A1.

Appendix 1

Denaturing Gradient Gel Electrophoresis as a fingerprinting tool for analyzing microbial communities in contaminated environments

Denaturing Gradient Gel Electrophoresis as a fingerprinting tool for analyzing microbial communities in contaminated environments

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(2004) *In* J.F.T. Spencer and A.L. Ragout de Spencer (eds.), Environmental Microbiology: Methods and Protocols. Methods in Biotechnology Series, Vol. 16, pp 407-417. Humana Press, Totowa, N.J., U.S.A.

A1.1. Introduction

A1.1.1. Advantages of DGGE/TGGE

The characterization of microbial communities has been very limited due to the lack of appropriate methods. The traditional selection of pure cultures and further study of their physiological and biochemical properties is not well adapted to the study of microbial communities. Indeed, it is assumed that nearly 99% of the microorganisms present in nature cannot be isolated and cultivated because of our ignorance of their physiological needs (1). Therefore, novel molecular techniques have been developed to compensate for the lack of cultivation methods.

One of the available molecular techniques is the construction of 16S ribosomal DNA (rDNA) libraries. Briefly, the DNA is extracted from the ecosystem under study and its 16S rDNA is amplified by PCR and ligated into plasmid vectors. Further sequencing of 16S rDNA fragments of the library and comparison of the sequences with other 16S rDNA sequences can provide information on the identity of the bacteria of the community. However, this technique is labor-intensive, time-consuming, and quite expensive. Furthermore, the evolution of the microbial composition over time cannot be easily monitored using this technique. For these reasons, a simple, rapid, and relatively cheap molecular method was needed. To meet these criteria, Muyzer and his co-workers (2) developed a method combining the amplification power of PCR on 16S rDNA of complex microbial populations together with the possibility of denaturing gradient gel electrophoresis (DGGE) to separate DNA fragments of the same size but differing according to their base-pair sequences.

A1.1.2. Principles of DGGE/TGGE

In DGGE systems, double-stranded DNA fragments are run through a polyacrylamide gel containing a linear gradient of denaturing agents (a mixture of urea and formamide) at a constant temperature (generally 60°C). During the migration in the gel, the fragment remains double stranded until it reaches a concentration of denaturing agents equivalent to a melting temperature that causes the lower melting part (also called “domain”) of the molecule to melt.

At this stage, the fragment changes from a helical structure to a partially melted molecule, which practically halts its migration through the gel.

In TGGE systems, the gradient of chemical denaturing agents is replaced by a gradient of temperature. The same principle governs the migration of the fragments. This way, two fragments in which an AT is replaced by a GC can be theoretically separated and visualized. However, it is basically estimated that only 50% of all the single-base-pair changes in a fragment of 50 to several hundred bp can be detected by DGGE (3). To overcome this limitation, the fragments to be separated can be previously amplified by PCR using a pair of "modified" primers. The modification consists in the adjunction of a GC-rich sequence (generally 40 bp) to the 5' end of one of the primers (4). This prevents the complete dissociation of the fragments during migration in the gel and therefore the loss of sequence-dependent gel migration upon complete strand separation (4). Bacterial and fungal communities can be analyzed by DGGE after PCR amplification of 16S rDNA and 18S rDNA genes, respectively. Primers are designed to specifically hybridize to conserved regions of the bacterial 16S rDNA or fungal 18S rDNA genes. By using group-specific primers, it is also possible to target well-defined communities, like the *Actinomycetes* (5) or the *Archaea* (6). Even though 16S rDNA or 18S rDNA are the genes mostly targeted, other specific genes can be amplified. Accordingly, Wawer et al. (7) analyzed the sequence diversity of the [NiFe] hydrogenase gene of *Desulfovibrio* species, an important group of sulfate-reducing bacteria. A common set of primers used to amplify the rDNA of bacterial and fungal communities are listed in Table 1.

