Published online March 12, 2007

Soil Microbial Community Analysis Using Denaturing Gradient Gel Electrophoresis

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The most biological diversity on this planet is probably harbored in soils. Understanding the diversity and function of the microbiological component of soil poses great challenges that are being overcome by the application of molecular biological approaches. This review covers one of many approaches being used: separation of polymerase chain reaction (PCR) amplicons using denaturing gradient gel electrophoresis (DGGE). Extraction of nucleic acids directly from soils allows the examination of a community without the limitation posed by cultivation. Polymerase chain reaction provides a means to increase the numbers of a target for its detection on gels. Using the rRNA genes as a target for PCR provides phylogenetic information on populations comprising communities. Fingerprints produced by this method have allowed spatial and temporal comparisons of soil communities within and between locations or among treatments. Numerous samples can be compared because of the rapid high throughput nature of this method. Scientists now have the means to begin addressing complex ecological questions about the spatial, temporal, and nutritional interactions faced by microbes in the soil environment.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction; T-RFLP, terminal fragment length polymorphism.

The enormous range of complexity in soil microbial communities has made it an incredibly challenging ecosystem to study (Torsvik and Øvreås, 2002; Torsvik et al., 2002). A number of molecular biological approaches are now being used to gain a better understanding of the ecology of soil microbial communities (Nakatsu, 2004). This has helped soil scientists to evaluate differences in microbial communities with respect to their environment. It has enabled advancement beyond the traditional laboratory cultivation approaches that were able to capture only about 1% of the community in the past (Staley and Konopka, 1985). The majority of molecular methods currently being used for community analysis examine nucleic acids. Some of these methods directly examine nucleic acids, whereas PCR amplification has been used to increase copies of a target gene for easier detection. Examples of methods being used to directly analyze nucleic acids are DNA:DNA reassociation kinetics (Torsvik et al., 1990), nucleic acid hybridization (Buckley et al., 1998), fluorescent in situ hybridization (Christensen et al., 1999; Ravenschlag et al., 2000), microarrays (Rhee et al., 2004; Small et al., 2001), and metagenome sequence analysis (Handelsman, 2004). The most common PCR-dependent approaches are DGGE (Muyzer et al., 1993), terminal fragment length polymorphism (T-RFLP; Liu et al., 1997), single strand conformational polymorphism (Schwieger and Tebbe, 1998), ribosomal intergenic spacer analysis (RISA; Ranjard et al., 2000) or automated RISA (ARISA; Cardinale et al., 2004), and sequence analysis of 16 rRNA gene clone libraries (Lane, 1991; Olsen et al., 1986). All of these approaches have strengths and weaknesses that need to be considered when choosing the most appropriate method for an application (Nakatsu, 2004). Comparisons of the information obtained using different community analysis methods have shown the value of the different information that they provide and the need to perform multiple methods to obtain a more complete picture of microbial communities (Casamayor et al., 2002; Joynt et al., 2006; Perkiomaki and Fritze, 2003). Often methods leading to a detailed view of a community are more expensive and time consuming and require greater technical expertise than those that produce a "snapshot" of a community represented as a genetic fingerprint. In general, genetic fingerprinting techniques such as DGGE, T-RFLP, and ARISA allow higher throughput and the comparative profiling of many samples, and thus facilitate the spatial and temporal analysis of microbial communities in ecosystems. This review covers one of these methods, PCR-DGGE. It includes the fundamental challenges that have been faced in performing PCR-DGGE, its strengths and weaknesses, and studies where it has been applied to examine soil microbial communities.

