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Multilocus Sequence Typing Has Better Discriminatory Ability for Typing *Vibrio cholerae* than Does Pulsed-Field Gel Electrophoresis and Provides a Measure of Phylogenetic Relatedness

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Twenty-two *Vibrio cholerae* isolates, including some from "epidemic" (O1 and O139) and "nonepidemic" serogroups, were characterized by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) by using three housekeeping genes, g*yrB*, *pgm*, and *recA*; sequence data were also obtained for the virulence-associated genes *tcpA*, *ctxA*, and *ctxB*. Even with the small number of loci used, MLST had better discriminatory ability than did PFGE. On MLST analysis, there was clear clustering of epidemic serogroups; much greater diversity was seen among *tcpA*- and *ctxAB*-positive *V. cholerae* strains from other, nonepidemic serogroups, with a number of *tcpA* and *ctxAB* alleles identified.

Vibrio cholerae is an environmental species that has been linked with seven pandemics of cholera since 1817 (22). Approximately 200 serogroups of *V. cholerae* have been identified to date, with classification based on epitopic variations in the heat-stable somatic O antigens of the strains (29). In the modern microbiology era, epidemic cholera has been associated with a limited number of closely related strains in O groups 1 and 139. All such epidemic strains carry genes for cholera toxin (encoded by the *ctxAB* genes) and the toxin-coregulated pilus (encoded by the *tcpA* gene) (10, 20). Recent studies have indicated that the *ctxAB* and *tcpA* genes may also be present in "nonepidemic" (i.e., other than O1 and O139) *V. cholerae* serogroups (7, 8, 9, 18, 23, 24, 26). While the genetic relatedness of O1 and O139 isolates has been well documented (1, 2, 3, 12), we know less about the genetic relatedness and phylogeny of *ctxAB*- and *tcpA*-positive isolates in other serogroups, and such data are critical for understanding why and how pandemic-causing *V. cholerae* strains emerge.

Several molecular typing approaches, including ribotyping (28), insertion sequence-based fingerprinting (3), amplified fragment length polymorphism (17), and pulsed-field gel electrophoresis (PFGE) (6), have been used to characterize the molecular epidemiology of *V. cholerae*, with PFGE reported (6, 8) to have the most discriminatory power among these methods. These techniques have less utility in defining underlying phylogenetic relationships (11). Multilocus enzyme electrophoresis (MLEE) is of value in this regard (12), although problems with band resolution and the fact that phenotypic expression of the enzyme under study can easily be altered in response to environmental conditions can adversely affect the reproducibility of MLEE results and complicate data analysis and interpretation.

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More recently, attention has turned to sequence-based approaches. Sequencing of a single gene (19, 31) is not optimal: because evolution occurs by a net-like process, gene trees based on a single gene may not permit accurate determination of the genetic relatedness among various isolates (15). Multilocus sequence typing (MLST), first described in 1998 (25), provides a balance between sequence-based resolution and informativeness and technical feasibility and has been used to characterize several pathogenic bacteria, including, in recent studies, *V. cholerae* (5, 13). The technique, as originally described, involves determining the nucleotide sequences of a series of housekeeping genes. MLST alleviates several problems associated with PFGE: MLST data, based as they are on nucleotide sequences, are unambiguously comparable among laboratories, and MLST detects all genetic variations within the amplified gene fragment while PFGE examines only those that are in specific restriction sites and those that involve large insertions or deletions of DNA. As variation occurs most commonly at the nucleotide level, MLST is likely to have better discriminatory ability than PFGE, as recently reported in studies with *Salmonella* (21).

The present study was undertaken with two goals: (i) to compare, at a methodological level, the discriminatory powers of MLST and PFGE for *V. cholerae* and (ii) to use MLST to assess phylogenetic clustering of *tcpA*- and *ctxAB*-positive isolates from epidemic and nonepidemic serogroups and evaluate relationships between these clusters and specific *tcpA* and *ctxAB* alleles.

Bacterial isolate collection. The 22 *V. cholerae* isolates used in this study (Table 1) have been previously described (23, 24), as have methods for isolate storage and propagation (30). The presence of the *tcpA* and *ctxAB* genes was initially determined by dot blot analysis (23), followed by sequencing studies, as described below.

