

## Multiple *Vibrio vulnificus* Strains in Oysters as Demonstrated by Clamped Homogeneous Electric Field Gel Electrophoresis

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**Clamped homogeneous electric field gel electrophoresis and a computer program for managing electrophoresis banding patterns (ELBAMAP) were used to analyze genomic DNA of 118 *Vibrio vulnificus* strains, isolated from three oysters by direct plating. Analysis with *Sfi*I resulted in 60 restriction endonuclease digestion profiles (REDP), while analysis with *Srf*I produced 53 different REDP. Similarities between REDP ranged from 7 to 93%. Principal-component analysis showed that the strains were heterogeneous.**

*Vibrio vulnificus* is an estuarine organism that occurs naturally in temperate and tropical climates. It is commonly found in seawater and sediment and is associated with various marine life forms (10, 16, 18, 19, 24, 26). It is considered one of the most invasive and rapidly lethal human bacterial pathogens known today. Human disease can occur from ingestion of raw seafood, mainly raw oysters (2, 13), or by infection of skin lesions. *V. vulnificus* poses the greatest risk to persons who are compromised, especially those with liver dysfunctions (2, 13, 23). The fatality rate is greater than 50% for individuals with primary septicemia (15). Because this at-risk population is ever growing, food safety precautions for such opportunistic pathogens are increasingly important. Although much is known of

the seasonal ecology of *V. vulnificus* and its relationship to temperature and salinity (9, 11, 16, 17, 24), there are no methods to monitor or track specific strains. Moreover, it is not known if a single oyster contains few or many different strains of *V. vulnificus*. The infectious dose of *V. vulnificus* is also unknown and is due in part to the inability to match clinical isolates with isolates from implicated lots of oysters and the lack of opportunities for epidemiologists to trace a human infection back to the implicated source.

Since classification of *V. vulnificus* at the species level (6, 21), various methods have been used to identify and characterize *V. vulnificus* strains (8, 16, 22, 27, 29, 30). However, these methods are not adequate for tracking or typing. Therefore, other

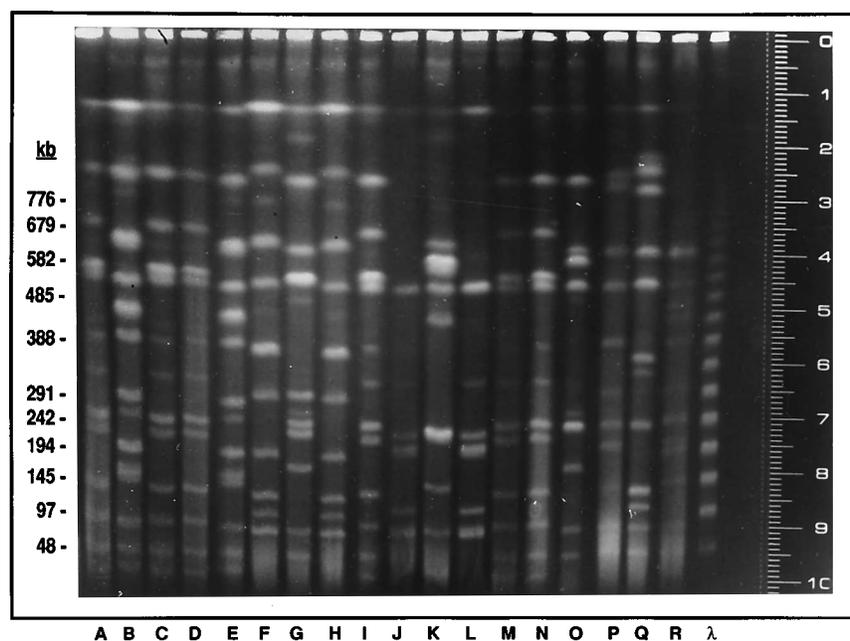


FIG. 1. DNA restriction patterns of *V. vulnificus* as determined by CHEF after cleavage with *Srf*I. Lane A, FRIK 236; lane B, FRIK 238; lane C, FRIK 239; lane D, FRIK 240; lane E, FRIK 241; lane F, FRIK 242; lane G, FRIK 243; lane H, FRIK 244; lane I, FRIK 245; lane J, FRIK 246; lane K, FRIK 247; lane L, FRIK 248; lane M, FRIK 249; lane N, FRIK 251; lane O, FRIK 252; lane P, FRIK 253; lane Q, FRIK 254; lane R, FRIK 232; lane  $\lambda$ , DNA size standards.

