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Molecular Characterization of *Vibrio cholerae* O1 Strains by Pulsed-Field Gel Electrophoresis

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Pulsed-field gel electrophoresis (PFGE) was performed on 180 isolates of *Vibrio cholerae* serogroup O1 representing 6 different multilocus enzyme electrophoresis (MEE) types and 27 rRNA restriction fragment length polymorphism types (ribotypes). Isolates were digested with the restriction enzyme *Not*I and were separated into 63 patterns on the basis of differences in band arrangements. In general, strains which were different by MEE or ribotyping also had different PFGE patterns. PFGE identified individual strains within a single MEE type or ribotype; isolates with one PFGE pattern were less frequently distinguished by ribotyping. All *V. cholerae* O1 isolates tested from the Latin American epidemic were indistinguishable by their MEE, ribotype, or PFGE patterns. PFGE could further distinguish strains of this same ribotype isolated in Africa, Europe, the South Pacific, or Southeast Asia. Although both MEE and PFGE could identify the strain from the Latin American epidemic, PFGE was more rapid and less labor intensive. PFGE also distinguished nontoxigenic isolates endemic to the U.S. Gulf Coast from unrelated nontoxigenic isolates. In the present study PFGE was more discriminating than other previously described subtyping assays for *V. cholerae* O1 and appears to be a useful epidemiologic tool.

The seventh pandemic of cholera began in 1961 and spread to much of Asia and Africa within a decade (4). Despite the potential for epidemic cholera in South America, no cases of domestically acquired cholera were identified there before 1991. Early that year, *Vibrio cholerae* serogroup O1 was isolated from the stools of people with severe diarrhea almost simultaneously in four coastal cities in Peru (20). The epidemic soon spread to other areas, eventually involving most of South and Central America. By the end of 1992, a total of 731,312 cases of cholera and 6,323 deaths were reported in 21 countries in the Western Hemisphere (8).

As the cholera epidemic spread in Latin America, it was of interest to determine whether infections in newly affected areas were due to the same strain of *V. cholerae* O1 as that identified in Peru. Traditional typing methods such as biotyping and serotyping could not distinguish Latin American isolates from seventh-pandemic isolates, although discrimination from U.S. Gulf Coast isolates was possible (22). However, the Latin American isolates appeared to be clonal and could be distinguished from other isolates by multilocus enzyme electrophoresis (MEE); they differed from seventh-pandemic isolates at one locus (leucine aminopeptidase) (23). In addition, all isolates related to the Latin American epidemic were ribotype 5, as were some *V. cholerae* O1 isolates associated with the seventh pandemic in other parts of the world (18).

Pulsed-field gel electrophoresis (PFGE) separates large DNA fragments created by digestion of total genomic DNA with restriction endonucleases that cut DNA infrequently. The patterns generated by PFGE have been used in the analysis of a variety of bacterial organisms, including *Pseudomonas* spp. (13), *Escherichia coli* (2), *Mycobacterium* spp. (25), and *Campylobacter* spp. (24). In the study described here, we used PFGE to characterize 180 isolates of *V. cholerae* O1, including 64 isolates from the current epidemic in Latin America. Analysis of *Not*I-digested total genomic DNA by PFGE allowed rapid identification of the Latin American strain and separated unrelated isolates more effectively than either MEE or ribotyping.

MATERIALS AND METHODS

Bacterial strains. One hundred eighty strains of *V. cholerae* O1 representing 6 MEE types and 27 different ribotypes were analyzed by PFGE. Sixty-four isolates were associated with the current epidemic in Latin America, including 30 which were imported into the United States from Latin America. The remaining 116 strains were isolated worldwide over the past 70 years. These strains include 24 nontoxigenic isolates, 13 isolates from the U.S. Gulf Coast, and 3 isolates from Australia.

