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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

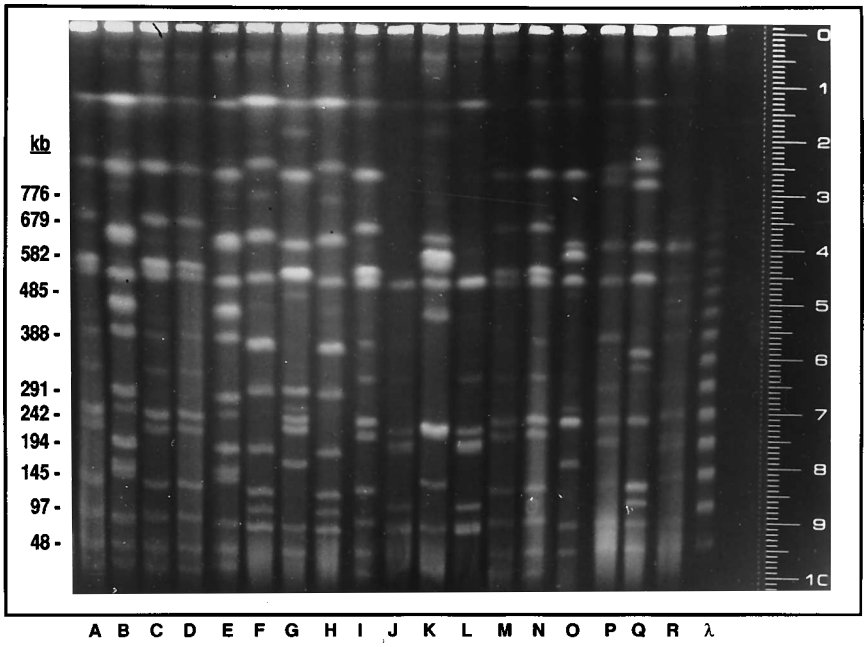

FIG. 1. DNA restriction patterns of *V. vulnificus* as determined by CHEF after cleavage with *Srf*I. Lane A, FRIK 236; lane B, FRIK 238; lane C, FRIK 239; lane D, FRIK 240; lane E, FRIK 241; lane F, FRIK 242; lane G, FRIK 243; lane H, FRIK 244; lane I, FRIK 245; lane J, FRIK 246; lane K, FRIK 247; lane 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 λ, DNA size standards.

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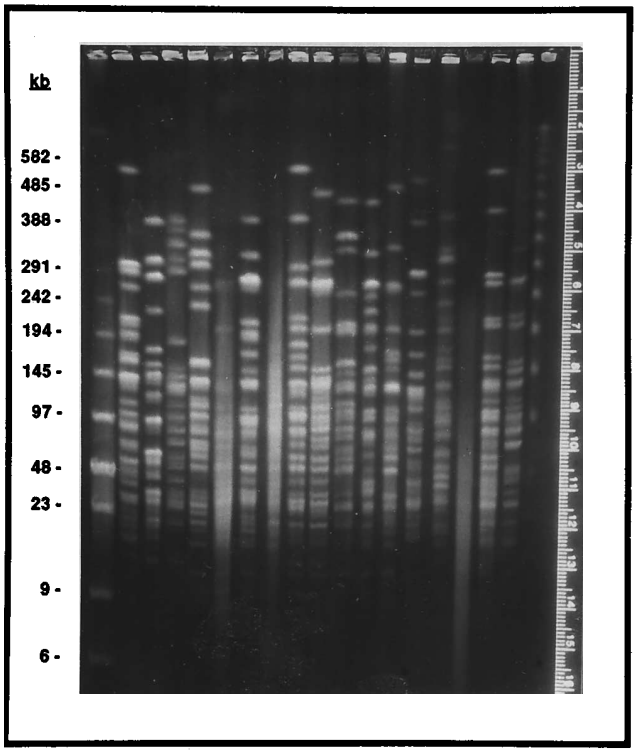

FIG. 2. DNA restriction patterns of *V. vulnificus* as determined by CHEF after cleavage with *Sfi*I. Lane A, FRIK 284; lane B, FRIK 285; lane C, FRIK 286; lane D, FRIK 288; lane E, FRIK 289; lane F, FRIK 290; lane G, FRIK 291; lane H, FRIK 293; lane I, FRIK 294; lane J, FRIK 251; lane K, FRIK 255; lane L, FRIK 301; lane M, FRIK 302; lane N, FRIK 310; lane O, FRIK 333; lane P, FRIK 334; lane Q, FRIK 232; lane λ, 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^2 (ca. 4.6-m^2) 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 μl 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;

