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Randomly Amplified Polymorphic DNA Analysis of Starved and Viable but Nonculturable *Vibrio vulnificus* Cells

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***Vibrio vulnificus* is an estuarine bacterium capable of causing a rapidly fatal infection in humans. Because of the low nutrient levels and temperature fluctuations found in the organism's natural habitat, the starvation state and viable but nonculturable (VBNC) state are of particular interest. A randomly amplified polymorphic DNA (RAPD) PCR protocol was developed previously for the detection of *V. vulnificus* strains grown in rich media and has been applied to starved and VBNC cells of *V. vulnificus* in the present study. As cells were subjected to starvation in artificial seawater, changes in the RAPD profile were detected as early as 15 min into the starvation period. Most noticeable was a uniform loss of RAPD amplification products. By 4 h of starvation, the cells were undetectable by the RAPD method. Cells that had been starved for up to 1 year again became detectable by the RAPD method when nutrients were added to the starvation microcosm. The same loss of signal, but at a lower rate, was also seen as cells entered the VBNC state. VBNC cells were resuscitated by a temperature upshift and were once again detectable by the RAPD method. The addition of chloramphenicol prevented the RAPD signal from being lost in both the starvation and VBNC states. This suggests that DNA binding proteins produced during starvation and entrance into the VBNC state may be responsible for the inability of the RAPD method to amplify *V. vulnificus* DNA in these states.**

Many members of the genus *Vibrio* have been implicated in both human disease and marine animal disease. Among these species, *Vibrio vulnificus* is responsible for greater than 95% of all seafood-related deaths (13). *V. vulnificus*, an opportunistic pathogen native to estuarine water, is capable of causing a rapidly fatal infection in humans. This infection can occur either after ingestion of raw or undercooked shellfish or through entry via a flesh wound (13). People vulnerable to infection include those with underlying chronic diseases involving elevated serum iron levels (13).

While *V. vulnificus* cells exist in their natural estuarine habitat, they are frequently subjected to a state of nutrient starvation. Under laboratory conditions, *V. vulnificus* has been observed to exist in a nutrient-starved state for greater than 7 years (12). It is known that many metabolic changes occur in *V. vulnificus* during nutrient starvation, including the synthesis of unique proteins (9). There are also distinct physical changes, such as the formation of dwarf cells, that occur as a response to starvation (11). Bacteria existing under nutrient starvation conditions become more resistant to subsequent stresses which may be incurred in the environment (19).

By standard methods, *V. vulnificus* is easily cultured from the environment during warm months, but during colder months attempts to culture this bacterium have been largely unsuccessful (14). It was determined that the temperature downshift occurring during colder months triggers a response termed the viable but nonculturable (VBNC) state. This response has been noted in at least 30 other species (16). As cells enter this state, they can no longer be cultured on routine media; thus, their viability can be ascertained only by the use of various direct viability assays which detect metabolic activity within the cell (14, 20). The VBNC state is thought to be a survival response to adverse environmental conditions, and in the case of *V. vulnificus* it can be induced by a temperature downshift (25). Physical and metabolic changes analogous to those seen in the starvation state also occur during the VBNC response (6, 7, 10, 14). Resuscitation of VBNC cells of *V. vulnificus* occurs when the temperature stress is removed (10, 14, 15, 17, 18, 23).

Randomly amplified polymorphic DNA (RAPD) is a technique related to PCR. The RAPD method employs a single, short oligonucleotide primer that binds to any region in the genome bearing the complementary sequence (3, 22, 24). PCR then results in amplification of these complementary genomic DNA sequences. The resulting products can be separated and visualized by agarose gel electrophoresis. The procedure is unique in that no prior sequence information of the genomic DNA sample is needed and whole-cell cultures can be used as the source of template DNA (5).

Because *V. vulnificus* cells exist in the natural environment under starvation conditions and at certain times under VBNC conditions, a method capable of reliable detection of these cells is needed. To this end, an RAPD method was developed to detect starved and VBNC cells.

