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Distribution of Virulence-Associated Genes and Genetic Relationships in Non-O1/O139 *Vibrio cholerae* Aquatic Isolates from China

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Non-O1/O139 *Vibrio cholerae* is naturally present in aquatic ecosystems and has been linked with cholera-like diarrhea and local outbreaks. The distribution of virulence-associated genes and genetic relationships among aquatic isolates from China are largely unknown. In this study, 295 aquatic isolates of *V. cholerae* non-O1/O139 serogroups from different regions in China were investigated. Only one isolate was positive for *ctxB* and harbored a rare genotype; 10 (3.4%) isolates carried several types of *rstR* sequences, eight of which carried rare types of toxin-coregulated pili (*tcpA*). Furthermore, 16 (5.4%) isolates carried incomplete (with partial open reading frames [ORFs]) vibrio seventh pandemic island I (VSP-I) or VSP-II clusters, which were further classified as 11 novel types. PCR-based analyses revealed remarkable variations in the distribution of putative virulence genes, including *mshA* (95.6%), *hlyA* (95.3%), *rtxC* (89.8%), *rtxA* (82.7%), IS1004 (52.9%), *chxA* (30.2%), SXT (15.3%), type III secretion system (18.0%), and NAG-ST (3.7%) genes. There was no correlation between the prevalence of putative virulence genes and that of CTX prophage or TCP genes, whereas there were correlations among the putative virulence genes. Further multilocus sequence typing (MLST) placed selected isolates ($n = 70$) into 69 unique sequence types (STs), which were different from those of the toxigenic O1 and O139 counterparts, and each isolate occupied a different position in the MLST tree. The *V. cholerae* non-O1/O139 aquatic isolates predominant in China have high genotypic diversity; these strains constitute a reservoir of potential virulence genes, which may contribute to evolution of pathogenic isolates.

Vibrio cholerae is the causative agent of cholera, a life-threatening diarrheal disease. Of the more than 200 known *V. cholerae* serogroups, only O1 and O139 are associated with epidemic and pandemic cholera (1). *V. cholerae* strains in other serogroups (non-O1/O139 *V. cholerae*) are often nonpathogenic or associated with only mild illness (2). However, depending in part on the virulence factors which they carry, they have been linked with more-severe, cholera-like illness and have been associated with sporadic cases and outbreaks of gastroenteritis and extraintestinal infections in both developing and developed countries (2–7).

Two genetic elements associated with virulence in pathogenic O1 and O139 *V. cholerae* are a lysogenic filamentous bacteriophage (CTX prophage), which encodes cholera toxin (CT) (8), and the toxin coregulated pilus (TCP) pathogenicity island, which encodes factors involved in intestinal colonization. The CTX prophage uses TCP as a receptor, allowing *V. cholerae* infection and prophage integration into the bacterial chromosome (8), resulting in the emergence of new toxigenic strains. Non-O1/O139 strains that carry the genes for the CTX prophage and TCP and express CT have been linked with occurrences of severe disease. Other factors that have been associated with virulence include heat-stable toxin (NAG-ST) and hemolysin (Hly) (9). Recently, several novel virulence mechanisms, including a type III secretion system (TTSS) and a type 6 secretion system (T6SS), have been identified in non-O1/O139 isolates (10, 11).

Non-O1/O139 *V. cholerae* strains are naturally present in aquatic ecosystems, such as rivers, estuaries, and coastal waters (4, 12). We hypothesize that carriage of these and other virulence factors by non-O1/O139 strains creates an environmental reservoir of critical virulence genes, which may contribute to evolution of pathogenic *V. cholerae*. To explore this hypothesis, we report here results of screening of a collection of 295 environmental non-O1/O139 strains, isolated from 2001 to 2010 from different regions and aquatic environments in China, to determine the frequency of carriage of virulence-associated genes and the underlying phylogenetic relationships on the basis of multilocus sequence typing (MLST) (13).

