

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

Derek Cody and Laura Leff, Department of Biological Sciences, Kent State University, Kent, Ohio 44240 USA

### 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<sub>2</sub> 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).

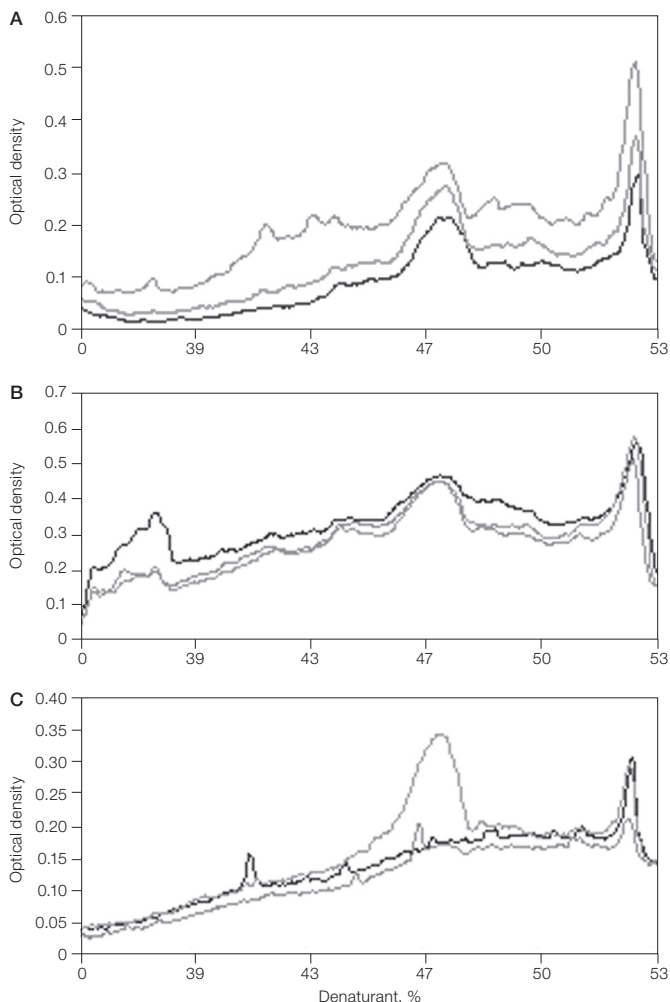
To obtain bacterial 16S rDNA, hot-start PCR reactions were performed using a bacteria-specific and a universal primer. The sequence for the forward primer, termed 68F, was 5'-TNANACATGCAAGTCGAKCG-3', and it aligned with nucleotides 65–85 of *E. coli* (Brosius et al. 1981). The sequence for the reverse 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<sub>2</sub>, 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



**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.

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.

## 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.

## References

- Brosius J et al., Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*, *J Mol Biol* 148, 107–27 (1981)
- Lemke MJ et al., The response of three bacterial populations to pollution in a stream, *Microb Ecol* 34, 224–231 (1997)
- Muyzer G et al., Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, *Appl Environ Microbiol* 59, 695–700 (1993)
- Tsai YL and Olson BH, Rapid method for direct extraction of DNA from soil and sediments, *Appl Environ Microbiol* 57, 1070–1074 (1991)
- Tsai YL and Olson BH, Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction, *Appl Environ Microbiol* 58, 2292–2295 (1992)

Excel and Microsoft are trademarks of Microsoft Corporation. Ilford is a trademark of Ilford Imaging. Nikon is a trademark of Nikon Corporation. SYBR is a trademark of Molecular Probes, Inc.

Practice of the polymerase chain reaction (PCR) may require a license.

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.

**BIO-RAD**

**Bio-Rad  
Laboratories, Inc.**

Life Science  
Group

Web site [www.bio-rad.com](http://www.bio-rad.com) USA 800 4BIORAD Australia 61 02 9914 2800 Austria 01 877 89 01 Belgium 09 385 55 11 Brazil 55 21 3237 9400  
Canada 905 364 3435 China 86 21 6426 0808 Czech Republic 420 241 430 532 Denmark 44 52 10 00 Finland 09 804 22 00 France 01 47 95 69 65  
Germany 089 318 84 0 Greece 30 210 777 4396 Hong Kong 852 2789 3300 Hungary 36 1 455 8800 India 91 124 4029300 Israel 03 963 6050  
Italy 39 02 216091 Japan 03 6361 7000 Korea 82 2 3473 4460 Mexico 52 555 488 7670 The Netherlands 0318 540666 New Zealand 0508 805 500  
Norway 23 38 41 30 Poland 48 22 331 99 99 Portugal 351 21 472 7700 Russia 7 495 721 14 04 Singapore 65 6415 3188 South Africa 27 861 246 723  
Spain 34 91 590 5200 Sweden 08 555 12700 Switzerland 061 717 95 55 Taiwan 886 2 2578 7189 United Kingdom 020 8328 2000