Preparation of bacterial DNA. Cultures were incubated in 15 ml of heart infusion broth at 37°C for 1 to 1.5 h with aeration until growth reached an optical density of 0.6 at 610 nm. Cells (10 ml) were harvested by centrifugation and were washed with 10 ml of wash buffer (1 M NaCl, 10 mM Tris [pH 8.0], 10 mM EDTA). Cells were resuspended in 1 ml of wash buffer and were warmed at 37°C for a few minutes. Bacterial suspensions were mixed with an equal volume of 1% chromosomal grade agarose (Bio-Rad, Richmond, Calif.) and were dispensed into a plug mold (Bio-Rad). Agarose plugs were allowed to solidify on ice for 10 min. Plugs were placed in clean tubes containing 3 ml of lysis buffer (1 M NaCl, 10 mM Tris [pH 8.0], 100 mM EDTA, 0.5% Sarkosyl, 0.2% sodium deoxycholate, 1 mg of lysozyme per ml, 2 μg of RNase per ml). Bacteria were lysed in the agarose plugs for 1 h at 37°C. The lysis buffer was removed, and the plugs were incubated overnight in 3 ml of ESP buffer (0.5 M EDTA, 1% Sarkosyl, 1 mg of proteinase K per ml) at 50°C. The next day, the plugs were

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rinsed briefly with deionized water. Plugs were washed twice in 2 ml of TE (10 mM Tris [pH 8.0], 1 mM EDTA) containing 30 μl of 0.1 M phenylmethylsulfonyl fluoride for 30 min each. Plugs were washed four times in 3 ml of TE for 30 min each time. If the plugs were not to be used immediately, only two washes in TE were performed; this was followed by overnight incubation in 5 ml of TE at 4°C. A small portion of the plug (2 by 7 mm) was sliced off and was incubated for 1 h in a microcentrifuge tube in 1 ml of *Not*I buffer (150 mM NaCl, 10 mM Tris [pH 8.0], 10 mM $MgCl_2$). The buffer was then replaced with 125 μl of fresh buffer containing 20 U of *Not*I (New England Biolabs, Inc., Beverly, Mass.), and the mixture was incubated for 4 h at 37°C.

Separation of *Not*I restriction fragments. Restriction fragments were separated in a 1% fast-lane agarose gel (FMC, Rockland, Maine) in 0.5× TBE (10× TBE is 89 mM Tris base, 89 mM boric acid, and 2.5 mM disodium EDTA) by using a CHEF-DR II system (Bio-Rad). Bacteriophage lambda DNA ladders (FMC) were used as molecular mass standards. A model 1000 mini chiller (Bio-Rad) was used to maintain the temperature of the buffer at 14°C. A ramp time of 5 to 50 s for 20 h at 200 V was used to maximize the separation of larger fragments. For longer gels, the run time was increased to 22 h. If separation of smaller fragments was necessary, a ramp time of 1 to 10 s for 12 h at 200 V was used. Following electrophoresis, gels were stained for 20 min with ethidium bromide (2 μg/ml in water), destained, and visualized on a UV light box.

Analysis of PFGE patterns. *V. cholerae* O1 isolates were separated into patterns on the basis of differences in band arrangements. Differences in the presence, absence, or intensity of a band among strains were given equal weights. Strains that differed by one band were assigned different pattern numbers. Pattern numbers were designated solely for discussion purposes and are not meant to imply relatedness between isolates or to fulfill a typing scheme. Table 1 lists the number, origin, year of isolation, and ribotype of each isolate tested within each PFGE pattern.

RESULTS

Representatives of the four major MEE types, designated by Wachsmuth et al. (23), of toxigenic El Tor *V. cholerae* O1 isolates were distinguishable by PFGE. Within each MEE type, multiple PFGE banding patterns were observed. The differences seen among isolates from Australia (electrophoretic type [ET] 1) and the U.S. Gulf Coast (ET 2) were rarely greater than two bands (Fig. 1A and B). Strains associated with the seventh pandemic (ET 3) showed much greater diversity (Fig. 2). The Latin American strain possessed a unique PFGE banding pattern as well as a unique MEE type (pattern 38; ET 4).

