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Mini Review

Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology

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Abstract

Here, the state of the art of the application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology will be presented. Furthermore, the potentials and limitations of these techniques will be discussed, and it will be indicated why their use in ecological studies has become so important.

Abbreviations: ARDRA – amplified ribosomal DNA restriction analysis; DGGE – denaturing gradient gel electrophoresis; DMSO – dimethylsulfoxide; PEG – polyethylene glycol; PCR – polymerase chain reaction; RAPD – randomly amplified polymorphic DNA; rDNA – ribosomal DNA; RDP – Ribosomal Database Project; RFLP – restriction fragment length polymorphism; rRNA – ribosomal RNA; SSCP – single strand conformation polymorphism; SSU – small-subunit; TGGE – temperature gradient gel electrophoresis

Introduction

It is now well recognised among microbiologists that only a small fraction of all bacteria have been isolated and characterised (Wayne et al., 1987; Ward et al., 1992). Comparison of the percentage of culturable bacteria with total cell counts from different habitats showed enormous discrepancies (summarised by Amann et al., 1995). One of the reasons for this difference might be the interdependency of different organisms upon each other, the most obvious example being the endosymbiotic bacteria in specific worms and molluscs (e.g., Fisher, 1990); another reason is certainly the lack of knowledge of the real conditions under which most of the bacteria are growing in their natural environment. So, to obtain a better understanding of the role of microbial diversity in the maintenance of ecosystems, other approaches, which complement the traditional microbiological procedures are needed. The application of molecular biological techniques to detect and identify microorganisms by certain molecular markers, such as 16S rRNA or its encoding gene (*'the rRNA approach';* Olsen et al., 1986; Amann et al., 1995), is now more and more frequently used to explore the microbial diversity and to analyse the structure of microbial communities (e.g., Muyzer and Ramsing, 1995, and references therein). The application of these techniques in microbial ecological studies has even become a discipline on its own, i.e. molecular microbial ecology (Akkermans et al., 1995). So far, most results with the molecular approach have been obtained by cloning of 16S rDNA fragments obtained either after reverse transcription of rRNA (e.g., Ward et al., 1990; Weller et al., 1991), or after enzymatic amplification of DNA extracted from different habitats, such as sediments (e.g., Gray & Herwig, 1996), soil (e.g., Liesack & Stackebrandt, 1992; Borneman et al., 1996), hot springs (e.g., Barns et al., 1994), and seawater (e.g., Giovannoni et al., 1990; Fuhrman et al. 1993). The results of these studies have shown

the enormous wealth of microbial diversity, and at the same time the limitations of traditional cultivation techniques to retrieve this diversity.

However, although successful, these studies have only focused on the exploration of microbial diversity, they have not given any information on the complex dynamics which microbial communities can undergo by diel and seasonal fluctuations or after environmental perturbations. As microbial ecology is the study of interactions among microorganisms and between microorganisms and their environment, microbial ecosystems have to be studied over longer time periods. For this purpose the cloning approach is not useful, because it is time-consuming and labour intensive, and hence impractical for multiple sample analysis. A better approach to investigate population shifts is the use of taxon-specific probes in dot-blot hybridisation of extracted rRNA (e.g., Stahl et al., 1988; Raskin et al., 1995) or in whole cell hybridisation (for an overview, see Amann et al., 1995, and references therein). These studies however only focus on particular microorganisms for which probes have been developed. Therefore, to study the complex structures of microbial communities and their dynamics other molecular biological techniques are needed.

Genetic fingerprinting techniques

Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community. One of the fingerprinting techniques that has been used in microbial ecology for more than a decade is the electrophoretic separation in high resolution polyacrylamide gels of low molecular weight rRNA molecules (5S rRNA and tRNA) extracted from natural samples (e.g., Höfle, 1988; 1990).

Recently, another genetic fingerprinting technique, *denaturing gradient gel electrophoresis* (DGGE) of PCR-amplified ribosomal DNA fragments has been introduced into microbial ecology (Muyzer et al., 1993). Within a short period of time this method has attracted the attention of many environmental microbiologists, and the technique is now used in many laboratories.

In this paper we describe the theoretical and practical aspects of DGGE and the related technique called *temperature gradient gel electrophoresis* (TGGE) and their application to the analysis of microbial communities. Furthermore, we will discuss the potentials and limitations of these approaches for studies in microbial ecology.

Theoretical and practical aspects of DGGE and TGGE

In DGGE (Fischer & Lerman, 1979, 1983; Myers et al., 1987) as well as in TGGE (Rosenbaum and Riesner, 1987; Riesner et al., 1991) DNA fragments of the same length but with different sequences can be separated. Separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient. The melting of DNA fragments proceeds in discrete so-called *melting domains:* stretches of base-pairs with an identical melting temperature. Once a domain with the lowest melting temperature reaches its melting temperature (*Tm*) at a particular position in the denaturing or temperature gradient gel, a transition of a helical to a partially melted molecule occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel.

By using DGGE or TGGE, 50% of the sequence variants can be detected in DNA fragments up to 500 bp (Myers et al., 1985). This percentage can be increased to nearly 100% by the attachment of a GC-rich sequence, a so-called GC-clamp, to one side of the DNA fragment (Myers et al., 1985; Sheffield et al., 1989). A sequence of guanines (G) and cytosines (C) is added to the 5′-end of one of the PCR primers, coamplified and thus introduced into the amplified DNA fragments (Sheffield et al., 1989; Sheffield et al., 1992). The GC-rich sequence acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands. The length of the GC-clamp can vary between 30 and 50 nucleotides (see Table 2 in Muyzer et al., 1997). As an alternative to GC-clamps, chemical clamps have been used (Führ, 1996). One of the PCR primers is labelled at its 5′-end with a photoactivatable compound, such as psoralene, which intercalates between the base plates of both DNA strands and will covalently link them together after UV irradiation. The use of a so-called *ChemiClamp* has the advantage that both primers have a similar length, but also has disadvantages. Firstly, DGGE bands with this clamp cannot be reamplified directly, because of the covalent

bond, and secondly, irradiation of the PCR products with UV might damage the amplified DNA causing multiple bands or even a smear in the DGGE analysis (Cariello et al., 1988; Führ, 1996).

