Microbial Ecology

Genotypic Microbial Community Profiling: A Critical Technical Review

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Abstract

Microbial ecology has undergone a profound change in the last two decades with regard to methods employed for the analysis of natural communities. Emphasis has shifted from culturing to the analysis of signature molecules including molecular DNA-based approaches that rely either on direct cloning and sequencing of DNA fragments (shotgun cloning) or often rely on prior amplification of target sequences by use of the polymerase chain reaction (PCR). The pool of PCR products can again be either cloned and sequenced or can be subjected to an increasing variety of genetic profiling methods, including amplified ribosomal DNA restriction analysis, automated ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, single strand conformation polymorphism, and denaturing high-performance liquid chromatography. In this document, we present and critically compare these methods commonly used for the study of microbial diversity.

Introduction

Uncultured organisms comprise the vast majority of the microbial world [3, 32, 48]. Although culturing has been indispensable for increasing our understanding of specific organisms [93], problems with using culturing for community analysis arise from the fact that an artificial homogenous medium typically allows growth of only a small fraction of the organisms. Culturing fails to reproduce the ecological niches and symbiotic relationships encountered in complex natural environments that are required to support the full spectrum of microbial diversity.

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Apart from selectively allowing growth of some species and suppressing growth of others, the community composition of the culturable fraction is distorted during culturing because replication times vary, with fast growing species efficiently outcompeting others. Furthermore, most culture media are extremely rich sources of carbon compared to the substrates normally encountered *in situ*, which may bias the cultured community composition toward copiotrophs [122]. It was frequently observed that direct microscopic counts exceed viable cell counts by several orders of magnitude, leading to the phrase "great plate count anomaly" [3, 118]. Because of the intrinsic constraints of culturing, species richness and evenness obtained by culturing cannot accurately capture the actual *in situ* diversity [3, 18, 129, 132].

The disparity between culturable and *in situ* diversity has increased the importance of culture-independent molecular approaches [3, 32, 38, 39, 92]. Initially, this transition was accomplished using fatty acid profiling [26, 135]; but recently, DNA has become the dominant signature molecule. DNA-based fingerprinting methods that phylogenetically dissect microbial communities have substantially increased our insight into microbial diversity. These methods have become indispensable tools not only in classical microbial ecology, but also in other areas of research because it is now accepted that many behavioral characteristics of individual species can only be explained in a community context.

Except for direct hybridization of sample DNA with probes, DNA-based methods generally employ polymerase chain reaction (PCR) amplification of genetic markers using universal primers capable of amplifying the target genes from a wide variety of different organisms. The predominant target for the assessment of microbial diversity has been the gene coding for 16S rRNA [38, 92]. Functional genes are the basis for studying subpopulations with certain physiological capabilities. Genes like the ones coding for ammonium monooxygenase (*amoA*)

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[36, 139], RNA polymerase B (*rpoB*) [87, 103], methane monooxygenase (*pmoA*) [37, 96], nitrogenase (*nifH*) [107, 137], nitrite reductase (*nirS*/*nirK*) [9, 99], dissimilatory sulfite reductase (*dsrAB*) [29, 78], or mercuric reductase (*merA*) [80, 115] have been successfully employed as genetic markers. These techniques share an initial direct extraction of total community DNA by any method that is optimal for the specific sample [85]. The DNA serves as template for PCR amplification of genetic targets with PCR products being amenable for profiling. As PCR products from conserved regions amplified by universal primers are often of very similar size, differentiation must be achieved on the basis of the nucleotide composition.

Suitable genetic targets should have both conserved and variable regions. The conserved regions serve as annealing sites for the corresponding PCR primers, whereas the variable regions can be used for phylogenetic differentiation. Ideally, primers should be nondiscriminating to amplify all the target sequences of the given population. Primer design, primer quality, and the ability to judge the integrative character of the primers depend heavily on the available sequence information of the particular target. In the absence of highly conserved regions in the target gene, regions with a lower degree of conservation can be chosen as annealing sites for degenerate primers. An increasing degree of degeneracy, however, also increases the risk of nonspecific amplification.

In this document, we compare the genotypic methods commonly used for the study of microbial diversity in ecosystems. An overview is given in Table 1. All methods described have been successfully employed in microbial ecology. With the exception of the cloning approach, many fragments can be analyzed simultaneously in a single run, providing snapshots of a community, even without knowing in detail the species contributing to the signals. By using these methods, the analysis of metagenomes provides valuable information concerning genetic diversity, species richness, and population structure. It also allows cross-comparisons of different communities, monitoring of temporal shifts resulting from changing environmental parameters [97], evaluation of bioremediation impacts [56], and ecological modeling. Rapidly growing databases increasingly enable researchers to predict with a high probability what species might be present in the corresponding community [3, 38].

