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Evolutionary Genetic Analysis of the Emergence of Epidemic *Vibrio cholerae* Isolates on the Basis of Comparative Nucleotide Sequence Analysis and Multilocus Virulence Gene Profiles

Yvonne A. O'Shea, F. Jerry Reen, Anne Marie Quirke, and E. Fidelma Boyd*

Department of Microbiology, University College Cork, National University of Ireland, Cork, Ireland

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***Vibrio cholerae*, the causative agent of cholera, is a natural inhabitant of the aquatic ecosystem. We examined a unique collection of *V. cholerae* clinical and environmental isolates of widespread geographic distribution recovered over a 60-year period to determine their evolutionary genetic relationships based on analysis of two housekeeping genes, malate dehydrogenase (*mdh*) and a chaperonin (*groEL*). In addition, the phylogenetic distribution of 12 regions associated with virulence was determined. Comparative sequence analysis of *mdh* revealed that all *V. cholerae* O1 and O139 serogroup isolates belonged to the same clonal lineage. Single-strand conformational polymorphism (SSCP) analysis of these O1 and O139 strains at *groEL* confirmed the presence of an epidemic clonal complex. Of the 12 virulence regions examined, only three regions, *Vibrio* seventh pandemic island 1 (VSP-I), VSP-II, and RS1, were absent from all classical *V. cholerae* isolates. Most *V. cholerae* El Tor biotype and O139 serogroup isolates examined encoded all 12 virulence regions assayed. Outside of *V. cholerae* O1/O139 serogroup isolates, only one strain, VO7, contained VSP-I. Two *V. cholerae* El Tor isolates, GP155 and 2164-78, lacked both VSP-I and VSP-II, and one El Tor isolate, GP43, lacked VSP-II. Five non-O1/non-O139 serogroup isolates had an *mdh* sequence identical to that of the epidemic O1 and O139 strains. These isolates, similar to classical strains, lack both VSP-I and VSP-II. Four of the 12 virulence regions examined were found to be present in all isolates: *hlyA*, *pilE*, MSHA and RTX. Among non-O1/non-O139 isolates, however, the occurrence of the additional eight regions was considerably lower. The evolutionary relationships and multilocus virulence gene profiles of *V. cholerae* natural isolates indicate that consecutive pandemic strains arose from a common O1 serogroup progenitor through the successive acquisition of new virulence regions.**

Vibrio cholerae is a natural inhabitant of the aquatic environment and is found associated with shellfish and crustaceans (12, 21, 29, 48). *V. cholerae* is the causative agent of the diarrheal disease cholera, and humans are the only known animal host. *V. cholerae* is an extracellular pathogen of the small intestine and causes significant human disease and death, particularly on the Indian subcontinent. A recent study has shown that the human host may contribute significantly to cholera epidemics, since passage through the human intestine was shown to induce a hyperinfectious state, which was perpetuated in the natural environment after release (43). Of the 200 O-antigen serogroups so far identified among *V. cholerae* isolates, only two serogroups, O1 and O139, are known to cause epidemic and pandemic cholera (34). The *V. cholerae* O1 serogroup can be further divided into two biotypes of epidemiological relevance, classical and El Tor, based on minor phenotypic differences. The first cholera pandemic, which began in 1817 in Asia, and subsequent pandemics, were probably caused by the classical biotype. In 1961, the seventh and present pandemic began, which was caused by the El Tor biotype (34). In 1992, for the first time in the recorded history of cholera a novel O-serogroup, O139 emerged to cause epidemic cholera (1). Significantly, exposure to O1 serogroup cholera does not protect against O139 cholera (45). The El Tor strain reemerged to overtake the O139 serogroup as the major cause of cholera by 1996 (23). However, the O139 serogroup is still present on the Indian subcontinent and, in some areas, is the predominant cause of cholera (23). Interestingly, several studies have proposed that the origin of the serogroup O139 strain was an El Tor strain that obtained the O139 biosynthesis genes (as well as the SXT element and a capsule) via antigenic switching from a donor strain (3, 44, 60, 65). Recently, it has been proposed based on comparative sequence analysis that an O22 serogroup maybe a possible donor for the O139 serogroup (17, 67). Sporadic cholera outbreaks caused by *V. cholerae* non-O1 and non-O139 isolates have been documented; for example, in 1968 in Sudan there was a cholera outbreak caused by an O37 serogroup isolate (21, 68).

The evolutionary genetic relationships among *V. cholerae* strains have been examined by multilocus enzyme electrophoresis (2, 11, 19, 20, 54, 58), single locus sequence analysis (7, 37, 38, 59), and multilocus sequence analysis of housekeeping genes (9, 36, 40). These analyses have given conflicting results regarding the ancestry of O1 serogroup classical and El Tor biotype strains. Several studies suggest that at least three pathogenic clones exist, consisting of classical and El Tor biotype strains and U.S. Gulf Coast strains (37, 63). Others have suggested that the three pathogenic clones are very closely

* Corresponding author. Mailing address: Department of Microbiology, UCC, National University of Ireland, Cork, Ireland. Phone: 353 21 4903624. Fax: 351 21 4903101. E-mail: f.boyd@ucc.ie.

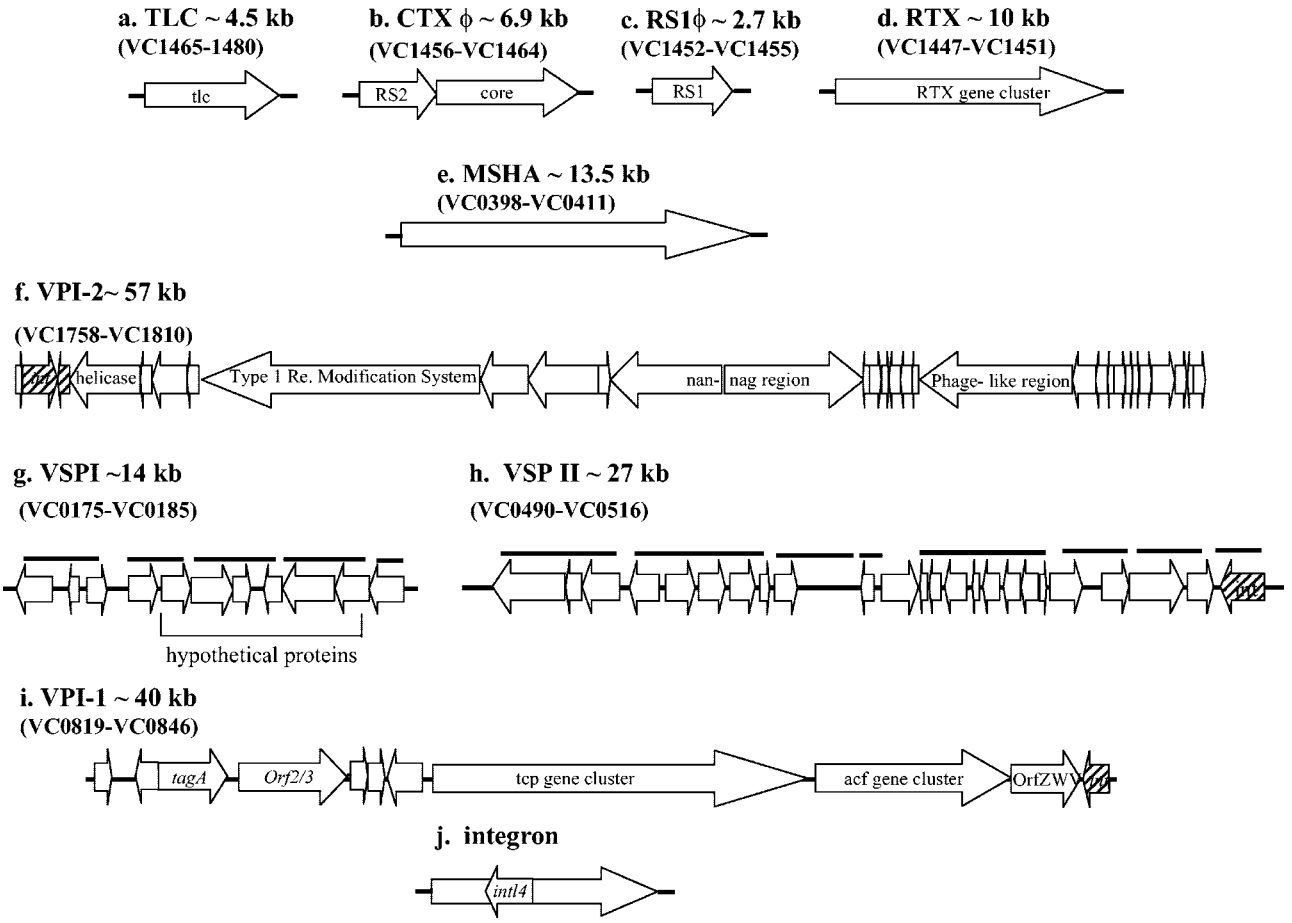

FIG. 1. Schematic representation of nine regions that are associated with pathogenesis in *V. cholerae*. The positions and directions of transcription of the open reading frames are indicated by the directions of the arrows. The black bold horizontal lines indicate the positions of the PCR primers.

related (2, 9, 18). In all studies, the epidemic *V. cholerae* isolates form a lineage separate from nonepidemic strains.