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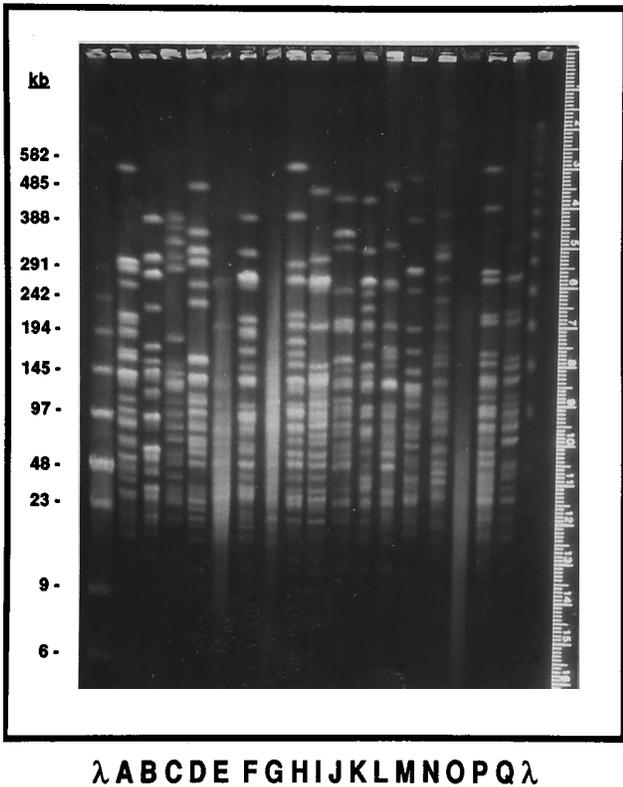


FIG. 2. DNA restriction patterns of *V. vulnificus* as determined by CHEF after cleavage with *Sfi*I. Lane A, FRIK 284; lane B, FRIK 285; lane C, FRIK 286; lane D, FRIK 288; lane E, FRIK 289; lane F, FRIK 290; lane G, FRIK 291; lane H, FRIK 293; lane I, FRIK 294; lane J, FRIK 251; lane K, FRIK 255; lane L, FRIK 301; lane M, FRIK 302; lane N, FRIK 310; lane O, FRIK 333; lane P, FRIK 334; lane Q, FRIK 232; lane  $\lambda$ , DNA size standards.

methods, particularly molecular techniques, have been applied to *V. vulnificus* (7, 20). Because epidemiological research requires methods that allow effective discrimination of strains and a basis for determining genomic relatedness among strains, we used clamped homogeneous electric field (CHEF) gel electrophoresis to analyze large chromosomal DNA restriction fragments of *V. vulnificus* strains isolated from three oysters.

This study was undertaken to determine molecular characteristics of *V. vulnificus* strains present in individual oysters and to investigate whether a few or multiple strains were present.