PFGE analysis. Plugs containing *V. cholerae* DNA were prepared (6), and *Ceu*I macrorestriction patterns were obtained after electrophoresis in the CHEF DR II apparatus (Bio-Rad

TABLE 1. Sources and years and places of isolation of *V. cholerae* strains examined, serogroups and PFGE and sequence types of the strains, and allele types of *ctxAB* and *tcpA* genes

Strain	Serogroup	PFGE type	Sequence type	Source	Place/year of isolation	Allele type designation for virulence gene(s):	
						tcpA	*ctxAB*
117-94	O35	P7	ST21	Environment	Korea/1994		
984-81	O89	P13	ST7	Diarrhea	India/1981		
AM2	O9	P2	ST6	Diarrhea	India/1995		
AM107	O144	P11	ST10	Diarrhea	India/1996		
NRT36-S	O31	P8	ST11	Diarrhea	Japan/1990		
981-75	O65	P1	ST17	Diarrhea	India/1975	T1	
8585	O53	P1	ST17	Diarrhea	Iraq/1969	T1	
8-76	O77	P12	ST5	Diarrhea	India/1976	T4	
1421-77	O80	P4	ST4	Diarrhea	India/1977	T4	
AQ1875	O48	P5	ST20	Food	Japan/1998	T9	
1322-69	O37	P5	ST18	Diarrhea	India/1969	T5	C5
N16961	O1 E1 Tor	P5	ST15	Diarrhea	Bangladesh/1975	T1	C3
365-96	O27	P6	ST16	Food	Japan/1996	T3	C1
NIH35A3	O1 classical	P10	ST14	Diarrhea	India/1941	T5	C2
507-94	O49	P5	ST12	Diarrhea	Thailand/1994	T10	C3
63-93 (MO 45)	O139	P9	ST13	Diarrhea	India/1992	T1	C3
153-94	O8	P5	ST19	Unknown	Unknown/1994	T8	C3
203-93	O141	P10	ST1	Diarrhea	India/1993	T6	C4
No. 63	O26	P10	ST2	Diarrhea	Japan/1991	T7	C1
506-94	O44	P5	ST3	Diarrhea	Thailand/1994	T1	C3
571-88	O105	P3	ST8	Diarrhea	China/1988	T3	C6
366-96	O191	P5	ST9	Diarrhea	Japan/1996	T2	C2

Laboratories, Hercules, Calif.) under the following conditions: voltage, 180 V; initial time, 2.2 s; final time, 64 s; and run time, 20 h. We elected to use *Ceu*I instead of, for example, *Not*I (which is more frequently used to type *V. cholerae*) because *Not*I restriction digestion of *V. cholerae* DNA generates numerous bands and it is very difficult to resolve multiple bands of similar sizes in *Not*I-generated PFGE patterns, which complicates data analysis, including the construction of rigorous dendrograms. *Ceu*I generates anywhere from 6 to 10 bands from *V. cholerae* DNA, and the bands can be separated very well by using the electrophoresis conditions used during our study. Also, *Ceu*I has been shown (27) to provide an excellent tool for rapidly examining the organization of genomes of various serovars and biovars of *V. cholerae*. A dendrogram (Fig. 1) was constructed with Fingerprinting DST Molecular Analyst software (Bio-Rad Laboratories). The patterns were compared by means of the Jaccard coefficient of band-based similarity by using the unweighted pair group method using averages (a tolerance of 3% in band position was applied). Thirteen PFGE types were identified among the 22 isolates examined (Table 1). PFGE types P1, P5, and P10 contained two, seven, and three isolates, respectively, with identical PFGE patterns, while each of the remaining 10 isolates had unique PFGE patterns. The isolates were grouped in three major PFGE clusters at the 64% similarity level (Fig. 1). Cluster A contained 5 isolates, cluster B contained 15 isolates (including the O1 and O139 serogroup isolates), and cluster C contained 2 isolates.

Within cluster B, serogroup O1 classical, O1 El Tor, and O139 isolates were grouped into distinct, but closely related, PFGE types (PFGE types P10, P5, and P9, respectively). However, PFGE did not differentiate among (i) the O1 classical, O141, and O26 serogroups, all of which were grouped in the P10 type, and (ii) the O1 El Tor, O37, O49, O191, O44, O48, and O8 serogroups, all of which were grouped in a single PFGE type, P5 (Fig. 1). Isolates from some serogroups that cluster by PFGE (e.g., O1 El Tor and O8) appear in different MLEE lineages (1). At the same time, some serogroups (e.g., O1 and O139) known to be very closely related genetically (1, 2, 3) were grouped in distinct types during our PFGE analysis. These observations suggest that conclusions about the genetic relatedness of *V. cholerae* isolates based on PFGE analysis must be interpreted with caution, as suggested previously for other bacteria (11).