MATERIALS AND METHODS

Sample location, isolation, and identification of non-O1/O139 *V. cholerae*. Samples were procured from estuary environments in eight coastal regions and two inland provinces in China from 2001 to 2010. Non-O1/O139 *V. cholerae* strains were isolated by previously described methods (14). All isolates were screened for the oxidase reaction and other biochemical tests (bioMérieux, Lyon, France) to identify them as *V. cholerae*. Non-O1/O139 isolates were identified as positive for species-specific *V. cholerae* outer membrane protein (*ompW*) by PCR (15) and negative for agglutination by specific polyvalent antisera against *V. cholerae* O1 and O139 (S & A Reagents Laboratory, Bangkok, Thailand). A total of 295 *V. cholerae* non-O1/O139 isolates were used in this study. The sources and times of isolation are shown in Table S1 in the supplemental material.

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TABLE 1 Distribution of CTX prophage and TCP genes[a]

Strain(s)	Province(s)	*ctxB* genotype	*rstR* genotype	*tcpA* genotype	*tcpI*
SC2008	Sichuan	8	ET		−
ZJ217-2	Guangdong		CL,VC06-18	CL (O1_CL, AF325733)	+
ZJ227-3	Guangdong		ET, *rstR6*	A199 (O56, EU362122)	+
ZJ234-1	Guangdong		*rstR-4***	523-80 (O115, AF452578)	+
ZJ259-1	Guangdong		*rstR-4***	153-94 (O8, AF452570)	+
ZJ260-2	Guangdong		*rstR-4***	A199 (O56, EU362122)	+
ZJ259-2, MJ-44, MJ-51	Guangdong, Fujian		*rstR6*	A199 (O56, EU362122)	+
MJ-34	Fujian		CL		−

[a] *tcpA* genotype data include reference strain identifiers followed by serogroup and GenBank accession numbers in parentheses. ET, El Tor; CL, classical; +, positive; −, negative.

PCR template preparation. Genomic DNA from *V. cholerae* was extracted with a genomic DNA purification kit (Tiangen Biotech, Beijing, China) in accordance with the manufacturer's instructions. DNA was dissolved in Tris-EDTA (TE) buffer and stored at −20°C until PCR assays were performed.

PCR and sequencing analysis. PCR assays were carried out using conventional PCR amplification. The target genes included cholera toxin B subunit (*ctxB*), different variants of the *rstR* repressor gene, including $rstR^{ET}$, $rstR^{class}$, $rstR^{calc}$, *rstR-4***, *rstR-5*, *rstR6*, *rstR232*, and $rstR^{VC06-18}$ of the CTX prophage, and the classical, El Tor-specific *tcpA* and *tcpI* genes of the TCP pathogenicity island. PCR was used to screen for five genes in the VSP-I cluster (VC0175, VC0178, VC0180, VC0183, and VC0185) and eight genes in the VSP-II cluster (VC0490, VC0493, VC0498, VC0502, VC0504, VC0512, VC0514, and VC0516). The putative accessory virulence genes included hemolysin (*hlyA*), heat-stable enterotoxin (ST), mannose-sensitive hemagglutinin (*mshA*), RTX toxin (*rtxA* and *rtxC*), *chxA*, SXT, IS1004, and TTSS genes. Table S2 in the supplemental material shows the primer sequences and their origins. Reference isolates for N16961 (O1 El Tor of 7th pandemic) and MO45 (O139 isolate isolated from India in 1993) were used as positive controls for PCR.

PCR products were sequenced commercially (TaKaRa, Dalian, China). Sequences were compared using BioEdit software (Ibis Biosciences, Carlsbad, CA). Clustal-W was used to perform multiple nucleotide alignments. The reference sequences of different types of *ctxB*, *rstR*, and open reading frames (ORFs) of VSP-I and VSP-II (VSP-I/II) were accessed from GenBank.

MLST. MLST was performed as described previously (13). Seven housekeeping genes were targeted for MLST analysis: *adk*, *gyrB*, *metE*, *mdh*, *pntA*, *purM*, and *pyrC* (see Table S2 in the supplemental material). The PCR products were directly sequenced in both directions (TaKaRa, Dalian, China). Contiguous nucleotide sequences were assembled with MEGA software, and sequence variants were designated allele profiles. Isolates with identical allelic profiles were assigned to the same sequence type (ST). eBURST (16) was used to identify clonal complexes (CCs). A minimum-spanning tree was constructed using the allelic differences between isolates of the seven housekeeping genes and BioNumerics software (Applied Math). The housekeeping genes of 21 reference *V. cholerae* strains (see Table S1 in the supplemental material) were extracted for MLST analysis. All new housekeeping sequences from this study were deposited in PubMLST and are accessible at http://pubmlst.org/vcholerae/.