The type strains of all 27 different ribotypes of *V. cholerae* O1 designated by Popovic et al. (18) were also distinguished by PFGE. A comparison of 157 isolates analyzed by both techniques indicated that PFGE and ribotyping results were in general agreement. However, isolates within 12 ribotypes could be further differentiated by PFGE. Isolates of ribotype 5 had the most diversity by PFGE (Fig. 2); 14 different PFGE patterns were identified among the 70 isolates of this ribotype, including the pattern for the Latin American strain (Fig. 2, lane 10). Twenty-one isolates from eight countries other than Latin America were ribotype 5, but they could be distinguished from the Latin American strain by PFGE. Within each of the 11 remaining ribotypes, an average of four PFGE patterns could be distinguished (Table 1).

Ribotyping separated isolates with the same PFGE patterns on six separate occasions (Table 1). Two isolates from an outbreak involving coconut milk from Thailand were indistinguishable by PFGE (pattern 24) but were of different ribotypes (7, 18, 21). One of three nontoxigenic isolates from the U.S. Gulf Coast with the same PFGE pattern (pattern 47) had the same ribotype as U.S. Gulf Coast toxigenic isolates (ribotype 2); the other two nontoxigenic isolates were of a different ribotype (ribotype 14). In both cases, the differences between ribotypes was only a single band (18). Ribotyping also discriminated between isolates in patterns 2, 29, 41, and 62 that were indistinguishable by PFGE (Table 1).

Classical isolates (Fig. 3) could be distinguished from El Tor strains by two sets of "ladder" banding patterns in ranges of 40 to 90 kb and 110 to 220 kb containing about eight bands each (patterns 1 to 14). Among Classical strains, 10 of 16 isolates differed by small restriction fragments at about 330 kb and/or additional bands located at about 100 kb. Two isolates had additional polymorphisms at about 75 and 125 kb. Five isolates (patterns 10 to 14) had greater differences from the other Classical isolates (Fig. 3, lanes 5 to 9). Among the seven ribotypes of Classical strains designated by Popovic et al. (18), three isolates with a unique ribotype also possessed a unique PFGE pattern. In addition, PFGE could further subdivide isolates within the three ribotypes made up of more than one strain each (Table 1).

All El Tor strains possessed identical banding patterns in a range of 25 to 75 kb. Isolates associated with the seventh pandemic or from Latin America (patterns 15 to 45) demonstrated the greatest amount of diversity in banding patterns (Fig. 2). The most apparent differences were among the larger restriction fragments (180 to 350 kb), which differentiated 27 patterns among seventh-pandemic and Latin American isolates. Additional polymorphisms at about 110 kb separated patterns 21 and 22, 29 and 30, and 35 and 36. Differences at about 150 kb separated patterns 25 and 26.

All isolates related to the current epidemic in Latin America were identical by PFGE (pattern 38; Fig. 2, lane 10) and were distinguishable from isolates from other areas of the world where cases of cholera are currently reported. To optimize the number of differences that could be detected, isolates were analyzed by PFGE by using two different sets of ramp times to maximize the separation of both larger and smaller restriction fragments (see Materials and Methods). Thirty-three toxigenic isolates of *V. cholerae* O1 from 15 different countries in Latin America and 29 toxigenic isolates from the United States that were epidemiologically linked to the Latin American epidemic (8, 11, 16) were indistinguishable. In addition, two nontoxigenic isolates, one from an outbreak of cholera on an airline flight originating in Latin America and one from El Salvador, were indistinguishable from the Latin American strain by PFGE and ribotyping (18). Fifteen toxigenic and 10 nontoxigenic isolates from Latin American countries possessed PFGE patterns different from that of the Latin American strain and appeared to be unrelated to the current epidemic (Table 1).

Ten toxigenic isolates of the strain endemic to the U.S. Gulf Coast and four nontoxigenic isolates with similar PFGE patterns (patterns 46 to 51) had only minor banding differences (Fig. 1). The first isolate from the U.S. Gulf Coast in 1973 (Fig. 1A, lane 1; Fig. 1B, lane 5) differed from other toxigenic isolates by having a single, slightly larger band rather than a doublet at about 110 kb. The two isolates in pattern 50 (Fig. 1A, lane 4) differed by having a band of about 210 kb rather than 220 kb, as was found for the other isolates. All four nontoxigenic isolates differed from most toxigenic isolates by the absence of a 40-kb band. Although this band could be seen by using the standard 5 to 50-s ramp time (Fig. 1A, lanes 1, 2,