Although the O-antigen is a major protective antigen in *V. cholerae* virulence and probably plays a role in host colonization, the two major virulence factors of *V. cholerae* are cholera toxin (CT), the main cause of the explosive rice watery diarrhea, and toxin-coregulated pilus (TCP), the main intestinal colonization factor (61). The *ctxAB* genes, which encode CT, are integral components of a novel filamentous phage CTXϕ (64), and the TCP biosynthesis genes are encoded on the *Vibrio* pathogenicity island (hereafter designated VPI-1) (35). A number of studies have found that the two main virulence factors, CT and TCP, are predominately associated with *V. cholerae* O1 and O139 serogroup strains and are only occasionally found in nonepidemic isolates (5, 7, 8, 10, 13, 25, 40, 47, 49, 50, 55).

Among *V. cholerae* El Tor isolates, CTXϕ is flanked by an additional filamentous phage RS1ϕ that is required for CTXϕ production (16, 22) (Fig. 1). The CTX prophage is also flanked by the toxin-linked cryptic plasmid (TLC), whose role in pathogenesis is unknown (56) (Fig. 1). A number of other gene clusters have also been identified that are found predominantly among epidemic *V. cholerae* isolates: the RTX toxin gene cluster (42), the mannose-sensitive hemolysin agglutination pilin (MSHA) (32), VPI-2 (31), hemolysin, and PilE pilin (28, 30) (Fig. 1). Recently, comparative genomic studies which used a *V. cholerae* DNA microarray among 11 epidemic isolates identified two regions, *Vibrio* seventh pandemic island I (VSP-I), encompassing VC0175 to VC0185, and VSP-II, encompassing VC0490 to VC0497, that were found exclusively among El Tor biotype isolates (18). The role of VSP-I and VSP-II in *V. cholerae* virulence remains undetermined.

To elucidate the steps and significance of virulence gene acquisition in the evolution of *V. cholerae* it is essential to know the underlying phylogenetic relationships among strains. In this study we examined a unique collection of 64 *V. cholerae* and 5 *Vibrio mimicus* isolates to determine their evolutionary genetic relationships and multilocus virulence gene profiles to elucidate the steps involved in the emergence of epidemic isolates. Our results show that *V. cholerae* serogroup O1 classical and El Tor biotype strains encompass a single epidemic clonal complex and that differences between biotype strains arose through the acquisition of additional virulence regions by El Tor isolates. The emergence of epidemic *V. cholerae* O139 serogroup strains was not a unique occurrence in the history of cholera, since *V. cholerae* O37 and O8 serogroup isolates phylogenetically cluster with O1 and O139 serogroup isolates, indicating antigenic switching.

MATERIALS AND METHODS

Bacterial isolates. A total of 64 *V. cholerae* isolates were examined in this study (Table 1). The 64 *V. cholerae* isolates belonged to 19 different serogroups, 3 isolates had no serogroup designation, and 23 isolates belonged to serogroup O1, of which 6 isolates were of the classical biotype and 15 isolates were of the El Tor biotype. The O139 serogroup was represented by 13 isolates, the O37 serogroup was represented by 7 isolates, and the O8 and O141 serogroups each were represented by 2 isolates. There were 14 serogroups represented by a single *V. cholerae* isolate. The *V. cholerae* isolates were recovered from six continents (North and South America, Asia, Europe, Australia, and Africa) over a 60-year period (1937 to 2000) (Table 1). In addition, our study also included five *V. mimicus* isolates, four O115 and one O41 serogroup isolates. Of the 69 strains examined, 51 were clinical isolates and 12 were environmental isolates (Table 1). All *Vibrio* strains were grown in Luria-Bertani (LB) broth and stored at −70°C in LB broth with 20% (vol/vol) glycerol.

DNA isolation. Chromosomal DNA was extracted from each *V. cholerae* and *V. mimicus* isolate by using the G-nome DNA isolation kit from Bio 101 (Vista, Calif.). Briefly, a single colony of each isolate was inoculated into 3 ml of LB broth and incubated overnight at 37°C with shaking at 150 rpm. The bacterial cells were pelleted at 3,000 rpm for 5 min, the supernatant was discarded, and the pellet brought to a final volume of 1.85 ml in cell suspension solution. The cells were lysed and treated with RNase and protease. DNA was extracted with Tris-EDTA buffer and ethanol and resuspended in Tris-EDTA buffer.

PCR amplification and nucleotide sequencing. PCR primers to amplify the chromosomal housekeeping gene malate dehydrogenase (*mdh*) were designed from the *mdh* sequence of *V. cholerae* strain N16961 (26). The following PCR cycle was used to amplify the *mdh* gene for each isolate: an initial denaturation step at 96°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, 30 s of primer annealing at 53.9°C, and 1.5 min of primer extension at 72°C. The primer pair mdh1-mdh2 amplified an 892-bp fragment, representing 84% of the *mdh* gene. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. After purification, an aliquot of 10 μl was used as a sequencing template. The *mdh* gene sequences were determined in both directions by MWG-Biotech based on the dye deoxy terminator method.

Phylogenetic analyses. The *mdh* gene sequences were aligned by using the CLUSTALW multiple-sequence alignment program (27). From the *mdh* sequence alignments, a 648-bp region was further analyzed by using the Molecular Evolutionary Genetics Analysis (MEGA) suite of programs, version 2.1 (39). Phylogenetic gene trees were constructed by the neighbor-joining method with the Jukes-Cantor distance method (33, 57). Bootstrap values were calculated for 1,000 trees. The proportions of synonymous (silent) substitutions per synonymous site (Ds) and nonsynonymous (replacement) substitutions per nonsynonymous site (Dn) were calculated.

PCR-SSCP. In conjunction with *mdh* sequencing, an additional 25 *V. cholerae* O1 and O139 serogroup strains were analyzed to confirm sequence identity at this locus within these two serogroups by PCR–single-strand conformational polymorphism analysis (PCR-SSCP), a simple and rapid method to determine point mutations within genes. Two oligonucleotide primers, mdh1 and mdh2 (Table 2), were used to amplify an 892-bp PCR product, which was then restricted with HindIII (Roche Molecular Biochemicals, East Sussex, United Kingdom) at 37°C to generate two fragments. Then 5 μl of the restricted DNA was mixed with 5 μl of denaturation buffer (5 mM EDTA, 0.05% bromophenol blue, and xylene cyanole in formamide), and the mixture was incubated at 95°C for 8 min. The sample was then placed directly in ice for 10 min before being loaded onto a nondenaturing 8% polyacrylamide gel. Samples (8 μl) were run at 100 V for 2 h. As a control, 4 μl of undenatured digested DNA (*mdh* gene) was used.

In addition, all epidemic *V. cholerae* O1 and O139 serogroup isolates were examined at the *groEL* locus by PCR-SSCP analysis. Primer pair groEL1A and groEL1B, designed from *V. cholerae* genome sequence (26) were used to PCR amplify a 1.6-kb band from 20 *V. cholerae* O1 and O139 serogroup isolates and 2 O37 serogroup isolates. PCR products were digested with BstYI at 60°C to generate four restriction bands. Restricted DNA (10 μl) was denatured as described above in 10 μl of denaturation buffer and electrophoresed at 175 V for 6 h.

After electrophoresis, the 8% polyacrylamide gels were silver stained with a DNA silver staining kit (Pharmacia Biotech). Briefly, the silver staining procedure was as follows. The gels were first fixed in 10% acetic acid for approximately 30 min at room temperature and washed with deionized water three times for 2 min. Color impregnation lasted for 20 min at room temperature. The gel was then washed for 5 to 10 s with deionized water, followed by color development for 6 min with a color development solution. The color reaction was stopped, and the bands were fixed. The gel was air dried for approximately 2 h. SSCP profiles were interpreted visually.

PCR analysis. PCR was used to assay 64 *V. cholerae* and 5 *V. mimicus* isolates for the presence of 12 regions associated with *V. cholerae* virulence. Of the 12 virulence regions examined, 10 regions were comprised of three or more genes (VSP-I, MSHA pilin, VSP-II, VPI-1, Repeat in toxin [RTX], RS1ϕ, CTXϕ, TLC, VPI-2, and class 1 integron) and 2 loci were single gene regions (*hlyA* and *pilE*). Of the 10 virulence gene clusters examined, 7 are associated with mobile genetic elements (Fig. 1). A total of 31 primer pairs were used to determine the distribution of the 12 regions among the 69 *Vibrio* isolates (Table 2). Five primer pairs were used to assay for the presence of VSP-I, four primer pairs were used to assay for MSHA, nine primer pairs were used to assay for VSP-II, three primer pairs were used to assay for VPI-1, four primer pairs were used to assay for the presence of CTXϕ, and one primer pair (each) was used to assay for the presence of *pilE*, *hlyA*, RTX, RS1ϕ, TLC, and *intl4* (Table 2). Gene fragments were amplified from chromosomal DNA isolated from the 64 *V. cholerae* strains and the 5 *V. mimicus* strains. PCR was performed in a 20-μl reaction mixture by using the following cycles: an initial denaturation step at 96°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, 30 s of primer annealing at 45 to 58°C, and 1 to 4 min of primer extension at 72°C (Table 2).

Southern blot analysis. To confirm negative PCR results, Southern hybridization analysis was carried out. DNA from each strain of interest was digested with the restriction enzyme EcoRI (Roche Molecular Biochemicals) and separated by electrophoresis in 0.6% (wt/vol) 1× Tris-borate-EDTA agarose. Separated DNA fragments were transferred to a nitrocellulose membrane for Southern hybridization. A single DNA probe was generated for each of the 12 regions by PCR amplification with *V. cholerae* strain N16961 as a template and labeled with horseradish peroxidase to verify the absence of a particular gene. Southern hybridization was carried out by using the enhanced chemiluminescence direct nucleic acid labeling and detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech). In all experiments, *V. cholerae* strain N16961 was used as a positive control.

