# Genetic fingerprinting of microbial communities – present status and future perspectives

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## ABSTRACT

The use of ribosomal RNA (rRNA) as molecular marker to identify microbial populations is now routinely used in microbial ecology. One of the strategies herein is cloning of PCR products obtained from environmental DNA to explore the diversity of microbial communities without the need of cultivation. Although successful, this approach is for practical reasons not suited to study the complex dynamics which microbial communities can undergo by diel or seasonal fluctuations or after environmental perturbations. For this purpose, genetic fingerprinting techniques are better tools. Here I present the current status of the application of genetic fingerprinting techniques in microbial ecology and give an outlook into the future.

## Introduction

Our understanding of microbial diversity, and concomitantly of species composition, of microbial communities is hampered by the inability to classify microorganisms. Microbes are small and, in general, without conspicuous external characters to classify them morphologically. In addition, classification based on physiological or biochemical features is often not possible because an estimated percentage of 99% of all microorganisms in nature can not be isolated. So, to obtain a better understanding of the role of microbial diversity in ecosystem functioning, other techniques, which complement the traditional microbiological methods are necessary. By the pioneering work of Woese [47] it is now clear that all organisms on Earth can be classified by comparative sequence analysis of their small sub-unit ribosomal RNA (SSU rRNA). Pace and co-workers [30] were the first to realize that this phylogenetic framework of rRNA sequences could be used to design primers and probes. Application of these in molecular biological techniques allows us to detect and enumerate microorganisms in their natural habitat and so to determine the structure, function and dynamics of bacterial communities (summarized in [26]). So far, cloning of PCR-amplified 16S rDNA is the most successful approach to explore microbial diversity and to determine the species composition of mixed microbial communities. However, because cloning is time-consuming and laborious, this approach is not suited to follow successional population changes in a microbial community, which is regarded as one of the most important goals in microbial ecology [21). For this purpose genetic fingerprinting techniques are better suited.

Genetic fingerprinting techniques provide a pattern or profile of the community diversity based upon the physical separation of unique nucleic acid species [39]. The methods are rapid and relatively easy to perform, but more importantly, they allow the simultaneous analysis of multiple samples, which makes it possible to compare the genetic diversity of

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microbial communities from different habitats, or to study the behaviour of individual communities over time. Here, I will give an overview of the application of genetic fingerprinting techniques to study the diversity and dynamics of microbial communities and will discuss the advantages and disadvantages of these methods. The methods can be divided into *direct methods*, whereby nucleic acids are extracted and directly analyzed, such as low-molecular-weight (LMW) RNA profiling, or into *indirect methods*, whereby the molecular marker first has to be amplified, which is the case for denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), single-stranded-conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF), bisbenzimide-polyethyleneglycol (Bb-PEG) electrophoresis, restriction fragment length polymorphism (RFLP) or amplified ribosomal DNA restriction analysis (ARDRA), and terminal RFLP (T-RFLP) or fluorescent RFLP (Flu-RFLP).

## Genetic fingerprinting techniques

### *LMW RNA fingerprinting*

A genetic fingerprinting technique that has been used for more than a decade is profiling of low-molecular-weight (LMW) RNA (5S ribosomal RNA {rRNA}and transfer RNA {tRNA}[9]. The technique (see [12] for a technical description) is straightforward; total RNA is extracted from an environmental sample, and separated by high-resolution polyacrylamide gel electrophoresis. The separation profiles of the 5S rRNA and tRNA (the 23S and 16S rRNA molecules are too large to enter the gel) can be visualized by silver staining or by autoradiography if the RNA was radioactively labelled. Subsequently, the profiles are scanned, and stored in an electronic database for comparison.
LMW RNA profiling has been used to monitor bacterial population dynamics in a set of freshwater mesocosms after addition of non-indigenous bacteria and culture medium [10]. The approach was also used to investigate the diversity and activity of bacterial populations in a stratified water column of the central Baltic Sea [11]. Bidle and Fletcher [2] used LMW RNA profiling to compare free-living and particle-associated bacterial communities from different depths and different sites in an estuary bay.

An advantage of the LMW RNA fingerprinting technique is the absence of an in vitro amplification step to produce sufficient material to be analyzed, because such an amplification step might create errors [46]. Another positive point is that individual bands can be sequenced [11] or profiles can be hybridized with specific probes to assess the identity of the community members. However, only limited phylogenetic information can be obtained from the small 5S rRNA (max. 131 nucleotides) and tRNA (max. 96 nucleotides). Another weak point is the rapid degradation of RNA, which might form additional bands in the profiles making the interpretation of results difficult.