Correlation analysis of virulence-associated genes. The relationships between genes were analyzed using Spearman's correlation with SPSS 17.0 software. Each pair of variables (genes) was compared by correlation measures. The correlation coefficients were obtained, and the correlation was considered significant at the 0.01 or 0.05 level. The gene analysis included CTX prophage elements, TCP genes, and VSP-I/II and putative accessory virulence genes.

Nucleotide sequence accession numbers. The representative new nucleotide sequences and predicted amino acid sequences for *ctxB*, *rstR*, and *tcpA* genes were deposited in GenBank with accession numbers KJ437653 (*ctxB*), KJ437633 to KJ437644 (*rstR*), and KJ437645 to KJ437652 (*tcpA*).

RESULTS

Distribution of CTX prophage and TCP genes. Of the 295 tested non-O1/O139 isolates, only one isolate (SC2008; Table 1) carried *ctxB* and was thus potentially toxigenic; we did not screen the strain to confirm that it actually expressed CT. Sequencing analysis indicated that SC2008 carried a rare *ctxB* type (genotype 8) (17) compared with the amino acid substitutions of known types (Fig. 1A and B). In addition, 10 isolates (3.4%) carried several types of *rstR* (Fig. 1C), some of which are found in environmental O1 and O139 isolates. Two of 10 *rstR*-positive isolates were positive for two types of *rstR*, indicating that at least two copies of the CTX prophage were present.

Because PCR showed that all 295 isolates were negative for El Tor and classical-type-specific *tcpA*, strains were screened by using a pair of primers that spanned the entire *tcpA* ORF (tcpA1185 [see Table S2 in the supplemental material]). Eight isolates (2.7%) yielded specific PCR products (1,185 bp), which were sequenced and identified as the environmental types (A199, 153-94 and 523-80) and classical type (Fig. 1D and Table 1) of *tcpA*. Meanwhile, the eight *tcpA*-positive isolates were positive for *tcpI* (Table 1), another ORF within the TCP pathogenicity island. Interestingly, all isolates with the pre-CTX prophage and TCP gene sequences were isolated from only two provinces (Table 1).

Characterization of VSP-I/II clusters and their relationship with elements of CTX prophage and TCP genes. As previously described (18), we used PCR to detect five ORFs in the VSP-I cluster and eight ORFs in the VSP-II cluster. A total of 16 (5.4%) non-O1/O139 isolates carried several VSP island genes, but none carried a complete VSP island. The isolates were positive for one or more than one ORF of VSP-I and/or VSP-II clusters (see Table S3 in the supplemental material). The positive PCR products were further sequenced and compared to those of *V. cholerae* O1 El Tor N16961. The VSP-I/II clusters were identified as 11 novel types and divided into three groups (Fig. 2): the partial ORFs of the VSP-I-positive types (group 1), the partial ORFs of the VSP-II-positive types (group 2), and the partial ORFs of the VSP-I- and VSP-II-positive types (group 3). The ORFs were 86% (VC0185) to 99% (VC0490 and VC0502) identical to ORFs from *V. cholerae* N16961. These results indicated that the isolates carried incomplete VSP-I/II types.

The combination patterns of CTX prophage, TCP genes, and VSP-I/II isolates were as follows: $CTX^{+}TCP^{+}(VSP\text{-}I)^{-}(VSP\text{-}II)^{+}$ ($n = 3$), $CTX^{+}TCP^{+}(VSP\text{-}I)^{-}(VSP\text{-}II)^{-}$ ($n = 5$), $CTX^{+}TCP^{-}(VSP\text{-}I)^{-}(VSP\text{-}II)^{-}$ ($n = 2$), $CTX^{-}TCP^{-}(VSP\text{-}I)^{+}(VSP\text{-}II)^{+}$ ($n = 2$), $CTX^{-}TCP^{-}(VSP\text{-}I)^{+}(VSP\text{-}II)^{-}$ ($n = 2$), and $CTX^{-}TCP^{-}(VSP\text{-}I)^{-}(VSP\text{-}II)^{+}$ ($n = 9$) (see Table S3 in the supplemental material).

