Analysis of Bacterial Assemblage Genetic Diversity in Environmental Samples Using the DCode™ System

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Introduction

Assessing bacterial genetic diversity in natural environments is problematic because of difficulties in culturing native bacteria and the large number of species that may be encountered. The morphological and physiological traits of most microbes are ambiguous and provide few clues as to their identity; nucleic acid sequence comparison is the most fundamental way to classify microorganisms. Molecular techniques like the polymerase chain reaction (PCR) have enhanced the ability to examine ribosomal genes of bacteria. Denaturing gradient gel electrophoresis (DGGE) has proven to be a powerful tool for analyzing PCR-amplified bacterial 16S rDNA fragments. The major advantage of DGGE is that it allows direct determination of bacterial genetic diversity, making it superior to cloning and subsequent sequencing.

Methods

Sediment samples were collected in triplicate from three sites in Four Mile Creek on the US Department of Energy Savannah River Site near Aiken, South Carolina, that differed in the extent of anthropogenic disturbance. DNA was extracted from samples using the Tsai and Olson method (Tsai and Olson 1991). Briefly, 10 g of sediment were mixed with 120 mM sodium phosphate buffer, then centrifuged at 6,000 x g for 10 min. Pellets were resuspended and incubated for 1.5 hr in 10 ml of lysis solution (0.15 M NaCl, 0.1 M Na_2 EDTA) containing 15 g/ml of freshly added lysozyme (Sigma Chemical) and heated at 37°C to aid in dissolution. Proteinase K (2 mg/ml, Sigma Chemical) was added to the samples followed by a 30 min incubation at 37°C. Next, 10 ml of lysis buffer (0.1 M NaCl and 0.5 M Tris-HCl) was added, and samples were subjected to three cycles of rapid freezing and thawing in a –70°C ethanol bath and a 70°C water bath. DNA was extracted once with Tris-saturated phenol; once with phenol, chloroform, and isoamyl alcohol (25:24:1); then once with chloroform and isoamyl alcohol (24:1) (Sigma Chemical). Each extraction was followed by a 10 min centrifugation at 6,000 x g. DNA was precipitated overnight in ice-cold isopropanol. The next day, samples were centrifuged at 10,000 x g and pellets were resuspended in 500 µl of Tris-EDTA buffer (TE), pH 8.0. Anhydrous ammonium acetate was added and samples were centrifuged for 30 min. Supernatant DNA was precipitated with 1.5 volumes of isopropanol for 2 hr. Samples were centrifuged at 10,000 x g for 10 min, and pellets were resuspended in 300 µl of TE buffer, pH 8.0. DNA extracts were purified using Sephadex G-200 (Sigma Chemical) spin columns (Tsai and Olson 1992).

To obtain bacterial 16S rDNA, hot-start PCR reactions were performed using a bacteria-specific and a universal primer. The sequence for the forward primer, termed 68F, was 5'-TNANACATGCAAGTCGAKCG-3', and it aligned with nucleotides 65–85 of *E. coli* (Brosius et al. 1981). The sequence for the reverse primer, termed 1392R, was 5'-ACGGGCGGTGTGTRC-3', and it aligned with nucleotides 1408–1423 of *E. coli* (Brosius et al. 1981); where N = adenine or thymidine, K = guanine or thymidine, R = adenine or guanine. Reaction mixtures were prepared in thin-walled microcentrifuge tubes (Fisher Scientific) and contained 1x Stoffel fragment buffer (PerkinElmer, Inc.), 5 µM bovine serum albumin, 200 µM of each primer, 4 mm $MgCl_2$, 1 µl (60–100 ng) template DNA, and 10 U of Stoffel fragment *Taq* polymerase (PerkinElmer, Inc.).

Site-specific genetic profiles were produced by analyzing PCR products with DGGE in the manner described by Muyzer et al. (1993). Six percent acrylamide stock solutions were prepared containing 0% and 100% denaturant. The 0% denaturant stock was prepared by adding 15 ml of 40% acrylamide (Fisher Scientific) plus 2 ml of 50x Tris-acetic acid-EDTA (TAE) buffer to 83 ml of distilled water. The 100% denaturant stock solution, defined as 7 M urea and 40% formamide, was prepared by mixing 15 ml of 40% acrylamide (Fisher Scientific) with 2 ml of 50x TAE buffer with 40 ml of molecular biology grade deionized formamide (Sigma Chemical) and 42 g of molecular biology grade urea (Sigma Chemical). Stock solutions were degassed under vacuum for 5–10 min and filtered through 0.45 µm filters. Parallel-style gels were cast using the Model 475 gradient former (Bio-Rad Laboratories, Inc.). Gels were run on the DCode universal mutation detection system in 1x TAE at 60°C for 9–12 hr at 170 V (Bio-Rad Model 200/2.0 power supply). Gels were subsequently stained with a 1:10,000 dilution of SYBR Green I (Molecular Probes, Inc.). Gels were photographed in a darkroom with a Nikon 35 mm camera and Ilford 400 black and white film, and the film negatives were digitized with a Hamamatsu CCD camera (Hamamatsu Corp.).

