JOURNAL OF BACTERIOLOGY, June 1995, p. 3191–3198 Vol. 177, No. 11
0021-9193/95/$04.00+0


# The Sixth and Seventh Cholera Pandemics Are Due to Independent Clones Separately Derived from Environmental, Nontoxigenic, Non-O1 *Vibrio cholerae*

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Received 28 November 1994/Accepted 31 March 1995

**The DNA sequences of the *asd* genes from 45 isolates of *Vibrio cholerae* (19 clinical O1 isolates, 2 environmental nontoxigenic O1 isolates, and 24 isolates with different non-O1 antigens) were determined. No differences were found within either sixth- or seventh-pandemic isolates; however, variation was found between the two forms and among the non-O1 isolates. O139 isolates had sequences identical to those of seventh-pandemic isolates. Phylogenetic trees with *Vibrio mimicus* as the outgroup suggest that the sixth-pandemic, seventh-pandemic, and U.S. Gulf isolates are three clones that have evolved independently from different lineages of environmental, nontoxigenic, non-O1 *V. cholerae* isolates. There is evidence for horizontal transfer of O antigen, since isolates with nearly identical *asd* sequences had different O antigens, and isolates with the O1 antigen did not cluster together but were found in different lineages. We also found evidence for recombination events within the *asd* gene of *V. cholerae*. *V. cholerae* may have a higher level of genetic exchange and a lower level of clonality than species such as *Salmonella enterica* and *Escherichia coli*.**

*Vibrio cholerae* is a normal inhabitant of aquatic environments (46). However, contaminated water supplies in some areas of the world have enabled some clones of the species to become pathogenic for humans. Seven pandemics of cholera have been recorded previously (12, 36). The seventh pandemic, beginning in 1961, is clonal (20, 22, 24, 37), is well defined, and has a known starting date, and previous studies (7, 22, 24, 37, 53) have shown it to have accumulated variation, with both random genetic drift and recombination possibly being involved. There are 140 known serotypes of *V. cholerae* (50). Until recently, all recorded pandemic and epidemic cases of cholera have been associated with the type O1 antigen; however, an epidemic which began in India late in 1992 and spread to several neighboring countries is caused by an O139 strain (1, 8, 33, 39). Studies have shown that the O139 strain is genetically very similar to strains of the seventh pandemic (13, 22, 37, 54). Karaolis et al. (22) have further shown that O139 isolates appear to have evolved from early seventh-pandemic isolates. Biochemically, it is nearly impossible to distinguish O1 and non-O1 *V. cholerae* (10, 38). *V. cholerae* non-O1 strains are far more frequently isolated from environmental sources than O1 strains and appear to constitute part of the normal microflora of prawns (31) and oysters (2, 4, 28, 47). Most environmental isolates do not possess cholera toxin genes, a heat-stable enterotoxin (31, 32), or toxin coregulated pilus (49, 54).

The population structure and molecular evolution of *V. cholerae* as a species have not been well characterized, and the relationship of the sixth- and seventh-pandemic isolates is not clear. We have investigated sequence variation within *V. cholerae* by examining variation in the *asd* locus, one of the few *V. cholerae* chromosomal housekeeping genes sequenced to date. Housekeeping genes are concerned with basic cell functions and hence are generally present in all strains of the species. The *asd* gene encodes aspartate-semialdehyde dehydrogenase (EC 1.2.1.11), which functions in the common pathway for biosynthesis of threonine, lysine, and methionine.

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We report sequence of the *asd* gene from 45 isolates of *V. cholerae* (21 O1 and 24 non-O1 isolates). We found no variation within the sixth- or seventh-pandemic clones; however, the two clones were different. The sixth-pandemic, seventh-pandemic, and U.S. Gulf isolates constituted three clones, apparently evolving independently from environmental, nontoxigenic, non-O1 El Tor organisms. Our results confirm previous findings that O139 isolates evolved from seventh-pandemic isolates. Horizontal transfer of O antigen genes was inferred, since isolates with near identical *asd* sequences had different O antigens, and isolates with the O1 antigen did not group together but were found in different lineages. Further analysis suggests that *V. cholerae* has a high level of genetic exchange and a relatively low level of clonality.

## MATERIALS AND METHODS

**Bacterial isolates.** We used isolates representing the major seventh-pandemic ribotypes found in a previous study (22) and included other O1 and non-O1 isolates from clinical and environmental sources (Table 1), and a *Vibrio mimicus* strain (ATCC 33653). *V. cholerae* and *V. mimicus* isolates were grown on thiosulphate citrate bile salt agar (Oxoid) at 37°C for 18 h to confirm their species status. The O antigen of *V. cholerae* O1 isolates was confirmed by agglutination with polyvalent antisera containing both Ogawa and Inaba (Murex Diagnostics) and specific-O-antigenic forms confirmed by testing the strains individually with monospecific Inaba and Ogawa antisera (Murex Diagnostics). Isolates were stored at −70°C and subcultured once onto nutrient agar when needed.

**Chromosomal DNA preparation.** For the seventh-pandemic isolates, colonies from nutrient agar plates (Oxoid) were picked, and chromosomal DNA for each strain was isolated as described previously (3). As an alternative, the chromosomal DNA from the remaining isolates was obtained by a simplified method whereby one isolated colony was suspended into 200 μl of sterile distilled water and boiled for 5 min and 5 μl of supernatant was used as the template in a PCR. The change reflects adoption of a simpler method and does not affect the outcome.

