Evaluating the Microbial Diversity of Soil Samples: Methodological Innovations

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ABSTRACT

This manuscript is a review of the innovative methodologies that enable more precise evaluations of soil microbial diversity. Highlighting the molecular approach, which does not require the isolation of microorganisms and allows the inclusion of non-culturable genotypes in the analyses, the described methodologies revolutionised the environmental microbiology and opened gateways for an accurate understanding of the ecology and diversity of microorganisms. The application of techniques based on soil total DNA extraction, PCR amplification of genes or gene fragments, and sequence analysis revealed that the microbial universe is far more complex than ever imagined. Examples of applications of the molecular approach to study the diversity of soil diazotrophic bacteria are given.

Key words: microbial diversity, DGGE, molecular methods, soil DNA, 16S rRNA.

INTRODUCTION

The study of microbial diversity is hampered by several problems, such as: 1) the microscopic dimensions of the subject; 2) incomplete taxonomic descriptions for many known species; and 3) lack of selective isolation and growth media for the majority of soil microorganisms. In addition, classical characterization methods based on microbial morphology and physiology are untrustworthy due to the great ability of microorganisms to adapt to a wide range of environmental conditions. The same genotype may reveal different cell shapes or behaviour when grown in the laboratory. There is an urgent need for faster and more efficient methodologies for the evaluation of microbial diversity. Table I lists current methods applied to study microbial diversity in the environment.

Until recently, the detection and identification of microorganisms in environmental samples was accomplished by their growth in artificial selective or non-selective media, or their direct observation using a microscope (Herbert, 1990). However, growth media are, to a greater or lesser extent, selective to particular groups of microorganisms. Even if a selective media for the target organism is available, some unculturable strains might be excluded from the analyses. Cell counts by microscopy can aid a quantitative assessment of the microbial populations, being less informative about their diversity (Pickup, 1991).

One of the methods applied to investigate the metabolic diversity of microbial communities is

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TABLE I
Methods to study the different levels of microbial diversity in the environment.

Method	Observations/References
I) Function based methods	
Colony counts	Herbert (1990)
Biolog	Heuer & Smalla (1997a)
Microbial biomass	Morgun & Winstanley (1997)
II) Community structure based methods	
PFLA ("Phospholipid fatty acid")	Morgun & Winstanley (1997)
"In situ" hybridization	Aman *et al.* (1990)
PCR/DGGE or TGGE	Muyzer *et al.* (1993) Rosado *et al.* (1998)
PCR/ARDRA	Smit *et al.* (1997)

based on the commercial kit named BIOLOG. The microtitre plates of BIOLOG are inoculated with bacterial cell suspensions extracted from soil samples. The wells of the BIOLOG kit are coated with different sources of carbon, nutrients and labelled with tetrazolium. The plates are then incubated and periodically monitored for substrate oxidation and tetrazolium reduction. This methodology was applied to study microbial communities from both soil and rhizosphere (Wünsche *et al.*, 1995), and to investigate the effects of the introduction of genetically modified organisms on soil microbial communities (Heuer & Smalla, 1997a).

During the last decade, the molecular approach to study microbial diversity has been progressively adopted and improved by microbiologists eager to avoid the *biases* associated with classical methods based on grosth in artificial media. One of these methods is based on the analysis of total phospholipid fatty acids (PFLA). This technique enables the structural analysis of microbial communities in the environment as well as the detection of shifts in microbial populations (Morgun & Winstanley, 1997). However, this type of analysis is not indicated to detailed structural evaluations of the soil microbial community.

Nucleic acids based techniques are an alternative to these methods. Torsvik *et al.* (1990) estimated the genetic diversity of a bacterial community by analysing the reassociation kinetics of DNA extracted from soils of a deciduous forest. Their results were compared with those obtained by conventional analysis of populations of bacteria isolated from the same soil samples. The authors found 4,000 different genomes in the soil samples when applying the molecular approach, an estimate 200-fold greater than that obtained by conventional analyses. This indicates that only a minute fraction of the soil microbiota is accounted for by the analysis of phenotypic variants of isolated bacteria.