Image analysis of DGGE gels was performed using quantitative analysis software, and lane intensity profiles measured by optical density highlighted the position of individual bands. Numerical representations of the profiles were exported to Microsoft Excel spreadsheet software, where the x-axis was modified to reflect the percent denaturant along the length of the lane.

Results

Replicate samples collected from the same location exhibited consistently similar DGGE profiles, demonstrating the degree of reproducibility of the results (Figure 1). DGGE banding patterns exhibited some similarities among sites, but the method was sensitive enough to detect some differences among sites. Analysis of the number of bands, the number of unique site-specific bands, and similarity in banding patterns provided a relatively complete representation of differences among sites.

In some cases, bands detected were less well-defined than other bands and appeared fuzzy on the gel images and as broad peaks on the gel profile graphs. Selection of PCR primers that amplify a smaller DNA fragment and use of a GC clamp is recommended to correct this problem.

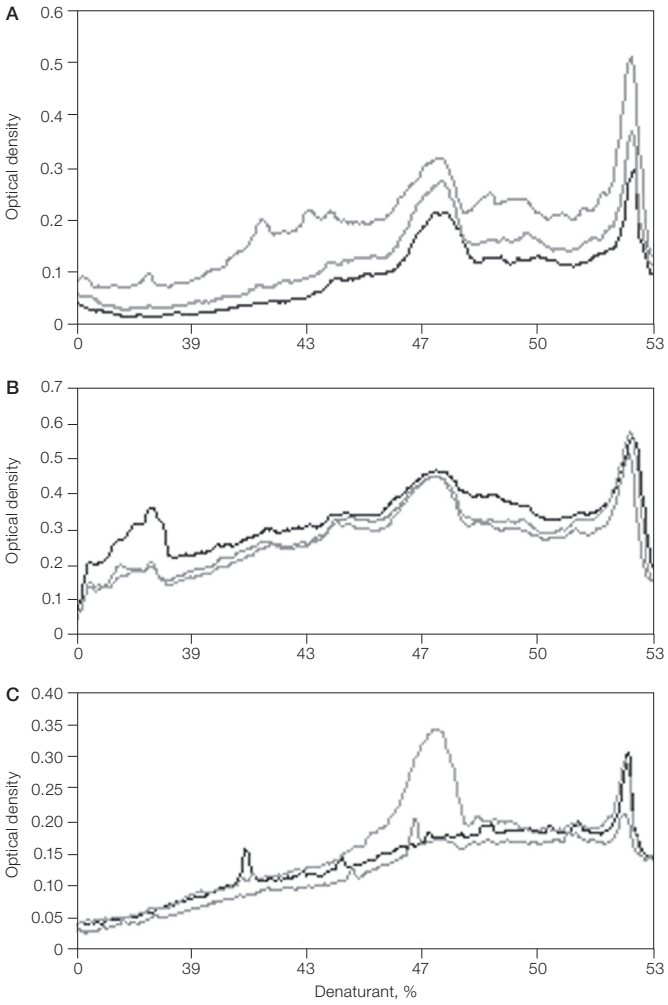

Fig. 1. Image analysis of DGGE profiles from August 1996 bank sediments. Graphs show replicate lane intensity profiles. Panels A, B, and C represent sites FOU1, FOU2, and FOU3, respectively. The three lines on each panel represent replicate samples from a given site. FOU1 is upstream from sources of mixed pollutant discharge; FOU2 and FOU3 are downstream from this discharge.

Discussion

DGGE analysis of PCR products demonstrated that in many ways bacterial assemblages differed among sites. In general, more total and more unique bands were detected at the polluted sites. Other methods used to examine the bacterial assemblages at these same sites also revealed site-specific differences (Lemke et al. 1997).

Interpretation of DGGE results should be performed with the knowledge that DGGE provides a relative measure of species diversity. As was the case in this study, DGGE was intimately dependent on the efficiency of the DNA extraction procedure and on an unbiased high-fidelity PCR. To more accurately understand the roles of bacterial populations to natural environments, it is necessary to be able to measure the microbial community structure and diversity as it relates to changing environmental factors. DGGE provides a more tractable way of assessing this diversity than other currently available methods.

Acknowledgement

This research was supported by a grant (#R823749-01-0) from the US Environmental Protection Agency, Office of Exploratory Research.

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Information in this tech note was current as of the date of writing (1998) and not necessarily the date this version (rev B, 2007) was published.

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