TABLE 1. PFGE banding patterns and other characteristics of the *V. cholerae* O1 isolates examined in the study

Biotype and pattern no.	No. of isolates	Origin	Year of isolation	Ribotype(s)
Classical				
1	1	India	1941	1c
2	1	India	1979	1a
	1	India	1980	1e
	1	Pakistan	1960	1e
3	1	Hong Kong	1939	1b
4	1	India	1960	1e
5	1	India	1941	1b
6	1	India	1940	1f
7	1	Burma	1961	1e
8	1	Iran	1960	1b
9	1	Bangladesh	1972	1d
10	1	Pakistan	1959	1b
11	1	Unknown	1922	1c
12	1	Japan	1921	1b
13	1	India	1960	1g
14	1	Bangladesh	1970	1b
El Tor				
15	2	Mexico	1991	6a
	4	Mexico	1992	6a
	3	Romania	1991	6a
16	1	Thailand	1990	5
	1	Southeast Asia	1988	5
17	1	Romania	1987	5
18	2	Truk Islands	1990	5
19	1	Truk Islands	1982	5
20	2	Malawi	1990	5
21	6	Costa Rica	1992	3
	1	Honduras	1992	3
22	1	Costa Rica	1992	3
23	2	Romania (1[a])	1987	5
24	2	Cambodia	1992	5
	1	Vietnam	1992	5
	2	Thailand	1991	3, 5
25	2	Burundi	1992	8
	1	Uganda	1992	8
	1	Zambia	1992	8
26	4	Zambia	1991	8
	1	Zambia	1992	8
27	1	Rwanda	1988	8
28	1	Gilbert Islands	1976	4
29	1	Philippines	1963	3
	1	Guam	1988	7
30	1	Indonesia	1961	3
31	1	Romania	1977	5
32	1	Pakistan	1992	6a
33	3	Philippines	1992	6c
34	2	Hawaii	1991	6b
35	1	Romania	1981	5
36	1	Romania	1981	5
37	1	Philippines	1987	5
38	21	Bolivia, Chile, Colombia, Ecuador, El Salvador (1[a]), Guatemala, Mexico, Peru, United States[b], Venesuela	1991	5
	43	Argentina (1[a]), Belize, Bolivia, Brazil, Chile, El Salvador, Honduras, Mexico, Nicaragua, Paraguay, Peru, United States[b]	1992	5
39	1	Brazil	1992	6a
40	1	Indonesia	1992	6c
41	1	Guinea Bissau	1988	3
	2	Romania	1991	5
42	1	India	1990	6a
43	1	Guam	1990	7
44	2	Bangladesh	1992	6a
45	1	Uganda	1992	5
46	1	Louisiana	1978	2
	1	Louisiana	1988	2
	1	Louisiana	1992	2
	1	Massachusetts	1990	2
	2	Texas	1981	2
47	1[a]	Florida	1980	14
	1[a]	Louisiana	1986	14
	1[a]	Georgia	1984	2
48	1	Texas	1973	2
49	1[c]	Florida	1986	2
50	1	Maryland	1984	2
	1	Texas	1992	2
51	1[a]	El Salvador	1991	14
52	2	Australia	1988	9
53	1	Australia	1977	10
54	1[a]	Alabama	1991	15
55	1[a]	Alabama	1991	16
56	1[a]	Guatemala	1991	15
57	1[a]	Louisiana	1991	15
58	4[a]	Colorado	1990	17
	1[a]	Bolivia	1991	17
59	1[a]	Texas	1992	6c
60	1[a]	Trinidad	1992	18
61	2[a]	Peru	1987	11
62	1[a]	Brazil	1978	13
	1[a]	Brazil	1992	19
63	2[a]	Mexico	1983	12

[a] The indicated number of strains were nontoxigenic.
[b] Imported isolates.
[c] One copy of *ctx* gene.

and 4), it was much more evident by using the 1- to 10-s ramp time (Fig. 1B, asterisk). The toxigenic strains possessing the 40-kb fragment hybridized with an oligonucleotide probe for the cholera toxin gene at this position (data not shown). Although nontoxigenic isolates in pattern 47 (Fig. 1A, lane 3) differed only by this 40-kb band from toxigenic isolates in pattern 46 (Fig. 1A, lane 2), they had at least two differences from isolates in other patterns seen among U.S. Gulf Coast isolates. One toxigenic isolate (Fig. 1A, lane 5; Fig. 1B, lane 6) was also missing this band and had an additional band at approximately 110 kb. The nontoxigenic isolate from El Salvador in pattern 51 (Fig. 1A, lane 6) had a larger band of about 300 kb rather than the one of 290 kb found in all other isolates.

