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Comparative Genomic Analyses of the Vibrio Pathogenicity Island and Cholera Toxin Prophage Regions in Nonepidemic Serogroup Strains of *Vibrio cholerae*

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Two major virulence factors are associated with epidemic strains (O1 and O139 serogroups) of *Vibrio cholerae*: cholera toxin encoded by the *ctxAB* genes and toxin-coregulated pilus encoded by the *tcpA* gene. The *ctx* genes reside in the genome of a filamentous phage (CTXϕ), and the *tcpA* gene resides in a vibrio pathogenicity island (VPI) which has also been proposed to be a filamentous phage designated VPIϕ. In order to determine the prevalence of horizontal transfer of VPI and CTXϕ among nonepidemic (non-O1 and non-O139 serogroups) *V. cholerae*, 300 strains of both clinical and environmental origin were screened for the presence of *tcpA* and *ctxAB*. In this paper, we present the comparative genetic analyses of 11 nonepidemic serogroup strains which carry the VPI cluster. Seven of the 11 VPI$^+$ strains have also acquired the CTXϕ. Multilocus sequence typing and restriction fragment length polymorphism analyses of the VPI and CTXϕ prophage regions revealed that the non-O1 and non-O139 strains were genetically diverse and clustered in lineages distinct from that of the epidemic strains. The left end of the VPI in the non-O1 and non-O139 strains exhibited extensive DNA rearrangements. In addition, several CTXϕ prophage types characterized by novel repressor (*rstR*) and *ctxAB* genes and VPIs with novel *tcpA* genes were found in these strains. These data suggest that the potentially pathogenic, nonepidemic, non-O1 and non-O139 strains identified in our study most likely evolved by sequential horizontal acquisition of the VPI and CTXϕ independently rather than by exchange of O-antigen biosynthesis regions in an existing epidemic strain.

Vibrio cholerae is a serologically diverse, environmental, gram-negative bacterial species. Although *V. cholerae* comprises more than 200 O-antigen-based serogroups (35), only the O1 and O139 serogroup strains are known to cause epidemics of cholera, a severe diarrheal disease. Ever since cholera epidemics caused by *V. cholerae* O139 Bengal surfaced in 1992 in the Bay of Bengal region (1, 31), there has been renewed interest in the pathogenesis and evolutionary mechanisms of non-O1 vibrios. Several studies have reported (8, 9, 11, 15, 28–30, 33, 36) the presence of the two virulence genes found in epidemic strains, *tcpA* and *ctxAB*, in environmental and clinical strains of serogroups other than O1 and O139. However, the mechanisms of origin of these strains are not completely understood at the present time.

Cholera toxin encoded by *ctxAB* is responsible for the severe diarrheal symptoms elicited by the bacterium (20), and toxin-coregulated pilus (TCP) encoded by *tcpA* is responsible for efficient colonization of the human intestinal tract by the bacterium (42, 43). In addition, the O antigen is the bacterium's major protective antigen, and therefore, changes in the O antigen of a preexisting epidemic strain may result in a new pathogen capable of causing disease in populations immune to the original epidemic strain (1, 31). For example, the *V. cholerae* O139 Bengal strain emerged from an O1 epidemic strain by genetic exchange of O-antigen biosynthesis regions (3, 26, 40), and O139 strains cause disease in persons immune to O1 strains (4, 27).

The *ctxAB* genes are carried in the genome of a filamentous, single-stranded DNA phage designated CTXϕ (44), and their dissemination to nonpathogenic strains may, therefore, occur via phage-mediated horizontal gene transfer. Since the discovery of CTXϕ, intra- and interspecies transfers of *ctxAB* genes in *V. cholerae* and *Vibrio mimicus*, via CTXϕ-mediated transduction, have been demonstrated under both laboratory and natural conditions (6, 7, 13, 14).

The TCP structural gene, *tcpA*, has been mapped (21) to a gene cluster designated the vibrio pathogenicity island (VPI), and the VPI has recently been proposed (22) to also be a filamentous phage, VPIϕ. Unlike for the CTXϕ, convincing data are lacking for the existence or the horizontal transfer of VPIϕ. At the present time, none of the 29 genes in the VPI (other than *tcpA*) has been assigned, based on experimental data, any function in the proposed phage's life cycle or phage transduction. However, TCP has clearly been demonstrated (32, 43, 44) to be the receptor for CTXϕ and to be the bacterium's colonization factor.

V. cholerae non-O1 and non-O139 strains have been occasionally isolated from humans with gastroenteritis, extraintestinal infections, and cholera-like illness (20, 34). Strains of serogroup O37 have been implicated in localized outbreaks of cholera in the past (2, 19). In addition, Mukhopadhyay et al. (28) reported the identification and genetic characterization of potentially pathogenic non-O1 and non-O139 *V. cholerae* strains from environmental samples in the Calcutta region.

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TABLE 1. Characterization of non-O1 and non-O139 *V. cholerae* strains with pathogenic potential[g]

Strain	History of isolation (country/year/source)	Serogroup	Presence of VPI		CTXϕ type			*ctx* core[h]
			Cluster[a]	*tcpA* type	*ctxA*	*ctxB*	*rstR*	
395	India/1966/diarrhea	O1 Cla	+	Cla	WT	Cla	Cla	+
N16961	Bangladesh/1975/diarrhea	O1 ET	+	ET	WT	ET	ET	+
153–94	Unknown/1994/CDC	O8	+▲/▼	O8	WT	ET	ET, O8[b]	+
No. 63	Japan/1991/diarrhea from travel in Thailand	O26	+▼	O26	WT	NT	Cla	+
506–94	Thailand/1994/diarrhea	O44	+▼	ET	WT	O26	ET, $O44^{NT}$	+
AQ1875	Japan/1998/tortoise imported from Taiwan	O48	+▼	O48			*rstR-4***[c], $O44^{NT}$	−
507–94	Thailand/1994/diarrhea	O49	+▲	O49	WT	ET	ET, O8[b]	+
8–76	India/1976/diarrhea	O77	+▼	O77				−
1421–77	India/1977/diarrhea	O80	+▼	O77				−
571–88	China/1988/diarrhea	O105	+▲/▼	O27[d]	NT[e]	NT	ET	+
523–80	United States/1980/diarrhea	O115	+▲	O115			ET	−
203–93	India/1993/diarrhea	O141	+▲/▼	O53G[f]	NT[e]	Cla	Cla	+
366–96	Japan/1996/prawn imported from Thailand	O191	+▲	O191	+	Cla	Cla	+
AM2	India/1995/diarrhea	O9						−
AM107	India/1996/diarrhea	O144						−
NRT36-S	Japan/1990/diarrhea	O31						−
117–94	Korea/1994/river water	O35						−
984–81	India/1981/diarrhea	O89						−

[a] +, presence of the entire VPI cluster based on restriction mapping and hybridization.
[b] *rstR*^{O8} is different from *rstR-4*** at the following three positions: $^{rstR\text{-}4}$**H2R^{O8}, $^{rstR\text{-}4}$**G5D^{O8}, and $^{rstR\text{-}4}$**R7H08.
[c] *rstR-4*** is SCE223 (28).
[d] Differs from *tcpA-env* allele of reference 28 at one position, ^{O27}V9D^{env}.
[e] A single amino acid substitution: ^{wt}S46N^{O37,O105,O141}.
[f] O53G is SCE5 from reference 29; *rstR*^{O44} is a novel allele, provisionally designated *rstR6.*
[g] Abbreviations: Cla, classical; ET, EI Tor; WT, wild type; NT, novel type; ▼, insertion; ▲, deletion; CDC, Centers for Disease Control and Prevention.
[h] Presence of CTXϕ genes other than *ctxAB*.

However, how frequently potentially epidemic non-O1 and non-O139 strains arise, and how widely they are distributed in nature, are not known at the present time.