RESULTS

Genetic variation at the *mdh* locus among *V. cholerae* isolates. To determine the evolutionary genetic relationships among our collection of *V. cholerae* isolates, we analyzed a 648-bp region of the housekeeping gene malate dehydrogenase (*mdh*) from 36 *V. cholerae* isolates and 5 *V. mimicus* isolates. Previous studies have shown that comparative nucleotide sequence analysis of the *mdh* locus is a reliable indicator of overall genetic relationships between strains (6). Within the 648-bp region from the 36 *V. cholerae* strains examined, there was a total of 44 polymorphic sites, which included two amino acid replacement sites (Table 3; Fig. 2). Of the 44 polymorphic sites, 22 were phylogenetically informative (at least two or more sequences contained the polymorphism) (Table 3). The average pairwise difference for the 36 *V. cholerae mdh* sequences was 1.03%, with a maximum pairwise difference of 4.61% observed between *V. cholerae* strain DK71, an environmental O66 serogroup strain from Germany, and *V. cholerae* strain 9581, a clinical O41 serogroup isolate from India. Eight epidemic *V. cholerae* O1 El Tor and O139 serogroup strains examined had identical *mdh* sequences which differed from classical biotype strains at a single site. Three *V. cholerae* O37 serogroup strains, V52, V53, and CO130, one O8 serogroup strain, V54, and one rough strain, V45, had *mdh* sequences identical to the El Tor O1 and O139 serogroup *mdh* sequence. Among the 23 *V. cholerae* non-O1 and non-O139 isolates examined at the *mdh* locus, there were a total of 43 polymorphic sites, which resulted in 42 synonymous polymorphic sites and 1 nonsynonymous polymorphic site (Table 3). An additional 25 *V. cholerae* O1 and O139 serogroup isolates were examined for sequence variation at the *mdh* locus by PCR-SSCP analysis (Table 1) (Fig. 3). The sensitivity of PCR-SSCP tends to de-

TABLE 1. Strains used in this study

Strain	Serogroup (biotype)	Source	Place of isolation	Yr of isolation
V. cholerae				
Sixth pandemic classical strains				
O395	O1 (classical)	Clinical	India	1964
569B	O1 (classical)	Clinical	India	1948
C1	O1 (classical)	Unknown	Unknown	1955
C14	O1 (classical)	Environmental	Unknown	1973
CA401	O1 (classical)	Clinical	India	1953
GP12	O1 (classical)	Unknown	India	1971
Seventh pandemic El Tor strains				
GP33	O1 (El Tor)	Unknown	Union of Soviet Socialist Republics	1971
GP43	O1 (El Tor)	Unknown	Australia	1972
GP155	O1 (El Tor)	Unknown	Australia	1979
N16961	O1 (El Tor)	Clinical	Bangladesh	1975
3038	O1 (El Tor)	Clinical	Vietnam	Unknown
F1873	O1 (El Tor)	Clinical	Rwanda	1994
F1875	O1 (El Tor)	Clinical	Goma, Zaire	1994
F1939	O1 (El Tor)	Clinical	Rwanda	1994
SM115	O1 (El Tor)	Clinical	Bahrain	1978
2125–98	O1 (El Tor)	Clinical	Bangladesh	1998
2164–78	O1 (El Tor)	Environmental	U.S. Gulf Coast	1978
Pre-seventh pandemic El Tor strains				
RV79	O1 (El Tor)	Clinical	Indonesia	1937
C5	O1 (El Tor)	Unknown	Indonesia	1957
Nontoxigenic El Tor strains				
2740–80	O1 (El Tor)	Environmental	U.S. Gulf Coast	1980
468–83	O1 (El Tor)	Clinical	United States	1983
O1 serogroup strains of unknown biotype				
1528–79	O1	Environmental	Louisiana	1979
917–84	O1	Clinical	Georgia (United States)	1984
O139 serogroup				
36054–98	O139	Clinical	Bangladesh	1998
SG20	O139	Clinical	Calcutta, India	Unknown
AS207	O139	Clinical	Calcutta, India	1996
AS209	O139	Clinical	Calcutta, India	1996
AS212	O139	Clinical	Calcutta, India	1996
AS213	O139	Clinical	Calcutta, India	1996
AS231	O139	Clinical	Calcutta, India	1996
AS259	O139	Clinical	Calcutta, India	1996
AS260	O139	Clinical	Calcutta, India	1996
35636–97	O139	Clinical	Bangladesh	1997
MO2	O139	Clinical	Madras, India	1992
MO10	O139	Clinical	Madras, India	1992
MO45	O139	Clinical	Madras, India	1992
Non-O1 and non-O139 serogroup strains				
151	O37	Environmental	Mexico	1998
CO476	O37	Clinical	Calcutta, India	1994
VO7	O37	Environmental	Varanasi, India	1988
CO130	O37	Environmental	Calcutta, India	1993
V45	Rough	Clinical	Bangladesh	1978
V46	O141	Clinical	United States	1978
V47	O141	Clinical	United States	1984
V52	O37	Clinical	Sudan	1968
V53	O37	Clinical	Sudan	1968
V54	O8	Clinical	Thailand	Unknown
SG3	O32	Clinical	Calcutta, India	1992–1993
SG6	O45	Clinical	Calcutta, India	1992–1993
SG7	O56	Clinical	Calcutta, India	1992–1993
SG8	O37	Clinical	Calcutta, India	1992–1993
SG9	O38	Clinical	Calcutta, India	Unknown
SG10	O69	Clinical	Calcutta, India	1992–1993
SG14	O54	Clinical	Calcutta, India	1992–1993
AM112	O39	Clinical	Calcutta, India	1996
VIG1613	O12	Clinical	Peru	Unknown
DK59	O70	Environmental	Germany	1994
DK67	O74	Environmental	Korea	1994
DK71	O66	Environmental	Germany	1994

Continued on following page

TABLE 1—*Continued*

Strain	Serogroup (biotype)	Source	Place of isolation	Yr of isolation
SCE4	O8	Environmental	India	1997
SCE188	O44	Environmental	India	1997
208	O11	Clinical	Thailand	1998
C43	NAG	Clinical	Unknown	Unknown
9581	O41	Clinical	India	1990
9582	Rough	Clinical	India	1990
V. mimicus				
PT5	O115	Clinical	Bangladesh	1985
PT48	O115	Clinical	Bangladesh	1985
9583	O115	Clinical	United States	1980
523–80	O115	Clinical	United States	1980
531–90	O41	Clinical	Japan	1990

crease with increasing fragment length, therefore the 892-bp amplicon was digested with HindIII to generate shorter fragments before PCR-SSCP analysis. Undenatured, digested *mdh* DNA of the *V. cholerae* strain produced two HindIII restricted bands of ~500 and ~300 bp. Denatured, digested *mdh* DNA produced 11 HindIII-restricted bands for all *V. cholerae* strains tested. Of the 25 strains analyzed, 23 exhibited PCR-SSCP profile 1, 1 classical strain, CA401, exhibited PCR-SSCP profile 2, and strain GP43 exhibited profile 3 (Fig. 3). There is a minor difference in the banding pattern of the three profiles, which could have resulted from a single nucleotide substitution. Overall, the *mdh* sequence and PCR-SSCP analyses indicate that the epidemic *V. cholerae* isolates at the *mdh* locus are highly homologous.

Genetic variation at the *mdh* locus between *V. cholerae* and *V. mimicus* isolates. Analysis of the *mdh* sequence from the five clinical *V. mimicus* isolates identified seven polymorphic sites, six synonymous polymorphic sites, and one nonsynonymous site among these isolates (Table 3). Clinical *V. mimicus* O115 serogroup strains PT5, PT48, 9583, and 523-80 all had identical *mdh* sequences, which differed from strain 531-90, a clinical O41 serogroup isolate recovered in Japan in 1990. Comparative nucleotide sequence analysis of the *mdh* locus between *V. cholerae* and *V. mimicus* isolates revealed a total of 91 polymorphic nucleotide sites, of which 81 sites were phylogenetically informative (Table 3). Of the 91 polymorphic sites, 45 were unique to *V. mimicus* isolates and resulted in two amino acid replacements (Fig. 2). The average pairwise difference for the 36 *V. cholerae* and 5 *V. mimicus mdh* sequences was 3.4%, the maximum difference of 12.03% was between the *V. mimicus* isolates and *V. cholerae* isolates, which is similar to the divergence between *Escherichia coli* and *Salmonella enterica* serovar Typhimurium isolates at the *mdh* locus.

Genetic variation at *groEL*. To elucidate further the relationships between *V. cholerae* O1 serogroup isolates, we examined 5 classical, 10 El Tor, 5 O139, and 2 O37 isolates by PCR-SSCP analysis at an additional locus, *groEL*. One of the most widely used techniques to localize mutations is PCR-SSCP, which is capable of detecting almost 100% of mutations. Alteration of the nucleotide sequence of the molecule by as little as a single base can reshape the secondary structure, with consequent changes in electrophoretic mobilities through a gel (52). The 1.6-kb *groEL* PCR amplicon was digested with BstYI, which resulted in four bands of 705, 435, 342, and 153 bp. Denatured, digested *groEL* DNA produced 11 bands representing profile 1 for all strains examined (Fig. 4). As can be seen from Fig. 4, *V. cholerae* classical and El Tor biotype strains gave identical banding patterns at *groEL*, indicating a lack of polymorphic sites in this gene among these isolates. In addition, *V. cholerae* O37 serogroup isolates V52 and V53 were also examined by PCR-SSCP at the *groEL* locus and, as expected, gave an identical banding pattern to the epidemic isolates, again confirming a common origin (data not shown). Taken together, the *mdh* sequence analysis and PCR-SSCP analyses at the *mdh* and *groEL* loci indicate that the *V. cholerae* O1 classical and El Tor biotypes and O139 serogroup strains are a highly homologous group of isolates representing a single clonal lineage.