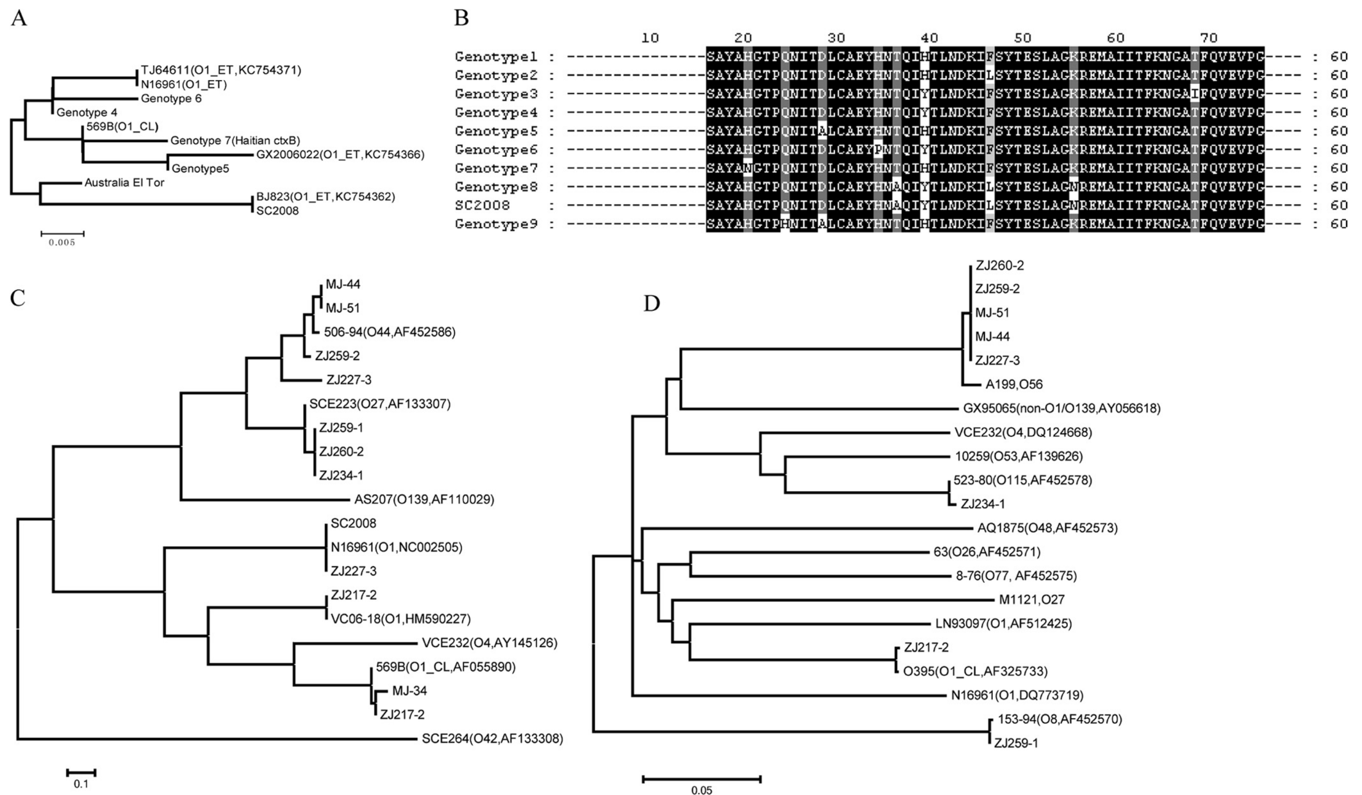

FIG 1 Phylogenetic trees and sequence alignments of *ctxB*, *rstR*, and *tcpA*. (A, C, and D) Phylogenetic trees of *ctxB*, *rstR*, and *tcpA*, respectively. (B) Alignment of deduced amino acid sequences of *ctxB* from *V. cholerae* strains. The serogroups and GenBank accession numbers of the reference sequences for *ctxB*, *rstR*, and *tcpA* are shown in parentheses in panels A, C, and D. Sequence characters in black on a white background represent rare substitutions; sequence characters in white on a black background represent identical sequences in all genotypes; sequence characters in white on a gray background represent common substitutions. El, El Tor biotype; CL, classical biotype.