Table 1. Set of primers

Pair of primers[a]	Size of the PCR product (bp)[b]	Target	Sequence	Reference
P63f-P518r	511	*Bacteria*	5'-CAGGCCTAACACATGCAAGTC-3' 5'-ATTACCGCGGCTGCTGG-3'	(31)
P338f-P518r	236	*Bacteria*	5'-ACTCCTACGGGAGGCAGCAG-3' 5'-ATTACCGCGGCTGCTGG-3'	(2)
Arch340f-Arch519r	233	*Archaea*	5'-CCCTACGGGG(C/T)GCA(G/C)CAG-3' 5'-TTACCGCGGC(G/T)GCTG-3'	(6)
Act243f-Act513r	342	*Actinomycetes*	5'-GGATGAGCCCGCGGCCTA-3' 5'-CGGCCGCGGCTGCTGGCACGTA-3'	(5)
NS2f-Fungr5r	230	Fungi	5'-GGCTGCTGGCACCAGACTTGC-3' 5'-GTAAAAGTCCTGGTTCCC-3'	(32)
GC clamp[c]			5'-CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGG-3'	(2)

[a] f, forward primer; r, reverse primer

[b] GC clamp included

[c] GC clamp attached to the 5' end of the reverse primer

A1.1.3. General Strategy

The general strategy to obtain DGGE/TGGE fingerprints and exploit the results is as follows (Figure 1): First of all, the DNA has to be isolated from the ecosystem, which can range from natural samples (sediments, soils, drinking water, air, and so on) to artificial systems (cultures, bioreactors, and so on). Because the DNA has to be amplified by PCR, it has to be clean enough to avoid any inhibition of the polymerase. This problem arises frequently with samples taken from soil matrices. Furthermore, the differences in cell adhesion and cell wall structure, together with the soil's characteristics, can affect the efficiency of the DNA isolation and purification. To tackle these problems, different protocols of extraction can be found in the literature, especially for soil matrices. Therefore it is recommended, once it has been experimentally confirmed that a specific protocol of extraction is efficient enough (in terms of extraction yield and purification suited for the PCR), to use the same one for all the subsequent extractions. Indeed, it has been demonstrated that different extraction protocols

can give different DGGE fingerprints and therefore different apparent bacterial community structures (8).