**PCR amplification and DNA sequencing of the *asd* genes.** The *asd* gene was amplified by PCR in 200-μl reaction mixtures essentially as described by Saiki et al. (45) using the PCR primers and annealing temperatures listed in Table 2. The *asd* primers were designed from the *asd* sequence (A. R. Avest Ter and R. Frits [GenBank accession no. X55363]). Segments of the *asd* gene were amplified by using primer pairs 414/416, 410/416, 414/411, 415/416, and 430/416. Primer pairs 410/416 and 414/411 were specifically designed to extend the *V. mimicus* sequence, with 410 designed after sequencing of the first 300 bp of *V. mimicus*. To successfully amplify the *asd* alleles from all isolates, a range of annealing temperatures was required (Table 2).

TABLE 1. Bacterial isolates used in this study

| Isolate | Yr isolated | Country or region of isolation | Source[a] | Serogroup | Presence or absence of cholera toxin |
|---|---|---|---|---|---|
| M642[b] | 1943 | India | C | O1 | + |
| M644[b] | 1947 | Egypt | C | O1 | + |
| M648[b] | 1970 | Bangladesh | C | O1 | + |
| M645[c] | 1931 | Saudi Arabia | C | O1 | + |
| M802[c] | 1937 | Indonesia (Celebes) | C | O1 | + |
| M543[c] | 1938 | Iraq | C | O1 | + |
| M640[c] | 1954 | Egypt | C | O1 | + |
| M793[c] | 1961 | Indonesia | C | O1 | + |
| M794[c] | 1974 | United States (Texas) | C | O1 | + |
| M796[c] | 1978 | United States (Louisiana) | C | O1 | + |
| M807[c] | 1966 | Vietnam | C | O1 | + |
| M825[c] | 1988 | Zaire | C | O1 | + |
| M826[c] | 1990 | Malawi | C | O1 | + |
| M811[c] | 1971 | Burma | C | O1 | + |
| M817[c] | 1974 | Chad | C | O1 | + |
| M812[c] | 1971 | Chad | C | O1 | + |
| M663[c] | 1992 | Indonesia (Bali) | C | O1 | + |
| M799[c] | 1989 | Hong Kong | C | O1 | + |
| M803[c] | 1961 | Hong Kong | C | O1 | + |
| M535[c] | 1992 | Thailand | E | O1 | − |
| M536[c] | 1992 | Thailand | E | O1 | − |
| M539[c] | 1994 | Thailand | C | O139 | + |
| M540[c] | 1994 | Thailand | C | O139 | + |
| M831[c] | 1993 | Bangladesh | C | O139 | + |
| M537[c] | 1994 | Thailand | C | O139 | + |
| M539[c] | 1994 | Thailand | E | O139 | + |
| M540[c] | 1994 | Thailand | E | O139 | + |
| M542[c] | 1994 | India | C | O139 | + |
| M545[c] | 1994 | India | C | O139 | + |
| M548[c] | 1993 | Germany | E | O25 | − |
| M549[c] | 1993 | Germany | E | O37 | − |
| M550[c] | 1993 | Germany | E | O99 | − |
| M551[c] | 1993 | Germany | E | O41 | − |
| M552[c] | 1993 | Germany | E | O103 | − |
| M553[c] | 1994 | Germany | E | O70 | − |
| M554[c] | 1994 | Germany | E | O83 | − |
| M555[c] | 1993 | Argentina | E | O97 | − |
| M556[c] | 1993 | Argentina | E | O74 | − |
| M557[c] | 1993 | Argentina | E | O6 | − |
| M558[c] | 1993 | Argentina | E | O66 | − |
| M559[c] | 1994 | Denmark | E | O52 | − |
| M560[c] | 1994 | Denmark | E | O39 | − |
| M561[c] | 1994 | Korea | E | O74 | − |
| M562[c] | 1994 | Korea | E | O10 | − |
| M563[c] | 1994 | Korea | E | O81 | − |
| M547[d] | Unknown | | C | NA[e] | |

[a] C, clinical isolate; E, environmental isolate.
[b] Classical.
[c] El Tor.
[d] *V. mimicus.*
[e] NA, not applicable.

With an annealing temperature of 50°C, primer pairs 414/416 and 415/416 gave amplification of all O1 and several non-O1 isolates. Amplification with primer pair 414/416 required 45°C for annealing with M558 and M547 and 40°C with M548, M549, M550, M551, M552, M554, and M562. For primer pair 415/416, isolates M553, M557, M558, M559, M560, and M562 required an annealing temperature of 45°C, while M548, M550, and M554 required 40°C. Neither M547 nor M552 could be amplified with primer pair 415/416; however, this region was successfully amplified when the *V. mimicus*-specific primer (410) was used at an annealing temperature of 40°C. For primer pair 430/416, all isolates were amplified at 45°C.