MOLECULAR PROBES

The need to monitor the release and fate of genetically modified microorganisms in the environment (Droge *et al.*, 1998; Kellenberger, 1994) fostered the development of a plethora of methods designed for the detection of all organisms. These methods are able to cope both with microorganisms in the non-cultured state, as well as cloned genes, which are amenable to transfer to other microbial populations (Giddings, 1998).

New strategies were developed and adapted to cope with the limitations of traditional culture-based approaches for the study of bacterial populations. Notable developments were achieved by nucleic acid based techniques. The use of molecular probes proved to be a versatile approach, allowing both the study of isolated and cultivated microorganisms as well as of specific organisms or

groups of organisms by use of nucleic acids extracted directly from environmental samples (Pickup, 1991).

A probe is a labeled fragment of DNA or RNA with a nucleotide sequence complementary to that of the target gene sequence of interest. Under controlled conditions (particularly ionic concentration and temperature), probe and single-stranded target gene sequence hybridize forming heteroduplexes. The detection of target gene sequences depends on the nature of the labeling system, which can be radioactively or non-radioactively labeled deoxynucleotides present in the probe sequence. The latter include a choice of labels, from enzymes (*e.g.*, alkaline phosphatase or horse radish peroxidase), to fluorescent molecules (*e.g.*, fluorescein), and biotin or digoxigenin (Stahl & Amann, 1991).

Nucleic acid probes can be constructed to target particular genes, and thus be used to detect organisms with the corresponding genotypes or biochemical pathways. Examples are probes directed to bacterial genes coding for antibiotic or heavy metal resistance, as well as for enzymes involved in biosynthetic or catabolic pathways, such as nitrogenase (Rosado *et al.*, 1998), and those involved in the metabolism of xenobiotic compounds. Probes can also be targeted to mobile genetic sequences, which can be transferred between chromosomal and plasmid DNA, such as transposons, or between organisms from different taxa, such as conjugative plasmids (Kellenberger, 1994; Hill & Top, 1998).

The use of molecular probes presents the following advantages (Stahl & Amann, 1991; Schleifer & Ludwig, 1994):

— can be designed to be highly specific and used under stringent conditions to detect a gene or nucleic acid sequence from a particular organism or group of organisms;

— can be used to detect and identify organisms without the need for cultivation and isolation in pure culture;

— probes targeted to regions of the rRNA molecule with different levels of variability may be designed and used to detect specific organisms or broader taxonomic groups;

— in general, the bacterial genome is highly stable and is not affected by growth conditions, contrary to antigenic molecules and phenotypic properties.

The application of nucleic acid probes to the study of natural microbial populations had a quantum leap after the development of the polymerase chain reaction technique (PCR; (Mullis & Faloona, 1987)). The PCR can significantly amplify rare nucleotide sequences against a background of diverse and more abundant sequences, thus enhancing the possibility of detecting rare organisms/sequences in heterogeneous mixtures. Sequences from particular microorganisms can be amplified using PCR primer pairs targeted at species-specific complementary sequences located in the 16S or 23S rDNA (Bej *et al.*, 1990; Roller *et al.*, 1992), or at genes coding for enzymes (Bej *et al.*, 1991), or even at some other genome fragments (Pooler & Hartung, 1995).

Over the last few years, a range of protocols based on the PCR have been reported for the detection of microorganisms in soil samples (Smalla *et al.*, 1993; Briglia *et al.*, 1996). The combined PCR/probing approach significantly enhances the sensitivity of detection protocols. Combined PCR/probe hybridization have raised approximately 1,000 times the sensitivity of detection of genetically engineered *Pseudomonas cepacia* strains in environmental samples (Steffan & Atlas, 1988).

