultured from three different freshwater lakes and ponds in the eastern part of Calcutta, India (4), were included in the study. These strains were previously shown to carry genetic variants of the TCP pathogenicity island with new alleles of several TCP island genes (30). Details of the strains analyzed in this study are listed in Table 1. Relevant characteristics of reference bacterial strains and properties of phages and plasmids used in this study are presented in Table 2.

The genetically marked phage MSF8.2Φ used in this study was a derivative of an El Tor type CTXΦ which carried a functional *ctxAB* operon as well as a kanamycin resistance (Km^r) determinant (16). MSF8.2Φ was prepared for the

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TABLE 1. Presence of major virulence-associated genes among *V. cholerae* non-O1, non-O139 strains isolated from environmental surface water, and susceptibility of the strains to a genetically marked derivative of CTXΦ

Strain	Serogroup	Presence of[a]:							Susceptibility to CTXΦ (frequency)	Competitive index of colonization
		tcpA[b]	*toxT*[b]	*toxR*	*lg1*	*rstR*	*RstC*	*ctxA*		
SCE4	O8	C	+	+	−	−	−	−	6.0×10^{-5}	0.35
SCE188	O44	Env	+	+	+	+	+	+	6.3×10^{-4}	0.80
SCE223	O27	Env	+	+	+	+	+	+	0	0.86
SCE225	O35	C	Env	+	−	+	+	−	0	0.31
SCE226	O35	C	Env	+	−	+	+	−	0	0.53
SCE227	O35	C	Env	+	−	+	+	−	0	0.20
SCE256	O42	C	Env	+	−	+	+	−	0	0.28
SCE258	O42	C	Env	+	−	−	−	−	0	0.28
SCE263	O10	C	Env	+	+	+	+	−	0	0.33
SCE264	O42	C	Env	+	+	+	+	−	0	0.42
SCE340	O69	E	Env	+	−	−	−	−	3.9×10^{-4}	1.26
SCE341	O69	E	Env	+	−	−	−	−	4.5×10^{-4}	0.29
SCE354	O27	Env	+	+	+	+	+	+	0	2.78
SCE359	O8	C	+	+	−	−	−	−	5.6×10^{-4}	0.82

[a] Presence of genes was detected by using DNA probes and PCR assays.
[b] Env refers to a new allele of the *tcpA* or *toxT* genes, different from those normally found in epidemic strains (30). C and E, classical and El Tor type *tcpA* genes, respectively.

present study from a culture of *V. cholerae* O395 carrying RF of the phage pMSF8.2. Aliquots of the culture supernatants were sterilized by filtration through 0.22-μm-pore-size filters (Millipore Corporation, Bedford, Mass.). The filtrate was titrated for infectious phage particles by incubating aliquots of the supernatants for 30 min at 30°C with the classical biotype strain RV508, which constitutively expresses TCP, and then selecting for colonies resistant to kanamycin.

The presence of virulence-associated genes was determined by using specific DNA probes or PCR assays. The gene probes used in this study included a 0.5-kb *Eco*RI fragment of pCVD27 (22) containing part of the *ctxA* gene and a 2.1-kb *Sph*I-*Xba*I fragment of pCTX-Km (37) containing the entire *zot* and *ace* genes and part of *orfU*. The *toxR* gene probe was a 2.4-kb *Bam*HI fragment of pVM7 (29). The $rstR^{ET}$ probe was a *Sac*I-*Xba*I fragment of pHK1 (26). Presence of the *rstC* gene was determined by a PCR assay with the two primers 5′ATG AGTTTGAAACCATACACTTT and 5′TTACAGTGATGG ATCAGTCAAT, as described previously (13). Presence of the *tcpA* and *acfB* genes were also tested by PCR assays described previously (14, 24). Colony blots or Southern blots were prepared by using nylon filters (Hybond; Amersham Biosciences, Uppsala, Sweden) and were processed by standard methods (28, 33). The probes were labeled by random priming (17) by using a random-primers DNA labeling kit (Invitrogen Corporation, Carlsbad, Calif.) and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). Southern blots and colony blots were hybridized with the labeled probes, and autoradiographs were developed as described previously (14).

The susceptibility of *V. cholerae* strains to the genetically marked derivative of CTXΦ was assayed under laboratory conditions and inside the intestines of infant mice by previously described methods (11, 12). Representative infected colonies were grown overnight in Luria-Bertani (LB) broth containing kanamycin (50 μg/ml), and cells were precipitated by centrifugation. The supernatant fluids of the cultures were titrated for the presence of MSF8.2Φ particles by using strain RV508 as the recipient. Total DNA or plasmids were extracted from bacterial pellets by standard methods (28) and purified by using microcentrifuge filter units (Ultrafree-Probind; Sigma).