DNA sequence was obtained from 20 bp before the ATG start codon to base 931 of the *asd* gene (951-bp total and 92% of the coding region). All oligonucleotides were synthesized with either universal forward or reverse M13 primer sequences attached to facilitate automated DNA sequencing. Double-stranded PCR DNA product was purified by using the Wizard PCR purification system (Promega) to remove excess PCR primers and was eluted in 25 to 30 μl of sterile distilled water, and the sequence was determined by the dye-labelled primer

TABLE 2. PCR primers used to amplify the *asd* genes from *V. cholerae* and *V. mimicus*

| Gene amplified and primer | Primer sequence (5′→3′) | Bases[a] | Annealing temp (°C) |
|---|---|---|---|
| *asd* | | | |
| 410 | GGCTCATTGGGCTCC | +237–251 | 40 |
| 411 | GGTGTGGGAAACCC | +651–664 | 40 |
| 414 | CCTTTGGCTAAACTCGG | −37–−21 | 40–50 |
| 415 | CGACTACGACATTCCTC | +306–322 | 40–50 |
| 416 | GTTATCCGCCACTACCC | +932–948 | 40–50 |
| 430 | ATGGACAACGGTTATAC | +616–632 | 45 |

[a] Primer locations relative to the ATG start codon, in which the A is position 1, in the *asd* gene (GenBank accession no. X55363).

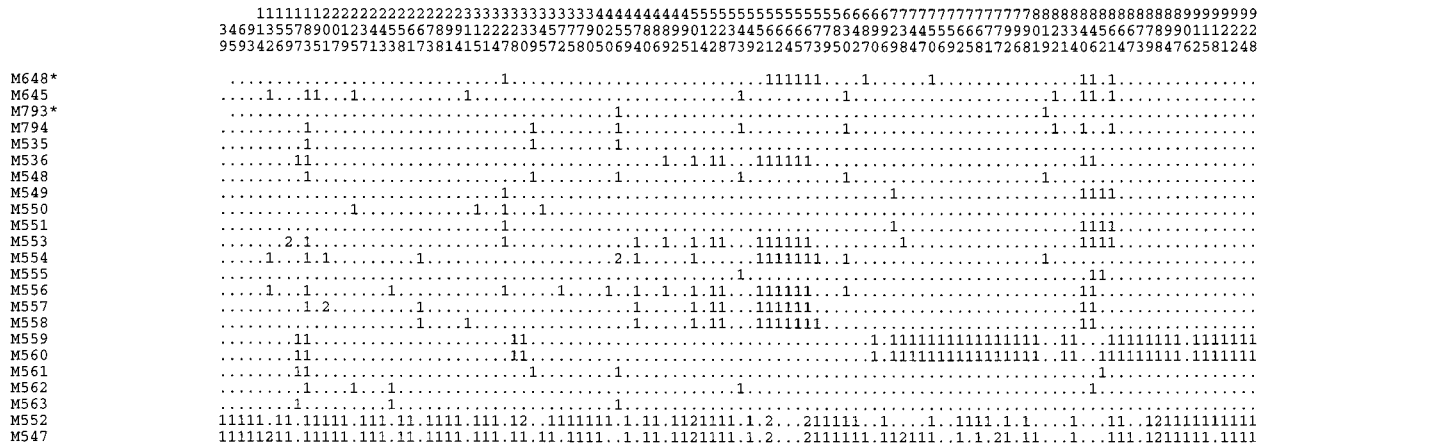

FIG. 1. Informative sites for the different *V. cholerae* and *V. mimicus asd* sequences. Individual sequences are shown, with the exception of the sixth- and seventh-pandemic strains, for which the sequence shown for M648* (sixth pandemic) also represents those for M642 and M644, while the sequence shown for M793* (seventh pandemic) also represents those for M802, M543, M640, M807, M825, M826, M811, M817, M812, M663, M799, M803, M539, M540, M831, M537, M542, and M545.

technique using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) and an automated 373A DNA sequencer (Applied Biosystems, Burwood, Victoria, Australia) according to the manufacturers' instructions.

**Computer analysis.** DNA sequences were analyzed using programs from the Australian National Genomic Information Service, at the University of Sydney (41), or the MULTICOMP package (40), which gives pairwise comparisons of DNA and derived amino acid sequences and incorporates programs such as those within the Phylip package (14a) and MacClade (25). It uses the Stephens method (51) to detect intragenic recombination and calculates nucleotide diversity ($\pi$) by the method described by Nei and Miller (34).

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the *asd* sequences are U25065 through U25087.

## RESULTS

**Nucleotide polymorphism of the *asd* gene.** The DNA sequence of a 951-bp region for 45 isolates was determined and included 931 bp of the *asd* gene. The sequence of M552 was very similar to that of *V. mimicus*, and M559 and M560 had a *V. mimicus*-like segment. They were omitted from calculations of variation within *V. cholerae*, which was based on the remaining 42 typical sequences. Sequencing revealed variation at 54 nucleotide sites within typical *V. cholerae* isolates, with the majority occurring at the 3′ end of the gene. There are eight nonsynonymous substitutions among the typical *V. cholerae*; most of these are in the center of the gene and are 2.58% of the 310 amino acids studied within the coding region. Within typical *V. cholerae* isolates, there were 31 phylogenetically informative sites (i.e., sites with at least 2 bases each present in two or more isolates), most of which are found at the 3′ end of the *asd* gene. Of the 54 polymorphic sites, 47 are at the third base of the codon, 3 are at the second base, and 4 are at the first base. There were an additional 80 polymorphic sites when *V. mimicus* was included (many also within M552, M559, and M560). There were 5 additional amino acid differences when *V. mimicus* was included. Of the 80 substitutions present only in *V. mimicus* or *V. mimicus*-like segments of M552, M559, and M560, 73 are at the third base, 1 is at the second base, and 6 are at the first base. Informative sites for all isolates are shown in Fig. 1.

