

DGGE/TGGE a method for identifying genes from natural ecosystems

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Five years after the introduction of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in environmental microbiology these techniques are now routinely used in many microbiological laboratories worldwide as molecular tools to compare the diversity of microbial communities and to monitor population dynamics. Recent advances in these techniques have demonstrated their importance in microbial ecology.

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Abbreviations

DGGE denaturing gradient gel electrophoresis
rRNA ribosomal RNA
TGGE temperature gradient gel electrophoresis

Introduction

Our knowledge of microbial diversity and its role in nature is poor, mainly because traditional microbiological techniques, such as microscopy and cultivation, have only a limited use for classification and identification of microorganisms. Classification on morphological traits is difficult, because microorganisms are small and look simple, lacking conspicuous external features for a reliable and robust grouping. Furthermore, classification of microorganisms on physiological and biochemical features is nearly impossible, because most, ~99%, of all microorganisms in nature can not be isolated in pure cultures mainly due to our ignorance of the culture conditions under which these microorganisms thrive in their natural environment [1]. Therefore, for a better understanding of microbial diversity and its role in ecosystem maintenance, other techniques, which complement the microbiological approach, are necessary.

The development of molecular biological techniques has allowed us to study microbial diversity at a different level, the genetic level. Microbes are grouped according to similarities in their genes, which also reflect their evolutionary relationship [2]. The most powerful approach to explore microbial diversity in natural samples is cloning and sequencing of 16S ribosomal RNA (rRNA) encoding genes [3]. By using this approach we now know that microbial diversity is much greater than previously anticipated, and that culture techniques are insufficient for exploring this enormous reservoir of hidden diversity. Although important, exploration of microbial diversity is just one aspect in microbial ecology; the study of successional population changes in microbial communities is another, and for this purpose the

cloning approach is not well suited, simply because it is too laborious, time consuming, and expensive. Hybridization techniques using specific oligonucleotide probes are more appropriate for studying population dynamics, but probes rely on sequence data and are either too specific, targeting only one particular population, or too general, overlooking closely related but ecologically different populations. So to determine the diversity of different microorganisms in natural ecosystems, and to monitor microbial community behavior over time, other approaches are needed. One such approach is genetic fingerprinting of complex microbial communities.

Genetic fingerprinting techniques provide a pattern or profile of the community diversity on the basis of the physical separation of unique nucleic acid species [4]. Several fingerprinting techniques can be used for comparison of microbial communities from different environments or to follow the behavior of one community over time [5]. The general strategy for genetic fingerprinting of microbial communities consists of first, the extraction of nucleic acids (DNA and RNA), second, the amplification of genes encoding the 16S rRNA, and, third, the analysis of PCR products by a genetic fingerprinting technique, such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) (Figure 1).

Separation of DNA fragments in DGGE and TGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient, which is created by two water baths attached to a cooling plate under the gel. Molecules with different sequences may have a different melting behavior, and will, therefore, stop migrating at different positions in the gel (for a more detailed description see Muyzer and Smalla [6]).

Since the first publication by Muyzer *et al.* [7] in 1993 an increasing number of studies in microbial ecology have used DGGE/TGGE. In this review I describe the recent developments of these techniques and discuss why they are so important for ecological studies.

Analyzing community diversity

DGGE/TGGE has been used to determine the genetic diversity of total bacterial communities or particular populations without further characterization of the individual inhabitants. Curtis and Craine [8] used DGGE to compare the diversity of total microbial communities present in different activated sludge plants. Heuer *et al.* [9] used a group-specific PCR and both DGGE and TGGE to analyze actinomycete communities in different soils. By using a special amplification strategy (i.e. firstly amplification