Three oysters were collected within a 50-ft<sup>2</sup> (ca. 4.6-m<sup>2</sup>) area in Apalachicola Bay, Fla., transported at 4°C, and examined within 24 h of harvest. The water temperature at the time of harvest was 23°C and the salinity was 10 ppt. Oyster shells were scrubbed with a brush under running water, opened with a sterile shucking knife, and transferred to a sterile blender jar. Three single oysters weighing more than 10 g each were diluted separately with an equal weight of sterile phosphate-buffered saline (PBS) and were homogenized (Polytron; Brinkmann Instruments). Serial 10-fold dilutions were prepared in sterile PBS, and 100  $\mu$ l of each dilution was plated on 10 plates of Trypticase soy agar (Difco, Detroit, Mich.) supplemented with 1% NaCl. After 4 h of incubation at 37°C, colonies were transferred to nylon membranes (Micron Separations Inc.) by overlaying agar plates with membranes. After approximately 2 min, the nylon membrane was inverted, placed on modified colistin-polymyxin B-cellobiose agar (25), and incubated at 40°C for 16 h. Typical *V. vulnificus* colonies were picked, and their identity was confirmed by enzyme-linked immunosorbent assay (ELISA) with a *V. vulnificus*-specific monoclonal antibody (25). A total of 118 *V. vulnificus* isolates (oyster I, 36 isolates;

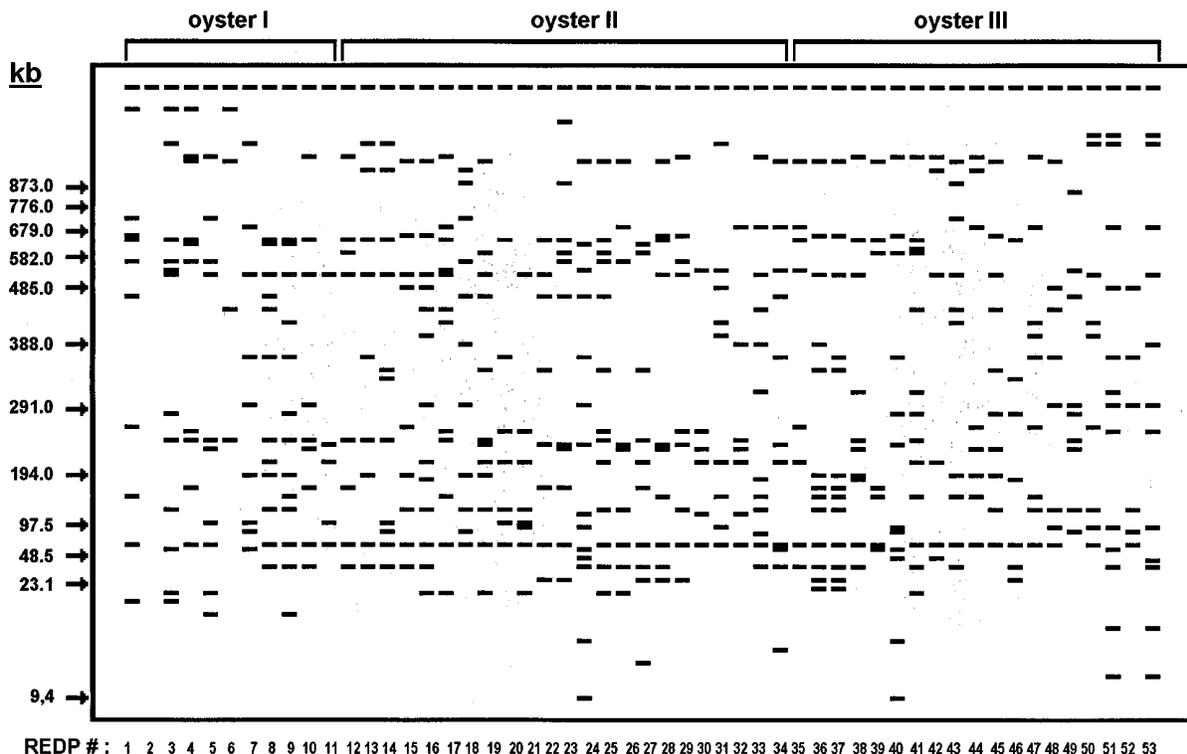


FIG. 3. Diagram of 53 representative *Srf*I REDP of 95 *V. vulnificus* strains.