**Amino acid polymorphisms in *asd*.** Of the 8 amino acid substitutions within typical *V. cholerae* isolates, there was not a single amino acid that was present in all of the O1 isolates which was not present in the non-O1 isolates and vice versa. In other words, O1 and non-O1 isolates share amino acid changes and are not distinct groups. Two amino acids within a 7-bp stretch distinguished the cluster containing the sixth-pandemic isolates, these resulting from two base changes within one codon that result in the amino acid change Gln-188→Asn and one base change that results in the change Ala-189→Thr.

**Sequence variation in the *asd* gene.** The average pairwise percentage difference for typical *V. cholerae* isolates (Fig. 2) over the fragment was 1.41% (only one of each sequence variant was included), the maximum difference being 2.74% (M645 and M554). No differences were found within sixth-pandemic or seventh-pandemic isolates, and O139 isolates had the same sequence as seventh-pandemic isolates. Sixth- and seventh-pandemic sequences differed at 1.62% of bases. Interestingly, sixth-pandemic isolates were most closely related to the environmental, nontoxigenic non-O1 El Tor isolates M549 and M551, with differences of 1.32 and 1.22%, respectively. Seventh-pandemic and O139 isolates were identical to two pre-seventh-pandemic El Tor isolates (M802 and M640) and otherwise most closely related to an environmental, nontoxigenic, non-O1 isolate, M535, with a 0.3% difference. The lack of variation in the *asd* genes of the seventh-pandemic strains isolated throughout a 31-year period is similar to our findings of no variation in the *gnd* genes and only one base change in the *mglB* genes of strains of the Sonnei clone of *Escherichia coli* isolated over a 41-year period (23).

**Comparison of *V. cholerae* and *V. mimicus* sequences.** There were 134 polymorphic bases between *V. mimicus* ATCC 33653 and typical *V. cholerae* isolates, most occurring at the second half end of the gene. There were 101 base differences between *V. mimicus* and seventh-pandemic isolates, 103 between *V. mimicus* and sixth-pandemic isolates and 102 compared with U.S. Gulf Coast isolates. The average percentage pairwise difference between *V. mimicus* and typical *V. cholerae* isolates was 10.43%, with the maximum difference (10.86%) being seen with M556 (Fig. 2). Isolate M552 resembles *V. mimicus* over the entire sequence, while M559 and M560 resemble typical *V. cholerae* isolates at the 5′ end (bases 1 to 728 [9.6% difference from *V. mimicus*]) but *V. mimicus* at the 3′ end (bases 729 to 931 [6.9% difference for M559 and 7.4% difference for M560]), indicating that their *asd* genes are the product of a recombination event.

| | M645 | M793 | M794 | M535 | M536 | M548 | M549 | M550 | M551 | M553 | M554 | M555 | M556 | M557 | M558 | M561 | M562 | M563 | M559 | M560 | M552 | M547 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M648 | 2.23 | 1.62 | 1.73 | 1.73 | 1.52 | 2.03 | 1.32 | 1.73 | 1.22 | 1.62 | 2.13 | 1.52 | 1.83 | 1.52 | 1.42 | 2.03 | 1.73 | 1.73 | 4.87 | 4.97 | 10.36 | 10.46 |
| M645 | | 1.62 | 0.91 | 1.52 | 2.34 | 1.42 | 1.52 | 1.73 | 1.42 | 2.64 | 2.74 | 1.32 | 2.44 | 2.34 | 2.44 | 1.83 | 1.12 | 1.62 | 4.87 | 4.97 | 10.36 | 10.56 |
| M793 | | | 0.91 | 0.30 | 1.73 | 0.41 | 0.91 | 0.71 | 0.81 | 2.23 | 1.83 | 0.51 | 2.23 | 1.73 | 1.83 | 0.61 | 0.71 | 0.41 | 4.06 | 4.16 | 9.95 | 10.25 |
| M794 | | | | 0.61 | 1.83 | 0.51 | 1.02 | 1.42 | 0.91 | 2.13 | 2.34 | 0.81 | 2.13 | 1.83 | 2.13 | 0.91 | 0.81 | 0.91 | 4.37 | 4.47 | 10.05 | 10.36 |
| M535 | | | | | 1.62 | 0.30 | 1.02 | 0.81 | 0.91 | 2.13 | 1.93 | 0.61 | 2.13 | 1.62 | 1.93 | 0.30 | 0.61 | 0.30 | 3.96 | 4.06 | 9.85 | 10.15 |
| M536 | | | | | | 1.93 | 1.83 | 2.03 | 1.73 | 0.91 | 1.83 | 1.62 | 0.91 | 0.61 | 0.91 | 1.73 | 1.62 | 1.52 | 4.97 | 5.08 | 10.36 | 10.66 |
| M548 | | | | | | | 1.32 | 1.12 | 1.22 | 2.44 | 1.83 | 0.71 | 2.23 | 1.93 | 2.23 | 0.61 | 0.71 | 0.61 | 4.26 | 4.37 | 9.95 | 10.25 |
| M549 | | | | | | | | 1.02 | 0 .10 | 1.73 | 2.54 | 0.61 | 2.13 | 1.83 | 1.93 | 1.12 | 1.02 | 1.12 | 4.16 | 4.06 | 10.25 | 10.36 |
| M550 | | | | | | | | | 0.91 | 2.34 | 2.44 | 0.81 | 2.34 | 2.03 | 2.13 | 1.12 | 0.81 | 0.91 | 4.37 | 4.47 | 10.05 | 10.15 |
| M551 | | | | | | | | | | 1.62 | 2.54 | 0.51 | 2.03 | 1.73 | 1.83 | 1.02 | 0.91 | 1.02 | 4.06 | 3.96 | 10.15 | 10.25 |
| M553 | | | | | | | | | | | 2.13 | 1.93 | 1.02 | 0.91 | 1.22 | 2.23 | 2.13 | 2.23 | 5.28 | 5.18 | 10.56 | 10.76 |
| M554 | | | | | | | | | | | | 2.23 | 1.73 | 1.32 | 1.52 | 2.23 | 2.23 | 2.03 | 5.58 | 5.69 | 10.56 | 10.76 |
| M555 | | | | | | | | | | | | | 2.13 | 1.62 | 1.73 | 0.71 | 0.41 | 0.71 | 4.16 | 4.06 | 10.05 | 10.36 |
| M556 | | | | | | | | | | | | | | 0.91 | 1.22 | 2.44 | 1.93 | 2.03 | 5.69 | 5.79 | 10.25 | 10.86 |
| M557 | | | | | | | | | | | | | | | 0.51 | 1.93 | 1.62 | 1.73 | 5.18 | 5.28 | 10.25 | 10.56 |
| M558 | | | | | | | | | | | | | | | | 2.23 | 1.93 | 2.03 | 5.48 | 5.58 | 10.46 | 10.76 |
| M561 | | | | | | | | | | | | | | | | | 0.91 | 0.41 | 4.06 | 3.96 | 10.15 | 10.46 |
| M562 | | | | | | | | | | | | | | | | | | 0.51 | 4.16 | 4.26 | 10.05 | 10.36 |
| M563 | | | | | | | | | | | | | | | | | | | 3.86 | 3.96 | 9.95 | 10.25 |
| M559 | | | | | | | | | | | | | | | | | | | | 0.10 | 8.93 | 8.53 |
| M560 | | | | | | | | | | | | | | | | | | | | | 9.04 | 8.63 |
| M552 | | | | | | | | | | | | | | | | | | | | | | 5.38 |

