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DGGE fingerprinting of culturable soil bacterial communities complements culture-independent analyses

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Abstract

Culture-dependent DGGE (CD DGGE) fingerprinting of the 16S rRNA gene was used to characterize mixed bacterial communities recovered on agar plates. Using R2A Agar as a growth medium, CD DGGE analysis resulted in clear banding patterns of sufficient complexity (16–32 major bands) and reproducibility to investigate differences in bacterial communities in a silt loam soil. Replicate CD DGGE profiles from plates inoculated with less-dilute samples (10^{-3}) had a higher band count and were more similar (72–77%) than profiles from more-dilute samples (51–61%). Different culture media and incubation conditions resulted in distinct community fingerprints and increased the cumulative number of unique bands detected. When CD DGGE fingerprints were compared to profiles constructed from 16S rRNA genes obtained from culture-independent clone libraries (CB DGGE profiles) 34% of the bands were unique to the culture-dependent profiles, 32% were unique to the culture-independent profiles and 34% were found in both communities. These data demonstrate that culture-independent DGGE profiles are supplemented by the distinct bands detected in culture-dependent profiles. CD DGGE can be a useful technique to follow the dynamics of distinct culturable fractions of the soil bacterial community.

Keywords: Culturable bacteria; DGGE; Plate wash; Bacterial community structure; Culture-dependent; Culture-independent

1. Introduction

A current paradigm in soil microbiology states that bacterial communities are highly diverse and only a small percentage of environmental bacteria can be isolated in pure culture using standard laboratory media (Janssen et al., 2002). As a result, various non-culturing methodologies that depend on nucleic acid extraction and gene amplification have superceded the isolation and identification of bacteria growing on agar plates and have become the preferred approach to study population dynamics and genetic diversity of soil microbial communities (Hugenholtz et al., 1998; Ellis et al., 2003). However, recent studies suggest that culture-dependent and culture-independent methods each recover and resolve distinct soil bacteria. This suggests that a suitable combination of these approaches would be useful to obtain a more comprehensive overview of the soil community. In general, culture-based methods better represent copiotrophs including high GC Gram-positive bacteria, whereas culture-independent methods provide better profiles of difficult-to-culture but widespread genera such as *Acidobacteria* and *Verrucomicrobia* (Barns et al., 1999; Smit et al., 2001; Lipson and Schmidt, 2004). Since many culturable bacteria exhibit fast growth rates and larger cell sizes (Bakken and Olsen, 1987; Bakken 1997) it is probable they also exhibit more short-term variation in community structure than slow-growing, difficult-to-culture bacteria. Ellis et al. (2003) have argued that culturable bacteria are important to soil ecosystem functions because of their higher total biomass and metabolic activity. Therefore, culturable bacteria may provide an ecologically relevant complement to culture-independent community characterizations and serve as responsive indicators of physical, chemical, and biological changes in the soil environment.

Significant impediments to routine characterization of culturable bacterial communities include isolation and identification of representative pure culture collections from many individual soil samples. We have approached

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this problem by pooling the bacterial cells growing on individual agar plates. Total DNA was extracted from this culture-dependent "community", followed by polymerase chain reaction (PCR) amplification of the 16S rDNA gene, and development of a community fingerprint using denaturing gradient gel electrophoresis Culture-dependent DGGE (CD DGGE) is complementary to the culture-independent DGGE methodology first proposed by Muyzer et al. (1993) and since widely employed to investigate the dynamics of soil microbial communities in relation to changing environmental factors (Muyzer and Smalla, 1998; Øvreås and Torsvik, 1998; Bruns et al., 1999; Smalla et al., 2001; McCaig et al., 2001; Girvan et al., 2003; Nicol et al., 2003; Salles et al., 2004).

The utility of CD DGGE for soil bacterial community characterization has not been systematically explored. Duineveld et al. (1998) and Ellis et al. (2003) previously used both culture-independent and CD DGGE to compare bacterial communities recovered from the rhizosphere of potted chrysanthemums and metal-contaminated soils, respectively. The two methods produced dissimilar community fingerprints; however, variable results obtained in the CD DGGE profiles suggested the need for better characterization and standardization of the technique.