Thus, the aims of our studies were to identify potentially pathogenic non-O1 and non-O139 strains in a large collection of *V. cholerae* isolates not examined previously and to determine the identified strains' evolutionary history. Here we report the identification and comparative genetic analyses of diverse non-O1 and non-O139 strains which possess VPI only or VPI and pre-CTXϕ (a precursor of CTXϕ which lacks *ctxAB* genes) (7) or CTXϕ prophage and have distinct lineages compared to the epidemic strains, thus indicating independent horizontal transfer and acquisition of the virulence regions. While this manuscript was under preparation, Boyd and Waldor (8) also reported the evolutionary and functional analyses of several novel TcpA alleles carried by non-O1 and non-O139 strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *V. cholerae* strains used in the preliminary screening for the presence of *tcpA* and *ctxAB* included 194 serogroup strains from the Shimada type culture collection (35), 36 clinical strains, and 70 clinical and environmental strains from the Smith collection (37). N16961 and

TABLE 2. List of primers

Primer	Gene, probe	Sequence (5′–3′)	Reference
Sulak 80	VPI-1, for-a*	GCA ACA GGA TGA GTA ATC GAG	This study
Sulak 81	VPI-1, rev-a*	CGA TTT CAC AAA GTA GCT CAC	This study
Sulak 84	VPI-3, for-c*	TGA GCC TGA AAT AAT CAC AGG	This study
Sulak 85	VPI-3, rev-c*	GAT GAT GAA GTG TAT ATC TAC	This study
Sulak 86	VPI-4, for-c**	TGG GGA AGA CTT TTG CTC AAG	This study
Sulak 87	VPI-4, rev-c**	ATA TCT TGA ATG GGC TTT ACC	This study
Sulak 121	VPI-5, for-b*	ACG GTT AGC TCT TCC ATC GAC	This study
Sulak 122	VPI-5, rev-b*	CAG AGC GTC TCA TCA AGA TTC AC	This study
Sulak 123	VPI-6, for-b**	GTG AAT CTT GAT GAG ACG CTC TG	This study
Sulak 124	VPI-6, rev-b**	GGT GAG CCA GGC TTA TTT GGG	This study
Sulak 76	*pgm,* for	AAA GAT ACT CAY GCS CTG TC	This study
Sulak 77	*pgm,* rev	AAC CAG CGT TTT ACC GAC GGC AAC A	This study
Sulak 78	*gyrB,* for	GAA GGB GGT ATT CAA GC	This study
Sulak 79	*gyrB,* rev	GAG TCA CCC TCC ACW ATG TA	This study
	rec, 1	GAA ACC ATT TCG ACC GGT TC	39
	rec, 2	CCG TTA TAG CTG TAC CAA GCG CCC	39
M 407	*rstR1*	GAC GTA GCG TGC GGA GTC GCG TTG	25
M 408	*rstR2*	TGA AGC ATA AGG AAC CGA CCA AGC	25
M 573	*rstA1*	ACT CGA TAC AAA CGC TTC TC	25
M 574	*rstA2*	AGA ATC TGG AGG TTG AGT G	25

TABLE 3. Classification of screened *V. cholerae* strains[a]

Genetic background	No. (serogroup[s]) of strains containing:				
	CTXϕ only	VPI only	VPI and RS1[b]	VPI and pre-CTXϕ[c]	VPI and CTXϕ[d]
Epidemic	0	0	0	2 (O53, O65)	2 (O27, O37)
Nonepidemic	0	2 (O77, O80)	1 (O115)	1 (O48)	7 (—[e])

[a] A total of 300 strains were screened, and 285 strains lacked VPI and CTXϕ.
[b] *rstR*$^+$ *rstA*$^+$.
[c] *rstR*$^+$ *rstA*$^+$ *ctx* core$^+$.
[d] *rstA*$^+$ *rstR*$^+$ *ctxAB*$^+$ *ctx* core$^+$.
[e] O8, O26, O44, O49, O105, O141, and O191.

395 represented the O1 El Tor and O1 classical strains, respectively. Other strains characterized in detail are described in Table 1. Bacterial strains were grown in Luria-Bertani medium, and other methods pertaining to bacterial growth and storage conditions have been described previously (38).

PCR. PCR amplification of various segments of the VPI and CTX regions to be used as probes in dot blot and restriction fragment length polymorphism (RFLP) analyses and PCR amplification of the *tcpA*, *rstR*, *pgm*, *recA*, and *gyrB* genes for sequencing were performed by using *Taq* DNA polymerase (Promega

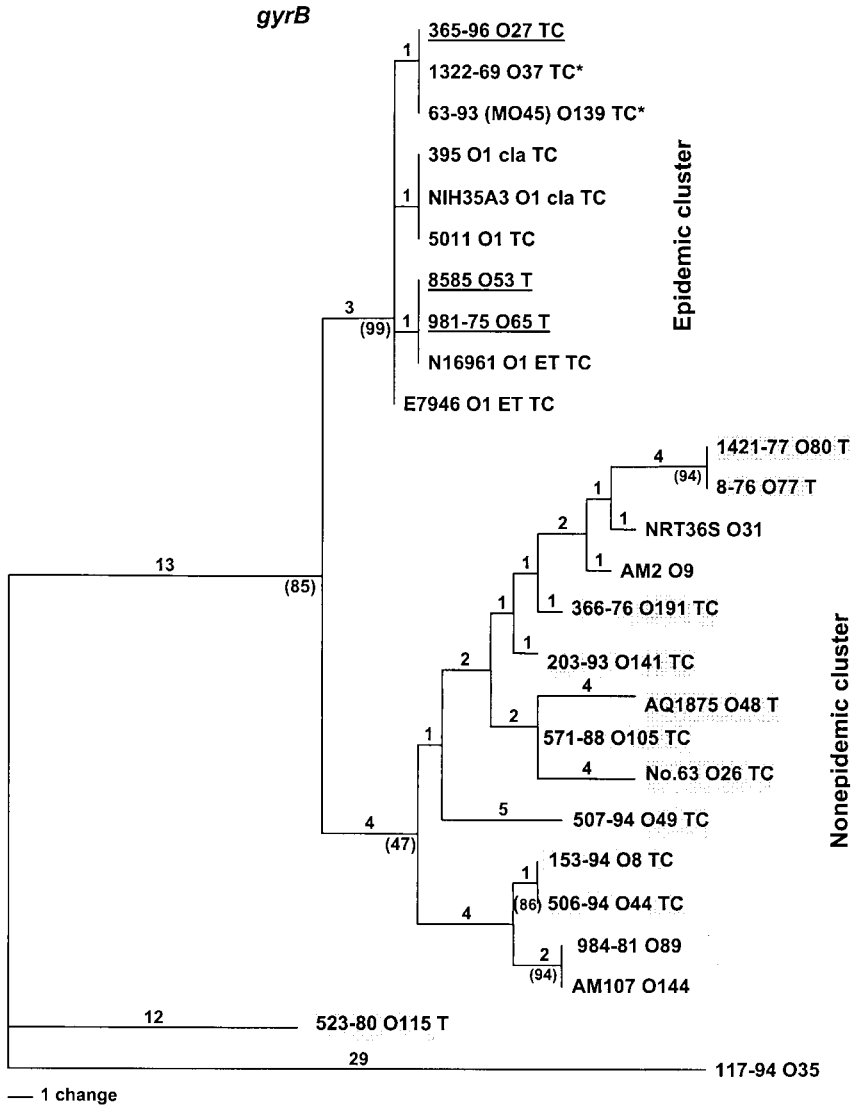

FIG. 1. Neighbor-joining tree constructed by the maximum-parsimony method with the nucleotide sequences of *gyrB* gene fragments of *V. cholerae* strains. Labels at the branch tips represent strain designation, serogroup, and the virulence gene (T for *tcpA* and C for *ctxAB*), if present. The horizontal length represents the genetic distance, and the vertical lengths are not meaningful. The numbers above the branch lines represent the number of changes, and the numbers in parentheses below the branch lines at the branch node are the bootstrap values. Strains for which O-antigen switching is the proposed mechanism are underlined, and strains in which O-antigen switching is supported by sequence evidence are indicated by asterisks. Strains which appear to have acquired VPI and CTXϕ or pre-CTXϕ by independent horizontal gene transfer are highlighted.