USE OF RIBOSOMAL RNA AS PHYLOGENETIC MARKERS

Ribosomal ribonucleic acids (rRNA) are considered to be the most adequate biopolymers for comparative studies in microbial molecular energy. Their coding genes, the rDNA, are universally distributed, being the molecule with the highest degree of conservation in the living world. Variability can accumulate to a greater or lesser extent in different regions of the molecule (Lane *et al.*, 1985). A major advantage of using rRNA sequence information is their availability in databases (RDP, GenBank, EMBL), in most cases accessible free-of-charge, along with services such as align-

ment and comparison of newly obtained sequences with the data set (Maidak *et al.*, 1997).

Automation of nucleic acid sequencing had a big impact on the use of 16S rRNA sequences as molecular markers, resulting in a large number of microbial rRNA sequences stored in the databases. These sequences can be used to build phylogenetic trees, depicting how the studied organisms relate to one another. Sequence alignments give precious information to aid taxa-specific oligonucleotide design. Specific oligonucleotides can be designed to different taxonomic levels, from universal (for example, encompassing all Eubacteria taxa) to family-, species-, or strain-specific probes or PCR primers. Microbial ecology research has benefited from a great variety of ribosomal probes specific to different genera, species or strains (Pace *et al.*, 1986).

Probes specific to the 16S rRNA of the nitrogen fixing actinomycetes *Frankia* were used to study the ecology of this microorganism in soil samples (Hahn *et al.*, 1990). Ecological and taxonomic studies of this genus have been hampered by their difficulty to grow in artificial media in the laboratory. The use of molecular probes enabled the identification of *Frankia* strains in *Alnus glutinosa* nodules.

A molecular ecology study based on the amplification and sequencing of 16S rRNA molecules amplified from DNA of Australian soils, followed by phylogenetic analysis and hybridization with taxon-specific probes revealed the occurrence of unique bacterial groups, which should be classified as new lines of descent under the domain *Bacteria* (Liesack & Stackebrandt, 1992). A similar approach was applied by Stackebrandt *et al.* (1993) to investigate the genetic diversity of streptomycetes in acid soils sampled from a subtropical Australian forest. Again, the results revealed a significant degree of microbial diversity. The majority of the 113 16S rRNA sequences analysed were new types of actinomycetes, unrelated to the known, culturable strains of these organisms.

The use of ribosomal probes were improved by Amann *et al.* (1990), who coupled fluorescent labels to the oligonucleotides. The fluorescent probes were used in *in situ* hybridization experiments, consisting of the immobilization and permeabilization of cells directly on the soil samples. The labelled probes were detected by fluorscent microscopy, revealing the target organisms. One could use, concomitantly, probes containing different fluorescent labels, allowing the discrimination of different species in soil samples.

Ribosomal probes can also be used to quantify microbial populations in soil samples. A quantitative PCR method was developed by Rosado *et al.* (1996) to detect *Paenibacillus azotofixans* strains in soils and wheat rhizosphere. The 16S rRNA gene regions were PCR amplified with specific primers, and hybridized to a homologous probe to an internal region of the amplified fragment. The method was shown to be adequate to evaluate the impact of water stress on the population of *P. azotofixans* in the soil. The primers and probes are highly specific and the method precludes the isolation or growth of the bacterial strains in the laboratory, as well as the use of other specific markers.

A similar approach, based on the hybridization of total soil rDNA against a taxon-specific probe, enables quantitative analysis of microbial taxa. The quantification is achieved by expressing the specific signal as a fraction of the hybridization signal obtained with a universal probe (Zheng *et al.*, 1996).

Microbial molecular diversity studies can focus on unique functional groups. The analysis of ribosomal sequences obtained from soil samples can reveal new species, modify concepts, and even reassemble the taxonomy of functional groups. Sequencing of ammonium oxidizing bacteria 16S rDNA fragments amplified from soil and sediment samples revealed that the vast majority of the sequences obtained were unique, meaning that the known culturable genera belonging to this functional group (*Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*) are not dominant in any of the analysed samples (Stephen *et al.*, 1996). These findings redefined this functional group, previously considered of low diversity, and highlights the importance of the molecular approach to studies of evaluation and monitoring of microorganisms in the environment.