Soil Sci. Soc. Am. J. 71:562–571
doi:10.2136/sssaj2006.0080
Received 19 Feb. 2006.
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Background of Denaturing Gradient Gel Electrophoresis

The medical research community first used DGGE for the identification of gene mutations (Borresen et al., 1988; Fodde and Losekoot, 1994; Hovig et al., 1991). Muyzer et al. (1993) were the first to adapt the use of PCR-DGGE for microbial community analysis. Initially, communities with expected limited diversity, such as deep-sea hydrothermal vents (Muyzer et

al., 1995) and hotsprings (Ferris et al., 1996) were examined. Subsequently, a number of research groups applied the method to soil community analysis (Duineveld et al., 1998; Heuer et al., 1997; Jensen et al., 1998; Kowalchuk et al., 1997a, 1997b). Due to the immense complexity of soil communities, researchers often select PCR primers to target specific populations known to be major inhabitants of soil, such as the actinomycetes (Heuer et al., 1997) and NH_3-oxidizing beta proteobacteria (Kowalchuk et al., 1997b), or limited their analysis to specific soil environments such as the rhizosphere (Duineveld et al., 1998). The acceptance of the PCR-DGGE approach for soil community analysis is evident in the steady increase in the number of studies since 1997 that have used it.

Theoretical Basis of Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis

Unlike most commonly used electrophoresis methods that separate nucleic acid fragments by size, DGGE separates DNA strands by their sequence composition (Hovig et al., 1991). Essentially, DNA fragments of about the same length are produced by amplification of a target gene by PCR. Then a gel composed of a linear gradient of denaturant is used to separate PCR products composed of different sequences. In DGGE, the chemical denaturants urea and formamide are used and in an analogous method, thermal gradient gel electrophoresis, temperature is used (Lessa and Applebaum, 1993). Differential migration occurs because more denaturant is needed to separate sequences with higher guanine (G) + cytosine (C) content due to differences in the number of H bonds between complementary nucleotides holding DNA strands together. There are three H bonds between guanine and cytosine, and only two between adenine and thymine. As DNA strands separate, their migration becomes retarded in the gel. To produce sharp bands, fragment migration is stabilized by adding a high GC sequence (GC clamp) to the end of one PCR primer, thereby preventing complete strand separation (Sheffield et al., 1989). If a mixture of PCR products with different sequences is amplified from a sample, then this type of gel will separate them during electrophoresis. The resulting genetic profiles or fingerprints represent the community structure, an approximation of the numbers of populations (represented by each band) and their relative abundance (represented by band intensity) within the amplified community. Because PCR is the first step in DGGE, the fingerprint profiles are representative of the proportion of PCR products, which may not directly correlate to the proportion of that population within the community (Chandler et al., 1997).

THE POLYMERASE CHAIN REACTION–DENATURING GRADIENT GEL ELECTROPHORESIS METHOD

The PCR-DGGE protocol consists of six major steps: sample collection, nucleic acid extraction, PCR amplification of the target gene, separation of PCR amplicons by DGGE, visualization of profiles, and data analysis (Fig. 1). In general, PCR-based methods are often chosen because large quantities of sample are not usually needed and they are conducive to high throughput and comparative analyses. The PCR results in an exponential increase in the number of copies of the initial target (Bej et al., 1991), which accounts for the small sample sizes needed.

Nucleic Acid Extraction

For community analysis, both DNA and RNA can be extracted from soil (Duineveld et al., 2001; Griffiths et al., 2000; Hurt et al., 2001) but because of the greater stability of DNA, it is usually the molecule of choice. The basics of nucleic acid extraction are fundamentally the same, but protocols with many technical variations for extraction are found throughout the literature (de Lipthay et al., 2004; Krsek and Wellington, 1999; Kuske et al., 1998; Miller et al., 1999; Niemi et al., 2001; Picard et al., 1992). There is no apparent consensus on a single method, but once a method is chosen for a project, it should be maintained to ensure reproducibility of results. The major factors influencing method choice are maximum cell lysis, minimal contamination from soil chemicals (especially humic acids), release of nucleic acids from soil, and minimal shearing of nucleic acids. Other factors that should be considered include the amount of soil required, time needed to perform the extraction, required technical expertise, and cost.