Evolutionary genetic relationships among *V. cholerae* natural isolates. From the 36 *V. cholerae* and 5 *V. mimicus mdh* sequences, we constructed a neighbor-joining tree based on synonymous polymorphic sites, which are sites in a codon predicted to not result in amino acid replacements and are therefore not under selective pressure (Fig. 5). The *mdh* gene tree groups *V. cholerae* O1 classical and El Tor isolates and O139 serogroup isolates together to form an epidemic clone complex (Fig. 5). Interestingly, several *V. cholerae* non-O1 and non-O139 serogroup isolates also clustered with this epidemic clone complex: three toxigenic *V. cholerae* serogroup O37 strains, V52, V53, isolated in Sudan in 1968, and CO130, isolated in India in 1993, and one toxigenic O8 serogroup strain, V54, recovered in Thailand. In addition, *V. cholerae* strain V45, a rough isolate clustered with the epidemic clone, as well as a nonagglutinable strain, C43, and a nontoxigenic clinical O1 serogroup strain, 468-83, isolated on the U.S. Gulf Coast in 1983 (Fig. 5). Comparative sequence analysis also demonstrates that strains of the same serogroup may belong to two or more widely divergent lineages (Fig. 5). Thus, for example, of the seven *V. cholerae* O37 serogroup strains examined, the remaining four strains (SG8, 151, CO476, and VO7), which are nontoxigenic clinical and environmental isolates (SG8, CO476, and VO7 were isolated in India and 151 was isolated in Mexico), were found on four separate branches of the *mdh* gene tree, indicating their diverse evolutionary origins (Fig. 5). A similar picture emerges from the analysis of two O141 strains (V46 and V47) examined; they are also found on divergent branches of the *mdh* tree, suggesting that serogroup designation is not an indicator of overall relatedness but represents

TABLE 2. PCR primers used in this study

Primer	Sequence (5′–3′)	Predicted PCR product size (bp)	Annealing temp (°C)	Reference or source
Housekeeping gene primers				
mdh1	ATGAAAGTCGCTGTTATT	892	53.9	7
mdh2	GTATCTAACATGCCATCC			
groEL1A	GATCCATATGGCTGCTAAAGACGTACG	1,600	58	This study
groEL1B	CTAGGTCGACTTACATCATGCGGCCCATGC			
Virulence gene primers				
VSP-1				
VC0175F	TGGATGCTCTCTTCTTCA	2,834	52	This study
VC0175R	CGCTCACTCACTAATACCGAG			
VC0178F	AGAGGCTTGTTTACTATCAG	2,053	50	This study
VC0178R	ATCGGTACTGTCAGGGCT			
VC0180F	GGATGAGCAAATACAGCTAAC	2,283	50	This study
VC0180R	CTAGGAAGAATTTTATCGGC			
VC0183F	CAGTAAGAGTGTAGCGTGCC	3,389	52	This study
VC0183R	CCTGCACATCGAGATGC			
VC0185F	AGGAGGCGTGTAAGTCATAGC	1,110	55	This study
VC0185R	AGACCACGAATACCTGCTCC			
MSHA				
msha398F	GGAACGTGGCACAAATG	3,000	49	This study
msha398R	TGACGTAAGTGAGCCGC			
msha400F	AAGATGAAATCGGGTTG	2,212	45	This study
msha400R	TATCTGGCGACGCTTGC			
msha403F	GAACCGATTTATCTGTAGGAG	3,874	51	This study
msha403R	TGACCGCCATTATCTGATAC			
msha406F	CGAGTATTAAGGTACTGAAGG	596	50	This study
msha406R	ATCGGTCAGCTTGATCG			
HlyA				
VC0489F	AGATCAACTACGATCAAGCC	1,677	54.2	This study
VC0489R	AGAGGTTGCTATGCTTTCTAC			
VSP-II				
VC0490F	CGTGAAGGGATATAGGAG	2,337	49.6	This study
VC0490R	TGCAGTTGTTGAATGGAC			
VC0493F	AATGCTTCTCAGGGGGGTCTT	3,600	57	This study
VC0493R	CGCTCTTCTTTCCACGCTTCA			
VC0498F	AGGTGGTATCGGGCTGGT	4,140	58	This study
VC0498R	TGCGGCTGGAATGGAGTCTG			
VC0502F	TCATCAGTTAGCACACGAAC	476	52	This study
VC0502R	GCTATCGTTATACTTGGCG			
VC0504F	CAGCAAAGGCGGAAGAGGTAG	3,240	55	This study
VC0504R	AGCCCGAAATGAATCCCAAAA			
VC0512F	CAGTGGCTTCGCAGAGGA	3,900	53	This study
VC0512R	CCCTCCACTGCTATTCCG			
VC0514F	TTATGATCCAAGGAGTAGGG	2,089	52	This study
VC0514R	AGGCTGAAAAACAACTTGAG			
VC0516F	GTTTTCTGCGTTGTTCGAG	965	52	This study
VC0516R	TCCTGATGTCTCTCTTGCCG			
VC0517F	CCCACTTCTTCCAGAGTATG	1,753	54.3	This study
VC0517R	CGCAGTCACAGCTTAAACAAC			
VPI-1				
tcpH1	AGCCGCCTAGATAGTCTGTG	2,176	51.7	51
tcpA4	TCGCCTCCAATAATCCGAC			
toxt1	AGGAGATGGAAGTGGTGTG	1,055	48.7	53
toxt2	CTTGGTGCTACATTCATGG			
acfB1	GATGAAAGAACAGGAGAGA	1,180	49	53
acfB2	CAGCAACCACAGCAAAACC			
PilE				
PilEF	CATACCTTTTGAGCATCGAC	3,087	50	This study
PilER	GTGGCAAGAAGGACTCG			
RTX				
rtxA1	GCGATTCTCAAAGAGATGC	1,366	53.8	42
rtxA2	CACTCATTCCGATAACCAC			
RS1ϕ				
rstC1	AACAGCTACGGGCTTATTC	238	52.4	66
rstC2	TGAGTTGCGGATTTAGGC			

Continued on following page

TABLE 2—*Continued*

Primer	Sequence (5′–3′)	Predicted PCR product size (bp)	Annealing temp (°C)	Reference or source
CTXϕ				
rstA1	ACTCGATACAAACGCTTCTC	1,009	53.7	66
rstA2	AGAATCTGGAAGGTTGAGTG			
orfU	CGTCACACCAGTTACTTTTCG	1,072	54.5	62
orfU	AGAATGTACGCCATCGC			
zot1	GGCTTAAACCTTGAACGC	1,036	54.7	24
zot2	AACCCCGTTTCACTTCTAC			
ctxA1	AGTCAGGTGGTCTTATGCC	1,037	51.2	This study
ctxB2	TTGCCATACTAATTGCGG			
tlc3	GGGAATGTTGAGTTCTCAGTG	1,548	55.5	56
tlc4	GTTGCGAAGTGGATTTTGTG			
intl4:3	CCTTCATTGGATCACTCG	597	51.9	This study
intl4:4	GACGGAAAAAGATAGTGCC			

lateral gene transfer of the O-antigen among strains. Of the remaining 17 *V. cholerae* non-O1 and non-O139 isolates examined at the *mdh* locus, strains SG7, V47, and DK71 formed the most divergent branches. The non-O1 and non-O139 serogroup strains formed separate lineages from the epidemic strains but in general are closely related to one another, hence, the very small branch lengths. Two clinical *V. cholerae* strains, AM112, an O39 serogroup isolate from India, and VIG1613, an O12 serogroup isolate from Peru, clustered together, indicating identity. In addition, an O45 serogroup strain, SG6, from India and an O70 serogroup strain, DK59, from Germany clustered together, as did strains VO7, V46, and 1528-79 (Fig. 5). These data suggest the occurrence of clones of wide geographic distribution.

We also identified two strains, 9581 and 9582, which were originally designated *V. mimicus* but clustered with *V. cholerae* non-O1 and non-O139 serogroup isolates on the *mdh* gene tree (Fig. 5). To determine the species designation of these isolates, we performed two biochemical tests previously used to differentiate *V. cholerae* and *V. mimicus* isolates: the Voges-Proskauer and corn oil tests. As expected, strains 9581 and 9582 were positive for both tests, similar to the control *V. cholerae* strains tested, indicating that these strains are indeed *V. cholerae*.

Evolutionary genetic relationships between *V. cholerae* and *V. mimicus*. As expected, the five *V. mimicus* isolates formed a separate divergent branch from the *V. cholerae* isolates on the *mdh* gene tree. Four *V. mimicus* isolates, PT5, PT48, 9583, and 523-80, clustered together, and strain 531-90 formed a separate divergent lineage (Fig. 5).