Corp. Madison, Wis). Primers used in PCRs were reported previously (25) and were designed based on the *V. cholerae* genome sequence (16). Five additional pairs of primers used to prepare PCR probes to characterize the left end of the VPI and the primers used to amplify fragments of *pgm* (phosphoglucomutase), *gyrB*, and *recA* for multilocus sequence typing (MLST) are listed in Table 2. For most PCRs, the amplification conditions were as follows: 92°C for 5 min, followed by 35 amplification cycles, each consisting of sequential incubation at 92°C (30 s), 55°C (1 min), and 72°C (1 to 2 min), and a final extension at 72°C (5 min). In some PCRs, optimal annealing temperatures were determined by gradient PCR analysis with a Robocycler Gradient 96 (Stratagene, Inc.) thermocycler.

MLST. A 900-bp fragment of *pgm*, a 650-bp fragment of *gyrB*, a 785-bp fragment of *recA*, a 1.8-kb fragment encompassing the *tcpA* gene, and a 1.5-kb fragment of the *ctxAB* genes were PCR amplified, and the amplified fragments were sequenced (in both directions) by using the BigDye terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) and either an ABI 377 Prism automated sequencer or an ABI 3700 DNA analyzer (Applied Biosystems, Inc.). Sequence alignments and dendrograms were generated as described previously (24) by using the Clustal-X (18) and PAUP (D. Swofford, Sinauer Associates, Sunderland, Mass.) programs, respectively. The *rstR* gene (750 bp) was PCR amplified with primers M407 (*rstR1*) and M408 (*rstR2*) (25), subcloned in the pCR2.1 vector (Invitrogen Life Technologies, Carlsbad, Calif.), and sequenced. When two different-sized fragments were obtained in a PCR of a single strain, the fragments were gel purified, cloned separately, and sequenced.

DNA dot blot and Southern analyses. Screening for the presence of *tcpA* and *ctxAB* was performed by DNA dot blot analysis as described previously (38). The presence of the *aldA*, *toxT*, *int*, *rstR*, and *rstA* genes was determined by Southern hybridization of the genomic DNAs with PCR fragments of these genes amplified from an El Tor strain, N16961. The *rstA* gene was PCR amplified with primers M573 (*rstA1*) and M574 (*rstA2*) (Table 2), and all of the other primers were reported earlier (25).

RFLP analysis. Two ~50-kb genomic segments encompassing the VPI and CTXϕ prophage regions were analyzed by RFLP. Genomic DNAs were restriction digested with *Xmn*I (for analysis of the VPI region) and with *Eco*RI or *Sph*I (for analysis of the *ctx* region), electrophoresed in an agarose gel, transferred onto a Zetaprobe membrane (Bio-Rad Laboratories, Hercules, Calif), and simultaneously hybridized with multiple nonradioactive probes prepared with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, N.J.). Hybridization conditions, the various probes, the lengths and order of the restriction fragments probed, and the exact endpoints of the two regions analyzed by RFLP were reported previously (25). The identity of the individual bands was confirmed by hybridizing the same blot with individual probes.

Nucleotide sequence accession number. The nucleotide sequences of the *rstR* (accession numbers AF452585 to AF452586),*tcpA* (accession numbers AF452570 to AF452580), *ctxA* (accession numbers AF452584, AF46340, and AF463401), *ctxB* (accession numbers AF463402 and AF452581 to AF452583), and *gyrB* alleles (accession numbers AF501888 to AF501913) of the non-O1 and non-O139 serogroup strains have been deposited in the GenBank database.

RESULTS

Identification of non-O1 and non-O139 strains containing VPI and CTXϕ. To determine the prevalence of *tcpA* and *ctxAB* in non-O1 and non-O139 *V. cholerae*, 300 strains were screened by DNA dot blot analysis for the presence of the genes. Fifteen non-O1 and non-O139 strains carried the *tcpA* gene, 9 of the 15 strains carried the *ctxAB* genes, and none of the 300 strains carried *ctxAB* alone. The 15 $tcpA^+$ strains were further analyzed, by Southern hybridization, for the presence of other genes in the VPI and CTX regions, and the results are summarized in Table 3. All of the $tcpA^+$ strains also carried three other genes, *aldA*, *toxT*, and *int*, from the left, middle, and right ends of the VPI cluster, respectively, which indicated that the entire VPI region might be present in these strains. Thirteen of the 15 $tcpA^+$ strains carried *rstR* and *rstA* of the CTXϕ genome, and two other strains (serogroups O77 and O80) did not carry any of the CTXϕ genes. Nine of the 13 $rstA^+rstR^+$ strains also carried *ctxAB*, one strain (serogroup O115) carried only *rstA* and *rstR,* and three strains (serogroups O48, O53, and O65) carried *rstR*, *rstA*, and the genes of the core region, except *ctxAB*. Li et al. (25) previously reported the genetic characterization of four of the 15 strains (serogroups O27, O37, O53, and O65) carrying the entire VPI and a CTXϕ or a pre-CTXϕ (7), i.e., CTXϕ lacking the *ctxAB* genes and an epidemic genetic backbone. In the present study, we examined the 11 remaining strains which have genetic backbones different from those of the epidemic strains, and we found that seven of the strains carried the CTXϕ, one carried the pre-CTXϕ, and one only had the repeat sequence element.

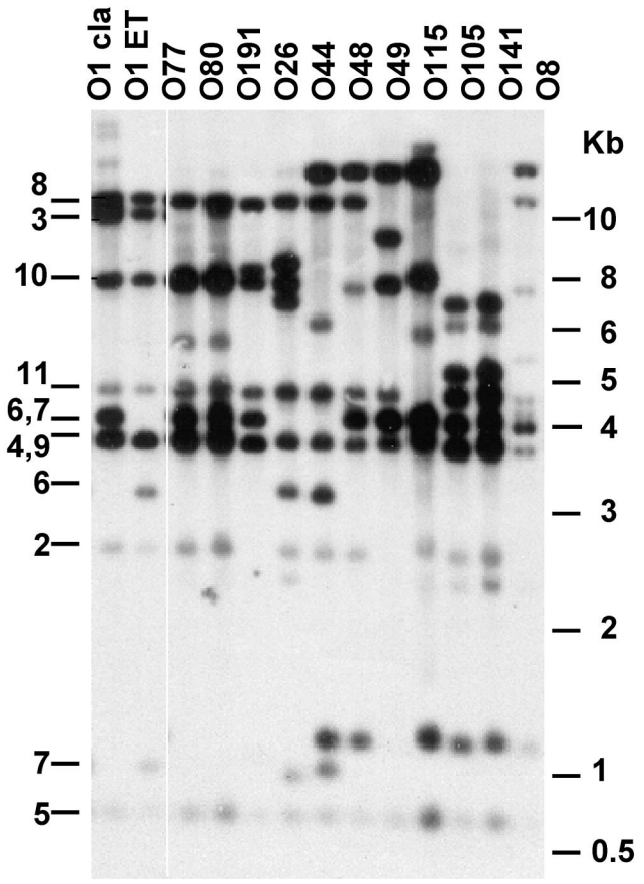

FIG. 2. RFLP analysis of the VPI region in various *V. cholerae* strains. Southern analysis of the *Xmn*I-digested genomic DNAs of the indicated strains was done by simultaneous hybridization with multiple probes. The probes used were b, d, e, g, h, and i, as shown in Fig. 3. The corresponding restriction fragments (shown in Fig. 3) of the El Tor (ET) VPI detected by these probes are indicated by the numbers on the left side of the figure. The size markers (in kilobases) are indicated on the right side of the figure. cla, classical.

