Denaturing Gradient Gel Electrophoresis (DGGE) Monitoring of Soil, Sediment and Composting Microbial Communities

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Introduction

The major portion of microorganisms present in the environment may not be readily cultivated by the contemporary available technologies. Indeed current estimates show that fewer than 1% of the microorganisms present in many environments can be cultivated with ease, reflecting that techniques based on laboratory cultivation could be significantly biased (71, 72). Microorganisms are typically plated on laboratory culture media, and different types of colonies are identified with biochemical or morphological methods (21). Microbial communities can also be analyzed with carbon-source utilization patterns through the application of environmental samples into BIOLOG microplates (63).

Application of molecular techniques to ecological studies has disclosed a wide diversity of microorganisms in natural and controlled communities, previously unknown to microbial ecologists. They have also devoted much effort to tracking biological interactions between species in the environment. The molecular techniques, including fatty acid methyl ester (FAME), phospholipid fatty acid ester (PLFA), $C_0t_{1/2}$ curve analysis, sequencing of gene encoding small subunit of ribosomal RNA and denaturing gradient gel electrophoresis (DGGE) have also been applied to analyze microbial communities with or without culturing microbes. Of these, DGGE is one of the most useful fingerprinting techniques and can process multiple samples without cultivation at a time. Virtually, culture-independent methods have revealed more complexity in the microbial populations of particular ecosystems than culture-based methods. Mostly these techniques are real-time based, sensitive, rapid, and effective.

In this review, denaturing gradient gel electrophoresis (DGGE), one of the most popular molecular biological techniques useful for the analysis of microbial community structures will be discussed. The discussion will be focused on use of the technique for the analysis of microbial communities such as soil, sediment and composting environments.

Concept of PCR-DGGE separation

Amplification of total DNA mixture from microbial communities using primers specific for 16S rRNA gene fragments of bacteria generates mixtures of PCR products. These mixtures have different base sequences so that they have different melting temperatures in the polyacrylamide gel containing a gradient of denaturants, typically a mixture of urea and formamide. The amplified DNA products first enter as double-stranded molecules and later they become melted and

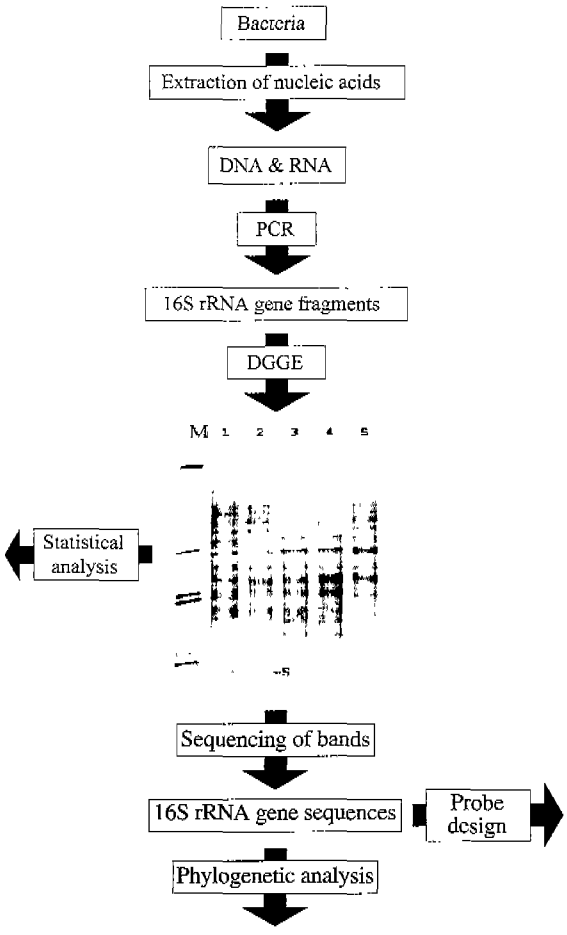

Fig. 1. A flow diagram of PCR-DGGE analysis of microbial communities (adapted from the references 2a and 62).

stop at different positions within the gel where different denaturant concentrations are met (Figure 1).