01	100
02	17 100
03	42 14 100
04	45 17 42 100
05	36 17 42 45 100
06	25 33 33 50 25 100
07	10 18 35 10 29 13 100
08	33 14 38 42 33 33 35 100
09	32 13 44 40 40 21 33 74 100
10	29 18 35 57 57 27 30 52 50 100
11	24 29 21 24 47 18 38 42 30 38 100
12	30 20 36 60 50 29 21 55 52 84 40 100
13	29 18 43 38 38 27 50 70 67 60 38 63 100
14	26 15 40 35 43 24 45 48 46 55 44 57 73 100
15	38 18 26 29 29 27 30 52 50 40 38 42 50 36 100
16	24 13 30 24 32 32 25 52 36 42 40 35 33 31 67 100
17	33 14 46 50 50 33 26 46 52 52 32 55 43 40 26 37 100
18	43 15 24 26 35 12 36 40 31 27 22 19 36 33 36 31 16 100
19	25 14 38 33 42 33 26 62 44 35 53 45 43 40 52 52 38 40 100
20	32 22 29 42 32 15 33 57 45 33 57 35 44 40 33 36 38 30 38 100
21	20 20 36 30 50 14 32 45 35 32 67 33 32 38 42 52 45 29 55 71 100
22	40 20 27 40 30 14 21 45 35 53 53 56 42 48 32 26 36 29 55 35 33 100
23	43 15 24 43 35 12 9 32 23 45 33 48 27 25 18 15 24 42 40 30 19 67 100
24	15 13 21 23 8 20 32 36 28 24 19 17 24 15 24 28 14 22 29 17 17 25 22 100
25	40 13 44 56 40 32 8 59 43 42 30 52 42 46 42 50 44 38 74 55 52 43 46 28 100
26	29 18 35 38 48 27 20 35 33 40 38 32 30 27 50 50 35 36 52 33 42 32 45 32 58 100
27	18 17 17 45 27 25 10 50 40 48 35 60 38 43 29 32 25 17 50 32 30 50 43 23 56 29 100
28	45 17 25 36 36 25 19 42 48 57 47 50 48 43 57 48 42 17 42 32 30 60 52 31 40 57 36 100
29	38 18 35 48 48 13 20 35 33 40 50 42 30 27 50 42 43 36 43 44 53 53 45 16 42 50 29 57 100
30	22 25 20 33 33 17 12 30 19 35 46 25 24 21 24 29 40 21 30 53 50 25 32 27 38 35 33 33 35 100
31	29 18 26 19 19 13 20 26 33 20 38 21 30 27 30 42 43 18 26 33 42 21 18 24 25 20 29 29 20 47 100
32	21 22 19 32 42 31 22 38 27 44 43 35 33 30 22 27 38 30 38 38 35 24 30 17 36 44 42 32 22 67 33 100
33	26 15 24 26 35 24 27 48 46 45 33 48 36 33 45 54 56 33 32 30 38 29 17 15 31 45 26 43 45 21 27 40 100
34	26 15 24 26 17 24 36 48 31 27 44 29 36 25 36 38 32 25 48 40 29 38 33 59 46 55 35 43 27 42 36 40 33 100
35	40 20 27 40 20 29 21 45 35 42 40 44 42 38 53 43 45 19 36 47 33 33 29 33 52 53 40 50 21 50 42 47 38 67 100
36	40 13 30 32 32 21 25 44 57 42 30 43 42 38 67 50 30 38 52 27 35 43 23 21 43 42 40 56 42 19 25 27 54 31 35 100
37	40 13 30 32 32 21 33 52 64 42 30 43 50 38 67 50 30 31 52 36 35 43 23 28 43 42 40 56 42 19 25 18 46 38 35 93 100