Genetic relatedness of the non-O1 and non-O139 strains to the epidemic strains. To determine the genetic relatedness of the 11 $tcpA^+$ nonepidemic strains to the $tcpA^+$ epidemic strains, MLST was performed. A 900-bp fragment of *pgm* (phosphoglucomutase), a 650-bp fragment of *gyrB*, and a 785-bp fragment of *recA* were sequenced. The nucleotide sequences were analyzed by using the maximum-parsimony and maximum-likelihood methods reported previously (17) to be well suited for determining phylogenetic relationships among various bacterial strains and species, and both methods gave consistent results for each of the three genes. The epidemic

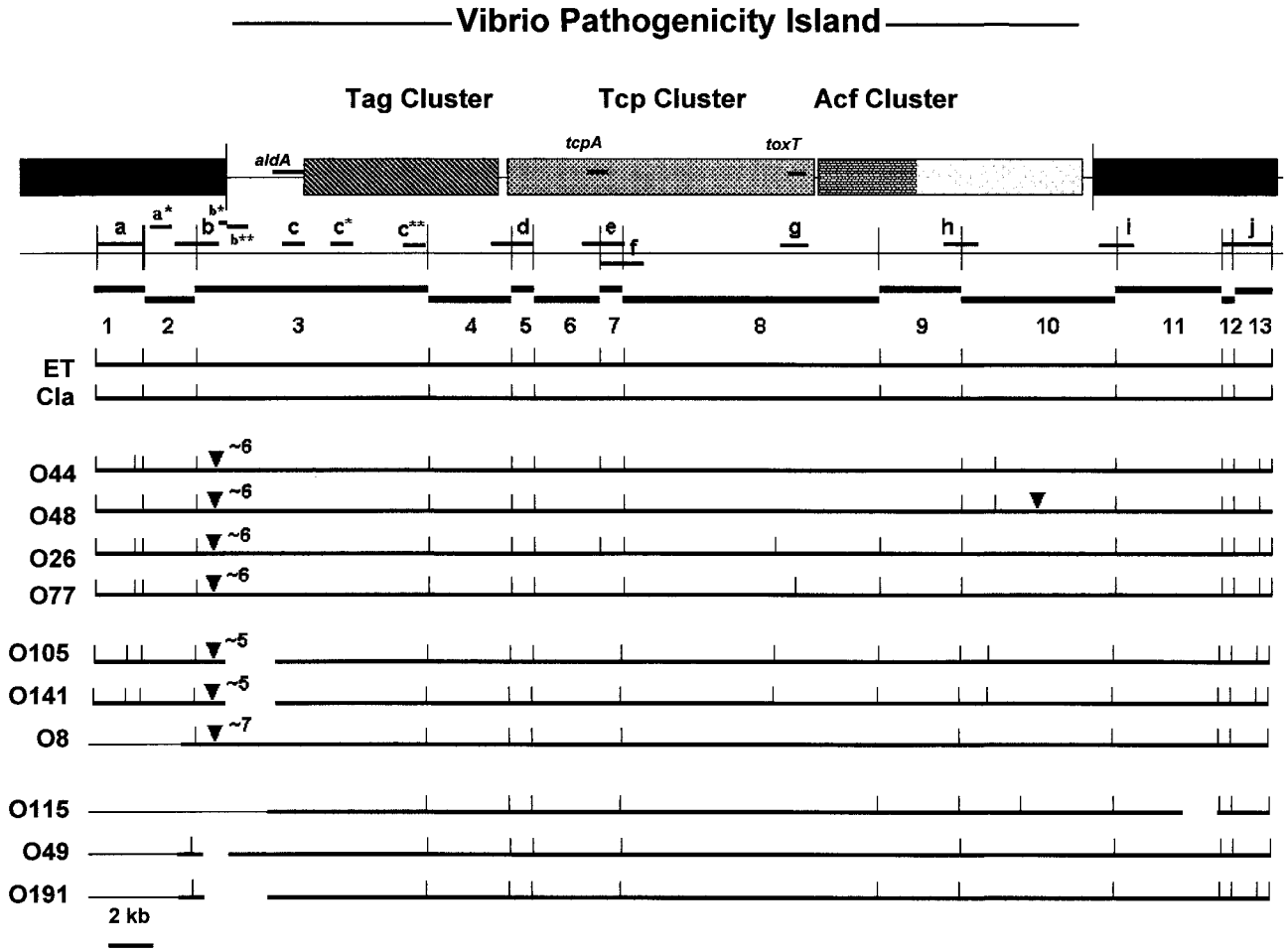

FIG. 3. Genetic organization of the VPI cluster. A schematic diagram of the VPI region in an O1 El Tor strain, based on the *V. cholerae* (16) genome sequence, is shown. The top bar represents the various subclusters within the VPI region, the filled bar indicates the junction segments of the VPI region, and the vertical lines mark the ends of the VPI cluster. The various probes are indicated in the second row (a through j). The predicted *Xmn*I fragments of the VPI region of the El Tor chromosome (numbered 1 to 13) are indicated in the third row. The observed *Xmn*I fragments of the VPI region deduced from hybridization analyses and the resulting genetic maps of the El Tor (ET), classical (Cla), and various non-O1 and non-O139 strains of *V. cholerae* are shown below. DNA insertions and their sizes in kilobases are indicated by triangles and the numbers above the triangles, broken lines represent deletions, and thin lines indicate the adjoining sequences on the chromosome. The El Tor type included the O53, O65, and O139 serogroups, the classical type included the O37 serogroup, and the O77 type included the O80 serogroup.

and nonepidemic strains were topologically separated into two major branches of the phylogenetic tree. The phylogram (Fig. 1) generated for one of the genes (*gyrB*) revealed a tight clustering of the strains of epidemic serogroups (O1 and O139) and strains of four other serogroups (O27, O37, O53, and O65), thus indicating the clonal nature of these strains. The branch node of the epidemic cluster was supported by a value of 99% in a 1,000-replicate bootstrap analysis. Similar clustering of epidemic strains was seen with the *pgm* and *recA* gene trees (data not shown), where the number of nucleotide changes per site among the strains was greater than with *gyrB*. The branch carrying 10 *tcpA*$^+$ non-O1 and non-O139 strains and several other non-O1 and non-O139 strains had a low bootstrap value (47%) (Fig. 1), which suggests that the strains in this group are nonclonal in nature and, accordingly, mostly formed independent secondary branches. Although a few strains within this group formed clusters with high bootstrap values (serogroups O77 to O80, O8 to O44, and O89 to O144) (Fig. 1), this clustering was not consistent with the *pgm*- and *recA*-based trees. The remaining one *tcpA*$^+$ non-O1 and non-O139 strain (serogroup O115) was a *V. mimicus* strain and was an outlier in the tree. Apparently, this strain has acquired the VPI region via interspecies horizontal transfer. Based on these data, we conclude that the 11 *tcpA*$^+$ non-O1 and non-O139 strains have distinct lineages and did not originate directly from epidemic strains by O-antigen switching.

Genetic organization of VPIs. The genetic organization of the VPI region in epidemic and nonepidemic strains was compared by RFLP analysis of genomic DNAs digested with *Xmn*I and hybridized with various probes derived from the VPI region (Fig. 2). The VPIs of the O1 classical and O1 El Tor strains had distinct RFLP patterns (Fig. 2, lanes O1 cla and O1 ET). Ten of the 11 non-O1 and non-O139 strains had distinct RFLP patterns that were different from those of the epidemic strains, with additional *Xmn*I sites and/or genetic rearrangements (insertions and deletions) at the left end of the VPI (Fig.

