

Randomly Amplified Polymorphic DNA Analysis of Clinical and Environmental Isolates of *Vibrio vulnificus* and Other *Vibrio* Species

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Vibrio vulnificus is an estuarine bacterium that is capable of causing a rapidly fatal infection in humans. A randomly amplified polymorphic DNA (RAPD) PCR protocol was developed for use in detecting *V. vulnificus*, as well as other members of the genus *Vibrio*. The resulting RAPD profiles were analyzed by using RFLPScan software. This RAPD method clearly differentiated between members of the genus *Vibrio* and between isolates of *V. vulnificus*. Each *V. vulnificus* strain produced a unique band pattern, indicating that the members of this species are genetically quite heterogeneous. All of the vibrios were found to have amplification products whose sizes were within four common molecular weight ranges, while the *V. vulnificus* strains had an additional two molecular weight range bands in common. All of the *V. vulnificus* strains isolated from clinical specimens produced an additional band that was only occasionally found in environmental strains; this suggests that, as is the case with the Kanagawa hemolysin of *Vibrio parahaemolyticus*, the presence of this band may be correlated with the ability of a strain to produce an infection in humans. In addition, band pattern differences were observed between encapsulated and nonencapsulated isogenic morphotypes of the same strain of *V. vulnificus*.

Many members of the genus *Vibrio* have been implicated in both human diseases and marine animal diseases. One member of this genus, *Vibrio vulnificus*, is ubiquitous in coastal waters and is responsible for more than 95% of all seafood-related deaths (11, 13). *V. vulnificus* infection can occur either after ingestion of raw or undercooked shellfish, particularly oysters, or through entry via a flesh wound (11). The people vulnerable to infection include those with chronic diseases involving elevated serum iron levels, immune function abnormalities, and other chronic disorders (11).

The same isolate of *V. vulnificus* has been observed to have two different colony morphologies when it is grown on solid nutrient media (14, 24). The first morphotype is termed opaque, and the surfaces of the cells are covered with a polysaccharide capsule. The translucent morphotype lacks a polysaccharide capsule. All virulent strains of *V. vulnificus* are opaque morphotype strains, which indicates that the capsule plays a role in the virulence of the organism (1, 6, 9, 14). Opaque strains of *V. vulnificus* have been observed to lose their capsule; they become translucent and lose their virulence (14). However, the reverse situation (translucent cells gaining a capsule) generally has not been observed. It has also been reported that more than 90% of environmental *V. vulnificus* strains are opaque morphotype strains (16), yet these strains have been found to be highly variable in terms of virulence (8, 15), suggesting that factors other than the presence of a capsule also contribute to the virulence of the organism. Because of the severity of *V. vulnificus* infections, a reliable method for rapid identification of virulent strains of this organism is needed.

Randomly amplified polymorphic DNA (RAPD) PCR (5, 19, 20, 21, 22) is a technique that is known to be a sensitive

method for detecting slight genetic differences between samples. We optimized a RAPD method suitable for distinguishing various *Vibrio* species from one another, as well as for differentiating between *V. vulnificus* strains. Furthermore, we investigated the ability of the RAPD method to detect genetic differences between opaque and translucent morphotypes of the same isolate of *V. vulnificus*. Finally, we investigated the possibility that clinical isolates of *V. vulnificus* may produce a unique RAPD band pattern that could be used to differentiate virulent strains from avirulent strains.

MATERIALS AND METHODS

Bacterial stains and culture preparation. A total of 16 *Vibrio* species (Table 1), as well as 39 clinical isolates and 30 environmental isolates of *V. vulnificus*, were used in the present study. Translucent morphotypes were isolated as spontaneous mutants of the opaque morphotypes in the laboratory of Ronald Siebling at Louisiana State University. All cells were grown to the stationary phase at 22°C with aeration in heart infusion (Difco Laboratories, Detroit, Mich.) broth. These cultures were used as the sources of template DNA for RAPD analysis.