### *DGGE and TGGE*

In 1993, Muyzer and co-workers introduced another genetic fingerprinting technique in microbial ecology, that of denaturing gradient gel electrophoresis (DGGE; [23]). A mixture of PCR products obtained with genomic DNA extracted from a complex assemblage of microorganisms and primers specific for a molecular marker, such as the 16S rRNA gene, is separated in a polyacrylamide gel containing a linear gradient of DNA denaturants (see [24]

for technical details). Sequence variation among the different DNA molecules influences the melting behaviour, and therefore molecules with different sequences will stop migrating at different positions in the gel. Another technique based on the same principle is temperature gradient gel electrophoresis (TGGE).

Within a short time, DGGE and TGGE have become very popular in microbial ecological studies, and they are now routinely used in many laboratories. The techniques are used to profile community complexity (e.g., [40]), to study population dynamics in microbial communities (e.g., [6, 34]), to study differential gene expression in mixed populations [45], to monitor enrichment cultures (e.g., [33, 37]), to compare DNA extraction methods (e.g. [8]), to screen clone libraries for redundancy (e.g., [13, 15]), and to determine rRNA operon microheterogeneity [28]. For an overview the reader is referred to Muyzer and Smalla [25].

One of the strongest points of the techniques is that bands can be excised from the gel and subsequently sequenced to reveal the phylogenetic affiliation of the community members. Furthermore, community profiles can be hybridized with group-specific probes to detect the presence of particular bacterial populations. One of the limitations of the techniques is the separation of relatively short (ca. 500 bp) DNA fragments, which limits the design of probes for hybridization analysis. Furthermore, it is not always possible to separate fragments with different sequences, because of similar melting behaviour of the fragments. In addition, the presence of double bands, which might be caused by primer degeneracies or of heteroduplex molecules might contribute to difficulties in the determination of community complexity.

### *SSCP*

Another fingerprinting technique, used in microbial ecology is single-stranded-conformation polymorphism (SSCP). In this technique DNA fragments, such as PCR products obtained with primers specific for the 16S rRNA gene, are denatured and directly electrophoresed on a non-denaturing gel. Separation is based on differences in the folded conformation of single-stranded DNA, which influences the electrophoretic mobility. Lee and coworkers [16] used this technique to study the structure and diversity of natural bacterial communities. Schwieger and Tebbe [36] used PCR-SSCP to analyze microbial communities in the rhizosphere of different plants. To overcome reannealing of DNA strands during electrophoresis, and the presence of multiple bands in the separation patterns, the authors used one phosphorylated primer in the PCR followed by specific digestion of the phosphorylated strands with lambda exonuclease. Scheinert et al. [35] used one biotinylated primer to perform magnetic separation of one single strand after denaturation, and so to exclude the possible reannealing of DNA strands during electrophoresis.

To assess the identity of the predominant bacterial populations in the community, bands can be excised and sequenced, or SSCP-patterns can be hybridized with taxon specific probes. Electrophoretic conditions, such as gel matrix, temperature, and addition of glycerol to the gel, can influence the separation. In addition, only short fragments (between 150 - 400 nucleotides) can be optimally separated.

### *RAPD and DAF*

Randomly amplified polymorphic DNA (RAPD) has been used to follow the response of different soil microbial communities to the application of 2,4-D [48]. Breen et al. [3] used a

similar approach, named DNA amplification fingerprinting (DAF) to compare microbial communities of different bioreactors, and to monitor population changes within one bioreactor. Both techniques use short (5-10 nucleotides) random primers, which anneal at different sites of the genomic DNA, generating PCR products of various lengths. The products are separated on agarose or acrylamide gels, and visualized by ethidium bromide or silver staining.

Reproducibility is the main problem of these techniques; differences in quality and quantity of template DNA and in the concentration of $MgCl_2$ and primer may result in different patterns [7]. Furthermore, no phylogenetic information can be obtained from the bands.