TABLE 2. Characteristics of *V. cholerae* reference strains, plasmids, and phages used in the study

Strain	Relevant characteristic(s)	Reference
MSF8.2Φ	Derivative of CTX-KmΦ in which the *ctxAB* operon was reinstated. This construct carries both a functional *ctxAB* operon and a kanamycin resistance cassette	16
O395	Classical Ogawa streptomycin-resistant strain	Laboratory collection
O395 (pMSF8.2)	Strain O395 carrying the RF of MSF8.2Φ	16
RV508	Derivative of classical biotype strain 569B that constitutively expresses CT, TCP pili, and other *toxR*-regulated gene products	36
Bah-2	Derivative of El Tor strain E7946 in which the entire CTX element as well as the attachment sequence *att*RS was deleted	25
TCP-2	Derivative of strain O395 which carries deletions in the *tcpA* and *ctxA* genes	35
SA-317	TCP-positive nontoxigenic *V. cholerae* O1 strain	12
P-27459	Toxigenic clinical *V. cholerae* O1 El Tor strain	Laboratory collection
MO10	Toxigenic *V. cholerae* O139 strain	Laboratory collection

The presence of the phage genome was verified by comparative Southern blot analysis of total DNA and plasmid preparations from the phage-infected strains and the corresponding native strains.

Colonization of infant mice by the environmental strains in competition with a reference strain was assayed as described previously (1, 18). Briefly, each *V. cholerae* test strain and the reference strain were grown to stationary phase at 30°C in LB broth. The test strain and the reference strain were mixed at a 1:1 ratio and diluted 1:1,000 in LB broth. Approximately 10^5 CFU of the bacterial mix in a 50-μl suspension was used to intragastrically inoculate groups of 5-day-old Swiss Albino mice. Each strain was inoculated in at least six mice, and the infections were allowed to proceed for 20 h. The mice were then sacrificed, and bacteria were recovered from the small intestines by homogenization in phosphate-buffered saline (pH 7.4). Serial dilutions of the homogenates were plated on appropriate antibiotic plates and on plates devoid of the antibiotic to determine the ratio of the number of organisms of the test strain to that of the reference strains. Competitive indices were calculated by dividing the output ratios by the inoculum input ratio of the test and reference strains. An in vitro analysis of the inoculum was also done to determine the precise ratio of test strain to reference strain and to determine the competitive indices in vitro. This was determined for each inoculum by plating serial dilutions, as described above, before and after 20 h of growth under in vitro laboratory conditions without antibiotic.

Production of CT by *V. cholerae* strains was determined by the G_{M1} ganglioside-dependent enzyme-linked immunosorbent assay (G_{M1}-ELISA) and the rabbit ileal loop assay, as described previously (8, 32). A toxigenic strain, P27459, and a nontoxigenic strain, SA-317, were included as positive and negative control strains in each round of assay.

Diarrheal response to the *V. cholerae* strains was assayed in adult rabbits by using the removable intestinal tie-adult rabbit diarrhea (RITARD) model (34) with New Zealand White rabbits, as described previously (16). Each strain was inoculated in at least five different rabbits. Rabbits were observed for overt diarrhea and for death, and stools or rectal swabs were cultured on gelatin agar plates and a duplicate plate containing kanamycin (50 μg/ml) when appropriate to monitor shedding of the challenge organisms. Observations were made at 6-h intervals during the 7 days following inoculation; the number of rabbits developing moderate to severe diarrhea arbitrarily was scored, and the number of deaths was recorded.