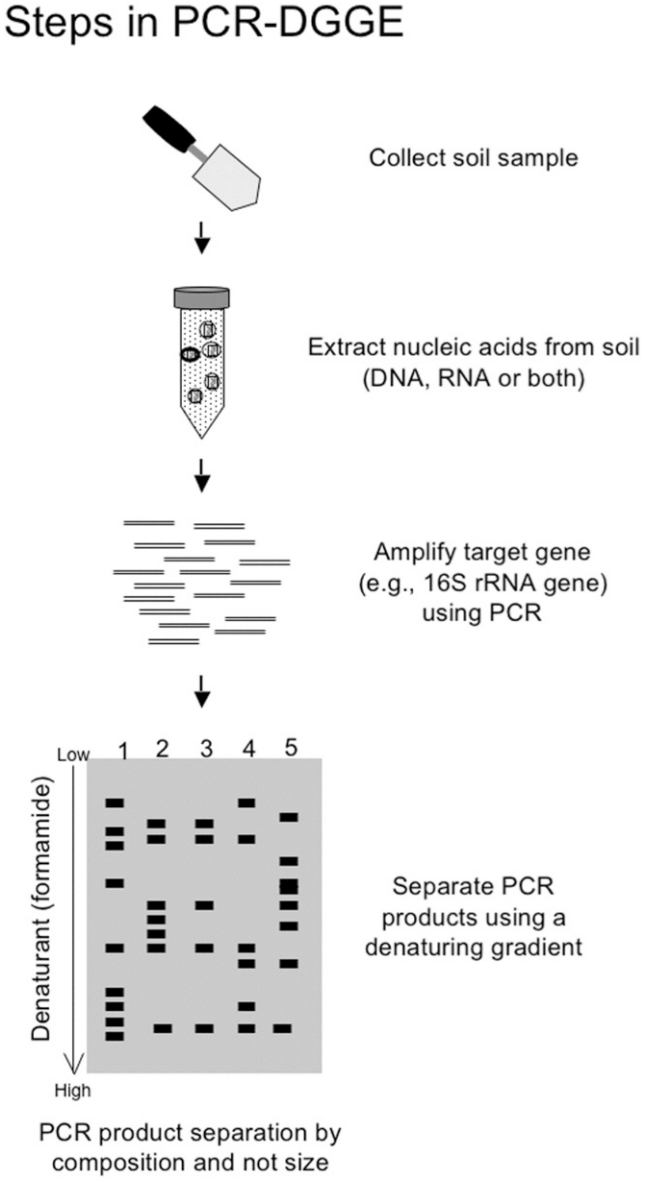

Fig. 1. Flow diagram of the steps for microbial community analysis using polymerase chain reaction (PCA)–denaturing gradient gel electrophoresis (DGGE).

The amount of soil sample needed usually depends on the microbial biomass present. Large amounts of soil are typically unnecessary for the nucleic acid extraction; commonly used kits for soil DNA extraction recommend 0.5 g of soil, and they yield sufficient recovery for community analysis (Mumy and Findlay, 2004). Due to the inherent heterogeneous nature of soils, however, larger quantities of soils are typically collected and homogenized to ensure that they are representative of that ecosystem, which must be confirmed by adequate replication (Kang and Mills, 2006). For spatial studies, even small soil aggregates have been successfully used for community analysis to understand their heterogeneity (Becker et al., 2006). An advantage of using commercially available kits for nucleic acid isolation is that they provide a rapid and standardized approach that can be quickly learned (for example see, MoBio Laboratories, Carlsbad, CA; MP Biochemicals-Qbiogene, Irvine, CA; or EpiCenter Biotechnologies, Madison, WI). Addition of an internal standard to nucleic acid extracts has been tested as a means to normalize quantities of DNA being used and to determine extraction efficiency (Mumy and Findlay, 2004; Park and Crowley, 2005).