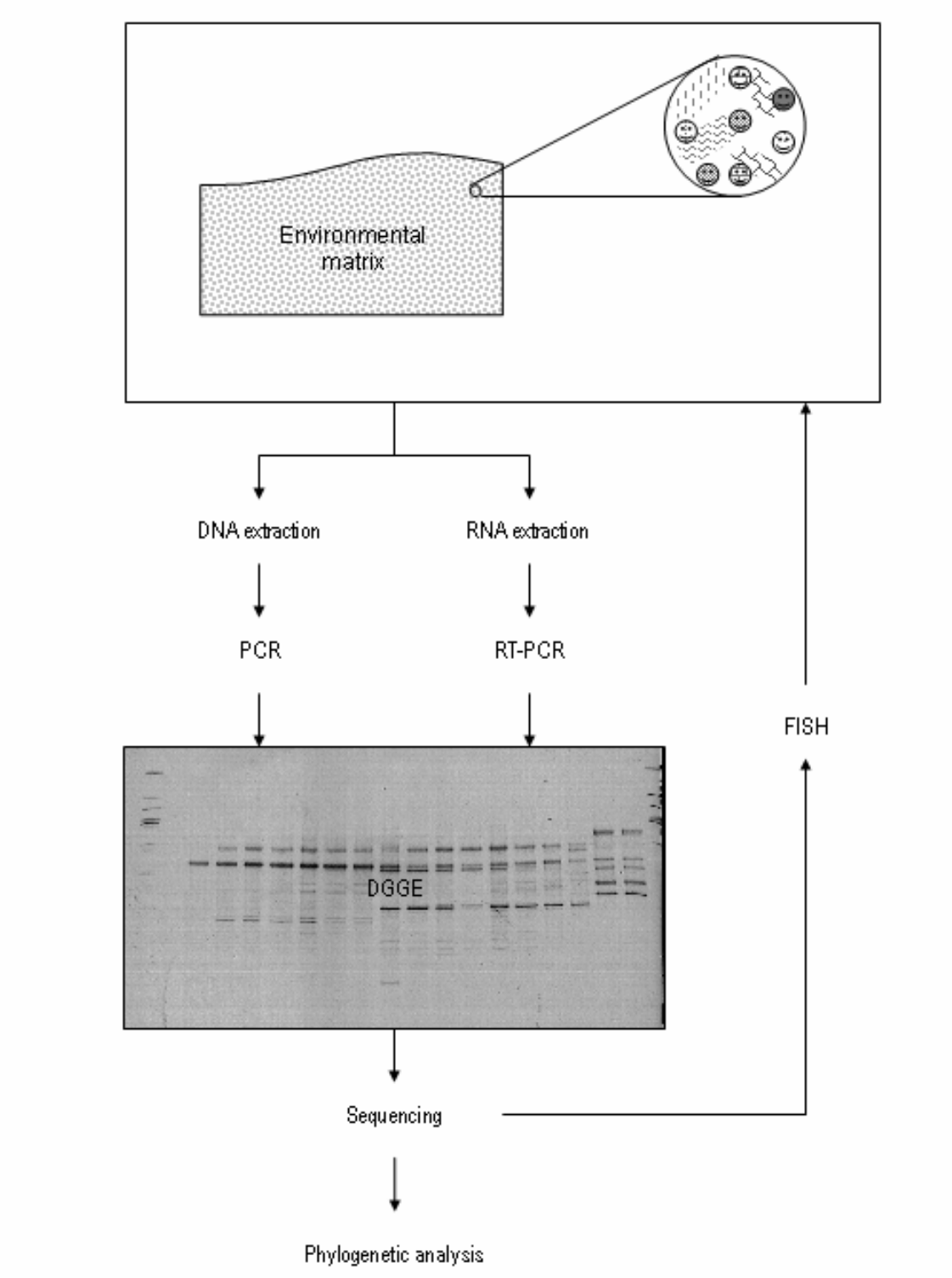

Figure 1. Flow-chart showing the successive steps required for the obtention of a DGGE/TGGE fingerprinting (sampling, nucleic acid extraction, PCR-amplification and DGGE/TGGE). Each band can be sequenced for further phylogenetic analysis and/or fluorescent in situ hybridization.

Alternatively to DNA, RNA can be isolated as an indicator of the level of activity of each microorganism in the environmental matrix under study, since the number of ribosomes per cell is roughly proportional to the growth rate of bacteria (9).

Once DNA or RNA is extracted and purified, the next step is respectively the PCR or RT PCR. The primers of interest can be selected based on the list that is given in Table 1. During the PCR program, the annealing temperature has to be properly chosen to avoid the amplification of nonspecific sequences. Different increasing annealing temperatures can be evaluated until non-specific amplifications are avoided. Alternatively to this trial-and-error method, a "touchdown" PCR can be used (10), which relies on incremental annealing temperature decrease during subsequent PCR cycles. When a sufficient quantity of PCR products are available, they can be loaded on a DGGE or TGGE. A detailed method to run DGGE/TGGE can be found in Subheading 3.

The microbial identity of each DGGE/TGGE band can be obtained after excision of the band of interest and further sequencing. This sequence can be compared with available 16S rDNA in the databases to identify the phylogenetic affiliation of each sequenced band. The Ribosomal Database Project (http://rdp.cme.msu.edu/html) offers the possibility to compare the sequence with other sequences available in the database and contains a useful package of tools for phylogenetic analysis (11) as well as probes/primers design (12). However, it has to be pointed out that these databases are incomplete and still growing. Therefore, if the sequence has a low similarity to known sequences, it is difficult to determine whether the sequence represents a novel microorganism or is part of known taxa for which no 16S rDNA sequences are available or of low quality (i.e., partial sequences or ambiguous sequences). Apart from this aspect of comparison, the sequence can also be used for the design of oligonucleotide probes for *in situ* monitoring of the corresponding bacterial population (fluorescent *in situ* hybridization [FISH] (13)).