Presence of virulence regions in *V. cholerae* and *V. mimicus*. In total, 64 *V. cholerae* strains were examined for the presence of 12 regions associated with virulence in *V. cholerae* by PCR assays with 31 primer pairs (Table 2). Of the six classical biotype strains assayed by PCR, all strains contained the same nine regions, MSHA, *hlyA*, VPI-1, *pilE*, RTX, CTXϕ, TLC, VPI-2, and *intl4* and lacked RS1ϕ, VSP-I, and VSP-II (Table 4). The 15 El Tor biotype strains analyzed were divided into three groups based on the year of isolation and the presence of *ctxAB* (Table 1). Of the 11 toxigenic seventh pandemic strains examined by PCR analysis, 7 strains contained all 12 virulence regions examined. Two toxigenic El Tor strains recovered from Australia in the 1970s, GP155 and GP43, lacked VSP-I and VSP-II and VSP-II, respectively, by PCR and Southern blot analyses (Table 4). In addition, strain GP33 lacked TLC by PCR and Southern blot analyses. PCR analysis with six primer pairs (Table 2) showed that the VSP-II region was larger than previously documented (18) and encompassed an additional 19.4-kb region from VC0498 to VC0516. From our PCR analysis, we estimate that the VSP-II region is an ~27-kb region encompassing VC0490 to VC0516 (Fig. 1). PCR assays indicated that VC0489 marked the 5′ flanking region and was

TABLE 3. Sequence variation at the *mdh* locus among *V. cholerae* and *V. mimicus* strains

No. of strains	Fragment size (bp)	Total no. of sites					ds ± SE	dn ± SE
		Polymorphic	Synonymous	Nonsynonymous	Informative	Singleton		
Within *V. cholerae*								
36	648	44	42	2	22	22	0.038 ± 0.008	0.0004 ± 0.0003
Within O1/O139 serogroups								
13	648	9	8	1	2	7	0.008 ± 0.003	0.0006 ± 0.0006
Within non-O1/non-O139 serogroups								
23	648	43	42	1	21	22	0.049 ± 0.010	0.0002 ± 0.0002
Within *V. mimicus*								
5	648	7	6	1	0	7	0.014 ± 0.006	0.001 ± 0.001
Between *V. cholerae* and *V. mimicus*								
41	648	91	87	4	81	10	0.144 ± 0.017	0.003 ± 0.001

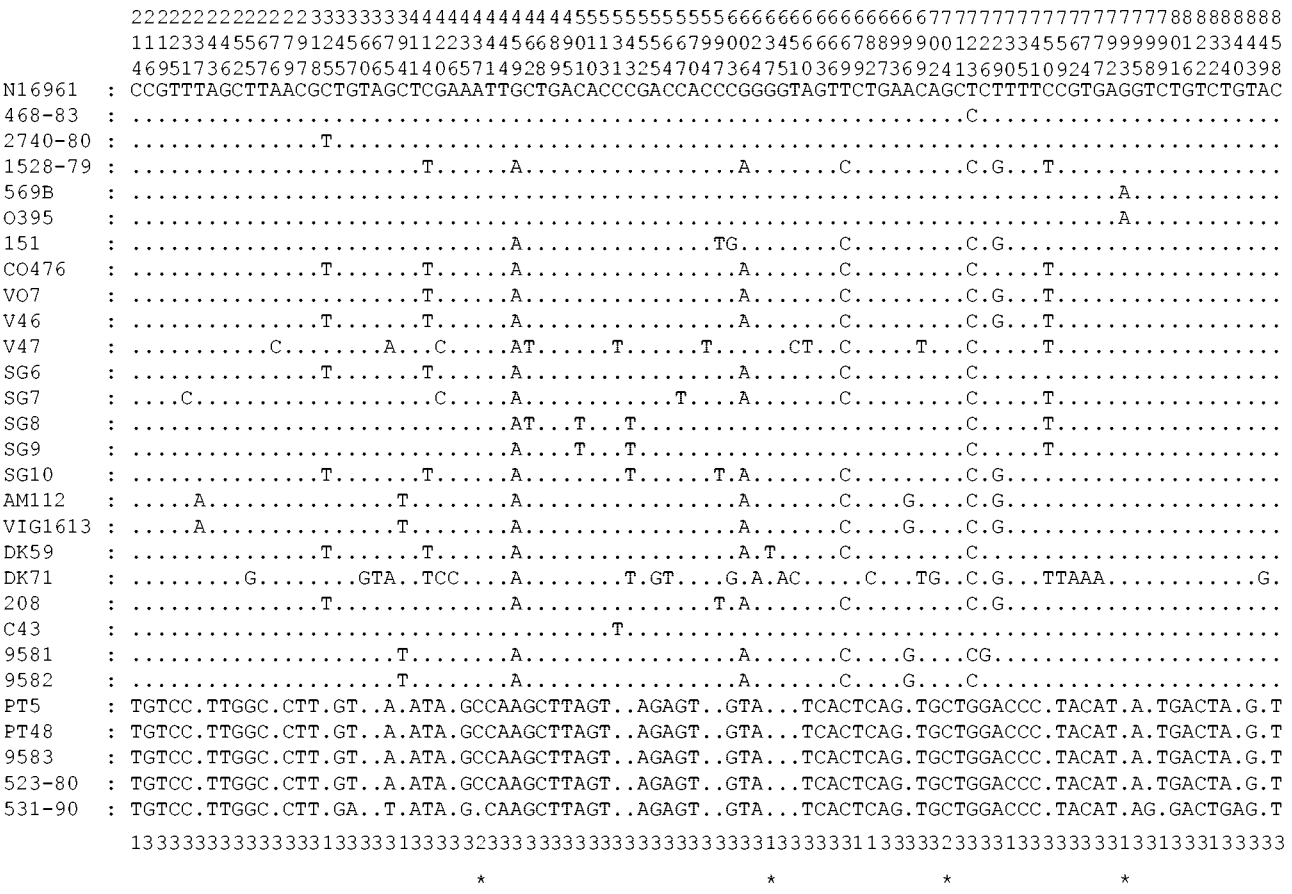

FIG. 2. Polymorphic sites within the *mdh* gene of *V. cholerae* and *V. mimicus* isolates. N16961 represents 3038, F1875, RV79, AS207, MO10, MO45, CO130, V45, V52, V53, and V54. The numbering of the polymorphic sites (vertical format) is from the first position of the sequence segment. The position within the codon for each polymorphic site is shown below the sequences. Asterisks indicate polymorphic sites that gave rise to an amino acid change.

found in all *V. cholerae* strains examined. Similarly, PCR assays with primer pair VC0517F-VCO517R showed that VC0517 marked the 3′ flanking region of VSP-II and was present in all *V. cholerae* strains examined. An environmental toxigenic El Tor isolate 2164-78, recovered in the United States in 1978, was shown not to contain VSP-I, VSP-II, and TLC by PCR and Southern blot analyses. A clinical pre-seventh pandemic El Tor strain RV79 isolated in Indonesia in 1937 lacked only three of the virulence regions examined: VSP-I, VSP-II, and VPI-2 (Table 4). The El Tor strain C5 isolated 20 years later in Indonesia contained 11 of the virulence regions with only RS1ϕ missing. Two nontoxigenic El Tor isolates, 468-83 and 2740-80, isolated in the United States in the early 1980s both lacked VSP-I, VSP-II, RS1ϕ, and CTXϕ; additionally, strain 468-83 did not contain VPI-1 and TLC (Table 4). Two *V. cholerae* O1 serogroup strains of unknown biotype, 1528-79 and 917-84, were examined. Neither contained VSP-I, VSP-II, nor RS1ϕ; strain 1528-79 also lacked VPI-1, CTXϕ, TLC, and VPI-2 (Table 4). The 13 *V. cholerae* O139 serogroup strains examined yielded positive PCR bands for all 12 virulence regions. However, as previously shown, there was a partial deletion of VPI-2 from 12 of the 13 O139 strains examined (31). These 12 O139 serogroup strains only contained a 20-kb 3′ region of VPI-2. Strain MO2 isolated in India in 1992 contained the entire VPI-2 region (Table 4) (31).

Twenty-eight *V. cholerae* non-O1 and non-O139 serogroup strains were assayed by PCR for the presence of 12 virulence regions (Table 4). None of the 28 strains examined by PCR and Southern blot analyses contained VSP-II, and only one strain, VO7, an environmental O37 serogroup isolate from India, contained VSP-I (Fig. 6). Only four regions MSHA, *hlyA*, *pile*, and RTX were present in all 28 *V. cholerae* non-O1 and non-O139 serogroup strains. The *intl4* region was absent in strain CO476. The three O37 serogroup strains, V52, V53, and CO130, which form part of the epidemic clone complex, lacked VSP-I and VSP-II; CO130 also lacked VPI-2. Similarly, strain V54 only lacked VSP-I and VSP-II, as did strain V45, which also lacked RS1ϕ (Table 4).

Among the five *V. mimicus* strains investigated, all gave negative PCR results for eight regions assayed: VSP-I, MSHA, *hlyA*, VSP-II, *pilE*, RTX, TLC, and *intl4*. Four *V. mimicus* strains, PT5, PT48, 9583, and 523-80, contained both VPI-1 and CTXϕ; two strains (PT5 and PT48) also contained RS1ϕ.