MATERIALS AND METHODS

Culture preparation. *V. vulnificus* C7184 (opaque) was employed throughout the studies. Cells were grown at 22°C to stationary phase with aeration in heart infusion (HI) (Difco, Detroit, Mich.) broth. These cultures were used to produce starvation and VBNC microcosms.

Preparation for starvation and VBNC studies. Stationary-phase cells were inoculated into fresh HI broth and were allowed to grow to mid-log phase (optical density at 660 nm, 0.15 to 0.20). These cells were then added as a 1% inoculum to an artificial seawater (ASW) microcosm containing either 200 μg of chloramphenicol (Sigma, St. Louis, Mo.) per ml or no antibiotic to produce a ca. 10^6-CFU/ml population. Starved cells were maintained at room temperature (22°C) and monitored periodically by means of plate counts on HI agar. VBNC microcosms were placed at 5°C and were similarly monitored on a daily basis for culturability by plate counts and by filtering a portion of the microcosm and plating the filter on HI agar. Once cells from a filtered 10-ml aliquot could no longer be cultured (<0.1 CFU/ml), viability was ascertained by a direct viability assay (see below).

Samples to be used for RAPD analysis were obtained by centrifuging 5 ml of the starvation or VBNC microcosm at 16,000 × *g* for 5 min. The cells were washed and resuspended in 25.0 μl of HI broth. Cells (5.0 μl) were added to 2.5 μl of 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM

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$MgCl_2$, 0.01% gelatin [Promega, Madison, Wis.]) and stored at −20°C until PCR was performed.

Viability assay. Cells starved at 5°C were initially monitored for cell numbers by plate counts and filtration. Once cells were no longer detectable, the CTC/DAPI technique (20) was employed to ascertain viability. Cells from the VBNC microcosm were centrifuged at 16,000 × *g* for 5 min. The pellet was washed twice with sulfate-free ASW and resuspended to a final volume of 1 ml. Cyanoditolyl tetrazolium chloride (CTC) (Polysciences, Inc., Warrington, Pa.) was added to produce a final concentration of 2 mM. These cells were incubated overnight. Cells were fixed with 37% formalin (Sigma) and counterstained for at least 1 h with 4′,6-diamidino-2-phenylindole (DAPI) (Polysciences, Inc.). The cells were filtered onto 25-mm black polycarbonate membrane filters (0.2-μm pores) (Poretics, Livermore, Calif.) and were observed and counted by epifluorescent microscopy on an Olympus model BH2-RFC microscope (Olympus Corporation, Lake Success, N.Y.) equipped with a 100-W mercury burner (Olympus Corporation). CTC- and DAPI-positive cells were observed and counted with the appropriate filter sets (Olympus Corporation).

Addition of nutrient to starvation microcosms. Starved cells in ASW microcosms were either subjected to the addition of an equal volume of HI broth or pelleted and resuspended in HI broth. Samples were taken for RAPD analysis in the manner described above.

Resuscitation of VBNC cells. Once cells were confirmed as VBNC by the viability assay described above, they were resuscitated through a temperature upshift to 22°C for a 12-h period. Samples were taken for plate counts and RAPD analysis in the manner described above.

RAPD analysis. Each 25.0-μl RAPD reaction mixture contained the following reagents: 2.5 μl of 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin [Promega]), 2.0 μl of sterile H_2O, 3.5 μl of $MgCl_2$ (25 mM) (Promega), 8.0 μl of deoxynucleoside triphosphates (5 mM) (Promega), 3.0 μl of primer (5′ GGATCTGAAC 3′; 25 mM) (Biosynthesis, Lewisville, Tex.), 5.0 U of *Taq* DNA polymerase (Promega), and 5.0 μl of cell culture (previously frozen starvation or VBNC samples stored in buffer). The reaction mixtures were overlaid with 20.0 μl of sterile mineral oil (Sigma) to seal the reactions and prevent evaporation in the thermal cycler. Thermal cycling was performed in a Techne PHC-3 thermal cycler (Techne, Princeton, N.J.). The cycling profile was as follows: 1 cycle of 95°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 of 72°C for 5 min. The RAPD products were electrophoresed with 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 μl at 10 mg/ml) and were photographed with a Polaroid Quick Shooter model QSP (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 applied to all strains a minimum of three times.