Performing Polymerase Chain Reaction

It is important to emphasize that PCR conditions must be optimized each time DNA extracts from a new community are being examined and when new PCR primers are being used. Variables most commonly optimized are primer-annealing temperature, concentration of the polymerase enzyme cofactor Mg, and nucleic acid template concentration (Boleda et al., 1996; Ishii and Fukui, 2001). Investigators are quickly alerted to a problem if no PCR products are produced from a sample. Although many believe this can only occur because of insufficient template, it can also occur if there is too much template. It is essential that nucleic acid extractions are quantified before any PCR is performed to aid in differentiating between the use of too little or too much template. Dilution of template nucleic acid can also aid in reducing concentrations of contaminating chemicals that can reduce PCR efficiency. When suboptimal PCR conditions create additional or fewer bands on DGGE gels, the aberrant results are not readily recognized without further analysis, such as nucleotide sequencing. Some PCR-generated errors are the formation of chimeras, preferential amplification of select targets, nonspecific amplification of non-targets, and the production of single-stranded products (Qiu et al., 2001; Speksnijder et al., 2001; Thompson et al., 2002; von Wintzingerode et al., 1997; Wang and Wang, 1996). Conducting replicate analyses and making comparisons of profiles under different PCR conditions may aid in the recognition of some these errors (Nakatsu et al., 2000).

Modified approaches have been used to improve specificity and sensitivity of PCR for DGGE. Methods such as touchdown PCR have been used to increase specificity by using an annealing temperature above the standard in the first few cycles to limit primer annealing to sequences with exact matches (Muyzer et al., 1993). If a population of interest constitutes a small fraction of the community, sensitivity can be improved using nested PCR, in which PCR is performed twice. The first round of PCR typically uses a primer set that may also amplify DNA from populations outside of the target group (Heuer et al., 1997; Nakatsu et al., 2000). This increases the number of potential target sequences in the sample. The second PCR is performed using primers that are specific for the target group. It is often necessary to optimize the cycle numbers of both steps to obtain the most representative results. An alternative approach has been to reduce or eliminate unwanted templates, such as plastid 16S rDNA from DNA extracted from plant roots, by preferential digestion with an endonuclease (Green and Minz, 2005). If PCR modifications are being used, then one must ensure precautions are taken to ensure PCR artifacts are minimized.

Target Genes for Polymerase Chain Reaction

Any gene can be used as a target for PCR amplification for DGGE analysis and the choice is dependent on the question being addressed. To be informative, any marker chosen should have both conserved and variable regions of sequence. The majority of community analysis studies have favored the phylogenetically informative 16S rRNA gene, but other markers that have been used are ribosomal intergenic spacer regions (Becker et al., 2004; de Oliveira et al., 2006; Janse et al., 2003), 5S rRNA gene (Stoner et al., 1996), and the RNA polymerase gene *rpo*B (da Mota et al., 2005; Dahllof et al., 2000; Peixoto et al., 2002). Different rRNA-specific primers for PCR have been used to discriminate communities at different phylogenetic levels. Primers have been determined for the amplification of all three domains: Bacteria (Baker et al., 2003; Muyzer et al., 1993; Watanabe et al., 2001), Eukarya (van Hannen et al., 1998), and Archaea (Baker et al., 2003; Nakatsu et al., 2000; Nicol et al., 2003a; Watanabe et al., 2004; Øvreås et al., 1997). Also, primers have been identified for specific groups of Bacteria: the β-subdivision NH_3 oxidizer (Kowalchuk et al., 1997b), Actinomycetes (Heuer et al., 1997), sulfate-reducing bacteria (Teske et al., 1996), methanotrophs (Jensen et al., 1998; Seghers et al., 2003; Wise et al., 1999), cyanobacteria (Boutte et al., 2006), and the genera *Acinetobacter* (Vanbroekhoven et al., 2004), *Sphingomonas* (Leys et al., 2004), *Pseudomonas* (Bergsma-Vlami, 2005), *Burkholderia* (Salles et al., 2004), and *Bacillus* species (Garbeva et al., 2003). The major group of Eukarya that have been targeted are the fungi (Brodie et al., 2003; Oros-Sichler et al., 2006; van Elsas et al., 2000), including both ectomycorrhizae (Pennanen et al., 2005) and arbuscular mycorrhizal fungi (de Souza et al., 2004; Ma et al., 2005), using primers for the rRNA genes and internal transcribed spacer (ITS; Anderson et al., 2003; Green et al., 2004; Viebahn et al., 2005). The ITS region is often targeted because of its greater discriminatory power, especially of fungi. For the detection of specific fungal genera, for example *Fusarium*, other gene targets such as the translation elongation factor-1 alpha has been used (Yergeau et al., 2005). Another common soil biota that has been monitored are nematodes (Waite et al., 2003). To examine genetic polymorphisms in functional genes within soil communities, nitrogenase reductase (Diallo et al., 2004; Rosado et al., 1998), denitrifying genes (Throback et al., 2004), ammonia monooxygenase (Avrahami et al., 2002; Avrahami and Conrad, 2003; Hornek et al., 2006), methane monooxygenase (Henckel et al., 1999, 2000; Hoffmann et al., 2002), and hydrogenase genes (Wawer and Muyzer, 1995), have been targeted. Before choosing primers for any study, their specificities and limitations should be understood (Baker and Cowan, 2004; Baker et al., 2003).