38	27 17 33 45 55 25 29 58 56 76 35 70 67 52 48 40 50 26 42 32 30 40 35 15 40 38 36 55 38 33 19 42 61 26 40 40 40 100
39	42 22 29 53 21 31 22 29 36 44 29 59 33 30 33 27 38 20 38 38 24 47 50 26 45 33 42 53 22 27 33 25 30 40 47 45 45 32 100
40	17 14 23 17 17 11 35 15 22 17 21 27 17 16 17 15 15 16 23 19 18 18 24 57 15 17 17 25 35 10 17 10 16 32 9 15 22 17 29 100
41	32 13 37 48 40 32 8 59 57 50 30 61 42 38 25 43 59 15 44 36 35 26 31 21 57 33 56 40 25 29 33 36 54 31 43 29 29 56 45 30 100
42	22 25 20 33 44 17 24 40 29 47 62 50 47 42 35 38 40 32 40 40 50 38 21 18 29 24 33 33 47 43 35 40 42 32 38 29 29 44 27 30 38 100
43	36 17 17 27 36 38 29 50 56 38 35 40 48 35 57 48 50 43 42 21 30 30 26 23 32 38 27 55 29 22 38 21 52 35 40 56 56 45 42 8 40 33 100
44	40 20 9 30 40 14 32 27 35 42 27 33 42 29 42 17 45 38 27 24 22 22 29 8 17 42 20 40 32 38 32 47 48 29 44 35 35 50 35 18 35 50 40 100
45	27 17 33 27 36 38 29 50 48 38 35 30 38 35 67 56 33 35 58 32 40 40 26 15 40 48 27 55 48 33 19 32 43 26 30 64 64 45 32 25 32 33 55 40 100
46	32 22 29 32 21 15 11 38 45 44 29 47 44 50 33 36 29 20 19 38 24 47 40 17 36 33 42 53 33 27 22 25 40 30 47 27 27 53 38 19 45 27 32 24 32 100
47	38 18 17 29 29 13 30 35 50 30 25 32 30 18 40 33 52 27 26 44 32 21 18 16 25 40 19 29 40 24 50 33 55 36 42 33 42 29 33 26 33 35 38 63 29 22 100
48	20 20 18 30 20 43 32 45 35 32 27 22 32 19 53 61 27 38 36 47 44 22 19 42 35 42 20 30 32 25 42 24 38 38 33 35 43 20 35 27 26 25 40 22 50 24 42 100
49	19 18 43 19 29 27 30 35 33 40 13 21 20 27 20 26 26 45 35 22 21 21 27 32 33 30 19 19 20 35 20 33 18 27 21 17 17 29 11 26 25 12 10 21 38 22 20 32 100
50	29 18 35 19 29 13 30 35 42 30 38 32 40 36 50 42 35 27 35 33 53 32 18 16 25 30 19 29 40 24 60 22 36 18 32 33 33 29 22 17 17 35 38 32 26 60 42 20 100
51	8 13 22 16 8 11 50 22 21 25 10 17 33 23 25 29 22 15 7 27 26 9 8 41 21 25 16 16 17 19 33 18 31 38 26 14 21 24 18 30 21 10 16 17 8 18 25 43 17 33 100
52	22 25 20 22 22 17 47 40 38 35 31 25 35 32 47 48 20 53 30 53 38 25 21 27 29 35 22 22 35 29 36 27 32 32 25 29 38 22 27 30 19 29 22 25 33 27 47 75 47 35 38 100
53	8 14 23 17 17 11 43 23 22 35 21 27 35 32 26 30 31 24 15 19 36 18 8 36 22 26 17 25 26 20 26 29 40 24 27 30 22 25 10 23 15 30 25 18 17 19 17 27 17 43 74 20 100