Isolates from Australia were very similar (Fig. 1A, lanes 7 and 8), having only minor banding differences at about 125 kb, which separated three isolates into two patterns (patterns 52 and 53, respectively). A diverse collection of nontoxigenic isolates (patterns 54 to 63) showed various PFGE patterns (Fig. 4) and did not resemble any nontoxigenic strains associated with U.S. Gulf Coast, seventh-pandemic, or Latin American isolates. These nontoxigenic isolates were very heterogeneous, having little similarity among themselves as well.

DISCUSSION

Traditional typing methods have limited ability to discriminate among *V. cholerae* O1 isolates. Phenotypic characteriza-

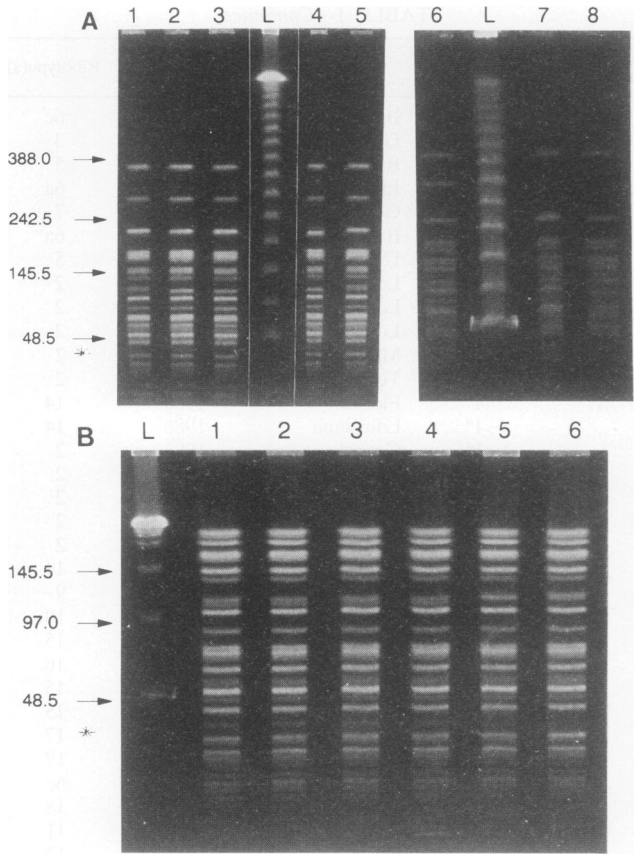

FIG. 1. (A) PFGE banding patterns of *Not*I-digested total cellular DNAs from representative *V. cholerae* O1 isolates from the U.S. Gulf Coast and Australia by origin (pattern number). Lane 1, Texas (pattern 48*); lane 2, Louisiana (pattern 46*); lane 3, Louisiana (pattern 47); lane 4, Maryland (pattern 50*); lane 5, Florida (pattern 49); lane 6, El Salvador (pattern 51); lane 7, Australia, 1988 (pattern 52); lane 8, Australia, 1977 (pattern 53). The PFGE ramptime was 5 to 50 s for 20 h at 200 V. (B) PFGE banding patterns of *Not*I-digested total cellular DNAs from representative toxigenic *V. cholerae* O1 isolates from the U.S. Gulf Coast by origin (pattern number). Lane 1, Louisiana (pattern 46*); lane 2, Texas (pattern 46*); lane 3, Maryland (pattern 50*); lane 4, Louisiana (pattern 46*); lane 5, Texas (pattern 48*); lane 6, Florida (pattern 49). The PFGE ramp time was 1 to 10 sec for 12 h at 200 V. Molecular mass markers (bacteriophage lambda DNA ladder) are given in lanes L (the numbers on the left are in kilobases). The asterisks mark the 40-kb fragment which hybridizes with the cholera toxin oligonucleotide probe as well as the strains containing this fragment.