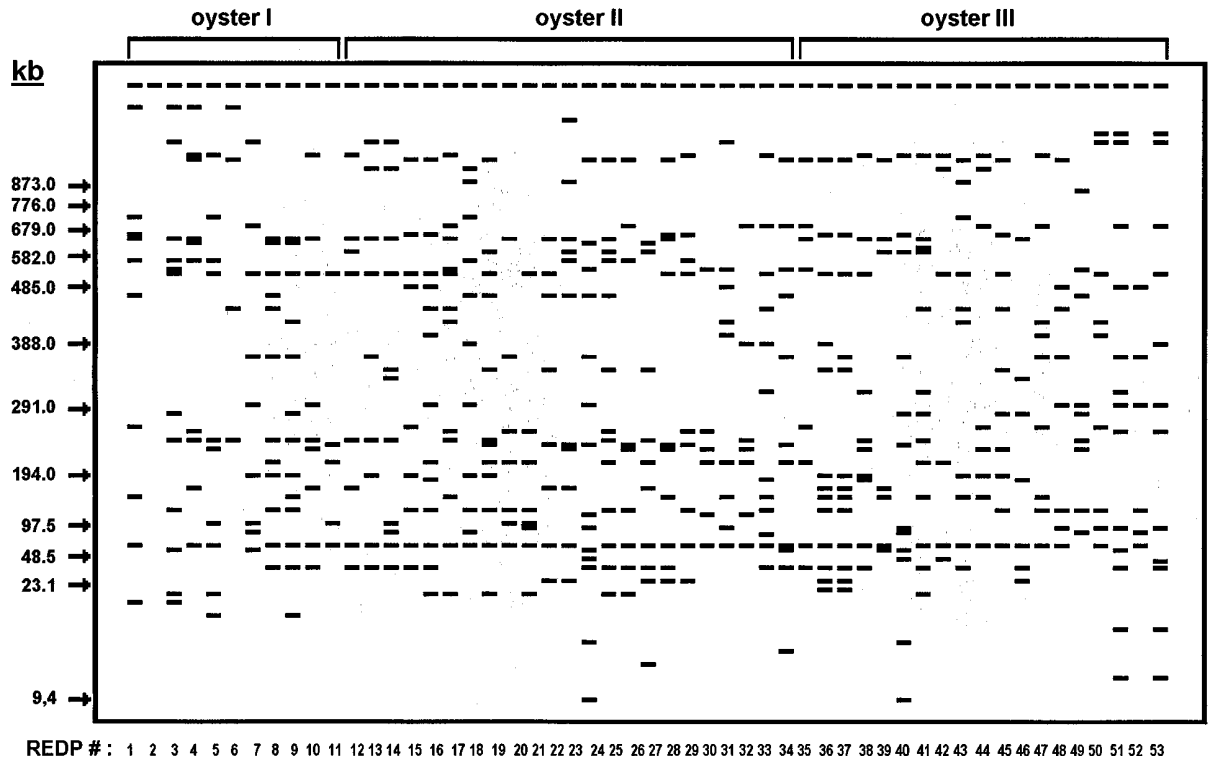

FIG. 3. Diagram of 53 representative *Srf*I REDP of 95 *V. vulnificus* strains.