2, lanes O77 through O8), and O77 and O80 serogroups had identical clusters. The deduced restriction maps of the VPI regions in the 10 strains are shown in Fig. 3. The insertions and deletions at the VPI's left end in the non-O1 and non-O139 strains were confirmed by hybridization of *Sal*I-digested genomic DNAs (Fig. 4). A *Sal*I-generated fragment of the expected size of 4,299 bp in length was seen in the wild-type VPI (Fig. 4b, lanes O1 cla and O1 ET). However, in the non-O1 and non-O139 strains, a fragment of >10 to 12 kb was observed (Fig. 4b, lanes O8 to O80, O191, O105, and O141), thus indicating a DNA insertion (~5 to 7 kb) in the VPI region. Also, the O49 and O115 strains did not hybridize with this probe, which indicates a deletion in this region. Thus, these non-O1 and non-O139 strains could be divided into three groups with respect to their VPIs' left ends. First, strains of serogroups O26, O44, O48, O77, and O80 contained an insertion of an approximately 6-kb fragment between the VPI left *att* site and the *Sal*I site within open reading frame (VC0817) at the left end of the VPI. Second, strains of serogroups O8, O105, and O141 carried a deletion and an insertion of an approximately 7-kb fragment (O8) or an approximately 5-kb fragment (O105 and O141) at the left end of the VPI. Third, strains of serogroups O49, O115, and O191 carried deletions of various lengths at the left end of the VPI.

To elucidate further the origin of the VPIs found in the above-described non-O1 and non-O139 strains, the *tcpA* genes located within the VPIs were sequenced. As observed earlier in other studies (8, 9, 15, 28–30), extensive divergence in the *tcpA* gene sequences of these strains was observed. The results are summarized in Table 1, and a phylogenetic tree based on the amino acid sequences (12 *tcpA* sequences from this study and 12 previously published *tcpA* sequences) is shown in Fig. 5. Four strains (serogroups O44, O53, O65, and O139) carried an El Tor allele, and strain 203-93 (serogroup O141) carried an allele very similar to the one found previously in strain 10259 (serogroup O53), except at three positions (^{O53}A190G^{O141}, ^{O53}P191T^{O141}, and ^{O53}G193S^{O141}) (15). The *tcpA* alleles in serogroups O27 and O105 were very similar to the recently described *tcpA-env* allele (28), except at one position (^{O27,O105}D9V$^{tcpA\text{-}env}$), and the O49 allele differed from the O27 and O105 alleles at one position (^{O27,O105}N56H^{O49}). Seven new alleles in serogroups O8, O26, O48, O49, O77, O141, and O191 were identified in this study. Taken together, these data suggest that the VPIs of a majority of the non-O1 and non-O139 strains differ from those of the epidemic strains (classical and El Tor) as do their *tcpA* genes.

Genetic organization of the *ctx* region. The organization of the *ctx* region in the seven non-O1 and non-O139 strains that were *ctxAB*$^+$ (serogroups O8, O26, O44, O49, O105, O141, and O191) was determined by RFLP analysis. *Eco*RI does not cut within the CTXϕ genome; thus, digestion of the genomic DNAs with *Eco*RI and hybridization with *ctxAB* probes revealed a single copy of the CTXϕ genome in strains of serogroups O8, O26, O44, O49, O105, and O191 and two copies in O141 (Fig. 6). Five other previously described (25) probes, *ctxrgn* 1 to 5, were used to scan the *ctx* region, and the genetic organization of that region in the non-O1 and non-O139 strains was found to be quite heterogeneous and distinct from that of the epidemic strains. Also, the non-O1 and non-O139 strains lacked pTLC, an element carried by all of the epidemic strains, and they possessed an El Tor-like RTX cassette (data not shown).

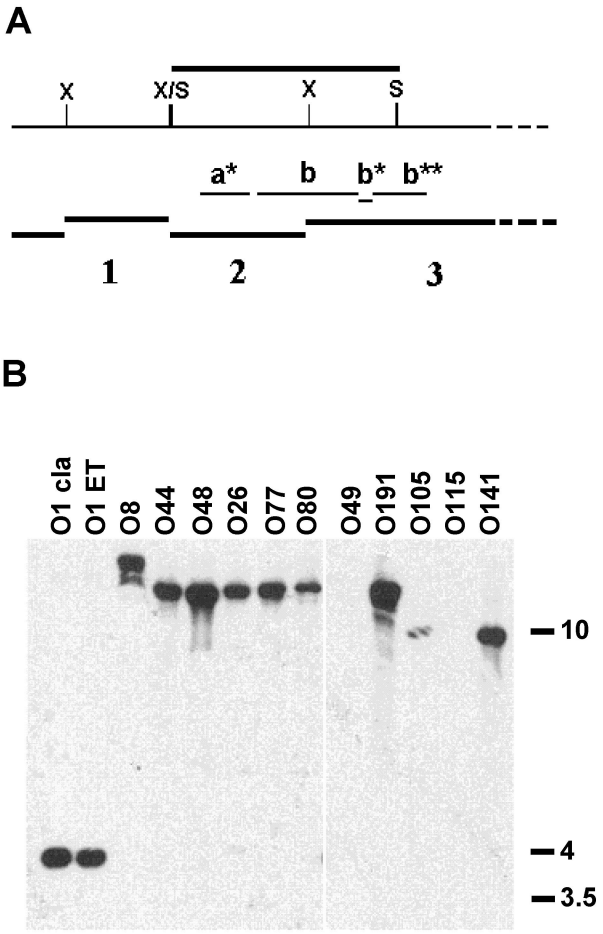

FIG. 4. RFLP analysis of the left end of the VPI region in *V. cholerae* strains. The schematic diagram of the left end of the VPI region and the expected *Sal*I- and *Xmn*I-generated fragments 1 to 3 as shown in Fig. 3 are indicated at the top (A). *Sal*I-digested genomic DNAs were hybridized with the b* probe (B). Strains of serogroups O49 and O115 have a deletion in the region of the probe. ET, El Tor; cla, classical.

To understand further the origin of the CTXϕs present in the non-O1 and non-O139 strains, the nucleotide sequences of the *rstR* and *ctxAB* genes were determined (the allelic types based on their sequences are listed in Table 1). The *rstR* sequences were diverse among the strains examined in this study. Four strains (serogroups O8, O44, O48, and O49) had two alleles (double lysogens), one of which was an El Tor type in the O8, O44, and O49 serogroups, three strains carried the *rstR*^{cla} allele (serogroups O26, O141, and O191), two strains carried only an *rstR*^{ET} allele (serogroups O105 and O115) and two new alleles were found, *rstR*^{O8} and *rstR*^{O44} (serogroups O8, O44, and O49). *rstR*^{O8} is a variant of the *rstR4*** type (28), and *rstR*^{O44} is a novel type, which we provisionally designate *rstR6*, and is similar to a sequence already available in the GenBank database (accession number AF302794). A ClustalX alignment of the nucleotide sequences of the *rstR* type alleles and two variants is shown in Fig. 7. Twenty-four *rstR* sequences