FIG. 2. Percentage difference of DNA between *asd* sequences of *V. cholerae* and *V. mimicus*. See the legend to Fig. 1 for details.

**Intragenic recombination in *asd*.** Evidence for recombination was sought by application of the Stephens method to informative sites only for all *V. cholerae* and *V. mimicus* isolates. There were three segments of clustered substitutions with a distribution suggesting a recombination event rather than a clustered mutation ($P$ values, $<0.00001$). One involves a segment of 203 bp (bases 729 to 931) with one form present in *V. mimicus* and M552 and two non-O1 isolates (M559 and M560). The second involves 7 bp (bases 561 to 567), while the third involves a stretch of 73 bp (bases 501 to 573) that includes the 7-bp segment.

**Phylogenetic analysis.** Phylogenetic analysis was carried out by using the neighbor-joining method. This analysis was complicated by the presence of the segments thought to have undergone recombination and was carried out on both the whole of the region sequenced (Fig. 3A and 3B) and also with the 203- and 7-bp segments omitted (Fig. 3C). The distribution of the two blocks has considerable influence on the details of the tree; however, in both cases the sixth-pandemic, seventh-pandemic, and U.S. Gulf strains clearly fall into separate groups, with non-O1 strains being closely associated.

## DISCUSSION

**The major pathogenic clones are independently derived.** Studies of *V. cholerae* by restriction fragment length polymorphism of rRNA genes, pulsed-field gel electrophoresis, and multilocus enzyme electrophoresis (MLEE) have together shown convincingly that the sixth and seventh pandemics constitute two distinct clones (7, 22, 24, 37, 48). Ribotyping also allowed us to gain some insight into evolution within the seventh pandemic; however, the evolutionary relationship of this clone to other major clones of *V. cholerae* could not be accurately determined, since the variation between the clones was too high to be identified in terms of specific loci and alleles (22). In the present study, we used the sequence of a chromosomal housekeeping gene, *asd*, which codes for an enzyme in branched-chain amino acid synthesis, to look at the relationship of these two clones to each other and other *V. cholerae* isolates.

We used 20 environmental isolates, 2 of O1, 2 of O139, and 16 others of different O groups, which were isolated from Europe or Asia. The two O139 isolates appear to be from the epidemic clone, but the two O1 isolates are not closely related to each other or the pathogenic clones. There are three groups of pathogenic strains, sixth-pandemic isolates, seventh-pandemic isolates plus O139 Bengal, and U.S. Gulf clinical isolates, with no variation within each group. The three are not closely related. The sixth- and seventh-pandemic isolates differ at 13 sites consisting of 4 isolated sites, one cluster of 6 sites within 13 bp, and another cluster of 3 sites within 22 bp. The results clearly suggest that the sixth-pandemic, seventh-pandemic, and U.S. Gulf isolates are not closely related but are independently derived from environmental clones. Cholera is confined to humans and is thought to be a relatively recent disease which was unknown to nomadic Palaeolithic humans but which developed when villages and village water supplies were established (15). However, *V. cholerae* is a species commonly found in marine and estuarine environments (46) and is also found closely associated with prawns (31) and oysters (2, 4, 28, 47). It appears from our data that the pathogenic forms must have arisen independently from such environmental forms.