Computer analysis of RAPD profiles. All gels were imaged with an ImageMaster DTS scanner (Pharmacia, Uppsala, Sweden). A 123-bp ladder was employed every three or four lanes on most gels as a standard molecular weight marker. Images were calibrated and data analysis was performed with RFLP-Scan, version 3.0, software (Scanalytics, Billerica, Mass.). A match tolerance of 1.0% of the molecular weight of each band was used.

RESULTS

The RAPD method described in our previous study (21) is capable of detecting various *Vibrio* species, including strains of *V. vulnificus*, grown in rich medium (HI). When this RAPD method was applied to various gram-positive and gram-negative organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*), no amplification products were observed, suggesting that the method developed is specific for members of the genus *Vibrio*. When cells were subjected to nutrient starvation in ASW at room temperature, initial detection was possible with the RAPD method, but our ability to amplify these cells was lost at an average of 4 h into starvation. Changes in RAPD profiles, including the loss of amplification fragments as well as the presence of unique amplification fragments, were noted as early as 15 min into starvation (Fig. 1, lanes 1 to 6). During this time, cell numbers were monitored by means of plate counts on HI agar and remained constant at ca. 10^5 CFU/ml. Addition of chloramphenicol to the microcosm at the initiation of nutrient starvation allowed the RAPD signal to be retained during the entire course of starvation (Fig. 1, lanes 7 to 12). Sampling of cells which had undergone 6 months of starvation showed that these cells were always detectable by the RAPD method. Once the signal was lost in starving cells, it could be regained in as little as 1 h by the addition of nutrient to the microcosm (Fig. 2). Cells starved for as long as 1 year were able to regain the ability to be amplified upon nutrient addition to the microcosm (data not shown). The same results were observed with both of the nutrient addition methods employed.

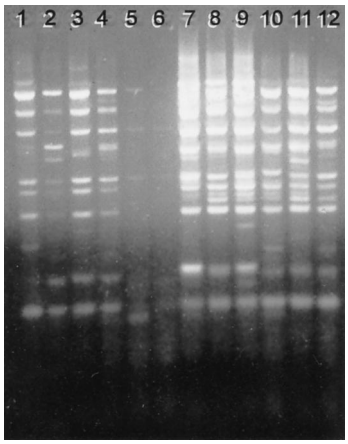

FIG. 1. RAPD profiles of starved cells of *V. vulnificus* C7184 with and without chloramphenicol addition. Lanes 1 through 6 contain cells starved for 0 min, 15 min, 30 min, 1 h, 2 h, and 4 h, respectively; lanes 7 through 12 contain starved cells with chloramphenicol added for 0 min, 2 h, 6 h, 12 h, 24 h, and 6 months, respectively.

V. vulnificus cells which were made VBNC (<0.1 CFU/ml) by placing them at 5°C lost their RAPD signal at an average of 7 days, although the timing of signal loss varied between 4 and 10 days (Fig. 3). Viability of these cells was verified by the CTC/DAPI viability assay. VBNC cells were resuscitated by a temperature upshift, and by 12 h they were once again detectable by the RAPD method (Fig. 4).

As was seen with starved cells, addition of chloramphenicol to the VBNC microcosms allowed the RAPD signal to be retained throughout the course of the VBNC state. This was observed for up to 1 year for cells which had been in the VBNC state (Fig. 5).