The objectives of this study were to: (1) compare and contrast CD DGGE profiles with DGGE fingerprints constructed from 16S rRNA genes obtained from culture-independent clone libraries, and (2) evaluate the complexity and reproducibility of DGGE fingerprints of the 16S rRNA genes from bacterial communities grown on agar plates. Our results indicate acceptable reproducibility with the CD DGGE method and the ability to discern statistical differences between bacterial communities in different soil samples. We also demonstrate that by combining soil pretreatments, incubation conditions, and various culture media it was possible to resolve unique DGGE bands that were not resolved in culture-independent profiles.

2. Methods

2.1. Soil sampling and experimental design

Soil samples were collected three times between June 2004 and August 2005 from market garden plots at the West Virginia University Organic Research Farm located in Morgantown, West Virginia, USA (39°39′0.18″N; 79°56′11.6″W). Four studies were conducted to address various technical questions about the CD DGGE procedure. Details of the studies conducted, the experimental design, and sampling strategy are presented in Table 1. Soils on the farm are Dormont and Guernsy silt loams: fine loamy, mixed, superactive, mesic, Oxyaquic Hapludalfs (Wright et al., 1982).

2.2. Culture-dependent bacterial communities

Twenty grams (dry weight) of composite soil samples from each plot were placed in sterile Waring blenders and agitated for one minute at high speed (3× with intermittent cooling on ice) in 180 ml of sterile Winogradsky's salts solution (WSS; Zuberer, 1994). Blended suspensions were serially diluted in sterile WSS prior to inoculation (100 µl) by spread plating onto 4 replicate agar plates. The media used included R2A Agar, Tryptic Soy Agar, Pseudomonas Isolation Agar (Difco, Detroit, MI), and Oil Agar prepared as described by Sexstone and Atlas (1977). Unless otherwise noted, 1000-fold (10^{-3}) diluted soil was routinely used to obtain heterotrophic soil bacterial communities grown on agar plates and incubated aerobically for 2 weeks at 25 °C (Fig. 1). In study 4 (Table 1, Fig. 4), plates were incubated anaerobically and/or the diluted soil was heat shocked (80 °C for 15 min) prior to inoculation of the agar plates.

2.3. Culture-dependent DNA extraction and PCR amplification of the V3 region of the 16S rRNA gene

Cultured cells were washed sequentially from 4 replicate plates using WSS (4 ml) and a sterile disposable inoculating loop. The resulting cell suspensions were vortexed and frozen (−20 °C). DNA was extracted from aliquots of each thawed cell suspension (1.8 ml) using the MoBio Microbial DNA extraction kit (MoBio Labs, Carlsbad, CA). The variable V3 region of the 16S rRNA gene from the domain *Eubacteria* was amplified using the PRBA338F primer with a GC clamp (5′-ACTCCTACGGGAGGCAGCAG-3′) and the PRUN518R primer (5′-ATTACCGCGGC-TGCTGG). The final 50-µl reaction mixture contained 1 × PCR buffer (Promega, Madison, WI), 3.2 mM of $MgCl_2$, 0.8 mM of deoxynucleotide triphosphates (Promega), 0.2% bovine serum albumin, 0.5 µM (each) of the forward and reverse primers (Integrated DNA Technologies, San Diego, CA), 2.5 units of *Taq* DNA polymerase (Promega), and ~50–200 ng of template DNA. The PCR protocol included a 5-min initial denaturation at 94 °C, 30 cycles of 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C. PCR products were cleaned and concentrated using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The amount of PCR product in the samples was determined by agarose gel electrophoresis of samples and a mass ladder (Fermentas Inc., Hanover, MD). Quantification of DNA in the PCR product was done by volume analysis using Quantity One Software (Biorad, Hercules, CA).

2.4. Culture-independent DGGE fingerprints derived from pooled 16S rDNA clone libraries

In a preliminary study (unpublished data) we constructed conventional culture-independent DGGE profiles by direct amplification of 16S rRNA genes from soil DNA (Muyzer et al., 1993). As observed by others, DGGE fingerprints constructed using this method had complex profiles and smeared regions that complicated the identification and scoring of bands (Øvreås and Torsvik, 1998;