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53

FIG. 4. Similarities (percent) of 53 representative REDP of 95 *V. vulnificus* isolates generated with *SrfI* and calculated with Dice's index.

oyster II, 42 isolates; and oyster III, 40 isolates) were obtained from a 0.5-g sample of each oyster.

A five-tube most-probable-number technique using alkaline peptone water enrichment (28) was also employed to determine the number of *V. vulnificus* organisms in each of the oyster homogenates. The alkaline peptone water was inoculated with 1.0 ml of the appropriate dilution and incubated for 16 h at 35°C. Turbid alkaline peptone water tubes were streaked on modified colistin-polymyxin B-cellobiose. Two *V. vulnificus*-like colonies were picked from each plate, and their identities were confirmed by ELISA.

For analysis by CHEF, a single colony of each isolate was inoculated into 10 ml of Trypticase soy broth supplemented with 1% NaCl and incubated for 5 h at 37°C. Cells were harvested, washed, and suspended in agarose plugs as previously described (3). The agarose plugs were incubated for up to 48 h in a proteinase K solution (EM Science, Cherry Hill, N.J.; 2 mg/ml in 0.5 M EDTA and 0.5% N-lauryl sarcosine; Sigma Chemical Co., St. Louis, Mo.) at 50°C. The plugs were

then treated with phenylmethylsulfonyl fluoride (Sigma) and washed with 0.1 M Tris-0.1 M EDTA buffer. The genomic DNA embedded within the agarose plugs was digested with restriction endonucleases obtained from New England Biolabs (Beverly, Mass.), Stratagene (La Jolla, Calif.), or Promega (Madison, Wis.), as recommended by the manufacturer. High-molecular-weight restriction fragments were resolved by a CHEF (CHEF-DR II; Bio-Rad Laboratories, Richmond, Calif.) pulsed-field system with 1.0% electrophoresis-grade agarose (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.). An electrophoretic regimen of 200 V for 24 h at a temperature of 18°C and a switching time from 1 to 40 s (*SfiI*) or 20 to 90 s (*SrfI*) were employed to fractionate fragments. As DNA size standards, a low-range lambda (New England Biolabs) and lambda concatemers (Promega) were used. In addition, *V. vulnificus* FR1K 232 (Food Research Institute—Kaspar culture collection strain 232), isolated from oyster I, was included on each gel as a standard profile for gel-to-gel comparisons and alignment. Furthermore, at least two common strains