THE EXTRACTION OF NUCLEIC ACID FROM SOILS AND SEDIMENTS

The study of microbial populations in the environment by using DNA or RNA sequences requires the extraction of the deoxyribonucleic acids from the samples. Various methods of extracting DNA or RNA from environmental samples are available in the literature, each adapted to the different categories of environmental sample (water, soil and sediments, among others). DNA extraction from water samples generally requires the concentration of the microbial biomass by means of filtration or centrifugation of large volumes of material (Somerville *et al.*, 1989). In the case of soils and sediments, both cellular extraction and direct cell lysis can be adopted (Fig. 1).

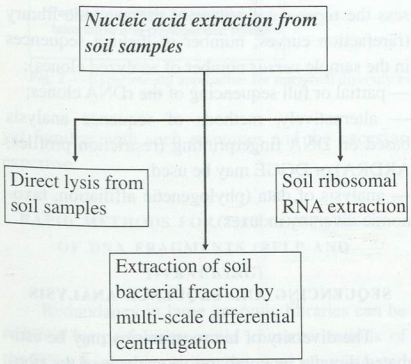

Fig. 1 — Major protocols for nucleic acid extraction from environmental samples.

The direct lysis approach is applied with increasing frequency to extract DNA from soils and sediments. It is based on the physical disruption of the cells in the soil or sediment matrix and subsequent purification of the released DNA (Ogram *et al.*, 1988). Despite being efficient, this process could result in partial lysis or widespread deterioration of the DNA. Therefore, careful standardization of the methodology for the different types of samples is necessary.

Soil and sediment samples also contain a large quantity of humic material, which is extracted along with the DNA. This occurs due to the polyanionic properties of humic acids, which are linked to free nucleic acids. The greater the organic carbon and clay contents of a soil, the more difficult it is to extract DNA of good quality (Wellington *et al.*, 1994). The humic acids are inhibitors of the constraining endonucleases and DNA polymerases, and may also interfere in the specific nature of the hybridization reactions. The addition of PVPP (polyvinyl polypyrrolidone) is recommended to remove, by adsorption, the phenolic compounds such as humic acids. Other compounds may be added to minimize oxidization of the phenols, such as the reducing agent sodium ascorbate (Holben *et al.*, 1988).

The total quantity of DNA (from procaryotes and eucaryotes) in soils and sediments ranges, on average, from 10 to 200 $\mu g\ g^{-1}$ of soil. However, the extraction yield depends on the nature of the sample (the soil type) and on the efficiency of the *in situ* cell lysis, which in turn depends on the composition of the bacterial populations and the lysis method utilized; Gram-positive bacteria, for instance, are more resistant to mechanical lysis. The extraction method used should minimize the period of time that the DNA is in contact with the soil matrix after the lysis has occurred, thus avoiding the adsorption of mineral particles, humic acids and the potential action of free nucleases.

Pillai *et al.* (1991) developed a simple method for purifying soil-extracted DNA before submitting it to PCR amplification. It consists of the separation of DNA from the bulk of the colloidal soil particles by centrifugation in a sucrose gradient before amplification by PCR. The authors demonstrated the potential of this method to extract bacterial DNA from a variety of soils.

A quick method for the direct DNA extraction from soils involves a lysozyme treatment followed by mechanical lysis by bead-beating coupled with detergent (SDS) at low temperatures. The nucleic acids are extracted using phenol and a cold process, followed by DNA precipitation with caesium chlorate and potassium acetate, and separation with HCl spermine or glass-milk (glass micro-beads). The DNA obtained using this methodology is suitable for PCR amplification (Smalla *et al.*, 1993).

An improvement of this method was achieved by van Elsas *et al.* (1997), who introduced a commercial product ("Wizard cleanup system", Promega) during the last stage of DNA purification, and in this way reduced total protocol time. Another simple and quick method for purifying DNA involves the addition of PVP (polyvinylpyrrolidone) to the agarose gel during the electrophoretic separation of the extracted DNA (Young *et al.*, 1993). The addition of PVP eliminates the joint migration of humic acids with the nucleic acids, by delaying the electrophoretic mobility of the phenol compounds.