Performing Denaturing Gradient Gel Electrophoresis

The greatest challenge in performing DGGE is making the gradient gel. The DGGE is performed using a vertical polyacrylamide gel electrophoresis apparatus similar to those used for protein analysis, although most laboratories use equipment specifically modified for DGGE (see Bio-Rad Laboratories, Hercules, CA, or C.B.S. Scientific Company, Del Mar, CA). The gradient is created using a standard two-cylinder gradient former or a specialized cam-operated gradient former is available (Bio-Rad Laboratories). Gel-to-gel variations that create challenges to between-gel comparisons occur when care is not taken to ensure that the denaturant gel solutions are being mixed and delivered into the gel plates at a steady and consistent rate. Standard markers, made from PCR products targeting the same gene from different organisms, should be included in all gels to monitor gel-to-gel variability. Sufficient numbers and diversity of organisms need to be chosen when making standards to ensure that marker bands will span the entire length of the denaturing gradients being used.

To determine the appropriate gradient, communities are first examined using a broad gradient range (e.g., 20–80% denaturant). This allows one to choose the ideal denaturant concentration range, which should include all the bands while maximizing their resolution. In some cases where the communities are complex and span a broad gradient range, it may be necessary to characterize communities using two or more different gradients to distinguish co-migrating bands (Joynt et al., 2006). Alternatively, bands have been excised and then run a second time using DGGE with a gradient spanning a narrower range to discriminate co-migrating bands (Gafan and Spratt, 2005). A possible new approach for more accurate separation and detection of PCR products is denaturing high performance liquid chromatography (dHPLC; Wagner et al., 1999; Xiao and Oefner, 2001). In this technology, the DNA binds to a chromatography column and is differentially eluted from the column with increasing concentrations of denaturant. An ultraviolet detector is used to automatically enumerate the products as they are released from the column. Most examples using this technology are currently for mutational analysis for medical application but with the availability of dHPLC instruments (WAVE System, Transgenomics, Omaha, NE), there are groups beginning to investigate its use for microbial analysis (Domann et al., 2003; Hurtle et al., 2002).

To optimize resolution between bands, the other important variables that must be determined are the time and voltage required for electrophoresis. The electrophoresis time should be determined empirically at the desired voltage using a time-interval experiment (Muyzer et al., 1996). A mixture of PCR products is loaded into the gel at set time intervals (e.g., every 30 min). Once the PCR products have reached their melting point, they will not migrate farther in the gel even with increasing electrophoresis time. The minimum time to reach this point should be chosen for electrophoresis times. A study comparing profiles of the same community using different voltages and run times concluded that maximum resolution is achieved by using high voltages for shorter electrophoresis times (Sigler et al., 2004). The nucleic acid stains used can also influence band resolution. The stains typically used for DGGE gels are SYBR Green, SYBR Gold, silver staining, and ethidium bromide. Comparisons of these stains for DGGE gels have not been done, but when tested for sensitivity of detection by ultraviolet transilluminators, the best stains were SYBR gold (Invitrogen, Carlsbad, CA) and silver staining followed by SYBR Green, then ethidium bromide (Tuma et al., 1999). The SYBR dyes and ethidium bromide have a one-step staining procedure, which is much simpler than using the multistep silver staining procedure; however, the type of sample being analyzed and the preference of the researcher should factor into the final decision.