tion, such as biotyping and hemolysin production, provides few subgroups, but the subgroups are stable over wide areas and for prolonged periods (3, 14). Serotyping divides isolates into two major groups, Inaba and Ogawa. However, antigenic switching may occur within a given strain because of mutations in the *rfbT* operon (19). Molecular characterization of *V. cholerae* O1 isolates has proved difficult as well. Most El Tor strains do not harbor plasmids, rendering plasmid analysis ineffective (10). Restriction fragment length polymorphisms are difficult to interpret because of the large number of fragments generated by the most commonly used enzymes. Hybridization of those fragments with specific probes such as cholera toxin and rRNA genes reduces the complexity of the restriction fragment length polymorphism pattern and has

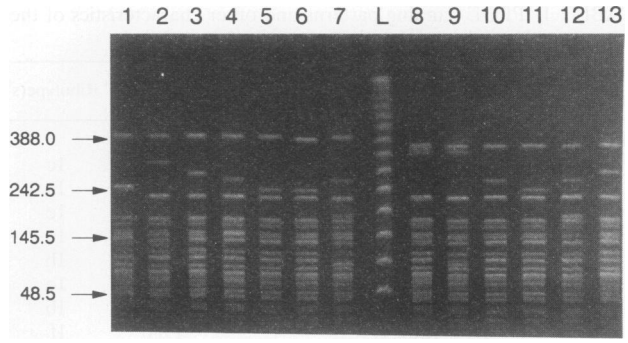

FIG. 2. PFGE banding patterns of *Not*I-digested total cellular DNAs from seventh-pandemic and Latin American isolates of *V. cholerae* O1 strains representing *Bgl*I ribotype pattern 5 from eight different countries (pattern numbers). Lane 1, Thailand (pattern 16); lane 2, Romania (pattern 17); lane 3, Truk Islands (pattern 18); lane 4, Malawi (pattern 20); lane 5, Romania (pattern 23); lane 6, Vietnam (pattern 24); lane 7, Romania (pattern 31); lane 8, Romania (pattern 35); lane 9, Romania (pattern 36); lane 10, Peru (pattern 38); lane 11, Romania (pattern 41); lane 12, Uganda (pattern 45); lane 13, Philippines (pattern 37). Molecular mass markers (bacteriophage lambda DNA ladder) are given in lane L (the numbers on the left are in kilobases).

been helpful in past investigations of epidemics (18). Ribotyping separates *V. cholerae* O1 into 27 types but does not distinguish the Latin American strain from strains associated with the seventh pandemic, which are distinguishable by other methods.

MEE, which analyzes stable "housekeeping" enzymes, was able to distinguish the Latin American outbreak strain from seventh-pandemic isolates but could not distinguish among widely distributed strains within the seventh pandemic (9, 23). A combination of ribotyping and MEE has provided useful epidemiologic information during investigations of the Latin American epidemic; however, both techniques are labor- and time-intensive.

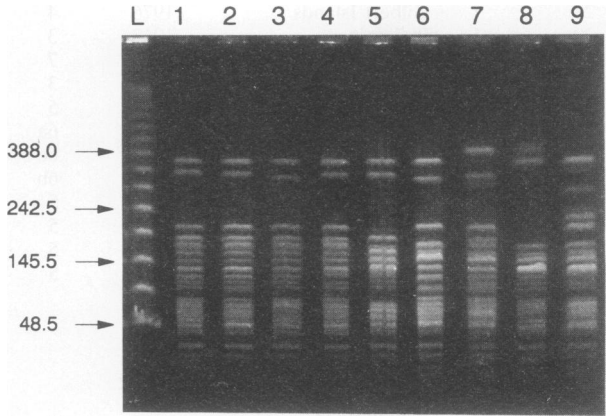

FIG. 3. PFGE banding patterns of *Not*I-digested total cellular DNAs from representative Classical isolates of *V. cholerae* O1 by country (pattern number). Lane 1, India (pattern 1); lane 2, India (pattern 4); lane 3, Burma (pattern 7); lane 4, Bangladesh (pattern 9); lane 5, Pakistan (pattern 10); lane 6, unknown (pattern 11); lane 7, Japan (pattern 12); lane 8, India (pattern 13); lane 9, Bangladesh (pattern 14). Molecular mass markers (bacteriophage lambda DNA ladder) are given in lane L (the numbers on the left are in kilobases).