MLST analysis. Six loci (Table 2) were sequenced, including regions from three housekeeping genes (the genes encoding the B subunit of DNA gyrase [*gyrB*], recombination protein [*recA*], and phosphoglucomutase [*pgm*]) and three virulence-related genes (those encoding the A and B subunits of cholera toxin [*ctxA* and *ctxB*, respectively], present in 12 isolates, and that encoding the toxin-coregulated pilus [*tcpA*], present in 17 isolates). The same primers were used for PCR amplification and sequencing, and they were designed with the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) and ClustalX (16) programs. Sequencing of amplified fragments was performed in both directions by using an ABI 3700 DNA analyzer (Applied Biosystems, Inc., Foster City, Calif.). The sequence type analysis and recombinational tests (START) program (http://outbreak.ceid.ox.ac.uk) was used to determine the G+C content, the numbers of alleles and polymorphic sites, and the proportions of nonsynonymous and synonymous base substitutions (d_N and d_S, respectively) for the genes examined during our study.

Sequence analysis of housekeeping genes. Analysis of sequence data for *gyrB*, *pgm*, and *recA* from our 22 isolates resulted in the identification of 12, 13, and 14 allelic variants,

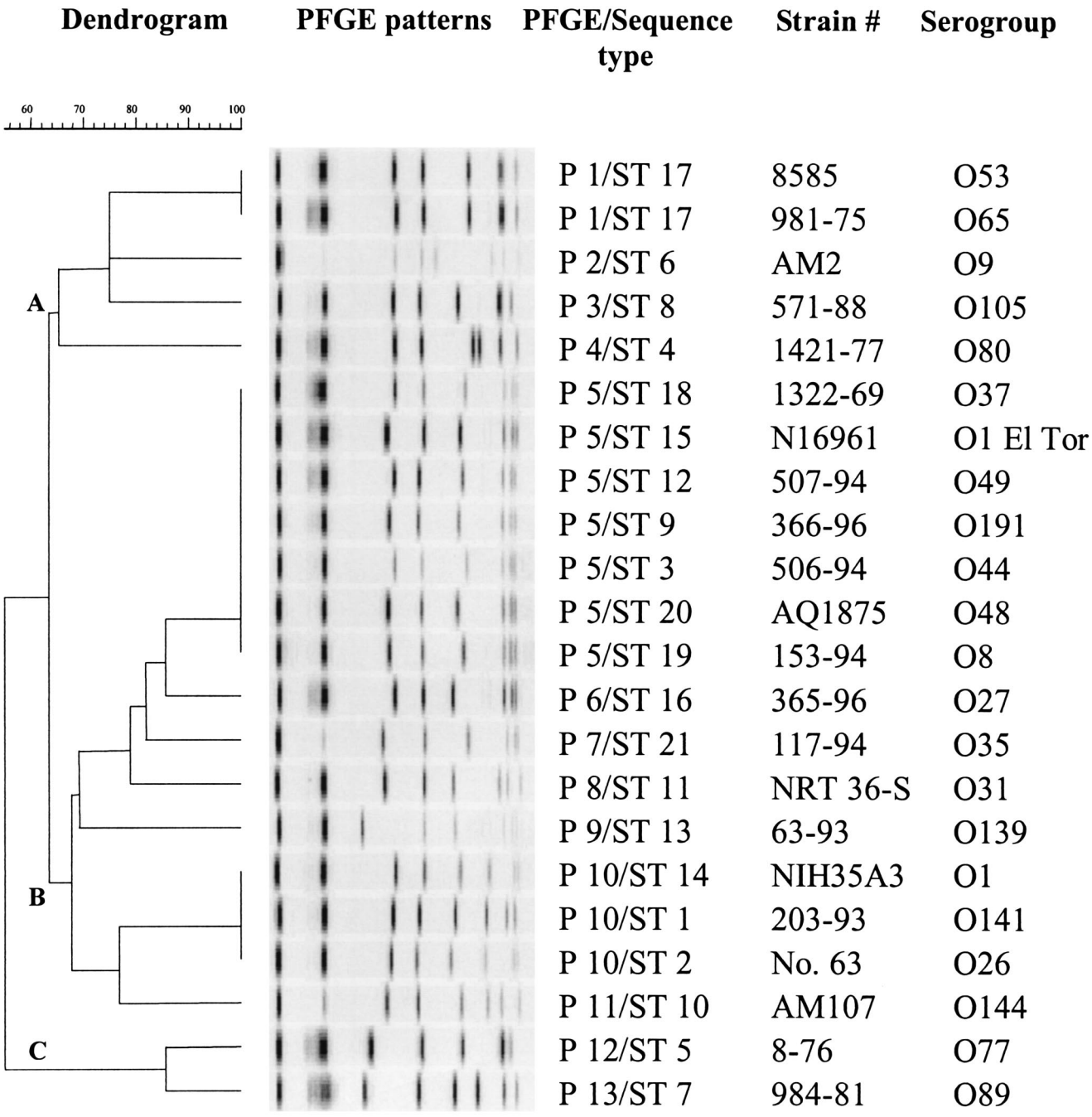

FIG. 1. Dendrogram portraying the genetic diversity of various *V. cholerae* isolates. The dendrogram is based on PFGE patterns obtained with *Ceu*I-digested *V. cholerae* DNA.

respectively. Twenty-one unique sequence types were identified among these 22 isolates, based on the allelic profiles (Table 1). The genetic relatedness of isolates based on various sequence types was determined by START by using the unweighted pair group method using averages. The resulting dendrogram revealed three clusters (Fig. 2), designated X, Y, and Z. Of note, the three epidemic serogroup isolates were found to cluster in a single subgroup within the largest cluster (cluster Y). The cluster also included several isolates from nonepidemic serogroups, some of which were *ctxAB* negative (e.g., isolate 981-75) or *tcpA* and *ctxAB* negative (e.g., isolate NRT36-S). Although the number of isolates analyzed was small, there was no obvious correlation between cluster patterns and dates or countries of isolation.