Data Analysis

Soils are composed of complex microbial communities with purported population richness exceeding several hundred phylotypes (Gans et al., 2005; Torsvik et al., 1996). In contrast, about 20 to 40 bands in a DGGE profile can be clearly resolved in a gel. Fluorescently labeled primers can be used to increase sensitivity (Neufeld and Mohn, 2005) but it is still impossible for this method to detect the entire diversity in most soil samples. Only PCR products estimated to comprise a minimum of 1% of the sample were observed as bands in a DGGE fingerprint (Gelsomino et al., 1999). Often the number of bands is counted to obtain an estimate of species richness but in most soils this represents only populations that are dominant in the community and not total richness. Band intensities reflect the relative density of different PCR products from a sample. This should not, however, be directly translated to a numerical assessment of populations in the original soil community (Chandler et al., 1997). The number of PCR products can be biased to specific groups (Baker and Cowan, 2004) or overestimated because of multiple rDNA copies per genome (Klappenbach et al., 2001).

The presence and absence of bands in two PCR-DGGE profiles have been used to create a binary matrix for quantitative comparisons between two communities (Kropf et al., 2004; Wilbur et al., 2002). These data have been obtained by visually scoring gels or aided by using commercially available software programs (for example Bionumerics, Applied Maths, Sint-Martens-Latem, Belgium, and Fingerprinting II Informatix, BioRad Laboratories, Hercules, CA). The simplest analysis has been to determine similarity indices, such as Dice (Dice, 1945). These data can be represented by cluster analysis, e.g., unweighted pair group method with arithmetic means (Sokal and Sneath, 1963), in which dendrograms have been used to illustrate the relationship between communities (Morgan et al., 2002). Alternatively, multidimensional scaling (Johnson and Wichern, 2003) has been used to obtain a scatterplot of the data (Feris et al., 2003). Multivariate analysis methods, such as principal components analysis (PCA; Pielou, 1969), have been used to analyze large data sets with greater sources of variation (Gremion et al., 2004; Joynt et al., 2006). A PCA calculates and ranks the contribution of each variable in a profile, and the approach can be used to identify the main sources of variation observed between profiles (Wilbur et al., 2002). For example, in DGGE profiles, the source (band) contributing the greatest variability can be statistically determined, then the bands can be extracted from the gel, and its nucleotide sequence determined to identify the population. Researchers are just beginning to quantitatively explore the complex relationships between microbial communities using data from DGGE analyses (Kropf et al., 2004). There is a need to move beyond the current methods and begin to obtain better resolution of very complex data sets using methods such as self-organizing maps and neural networks (Dollhopf et al., 2001).

Limitations and Challenges in the Denaturing Gradient Gel Electrophoresis Method

Although there are many advantages to using PCR-DGGE for microbial community analysis, method limitations must be recognized for correct result interpretation. The greatest challenge in performing DGGE and attaining reproducible results is minimizing variation between gel gradients (Fromin et al., 2002). It is very difficult to exactly reproduce gel gradients; this must be kept in mind when performing between-gel comparisons. The inclusion of DGGE markers on all gels has been used to aid between-gel comparisons and to assess gradient variations. Ideally, a marker should be chosen with a sufficient number of bands to span the entire gradient, since there is variation within a gradient.

Another limitation of DGGE is that a complex community (e.g., soil) may be comprised of numerous populations (from >100 and possibly $>10^8$) in relatively equivalent proportions, thus resulting in a smear of bands, which makes it difficult to identify individual populations (Nakatsu et al., 2000). It is still possible to qualitatively state that two communities are different if the smear of bands looks different; however, the converse may not be true. The number of bands observed in a profile cannot be interpreted to be the exact numbers of populations in a community. In some cases, investigators have found that a single laboratory isolate can produce multiple bands by DGGE (Nübel et al., 1996; Satokari et al., 2001), and conversely, a single band may represent multiple populations (Yang and Crowley, 2000). Combining DGGE profiling with other techniques, such as sequencing of bands or hybridization with probes, can reduce the ambiguity of band identification (Stephen et al., 1998). Nevertheless, the method can provide an estimate of richness that enables researchers to determine subsequent analyses that can be conducted and a means of choosing samples representing unique or representative communities (Joynt et al., 2006).