DNA bands in DGGE and TGGE profiles can be visualised using ethidium bromide. Recently, SYBR Green I was introduced as an alternative to ethidium bromide (Muyzer et al., 1997). The advantage of SYBR Green I is the lack of background staining, which makes it possible to observe less dominant DNA fragments. A more sensitive detection method is silver staining (Felske et al., 1996). However, silver staining also stains single stranded DNA, and silver stained gels cannot be used for subsequent hybridization analysis (Heuer & Smalla, 1997).

Prior to DGGE or TGGE analysis of DNA fragments it is necessary to determine the melting behaviour of the DNA fragments. Furthermore, to obtain the best separation of different DNA fragments, it is necessary to optimise the gradient and the duration of electrophoresis.

The melting behaviour of DNA fragments, as well as the optimal gradient can be determined experimentally with perpendicular gradient gels. Perpendicular gels have an increasing gradient of denaturants or temperature from left to right, perpendicular to the direction of electrophoresis. The sample is applied across the entire width of the gel and electrophoresed for about 3 hours at 200 Volts. After staining the gel with ethidium bromide and UV-transillumination, the electrophoretic pattern will appear as a sigmoid-shaped curve. DNA molecules at the left side of the gel, where the concentration of denaturants or the temperature is low, will migrate as double-stranded DNA. At the other side of the gel, where the concentration of denaturants or temperature is high, the molecules melt into branched molecules as soon as they enter the gel and therefore halt. At intermediate concentrations of denaturants, the molecules have different degrees of melting, and concomitantly different mobilities. A steep transition in mobility occurs at the denaturant concentration corresponding to the melting temperature of the lowest melting domain of the fragment. Perpendicular gels are used to determine the melting behaviour of the DNA fragments. In addition, from these gels the optimal gradient can be determined for multi-lane analysis in parallel gels.

The optimal time of electrophoresis is determined by parallel gradient electrophoresis. Parallel gradient gels have an increasing gradient of denaturants or temperature from top-to-bottom, parallel to the direction of electrophoresis. They are used for analysing multiple samples on the same gel. Before analysing samples on a parallel gel, the duration of electrophoresis must be determined to obtain maximum resolution between the different DNA fragments. For this purpose, individual samples are loaded onto the parallel gel at constant time intervals, a so-called *time travel* experiment.

DGGE equipment can be obtained from different commercial companies, such as Bio-Rad (Hercules, USA), INGENY (Leiden, The Netherlands), and C.B.S. Scientific Co., Inc. (Del Mar, USA). TGGE equipment originally sold by Diagen GmbH (Germany), can now be purchased from Biometra (Germany)

Applications of DGGE and TGGE in microbial ecology

Studying community complexity

DGGE of PCR-amplified 16S rDNA fragments was first used to profile community complexity of a microbial mat and bacterial biofilms (Muyzer et al., 1993). For this purpose bacterial genomic DNA was extracted from natural samples, and segments of the 16S rRNA genes were amplified in the polymerase chain reaction (PCR; Saiki et al., 1988). This resulted in a mixture of PCR products obtained from the different bacteria present in the sample. The individual PCR products were subsequently separated by DGGE. The result was a pattern of bands, for which the number of bands corresponded to the number of predominant members in the microbial communities. To obtain more detailed information about some of the community members, DGGE profiles were blotted onto nylon membranes and hybridised with a radioactively-labelled oligonucleotide probe specific for sulfate-reducing bacteria (Amann et al., 1992). In a subsequent study, Muyzer and de Waal (1994) were able to identify community members by sequencing of DNA eluted from excised DGGE bands. Figure 1 gives a flow chart of the different steps in this strategy.

Muyzer et al. (1995) used DGGE analysis of PCR-amplified rDNA fragments to provide information on the genetic diversity of microbial communities found around hydrothermal vents. Denaturing gradient gel electrophoresis of DNA fragments obtained after enzymatic amplification of the 16S rDNA using genomic DNA extracted from 2 different hydrothermal vent samples and bacterial primers, showed only 1 band for