### *Bb-PEG electrophoresis*

Wawer and co-workers developed a simple and rapid electrophoresis method to detect sequence variation in PCR products from bacterial cultures [43] and environmental samples [44]. Electrophoresis is performed in agarose gels containing the DNA ligand bisbenzimide to which long chains of polyethyleneglycol (PEG) are covalently coupled. Bisbenzimide binds to adenine and thymine (A+T) rich sequence motifs in the DNA. Therefore, being loaded with the Bb-PEG conjugate, the A+T-rich DNA molecules are more retarded in the gel than the molecules which are low in A+T, and so separation is achieved.

A strong point is that no expensive electrophoresis equipment is needed; Bb-PEG electrophoresis can be performed in a normal agarose electrophoresis apparatus. Furthermore, identification of community members by sequencing of individual bands or by hybridization analysis is possible. A disadvantage is the limited resolution, because of thick and fuzzy bands, and the price of the Bb-PEG conjugate.

### *RFLP and ARDRA*

Restriction fragment length polymorphism (RFLP) of PCR-amplified rDNA fragments, otherwise known as amplified ribosomal DNA restriction analysis (ARDRA) has also been used to characterize microbial communities (see [22] for a technical description of the method). 16S rDNA fragments are generated by PCR using general primers, digested with restriction enzymes, electrophoresed in agarose or acrylamide gels, and stained with ethidium bromide or silver nitrate. Martinez-Murcia et al. [19] used RFLP analysis of PCR-amplified 16S rDNA fragments to estimate the prokaryotic diversity in hypersaline ponds. Smit et al. [38] used ARDRA to monitor community shifts after copper contamination.

A problem of estimating microbial diversity by ARDRA is that the number of bands in the profile is greater than the number of amplified DNA fragments and therefore overestimates the number of community members. However, this problem was overcome by the use of fluorescently-labelled PCR products as described in the next section.

### *T-RFLP*

This technique, which is in principle similar to RFLP or ARDRA, makes use of fluorescently-labelled PCR products. These products are terminally labelled during the amplification process by the use of one fluorescent primer in the PCR reagent mixture. The amplified genes are digested with a restriction enzyme and subsequently assayed on an automatic DNA sequencer. Only those bands carrying the fluorescent label, the terminal restriction fragments are detected.

Avaniss-Aghajani and co-workers [1] were the first to use this approach to identify different bacteria in complex mixtures. Instead of an automatic DNA sequencer to detect the fluorescently-labelled restriction fragments, they used capillary electrophoresis with a laser-induced fluorescence (LIF) detector. It took more than 3 years until the next paper appeared on the use of this approach in the study of bacterial mixtures. Liu et al. [17] used terminal restriction fragment length polymorphism (T-RFLP) to determine the genetic diversity of microbial communities from activated and bioreactor sludge, aquifer sand, and termite guts. 36 bands could be detected in the termite guts, but as many as 72 bands were detected in the community profiles obtained from the 3 other environments. The same authors used the approach to analyze microbial [18], and eukaryal [20] community diversity in activated sludge. Van der Maarel et al. [41] used T-RFLP to study marine Archaea in the digestive tract and faeces of the flounder. Bruce [4] used the same approach, but under a different name, fluorescent-PCR-restriction fragment length polymorphism (FluRFLP), to analyze bacterial communities in mercury-polluted and pristine soil and sediment samples. Instead of using 16S rRNA, the *mer* (mercury resistant) gene was used as a molecular marker.

Advantages of the technique are (1) the high resolution of separation on an automatic DNA sequencer, (2) the use of intra-lane markers with a different fluorescent label, which facilitates sample-to-sample comparison, and (3) the possibility to quantify bands or peaks directly. Unfortunately, hybridization analysis or sequencing of excised bands for further identification of community members is not possible, because fragments can not be excised or collected. Furthermore, the automatic sequencer needed to perform T-RFLP is very expensive.

## Discussion

The use of genetic fingerprinting techniques has attracted the interest of many scientists working in the field of environmental microbiology. The reason for this is the simplicity and speed to compare the genetic diversity of different communities and to monitor changes in bacterial populations after a shift in environmental conditions. These tasks are possible with all of the described techniques, however, only a few of them, such as LMW RNA profiling, DGGE and SSCP, allow a further characterization of the community members either by hybridization analysis or by sequencing of excised bands. With the exception of LWM RNA fingerprinting, all of the other techniques, i.e., DGGE/TGGE, SSCP, BbPEG, ARDRA, T-RFLP are based on analyzing DNA fragments obtained by PCR amplification of mixtures of bacterial genomes. On the one hand PCR amplification is an excellent means to obtain sufficient material for further analysis, but on the other hand it creates problems, such as chimera formation and preferential amplification. The pitfalls of PCR-based rRNA analysis have been summarized by Wintzingerode et al. [46].