As mentioned before, DGGE/TGGE is a powerful tool to monitor the response of microbial communities to stimuli and/or stresses by comparison of the fingerprint patterns before and after the external perturbation. Statistical analysis can be performed on these fingerprints (provided a numerical image of the gel is available) to determine whether a significant change occurred because of the stimulus/stress. Two statistical methods are commonly used, principal coordinate analysis and canonical variate analysis (14).

A1.1.4. Applications of DGGE/TGGE

Since the first application of DGGE/TGGE in natural ecosystems (2), it has found many applications for the monitoring of microbial populations in complex matrices, from natural to artificial systems. This section will focus on some of these applications.

As aforementioned, molecular techniques avoid the need for cultivation, which is an important selection factor. By using a library of clones containing a fragment of 16S rDNA together with TGGE, Felske and co-workers (15) discovered an uncultured member of the class of the *Actinobacteria* in grassland soils.

Analysis of DGGE/TGGE fingerprints is possible on a wide range of natural and artificial matrices (provided that sufficient DNA/RNA can be recovered and purified), from soils (16), wall painting (17), to wastewater systems for the bioremediation of phenol (18) or biotrickling filters containing styrene degrading biofilms (19). Furthermore, as described above, it is possible to study part of the microbial community structure by the use of primers targeting specific groups.

Thanks to the ability of this technique to analyze the evolution of microorganism composition, MacNaughton et al. (20) evaluated the microbial population changes that occurred during bioremediation of an experimental oil spill. They discovered that the treatment of the contaminated soil promoted the growth of Gram-negative bacteria in the α-protobacteria and *Flexibacter-Cytophaga-Bacteroides* phylum. As previously mentioned, group-specific populations can be analyzed in very complex communities. In this way, Henckel et al. (21) used PCR-DGGE type I and type II to monitor methylotrophs in rice-field soil, along with the functional genes for particulate methane monooxygenase and methanol dehydrogenase, which are important enzymes for the catabolism of all methanotrophs. They found that there was a pronounced shift in the methanotroph community when CH_4 oxidation began.

A1.1.5. Limitations of DGGE/TGGE

Even if DGGE/TGGE is common and one of the most widely utilized methods to analyze microbial communities, it has to be emphasized that the technique does have some limitations, but also that there are solutions to overcome these limitations. First of all, the way a sample is stored can affect the microbial community. For example, anaerobic or aerobic storage or direct freezing of the samples can result in the identification of different microbial communities (22). In addition, insufficient or preferential disruption of cells can introduce a bias during subsequent DGGE analysis.

Several biases can also occur during the PCR (23). Suzuki and Giovannoni (24) detected a preferential amplification of rRNA genes in mixed microbial populations. The authors discovered that this preferential amplification can be limited by an appropriate choice of primers and by decreasing the number of cycles of replication. However, they postulated that this phenomenon would be minor if the sample contains a high diversity of templates.

Another bias is the formation of chimeric molecules during PCR. Chimeric molecules are composed of parts of two sequences originating from templates with a high sequence similarity. These templates compete with primers during the annealing step of the PCR. The percentage of chimeric molecules can be very important, depending on the number of PCR cycles and sequence similarities between templates. For example, Wang and Wang (25) observed up to 30% chimeras after 30 cycles with templates showing a 99.3% sequence similarity. They also suggested an increasing of the elongation time (2 to 5 min) and a diminution of the number of cycles of replication to reduce the formation of chimera. If chimeric molecules are still formed, the Ribosomal Database Project (11) offers the possibility to screen sequenced fragments for possible chimera.