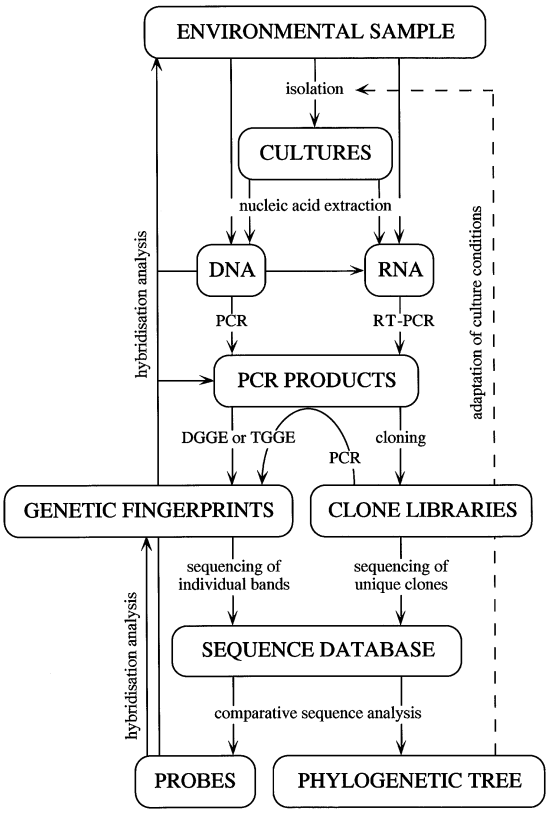

Figure 1. Flow diagram of the different steps in the study of the structure and function of microbial communities. Genetic fingerprinting by DGGE or TGGE of molecular markers is the heart of a strategy to study the presence (DNA) and activity (rRNA or mRNA) of bacterial populations in complex mixtures. Additional information about particular bacterial populations within the community can be obtained by hybridisation analysis with taxon-specific probes. Furthermore, individual bands can be excised from the gels and sequenced to identify the community members. These techniques are also used to monitor the success of isolation of bacteria in pure cultures, and to screen clone libraries for redundancy.

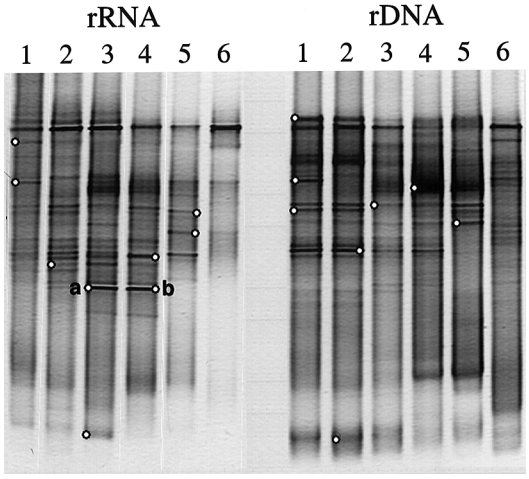

Figure 2. DGGE profiles of ribosomal DNA fragments obtained after enzymatic amplification of either DNA or RNA extracted from Mariager Fjord water column and sediment samples. PCR products obtained from DNA reflect the presence of bacteria, while those obtained from rRNA show the metabolically active populations. Band a and b are in this respect regarded as minor, but highly active bacterial populations. The small circles indicate gel portions that were excised from the gel for sequencing. Adapted from Teske et al., 1995.

one sample and 3 bands for the other sample. These results indicated a low number of dominant bacterial species in this habitat, which was also found by other investigators (Moyer et al., 1994, 1995) for a microbial community from another vent site. Sequencing of excised DGGE bands revealed sequences similar to those of members of the genus *Thiomicrospira*, sulfur-oxidizing bacteria, which had been isolated from similar habitats before. The sequence of another DGGE band gave the highest similarity value with the sequence of the sulfate-reducer *Desulfovibrio salexigens.*

DGGE analysis of 16S rDNA fragments has been used to study the presence and activity of sulfate-reducing bacteria in a stratified water column of Mariager Fjord in Denmark (Teske et al., 1996a). The concept behind this was that PCR products obtained from environmental DNA would demonstrate the presence of different bacterial populations, i.e. biodiversity, and that PCR products obtained after amplification of ribosomal copy DNA (rcDNA) would indicate which of these bacterial populations were active. DGGE comparison of products obtained by PCR and those obtained by RT-PCR with nucleic acids from different depths showed the presence of two bands in the DGGE patterns for rRNA, which were not visible in the pattern for DNA (Figure 2, lanes 3 and 4). From this result the authors concluded that there might be two active bacterial populations present, which were in low numbers.

Similar observations were made by Felske et al. (1996) for soil microbial communities. TGGE profiles of PCR products obtained from rRNA and rDNA extracted from the same soil sample were shown to be different, indicating various active bacterial populations.

DGGE of PCR-amplified 16S rRNA gene fragments has been applied to profile the distribution of microbial populations inhabiting regions with different temperatures in a hot spring cyanobacterial com-

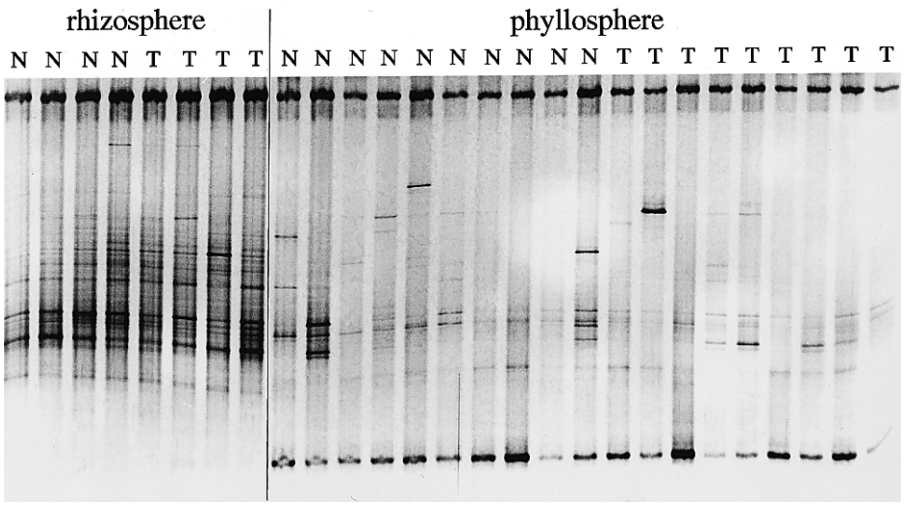

Figure 3. TGGE profiles of 16S rDNA fragments from the rhizophere and phyllophere of several transgenic (T) and non-transgenic (N) potato plants.

munity (Ferris et al., 1996). DGGE profiles of samples taken from sites with the same temperature were similar, indicating the reproducibility of DNA extraction, PCR amplification, DGGE analysis, and the presence of similar bacterial populations. However, different profiles were found for samples from sites with different temperatures indicating different populations. Sequencing of individual bands from the different profiles revealed known but also new bacterial phylotypes.