Figure 1

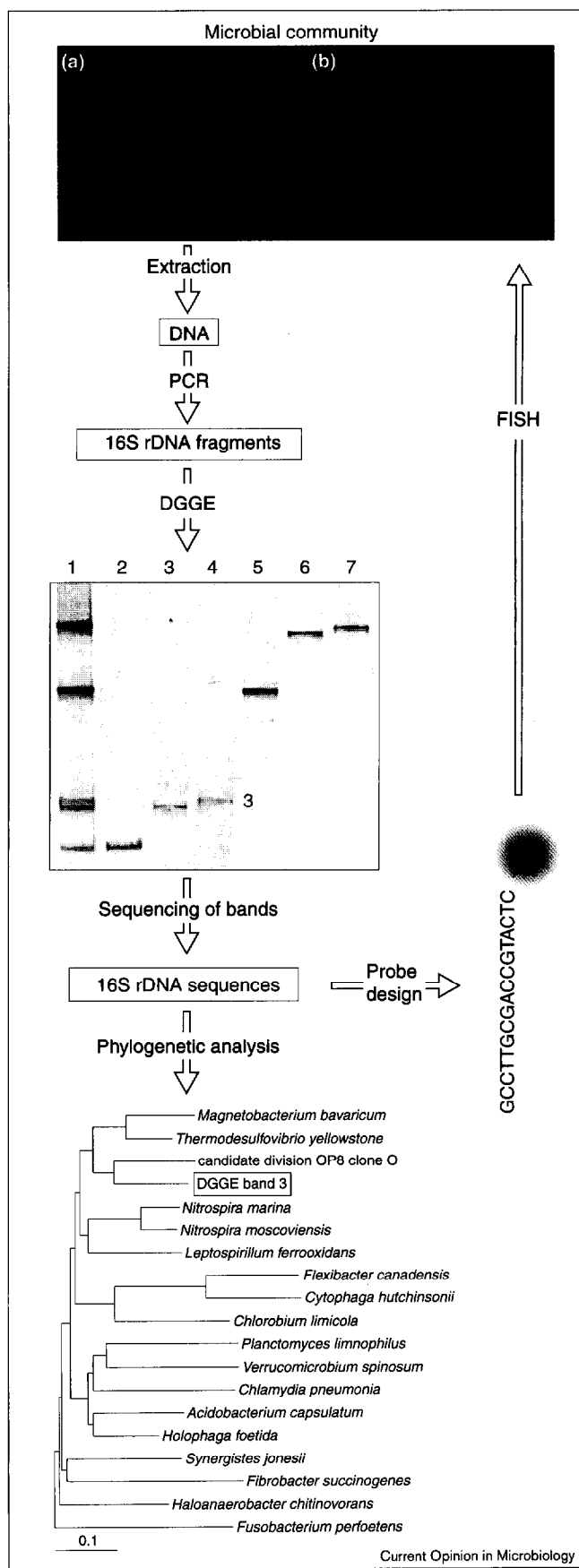


Figure 1

Flow diagram showing the different steps in the analysis of microbial community structure by PCR-DGGE. DNA is extracted from an environmental sample and used as template in the polymerase chain reaction (PCR) to amplify the 16S rRNA encoding genes of bacteria. Thereafter, the PCR products are separated by denaturing gradient gel electrophoresis (DGGE) (lane 1). The phylogenetic affiliation of the predominant community members, as represented in the DGGE band pattern, can be inferred by comparative analysis of sequences from excised and re-amplified DNA fragments (lanes 2–7) and sequences stored in nucleotide databases. Moreover, the sequence information can be used to design an oligonucleotide probe for the detection of a specific bacterial population by fluorescence *in situ* hybridisation (FISH). (a) A photograph of a microbial community stained with the DNA-binding fluorochrome DAPI resulting in blue light emission from all bacteria after UV illumination. (b) The same microbial community after incubation with a red fluorochrome-labelled oligonucleotide probe specific for the bacterial population represented by the sequence obtained from band 3 in the denaturing gradient gel.

with actinomycete-specific primers followed by a 'nested' PCR with bacterial primers) the authors could estimate the representation of actinomycetes within the bacterial community. Both DGGE and TGGE analysis of the amplified PCR products gave similar results.