RAPD analysis. Ten 10-bp oligonucleotide primers (Genosys Biotechnologies, Inc., The Woodlands, Tex.) with G+C contents of 50% were screened for the ability to provide a suitable band pattern with various *V. vulnificus* strains. The primer selected had the following sequence: 5'GGATCTGAAC3'. Each 25.0- μ l RAPD reaction mixture contained the following reagents: 2.5 μ l of 10 \times reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin) (Promega, Madison, Wis.), 2.0 μ l of sterile H₂O, 3.5 μ l of 25 mM MgCl₂, 8.0 μ l of a solution containing each of the deoxynucleoside triphosphates (Promega) at a concentration of 5 mM, 3.0 μ l of primer (Biosynthesis, Lewisville, Tex.), 5.0 U of *Taq* DNA polymerase (Promega), and 5.0 μ l of cell culture. The reaction mixtures were overlaid with 20.0 μ l of sterile mineral oil (Sigma Chemical Co., St. Louis, Mo.) to seal them and to prevent evaporation in the thermal cycler. Thermal cycling was performed with a model PHC-3 thermal cycler (Techne, Princeton, N.J.). The cycling profile was as follows: one cycle consisting of 94°C for 5 min, 45 cycles consisting of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, and a final cycle consisting of 72°C for 5 min. The RAPD products were electrophoresed by using a Fisher Biotech Small Horizontal Gel System (Fisher Scientific, Pittsburgh, Pa.) at 60 V for approximately 3 h on a 2.0% agarose gel containing ethidium bromide (2.5 μ l of a 10-mg/ml solution) and were photographed with a Polaroid model ASP Quick Shooter camera (International Biotechnologies, Inc., New Haven, Conn.) under UV light. A 123-bp ladder (Sigma) was used as a molecular weight marker. The RAPD method was used with all strains at least three times.

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TABLE 1. *Vibrio* species used for RAPD analysis

Strains used
<i>V. aestuarianus</i> OSU-65
<i>V. alginolyticus</i> ATCC 17749
<i>V. anguillarum</i> ATCC 19264
<i>V. cholerae</i> 2741-80
<i>V. fluvialis</i> 5440
<i>V. fischeri</i> B64
<i>V. furnisii</i> 8760
<i>V. harveyi</i> ATCC 25919
<i>V. leiognathi</i> B474
<i>V. metschnikovii</i> ATCC 7708
<i>V. mimicus</i> ATCC 33653
<i>V. natriegens</i> ATCC 14048
<i>V. nigripulchritudo</i> ATCC 27043
<i>V. parahaemolyticus</i> ATCC 17802
<i>V. pelagius</i> 25916
<i>V. proteolyticus</i> ATCC 15538
<i>V. vulnificus</i> ^a

^a A total of 70 *V. vulnificus* strains from clinical and environmental sources were used.

Computer analysis of RAPD profiles. All of the gels were scanned with an ImageMaster DTS scanner (Pharmacia, Uppsala, Sweden). A 123-bp ladder was included every three or four lanes on all gels as a standard molecular weight marker. Images were calibrated and data analysis was performed by using RFLP-Scan software (Scanalytics, Billerica, Mass.). A match tolerance equivalent to 1.0% of the molecular weight of each band was used.

RESULTS

A RAPD method was developed and optimized for use with *Vibrio* species, including *V. vulnificus*. When this method was applied to various gram-positive and gram-negative organisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*), no RAPD profiles were observed. All of the *Vibrio* species tested produced unique RAPD profiles. Representative profiles of various vibrios are shown in Fig. 1. Four bands in specific molecular weight ranges were identified as bands that were common to all species of this genus (Table 2). Furthermore, when 70 *V. vulnificus* isolates were analyzed (Fig. 2), bands in an additional two molecular weight ranges (492 to 464 and 264 to 244 bp) were produced by all of the isolates of this species. While all of the *V. vulnificus* strains examined produced bands in these six ranges, all of the *V. vulnificus* isolates could be clearly distinguished from one another based on their RAPD profiles. In addition to the two unique *V. vulnificus* bands, one additional molecular weight range band was present in all 31 clinical isolates tested. This band, at 200 to 178 bp, was found in only 3 of the 39 environmental isolates tested.

RAPD analysis of opaque and translucent isogenic morphotypes of 12 *V. vulnificus* strains revealed differences in the RAPD profiles obtained for each pair of morphotypes (Fig. 3). Occasionally, the two morphotypes produced nearly identical profiles, but in most cases there were detectable differences between the two morphotypes. The opaque morphotypes always produced complete band profiles, while the translucent morphotypes often produced band profiles with fewer bands than the opaque morphotype profiles, as well as unique bands not found in the opaque profiles. As was the case with all of the *Vibrio* species and *V. vulnificus* isolates examined, the RAPD profiles of the opaque and translucent morphotypes were consistently reproducible.