The extraction of ribosomal RNA from soil samples can be useful, since it enables information to be obtained on the metabolically active members of the microbial population in the environment. Recently, a protocol to obtain DNA or RNAr simultaneously was developed by Duarte *et al.* (1998). The protocol enables the extraction of large quantities of both nucleic acids from different soil samples and the subsequent amplification of them using (RT)-PCR ("Reverse Transcriptase-Polymerase Chain Reaction").

rDNA SEQUENCE ANALYSIS

Several approaches can be applied to evaluate microbial diversity by use of rDNA sequences amplified from environmental samples. In general, the choice of approach must take into account the aim of the study, the availability of suitable probes and primers for the subject taxa, laboratory infrastructure, expertise and time.

The automation of sequencing techniques, combined with PCR, led to a rapid increase in the number of rDNA sequences obtained from cultured microorganisms and environmental samples, which are stored in the databases and available for phylogenetic analysis. Ideally, a comprehensive evaluation of microbial diversity should involve the quantification and sequencing of all representative rDNA molecules present in the sample, which is certainly not a practical task to be performed. If one considers the need for replicating samples in order to attain reliable results, then it becomes a daunting task. Thus, the alternative is to analyze only a fraction of the rDNA molecules obtained.

The methods that follow are all based upon PCR-amplified rDNA sequences, which aim at the estimation of microbial diversity and phylogenetic analysis through the evaluation of a moiety of the rDNA sequences present in the samples. A basic protocol for the study of microbial diversity in environmental samples would involve the following steps (Fig. 2):

— extraction and purification of DNA from the sample;

— partial or full amplification of rDNA sequences (16S and/or 23S rDNA, or intergenic regions) using primers with the desired specificity;

— cloning of the PCR products in plasmids/phage and construction of a genomic library representative of the amplified rDNAs;

— if necessary, primary analysis of the cloned sequences (*e.g.*, using *T-tracking* sequencing) to assess the representativeness of the genomic library (rarefaction curves; number of distinct sequences in the sample *versus* number of analyzed clones);

— partial or full sequencing of the rDNA clones;

— alternatively, methods of sequence analysis based on DNA fingerprinting (restriction profiles, ARDRA) or DGGE may be used;

— analysis of data (phylogenetic affiliation, taxonomic diversity indexes).

SEQUENCING AND SEQUENCE ANALYSIS

The diversity of microorganisms may be estimated directly by comparative analysis of the ribosomal sequences obtained from the environmental sample. Partial or full sequence analysis of 16S rDNA or bacteria and archaea provides a comprehensive framework for such studies. An additional advantage is the fact that the 16S rDNA molecule may provide information at both supra- and infraspecific levels as different portions of the molecule present different degrees of variability, and thus may have different levels of taxonomic resolution.

Usually, diversity studies based on 16S rDNA sequence analysis depend on the availability of a good sequencing infrastructure. High throughput and automated sequencing are the options of choice for conducting such studies, though many microbiology laboratories around the globe are not

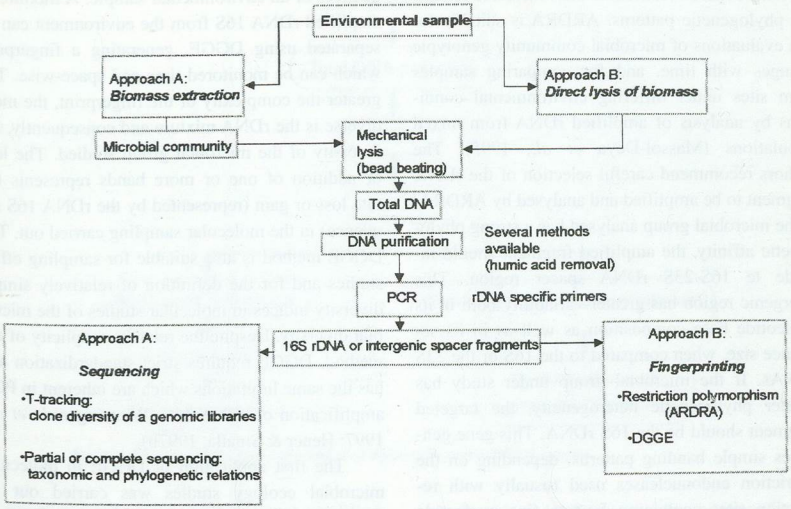

Fig. 2 — Experimental approaches for microbial diversity evaluations through rDNA analysis.