Another problem that might be difficult to overcome, is the limitation of resolution of patterns obtained from very complex, for instance, soil communities. Fractionation of cells (e.g. [14]) or of genomic DNA (e.g. [29]) prior to PCR amplification can reduce complex banding patterns. Simpler profiles can be obtained by the application of specific primers to amplify the 16S rRNA genes of one bacterial group. Also hybridization analysis of complex profiles might give easily interpretable results on the presence of particular bacterial populations.

Another means to obtain simpler profiles is the use of primers that amplify protein-encoding genes. The use of protein-encoding genes opens up the exciting possibility to study gene expression of bacteria in environmental samples [45]. It might reveal differences between related, but ecologically different microorganisms, because the sequences of these functional genes might be more discriminative then genes encoding rRNA [31]. So far, protein-encoding genes were only used in a few genetic fingerprinting studies. Wawer and co-workers [42, 45] used the (NiFe) hydrogenase gene to study the diversity and gene expression of *Desulfovibrio* species in environmental samples. Rosado et al. [32] used the dinitrogenase reductase gene (*nif*H) to study the genetic diversity in *Paenibacillus azotofixans* strains in soil samples. Bruce [4] used the *mer* (mercury resistant) gene to analyze different subclasses within bacterial communities in soil and sediment.

During recent years most papers have dealt with the technical aspects of genetic fingerprinting techniques. However, this has changed, and genetic fingerprinting methods are now routine tools. A comparison of two meetings on genetic fingerprinting of microbial communities nicely shows this difference. The first meeting, held in Braunschweig in 1995, was dominated by discussions on problems and limitations of genetic fingerprinting methods, but the next meeting held in Bremen (Germany) in 1998 focussed on the results generated with these techniques. One aspect that certainly needs more attention is the analysis and interpretation of the fingerprinting results. So far, only a few studies have used statistical methods, such as UPGMA, to interpret community profiles (e.g., [5, 17]). Noble et al. [27] used a back-propagating neural network and cluster analysis to interpret complex

**Table 1**. Overview of strong and weak points of genetic fingerprinting techniques used in microbial ecology

| Technique[a] | Strong points | Weak points |
|---|---|---|
| LMW RNA | straightforward;<br>no in vitro amplification step necessary | rapid degradation of RNA;<br>limited phylogenetic information and length variation of the LMW RNA |
| DGGE/TGGE | identification of community members possible | only short (ca. 550 bp) fragments;<br>double bands and heteroduplex molecules |
| SSCP | identification of community members possible | only short (150-400 nt) fragments;<br>problems of reproducibility |
| RAPD/DAF | no special primer design required | no phylogenetic information obtained;<br>problems of reproducibility |
| Bb-PEG | simple;<br>no expensive equipment | low resolution;<br>availability and price of Bb-PEG dye |
| RFLP/ARDRA | straightforward;<br>no expensive equipment | number of bands not directly related to number of community members |
| T-RFLP/Flu-RFLP | high resolution; intra-lane markers;<br>direct quantification of fragments | no phylogenetic information obtained;<br>expensive equipment |

[a] LMW RNA, low-molecular-weight RNA profiling; DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; SSCP, single-stranded-conformation polymorphism; RAPD, randomly amplified polymorphic DNA; DAF, DNA amplified fingerprinting; Bb-PEG, bisbenzimide polyethyleneglycol electrophoresis; RFLP, restriction fragment length polymorphism; ARDRA, amplified ribosomal DNA restriction analysis; T-RFLP, terminal RFLP; Flu-RFLP, fluorescent RFLP.

LMW RNA banding patterns from free-living and particle-associated microbial assemblages.

In the coming years we can expect a steep increase in the use of molecular fingerprinting techniques, such as DGGE/TGGE and T-RFLP, and in the use of fluorescent PCR products in these techniques. However, although successful in the study of community dynamics, we have to keep in mind that none of these methods is perfect and that they all have their strong and weak points (see Table 1). In this respect only a polyphasic approach combining different molecular biological techniques, microbiological methods, and methods to determine the environmental parameters will lead to an unbiased understanding of the role of microorganisms in their environment.

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