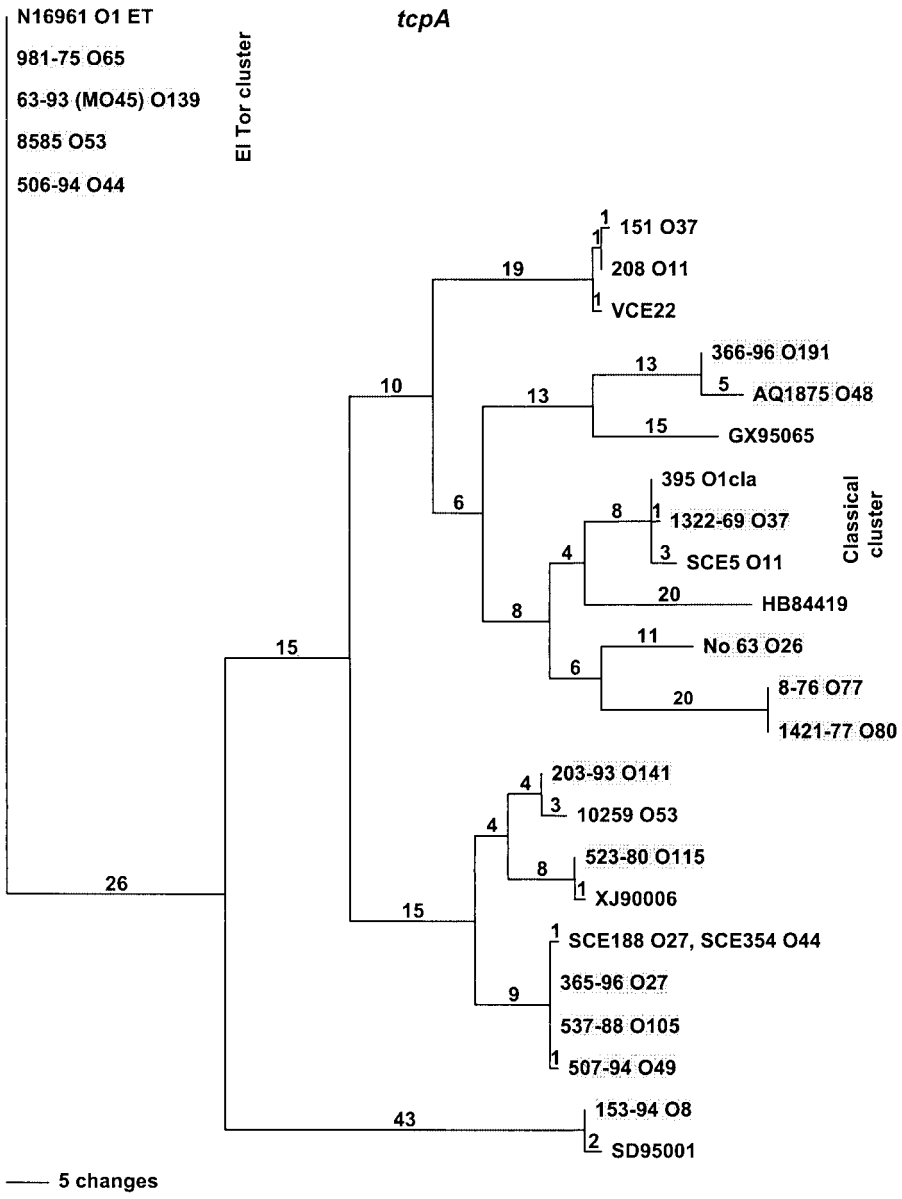

FIG. 5. Neighbor-joining tree constructed by the maximum-parsimony method with the amino acid sequences of the *tcpA* gene fragments of *V. cholerae* strains. The labels at the branch tips represent the strain designation and serogroup, if known. A total of 28 *tcpA* sequences, which included alleles described in this study (highlighted) and in previously published studies, were used for the analysis. The strain designations of published sequences are as follows (serogroups, if known, and GenBank accession numbers are in parentheses): N16961-ET (O1, AF325734), 395 classical (O1, AF325733), 151 (O37, AF030546), 208 (O11, AF030309), VCE22 (AF414371), SCE5 (O11, AB012946), 10259 (O53, AF139626), SCE188, SCE354 (O27, O44, AF208385), GX95065 (AY056618), HB84419 (AY052830), XJ90006 (AY056619), SD95001, (AY052831), and 365-96 (O27, AF390571). The strain designations of novel alleles reported in this paper are as follows (serogroups, if known, and GenBank accession numbers are in parentheses): 366-96 (O191, AF452580), AQ1875 (O48, AF452573), 1322-69 (O37), No. 63 (O26, AF452571), 8-76 (O77, AF452575), 203-93 (O141, AF452579), 523-80 (O115, AF452578), 507-94 (O49, AF452574), 153-94 (O8, AF452570), 571-88 (AF452577), 1421-77 (O80, AF452576), and 506-94 (O44, AF452572). ET, El Tor; cla, classical.

available in the GenBank database were aligned. They fall into 5 *rstR* types: El Tor type (5 sequences), classical type (4 sequences), Calcutta type (4 sequences), *rstR4*** type (9 sequences), and *rstR6* (2 sequences). The sequences upstream and downstream of *rstR* (*ig-1* and *rstA*) are highly conserved in all of the alleles while the *rstR* region showed extensive variations, as do their respective RstR proteins.

The CtxA protein was conserved in all seven strains, except at one position in two strains ($^{wt}S46N^{O105,O141}$). The CtxB protein was of the El Tor type in serogroups O8 and O49 and was of the classical type in serogroups O141 and O191. The classical and El Tor CtxB alleles differed from each other at two positions ($^{ET}Y39H^{cla}$ and $^{ET}I68T^{cla}$). In the O26, O44, and O105 serogroups, novel alleles with four amino acid substitutions ($^{wt}T36A$, $^{wt}F46L$, $^{wt}K55N$, and $^{ET}I68T^{cla,O26,O44,O105}$) were found.

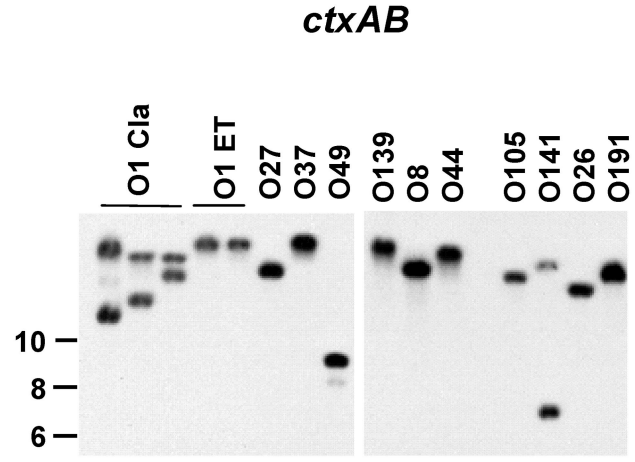

FIG. 6. Analysis of the CTXϕ prophage in *V. cholerae* strains. Southern blot analysis of *Eco*RI-digested genomic DNAs probed with the *ctxAB* gene probes is shown; each band represents one copy of the CTXϕ prophage genome. ET, El Tor; Cla, classical.

DISCUSSION

In this study we utilized a panel of 300 clinical and environmental strains of *V. cholerae* to identify and characterize 15 novel pathogens which belong to serogroups traditionally considered nonpathogenic and nonepidemic and which carry the VPI or the VPI and CTX prophage (regions originally thought to be exclusive to O1 and O139 serogroups). Mukhopadhyay et al. (28) reported the identification and characterization of similarly unusual *V. cholerae* pathogens (serogroups O8, O10, O11, O27, O35, O42, and O69) isolated from environmental samples from the Calcutta region, and very recently, Boyd and Waldor (8) reported several additional isolates (serogroups O8, O37 and O141). Thus, our study further expands the repertoire of novel *V. cholerae* serogroups (O26, O37, O44, O48, O49, O53, O65, O77, O80, O105, O115, O141, and O191) found to carry virulence genes.