All three pathogenic clones have the same virulence package (*ctxA ctxB zot ace*, etc.) on a transposon-like element (17) and

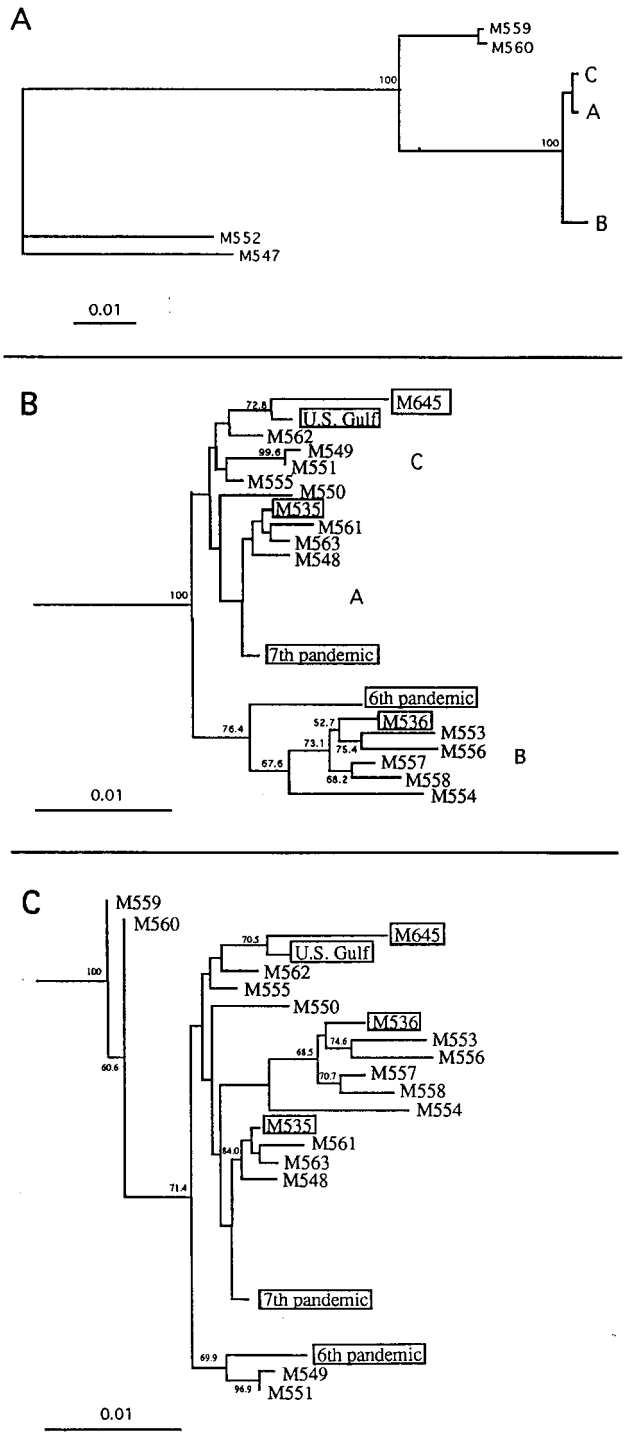


FIG. 3. (A) General phylogenetic tree generated by the neighbor-joining method for bases 1 to 931 of the *V. cholerae asd* gene; (B) detail of panel A showing strains in groups A, B, and C; (C) detailed tree showing all strains other than M552 and M547 (which had the same arrangement as in panel A) after omission of the 7- and 203-bp segments involved in recombination. Bootstrap values are percentages of 1,000 replications; branches with values of less than 50 are not shown. Individual strains are shown, except for the seventh-pandemic clone (including O139 strains and the pre-seventh-pandemic El Tor strains M802, M543, and M640), the sixth-pandemic clone, and the strains from the U.S. Gulf coast. ▭, *V. cholerae* strains with the O1 antigen.

the *tcp* cluster which maps elsewhere. None of these genes are generally present in nonpathogenic *V. cholerae* isolates. Wachsmuth et al. (52) found three very similar sequences for the *ctxB* gene, one present in remnants of the sixth pandemic (1921 to 1970) and in U.S. Gulf isolates (1973 to 1990), a second which differed at one base in isolates from Australia (1977 and 1988) (29), and a third from seventh-pandemic isolates (1963 to 1991) which differed from the first at two sites contained. The finding of very little sequence variation in *ctxB* contrasts with the variation in *asd* and suggests that the same package of virulence genes has been acquired relatively recently by three different clones. In this way, pathogenic *V. cholerae* isolates may not be very different from pathogenic strains of *E. coli*, such as enterotoxigenic strains that have acquired toxin genes present on a plasmid.

There are some discrepancies between the ribotyping and *asd* sequence data. Ribotyping (22) showed that the 1931 Saudi Arabian, the 1937 Indonesian, and the 1954 Egyptian El Tor isolates are quite different from each other and from the seventh-pandemic strains. However, sequencing suggests that the 1937, 1938, and 1954 isolates may be related to the seventh-pandemic clone. It is too early to speculate on the significance of this observation, but further analysis may reveal the relationships between these strains and the seventh pandemic.

**Origins of pathogenic *V. cholerae*.** How did the disease known as cholera arise? The natural niche of *V. cholerae* may well include crustacea or molluscs, since this species is often found there (9, 27). It is otherwise difficult to explain the almost universal presence of a hemolysin gene in *V. cholerae* which presumably is effective only on eukaryotic cells. It is not required for pathogenesis in humans and is evidenced by the loss of expression of the gene in sixth-pandemic isolates and the progressive loss during the seventh pandemic. There is a related species, *Vibrio fischeri*, which is found in association with marine organisms, in particular sepiolid squids and monocentrid fish (16, 44). The release of the *V. fischeri* strain associated with squids is the source of the organisms found in the ambient seawater. Perhaps a similar situation applies to *V. cholerae*, which could have its primary location in association with crustacea and molluscs, with the hemolysin being involved in this association.