Data analysis of PCR-DGGE patterns

DGGE patterns from mixed microbial communities may be quite complex. Various kinds of information can be obtained from the patterns such as number, position (absence or presence of particular bands) and relative intensity of bands. Nucleotide sequence information of the bands can also be additional. The analysis of DGGE patterns require statistical methods, including unweighted pair-wise grouping with mathematical averages (UPGMA) and multidimensional scaling (MDS) to set up a binary matrix that is representative of the bands occurring in a set of DGGE patterns (Figure 2).

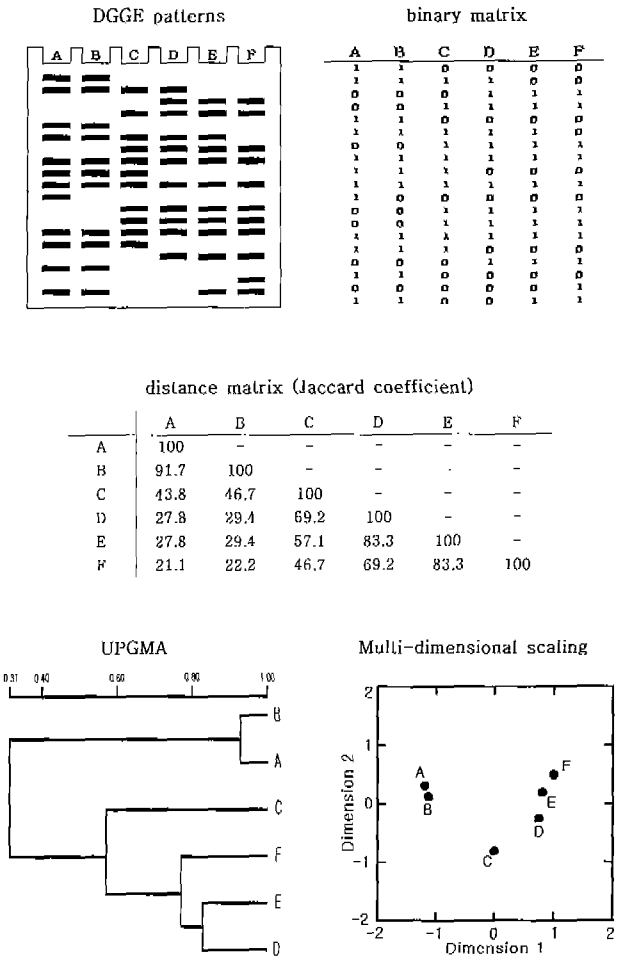

Fig. 2. A scheme of statistical analysis of DGGE profiles. The presence (1) and absence (0) of DGGE bands in various samples are scored in a binary matrix. The binary matrix is transformed into a distance matrix using a similarity coefficient (e.g., Jaccard coefficient) that is used for UPGMA or MDS (redrawn from the reference 62).

PCR-DGGE to monitor population shifts after environmental stress and perturbation

Here we describe various applications of the PCR-DGGE technique to analysis of pristine and polluted microbial ecosystems. The former will include natural soil, sediment and composting microbial communities while the latter will cover several polluted soil and sediment microbial ecosystems.

Natural aquatic and soil microbial communities

Over the last 10 years several molecular techniques have been developed in order to study natural samples (44). These techniques can help identify microorganisms without isolation (1, 28) and have elucidated the enormous extent of microbial diversity (54). Moreover, new molecular approaches have been proposed recently in order to link microbial processes with the organisms involved (2, 5). Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes is a molecular technique that is used to study the dynamic behavior of complex microbial assemblages (44, 46) and to isolate microorganisms in pure culture (61, 70).

Casamayor et al. (4) have analyzed the microbial assemblages of Lake Ciso and Lake Vilar (Banyoles, northeast Spain) that were analyzed in space and time by microscopy and by performing PCR-denaturing gradient gel electrophoresis (DGGE) and sequence analysis of 16S rRNA gene fragments. Samples obtained from different water depths and at two different times of the year (in the winter during holomixis and in the early spring during a phytoplankton bloom) were analyzed. Sequences obtained from Lake Ciso samples were related to gram-positive bacteria and to members of the division Proteobacteria. Sequences obtained from Lake Vilar samples were related to members of the *Cytophaga-Flavobacterium-Bacteroides* phylum and to cyanobacteria. Euryarchaeal sequences (i.e., methanogen- and thermoplasma-related sequences) also were present in both lakes. These data showed that the sequences obtained from the DGGE fingerprints corresponded to the microorganisms that were actually present at higher concentrations in the natural system.