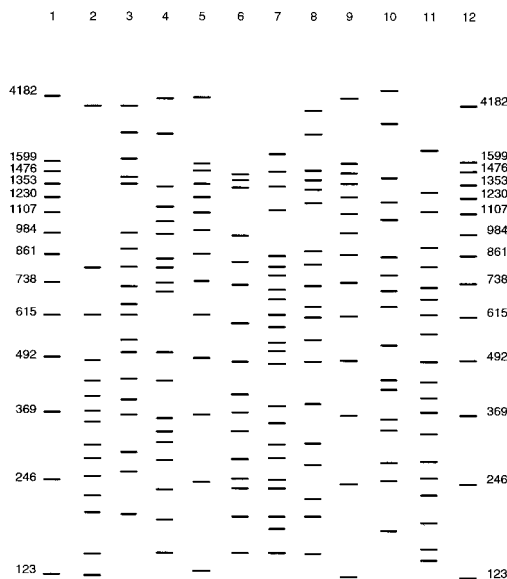


FIG. 1. Representative RAPD profiles obtained for various *Vibrio* species. Lanes 1, 5, 9, and 12, 123-bp ladder; lane 2, *V. parahaemolyticus*; lane 3, *V. nigripulchritudo*; lane 4, *V. anguillarum*; lane 6, *V. fluvialis*; lane 7, *V. alginolyticus*; lane 8, *V. natriegens*; lane 10, *V. mimicus*; lane 11, *V. harveyi*.

DISCUSSION

When the RAPD method was used, all of the *Vibrio* species could be clearly differentiated from one another. Furthermore, all isolates could be identified as members of the genus *Vibrio* based on the presence of amplification fragments whose molecular weights were within four molecular weight ranges. *V. vulnificus* isolates produced a unique band profile with two additional molecular weight range bands, suggesting that the RAPD method may be an important tool for differentiating *V. vulnificus* from other members of the genus *Vibrio*. The finding that each *V. vulnificus* isolate produced a unique RAPD profile also suggests that *V. vulnificus* isolates are genetically very heterogeneous, which is consistent with the results of previous studies of *V. vulnificus* in which researchers used arbitrarily primed PCR (3), ribotyping (7), clamped homogeneous electric field gel electrophoresis (4), pulsed-field gel electrophoresis (17), and amplified fragment length polymorphisms (2).

Previous studies have revealed no significant differences between clinical and environmental isolates of *V. vulnificus* based on biochemical test results, antimicrobial susceptibility patterns, or virulence characteristics (15, 18). Other studies have shown that a capsule is required for *V. vulnificus* virulence (1, 6, 9, 14) and that 90% of environmental isolates have capsules (16). Nevertheless, the virulence of these isolates is variable (8,

TABLE 2. Common molecular weight range bands in *Vibrio* species as determined by computer analysis

Mol wt range (bp)	Organisms found in
853-714.....	All <i>Vibrio</i> species
706-509.....	All <i>Vibrio</i> species
426-328.....	All <i>Vibrio</i> species
319-273.....	All <i>Vibrio</i> species
492-273.....	<i>V. vulnificus</i> (all isolates)
264-244.....	<i>V. vulnificus</i> (all isolates)
200-178.....	<i>V. vulnificus</i> (clinical isolates only)

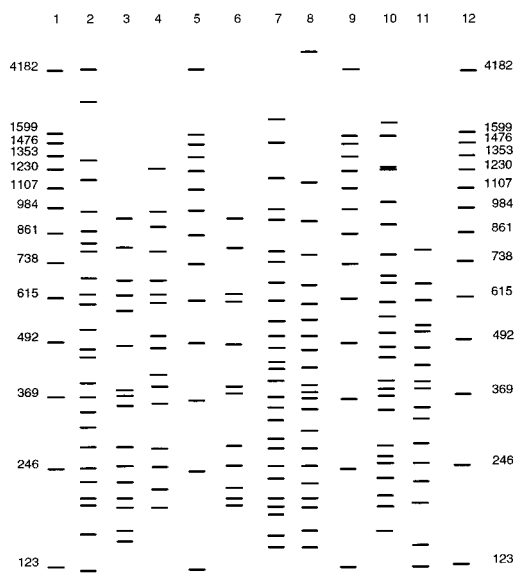


FIG. 2. Representative RAPD profiles of clinical and environmental *V. vulnificus* isolates. Lanes 1, 5, 9, and 12, 123-bp ladder; lane 2, strain EDL174 (clinical); lane 3, strain E4125 (clinical); lane 4, strain 371 (environmental); lane 6, strain M06 (clinical); lane 7, strain CVD713 (clinical); lane 8, strain H3308 (clinical); lane 10, strain B9629 (clinical); lane 11, strain 890 (environmental).