yet familiar with such resources and the necessar expertise.

RAPID METHODS FOR THE COMPARISON OF DNA FRAGMENTS (RFLP AND T-TRACKING)

Redundancy in large genomic libraries can be reduced by performing rapid characterizations of the DNA fragments prior to the more laborious and expensive analyses (*e.g.*, full sequence determination). One commonly used approach is based on the restriction enzyme profiles obtained after digesting the DNA fragments with restriction enzymes, that are selected based on their ability to reveal restriction length polymorphisms (RFLPs) in the DNA fragments analysed. Usually, enzymes that have short recognition sites (4- or 5-cutters), such as *Alu* I, *Dde* I, *Hinf* I, *Msp* I, *Rsa* I, *Sau*3 A and *Taq* I, are selected for RFLP analyses.

Another approach, denominated *T-tracking*, is based on partial sequence analysis of DNA fragments by using only one single terminator dideoxynucleotide (ddTTP) in the sequencing reactions, which are submitted to electrophoresis in sequencing gels. The results are profiles comprised of bands corresponding to the T positions of the DNA fragment and larger gaps corresponding to the remaining DNA bases. The profiles obtained can be directly compared to those obtained for other DNA fragments. The diversity of patterns represented in the sample of fragments analysed can be correlated to the diversity of the population (Stephen *et al.*, 1996). *T-tracking* is usually applied to construct rarefaction curves to estimate how many clones need to be analyzed in order to have a representative sampling of a given population of PCR-amplified/cloned DNA fragments.

ARDRA

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is a technique based on the restriction enzyme digestion of amplified rDNA fragments followed by the electrophoretic separation of the resulting fragments in agarose gel. This methodology was originally applied to genome characterization as well as to comparative studies of isolated strains (Vaneechoutte *et al.*, 1992). The technique is based on the degree of conservation of

the rRNA operon restriction sites, therefore reflecting phylogenetic patterns. ARDRA is suitable for fast evaluations of microbial community genotypic changes with time, and for comparing samples from sites under differing environmental conditions by analysis of amplified rDNA from mixed populations (Massol-Deya *et al.*, 1995). The authors recommend careful selection of the rDNA fragment to be amplified and analysed by ARDRA. If the microbial group analysed has a strong phylogenetic affinity, the amplified fragment should include te 16S-23S rDNA spacer region. This intergenic region has greater variability both in its nucleotide base composition as well as in its sequence size, when compared to the 16S or the 23S rDNAs. If the microbial group under study has greater phylogenetic heterogeneity, the targeted fragment should be the 16S rDNA. This gene generates simple banding patterns, depending on the restriction endonucleases used (usually with restriction sites containing four to five nucleotide bases). This technique was applied recently to evaluate the effects of copper contamination on soil microbial diversity (Smit *et al.*, 1997).