Recently, Murray et al. (1996) used DGGE of PCR-amplified 16S rDNA fragments to compare the phylogenetic diversity of bacterioplankton assemblages from two estuaries. They found that the two assemblages were different in species composition, probably as a result of the availability of different kinds of organic substrates. Their findings were consistent with results obtained from earlier studies which had focused on the fate of thymidine, and the use of different carbon sources (Hollibaugh, 1994).

DGGE of enzymatically amplified rDNA fragments has also been used to identify bacteria in a biodegraded wall painting (Rölleke et al., 1996). Sequencing of individual DGGE bands demonstrated the presence of close relatives of the genera *Halomonas, Clostridium* and *Frankia*, while enrichment techniques favoured the growth of other bacteria.

Nübel et al. (1997) designed a primer pair for the specific amplification of 16S rDNA fragments from cyanobacteria. Combined with DGGE analysis of these fragments it was possible to determine the cyanobacterial diversity in nonaxenic cultures, lichens and complex microbial assemblages, such as those present in microbial mats, and seawater samples.

Zwart et al. (1997) used DGGE to determine the presence of different members belonging to the *Verrucomicrobiales* in a temperate freshwater lake in the Netherlands. Comparative DGGE analysis of DNA fragments obtained from water samples, and by a so-called *nested* PCR from full length cloned rDNA fragments which were obtained from the same water samples, demonstrated the presence of these bacteria in the lake throughout the year.

TGGE analysis of PCR-amplified 16S rDNA fragments has been applied to compare bacterial populations inhabiting the rhizosphere and phyllosphere of transgenic potato plants expressing T4-lysozyme and non-transgenic potato plants (Figure 3; Heuer & Smalla, 1997). TGGE patterns of rDNA fragments from the rhizosphere were complex, but identical between transgenic and non-transgenic plants. In contrast, profiles obtained from the phyllosphere samples were less complex, but showed much more variation between plants.

Heuer et al. (1997) used DGGE and TGGE to study the genetic diversity of actinomycetes in different soils, and to monitor shifts in their abundances in the potato rhizosphere. In this study, the authors used two amplification strategies, i.e., a direct amplification of the actinomycetes 16S rDNA using group-specific primers, and an indirect amplification approach, whereby actinomycetes specific DNA fragments were generated with a forward group-specific

primer and a reverse bacterial primer, followed by a second, so-called *nested* PCR with two bacterial primers. By using the direct PCR, the genetic diversity of the actinomycetes could be investigated rapidly by gradient gel electrophoresis (DGGE or TGGE). Simultanous gradient gel electrophoresis of products obtained with the nested PCR and those obtained after amplification of the environmental DNA with the bacterial primers directly, made it possible to estimate the abundance of the actinomycetes populations relative to the abundance of the other bacteria present in the soil. DGGE analysis of PCR products generated by this strategy showed that the actinomycetes were only present in low numbers, and that their template DNAs were therefore outcompeted in the amplification process with bacterial primers by template DNAs of bacteria which were present in greater numbers.

PCR-products obtained from total community DNA, from bacterial isolates and from cloned rDNA inserts all from the same environment have been compared by TGGE to investigate microbial communities in zinc-contaminated soils (Konny Smalla, pers. comm.). The TGGE band of the most frequently isolated *Arthrobacter*-like strain was at the same position in the gel as the major band in the TGGE profile derived from total community DNA as well as a band obtained from one of the clones. This result indicated the dominance of this strain in the enviromental sample. However, only sequencing would prove the presence of the same strain.

PCR-DGGE and sequencing of cloned 16S rDNA molecules were used to study ammonia-oxidizing bacteria in Dutch coastal sand dunes (Kowalchuk et al., 1997). Comparative DGGE analysis of PCR products from environmental DNA and from cloned inserts demonstrated the presence of sequences affiliated to the genus *Nitrosomonas* in dunes relatively close to the sea, while sequences affilated to the genus *Nitrosospira* were detected in samples from all sites, although different *Nitrosospira* sequence types were detected in dune soils with different pH.

So far, most studies in the application of genetic fingerprinting techniques have focused on the analysis of 16S rRNA or its encoding gene, but PCR products obtained from functional genes can also be used. Wawer and Muyzer (1995) designed PCR primers to amplify the [NiFe] hydrogenase gene from *Desulfovibrio* species. PCR products obtained from different *Desulfovibrio* strains could easily be separated by DGGE. In addition, PCR products obtained with bacterial DNA extracted from a microbial mat and from different bacterial biofilms demonstrated a greater genetic diversity of *Desulfovibrio* species in the natural microbial mat than in the bacterial biofilms from the man-controlled bioreactors (Wawer and Muyzer, 1995; Wawer, 1996).

Studying community changes

Microbial ecological studies often require the sampling at different time points over a long period. As mentioned in the introduction, cloning techniques are not suited for the analysis of many different samples. By using DGGE or TGGE many samples taken at different time intervals during the study can be simultaneously analysed. This makes the techniques a powerful tool for monitoring community behaviour after environmental changes.

Donner and coworkers (1996) followed the succession of bacterial community structure and activity in a pelagic chemocline which changed from a static over a turbulent into a euthropic situation. Changes in the enzymatic activity of cellulases and esterases coincided with changes in the DGGE profiles of 16S rDNA fragments obtained after PCR amplification of bacterial DNA extracted from the water samples taken at different time points.