DISCUSSION

In this study we show that clinical epidemic *V. cholerae* O1, O139, and O37 serogroup isolates form a highly uniform clone and that the emergence of the sixth and seventh cholera pan-

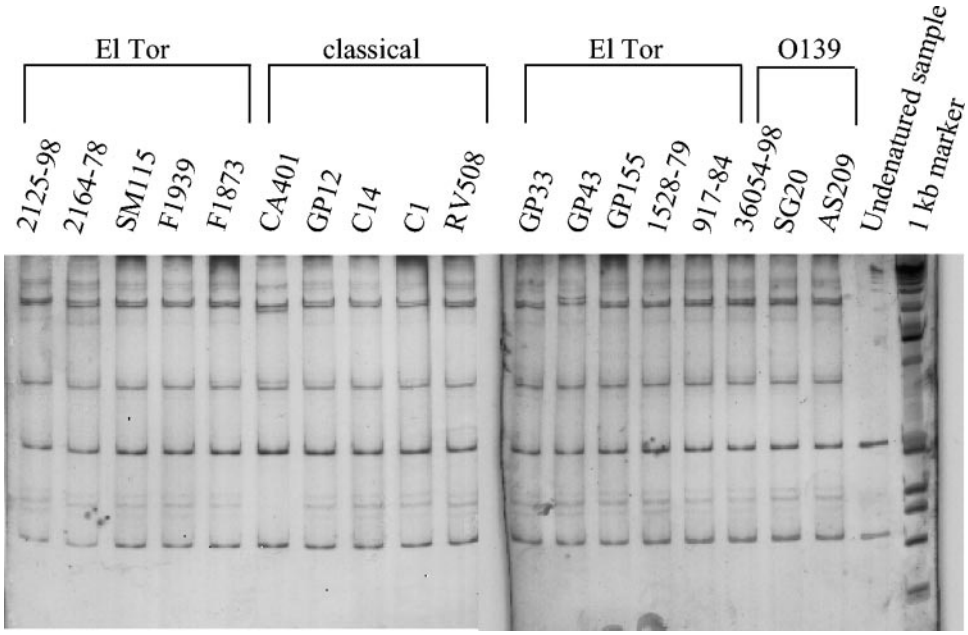

FIG. 3. PCR-SSCP profiles of *mdh* fragments after HindIII digestion of 18 strains (25 strains profiled, 7 strains not shown). PCR-SSCP profiles are as follows. Profile 1, 2125-98, 2164-78, SM115, F1939, F1873, GP12, C14, C1, RV508, GP33, GP155, 1528-79, 917-84, 36054-98, SG20, and AS209 (AS212, AS213, AS231, AS259, AS260, 35636-97, and MO2 are not shown); profile 2, CA401; profile 3, GP43.

demic strains resulted from the successive acquisition of virulence regions.

Genotypic and phenotypic analysis of two pre-seventh pandemic isolates, RV79 and C5, isolated in Indonesia in 1937 and 1957, respectively, give some interesting insights into a possible scenario for the evolution of epidemic isolates. El Tor strain RV79 is identical to other O1 serogroup strains at *mdh* and *groEL* and lacks only 3 of the 12 virulence regions, VSP-I, VSP-II, and VPI-2, examined in this study. El Tor strain C5, similar to RV79, is identical to other O1 serogroup strains, isolated 20 years later lacks only 1 of the 12 regions examined, RS1ϕ. Since classical biotype strains were still circulating in the human population prior to the emergence of the seventh cholera pandemic El Tor strain, the question arises as to whether El Tor seventh pandemic isolates arose from a classical progenitor strain via the acquisition of RS1ϕ, VSP-I, and VSP-II

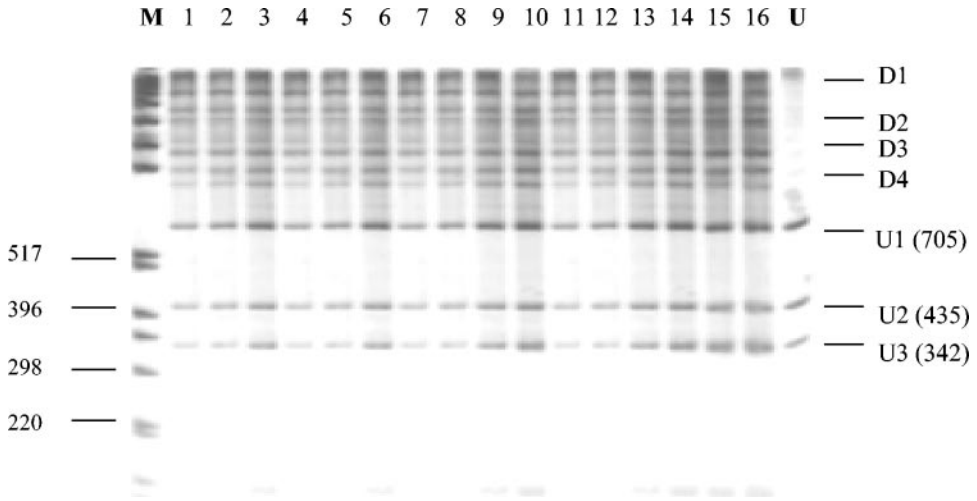

FIG. 4. PCR-SSCP profiles of *groEL* restriction fragments after BstYI digestion from three *V. cholerae* serogroups separated on a 0.8% nondenaturing polyacrylamide gel. The strains and respective serogroups are as follows: lanes 1 to 8, N16961, 2740-80, F1875, 3038, GP33, 2125-98, 2164-78, C5, O1 serogroup, biotype El Tor; lanes 9 to 12, CA401, O395, 569B, C14, O1 serogroup, biotype classical; lanes 13 to 15, MO10, MO45, 36054-98, O139 serogroup; lane 16, V52, O37 serogroup; lane M, Gibco-BRL 1-kb marker (with sizes in base pairs noted on the left); lane U, undenatured *groEL* restriction fragments from N16961. D1 to D4, single-stranded band pairs of the denatured 705-, 435-, 342-, and 153-bp BstYI *groEL* restriction fragments; U1 to U3, renatured double-stranded BstYI *groEL* restriction fragments (with sizes in base pairs noted in parentheses). The smaller 153- and 112-bp fragments were too faint to be presented here.

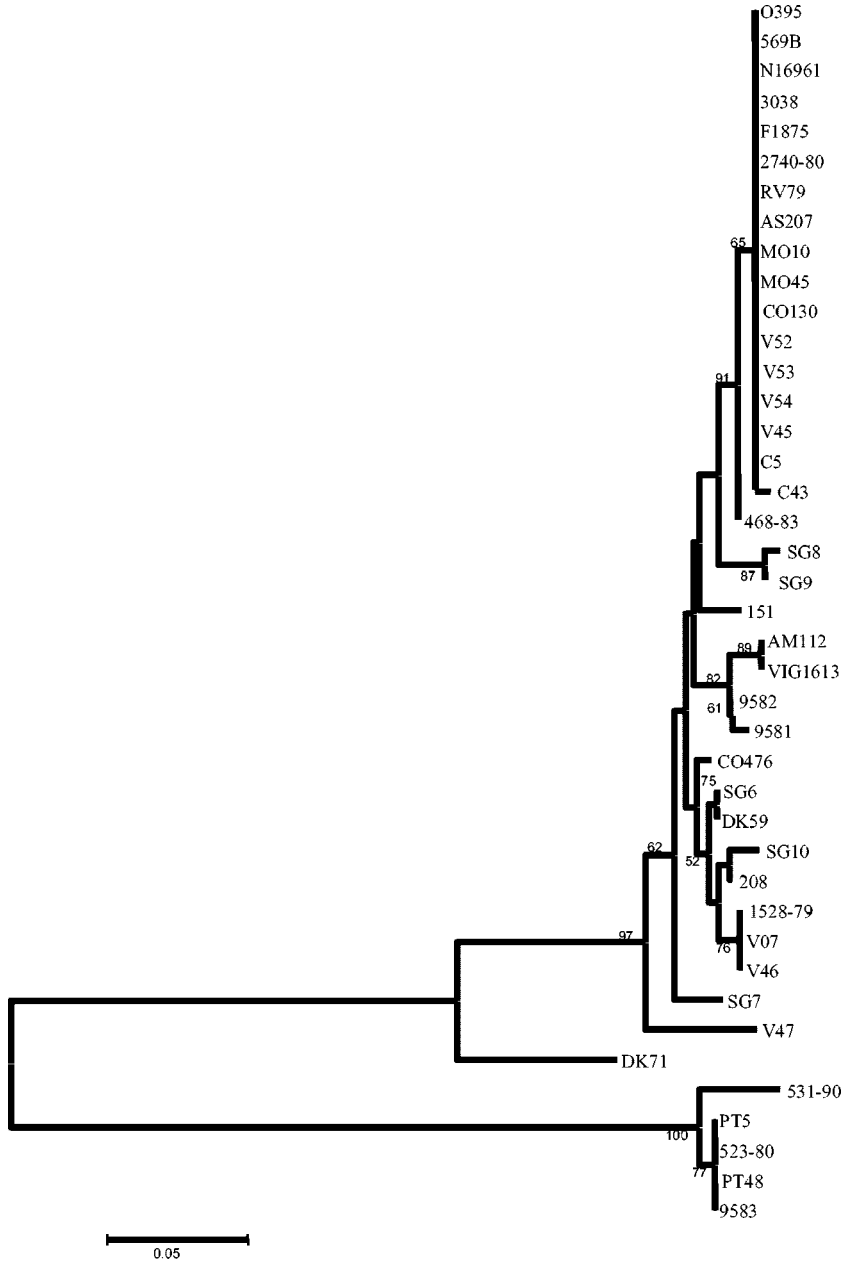

FIG. 5. Neighbor-joining tree constructed by the Jukes-Cantor method with the nucleotide sequences of *mdh* gene fragments of *V. cholerae* strains. Construction and bootstrapping of the trees were carried out with the MEGA suite of programs. One thousand bootstrap replicates were performed for each analysis, and bootstrap values are given at the nodes.

or, alternatively, whether they arose from an RV79 and C5 progenitor-like isolate. The most parsimonious scenario (one requiring the least number of steps) is that an O1 isolate acquired CTXϕ, VSP-I, VSP-II, and RS1ϕ (Fig. 7).