APPLICATIONS OF DENATURING GRADIENT GEL ELECTROPHORESIS IN TERRESTRIAL MICROBIAL ECOLOGY

Investigating the biology and ecology of microscopic organisms presents many challenges to researchers (Morris et al., 2002a). The heterogeneity of soils adds to the complexity of studying and understanding soil communities. The PCR-DGGE technique has provided a relatively rapid means to study and compare the community structure of different systems without the use of (or in addition to) cultivation-based approaches (Torsvik and Øvreås, 2002). Researchers have been using PCR-DGGE to investigate basic aspects of soil communities, such as changes or differences in community structure with respect to environment, space, time, and perturbations. In addition, nucleotide sequencing of bands has been used to identify populations associated with these community changes or differences. Investigators choosing PCR-DGGE as a community analysis method must remember that it is just a good starting point for more in-depth studies.

Community Structure Analysis

A number of earlier and more recent studies have used PCR-DGGE to obtain a "snapshot" of community structure, an approximation of the numbers of populations and their proportional representation within the total community. Bacterial communities have been examined by PCR-DGGE from almost every soil environment imaginable, including agricultural soils (Nakatsu et al., 2000), plant rhizospheres (Duineveld et al., 1998; Marschner et al., 2001; Yang and Crowley, 2000), forests (Jaatinen et al., 2004; Laverman et al., 2005; Marschner and Timonen, 2005), grasslands (Felske and Akkermans, 1998; Griffiths et al., 2003; Ritz et al., 2004), upland (Dilly et al., 2004; Sun et al., 2004), rice paddy agricultural soils (Weber et al., 2001), oil-contaminated paddy soil (Zhang et al., 2005), orchards (Yao et al., 2005), wetlands (Ibekwe et al., 2003; Jaatinen et al., 2005; Wartiainen et al., 2003), industrial sites (Zocca et al., 2004), desert soil crusts (Nagy et al., 2005), sand dunes (Kowalchuk et al., 1997b; Smith et al., 2004), landfills (Wise et al., 1999), nematode egg masses (Papert et al., 2004), and plant endophytes (Kuklinsky-Sobral et al., 2005; Seghers et al., 2004). These studies were able to illustrate and compare the complexity of communities in these various environments. Because DGGE does not depend on cultivation, it has revealed greater diversity than previously uncovered using traditional cultivation methods (El Fantroussi, 2000; Nakatsu et al., 2005; Wise et al., 1999).

Monitoring Changes or Differences in Community Structure

A strength of the PCR-DGGE method is its use in determining the effect of different soil treatments on community structure. Some treatments that have been examined are: agronomic management practices (Alvey et al., 2003; Backman et al., 2003), sewage sludge application (Gray et al., 2003), land use (Avrahami and Conrad, 2003), pesticide application (Bending et al., 2003; El Fantroussi et al., 1999), hydrocarbon contamination (Duarte et al., 2001; Juck et al., 2000), wood ash fertilization (Yrjala et al., 2004), and heavy metal exposure (Joynt et al., 2006; Kozdroj and van Elsas, 2001). The number of variables in the field can complicate data interpretation, and therefore, many have used laboratory soil microcosms to analyze the impact of specific treatments (Duarte et al., 2001; Nakatsu et al., 2005; Saeki and Toyota, 2004). These studies found that treatments that selectively enriched specific populations resulted in community structure differences, which could be seen in the PCR-DGGE profiles. Researchers do need to exercise caution when interpreting DGGE profiles. Often researchers interpret the disappearance of bands as a complete disappearance of that species from the community. In fact, it may only be reflecting a change in the relative densities between populations within the community, where the increase in some populations puts other populations below the detection limit of DGGE.