The results observed were reproducible over time; however, the timing of signal loss and gain had slight variability between microcosms. Each experiment was performed with a minimum

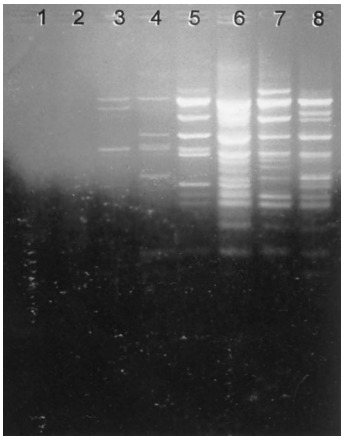

FIG. 2. RAPD profiles of starved cells of *V. vulnificus* C7184 with nutrient added to the microcosm. Lane 1 contains cells starved for 24 h; lanes 2 through 8 contain starved cells with nutrient added to the microcosm for 0 min, 15 min, 30 min, 1 h, 2 h, 6 h, and 24 h, respectively.

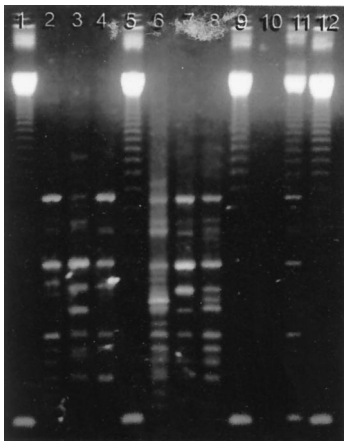

FIG. 3. RAPD profiles of *V. vulnificus* cells as they enter the VBNC state. Lanes 1, 5, 9, 11, and 12 each contain a 123-bp ladder. Lanes 2 through 4 contain cells entering the VBNC state at 0, 1, and 2 days, respectively. Lanes 6 through 8 contain cells entering the VBNC state at 3, 4, and 5 days, respectively; and lane 10 contains cells entering the VBNC state at 6 days.

of three separate microcosms. In all cases, the same general trends were observed.

DISCUSSION

We have developed an RAPD method suitable for detection and differentiation of various *Vibrio* species, including *V. vulnificus* (21). This method has been applied to both starved and VBNC cells of *V. vulnificus* in the present study. Initial detection of *V. vulnificus* cells was accomplished in both cases, but our ability to amplify cells by the RAPD method under these stress conditions was eventually lost. In addition, variations in the RAPD profiles occurred during the first few days of entrance into the VBNC state before the signal was lost. This finding agrees with the results of another study employing arbitrarily primed PCR (AP-PCR) to analyze VBNC cells of *V. vulnificus* (1). We have also observed similar results with VBNC studies performed on enterohemorrhagic *E. coli* strains (8). Previous studies in our lab reported a similar loss of signal when PCR was performed on the hemolysin gene of VBNC cells of *V. vulnificus* (2). In that study, it was found that as much as 500 times more DNA, whether extracted or from whole-cell lysates, was required for amplification of VBNC cells. In a subsequent study in which PCR conditions were optimized for VBNC cells, 100 times more VBNC cells than culturable cells was still required in order to obtain PCR amplification (4). We have attempted to compensate for this need by modifying the methodology so that an excess of cells is present in the RAPD reaction mixture.

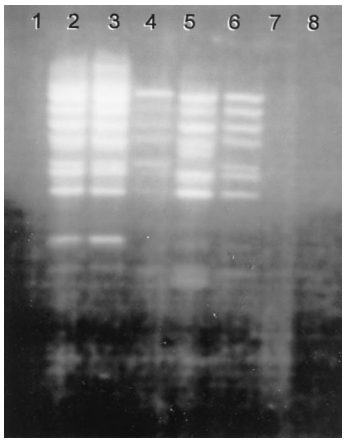

FIG. 4. RAPD profiles of resuscitation of VBNC cells in five separate microcosms. Lanes 1, 7, and 8 are blank. Lanes 2 through 6 each contain a resuscitated sample from a single VBNC microcosm.