In 1968 there was a large outbreak of cholera in Sudan caused by an O37 serogroup isolate (68). Interestingly, Bik and colleagues (4) determined by IS*1004* fingerprinting that this O37 serogroup strain from Sudan is closely related to classical O1 strains and may have acquired the O37 biosynthesis genes via lateral gene transfer. Beltran et al. (2) confirmed the identity of the O37 strain to O1 strains by multilocus enzyme electrophoresis analysis and identified an additional O37 serogroup strain from India that was similar to O1 classical strains. Recently, an analysis of the O-antigen biosynthesis region also identified an O37 serogroup strain from India that had an O1 serogroup core genome (41). In our study, two *V. cholerae* O37 serogroup isolates (isolated in Sudan in 1968) had *mdh* sequences and PCR-SSCP profiles for *groEL* identical to those of the O1 and O139 strains, indicating that these have an O1 serogroup core genome. These strains likely arose by modification of an O1 strain similar to the emergence of the O139 serogroup clone as previously suggested by Bik et al. (4). Based on multilocus virulence gene profiles of the O37 and O8 serogroups, it is likely that strains V45, V52, V53, and V54 arose from a classical-like progenitor, since they lack only VSP-I and VSP-II; in addition, V45 lacks RS1ϕ, similar to classical

TABLE 4. Distribution of 12 regions associated with virulence among *V. cholerae* natural isolates as determined by PCR analysis[a]

Strain type and name	VSP-I (0175–0185)	MSHA (0398–0411)	hlyA (0489)	VSP-II (0490–0516)	VPI-I (0819–0847)	PilE (0857)	RTX (1447–1451)	RS1 (1452–1455)	CTXϕ (1456–1464)	TLC (1465–1480)	VPI-2 (1758–1809)	intI4 (vca0291)
O1 classical												
O395	−	+	+	−	+	+	+	−	+	+	+	+
569B	−	+	+	−	+	+	+	−	+	+	+	+
C1	−	+	+	−	+	+	+	−	+	+	+	+
C14	−	+	+	−	+	+	+	−	+	+	+	+
CA401	−	+	+	−	+	+	+	−	+	+	+	+
GP12	−	+	+	−	+	+	+	−	+	+	+	+
O1 E1 Tor												
GP33	+	+	+	+	+	+	+	+	+	−	+	+
GP43	+	+	+	−	+	+	+	+	+	+	+	+
GP155	−	+	+	−	+	+	+	+	+	+	+	+
N16961	+	+	+	+	+	+	+	+	+	+	+	+
3038	+	+	+	+	+	+	+	+	+	+	+	+
F1873	+	+	+	+	+	+	+	+	+	+	+	+
F1875	+	+	+	+	+	+	+	+	+	+	+	+
F1939	+	+	+	+	+	+	+	+	+	+	+	+
SM115	+	+	+	+	+	+	+	+	+	+	+	+
2125–98	+	+	+	+	+	+	+	+	+	+	+	+
2164–78	−	+	+	−	+	+	+	+	+	−	+	+
Pre-seventh pandemic E1 Tor strains												
RV79	−	+	+	−	+	+	+	+	+	+	−	+
C5	+	+	+	+	+	+	+	−	+	+	+	+
Nontoxigenic El Tor strains												
2740–80	−	+	+	−	+	+	+	−	−	+	+	+
468–83	−	+	+	−	−	+	+	−	−	−	+	+
O1 strains of unknown biotype												
1528–79	−	+	+	−	−	+	+	−	−	−	−	+
917–84	−	+	+	−	+	+	+	−	+	+	+	+
O139												
36054–98	+	+	+	+	+	+	+	+	+	+	+*	+
SG20	+	+	+	+	+	+	+	+	+	+	+*	+
AS207	+	+	+	+	+	+	+	+	+	+	+*	+
AS209	+	+	+	+	+	+	+	+	+	+	+*	+
AS212	+	+	+	+	+	+	+	+	+	+	+*	+
AS213	+	+	+	+	+	+	+	+	+	+	+*	+
AS231	+	+	+	+	+	+	+	+	+	+	+*	+
AS259	+	+	+	+	+	+	+	+	+	+	+*	+
AS260	+	+	+	+	+	+	+	+	+	+	+*	+
35636–97	+	+	+	+	+	+	+	+	+	+	+*	+
MO2	+	+	+	+	+	+	+	+	+	+	+	+
MO10	+	+	+	+	+	+	+	+	+	+	+*	+
MO45	+	+	+	+	+	+	+	+	+	+	+*	+
Non-O1/non-O139 strains												
151	−	+	+	−	+	+	+	−	+	−	+	+
CO476	−	+	+	−	−	+	+	−	−	+	−	−
VO7	+	+	+	−	+	+	+	−	−	+	−	+
CO130	−	+	+	−	+	+	+	+	+	+	−	+
V45	−	+	+	−	+	+	+	−	+	+	+	+
V46	−	+	+	−	+	+	+	+	+	−	+	+
V47	−	+	+	−	+	+	+	+	+	−	+	+
V52	−	+	+	−	+	+	+	+	+	+	+	+
V53	−	+	+	−	+	+	+	+	+	+	+	+
V54	−	+	+	−	+	+	+	+	+	+	+	+
SG3	−	+	+	−	−	+	+	+	+	−	−	+
SG6	−	+	+	−	−	+	+	−	−	+	−	+
SG7	−	+	+	−	−	+	+	−	−	+	−	+
SG8	−	+	+	−	−	+	+	−	−	+	−	+
SG9	−	+	+	−	−	+	+	−	−	−	−	+
SG10	−	+	+	−	−	+	+	+	−	+	−	+
SG14	−	+	+	−	−	+	+	+	−	+	+	+
AM112	−	+	+	−	−	+	+	−	−	+	+	+
VIG1613	−	+	+	−	−	+	+	−	−	−	+	+
DK59	−	+	+	−	−	+	+	−	−	−	−	+
DK67	−	+	+	−	−	+	+	−	−	−	−	+
DK71	−	+	+	−	−	+	+	−	−	−	+	+
SCE4	−	+	+	−	+	+	+	−	−	−	+	+
SCE188	−	+	+	−	+	+	+	+	+	+	+	+
208	−	+	+	−	+	+	+	+	+	−	−	+
C43	−	+	+	−	−	+	+	−	−	−	+	+
9581	−	+	+	−	+	+	+	−	−	−	+	+
9582	−	+	+	−	+	+	+	−	−	−	+	+

[a] The numbers in parentheses. refer to the genetic organization, e.g., 0175 is VC0175. Asterisks denote strains that only contain the phage-like region of VP1–2; the nan-nag region and restriction modification system are absent. +, present; −, absent.