Although none carried complete VSP-I and -II islands, there was a correlation between the presence of VSP-I and the presence of VSP-II; only the presence of VSP-II correlated with the presence of TCP and CTX prophage (see Table S4 in the supplemental material).

Prevalence of putative virulence genes and relationship with CTX prophage/TCP genes. PCR-based analyses revealed remarkable variations in the distribution of putative virulence genes, including *mshA* (95.6%), *hlyA* (95.3%), *rtxC* (89.8%), *rtxA* (82.7%), IS1004 (52.9%), *chxA* (30.2%), SXT (15.3%), TTSS (18.0%), and NAG-ST (3.7%) (see Table S5 in the supplemental material). There was no correlation between the prevalence of putative virulence gene(s) and the prevalence of CTX prophage or TCP genes, whereas there were correlations among those nine putative virulence genes (see Table S4 in the supplemental material). *rtxA* correlated to six other putative virulence genes (*mshA*, *hlyA*, *rtxC*, IS1004, *chxA*, and TTSS); *hlyA* correlated to five others (*rtxC*, *chxA*, *mshA*, *rtxA*, and IS1004). *mshA*, IS1004, and *chxA* correlated to four others. *rtxC* correlated to three others, and TTSS correlated to two others. SXT and ST had no significant correlation to other putative virulence genes.

MLST. We selected 70 non-O1/O139 isolates for MLST analysis. The isolates were selected based on their different gene characters (types of CTX prophage, TCP genes, and VSP-I/II and putative accessory virulence genes) and sources (different years and provinces or regions). All 70 isolates represented the different types of non-O1/O139 isolates used in this study. The housekeeping genes of 29 reference O1, O139, and non-O1/O139 isolates (see Table S1 in the supplemental material) were also included, resulting in a total of 99 isolates. Excluding two isolates (ZJ193-1 and ZJ194-1), there were 68 non-O1/O139 isolates that had a unique sequence type (ST) and showed high diversity (Fig. 3). Most STs differed from each other by three or more loci. There was no evidence of ST clustering by year or geographic location.

Six *ctxB*-positive isolates were included in the MLST analysis. O1 El Tor (N16961) and O139 (4260B) isolates were grouped into one ST, while O395 (O1 classical serogroup), V52 (O37), and SC2008 (non-O1/O139 from the current study) and four other *ctxB*-negative O1 El Tor isolates formed one clonal complex (CC). The only *ctxB*-positive isolate from the current study, SC2008, had a unique ST. In contrast, V51, an O141 *ctxB*-positive strain, was far from all *ctxB*-positive isolates in the MLST tree, forming its own ST.

Unlike the *ctxB*-positive isolates, all *ctxB*-negative isolates of the O1, O139, or non-O1/O139 types presented highly diverse single nucleotide polymorphisms (SNPs) in the seven housekeeping genes (see Table S6 in the supplemental material). The nine pre-CTX prophage (*ctxB*-negative but *rstR*-positive) isolates and the 16 VSP-I/II-positive isolates were grouped into different positions on the MLST tree and formed STs of their own. Except for two *ctxB*-negative O139 isolates, the STs of six *ctxB*-negative O139 isolates formed a CC and also belonged to a different CC with *ctxB*-positive O139 isolates (Fig. 3). However, the six *ctxB*-nega-