Sequence analysis of virulence genes. Ten allele types were identified among the 17 *tcpA*-positive isolates, and six allele types were identified among the 12 *ctxAB*-positive isolates (Table 1). *tcpA* had the largest number of polymorphic sites among the six genes that we analyzed (Table 2), which is in agreement with previously reported data (4, 24) about the genetic vari-

TABLE 2. Primers used for MLST of *V. cholerae* strains, numbers of alleles and polymorphic sites identified per gene, and d_N/d_S ratios for various genes

Gene	Primer set (5′ → 3′)	No. of strains	Fragment length (bp)		No. of alleles	No. of polymorphic sites	Mean G+C content (%)	d_N/d_S
			Amplified	START analyzed				
ctxA	GGCTGTGGGTAGAAGTGAAACGG, CTAAGGATGTGGAATAAAAACATC	12	1,140	770	6[b]	1	38.5	0.0000
ctxB[a]		12		360		4	33.6	0.0087
tcpA[c]	AAAACCGGTCAAGAGGG, CAAAAGCTACTGTGAATGG, CAAATGCAACGCCGAATGG	17	600	300	10	140	43.6	0.0579
gyrB	GAAGGBGGTATTCAAGC, GAGTCACCCTCCACWATGTA	22	560	460	12	16	51.2	0.000
pgm	AAAGATACTCAYGCSCTGTC, AACCAGCGTTTTACCGACGGCAACA	22	730	540	13	19	49.2	0.0262
recA	GAAACCATTTCGACCGGTTC, CCGTTATAGCTGTACCAAGCGCCC	22	700	380	14	35	49.5	0.0069

[a] The *ctxAB* primers have been described in reference 23. The amplified 1,140-bp region includes *ctxA* and *ctxB* loci.
[b] The allele number shown here and the *ctxAB* allele numbers and designations used throughout the text, Fig. 2, and Table 1 are based on the combined sequence of the *ctxA* and *ctxB* loci.
[c] A mixture of two previously described (20) reverse primers was used to amplify the *tcpA* gene.

ability of *tcpA* genes. In contrast, the numbers of polymorphic sites were very low in *ctxA* and *ctxB* (one and four, respectively). There were differences in the G+C contents of the *ctxA* and *ctxB* regions (38.5 and 33.6%, respectively; Table 2).

Four of the five *tcpA* T1 alleles were within the Y cluster in the dendrogram, as were both T5 alleles; both *tcpA* T4 alleles were in cluster X. At the same time, the T3 allele was found in cluster Y and in a second isolate outside of all three clusters. As expected, given that *tcpA* is a receptor for the *ctx* phage, the *ctx* gene was found only in *tcpA*-positive strains. The *ctxAB* allele C3 was associated with the *tcpA* T1 allele in three instances (the O139 and O1 El Tor isolates in cluster Y and an O44 isolate in cluster X). In all other instances, the same *ctxAB* allele was associated with different *tcpA* alleles.