The rhizosphere is the volume of soil adjacent to and influenced by the plant root (24). Roots are known to excrete several forms of organic materials. The amounts and composition of these organic materials are different in different plant species and cultivars, change during plant development, and are different in old and young parts of the root system (20). As a result, the bacterial communities in the

rhizosphere, which can use these organic materials as a substrate, will differ in composition and density (6). This may result in the buildup of a microflora specific to a particular plant species and genotype (48), as well as to the plant developmental stage and the root part (base or tip) (37). The study of the diversity of bacterial communities in the soil or rhizosphere is inherently difficult, since all methods developed to date have limitations (31, 74).

Recently, a novel method, PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE), was proposed for the study of the phylogenetic diversity of bacterial populations in environmental samples (44). Duineveld et al.(9) have used PCR-DGGE in conjunction with cultivation-based methods to analyze the diversity of the bacterial community in the rhizosphere during the growth of chrysanthemum plants. Information on both the dynamics of the total bacterial community and the metabolic potential of populations may be important for the identification of suitable biocontrol agents. In this study they intended to obtain a better understanding of root effects on the total and the culturable bacterial community in soil on a general level without dealing with specific groups of bacteria. The DGGE patterns showed that the bacterial communities as determined from direct rhizosphere DNA extracts were largely stable along developing roots of the chrysanthemum, with very little change over time or between root parts of different ages. Results obtained with the sole carbon source utilization tests showed that the metabolic profile of the bacterial communities in the rhizosphere of the root tip did not change substantially during plant growth. This suggests selective development of specific bacterial populations by the presence of a root tip.

Furthermore, the soil type, growth stage, cropping practices (such as tillage and crop rotation), and other environmental factors (7, 19, 27, 36, 38, 79) seem to influence the composition of the microbial community in the rhizosphere. Rhizosphere microorganisms can exert strong effects on plant growth and health by nutrient solubilization, N_2 fixation, or the production of plant hormones (26, 55). However, to fully exploit the potentials of biological control agents, a better understanding of the structural and functional diversity of microbial populations in the rhizosphere and their succession during plant development is required (75).

DGGE profiles of the rhizoplane more closely resembled those in the soil than the profiles found in the root tissue

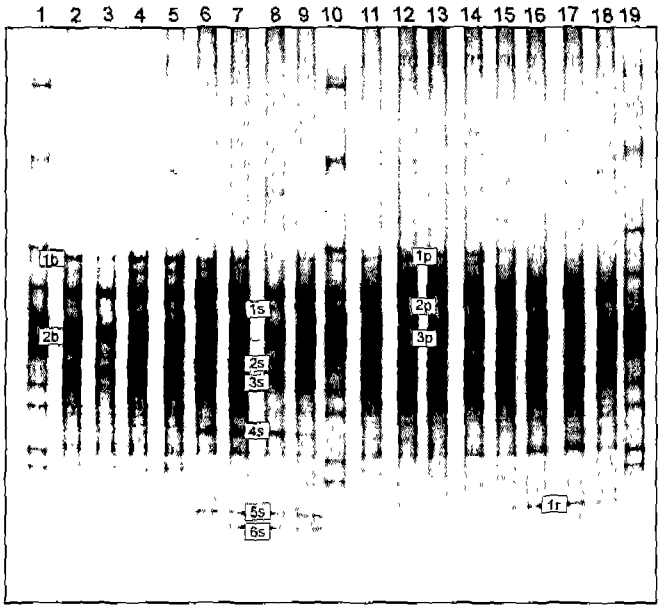

Fig. 3. Comparative analysis of DGGE profiles of 16S rDNA fragments amplified from bulk soil DNA (lanes 2 to 5) or from rhizosphere DNA of strawberry (lanes 6 to 9), potato (lanes 11 to 14), or oilseed rape (lanes 15 to 18) at sampling time (3rd month of 2nd year); lanes 1, 10, and 19, standard. The numbered bands in the gel profile shows representative fingerprinting bands from which a DNA sequence was identified (from the reference 64).