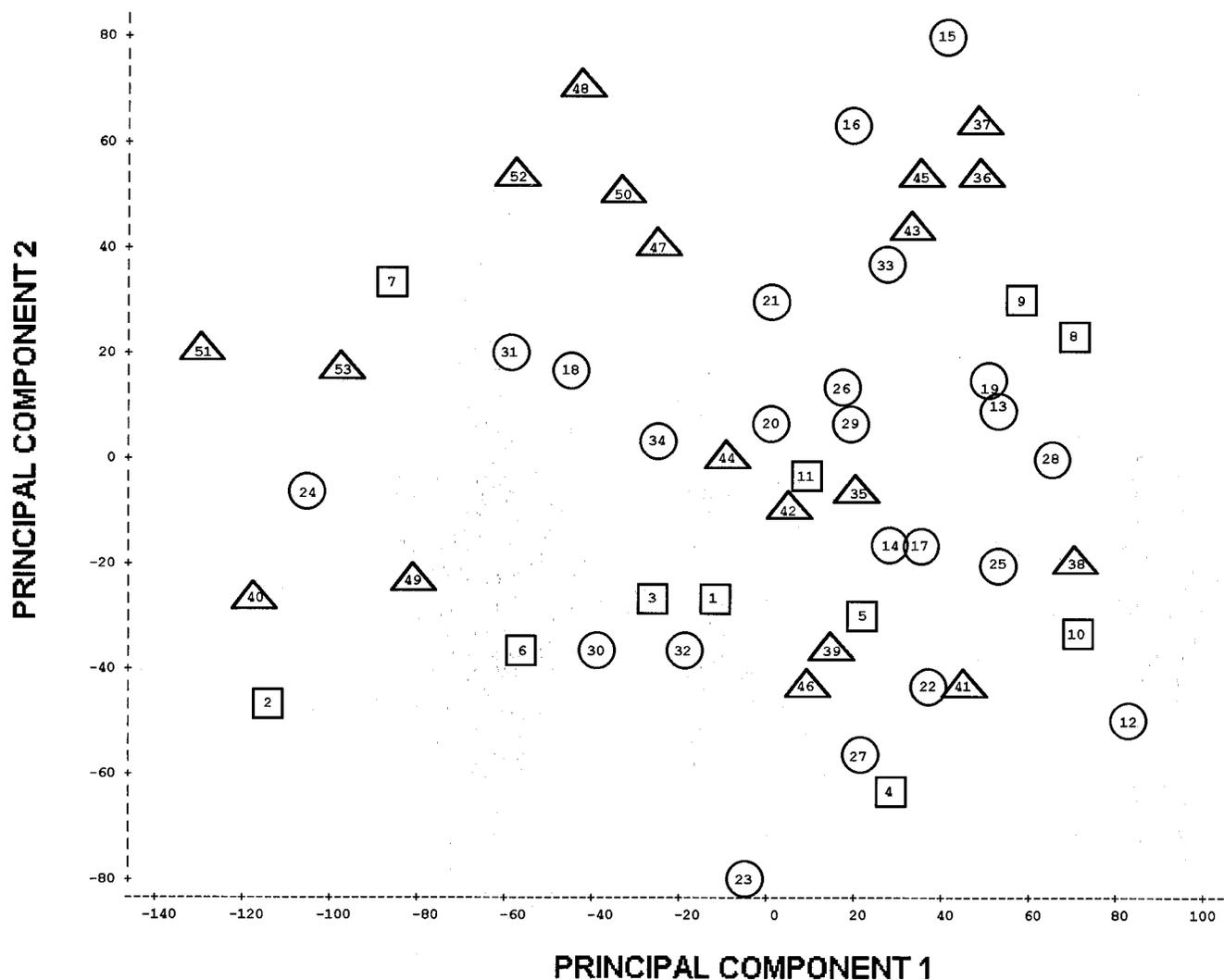


FIG. 5. Plot of the first and the second principal components obtained by principal-component analysis of Dice similarity data of 53 representative *SrfI* REDP of 95 *V. vulnificus* isolates. REDP numbers correspond with numbers given in Fig. 3 and 4. REDP were from strains isolated from oyster I (□), oyster II (○), and oyster III (△).

on each gel were overlapped to normalize band positions on gels. The gels were stained in ethidium bromide (Sigma) solution and photographed under short-wave UV light.

The genomic patterns of all strains were visually compared, and the presence or absence of bands was recorded in binary scores for calculating similarity indices and diagramming restriction endonuclease digestion profiles (REDP) by using a computer program for managing electrophoresis banding patterns, ELBAMAP 2.2 (12). The program plots REDP on the basis of the entered database in which the highest- and lowest-molecular-weight bands set the limits for plotting. In addition, a similarity index was calculated from the REDP patterns by pairwise comparisons. The similarities of the profiles were calculated by using Dice's coincidence index (5): DNA fingerprint similarity for strains  $x$  and  $y$  ( $S_{xy}$ ) is the number of common bands in their DNA profiles ( $n_{xy}$ ) divided by the average number of bands exhibited by both strains [ $S_{xy} = 2n_{xy}/(n_x + n_y)$ ].

To more clearly visualize genomic relationships among the *V. vulnificus* strains, principal-component analysis of the simi-

ilarity coefficients was performed by using the Statistical Analysis System (SAS Institute, Cary, N.C.), essentially as described by Chen et al. (4).