Of 14 environmental strains included in the present study, 12 strains carried one or more new alleles of the TCP island genes (30). This included the *tcpA* gene, which encodes the major structural subunit of TCP, and the major virulence regulatory gene *toxT*, which controls the expression of both CT and TCP. Other genetic variation was found in the *tcpF* genes or in a regulatory sequence upstream of the *toxT* gene (30). Since TCP is the major colonization factor of *V. cholerae* and a crucial factor for successful infection, we tested the ability of the strains to infect infant mice in competition with a known TCP-positive, CT-negative strain, Bah-2 (25). The reason for choosing the CT-negative strain was that most of the environmental strains tested were also negative for CT. It may be mentioned that previous studies have indicated that CT-positive strains are more efficient colonizers than the corresponding CT-negative mutants (2, 15). The reference strain Bah-2 was resistant to streptomycin, and this allowed us conveniently to differentiate the reference strain from the test strains. The proportions of test strain and reference strain recovered from the mouse intestine 20 h after intragastric inoculation of groups of infant mice with a mixture of the two strains are shown in Fig. 1. While the inoculum contained the two strains at an approximately 1:1 ratio, the ratio in the recovered samples varied between 0.33 and 3.7. In the in vitro assay, no major change was observed in the ratio of the two strains. The competitive index of colonization varied from 0.28 to 2.78 (Table 1). The TCP-negative control strain TCP-2 included in the study, however, was completely outcompeted by the reference TCP-positive strain (competition index, 0.007). This suggested that the test strains competed with the reference strain with various efficiencies for infecting infant mice. All strains included in the study carried the *tcpA*, *toxT*, and *acfB* genes or their homologs (Table 1) and, presumably, the entire TCP pathogenicity island. Hence, the observed moderate to high colonization efficiency compared to that of the reference strain may be attributed to the production of TCP, unless these strains produce any previously undiscovered colonization factors. It may be mentioned that at least one of these strains has previously been found to produce a pilus (4), and most of the strains demonstrated autoagglutination, a property attributed to the production of TCP by *V. cholerae*. The results of the present study indicate that the TCP produced by these environmental *V. cholerae* strains is a biologically active pilus and can contribute to colonization of the mouse intestine.

TCP is also used by CTXΦ as its receptor for invading *V. cholerae* cells. We therefore tested the susceptibility of the environmental *V. cholerae* strains to CTXΦ. This was done by using a genetically marked phage which carried a Km^r determinant as well as a functional *ctxAB* operon (16). The infected strains were initially selected by their resistance to kanamycin and were later tested for the presence of CTXΦ-specific genes. The results showed that 5 of 14 environmental strains tested were infected by the phage. These five strains included two strains carrying classical type *tcpA* gene, two strains with El Tor type *tcpA* gene, and one strain with a new variant (Env type) *tcpA* gene (Table 1). We tested the susceptibilities of the strains both under in vitro laboratory conditions and inside the intestines of infant mice. Previous studies with CTXΦ showed that the efficiency of transduction was considerably higher in vivo, and this was attributed to more adequate expression of the phage receptor TCP in vivo than under laboratory conditions (11, 12). In the present study, we did not detect any Km^r transductants of the environmental strains in the in vitro assay, and infection with MSF8.2Φ was detectable only in the infant mouse assay. This suggested that infection of these *V. cholerae* strains by CTXΦ was possibly TCP dependent. Susceptibility of the environmental strains was low (the mean frequency of infection was between 5.5×10^{-5} and 6.5×10^{-4}) compared to that of the control CTXΦ-negative *V. cholerae* O1 strain SA-317 that was included in the study. The susceptibility of this O1 strain varied between 2.1×10^{-2} and 6.3×10^{-2} in vitro and between 8.6×10^{-2} and 19.2×10^{-2} in the in vivo assays. Nevertheless, this is the first demonstration of environmental