The 15 non-O1 and non-O139 strains carrying the VPI cluster could be divided into two groups, based on comparative genomic analyses (MLST and RFLP) (Fig. 1 and 2 to 4, respectively). First, strains of serogroups O27, O37, O53, and O65 have very similar genetic backgrounds and cluster with the epidemic strains, and they most likely emerged by horizontal transfer and exchange of O-antigen biosynthesis regions. In that regard, previous studies (5, 26) and the results we obtained during our sequencing of the O-antigen biosynthesis region in an O37 serogroup strain (25) support that emergence mechanism. Second, strains of serogroups O8, O26, O44, O48, O49, O77, O80, O105, O115, O141, and O191 have distinct genetic backgrounds and virulence regions compared to the epidemic strains, and they most likely arose by independent acquisition of the VPI. Also, several strains in the second group subsequently acquired a CTXϕ or a pre-CTXϕ. None of the 300 strains screened in our study had the CTXϕ alone, which supports the two-step sequential model for the acquisition of VPI and CTXϕ (7). Also, as previously reported (7), intermediates in the evolution of the CTXϕ have also been found, i.e., a pre-CTXϕ lacking *ctxAB* has been found in three different non-O1 serogroups (O48, O53, and O65).

The *tcpA*, *rstR*, and *ctxAB* diversity observed in this study is similar to that reported in previous publications. For example, the two new *rstR* alleles identified in the present work ($rstR^{O8}$ and $rstR^{O44}$) are very similar to the *rstR4* allele (28) and to another sequence deposited in the GenBank database (accession number AF302794). In addition, an insertion and deletion observed at the left end of the VPIs in some non-O1 and non-O139 strains has been reported by Mukhopadhyay et al. (28). However, the sizes of the inserted fragments observed by us are much larger than the 1.6-kb segment of chromosome II reported, in the above-referenced study (28), to have been translocated to the left end of the VPI on chromosome I in place of a 300-bp segment. The genetic contents of the inserts and the deletion end-points need to be determined in order to understand whether translocation of the same region has occurred in the non-O1 and non-O139 strains described in this paper.

Extensive site-directed mutagenesis analysis of the *tcpA* gene (10, 23, 41) has yielded useful insights into the structure-function relationship of the TCP. Based on functional analysis of mutant TcpA pilins, the TcpA protein has been proposed to have three distinct domains, N-terminal, C-terminal structural, and C-terminal interaction domains (10, 23, 41). As observed earlier (8), the various *tcpA* alleles identified in our study have variations in the amino acid residues previously demonstrated (23, 41) to have functional significance, i.e., the majority of the changes are found in the C-terminal structural and interaction domains (Fig. 8). The novel finding, however, is further delineation of the region between amino acid residues 50 and 120, which carries two mutational hotspots, residues 53 to 75 and 90 to 105, where multiple variations are seen (Fig. 8). The role of this region in the structure-function relationship of TcpA pilin remains to be determined. Since there are multiple variations within any given *tcpA* allele, assessing the effect of these changes may be difficult, especially if the changes in one domain are the result of intragenic suppression of mutations elsewhere in the gene. Site-directed mutagenesis studies of this region (residues 50 to 120) in a wild-type *tcpA* may reveal the functional significance of this domain.

Horizontal gene transfer plays an important role in the evolution of pathogenic bacteria, including *V. cholerae*, which has become a paradigmatic organism for studying horizontal gene transfer mechanisms. All three of the major known *V. cholerae* virulence markers (CTXϕ, VPI, and O-antigen biosynthesis regions) are believed to have been acquired by horizontal gene transfer. Two of these markers (CTXϕ and VPI) have been introduced into the *V. cholerae* chromosome via phage-mediated transduction (22, 44); the mechanisms responsible for acquiring the O-antigen biosynthesis regions are unknown at the present time. However, acquisition of VPI and CTXϕ appears to be more frequent than exchange of O antigens in epidemic serogroups, which may reflect mechanistic differences in their horizontal transfer. Acquisition of VPI and CTXϕ involves single-stranded phages and a site-specific recombination process, whereas acquisition of the O-antigen region probably involves generalized transducing phages and a homologous recombination mechanism. Hence, it is tempting to speculate that horizontal transfer of O-antigen regions might be subjected to DNA restriction and recombination barriers.