Recombinational basis of genetic diversity of loci analyzed by MLST. With the exception of the *ctxA* and *ctxB* regions, which were relatively homogeneous, considerable sequence variability was observed within the sequenced gene regions (Table 2). The d_N/d_S ratios were less than one for all of the genes examined, which suggests that selective pressure for nonsynonymous nucleotide substitutions was not the primary driving force during the diversification of the genes. Thus, we used Splitstree analysis (http://bibiserv.techfak.uni-bielefeld.de/splits) to determine whether recombinational changes were likely to have contributed to the genetic heterogeneity of the genes that we analyzed.

In the Splitsgraph, recombination is depicted by parallelograms; i.e., the observation that parallelograms are formed during Splitstree analysis indicates that recombination may have been involved in the evolution of the analyzed gene. During our analysis, we observed parallelograms for five of the six genes examined (the *ctxA* analysis did not reveal parallelograms and, thus, any supportive evidence of recombination in the gene) (data not shown). Genetic recombination has been described (5) to occur in other housekeeping genes of *V. cholerae*, and our Splitstree results suggest that it has also occurred in the three housekeeping genes analyzed during our study.

Isolates 981-75 and 8585 had identical sequence types and the same *tcpA* allele type (T1), were both *ctxAB* negative, and were grouped into a single PFGE type (P1); however, they belong to two distinct serogroups (O65 and O53, respectively). This observation is in keeping with other reports (3, 23) of O-antigen switching among isolates with a common genetic background, consistent with the hypothesis that the O-antigen-encoding genes are readily transferred among isolates via genetic recombination.

Discriminatory ability of MLST versus that of PFGE. Thirteen PFGE types were identified among the 22 isolates analyzed during our study, whereas the number of sequence types was larger (21 sequence types; Table 1), an observation which suggests that the discriminatory ability of MLST is better than that of PFGE. The O1 classical, O1 El Tor, and O139 isolates of *V. cholerae* examined during our study were differentiated by both PFGE and MLST. However, several isolates within the same PFGE types (i.e., indistinguishable by PFGE typing) were differentiated into separate sequence types by MLST (but not vice versa). For example, each of the seven *V. cholerae* isolates (1322-69, N16961, 507-94, 366-96, 506-94, AQ1875, and 153-94) grouped together in PFGE type P5 (Fig. 1) had a unique sequence type (Table 1). At the same time, the two