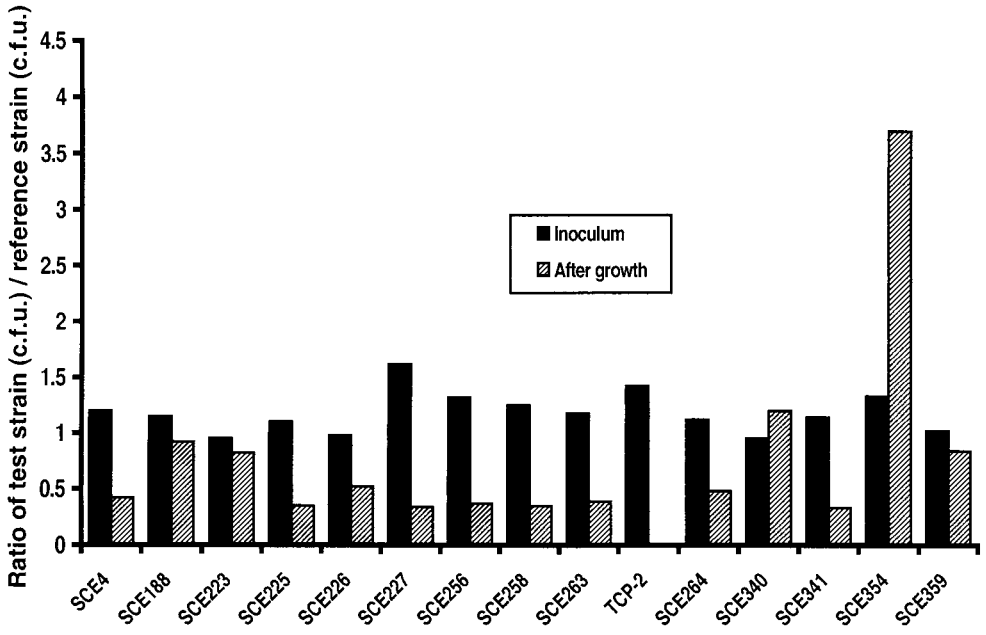

FIG. 1. Colonization of infant mice by environmental *V. cholerae* strains in competition with a reference TCP-positive, nontoxigenic *V. cholerae* strain, Bah-2. The TCP-negative strain TCP-2 was used as a negative control.

V. cholerae strains carrying new alleles of the TCP island genes being infected by CTXΦ.

Previous studies have described heteroimmunity among CTX phages mediated by widely diverse CTXΦ repressors encoded by different *rstR* genes. (7, 26). The existence of at least three different *rstR* genes carried by different CTX phages, namely, phages $CTX\Phi^{ET}$, $CTX\Phi^{class}$, and $CTX\Phi^{Calc}$, has been recognized (7). More recently, some of the environmental strains included in the present study have been reported to carry novel *rstR* homologs (30). The genetically marked phage MSF8.2Φ used in our study carried an El Tor type *rstR* gene ($rstR^{ET}$). To investigate the reasons for the resistance of nine environmental strains to the phage, we analyzed the strains for the possible presence of an $rstR^{ET}$ gene. This showed that all strains which were resistant to CTXΦ infection in the present study carried one or more copies of the $rstR^{ET}$ gene, although all except one strain (SCE188) were nontoxigenic. It is interesting that nine strains carried the *rstR* gene independently of the other CTXΦ genes (core genes). We have previously demonstrated the horizontal transfer of the RS1 element of *V. cholerae* as a filamentous phage which also carries the *rstR* gene (13). To examine whether these environmental strains carried the RS1 element, we analyzed these strains for the RS1-specific gene *rstC*, which is not present in the related genetic element RS2, an integral part of the CTXΦ genome (38). PCR analysis of the environmental *V. cholerae* strains for *rstC* confirmed the findings of a previous investigation (30) that strains which were positive for *rstR* were also positive for *rstC*. This suggested that the environmental strains harbored the RS1 element carrying an $rstR^{ET}$ gene, which accounted for the immunity of these nine strains to further infection by an El Tor type CTXΦ.

Production of CT was initially studied in vitro by G_{M1}-ELISA by using an antibody against the B subunit of CT (Table 3). Of the 14 strains tested, strain SCE 188 was toxigenic and produced CT, whereas the remaining 13 strains were nontoxigenic. However, environmental strains which were infected by CTXΦ in our study produced CT after transduction by the phage. To further ascertain whether the toxin produced by these transductants was biologically active, we used the ligated ileal loop assay in rabbits and observed fluid accumulation. All culture supernatants which were positive for CT in the ELISA also caused fluid accumulation in the ileal loops of rabbits, confirming that these strains produced biologically active CT. The pathogenic potentials of these strains were further assessed by using the adult rabbit diarrhea model. While rabbits challenged with the CTX-negative strains did not show a diarrheal response (Table 3), *V. cholerae* strains carrying the MSF8.2Φ genome, as well as the native toxigenic strain SCE188 and the reference virulent strains P-27459 and MO10, produced severe diarrhea in rabbits. The environmental strains were less virulent than the control strains, since none of these strains caused fatal diarrhea. However, they caused mild to moderate diarrhea, and the test strains were excreted by challenged rabbits for 2 to 9 days following inoculation. The prolonged shedding of the challenge organisms suggested that the strains colonized the intestines of the rabbits, and the data supported the results of the mouse colonization assay.