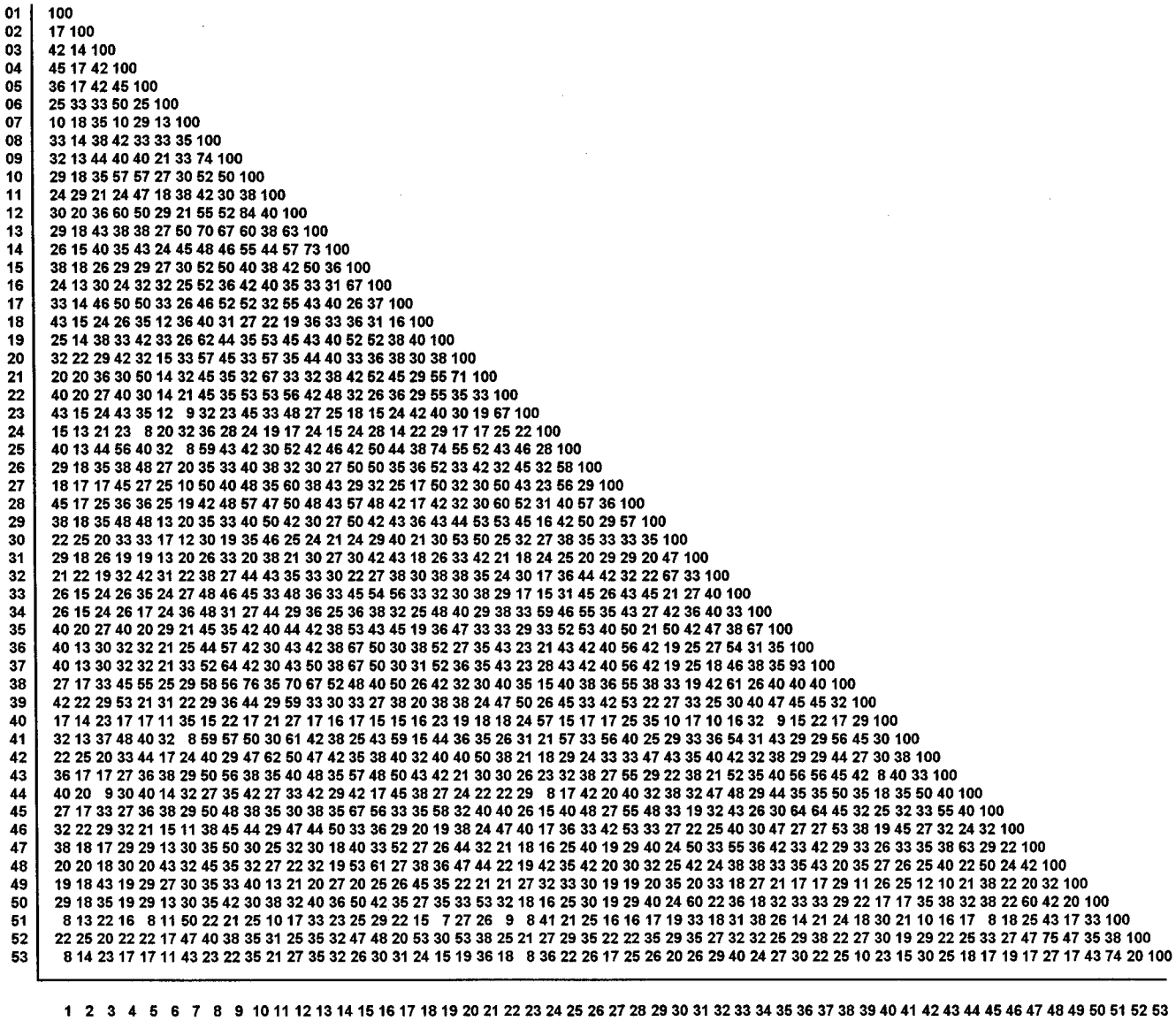

FIG. 4. Similarities (percent) of 53 representative REDP of 95 *V. vulnificus* isolates generated with *Srf*I and calculated with Dice's index.

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 (*Sfi*I) or 20 to 90 s (*Srf*I) 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* FRIK 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