Santegoeds et al. (1997) have combined microsensors and PCR-DGGE to monitor successional changes, such as the development of anoxic zones, the start of sulfate reduction and population changes, in a growing bacterial biofilm. Concomitantly with the development of the biofilm an increasing number of bands was observed in the DGGE profiles, indicating an increase in bacterial species.

DGGE analysis was also used to follow the spatial-temporal distribution of sulfate reducing bacterial populations in microbial mat samples taken at 3 different time points of the day, i.e. at 5 a.m., when the mat was anoxic, at 12 a.m., when the top layers of the mat became oxic through photosynthetic activity of the cyanobacteria, and at 5 p.m., when the top layers of the mat were exposed to oxygen during a full day (Teske, 1995). Hybridisation analysis of DGGE patterns of 16S rDNA fragments from different layers of the microbial mats with a oligonucleotide probe specific for the filamentous, sulfate reducing bacterium *Desulfonema* demonstrated the presence of this bacterium in the top layers of the microbial mat at 5 a.m. and 12 a.m., but not at 5 p.m., at which time point *Desulfonema* was not present in the upper 2 mm of the mat.

Recently, Ferris et al. (1997) used PCR-DGGE to study the re-establishment of a microbial mat after removal of the entire cyanobacterial layer. The results showed that previously undetected cyanobacteria colonized the remaining part of the mat, and that other cyanobacteria which were present before the disturbance remained undetected for up to 40 days.

In a subsequent study, DGGE was used to evaluate seasonal distributions of bacterial populations along a thermal gradient in a hot spring microbial mat (Ferris & Ward, 1997). Similar DGGE patterns were found for samples collected at the same site and for sites with the same temperature, regardless of the season. However, different profiles were seen for samples from sites with different temperatures.

Monitoring the enrichment and isolation of bacteria

Although DGGE analysis was originally used to study community complexity, the techniques are also suited to monitor simpler mixtures of microorganisms. DGGE was used to monitor enrichment cultures of aerobic chemoorganotrophic bacteria from hot spring cyanobacterial mats (Santegoeds et al., 1996; Ward et al., 1996).

A polyphasic approach, including phenotypical and phylogenetical analyses, was used to investigate the diversity of geographically distant field populations and cultured strains of the cyanobacterium *Microcoleus chthonoplastes* (Garcia-Pichel et al., 1996). Identical DGGE patterns of 16S rDNA fragments from the field populations and cultures as well as similar morphology, and the presence or absence of certain biochemical markers, demonstrated that *M. chthonoplastes* represented a single, well-defined taxon with a ubiquitous distribution.

Recently, Teske et al. (1996b) used DGGE of rDNA fragments as a tool to analyse the constituents of a coculture. After identification of the two constituents by sequencing of their DGGE bands the authors were able to design more selective conditions and succeeded in the isolation of both strains, *i.e.* a *Desulfovibrio* and an *Arcobacter,* in pure culture.

A similar strategy was applied by Brinkhoff and Muyzer (1997) to determine the success of isolation of the sulfur-oxidizing bacterium *Thiomicrospira* from different habitats. By using a specific PCR the authors first screened several habitats for the presence of *Thiomicrospira* species and than attempted to isolate these species from the environments by enrichment cultures and selective plating. The success of isolation of *Thiomicrospira* in pure culture was monitored by hybridisation analysis of DGGE patterns of 16S rDNA PCR products obtained with bacterial primers which were subsequently hybridised with a *Thiomicrospira*-specific oligonucleotide probe for which the target site was located within the amplified fragment. By using this combined molecular and microbiological approach the authors were able to isolate 7 new *Thiomicrospira* strains from several different habitats.

Buchholz-Cleven et al. (1997) and Wichels (1996) used DGGE analysis of PCR-amplified 16S rDNA fragments as a first rapid means to screen the genetic diversity of different bacterial isolates after which a more detailed analysis, i.e., sequencing of the total 16S rRNA encoding gene of a few unique isolates was performed. By PCR-DGGE analysis Buchholz-Cleven and coworkers (1997) could group 12 bacterial isolates into 3 clusters (A-C) from which 5 strains (one strain from each A and B, and three strains from C) were selected for further physiological and phylogenetical characterization. The three members from group C showed 99.5% or more sequence similarity among each other, while there was only 94% and 84% sequence similarity with the sequences of members of group A and B, respectively. The authors indicated that the DGGE approach is an easy, and time-saving means for screening large numbers of bacterial isolates, and so to discriminate between distantly related bacteria. Also Wichels (1996) could group 18 bacterial isolates into 3 clusters by PCR-DGGE. Phylogenetic analysis of partial 16S rDNA sequences of members of the 3 groups confirmed the clustering by DGGE analysis.

TGGE analysis of 16S rDNA fragments has also been used to follow the efficiency and reproducibility of protocols used for the physical separation of bacteria from soil matrices and the rhizosphere (Heuer & Smalla, 1997). Reproducible patterns of PCR-amplified 16S rDNA fragments were obtained from bacterial cells dislodged from duplicate soil samples.

Recently, Jaspers and Overmann (1997) used PCR-DGGE analysis to check the fractionation of mixtures of different bacteria by isoelectric focussing (IEF).

Detection of microheterogeneity in rRNA encoding genes

One of the observations in analysing PCR products from pure bacterial cultures was the presence of more than one band in the DGGE/TGGE pattern. By using TGGE Nübel et al. (1996) found a pattern of ten different bands obtained after PCR amplification of the

16S rRNA genes from a pure culture of *Paenibacillus polymyxa*. A more detailed analysis of this observation revealed microheterogeneity in the different rRNA operons present in this species. As indicated by the authors this finding has important consequences for the use of 16S rRNA sequence data for biodiversity estimates, phylogenetic reconstruction and the design of taxon-specific oligonucleotide probes.