More information about the identity of community members can be obtained by hybridization analysis of DGGE/TGGE patterns with taxon-specific oligonucleotide probes or with polynucleotide probes to hypervariable regions of the 16S rRNA [10,11]. The latter probes are dioxigenin-labeled by enzymatic amplification of the rDNA of a particular bacterial strain [10] or the rDNA of excised TGGE bands [11] using universal primers. These probes are then used, under very stringent hybridization conditions to obtain enough specificity, in hybridization analysis of DGGE/TGGE patterns. The advantage of this strategy is that no specific sequence information is needed to create the probe. PCR-DGGE followed by hybridization analysis using genus- and cluster-specific oligonucleotide probes was used to investigate the influence of soil pH on the composition of ammonia oxidizers [12].

Other studies use PCR-DGGE as well as cloning and sequencing to obtain more information about the identity of the microbial community members, such as those present in a bacterial biofilm on the shells of a bivalve mollusc [13]. Kowalchuk *et al.* [14] used this approach to study the distribution of ammonia-oxidizing bacteria in coastal sand dunes, and found that members of the genus *Nitrosomonas* were present in dunes relatively close to the sea, whereas members of the genus *Nitrospira* were detected in samples from all sites. Cloning and TGGE analysis of 16S rDNA fragments was also used to determine the diversity and phylogenetic affiliation of predominant bacteria in the human gastrointestinal tract [15]. Comparison of TGGE patterns of PCR products obtained from rDNA and rRNA of 16 individuals showed stable and host-specific microbial communities in which most bacteria were metabolically active and affiliated to known members of different

Clostridium clusters. The authors optimized the nucleic acid extraction, template concentration, and the number of amplification cycles to minimize the potential biases of the strategy. The same approach was also used to identify a highly physiologically active uncultured microorganism affiliated to the Actinobacteria in a Dutch grassland soil [16].

A more comprehensive approach, including molecular and microbiological methods and chemical analysis, was used to study the effect of marine fish farming on the species composition and activity of ammonia-oxidizing bacteria in the underlying sediments [17]. Hybridization analysis of DGGE patterns with oligonucleotide probes for different ammonia oxidizers and sequencing of cloned 16S rDNA inserts showed the presence of a novel marine *Nitrosomonas* population. This population was most abundant in sediments directly beneath the fish cages where the nitrogen-rich organic pollution from excess food and fecal material was maximal.

Øvreås *et al.* [18*] were the first to perform DGGE of archaeal rDNA. They used domain-specific sets of primers to study the distribution of Bacteria and Archaea in a meromictic lake in Norway. Opposing results were found for members of both domains; bacterial diversity decreased with depth, whereas archaeal diversity increased. Hybridization analysis of the DGGE patterns with group-specific oligonucleotide probes showed the presence of sulfate-reducing bacteria and methanogens.

So far, only a few studies have used PCR-DGGE/TGGE to study the diversity of eukaryotic microorganisms. PCR-DGGE analysis of genes coding for 18S rRNA was used to study fungal infections of the *Ammophila arenaria*, a sand-stabilizing grass species in coastal dune areas [19*]. Comparison of sequences of excised DGGE bands with sequences of fungal isolates revealed as yet unknown diversity. Van Hannen *et al.* [20] used DGGE of 18S rDNA fragments to compare eukaryotic diversity of different water bodies in a Dutch freshwater lagoon system. Specific community profiles correlated well with certain environmental conditions (e.g. dissolved organic matter and algal pigment concentrations). To match the sequences of different eukaryotic microorganisms primers with several, up to four, degeneracies were needed, which might lead to a biased picture of diversity due to preferential amplification of certain community members [21**]. In combination with DGGE/TGGE, the use of degenerate primers might result in multiple bands and thus to an overestimation of diversity [14]. The use of degenerate primers in microbial ecological studies and especially in those using DGGE/TGGE is, therefore, strongly discouraged, and, if it is not possible to avoid, the results need to be carefully interpreted.

Most DGGE/TGGE studies focussed on the number of bands as an estimate of community diversity, while little attention was given to quantification of band intensity. Recently, Nübel *et al.* [22**] used DGGE analysis of 16S

rDNA fragments to quantify the diversity of oxygenic phototrophs in eight hypersaline microbial mats. The number of bands in the gel was a measure for 'richness', whereas the proportional abundance ('evenness') of the different sequence-defined populations was calculated from the intensity of the bands. Different diversity indices were found for different communities. These results were supported by similar diversity indices for the different mats obtained by two other cultivation-independent techniques (i.e. microscopical observation of morphotypes and HPLC analysis of carotenoids). The study showed for the first time the quantification of microbial diversity in natural habitats.