However, it is not clear from this study why the environmental strains appeared to be less virulent in the rabbit assay than the *V. cholerae* O1 and O139 strains used as positive controls. Remarkably, all environmental strains carrying a combination of the classical type *tcpA* gene and the new allele of the *toxT* gene were completely resistant to CTXΦ. The competitive indices of colonization of mice by these strains were also lower than those of most other strains (Table 1). If this difference in

TABLE 3. Production of cholera toxin by environmental *V. cholerae* strains and their derivatives (carrying the genome of a genetically marked CTX phage, MSF8.2Φ), and diarrheal response of adult rabbits to these strains

Strain	Cholera toxin production by:		Diarrheal response of adult rabbits by the RITARD model			
	GM1-ELISA[a]	Fluid accumulation in rabbit ileal loops (ml/cm of loops)[b]	No. of animals challenged	No. responding with fatal diarrhea[c]	No. developing nonfatal diarrhea[d]	Mean duration of shedding of strain in stools (days)
SCE4	UD	0	5	0	0	3.6 ± 1.3
SCE4 (MSF8.2)	2.19 ± 0.61	2.06 ± 0.46	6	0	5	4.8 ± 1.7
SCE188	2.92 ± 0.56	2.27 ± 0.82	5	1	4	4.8 ± 0.8
SCE188 (MSF8.2)	3.96 ± 0.79	2.95 ± 0.78	6	2	4	5.3 ± 1.5
SCE340	UD	0	5	0	0	4.6 ± 1.6
SCE340(MSF8.2)	2.65 ± 0.45	2.09 ± 0.51	4	0	4	6.4 ± 0.8
SCE341	UD	0	5	0	0	3.8 ± 0.8
SCE341 (MSF8.2)	2.71 ± 0.56	2.13 ± 0.64	6	0	5	3.6 ± 1.2
SCE359	UD	0	5	0	4	6.0 ± 1.3
SCE359 (MSF8.2)	2.93 ± 0.51	2.12 ± 0.73	5	2	2	7.2 ± 1.6
SA-317	UD	0	5	0	1	5.6 ± 2.1
P-27459	2.97 ± 0.81	2.55 ± 0.79	6	3	2	6.3 ± 1.6
MO10	3.16 ± 0.73	2.32 ± 0.65	6	4	2	6.1 ± 1.4

[a] Toxin amounts are expressed in micrograms per unit of optical density of the culture at 600 nm. Values represent the averages of results of five independent observations. UD, undetectable (toxin amounts were less than 0.01 μg/ml, which was the lowest concentration of purified toxin used as control).
[b] Values represent the averages of results of five independent observations made in different rabbits.
[c] Rabbits developing fatal diarrhea died within 12 h after the initiation of diarrhea.
[d] The duration of diarrhea was between 4 and 6 days for rabbits challenged with strains carrying the CTXΦ genome.

colonization was due to inadequate expression of TCP, this would mean that optimum expression of classical type TCP requires the epidemic type ToxT. Hence, the epidemic type *toxT* gene may have evolved from an environmental *toxT* driven by a need to upregulate the expression of TCP, given that the classical biotype of *V. cholerae* O1 was responsible for the sixth pandemic, and possibly the earlier pandemics, of cholera (15).

Demonstration of the presence of the TCP pathogenicity island in environmental strains, and particularly the genetic diversity of TCP island genes, can provide clues about the origin of the TCP pathogenicity island. Recent studies have shown that virulence genes or their homologs are dispersed among environmental *V. cholerae* strains belonging to diverse serogroups, whereas most previous studies suggested that virulence genes, such as the TCP island genes, are carried only by clinical isolates. This assumption was made because the studies overlooked the possibility of genetic variation within the virulence genes to the extent that the variants might escape detection with PCR or probes that were designed strictly based on the sequences of the corresponding genes found in clinical strains. For example, in a previous study, some of the same environmental strains were reported to be negative for TCP genes (4), whereas a more recent study (30) confirmed that these strains carried the TCP island, but the sequence of some of the genes differed from previously reported sequences of these genes.

In the present study, we have demonstrated that virulence gene homologs carried by environmental strains are functional genes, and such strains are potential pathogens, although their virulence potential may be somewhat lower than that of epidemic strains. The results reported here indicate that functional virulence genes possessed by clinical strains may have evolved from environmental genes. Thus, new insight is provided into the origin and evolution of virulence genes in *V. cholerae*. Characterization of these genes will contribute to the understanding of the ecological significance of the occurrence of virulence gene homologs in environmental strains and their relationship with virulence-associated functions. It has been suggested that virulence factors, including colonization factors and CT, may have a crucial function in the symbiotic and/or commensal association between *V. cholerae* and specific aquatic organisms (5, 6). It is possible that gene products described as virulence factors in the context of human infection may have additional roles while *V. cholerae* persists in the environment. Nonetheless, results of the present study have revealed that the environmental strains of *V. cholerae* not only carry variants of the TCP island genes, but also that these gene variants are functional and capable of producing TCP. Further studies will be done to understand more definitively the role of these virulence-associated factors in the natural environment as well as the environmental selection pressures for *V. cholerae* to carry virulence genes or their homologs.

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