Comparison of different DNA extraction protocols

DGGE and TGGE of PCR-amplified 16S rRNA fragments have been used to compare the efficacy and reproducibility of different DNA extraction protocols (e.g., Führ, 1996; Heuer & Smalla, 1997; Liesack et al., 1997). The second study showed that TGGE patterns of PCR products from bacterial genomic DNA extracted from soil samples using a harsh lysis method (i.e. lysozyme, SDS and bead beating) gave more bands and more intense bands than profiles of PCR products obtained from the same sample by using a soft lysis method (i.e., lysozyme, and alkaline SDS). Differences in DGGE patterns were also found by Liesack et al. (1997) who compared two different lysis methods, i.e., bead-mill homogenization alone versus a combination of freeze-thawing, lysozyme and SDS treatment, and bead-mill homogenization.

Screening of clone libraries

Several different strategies, such as colony hybridization and restriction fragment length polymorphism (RFLP) of cloned rRNA inserts (Moyer et al., 1996) have been applied to screen clone libraries. TGGE (Felske et al., 1997) and DGGE (Kowalschuk et al., 1997; Schäfer, 1997) have also been used to determine the redundancy in clone libraries, and to estimate the abundance of particular cloned 16S rDNA inserts in the natural environment. For this purpose, the nearly complete 16S rRNA genes are amplified and cloned in suitable vectors. Subsequently, the inserts are reamplified with *nested* primers, and the PCR products are analysed by DGGE or TGGE. By using this strategy the numerous clones can be clustered in groups and the inserts of one representative of every group can be sequenced (see also Figure 1). In addition, simultaneous DGGE analysis of PCR products from cloned inserts and those obtained from environmental DNA might give an indication of the representative members in the natural microbial community. However, it must be stated that bands at the same position in the gel have the same melting behaviour, but not necessarily the same sequence. Only sequencing of bands can proof sequence identity.

Determining PCR and cloning biases

DGGE has been used to determine the error rate of different DNA polymerases during DNA synthesis (Keohavong & Thilly, 1989). In this respect both DGGE as well as TGGE have also been used to study PCR and cloning biases in microbial ecological studies. Konny Smalla (pers. comm.) for example used DGGE and TGGE to detect preferential amplification of rRNA genes from bacterial DNA extracted from different environmental samples. Comparative analysis of PCR products obtained from environmental DNA and those obtained after amplification of 1.5 and 4 kb PCR products with nested DGGE/TGGE primers showed in some samples other bands for the 4 kb PCR products indicating preferential amplification.

Limitations of molecular techniques in microbial ecological studies

It must be emphasised that as with every method, also the molecular techniques are not free from errors and biases. Biases may already be introduced by sample handling. Rochelle et al. (1994) found that different sample handling procedures, such as aerobic or anaerobic storage or direct freezing of the samples, greatly affected the species composition found by 16S rRNA sequence analysis. For instance, a sediment sample taken under anaerobic conditons, but stored aerobically for 24 hours before freezing was dominated by sequences belonging to beta- and gamma-*Proteobacteria*, while a duplicate sample which was kept anaerobically and frozen within 2 hours after sampling showed a greater diversity with sequences from alpha-, gamma-, and delta-*Proteobacteria* and Gram-positive bacteria.

The next step in the molecular characterization of microbial communities is the extraction of nucleic acids from bacterial cells present in the samples; also this step is not free from biases. Problems are encountered with the reliable and reproducible lysis of all bacterial cells as well as with the extraction of intact nucleic acid, and the removal of substances, such as humic acids and bacterial exopolysaccharides, which may inhibit DNA digestion with restriction enzymes and PCR amplification (e.g., Wheeler & Stahl, 1996).

PCR itself is an important source of errors and biases in molecular studies of environmental samples. Amplification efficiency of genes using whole bacterial cells as template instead of extracted DNA can be affected by the physiological state of the cells (Silva & Batt, 1995). Differential or preferential amplification of rRNA genes by PCR has been described by Reysenbach et al. (1992). Recently, Suzuki and Giovannoni (1996) found that preferential amplification might be caused by reannealing of the template DNA thereby inhibiting primer binding. Addition of acetamide to the PCR reaction was used to facilitate template denaturation and to prevent preferential amplification (Reysenbach et al., 1992). Cosolvents, such as glycerol and dimethylsulfoxide (DMSO) have also been used for this purpose (Smith et al., 1990; Shen & Hohn, 1992; Varadaraj and Skinner, 1994). Farrelly et al. (1995) demonstrated the effect of genome size and the copy number of 16S rRNA genes on the quantities of PCR products. Another problem in the use of PCR to amplify mixed target DNAs is the formation of so-called *chimeric molecules* (Liesack et al., 1991; Kopczynski et al., 1994). Computer algorithms, such as the CHECK_CHIMERA option in the Ribosomal Database Project (RDP; Maidak et al., 1996), the Aligned Similarity Method (Robison-Cox et al., 1995), and the Chimeric Alignment Method (Komatsoulis & Waterman, 1997) have been developed to detect chimeric sequences. Also the cloning approach is not free from biases. Rainey et al. (1994) described different cloning efficiencies for different cloning vectors and with different primer pairs.