Absolute quantification of TGGE band intensities was performed using competitive reverse transcription (RT)-PCR of community 16S rRNA and known concentrations of standard template [23].

Study of community dynamics

One of the strongest points of the application of DGGE/TGGE in microbial ecology is the simultaneous analysis of multiple samples, which allows monitoring of the complex dynamics that microbial communities may undergo by diel and seasonal fluctuations or after environmental perturbations. Ward and coworkers [24**] were among the first who used DGGE of 16S rDNA fragments to study population changes in microbial communities. They examined the seasonal distribution of community members in a hot spring microbial mat community [25], and the recolonization of bacterial populations after removal of the top 3 mm of the community [26]. PCR-DGGE of 16S rDNA fragments has also been used to examine seasonal changes in bacterioplankton in coastal waters of Antarctica [27], and to study the composition and dynamics of bacterial populations in the rhizosphere of the plant chrysanthemum [28]. Recently, van Hannen *et al.* [29] used PCR-DGGE to monitor changes in the community composition of bacterial and eukaryal microorganisms after viral lysis.

Microbiological, molecular and biogeochemical approaches were combined to investigate the diurnal behavior of sulfate-reducing bacteria in the top layer of a microbial mat community [30]. Hybridization analysis of 16S rDNA DGGE patterns showed a filamentous migrating sulfate reducer affiliated to *Desulfonema*, which is assumed to be obligatory anaerobic, within and below the oxic surface layer.

Santegoeds *et al.* [31*] combined molecular techniques and microsensors to study the presence and activity of sulfate-reducing bacteria in biofilms from an activated sludge basin of a wastewater treatment plant. Microsensor measurements indicated that anaerobic zones developed within one week of biofilm formation, but that sulfate reduction did not occur until after six weeks. Hybridization analysis of 16S rDNA DGGE profiles showed that *Desulfobulbus* and *Desulfocibrio* populations were the main sulfate-reducing bacteria, and that different populations came up at the onset of the sulfate reduction.

An excellent study by Watanabe and coworkers [32**] used a combination of molecular biological and microbiological methods to detect and characterize the dominant phenol-degrading bacteria in activated sludge. TGGE analysis of PCR products of 16S rDNA and of the gene encoding phenol hydroxylase (LmPH) showed a few dominant bacterial populations after a 20 day incubation with phenol. Comparison of sequences of different bacterial isolates and excised TGGE bands revealed two dominant bacterial strains responsible for the phenol degradation.

An integrated approach including metabolic and genetic fingerprinting as well as conventional ecotoxicological testing procedures was used to follow the impact of pesticide treatment on the structure and function of bacterial soil communities [33*]. The application of the herbicide Herbogil showed the greatest impact on community composition and metabolic activity. BIOLOG (a substrate utilization assay) and TGGE analysis showed differences in substrate utilization patterns, and in the number and intensities of bands, respectively. The ecotoxicological testing procedures showed a reduction of substrate-induced respiration and dehydrogenase activity, and an increase in nitrogen mineralization. Sequencing of excised TGGE bands showed the phylogenetic affiliation of community members that were most responsive to herbicide treatment.

'Hunting for microbes' with molecular tools

Although we recognize the short-comings of traditional culture techniques in isolating most of the microorganisms in nature, we certainly need these isolates for a better understanding of their physiology and role in the cycling of chemical elements. Molecular tools can be helpful in monitoring enrichment cultures and in facilitating the successful isolation of ecologically relevant bacterial populations. PCR-DGGE/TGGE is well suited for this purpose, because it allows the rapid and simultaneous analysis of mixed cultures grown under different conditions together with the environmental samples from which the inocula for these cultures were taken [34–36]. Recently, Smalla *et al.* [37*] used DGGE and TGGE analysis to determine the bacterial populations contributing to BIOLOG substrate utilization patterns. Two microbial communities were tested, one from the rhizosphere of potatoes, and the other from an activated sludge reactor fed with glucose and peptone. The DGGE/TGGE results showed the enrichment of specific bacterial populations in the inocula of both communities. Enriched strains from the rhizosphere sample could not be found in the banding pattern of the original inoculum, whereas the enriched strains from the activated sludge sample could be found in its inoculum. Hybridization analysis of the DGGE/TGGE patterns indicated the enrichment of strains affiliated to the γ -subclass of the *Proteobacteria* that were dominant community members in the activated sludge reactor, but only minor constituents of the rhizosphere microbial community. PCR-DGGE can also be used as a tool to follow the successful isolation of bacterial strains in pure cultures