Chimeric structures are not the only factor that can affect the interpretation of DGGE/TGGE fingerprints and/or lead to an overestimation of the number of microbial species. Indeed, bacteria can have more than one 16S rRNA gene (*rrn* operons) on their chromosome. The number of *rrn* operons can differ widely, from one copy for *Bradyrhizobium japonicum* (26) to 10 copies for *Bacillus subtilis* (27). In addition, these copies can show variable sequence heterogeneities, as shown by the detection of up to 10 variants in *Paenibacillus polymyxa*

when a 347-bp fragment of its 16S rRNA was amplified by PCR and then loaded on a TGGE (28).

In addition to storage and PCR bias, the interpretation of DGGE/TGGE fingerprints themselves can be ambiguous when very complex communities are analyzed. It is estimated that bacterial populations that compose more than 1% of the total community can be detected by PCR-DGGE (2). This limit can be overcome by bacterial fractionation (29), differential centrifugation of extracted DNA bound to bisbenzimidazole in CsCl gradients according their G+C content (30) or by using group-specific primers.

A1.2. Materials

A1.2.1. DNA Extraction and Purification

There are several protocols available in the literature. However we would highly recommend the kit developed by Mo Bio for environmental samples (Mo Bio UltraClean Soil DNA Kit, Solana Beach, CA). The kit is very fast and yields relatively high amounts of clean DNA suitable for PCR.

A1.3. Methods

A1.3.1. PCR Conditions

A 2-mL volume of the extracted DNA is amplified by PCR with a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). The PCR mixture contains 0.5 m*M* of each primer, 100 m*M* of each deoxynucleoside triphosphate, 10 mL of 10X PCR buffer, 2 U of Expand high-fidelity DNA polymerase (Boehringer, Mannheim, Germany), 400 ng of bovine serum albumin (Boehringer) per mL, and sterile water to a final volume of 100 mL. Other polymerases could be used but one should keep all the conditions standard to avoid any biases. Samples are amplified as follows: 94°C for 5 min, followed by 30 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

A1.3.2. DGGE

DGGE based on the method cited by Muyzer et al. (2) is performed with the D Gene System (Bio-Rad, Hercules, CA). PCR samples are loaded onto 6% (w/v) polyacrylamide gels in 1X TAE (20 m*M* Tris, 10 m*M* acetate, 0.5 m*M* EDTA [pH 7.4]) for the primer set P63f plus P518r, or 8% polyacrylamide gels for the set P338f plus P518r. The polyacrylamide gels were made with a denaturing gradient ranging from 40 to 60% (where 100% denaturant contains 7 *M* urea and 40% formamide). For more details concerning the percentage of denaturants the reader is referred to any basic molecular biology handbook. The electrophoresis could be run overnight at 60°C at 75 V for the primer set P63f plus P518r, or at 35 V for the set P338f plus P518r. Shorter times with higher voltages could be used as well. After the electrophoresis, the gels are soaked for 30 min in SYBR Green I nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, ME). The stained gels are immediately photographed on a UV transillumination table with a video camera module (Vilbert Lourmat, Marne la Vallée, France). Alternatively, ethidium bromide can be used. Statistical comparison of different DGGE patterns, run on the same gel, are done with GelCompar software 4.1 (Applied Maths, Kortrijk, Belgium) or any other available software designed for electrophoresis gel analysis.

A1.3.3. Sequencing of DGGE Fragments

DNA fragments to be sequenced are excised from the gel, placed into sterile Eppendorf tubes containing 25 mL of sterilized water, and incubated overnight at 4°C. A 4-mL volume of the DNA diffused in water serves as a template for PCR amplification. The amplified products are subjected to a new DGGE step to confirm their electrophoretic mobility. The PCR products are purified with a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) before sequencing. However, it is important to note that this strategy doesn't always work. Therefore, it is advisable to clone the PCR products into a cloning vector before sequencing. The Topo TA cloning kit from InVitrogen (San Diego, CA) is recommended. The sequences are aligned to 16S rRNA sequences available in the National Centre for Biotechnology Information database by using the BLAST 2.0 search program.

A1.4. References

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