or on the seed, suggesting that rhizoplane bacteria primarily originated from the surrounding soil (51). No change in bacterial community composition was observed in relation to plant age. Pregermination of the seeds for up to 6 days improved the survival of seed-associated bacteria on roots grown in soil, but only in the upper, nongrowing part of the rhizoplane

The bacterial rhizosphere communities of three host plants of the pathogenic fungus *Verticillium dahliae,* field-grown strawberry (*Fragaria ananassa* Duch.), oilseed rape (*Brassica napus* L.), and potato (*Solanum tuberosum* L.), were analyzed (64). The DGGE fingerprints showed plant-dependent shifts in the relative abundance of bacterial populations in the rhizosphere which became more pronounced in the second year. DGGE patterns of oilseed rape and potato rhizosphere communities were more similar to each other than to the strawberry patterns. In both years seasonal shifts in the abundance and composition of the bacterial rhizosphere populations were observed (Figure 3). *Bacillus megaterium* and *Arthrobacter* sp. were found as predominant populations in bulk soils. Sequencing of dominant bands excised from the rhizosphere patterns revealed that 6 out of 10

bands resembled gram-positive bacteria. *Nocardia* populations were identified as strawberry-specific bands.

Composting microbial communities

Composting turns easily degradable organic matter into stable matter containing a humic-like substance by passing through a thermophilic stage (14, 18). This waste treatment is useful in addressing environmental problems such as global warming, due to the accumulation of man-made greenhouse gases in the atmosphere, because the process emits less CO_2 than burning. Monitoring of the microbial succession is important in effective management of the composting process as microbes play key roles in the process and the appearance of some microbes reflects the quality of maturing compost (32).

Although workers have examined nitrogen balance during composting (51, 53), this parameter has not been described well yet. Inbar et al. (30) qualitatively characterized nitrification as a normal process in compost that depends on the composting conditions used. Nodar et al. (49) reported the presence of "very few ammonium oxidizers and nitrite oxidizers" belonging to undetermined genera in a poultry dung-pine sawdust mixture, and the number of nitrifiers decreased after prolonged storage of poultry slurry (50). Thus, although nitrifiers have been recognized as potentially important organisms in composts and composting materials, their species composition, distribution, and activity have not been assessed yet. The lack of data concerning ammonia oxidizers in compost may be due to the difficulties encountered in studying this specialized group of organisms by conventional culture-based techniques. Ammonia oxidizers have low maximum growth rates and produce low biomass yields, and pure-culture isolation is extremely time-consuming and unrepresentative (57). The monophyletic nature of the beta-subgroup proteobacterial ammonia oxidizers has facilitated the design of PCR primers and oligonucleotide probes that target the 16S rRNA gene in this group at different taxonomic levels, and the use of these primers and probes has led to a recent progress in the analysis of ammonia oxidizer populations (25, 43, 67, 76).

Denaturing gradient gel electrophoresis (DGGE) has been a powerful tool for analyzing microbial communities. It has been used to separate mixed PCR products after amplification of 16S ribosomal DNA (rDNA) fragments (45), and this technique has been applied to the study of ammonia-oxidizing bacteria (35). DGGE band patterns can be characterized by hybridization with specific oligonucleotides that target internal sites, as demonstrated for broad taxonomic groups within the domain Bacteria (69) and the seven recognized sequence clusters within the beta-subgroup proteobacterial ammonia oxidizers (66, 67).

A study (35) was carried out to investigate the distribution and community composition of beta-subgroup proteobacterial ammonia oxidizers in different types of compost and composting materials in order to determine to what extent these organisms might be responsible for nitrogen transformations in these substrates. Here ammonia oxidizer-like 16S rDNA was detected in almost all of the materials tested, including industrial and experimental composts, manure, and commercial biofertilizers. A comparison of the DGGE and hybridization results after specific PCR and RT-PCR suggested that not all of the different ammonia oxidizer groups detected in compost are equally active. 16S rRNA was also simultaneously targeted by reverse transcriptase PCR (RT-PCR) to determine which of the ammonia oxidizer populations detected were most active in the composting materials tested (11, 56, 77).

Ishii et al. (32) monitored the composting microbial succession in the S phase. Some fermenting bacteria, such as lactobacillus, were present with the existing organic acids; in the T phase thermophilic bacillus appeared and, after the C phase, bacterial populations were more complex than in previous phases and phylogenetic positions of those populations were relatively distant from strains so far in the DNA database. Here, the DGGE method has been useful to elucidate microbial succession during a composting process.