In addition, the formation of heteroduplex molecules during the amplification process might contribute to difficulties in the interpretation of community complexity from DGGE or TGGE patterns (Myers et al., 1989; Ferris et al., 1997b). A heteroduplex DNA molecule has strands from two different PCR products, which can be formed by re-annealing of denatured PCR products. Because of mismatches between the double-strands in the heteroduplex molecules the melting temperature is lower than for homoduplex molecules. DGGE analysis of two PCR products after induction of heteroduplex formation will result in four bands two heteroduplex and homoduplex molecules. Although heteroduplex analysis is frequently used in DGGE and TGGE analysis to increase the resolution in the detection of two DNA fragments with nearly identical sequences, it can be problem for the analysis of mixed bacterial populations, because it overestimates the real number of community constituents. However, Murray et al. (1996) concluded that the formation of heteroduplex molecules in PCR-DGGE analysis of mixed microbial populations is not a significant problem. When heteroduplex molecules are expected then the relative formation of heteroduplex DNA in the PCR can be reduced by using a higher ionic strength, higher primer concentrations, and a lower annealing temperature as well as by decreasing the number of amplification cycles (Jensen & Straus, 1993). Furthermore, PCR products can be treated with a single-strand endonuclease, such as mung bean nuclease, prior to electrophoretic analysis.

Limitations of DGGE and TGGE

One of the limitations is the separation of only relatively small fragments, up to 500 basepairs (Myers et al., 1985). This limits the amount of sequence information for phylogenetic inferences as well as for probe design.

It has been demonstrated that it is not always possible to separate DNA fragments which have a certain amount of sequence variation. Vallaeys et al. (1997) found that 16S rDNA fragments obtained from different methane-oxidizing bacteria could not be resolved by DGGE although they had substantial sequence variation. A similar result was described by Buchholz-Cleven et al. (1997) who demonstrated that it was not possible to separate rDNA fragments differing in two to three nucleotides under the electrophoretic conditions they used. In contrast to these failures of separation, Nübel et al. (1996) could separate DNA fragments from different *rrN* operons, some of which were only differing in one basepair. Furthermore, Kowalchuk et al. (1997) nicely demonstrated that double bands in the DGGE patterns were a result of the presence a so-called *wobble base* (either a C or a T) in the reverse primer. When a mixture of the reverse primers was used, two bands were visible in the DGGE pattern, while when the two primers were used in separate PCR reactions only one band per reaction was found.

So, the use of different regions of the 16S rRNA and different DGGE or TGGE conditions might result in different resolutions of separation. As the melting behaviour and the mobility in denaturing gradient gels of rDNA fragments for which sequences are known can be predicted by using computer algorithms (Lerman & Silverstein, 1987), it might be helpful to perform a comparative analysis of the different 16S rRNA sequences present in the databases to find those regions for which an optimal separation in DGGE or TGGE can be expected. Similar computer simulations

have been performed to identify the optimal combination of different tetrameric restriction enzymes for RFLP screening of SSU rDNA clone libraries (Moyer et al., 1996), or to analyse the hybridisation potential between primers and probes and SSU rRNA sequences (Brunk et al., 1996).

Related to the problem of resolution might be the maximum number of different DNA fragments which can be separated by DGGE or TGGE. For instance, by using DNA-DNA reannealing experiments Torsvik et al. (1990a,b) found that there might be as many as 10^4 different genomes present in soil samples. It will be obvious to the reader that DGGE or TGGE cannot separate all of the 16S rDNA fragments obtained from such a variety of microorganisms. In general, these electrophoretic techniques will only display the rDNA fragments obtained from the predominant species present in the community. Several different studies revealed that bacterial populations that make up 1% or more of the total community can be detected by PCR-DGGE (Muyzer et al., 1993; Murray et al., 1996). A similar value has been found by Lee et al. (1996) using PCR-SSCP to characterize bacterial community structures.

Furthermore, co-migration of DNA fragments can be a problem for retrieving clean sequences from individual bands. Another problem in the study of community diversity on the basis of 16S rRNA genes, using DGGE, TGGE or cloning strategies is the presence in some bacteria of multiple *rrN* operons with sequence microheterogeneity DGGE and TGGE can visualise this sequence heterogeneity (Nübel et al., 1996) which might lead to an overestimation of the number of bacteria within natural communities. The same is true for the double bands in the DGGE or TGGE patterns which were produced by the use of degenerate primers in the PCR reactions (Kowalchuk et al., 1997).

Means to obtain useful information from very complex communities

Nevertheless, substantial information about the species composition can be obtained from very complex microbial communities by DGGE or TGGE analysis. Bacterial cells can be dislodged from soil (e.g. Priemé et al., 1996) and fractionated prior to the amplification process (e.g. Jaspers & Overmann, 1997). Furthermore, community DNA can be fractionated according to its %G+C using bisbenzimide (Hoechst 33258) and centrifugation in CsCl gradients (Holben et al., 1993; Holben & Harris, 1995). Øvreas et al. (1995) used this approach to fractionate complex mixtures of DNA extracted from soil samples prior to PCR amplification and DGGE analysis.

Recently, Wawer et al. (1995) used agarose gels containing bisbenzimide to which long chains of polyethylene glycol (PEG) were covalently coupled to separate PCR products. This electrophoretic approach has also been used to fractionate bacterial genomic DNA (Gerard Muyzer, unpublished results), and might so be another means to reduce the complexity of template DNAs in the analysis of bacterial communities by PCR.

Another useful approach to obtain information of complex DGGE or TGGE profiles is hybridization analysis of the patterns with group-specific oligonucleotide probes (Muyzer et al., 1993; Teske et al., 1996a; Brinkhoff & Muyzer, 1997). Heuer et al. (1995) used digoxigenin-labelled polynucleotide probes under stringent hybridisation conditions to detect particular microorganisms, e.g. *Agrobacterium tumefaciens*, in DGGE or TGGE patterns. These probes were produced by enzymatic amplification of the hypervariable V6 region (Neefs et al., 1990) (from position 971 to position 1057 in *E. coli*) of the 16S rRNA of particular bacterial strains using universal primers flanking this region. The advantage of this strategy is that no sequence information is required to produce the probes.