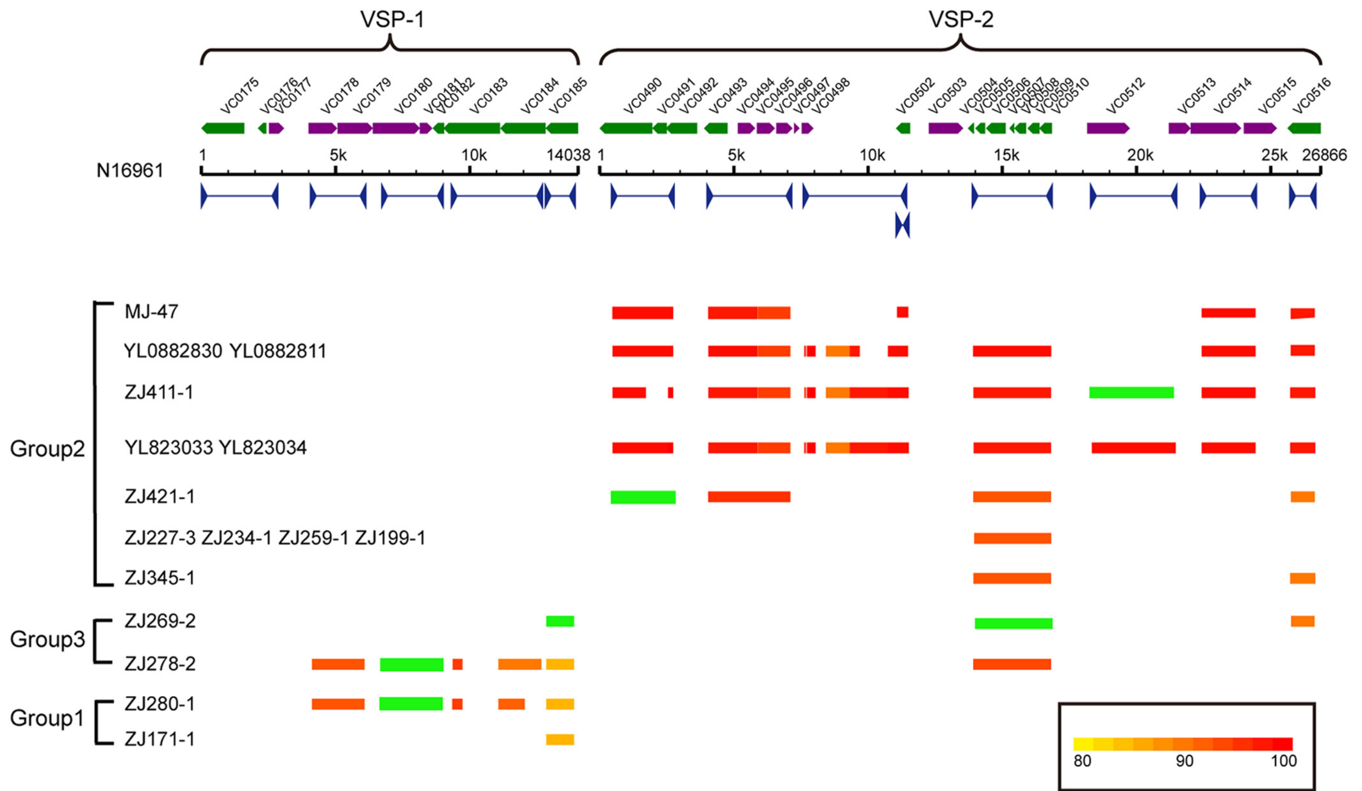

FIG 2 Characterization of VSP-I/II clusters in non-O1/O139 *V. cholerae*. PCR primers are shown as blue arrows, and the color gradient from yellow to red indicates the similarity of VSP-I/II clusters in non-O1/O139 *V. cholerae* and strain N16961 (reference). Green rectangles indicate the ORFs amplified by PCR.

tive O139 isolates were sourced from different years and provinces in China.

DISCUSSION

We found a wide range of putative virulence genes present in our collection of non-O1/O139 strains. Only one strain carried the *ctxB* gene, which, interestingly, had an unusual genotype, matching *ctxB* genes previously identified among environmental O1 isolates in China. To date, based on amino acid residue substitutions, nine genotypes of *ctxB* have been identified (Fig. 1A and B). Genotypes 1, 2, and 3 have been linked with O1 and O139 *V. cholerae* isolates (19): genotype 3 is responsible for the 7th pandemic, genotype 2 has been found only in El Tor isolates from Australia, and the first report of an atypical El Tor biotype that consisted of classical *ctxB* (genotype 1) and emerged between 1991 and 1994 in Matlab, Bangladesh, was published in 2002 (20). Other *ctxB* variants have been reported in association with O139 strains in Bangladesh (21) and El Tor O1 strains in China (17). Here, the *ctxB* of our SC2008 strain matched that from previously reported Chinese environmental O1 El Tor isolates (17). Factors which select for *ctxB* gene variants are not well understood. There is a suggestion that the recent emergence of a classical *ctxB* variant in the predominant global El Tor lineage (22, 23) is associated with increased clinical virulence, linked with a possible increase in CT production (24).