DGGE

Another technique recently developed is the DGGE method (*denaturing gradient gel electrophoresis*), which has been successfully applied to detect variations in the sequences of various microbial genes. This methodology enables the separation of DNA fragments of same length, differing in their nucleotide sequences. This separation is achieved based on the differential mobility of DNA molecules subjected to electrophoresis in polyacrylamide gels under a gradient provided by a denaturing agent. Partially denatured DNA molecules have a lower mobility when compared to fully paired double stranded molecules. Therefore, the electrophoretic migration of DNA fragments with different sequences ceases at different positions in a DGGE gel, depending on base composition, generating fingerprints of the mixture of genes analyzed (Fischer & Lerman, 1983). This technique can be applied to the study of microbial diversity based on the differences between the rDNA sequences of an environmental sample. A mixture of amplified rDNA 16S from the environment can be separated using DGGE, generating a fingerprint which can be monitored time and space-wise. The greater the complexity of the fingerprint, the more diverse is the rDNA mixture and consequently, the diversity of the microbial group studied. The loss or addition of one or more bands represents the rate loss or gain (represented by the rDNA 16S sequence) in the molecular sampling carried out. The DGGE method is also suitable for sampling effort studies and for the definition of relatively simple diversity indices in molecular studies of the microbial diversity. Despite the relative simplicity of the method, DGGE requires strict standardization and has the same limitations which are inherent in PCR amplification of rDNA 16S (Wintzingerode *et al.*, 1997; Heuer & Smalla, 1997b).

The first application of DGGE in molecular microbial ecology studies was carried out by Muyzer *et al.* (1993). The objective of the authors was to analyze the genetic diversity of microbial populations. They observed the presence of up to 10 different rDNA fragments in microbial communities obtained from different origins. The specific amplification of DNA fragments associated with microbial strains or defined taxonomic groups, allied with DGGE, is a strategy which enables direct detection of the presence and relative abundance of different microbial species in environmental samples for the first time (Muyzer *et al.*, 1993). The DNA contained in the DGGE gel can be transferred to nylon membranes and hybridized with specific probes for certain microbial groups or the PCR bands can be eluted from the gel, re-amplified and sequenced to enable phylogenetic positioning of the members of the community (see diagram in Fig. 3).

The DGGE technique has been frequently used to study the structure of natural microbial communities. For this purpose, the ribosomal gene 16S has been most frequently used in the published protocols (Muyzer *et al.*, 1993). However, other preserved genes can also be used in DGGE systems (Rosado *et al.*, 1997). Waver & Muyzer (1995) used DGGE to study the genetic diversity of *Desulfovibrio* species by analyzing fragments of

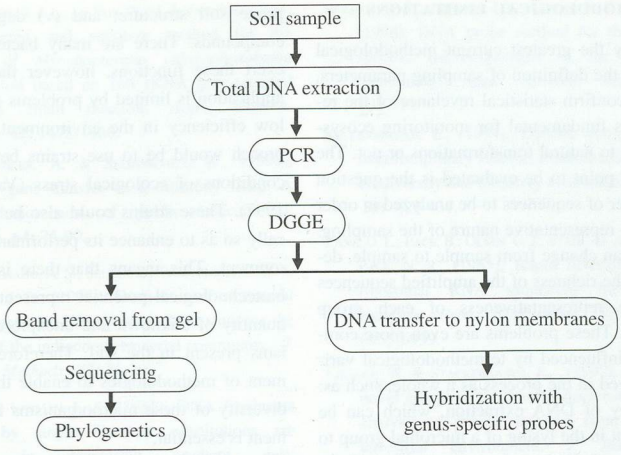

Fig. 3 — The use of DGGE to study microbial diversity in environmental samples.

the functional NiFe gene (hydrogenase) amplified using PCR.

A DGGE system was recently developed based on *nifH* genes from *P. azotofixans* (Rosado *et al.*, 1998). The *nifH* gene was selected because it has preserved and variable regions, useful for the development of primers for PCR. It was first necessary to obtain information on the *nifH* gene from *Paenibacillus* sp., since there was no information available in the data bank. For this purpose, a methodology using degenerate primers was used (Zehr & McReynolds, 1989) to amplify part of the *nifH* gene from *Paenibacillus* nitrogen-fixing species. This methodology proved to be simple and quick. The PCR products obtained were cloned and sequenced. The alignment and analysis of the *Paenibacillus nif*H sequences using parsimony and matrix methods (neighbor joining), enabled the construction of a phylogenetic tree based on the similarities found. The phylogenetic tree suggests that the *Paenibacillus* species are closely grouped amongst themselves and that the *P. azotofixans* strains have sequence deviations but form a very interrelated group. What is even more surprising is the presence of copies of the *nif* gene in *P. azotofixans* strains that are grouped with sequences of an alternative nitrogenase system (*anf*), which suggests the existence of an alternative nitrogen fixing system in *P. azotofixans*. In the future, it will be interesting to study the different genic expressions of the two nitrogen fixing systems in *P. azotofixans*.