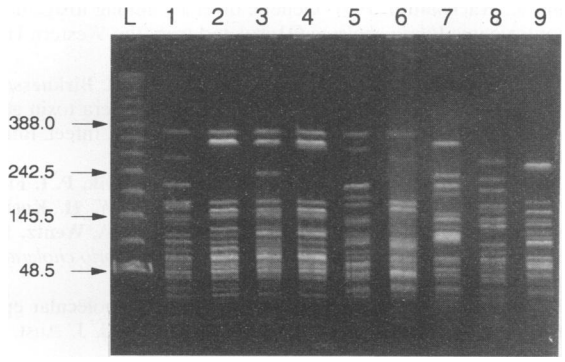

FIG. 4. PFGE banding patterns of *Not*I-digested total cellular DNAs from representative nontoxigenic *V. cholerae* O1 isolates by location (pattern number). Lane 1, Florida, U.S. Gulf Coast (pattern 47); lane 2, Alabama (pattern 54); lane 3, Guatemala (pattern 56); lane 4, Louisiana (pattern 57); lane 5, Bolivia (pattern 58); lane 6, Texas (pattern 59); lane 7, Trinidad (pattern 60); lane 8, Peru (pattern 61); lane 9, Brazil (pattern 62). Molecular mass markers (bacteriophage lambda DNA ladder) are given in lane L (the numbers on the left are in kilobases).

PFGE banding patterns distinguished between and identified diversity within each of the four major MEE types described previously (23). Isolates related to the seventh pandemic, as determined by MEE, had more diverse PFGE binding patterns than those from the separate environmental reservoirs of the U.S. Gulf Coast and Australia. Banding pattern alterations may be more likely to occur with the exposure of a pandemic strain to various selective pressures over 30 years, whereas isolates associated with local environmental reservoirs and limited spread would have fewer banding pattern alterations because they are subject to fewer selective pressures. Although the Latin American strain (ET 4) is subject to the same type of selective pressures to which seventh-pandemic isolates are subjected, the current epidemic has been present for only 2 years. Over time, the Latin American strain may evolve and have multiple PFGE banding patterns.

The groupings obtained by separation of strains by PFGE patterns largely agreed with the groupings obtained by ribotyping. Fifteen ribotypes had unique PFGE patterns. PFGE identified more than one pattern among isolates within each of the remaining 12 ribotypes. The great amount of diversity seen among ribotype 5 isolates—14 different patterns among isolates from 22 countries, including countries in Latin America (Fig. 2)—is particularly important because ribotype 5 is common among current seventh-pandemic strains as well as the Latin American strain. In general, PFGE was as discriminating or more discriminating than ribotyping. However, ribotyping was able to differentiate between isolates with the same PFGE pattern in six cases.

Twenty-five isolates from Latin America were unrelated to the epidemic, as determined by their PFGE patterns and ribotypes (18). Ten of these isolates were nontoxigenic and represented both human and environmental isolates; 5 of the 10 were isolated before the current epidemic (Table 1). Six toxigenic isolates from a rural village in Mexico (pattern 15) and seven toxigenic isolates from a single household in Costa Rica (patterns 21 and 22) formed two distinct clusters of patterns that were different from the pattern of the Latin American strain. Without laboratory data, these isolates would have been considered part of the Latin American epidemic. The two remaining toxigenic isolates, from Honduras (pattern 21) and Brazil (pattern 39), may also represent imported isolates that were detected as a result of the increased surveillance brought about by the Latin American epidemic or may be derivatives of the Latin American strain.