In this study, the pre-CTX prophage and TCP genes identified demonstrated a variety of different *rstR* and *tcpA* alleles suggestive of ongoing genetic recombination among non-O1/O139 strains in environmental reservoirs. Non-O1/O139 strains carrying pre-CTX prophage and TCP genes were isolated from only two provinces, consistent with localization of these genes in the available gene pool in a single region. VSP-I and VSP-II are linked with the pandemic potential of seventh pandemic El Tor isolates (25). We found that VSP-I/II gene segments were present in 5.4% of our non-O1/O139 isolates from aquatic environments, although, in all instances, the gene cluster was incomplete, in keeping with studies of environmental non-O1/O139 strains from other areas (26, 27). Environmental non-O1/O139 isolates are also known to carry a variety of other putative accessory virulence genes. Although they were from aquatic environments, the non-O1/O139 isolates analyzed in this study contained roughly the same rates of *hlyA* and *mshA* as isolates from diarrheal patients in India (4). The rate of TTSS genes (<20%) was significant lower than in patients from India (4) but a little higher than in environmental isolates from Dhaka, Bangladesh (26). TTSS translocates a number of TTSS effectors, such as VopF and VopE, which interfere with host cell signaling pathways (28): a functional TTSS has been shown to be essential for the pathogenicity of the non-O1/O139 AM-19226 strain (29).

As demonstrated by MLST analysis, the environmental non-O1/O139 population in our sample of aquatic environments in China was phylogenetically heterogeneous. These findings are consistent with a recent report by Octavia et al., in which 77 clinical and environmental non-O1/O139 isolates from a number of different countries were found to form 66 STs (13). The majority of these STs were unique, and only three clonal complexes were formed; as in our study, the clonal complexes cen-