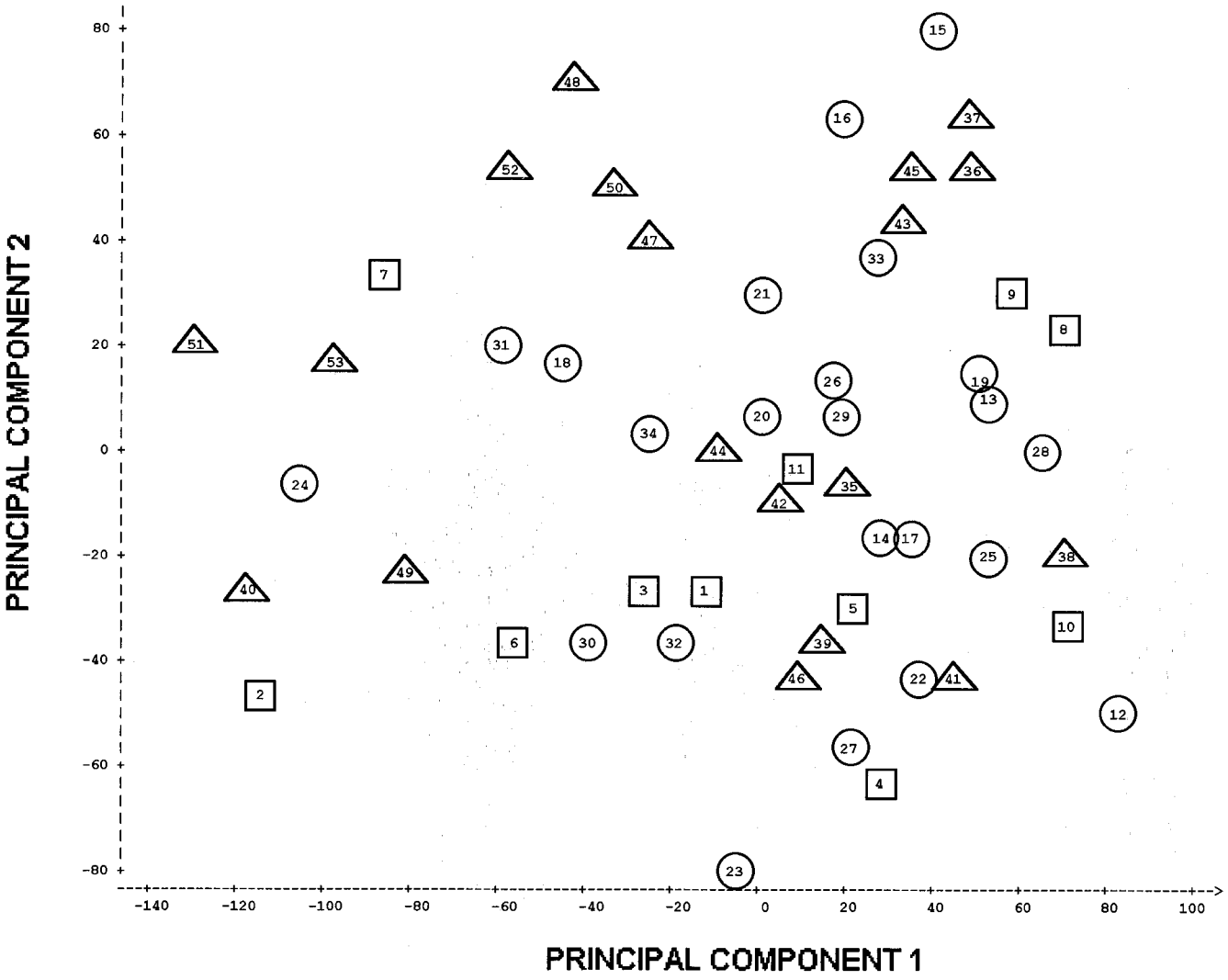

FIG. 5. Plot of the first and the second principal components obtained by principal-component analysis of Dice similarity data of 53 representative *Srf*I REDP of 95 *V. vulnificus* isolates. REDP numbers correspond with numbers given in Fig. 3 and 4. REDP were from strains isolated from oyster I (□), 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 diagraming 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 similarity 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 (*Not*I, *Sfi*I, *Asc*I, and *Srf*I) or 6-pair recognition sequences (*Sma*I, *Apa*I, and *Xba*I) 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, *Sfi*I (GGCCNNNNNGGCC) and *Srf*I (GCCCGGGC) were selected because they generated the most appropriate number and best distribution of restriction fragments in *V. vulnificus. Sfi*I and *Srf*I 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 *Sfi*I and 53 different REDP types with *Srf*I. Thirty-six isolates (five nontypeable) from oyster I yielded 12 different REDP with *Sfi*I and 11 REDP with *Srf*I. 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 *Sfi*I and *Srf*I, 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 (*Sfi*I) or 600 (*Srf*I) for oyster I, 660 (*Sfi*I) or 570 (*Srf*I) for oyster II, and 940 (*Sfi*I) or 1,000 (*Srf*I) for oyster III.

Visual comparisons of *Sfi*I and *Srf*I gels revealed that no specific grouping of strains was possible. For more precise comparisons, a computer program for managing banding patterns was applied to *Srf*I genomic patterns. *Srf*I 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 *Srf*I 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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