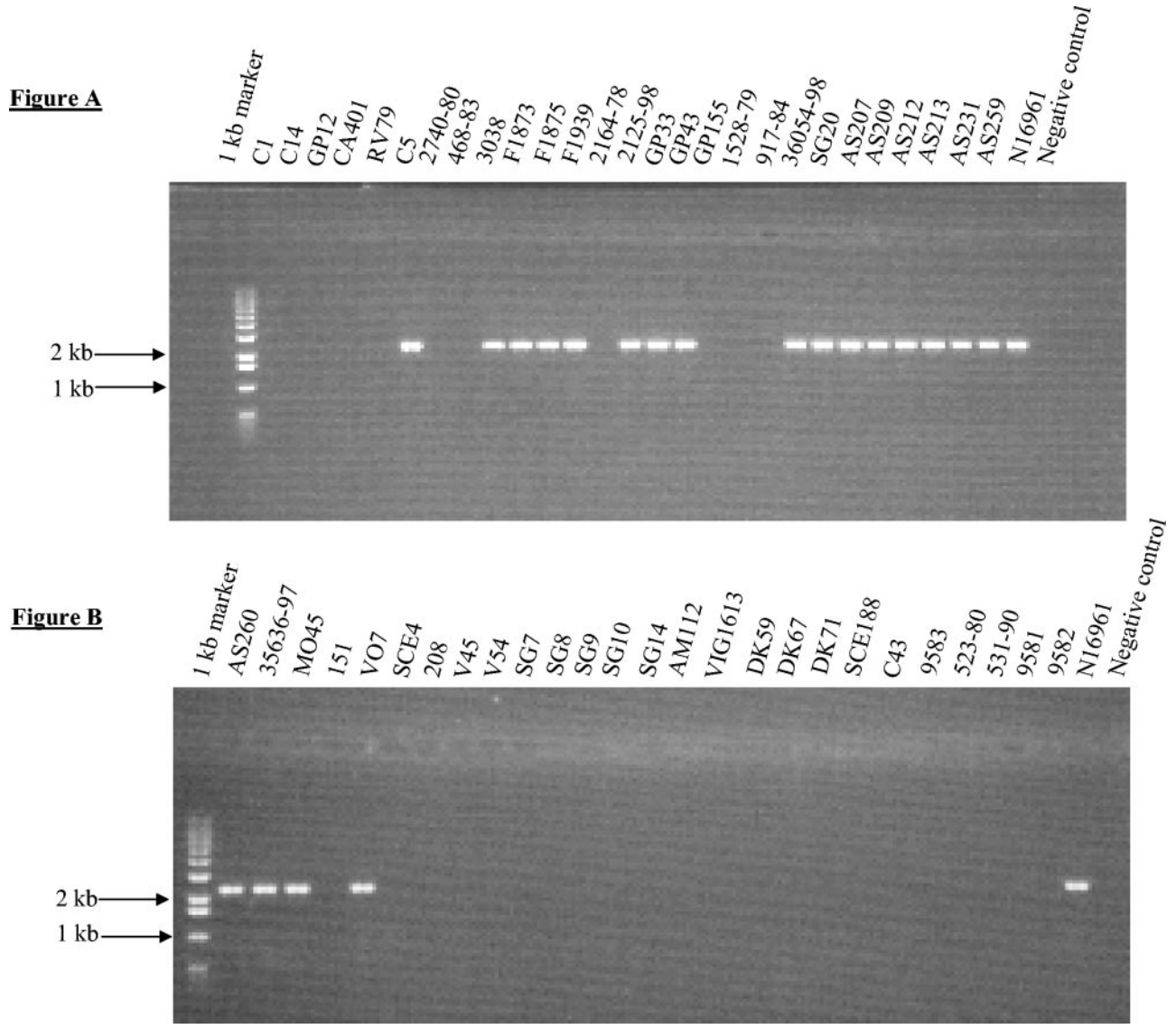

FIG. 6. PCR amplification of gene VC0180, which is part of VSP-I. (A) Lane 1, molecular size ladder; lanes 2 to 29, PCR amplicon with primer pair VC0180F-VC0180R. (B) Lane 1, molecular size ladder; lanes 2 to 27, PCR amplicon with primer pair VC0180F-VC0180R.

strains. Interestingly, a recent study with the infant mouse cholera model has shown that several non-O1 and non-O139 serogroup isolates, including O37 serogroup strains are efficient intestinal colonizers (8). Morris et al. (46) also demonstrated that a non-O1 and non-O139 *V. cholerae* strain was capable of causing severe diarrheal disease in humans.

In contrast to the data for O1 and O139 serogroup strains, which all belong to a single unique epidemic clone, our analyses indicate that strains from the same serogroup can belong to divergent lineages and that strains with different serogroup designations can belong to the same lineage, which is expected for regions, such as the O-antigen, that can be acquired by lateral gene transfer (2, 4, 9, 37, 41, 59).

Multilocus virulence gene profile analysis demonstrates the cooccurrence of several virulence regions among *V. cholerae* isolates (Table 5). For example, with the exception of SG3, CTXϕ was only found in strains containing VPI-1, which is to be expected, since it encodes the CTXϕ receptor TCP (64). In addition, RS1ϕ was mainly present in isolates that also contained CTXϕ, which again is to be expected, since recent data suggest that both elements require each other for transfer (22). Two strains, SG10 and SG14, however, contained only RS1ϕ. This observation may be explained by a recent finding that described an alternative mechanism for RS1ϕ transfer via a novel filamentous phage named KSF-1ϕ (23). Consistent with previous studies, we found that all classical strains examined in this study lacked RS1ϕ, VSP-I, and VSP-II and that these three regions are all present in El Tor strains (Table 4) (14, 18). Dziejman et al. (18) recently found VSP-I and VSP-II present only in El Tor isolates. In our study, we found VSP-I in an O37 serogroup strain and both VSP-I and VSP-II were absent from several El Tor isolates. Furthermore, Dziejman and colleagues found that VSP-II encompassed open reading frames VC0490 to VC0497; however, we found that VSP-II

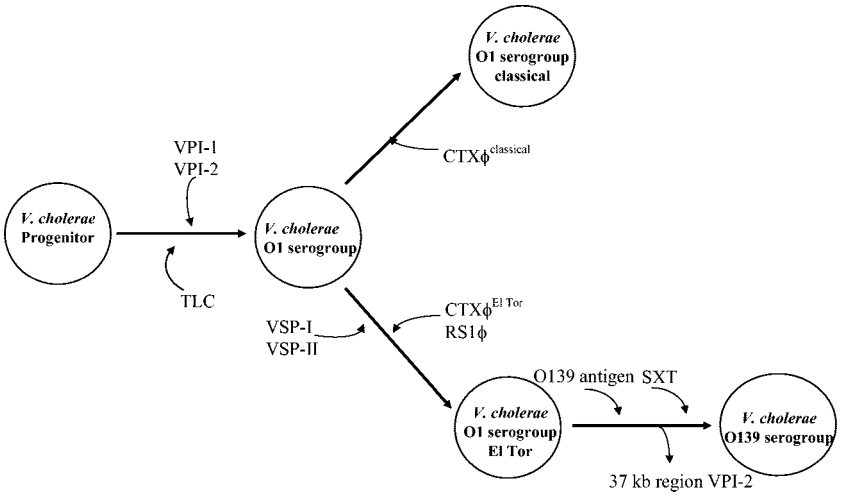

FIG. 7. Hypothetical evolutionary scenario for the emergence of epidemic *V. cholerae* O1 and O139 isolates. From the left, the parsimonious evolutionary steps beginning with the *V. cholerae* progenitor to the right to the contemporary states are indicated. The model begins with a *V. cholerae* ancestor that most resembles the present day *V. cholerae* isolates in metabolic functions. From this ancestral state, *V. cholerae* natural isolates diverged from one another through mutation. The *V. cholerae* O1 serogroup strains appear to have only arisen once and given rise to the highly successful epidemic classical and El Tor biotype isolates through the independent acquisition of CTXϕ by these isolates. The *V. cholerae* El Tor biotype, which is responsible for the present seventh cholera pandemic, acquired additional virulence regions including RS1ϕ, VSP-I, and VSP-II. The *V. cholerae* O139 serogroup, which arose in 1992, acquired a new O-antigen as well as the SXT element.

spanned a larger region encompassing VC0490 to VC0516. Nonetheless, consistent with their results, we found that VSP-II is confined to seventh pandemic strains.

Four of the virulence regions, MSHA, *hlyA*, *pilE*, and RTX, were present in all *V. cholerae* isolates and absent from all *V. mimicus* strains examined, indicating that these regions were acquired after *V. cholerae* and *V. mimicus* diverged from their most recent common ancestor. Among the *V. mimicus* isolates examined, only three regions, VPI-1, CTXϕ, and TLC, were present. Previous studies of VPI-1 and CTXϕ have indicated recent interspecies lateral transfer between *V. cholerae* and *V. mimicus*, suggesting that transfer of virulence factors among isolates is an ongoing process.

Initially, a *V. cholerae* O1 serogroup strain first acquired the pathogenic island VPI-1, which encodes TCP, an essential colonization factor and the receptor for CTXϕ. This proposition is supported by the near sequence identity between classical and El Tor biotype strains across most of the VPI-1 region (36). The hypervariability documented at the *tcpA* gene is likely the result of positive Darwinian selection in this region (8). A second pathogenic island, VPI-2, which encodes genes involved in restriction modification and *N*-acetyl neuraminic acid utilization, is found predominantly among O1 and O139 epidemic *V. cholerae* isolates (31) and was most likely present in an O1 serogroup strain that gave rise to classical and El Tor biotype strains. Following the acquisition of VPI-1 and VPI-2 by an O1 serogroup progenitor strain, classical and El Tor biotype isolates emerged and diverged from one another through the acquisition of VSP-I, VSP-II, and RS1ϕ. Studies based on comparative nucleotide sequence analysis of CTXϕ genes indicate that this region was acquired independently in classical and El Tor biotype isolates (7). The *V. cholerae* classical biotype was responsible for the sixth cholera pandemic, which began in 1899, and presumably previous cholera pandemics. *V. cholerae* El Tor biotype isolates, which are responsible for the ongoing seventh cholera pandemic, which began in 1961, acquired at least three regions in addition to CTXϕ: RS1ϕ, which facilitates CTXϕ production, and VSP-I and VSP-II, whose roles in *V. cholerae* virulence are unknown (18). The *V. cholerae* O139 strains that emerged in 1992 were derived from an El Tor progenitor by O-antigen switching likely facilitated by bacteriophages as well as the acquisition of a novel CTXϕ and SXT constin (4, 15, 66).

Since the beginning of the modern era of cholera pandemics,

TABLE 5. Cooccurrence of virulence regions among *V. cholerae*

Serogroup (biotype)	Total no. of strains	No. of strains containing:								
		VSP-I	VSP-II	VPI-I	PilE	RTX	RS1	CTXϕ	TLC	VPI–2
O1 (classical)	6	0	0	6	6	6	0	6	6	6
O1 (El Tor)	15	10	9	14	15	15	12	13	12	14
O1	2	0	0	1	2	2	0	1	1	1
O139	13	13	13	13	13	13	13	13	13	13[a]
Non-O1/non-O139	28	1	0	14	28	28	11	11	14	16

[a] These strains only contain the phage-like region of VP1–2; the *nan-nag* region and restriction modification system are absent. MO2 is an exception, it contains the entire VPI-2.

all epidemic *V. cholerae* isolates appear to have a highly conserved core genome onto which additional DNA was added via lateral transfer, facilitating pathogenesis. In addition, the sequence identity at *mdh* and *groEL* among *V. cholerae* epidemic O1, O139, and O37 isolates suggests that these strains have emerged recently, evolutionarily speaking, which is also indicated by the fact that humans are the only known animal hosts for *V. cholerae*.

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