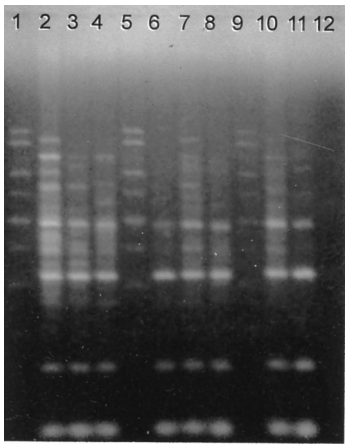

FIG. 5. RAPD profiles of *V. vulnificus* cells entering the VBNC state with the addition of chloramphenicol to the microcosm. Lanes 1, 5, 9, and 12 each contain a 123-bp ladder. Lanes 2 through 4 contain cells entering the VBNC state with chloramphenicol added for 0, 1, and 2 days, respectively; lanes 6 through 8 contain cells entering the VBNC state with chloramphenicol added for 3, 4, and 5 days, respectively; and lanes 10 and 11 contain cells entering the VBNC state with chloramphenicol added for 6 and 7 days, respectively.

We hypothesize that either DNA binding proteins or chromosomal supercoiling, or a combination of both, prevents detection of *V. vulnificus* DNA by the RAPD method under both starvation and VBNC conditions. Supercoiling alters the state of the chromosome in a manner such that sequences complementary to the primer or DNA polymerase binding sites may become inaccessible, thus explaining our inability to amplify these cells during stress conditions. Preliminary studies in our lab utilizing the antibiotic ciprofloxacin, a supercoiling inhibitor, have shown similar results regarding the ability to maintain a RAPD signal (data not shown).

From two-dimensional protein gel analysis, it is known that at least 21 unique proteins are synthesized in the first 20 min of nutrient starvation of *V. vulnificus* (9). In the case of VBNC cells of *V. vulnificus*, over 40 cold shock proteins are produced during the temperature downshift required to initiate the VBNC response (7). Stress proteins produced under these conditions may also be DNA binding proteins and may block primer or polymerase binding sites, thus preventing amplification by the RAPD method. By adding chloramphenicol to our microcosms in order to inhibit protein synthesis, we have been able to retain RAPD signals in both starvation and VBNC states for periods as long as 1 year. This illustrates the important role of DNA binding proteins in the starvation and VBNC responses of *V. vulnificus*. These results suggest that a combination of supercoiling and DNA binding proteins may play a role in the VBNC response.

Starved cells of *V. vulnificus* can once again be detected by RAPD when nutrient is added to the starvation microcosm. This may be due to the fact that once the starvation stress is eliminated, starvation proteins are degraded and the chromosome is again free for binding with RAPD primers and polymerase. VBNC cells also regain RAPD signals (although the

profiles sometimes show reduced or modified banding) when allowed to resuscitate at room temperature for approximately 12 h. Again, this suggests that once the temperature stress is removed the proteins involved in the VBNC response are degraded, and the chromosome is free for primer and polymerase binding. These results are in agreement with a recent study (1) utilizing AP-PCR to analyze resuscitated VBNC cells of *V. vulnificus* in which it was found that reversal of the VBNC state caused a restoration of the original DNA fingerprint.

V. vulnificus cells exist in the natural environment under a variety of stress conditions, including low nutrient levels and low temperatures. It is important to develop molecular methods capable of detecting cells under these conditions in the natural environment. We have developed an RAPD method for detection of cells under nutrient-rich conditions, but when applied to typical environmental conditions this method eventually could not detect these cells. Our finding that the addition of nutrient to a starvation microcosm, as well as resuscitation of VBNC microcosms, led to a renewed ability to detect cells with the RAPD method suggests that stress proteins produced under these conditions may bind the DNA such that the chromosome is blocked, thus preventing RAPD amplification. Further studies will continue to investigate the roles of supercoiling and stress proteins in the effects observed in this study so that a method for the detection of *V. vulnificus* cells under naturally imposed stress conditions may be developed.

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