By this hypothesis, the organisms in the substratum and water would be released from the primary location and would be waiting for an opportunity to colonize a new host. If this is the case, one can envisage the origin of the pathogenic mode by the gradual development of the capacity to survive and multiply in the guts of animals which eat molluscs or crustacea (which clearly includes humans from early times). Once released through feces back into the water, the organism would reenter its normal life cycle. The capacity for human-to-human infection has apparently arisen only rarely and depends on poor hygiene and crowded living conditions. This capacity may have been gained by sequential acquisition of necessary virulence genes, since the virulence cassette and *tcp* are both needed for virulence but are separated on the chromosome.

**The U.S. Gulf Coast isolates.** The role of the virulence package in the U.S. Gulf isolates is not clear. The cholera toxin (CT)-positive isolates all contain the full virulence cassette (19a). There are CT-negative isolates from the U.S. Gulf coast which are almost identical to CT-positive isolates by MLEE (53); this situation may have arisen by recombination among the RS1 elements that flank this cassette, causing its elimination. Given the correct conditions, these isolates could have pandemic potential, and one possibility is that they are remnants of a pandemic strain that predates the fifth pandemic. If our speculation on the ecological niche occupied by *V. cholerae*

is correct, the virulence package may serve to allow occasional infection of mollusc- or crustacean-eating animals, with subsequent release of the organisms into the water.

**Origins of the O139 Bengal isolates.** O139 and seventh-pandemic isolates have identical *asd* genes, adding to the evidence of ribotyping (13, 22, 37), restriction fragment length polymorphism for *tcpA* (54), restriction length polymorphism on iron-regulated genes (6), and expression of cholera toxin (54) for the O139 clone being very close to those for the seventh-pandemic clone. We have shown by ribotyping that O139 isolates have evolved from an early seventh-pandemic isolate (22). They have acquired sufficient genetic information to provide a novel O antigen.

**Relationship between *V. mimicus* and *V. cholerae*.** *V. mimicus* can be pathogenic for humans, producing gastroenteritis or wound infections, and it is associated with oysters, prawns, and seawater. Biochemical and physiological tests (30) show that *V. mimicus* and *V. cholerae* are very similar. Some *V. mimicus* isolates have a high percentage DNA relatedness (80% DNA-DNA hybridization) to *V. cholerae* (11). *V. mimicus* was formerly included in *V. cholerae* non-O1 and is biotypically distinguishable by its inability to ferment sucrose and its negative Voges-Proskauer reaction (19). An MLEE study (48) of *V. cholerae* and *V. mimicus* also indicates a close relationship between the species.

From our results and those of others (11, 30), we feel that the designation of separate species status for *V. cholerae* and *V. mimicus* may need further consideration. We find only 10% difference in nucleotide sequence, and only one amino acid of *asd* is unique to the *V. mimicus* isolate. This is within the expected variation occurring within a species. The value is similar to the level of variation found between the subspecies of *S. enterica* with subspecies V and I, which differ by up to 8% (5, 35). Furthermore, of the 22 sequence variants of the *V. cholerae asd* gene, one had essentially the *V. mimicus* sequence and two had a 203-bp segment closely related to it. This is a high level of recombination, again suggesting the possibility that we are dealing with subspecies rather than species.

**Relationship among O1 and non-O1, classical, and El Tor isolates.** It is strikingly clear from our data that isolates with the O1 antigen do not cluster together. Although the O1 antigen is correlated with pandemic cholera, it is present in several different lineages and does not appear to identify a separate cluster; rather, it appears to have undergone horizontal transfer within *V. cholerae*. While our results need to be confirmed by studies of other genes, the evidence strongly suggests that the distinction often made between O1 and non-O1 is not useful. This is supported by Heiberg (18), who described six biogroups of *V. cholerae* and cholera-like vibrios on the basis of fermentation reactions in sucrose, mannose, and arabinose. All O1 isolates belonged to his biogroup I, while there were isolates with non-O1 antigen also belonging to this biogroup.

We also need to review the distinction often made between classical and El Tor biotypes. Although they are traditionally distinguished by five characters, the El Tor form of three characters can now be seen to be typical of *V. cholerae* in general. The hemolysin-negative, Voges-Proskauer-negative, and hemagglutinin-negative characters of classical strains all represent loss of function by the sixth-pandemic clone. In fact, as the seventh pandemic has progressed, there has been an increasing number of isolates of the clones that have lost hemolysin expression (42, 43), and Voges-Proskauer-negative El Tor isolates have also been reported (14). The basis of the variation in polymyxin B sensitivity is not known, and the phage susceptibility difference simply says that they belong to different phage types. Thus, the most useful distinction is between the two clones which gave rise to the sixth and seventh pandemics. All three classical strains isolated from 1943 to 1970 have the same sequence, and it is probable that all isolates commonly referred to as classical are remnants of the sixth pandemic.