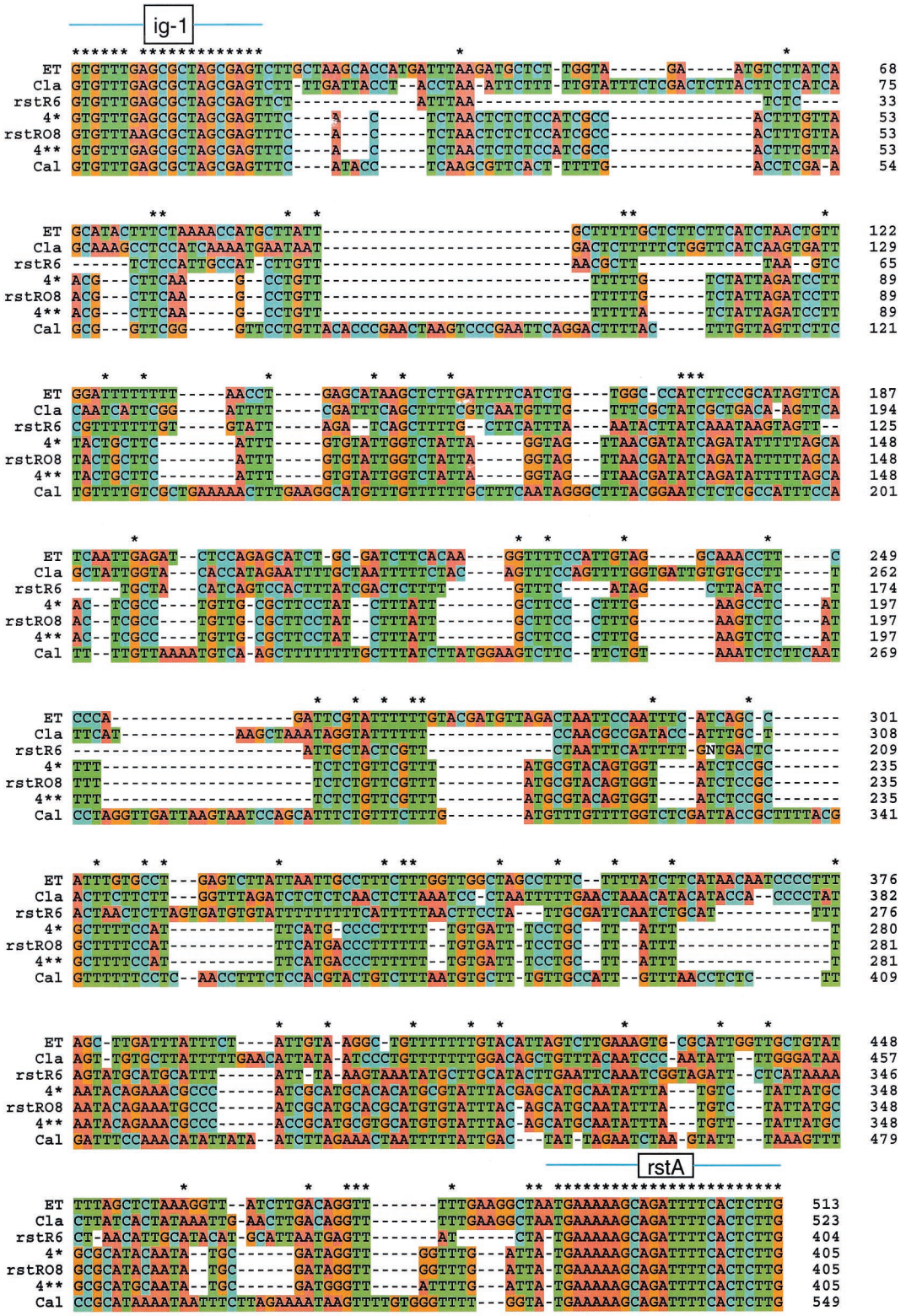

FIG. 7. Clustal-X alignment of *rstR* sequences. A total of 24 *rstR* sequences available in the GenBank database, which included alleles described in this study, were used for the analysis. Only the *rstR* type sequences that encode different RstR proteins (5 type sequences, i.e., El Tor [ET], classical [Cla], Calcutta [Cal], *rstR4***, and *rstR6*) and two variants are shown in the figure. One sequence (accession number AF133308, designated *rstR5* from strain SCE264) of the Calcutta type shows extensive variations, its RstR protein exhibited the least similarity to all of the other alleles (28), and hence, it was not included in the alignment. The strain designations of the published sequences are as follows (the serogroup [if known] and the GenBank accession numbers are in parentheses: El Tor (ET) type, E7946 (O1 ET, U83795), E7946 (O1 ET, U83796), SC8511 (O1 ET, AF511000), N16961 (O1 ET, AE004224), and JS9803 (O139, AY101180); classical (Cla) type, O39 (O1 Cla, AF262318), SC9773 (O1 ET, AF510999), 569B (O1 Cla, AF05890), and 86015 (O1 ET, AF220606); Calcutta (Cal) type, AS207 (O139, AF110029), SCE188 (O44, AF133310), FJ98352 (O139, AF511001), and SCE264 (O42, AF133308); *rstR4*** type, SCE223 (O27, AF133307), 365-96 (O27, AF390570), JX94484 (O139, AF511001), VCE22 (O36, AY145124), VCE228 (O27, AY145125), VCE232 (O4, AY145126), VCE233 (O27, AY145127), 153-94 (O8, AF452585), and SCE263 (O10, AF133309); Novel type, 506-94 (O44, AF452586) and 9803 (O139, AF302794). Nucleotides CT and AG are in green and red, respectively. ig-1, intergenic region 1; rstA, the 5′ end of the *rstA* gene. Nucleotides that are identical in all sequences are indicated by asterisks.

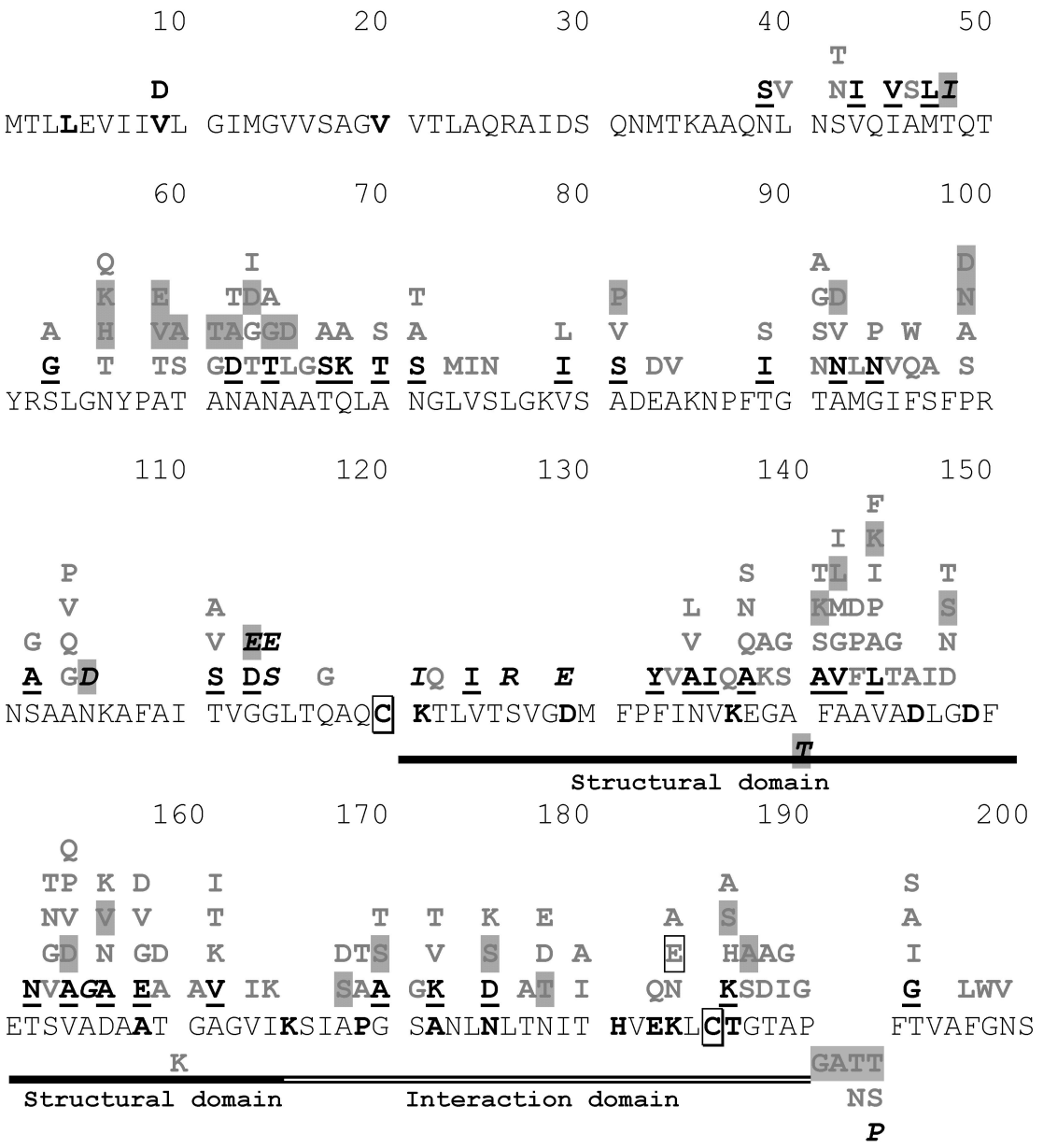

FIG. 8. Domain structure of TcpA. The primary amino acid sequence of the El Tor TcpA is shown. The residues shown to have functional significance, based on site-directed mutagenesis (10, 23, 41), are indicated in boldface type, the two cysteine residues predicted to be essential for the structure and function of TcpA pilin are indicated by boxes, and the proposed domains are indicated by bars (10, 23, 41). The variations in the amino acid residues found in various *tcpA* alleles described in other studies (9, 15, 28–30) and in this work (highlighted) are indicated above the primary sequences. The variations seen in the classical allele are underlined. The changes unique to a single strain are indicated in italics. The O37 serogroup has a classical TcpA with a single change (K184 to E) indicated by a shaded box.

The present study provides further support to the growing body of evidence that the classical *V. cholerae* virulence markers, *ctxAB* and *tcpA*, are not unique to epidemic strains and that they are found in at least some nonepidemic, non-O1 and non-O139 *V. cholerae* serogroups. However, the epidemic potential of these strains is not clear at the present time, especially since—and in clear contrast to O1 and O139 isolates—none of the non-O1 and non-O139 strains has been associated with past epidemics and/or pandemics of cholera. However, these observations raise the intriguing possibility that the presence of *ctxAB* and *tcpA* in *V. cholerae*, while critical, is not sufficient for the full-blown epidemics of cholera. In this context, Dziejman et al. (12) recently reported comparative genome analyses of 11 *V. cholerae* strains of epidemic serogroups, and they identified two clusters of genes differentiating pandemic strains from other strains. Similar comparative genomic analysis of epidemic strains with the nonepidemic, non-O1 and non-O139 strains identified in this study is likely to provide invaluable information regarding the mechanisms responsible for the emergence of the epidemic *V. cholerae* serogroups and strains.

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