DGGE results using the product of PCR *nifH* from *P. azotofixans* indicated that this gene is present with several copies in the bacterial genome and that there is a great diversity amongst the different copies and also between different strains. The use of the DGGE *nifH* system with DNA extracted from environmental samples demonstrates that the DGGE system is a powerful tool for the study of the genetic diversity in natural *Paenibacillus* populations (Rosado *et al.*, 1998).

A DGGE system was also applied to evaluate the diversity of *Rhizobium* species in soils subjected to different types of agricultural handling (direct planting, conventional planting, and the use of a soil insecticide) (Oliveira *et al.*, 1998). The same group described the design of specific primers for sites in the ribosomal spacing region (rDRNA 16S-rDNA 23S) of the *Rhizobium leguminosarum* and *R. tropici* species and its use to amplify fragments of the ribosomal genic region of *Rhizobium* species from soil extracted DNA from the "Cerrado" region in the northern part of the State of São Paulo (Oliveira *et al.*, 1999).

METHODOLOGICAL LIMITATIONS

Possibly the greatest current methodological limitation is the definition of sampling parameters, in order to confirm statistical revelance of the results. This is fundamental for monitoring ecosystems subject to natural transformations or not. The fundamental point to be evaluated is the question of the number of sequences to be analyzed in order to define the representative nature of the sampling. This value can change from sample to sample, depending on the richness of the amplified sequences and relative representativeness of each group (abundance). These problems are even more complex, being influenced by te methodological variations involved in the process as a whole, such as: the efficiency of DNA extraction, which can be more efficient in the lysing of a microbial group to the detriment of another; the degree of specificity of the primers or genetic probes, defined as a function of the sequences representing only known and already sequenced microorganisms (a minimal fraction of the environmental diversity); the amplification procedure using PCR, which can exhibit preferential amplification of some sequences and the formation of technical artifacts (*e.g.* chimeric molecules). To this, the problems with respect to sequential variations in the RNAr 16S molecules due to the heterogeneous nature of the rrn operon can be added. The possible limitations of using PCR and DGGE in environmental samples were recently reviewed by Wintzingerode *et al.* (1997) and Heuer & Smalla (1997b), respectively.

GENERAL CONCLUSIONS

Molecular microbial ecology is a multidisciplinary theme covering the sciences of microbiology, ecology, molecular biology, statistics and bioinformatics, among others. Its tools aid the investigation of microbial diversity and the advantages and risks involved in environmental applications of microorganisms. The development of agricultural and environmental biotechnology led to a greater demand for microbial strains that exert important functions, such as: (i) supplying nutrients to plants; (ii) promote plant growth; (iii) control or inhibit phytopathogenic activity; (iv) improve soil structure; and (v) degrade polluting compounds. There are many bacterial species to exert these functions, however their widespread application is limited by problems of survival and low efficiency in the environment. The best approach would be to use strains better adapted to conditions of ecological stress (Van Veen *et al.*, 1997). These strains could also be altered genetically so as to enhance its performance in the environment. This means that there is an enormous biotechnological potential represented by the vast quantity of unknown and unexplored microorganisms present in the soil. Therefore, the development of methodologies to enable the study of the diversity of these microorganisms in the environment is essential.

A polyphasic approach to the study of microbial diversity, integrating conventional and molecular techniques, is necessary. In addition, the development of new and improved methods and approaches would benefit dearly by a greater interaction among microbiologists, ecologists, statisticians, and professional of the different disciplines involved in microbial diversity studies. The emphasis of those studies should be on the evaluation of the current paradigms and theoretical models and on the importance of microbial diversity to the biological processes.

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