[38,39]. Such examples demonstrate how the combined use of PCR-DGGE/TGGE together with existing and new isolation strategies (e.g. the serial dilution technique [40]) can provide a new impulse in the isolation of ecologically relevant microorganisms.

Studying niche differentiation

An exciting new direction in molecular microbial ecology is the analysis of enzyme encoding genes. Generally these genes have more sequence variation than the relatively conserved 16S rRNA encoding genes, and might, therefore, be better molecular markers to discriminate between closely related but ecologically different populations [41**]. Moreover, the use of functional genes makes it possible to study the specific activities of bacterial populations.

Wawer *et al.* [42**] were the first to use DGGE analysis of [NiFe] hydrogenase gene fragments of *Desulfovibrio* species, an important group of sulfate-reducing bacteria. By comparative analysis of PCR products obtained from genomic DNA and mRNA extracted from bioreactor samples incubated with hydrogen, the substrate for the [NiFe] hydrogenase enzyme, the authors could demonstrate the presence of different *Desulfovibrio* populations, but only the preferential expression of the [NiFe] hydrogenase gene by one population. It was concluded that this population might be better adapted to growth on hydrogen than other *Desulfovibrio* populations suggesting a niche differentiation of closely related bacterial populations performing different functions in the community. As more sequences for other functional genes become available in the future, we will soon be able to use PCR-DGGE/TGGE to relate community structure and function.

Conclusions

Today DGGE/TGGE is a well-established molecular tool in environmental microbiology that allows the study of complexity and behavior of microbial communities. The technique is reliable, reproducible, rapid and inexpensive. DGGE/TGGE allows the simultaneous analysis of multiple samples making it possible to follow community changes over time. An additional strong feature of these techniques is the possibility of identifying community members by sequencing of excised bands or by hybridization analysis with specific probes, which is not possible with other fingerprinting techniques, such as terminal restriction fragment length polymorphism (T-RFLP) [43*]. Moreover, probes can be designed after sequencing of excised DGGE/TGGE bands and used in hybridization analysis [44] (see also Figure 1), or even be generated without prior sequence knowledge by 'nested' amplification of excised bands [11]. DGGE and TGGE, however, also have their limitations. Apart from the general potential biases, which most of the molecular techniques in microbial ecology face (e.g. those produced by sample handling, uneven cell lysis or PCR [21**]), DGGE/TGGE also have some specific limitations [6**]; for instance, the detection of heteroduplex molecules [25] and molecules produced by

different rRNA operons of the same organism [45]. Furthermore, the separation of relatively small DNA fragments, the co-migration of DNA fragments with different sequences [46], and the limited sensitivity of detection of rare community members can cause problems. Some of these limitations, such as the presence of heteroduplex molecules are not commonly found [22**], whereas other limitations such as the limited sensitivity can be improved by hybridization analysis [47] or by the application of a group-specific PCR [9].

Future innovations might include the use of double-gradient DGGE/TGGE (i.e. the combined application of a gradient of acrylamide and a gradient of denaturants or temperature to obtain a better resolution), and the use of terminally labeled fluorescent PCR products and the addition of fluorescent intra-lane standards for detection of rare community members and an accurate sample-to-sample comparison. Also, the routine use of functional genes as molecular markers could be used to discriminate between closely related but ecologically different populations.

There is a clear trend to combine PCR-DGGE/TGGE and other molecular techniques as well as microbiological and geochemical methods [17,27,30,33*,47,48*]. This is important to reduce potential biases and limitations of the different techniques, and hence to obtain a more realistic picture of microbial community structure and function.

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