One of the advantages of PCR-DGGE over methods such as T-RFLP is that the nucleotide sequence of bands directly extracted from gels can be determined (Øvreås et al., 1997). Sequence analysis was used in a number of studies utilizing DGGE to identify populations potentially responsible for observed functional changes (Backman et al., 2003; Bending et al., 2003; Joynt et al., 2006; Juck et al., 2000; Nakatsu et al., 2005; Saeki and Toyota, 2004). As another caution in data interpretation, however, it is important to realize that a correlation between community structural change and functional response does not necessarily mean that all intense bands in a profile represent populations responsible for the specific function of interest. In fact, subsequent cultivation and physiological

testing of culturable bacteria has found that some and not all populations enriched in a DGGE profile are responsible for the function being tested (Nakatsu et al., 2005). Additional approaches, such as analysis of functional genes, are still needed because interactions between biota in an ecosystem are very complicated and cannot be deciphered solely by examining community structure or cultivation. Investigators must also remember that the length of sequences (typically 200–500 base pairs) from DGGE bands only represents a fraction of the rRNA gene, thus constraining identification to higher taxonomic levels and not species. A few studies have noted some of the challenges faced in extracting and reamplifying targeted bands from DGGE gels for nucleotide sequencing (Nikolausz et al., 2005; Zhang et al., 2005).

Active vs. Persistent Populations

To gain a better understanding of the active populations within a community, RNA has been used instead of DNA as a template for PCR. For clarity, in this section rDNA will refer to the use of a DNA template for PCR amplification of the rRNA gene and rRNA when an RNA template is used. Cell activity (growth rate) has been coupled to an increase in total RNA (ribosomes) (Rosset et al., 1966). Shifts in community structure resulting from a perturbation can be detected earlier using rRNA as a template instead of rDNA (Morgan et al., 2002). With continuous exposure to the same treatments that increases the biomass of the active population, however, the community structures using rRNA or rDNA can become more similar. Active populations (rRNA-based DGGE profile) have been examined in the rhizosphere and were found to be composed of a subset of the residential community (rDNA-based DGGE profile) (Duineveld et al., 2001; Sharma et al., 2005). In most cases, it is still impossible to identify the active role potentially being played by these microbes unless the function is limited to a small group of organisms. For example, anaerobic microcosms constructed from grassland rhizosphere soils found that Euryarchaeal reverse transcriptase PCR DGGE products that coincided with detectable methane production is a function probably limited to this group (Nicol et al., 2003b).

Linking Community Structure and Function

In general, although the use of rRNA as template can distinguish active from persistent members of a community, it still cannot link an organism to a function. Stable isotope probing (SIP) is a promising method that enables phylogenetic identification of communities responsible for utilization of a substrate (Dumont and Murrell, 2005; Radajewski et al., 2000). After addition of a C substrate labeled with ^{13}C, nucleic acids of organisms using that substrate will be heavier, giving them a different buoyant density when separated by Cs gradient centrifugation. Typically, RNA is extracted to ensure that the most active populations are being identified. Most commonly, the rRNA is sequenced to identify the functional populations (Manefield et al., 2002; Morris et al., 2002b), but recently SIP has been used in conjunction with PCR-DGGE analysis to unravel specific roles of specific populations within an active community. For example, only a subset of the active community in a plant rhizosphere was found to be using organic C from root exudates (Rangel-Castro et al., 2005). The combination of these methods is beginning to link structure and function, thus showing great promise toward unraveling the complexity of interactions in soil ecosystems.

SUMMARY

The application of molecular techniques, such as PCR-DGGE, has provided microbiologists a means of examining microbial communities in soils. A combination of many approaches has shown us that our current knowledge represents only a small fraction of the vast diversity and function harbored within the soil community. Molecular techniques such as PCR-DGGE, however, are providing cost-effective means to examine and compare a large number of samples within short time frames.

ACKNOWLEDGMENTS

This work was supported by the USDA Hatch Grant IND050025, the National Science Foundation (Award no. 0302645-BES), and the Department of Energy's Natural and Accelerated Bioremediation Research (DOE-NABIR) program (Award no. DE-FG02-98ER62681). Special thanks for critical review of this manuscript and very useful discussions by D. Thompson and M. Savin.

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