15), suggesting that the presence of a capsule is not the sole requirement for virulence. Attempts in our lab to correlate particular capsular types with virulence have been unsuccessful (10). The fact that the 200- to 178-bp segment was always present in the RAPD profiles of clinical *V. vulnificus* isolates and only occasionally present in the RAPD profiles of environmental isolates suggests that this segment of DNA may in some way influence the virulence of an isolate and thus the ability of the organism to infect human hosts. A recent study performed with *V. vulnificus* resulted in a similar conclusion; most clinical isolates contained a certain iron acquisition gene (*viuB*), while most environmental strains did not contain this gene (3). This gene may also serve as a target for identification of virulent strains of *V. vulnificus*.

Additional studies in our laboratory are concentrating on the role that the fragment from clinical isolates may play in virulence. It is possible that this fragment could be used as a probe to determine whether environmental strains are virulent. The fact that only about 8% of the environmental strains contained this DNA fragment suggests the possibility that, like strains of *Vibrio parahaemolyticus* (12), only certain strains of *V. vulnificus* may be capable of causing human infection. If this is the case, then the commonly employed mouse models used to predict the virulence of *V. vulnificus* strains (23) may not be valid. These models often predict that nearly all strains are virulent.

Differences in the RAPD profiles of opaque and translucent isogenic isolates may eventually lead to an understanding of the genetic mechanism responsible for the change from opaque morphotype to translucent morphotype. This mechanism is poorly understood, and various attempts to determine the trigger for this event have been unsuccessful. It has been suggested previously that the trigger for the switching event may be a chromosomal rearrangement or genetic inversion (16). Our results indicate that this phenomenon is influenced by permanent genetic changes which can be detected by the production of unique RAPD profiles by isogenic morphotypes of the same

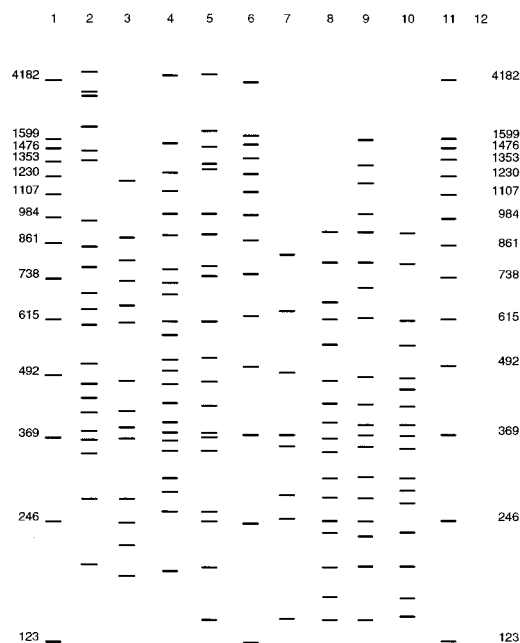


FIG. 3. Representative RAPD profiles of opaque and translucent morphotypes of *V. vulnificus* isolates. Lanes 1, 6, and 11, 123-bp ladder; lane 2, strain 549 (opaque); lane 3, strain 549 (translucent); lane 4, strain 1007 (opaque); lane 5, strain 1007 (translucent); lane 7, strain 707 (opaque); lane 8, strain 707 (translucent); lane 9, strain 1002 (opaque); lane 10, strain 1002 (translucent).

isolate of *V. vulnificus*. The presence of such dramatic differences between opaque and translucent morphotypes further suggests that global changes may occur instead of a single, simple event.

The findings of this study suggest that the RAPD method is indeed a useful tool for identification and differentiation of various *Vibrio* species, as well as for differentiation of clinical and environmental *V. vulnificus* isolates. The genetic differences between opaque and translucent isolates of *V. vulnificus* will be explored further. The finding that the 200- to 178-bp segment of DNA is found in all clinical isolates and in only a few environmental strains should serve as a focus for further studies involving sequencing and probe production.

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