Complex banding patterns can also be reduced by the analysis of PCR products obtained with group-specific primers, such as those for cyanobacteria (Nübel et al., 1997), beta-ammonium oxidizers (Kowalchuk et al., 1997), agrobacteria/rhizobia (Konny Smalla, pers. comm.) and actinomycetes (Heuer et al., 1997). A second strategy is the amplification of DNA fragments obtained with group-specific primers, such as those for the beta-ammonium oxidizers (McCaig et al., 1994; Voytek & Ward, 1995) followed by a reamplification of these PCR products with nested DGGE primers. This approach makes it possible to determine the ecological importance of particular bacteria within microbial communities. Another advantage of the use of group-specific primers is the increased sensitivity of detection (Brinkhoff & Muyzer, 1997).

Furthermore, simpler DGGE patterns can be obtained using PCR primers for functional genes, which are only present in particular bacterial populations (see under *Studying community complexity*; Wawer and Muyzer, 1995).

Perspectives of DGGE and TGGE in microbial ecology

The use of DGGE and TGGE in microbial ecology is still in its infancy, but their future perspectives are promising. Combined with PCR amplification of marker genes or their transcripts (rRNA and mRNA) DGGE and TGGE can give a direct display of the predominant constituents in microbial assemblages. In this respect they are excellently suited to investigate the temporal and spatial distribution of bacterial populations. This aspect is certainly the most important reason for the popularity of these techniques in microbial ecological studies. Other aspects are their easiness, reproducibility, reliability, and speed. Furthermore, the techniques are well sorted out; a lot of experience, especially with DGGE, has been gathered in detecting sequence variation in genes related to diseases. In addition, the theoretical background of how these techniques function, e.g. the principle on the thermodynamics of melting behaviour of double-stranded DNA in solution and gels is well understood.

The use of these techniques might be especially interesting to answer questions on the fate of microbial communities or certain 'indicator microorganisms' after environmental perturbations, such as the addition of toxic compounds or the release of genetically modified microorganisms.

An exciting new direction within the field of molecular microbial ecology is the use of functional genes as molecular markers to perceive metabolic activity (Jeffrey et al., 1996; Paul, 1996). Recently, we extended the application of DGGE to determine the differential expression of the [NiFe] hydrogenase gene by different *Desulfovibrio* populations in experimental bioreactors (Wawer et al., 1997). By comparative analysis of DGGE patterns of PCR products obtained from genomic DNA and mRNA extracted from bioreactor samples incubated with hydrogen, we could demonstrate the presence of at least 2 different *Desulfovibrio* populations, but only the preferential expression of the [NiFe] hydrogenase gene by one *Desulfovibrio* population. Our preliminary conclusion of this result is that this particular population might be better adapted to the growth on hydrogen than the other *Desulfovibrio* population. This might point to a niche differentiation of closely related bacterial populations performing similar functions in the community under different environmental conditions. As more PCR assays for functional genes have been developed, such as for the ammonia monooxygenase (*AMO*) gene (Sinigalliano et al., 1995) and for the sulfite reductase gene (Karkhoff et al., 1995), we will soon be able to use DGGE and other fingerprinting techniques to relate structure to function, and to obtain a more detailed view of the organisation of microbial communities and their performance in the biogeochemical cycling of elements.

A positive spin-off of the introduction of DGGE in microbial ecology is the revived interest in genetic fingerprinting of microbial communities. This has resulted in the development of other genetic fingerprinting techniques to profile the complexity of mixed microbial populations. Lee and coworkers (1996) described the use of single-strand-conformation polymorphism (SSCP; Orita et al., 1989) of PCR-amplified 16S rRNA genes for studying the diversity of natural bacterial communities. Furthermore, SSCP of the PCR-amplified 16S-23S rRNA spacer region has been used to analyse mixtures of bacteria (Scheinert et al., 1996). Amplified ribosomal DNA restriction analysis (ARDRA) has also been used to study the genetic diversity of mixed microbial populations (e.g., Massol-Deya et al., 1995; Martínez-Murcia et al., 1995), or to monitor community shifts after environmental perturbation, such as copper contamination (Smith et al., 1997). Xia et al. (1995) have used randomly amplified polymorphic DNA (RAPD; Williams et al., 1990) fingerprinting to follow the response of different soil microbial communities to the application of 2,4-dichlorophenoxyacetic acid (2,4-D). Wawer et al. (1995) used a simple and rapid electrophoresis method to detect sequence variation in [NiFe] hydrogenase gene fragments obtained after enzymatic amplification of bacterial DNA from pure cultures and environmental samples (Wawer, 1996). Important in this respect is that sequencing of individual bands separated by ARDRA, DGGE, TGGE or SSCP can reveal phylogenetic information, while sequencing of bands obtained by RAPD analysis might not.

The use of fluorescent dye labelled primers, which are nowadays routinely used in DNA sequencing procedures will also be used in the genetic fingerprinting of mixtures of microbial populations (Scheinert et al., 1996). The application of different fluorescent labels makes the addition of 'intra-lane' standards possible, which facilitates gel-to-gel comparison, and image analysis of the gels. Furthermore, the use of fluorescent PCR products combined with capillary electrophoresis (CE) will reduce the analysis time, improve the resolution and sensitivity by 'on line' detection, and will hopefully result in a rapid and straightforward collec-

tion of separated PCR products, which can than be further characterized by DNA sequencing.

However, regarding the biases and limitations of all methods only an integrated approach combining molecular techniques, new isolation strategies and physiological characterization of the obtained isolates will reveal the role of microbial diversity in ecosystem functioning.

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