**Level of clonality in *V. cholerae*.** The bootstrap values for some branches in the phylogenetic trees were low, and we suspected the presence of recombination events other than those detected by the Stephens method. Bacterial species vary in frequency of recombination and hence in the level of clonality as revealed by MLEE or sequence variation (26). We used the program MacClade to estimate the proportion of parallel or reverse changes required to fit the data to a tree. Even with the segments involved in the two obvious recombination events removed, the *V. cholerae asd* gene has 49 apparent reverse or parallel substitutions for 34 sites (4 with 3 bases present), giving a tree length of 87. This seems a very high proportion of reverse or parallel mutations; therefore, we looked at other genes. The *Salmonella enterica gapA* and *mdh* genes and the *E. coli mdh* gene have a much smaller proportion of reverse and parallel mutations (Table 3). The *V. cholerae asd* sequence is the longest, with 931 bp under consideration; however, the others are similar (range, 864 to 924 bp). The most likely explanation is a higher level of recombination in *V. cholerae*. These findings were supported by analysis by application of the Maynard Smith algorithm (26) to the MLEE data described by Salles and Momen (48).

It appears that *V. cholerae* is not as strongly clonal as *S. enterica* or *E. coli*. However, although the recombination rate appears to be higher in *V. cholerae*, leading to a relatively low level of clonality, it is important to note that this applies to comparisons of independent environmental isolates. The rate of recombination must still be low in absolute terms, since the *asd* gene shows no variation throughout the life of the seventh-pandemic clone. The low level of clonality in the environmental isolates makes it difficult to have confidence in the details of the trees shown in Fig. 3. Nonetheless, the major conclusion that the three pathogenic clones are independently derived

TABLE 3. Numbers of reverse and/or parallel mutations expected for the *asd* gene of *V. cholerae* after comparisons to the *mdh* and *gapA* genes in *S. enterica* and *mdh* in *E. coli*

| Gene (species) | Tree length | No. of characters[a] | No. of reverse or parallel mutations[b] | No. of bases | No. of sequences |
|---|---|---|---|---|---|
| *mdh* (*S. enterica*) | 211 | 161 (127 + 34) | 50 (20) | 864 | 23 |
| *gapA* (*S. enterica*) | 168 | 129 (108 + 21) | 39 (20) | 924 | 16 |
| *mdh* (*E. coli*) | 49 | 33 (29 + 4) | 16 (28) | 864 | 21 |
| *asd* (*V. cholerae*) | 87 | 38 (34 + 4) | 49 | 931 | 21 |

[a] Values in parentheses are numbers of characters making up the tree plus the second and third most common sites.
[b] Numbers in parentheses refer to the numbers of reverse or parallel mutations expected in *asd* compared with those that are found in the *mdh* and *gapA* genes for *S. enterica* and the *mdh* gene of *E. coli*.

from environmental, nontoxigenic, non-O1 isolates is still valid.

**Relevance to vaccine development.** There are proposals for use of attenuated *V. cholerae* strains for cholera vaccines. Our data indicate that *V. cholerae* may have a recombination rate which is high relative to species such as *E. coli*. This is at present only an indication, but even if confirmed, it will perhaps not detract from the use of such vaccines. Firstly, the recombination rate is certainly not so high as to have resulted in any change in the *asd* gene of the seventh-pandemic clone in 30 years, and, secondly, recombination is likely at most to generate a single pathogenic cell in a situation in which a toxigenic strain is already present. As Kaper et al. state (21), there would be minimal if any adverse consequences with one more toxigenic strain of *V. cholerae* in an environment in which a single patient can excrete up to 20 liters of watery stool containing $10^7$ to $10^8$ toxigenic *V. cholerae* cells per ml per day.

**Concluding comments.** *V. cholerae* is a diverse species, as is shown by the many serogroups present; however, the number of pathogenic clones is small. Only a few pathogenic clones have caused disease on a worldwide scale, but until the present, the evolutionary relationship of the pandemic clones was not clear. Our sequencing results indicate that the sixth-pandemic, seventh-pandemic, and U.S. Gulf isolates are three independent clones. Unfortunately, there are probably no surviving strains from earlier pandemics. We propose that the sixth-pandemic, seventh-pandemic, and U.S. Gulf isolates are independent adaptations to human pathogenesis of nontoxigenic, environmental, non-O1 *V. cholerae* isolates which also were positive for hemolysin, Voges-Proskauer, and hemagglutinin. However, the virulence cassette and quite possibly other factors may well have been passed from the sixth-pandemic to the seventh-pandemic clone by recombination. It is also probable that only some O antigens are compatible with pathogenesis and that the O antigen genes were also transferred from the sixth- to the seventh-pandemic clone. This has major implications for cholera epidemiology and provides important information as to how epidemics and pandemics of cholera can arise. Our data support previous studies which indicated that the O139 Bengal clone evolved from the seventh-pandemic clone, since O139 isolates, regardless of their source, were identical to seventh-pandemic isolates in *asd* sequence. Although the O1 antigen is commonly found in pandemic isolates, it is not a prerequisite for pathogenesis, since O139 isolates also have pandemic potential. We have found several recombination events within the *asd* gene and it appears that *V. cholerae* as a species has a high rate of genetic transfer and has a low level of clonality. Isolates with nearly identical sequences but with different O antigens were found, and O1 isolates did not cluster together but were found in a variety of isolates of several lineages, suggesting horizontal transfer of the O1 antigen.

## ACKNOWLEDGMENTS

We thank T. Shimada from the Department of Bacteriology at the National Institute of Health, Tokyo, Japan, for kindly supplying strains M548 to M563.

This work was supported by a grant from the Australian Research Council.

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