Polluted soils and sediments

As sensitive molecular biological techniques are being developed, it is now becoming possible to define the causes of time-dependent changes in the health of a stressed ecosystem on the basis of the structural composition of the ecosystem population (22). The measurement of lipid biomarkers, specifically, phospholipid fatty acids (PLFA), combined with nucleic acid-based molecular techniques for fingerprinting the 16S ribosomal DNA (rDNA) component of microbial cells has been a powerful technique for elucidating the microbial ecology of actively bioremediating communities (68). Lipid biomarker-based techniques measure the lipid profiles of microbes in the environment irrespective of culturability, thereby avoiding culture bias (80, 81). Microbial communities within contaminated ecosystems could be dom-

inated by the organisms capable of utilizing and/or surviving toxic contamination. As a result, these communities are typically less diverse than those in non-polluted systems, although the diversity may be influenced by the complexity of chemical mixtures present and the length of time the populations have been exposed.

Garland and Mills (16) used the Biolog redox technology based on community-level carbon source utilization patterns to characterize and classify microbial communities from environmental (soil, aquatic, and rhizosphere) samples. In a comparative study of rhizosphere bacterial communities and hydrocarbon-polluted environments, Garland and Mills (17) and Wunsche et al. (83) demonstrated that substrate utilization patterns could be used as an indicator of community structure and function. Ibekwe et al. (29) examined the ability of soil microbial communities to recover after treatment with fumigants using culture-dependent (Biolog) and culture-independent (phospholipid fatty acid [PLFA] analysis and denaturing gradient gel electrophoresis [DGGE] of 16S ribosomal DNA [rDNA] fragments amplified directly from soil DNA) approaches. Here, the effect of MeBr on heterotrophic microbial activities was most severe in the first week and thereafter the effects of MeBr and the other fumigants were expressed at much lower levels. High diversity indices were maintained between the control soil and the fumigant-treated soils, except for MeBr. Sequence analysis of clones generated from unique bands showed the presence of taxonomically unique clones that had emerged from the MeBr-treated samples and were dominated by clones closely related to *Bacillus* spp. and *Heliothrix oregonensis*. Variations in the data were much higher in the Biolog assay than in the PLFA and DGGE assays, suggesting a high sensitivity of PLFA analysis and DGGE in monitoring the effects of fumigants on soil community composition and structure.

In general, the effect of herbicides on soil microbial communities has often been investigated by conventional methods based on cultivation of the microbial communities and on measurements of their metabolic activities (65, 78). Of the 16S rDNA-based methods used for studying complex microbial populations, denaturing gradient gel electrophoresis (DGGE) has received the most attention and has been successfully applied to several natural habitats (33, 47, 69, 73). The influences of three phenyl urea herbicides (diuron, linuron, and chlorotoluron) on soil microbial communities were studied by using soil samples with a 10-year history