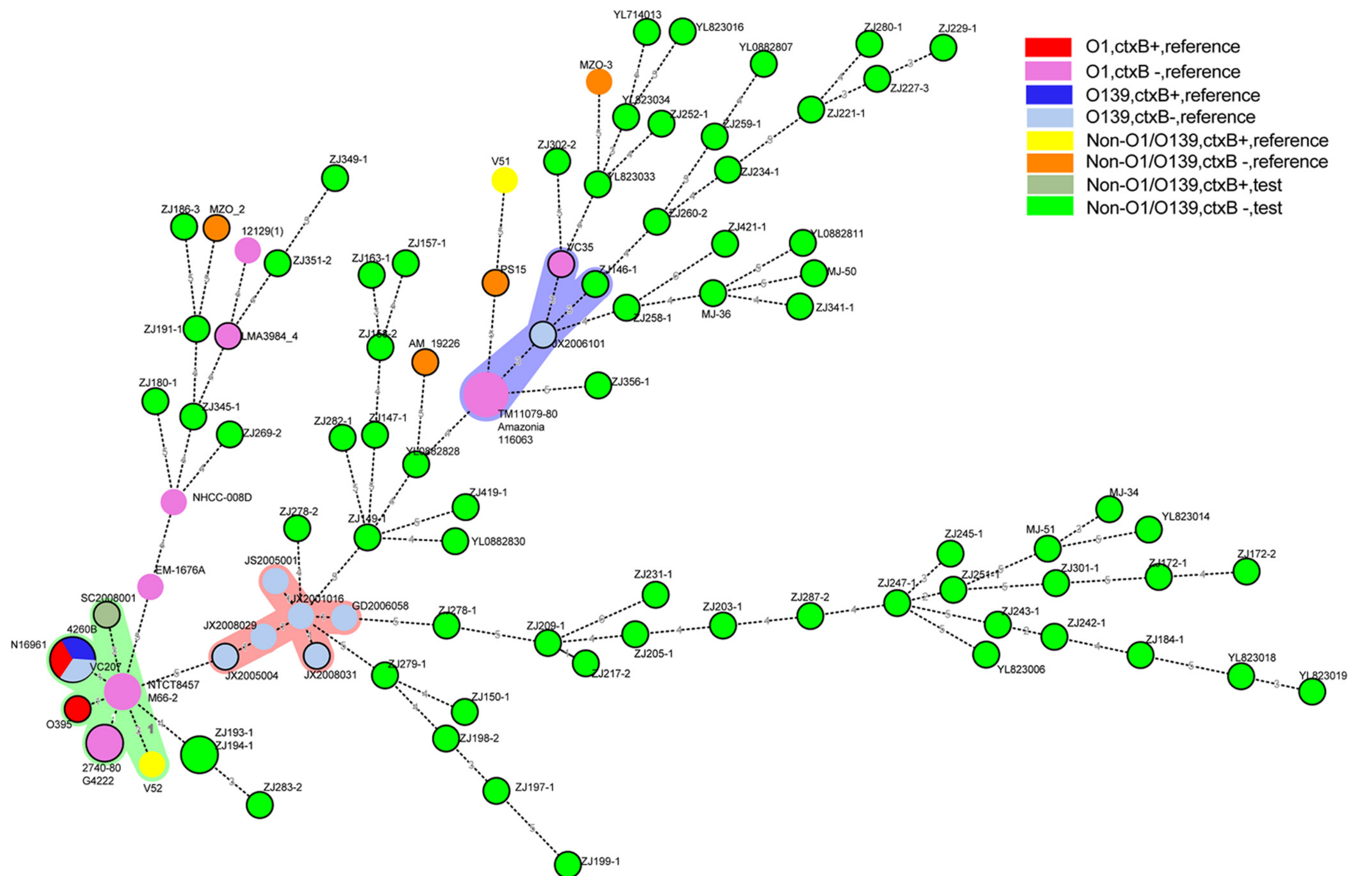

FIG 3 Genetic relationships between 99 *V. cholerae* isolates were analyzed by a minimum-spanning tree. Each circle in the tree represents 1 sequence type (ST), and the size of the circle reflects the number of isolates belonging to each ST. The digits on the lines between two circles represent the different numbers of types. The colors of the halos surrounding the ST denote the types that belong to the same clonal complex (CC). The species within different serogroups are represented by different colors, as indicated. Detailed MLST profiles are shown in Table S6 in the supplemental material.

tered on epidemic or pandemic, *ctx*-positive strains, highlighting the close phylogenetic relationship among such strains, in contrast to the diversity seen among non-O1/O139 strains. The nontoxigenic O1 isolates contained STs similar to those seen with the non-O1/O139 isolates, which also scattered into different positions in the MLST tree and showed high diversity. In contrast, the six nontoxigenic O139 isolates from different years and regions formed a CC, consistent with a more recent common origin.

The virulence of non-O1/O139 isolates is multifactorial and combinatorial, with a range of virulence factors involved in disease causation (30). Strains producing cholera toxin have been clearly linked with illness, and, in human volunteer studies, a non-O1/O139 strain that produced NAG-ST (but which did not have the *ctx* or *tcpA* gene) caused a major diarrheal purge (30, 31). While other virulence factors have been identified in strains from patients with diarrhea, in the absence of strong epidemiologic data or volunteer studies caution must be taken in assuming that these factors, by themselves, are responsible for illness (31). In this setting, it is difficult to know how to assess the public health risk posed by the environmental strains evaluated in this study. Strain SC2008, with its *ctxB* gene and position within the same CC as known epidemic strains, is likely to have been pathogenic and may have evolved from an epidemic strain. In contrast, our environmental O1/O139 strains do not appear to share a phylogenetic background with epidemic or pandemic *V. cholerae* strains (or with each other), suggesting that the virulence genes and gene fragments identified in these strains were acquired through horizontal gene transfer. While we cannot be certain that acquisition of one or more of these putative virulence genes has resulted or will result in human virulence, it remains a theoretical possibility in the appropriate strain background.

In summary, we described the distribution of virulence factors in *V. cholerae* non-O1/O139 strains from aquatic environments. The findings indicate that strains in aquatic environments are reservoirs for multiple *V. cholerae* virulence genes, with ongoing recombination events and mutations leading to acquisition and emergence of genes, gene fragments, and new variant genes. Further surveillance and investigation are required to understand the molecular evolution, epidemiology, and pathogenicity of non-O1/O139 isolates of environmental origin.

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