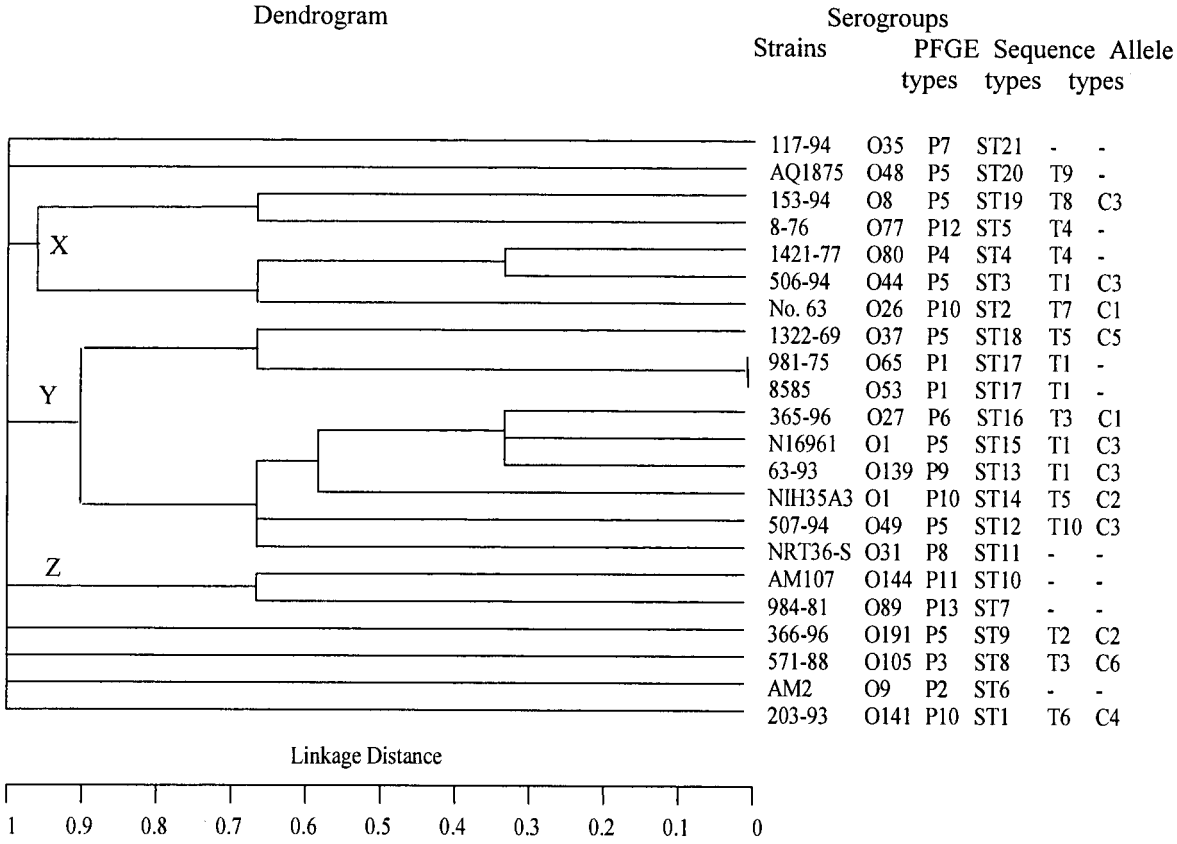

FIG. 2. Dendrogram portraying the genetic diversity among various *V. cholerae* sequence types, constructed by the unweighted pair group method using averages. Allele types are for *tcpA* and *ctx*, respectively.

isolates (981-75 and 8585) with identical sequence types (ST17) could not be differentiated with PFGE (Table 1). The increased discriminatory power of MLST was apparent even though we used only three housekeeping genes; the addition of more loci will only increase the discriminatory power of the method.

In order to compare further the discriminatory abilities of MLST and PFGE, we analyzed the linkage distances among the isolates grouped in each of the three PFGE types containing more than one isolate (i.e., PFGE types P1, P5, and P10). Linkage distances were consistently larger than 0.35 (the minimal linkage distance identified in the dendrogram for the isolates in distinct sequence types) (Fig. 2); e.g., linkage distances for the seven isolates grouped in PFGE type P5 varied from 0.65 to 1.

Summary. At a methodological level, and in agreement with findings with other species (21), our data suggest that MLST has better discriminatory ability than PFGE for typing *V. cholerae*. Our secondary objective was to further explore relationships among virulence marker-carrying isolates in epidemic and nonepidemic serogroups. To do this, we looked at phylogenetic relationships among isolates as defined by sequence types (based on allelic variants of three housekeeping genes) and then assessed allelic variation of *tcpA* and *ctxAB* genes in the context of the resulting phylogenetic tree. While there was definite clustering of the epidemic O1 and O139 isolates based on MLST analysis of housekeeping genes, no other clear patterns emerged: *tcpA* and *ctxAB* genes were present in nonepidemic serotypes with a variety of MLST sequence types, suggesting that acquisition of these virulence markers is not restricted to a subset of closely related *V. cholerae* strains. There were suggestions that certain *tcpA* allelic types were more common among epidemic isolates and clusters. However, there was also fairly extensive mixing of sequence types, *tcpA* allelic types, and *ctxAB* allelic types, consistent with the reported mobility of certain elements (i.e., *ctxAB* [32]) and the hypothesis that recombinational events are not an infrequent occurrence among *V. cholerae* strains.

Nucleotide sequence accession numbers. The DNA sequences have been deposited in GenBank under accession numbers AF501888 through AF501909 for the *gyrB* sequences; AF536843 through AF536864 for the *pgm* sequences; AF301032, AF301042, AF301050, AF301052, AF301055, AF301063, AF301080, AF301086, AF301105, AF301123, AF301131, AF501905, AF536832 through AF536838, and AF536840 through AF536842 for the *recA* sequences; AF390572, AF452584, AF463400, and AF463401 for the *ctxA* gene sequences (isolate NIH35A3 sequences are identical to those of previously characterized isolate 569B [accession number X58785], and the sequences of the remaining isolates are identical to those of isolate N16961 [accession number AE004224] [14]); AF390572, AF452581, AF452582, AF452583, and AF463402 for the *ctxB* sequences (isolates NIH35A3, 203-93, and 366-96 have sequences identical to those of isolate 569B [accession number X58785], and isolates 507-94, 63-93, and 153-94 have sequences identical to those of isolate N16961

[accession number AE004224] [14]); and AF390571, AF452570 through AF452577, AF452579, AF452580, and AF536865 through AF536870 for the *tcpA* sequences.

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