Isolates from the U.S. Gulf Coast and Australia compose two separate groups of environmental reservoirs of cholera (6, 12). Phenotypic and genotypic characterizations have distinguished these strains from all other strains (9, 14, 18, 23), including the seventh-pandemic and Latin American strains. Although general similarities in PFGE patterns exist between these different groups of strains, the banding patterns of no seventh-pandemic or Latin American isolate matched the banding patterns of U.S. Gulf Coast or Australian isolates (Fig. 1A and 2). These results are supported by MEE data, indicating the separate clonality of Australian and U.S. Gulf Coast strains (23).

Isolates from the U.S. Gulf Coast collected over a 15-year period (5) had nearly identical PFGE patterns, indicating the relative stability of PFGE patterns over time. The separation of U.S. Gulf Coast isolates was largely due to minor shifts in banding patterns. The toxigenic isolate from Florida, which lacked a 40-kb band that hybridized with an oligonucleotide probe to the cholera toxin gene (pattern 49; Fig. 1A, lane 5; Fig. 1B, lane 6), possessed one copy of the cholera toxin gene, whereas two copies of the gene were found in other U.S. Gulf Coast isolates (15). A nontoxigenic strain (pattern 51) isolated from river water in El Salvador (Fig. 1A, lane 6) possessed a pattern remarkably similar to the patterns of other U.S. Gulf Coast isolates. The ribotype and MEE type of this strain also categorized it as a U.S. Gulf Coast strain (18, 23), suggesting that it may have originated from the same environmental reservoir.

Nontoxigenic isolates associated with the U.S. Gulf Coast strain (Fig. 1A, lane 3) as well as the strain from El Salvador (Fig. 1A, lane 6) lacked a restriction fragment at approximately 40 kb that was present in all toxigenic U.S. Gulf Coast isolates (Fig. 1A, lanes 1, 2, and 4; Fig. 1B) except the one from Florida. This "missing" fragment was the only difference between isolates in patterns 46 and 47. When toxigenic isolates were probed with an oligonucleotide to the cholera toxin gene (1), a band at this approximate location hybridized with the probe (data not shown). These data suggest that the 40-kb fragment carries the cholera toxin gene. Since the cholera toxin gene is carried on a transposon-like element (17), it is possible that these nontoxigenic isolates represent toxigenic strains from which the gene was lost. While the absence of the cholera toxin gene may be detectable by PFGE in the U.S. Gulf Coast isolates, two nontoxigenic isolates associated with the Latin American epidemic had the same PFGE pattern as the toxigenic strain.

A group of nontoxigenic strains unrelated by geographic location, epidemiologic association, or phenotypic characterization to other isolates comprises a heterogeneous group of isolates affiliated only by their lack of a cholera toxin gene. These isolates clearly differed from nontoxigenic isolates associated with the U.S. Gulf Coast strain by PFGE (Fig. 4). They also had little similarity to nontoxigenic strains related to the Latin American epidemic or the seventh pandemic. Characterization by MEE typing (9, 23) and ribotyping (18, 23) also demonstrates the genetic diversity seen among these isolates.

Although the PFGE patterns of *V. cholerae* O1 may be too numerous and analysis of these patterns may be too complex to be used in a general typing scheme, the variety that they offer is of particular value in investigations of epidemics. Isolates from the current epidemic in Latin America imported into the

United States were readily distinguished from isolates imported from other countries. Six isolates from Mexico were distinguishable from isolates from the concurrent Latin American epidemic, indicating a previously unknown, independent source of cholera. These same isolates are identical to some from Romania, although no known epidemiologic connection is apparent. Isolates endemic to the U.S. Gulf Coast were easily distinguished from other strains. Nontoxigenic isolates associated with the U.S. Gulf Coast strain were clearly similar to these toxigenic isolates and were markedly different from other nontoxigenic strains not associated with the U.S. Gulf Coast strain. PFGE appears to be the most discriminating of several molecular subtyping methods used in our studies of *V. cholerae* O1, particularly recent studies of the isolates involved in the Latin American cholera epidemic. It is also reproducible, relatively stable over time and is relatively rapid in comparison with MEE and methods requiring DNA hybridizations.

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