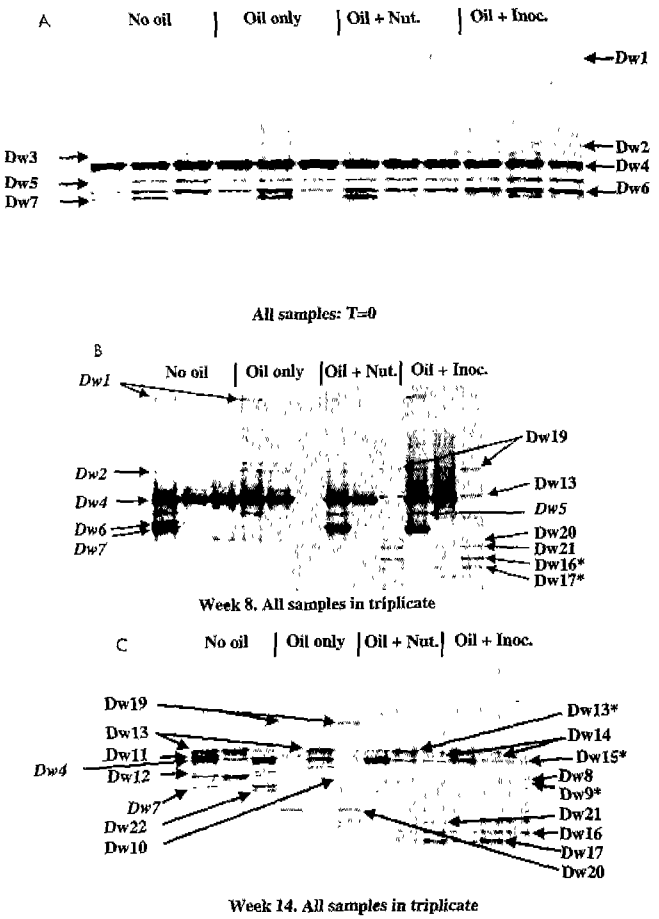

Fig. 4. DGGE profiles of bacterial communities at three time points. Amplified products were electrophoresed on a gradient of 15 to 55% denaturant. All labeled bands were excised from the gel, reamplified, and subjected to sequence analysis. Italicized labels point out bands that were also derived from earlier time points and are noted to allow visual comparison between gels. (A) Community structures at time zero. Time zero was considered as 4 days after oil was added to the plots and was the time point at which accelerated remediation techniques were initiated (amendment with nutrients [Nut.] or nutrients plus inoculum [Inoc.]). (B) Community structures after 8 weeks of treatment. (C) Community structures after 14 weeks of treatment. The banding profiles of all oiled plots were complex compared to unoiled plots. Two bands (Dw8 and Dw9) were observed only in oiled plots that had also received nutrient amendment (from the reference 39).

of treatment (60). Similarity cluster analysis demonstrated that the microbial community structures of the herbicide-treated and nontreated soils were significantly different. Moreover, the bacterial diversity seemed to decrease in soils treated with urea herbicides, and sequence determination of several DGGE fragments showed that the most affected species in the soils treated with diuron and linuron belonged to an uncultivated bacterial group. In addition, principal-component analysis performed on BIOLOG data showed that

the functional abilities of the soil microbial communities were altered by the application of the herbicides.

A study was undertaken to gain insight on the progress of natural attenuation and enhanced bioremediation during a controlled oil spill field experiment in Delaware (39). Three crude oil bioremediation techniques were applied in a randomized block field experiment simulating a coastal oil spill. Here, four treatments (no oil control, oil alone, oil plus nutrients, and oil plus nutrients plus an indigenous inoculum) were set up. The results of PLFA analysis demonstrated a community shift in all plots from primarily eukaryotic biomass to gram-negative bacterial biomass with time. PLFA profiles from the oiled plots suggested increased gram-negative biomass and adaptation to metabolic stress compared to unoiled controls. PCR-DGGE analysis of untreated control plots showed a simple, dynamic dominant population structure throughout the experiment. This DGGE pattern disappeared in all oiled plots, reflecting that the structure and diversity of the dominant bacterial community changed substantially. No consistent differences were detected between nutrient-amended and indigenous inoculum-treated plots, but both differed from the oil-only plots. Representative bands were excised for sequence analysis and indicated that oil treatment encouraged the growth of gram-negative microorganisms within the alpha-proteobacteria and *Flexibacter- Cytophaga-Bacteroides* phylum (Figure 4).

Recently Duarte et al. (8) studied the selective effects of sulfur-containing hydrocarbons, with respect to changes in bacterial community structure and selection of desulfurizing organisms and genes in the soil environment. Generally, counts of dibenzothiophene (DBT) degraders were 10- to 100-fold lower than the total culturable counts. PCR-DGGE, however, demonstrated that the numbers of bands detected in the molecular community profiles decreased with increasing oil content of the soil. Analysis of the sequences of three representative bands of the profiles generated from the highly polluted soil samples suggested that the underlying organisms were related to *Actinomyces* sp., *Arthrobacter* sp., and a bacterium of uncertain affiliation. PCR-DGGE applied to sequential enrichment cultures in DBT-containing sulfur-free basal salts medium prepared from the A and treated FSL soils revealed the selection of up to 10 distinct bands. Sequencing a subset of these bands provided evidence for the presence of organisms related to *Pseudomonas putida*, a *Pseudomonas* sp., *Stenotrophomonas maltophilia*, and *Rhodococcus erythropolis*. Several of 52 colonies obtained from the A and FSL soils on agar plates with DBT as the sole sulfur source produced bands that matched the migration of bands selected in the enrichment cultures. These results indicate the dominant DBT degraders could be detected and monitored by the PCR-DGGE technique.