A total of 118 *V. vulnificus* isolates from three oysters was examined. The most probable number for each oyster was 310/g for oyster I, 220/g for oyster II, and 3,300/g for oyster III. DNA from *V. vulnificus* isolates was subjected to CHEF gel electrophoresis after digestion with restriction endonucleases possessing 8-base recognition sequences (*NotI*, *SfiI*, *AscI*, and *SrfI*) or 6-pair recognition sequences (*SmaI*, *ApaI*, and *XbaI*) that are likely rare in bacterial genomes (14) having GC contents of 46 to 48%, as does the *V. vulnificus* genome (1). Among the enzymes tested, *SfiI* (GGCCNNNNNGGCC) and *SrfI* (GCCCCGGGC) were selected because they generated the most appropriate number and best distribution of restriction fragments in *V. vulnificus*. *SfiI* and *SrfI* digests yielded up to 28 and 14 visible bands, respectively (Fig. 1 and 2).

CHEF gel electrophoresis was a highly discriminatory and reproducible method for *V. vulnificus*; however, the DNA of some of the isolates consistently produced smears when exam-

ined by CHEF, and no REDP could be determined (Fig. 2, lanes E, G, and O). Although the utmost care was taken in DNA preparation, 23 (19.5%) of the 118 isolates could not be typed in repeated (up to seven times) trials. The degradation of DNA might have been caused by nucleases or other properties specific to these strains. Further studies to overcome this problem are underway. The analysis of the 118 isolates resulted in 95 typeable isolates which could be divided into 60 different REDP types with *SfiI* and 53 different REDP types with *SrfI*. Thirty-six isolates (five nontypeable) from oyster I yielded 12 different REDP with *SfiI* and 11 REDP with *SrfI*. Forty-two isolates (12 nontypeable) from oyster II yielded 28 and 24 REDP, and 40 *V. vulnificus* isolates (6 nontypeable) from oyster III yielded 19 and 20 REDP with *SfiI* and *SrfI*, respectively. No REDP was common among isolates from the three oysters. On the basis of the test of undiscovered proportion (singletons divided by sample size), the upper limits of different strains per oyster could be 660 (*SfiI*) or 600 (*SrfI*) for oyster I, 660 (*SfiI*) or 570 (*SrfI*) for oyster II, and 940 (*SfiI*) or 1,000 (*SrfI*) for oyster III.

Visual comparisons of *SfiI* and *SrfI* gels revealed that no specific grouping of strains was possible. For more precise comparisons, a computer program for managing banding patterns was applied to *SrfI* genomic patterns. *SrfI* REDP were selected for this analysis because the average number of bands was relatively small and easy to manage (Fig. 3). To define genetic relatedness among strains, similarity matrices were calculated (Fig. 4) by pairwise comparison of the REDP data from *V. vulnificus* strains representing the 53 *SrfI* REDP with the Dice coincidence index. The results showed that the profile similarities ranged from 7 to 93% for all the REDP analyzed. The REDP similarities were 10 to 57% for strains isolated from oyster I, 8 to 71% for strains isolated from oyster II, and between 7 and 93% for strains isolated from oyster III. The strains isolated from one oyster did not show higher similarity indices than the strains isolated from different oysters. Only 0.7% of the Dice coincidence indices was between 70 and 93%. For example, profiles 36 and 37 had a similarity of 93%, and REDP 12 and 13 had a Dice coefficient of 84%. Eighty-nine percent of the similarity coefficients were 50% or less (Fig. 4).

In addition, principal component analysis for detection of strain clusters was conducted. The first component of the principal-component analysis accounted for 23% and the second component accounted for 15% of the total variance (Fig. 5). The observation that the first two principal components account for only 38% of the total variance indicates the heterogeneity within the REDP. Again, no specific clustering of strains was detected. Also, strains from an individual oyster did not cluster (Fig. 5).

These data indicate that great genomic diversity exists among *V. vulnificus* organisms in individual oysters. These results support two hypotheses related to human *V. vulnificus* infections: (i) infections are caused by mixed populations of *V. vulnificus*, or (ii) only a few of these different *V. vulnificus* strains are pathogenic. Further studies to determine whether a correlation exists between certain profiles and strain virulence are underway.

These results further emphasize the need for refined molecular typing methods in epidemiological and ecological investigations of phenotypically similar *V. vulnificus* strains and for implicating a specific source in infections.

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