TGGE techniques

Methods such as denaturing gradient gel electrophoresis (15) or temperature gradient gel electrophoresis (TGGE) (58) have been developed to analyze microbial communities rapidly, based on sequence-specific separation of 16S rDNA amplicons (10, 45).

A group-specific primer, F243 (positions 226 to 243, *Escherichia coli* numbering), was developed by comparison of sequences of genes encoding 16S rRNA (16S rDNA) for the detection of actinomycetes in the environment with PCR and TGGE or DGGE (21). The specificity of the forward primer in combination with different reverse ones was tested with the genomic DNAs from a variety of bacterial strains. Most actinomycetes investigated could be separated by TGGE and DGGE, with both techniques giving similar results.

The diversity of the predominant bacteria in the human gastrointestinal tract was studied by using 16S rRNA-based approaches. PCR amplicons of the V6 to V8 regions of fecal 16S rRNA and ribosomal DNA (rDNA) were analyzed by TGGE. (84). Here a temperature gradient from 36-45°C was applied parallel to the electrophoresis running direction. The sequences matching 15 bands in the TGGE pattern showed 91.5 to 98.7% homology to sequences derived from different *Clostridium* clusters. The results indicate that the combination of cloning and TGGE analysis of 16S rDNA amplicons can be a reliable approach to monitoring different microbial communities in fecal environments.

A greenhouse study with soil-plant microcosms has been conducted in order to compare the effect of crop species, soil origin, and a bacterial inoculant on the establishment of microbial communities colonizing plant roots (42). It turned out that the influence of soil was of minor importance, while a modification of the alfalfa-associated microbial community structure after inoculation with *Sinorhizobium meliloti* L33 was only consistently observed by using TGGE.

Limitations of PCR-DGGE approach

It is highly probable that molecular techniques provide a biased view of microbial diversity. For instance, many of the procedures rely on PCR, a technique in which biases

have been shown to exist, and on cloning, which can act in a selective way (82). Similarly, it is not clear whether bacterial cells in nature possess different degrees of resistance to cell breakage, which is required for nucleic acid extraction. All taken, it is rather hard to ascertain whether the collection of sequences obtained from an environment represents the natural assemblage accurately. As with all other molecular biological applications that rely on PCR as an initial step, DGGE fingerprinting is potentially afflicted with the PCR inherent biases.

Another side of DGGE limitations is that only short sequence fragments can be used (up to ca. 500bp), hereby limiting the amount of sequence information for subsequent identification by comparative sequence analysis. Furthermore, the resolution of different sequences is not always accomplished and recognition of features as bands or not bands can also be difficult in some cases. Other biases can also generate artifacts with other molecular approaches. Bacteria may carry more than one copy of the 16S rRNA encoding gene, with heterogeneous sequences, giving rise to more than one band on DGGE (52).

Furthermore, dissimilar sequences may co-migrate to the same position in a DGGE gradient (3, 13, 35, 59), causing a band to be a mixture of more than one sequences and preventing recovery of a clean sequence after re-amplification. These problems potentially interfere with the reliable estimation of the number of different phylotypes (i.e., richness) by all genetic fingerprinting methods. Futhermore, artificial bands may be due to hetroduplex molecules (12), which may form between single strands of two similar, but cannot interfere with conclusions based on the comparison of patterns from different samples. Murray et al. (47), however, noted that heteroduplex formation did not significantly interfere with DGGE analysis of complex communities. To reduce misinterpretation of results due to biases or limitations of the techniques used, it may be of advantages if other molecular, microbiological and geochemical measurements are made at the same time.

Conclusions

Numerous studies in microbial ecology have used PCR-DGGE fingerprinting for the analysis of microbial community composition up to now. It has been shown by several studies that the approach is reproducible and sensitive. These beneficial features as well as the simpleness of DGGE techniques will surely attract even more scientists to adapt this relatively inexpensive technique as a new tool in analyzing various unexplored microbial communities in the future. The limitations of the techniques can be overcome by employing conventional microbiological methodologies, compensating molecular techniques, and the microbial genomics techniques including DNA microarray and other adranced molecular techniques.

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