Cloning and Sequencing

Cloning of PCR-amplified sequences is the first step for subsequent species identification by sequencing. Some cloning strategies make use of an overhanging 3′-A added to PCR products by the *Taq* polymerase. The "sticky end" allows efficient ligation into vectors with an overhanging 3′-T [50, 61].

After cloning into the vectors, sequencing of the inserts is the method offering the highest phylogenetic resolution, allowing either species identification or determination of similarity to already known species through the use of extensive and rapidly growing sequence databases. Sequencing of clone libraries from environmental samples has led to a wealth of information about prokaryotic diversity [116]. The Ribosomal Database Project (RDP) on DNA currently has more than 253,000 entries (as of July 2006) and is frequently updated (http://rdp.cme.msu.edu/index.jsp). Sequencing is the basis for construction of phylogenetic trees and for other comparative studies. Advances in sequencing technology and the cost-effectiveness of high-throughput systems in large genomic service facilities have increased the popularity of direct sequence analysis of clones as an alternative to laborious screening of clones by restriction analysis.

A high degree of automation is possible with 96- and 256-well plate technology, beginning at the level of colony picking. Automation increasingly allows direct sequencing of clones without prior screening, even with highly redundant communities (which is often the case in natural environments). Screening (e.g., by applying amplified ribosomal DNA restriction analysis [ARDRA]) to limit sequencing to a number of selected operational taxonomic units has been very common because a large number of clones must be sequenced to detect rare organisms against a background of a few dominant species. For this reason the collection of detailed sequence information was normally only possible for a limited number of samples because of the labor intensity of the approach [123].

An alternative to the sequencing of specific conserved genes is the sequencing of randomly cloned community DNA, so-called "whole-genome shotgun cloning." The sequencing templates are relatively small pieces of genomic DNA produced by physical shearing and size fractionation [28]. Advances in sequencing technology will increasingly encourage massive efforts to capture the genomes of the total microbiota of a specific environment, termed metagenome [105, 106]. A revolutionary step was taken by Venter et al. [126] who reported more than one million kilobase pairs of nonredundant sequence from their attempt to sequence the entire metagenome of the Sargasso Sea. The same shotgun sequencing approach was used to elucidate the metagenome of a natural acidophilic biofilm [125]. The interpretation of the resulting wealth of genomic information will rely on powerful computational tools. Such holistic sequencing of environmental genomes provides deeper insight into microbial diversity and might allow us to better understand the metabolic and biogeochemical potential of the examined community [105], thereby narrowing the gap between diversity and function.

However, due to our limited knowledge of the relationship between gene homology and actual function (and activity) these data have to be interpreted with caution.

Amplified Ribosomal DNA Restriction Analysis

Amplified ribosomal DNA restriction analysis, also known as restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes, is a simple method based on restriction digestion of the PCR-amplified ribosomal community DNA followed by electrophoretic separation of restriction fragments on high percent agarose or acrylamide gels [51, 65, 70]. Because one single restriction enzyme sometimes does not provide sufficient genotypic resolution (different species can yield identical patterns), multiple restriction enzymes are used either separately or in combination. Moyer et al. [71] evaluated the selection of tetrameric restriction enzymes for 16S rRNA gene analysis.

Amplification products are either processed as a pool [1, 64] or cloned to achieve separation of individual sequences for further analysis [91]. In the case of restriction digestion of a pool of PCR products, the subsequent separation can result in complex patterns. The fact that a single species can produce four to six restriction fragments using a 4-base cutting enzyme makes community patterns very complex and difficult to compare. This, together with the limited staining sensitivity using DNA binding dyes, results in a suppression of bands from less abundant community members and thus in a loss of phylogenetic information [123]. The use of 6-base cutting enzymes has been suggested to reduce the number of restriction fragments per species [45] and might make ARDRA also applicable to more complex species-rich samples. The construction of a clone library and individual processing of clones avoids this limited sensitivity and resolution but requires individual reamplification of cloned inserts and restriction of the resulting PCR products. Although this approach is more labor- and time-intensive, it has been successfully chosen for examining the microbial diversity associated with the seagrass *Halophila stipulacea* [133]. Sequence analysis of clones with identical ARDRA patterns confirmed that the group members were closely related to each other. Amplified ribosomal DNA restriction analysis is also commonly used to cluster-isolated bacterial species into genotypic groups. Pan et al. [94] succeeded in grouping 165 halophilic archaea from three different hypersaline lakes in Inner Mongolia into 14 genotypes. The importance of carefully choosing the restriction enzymes is exemplified by a study examining the pathogenic strains associated with hazelnut decline in Greece and Italy [110]. Only one enzyme out of nine was useful for grouping 53 isolated *Pseudomonas avellanae* strains. All other enzymes failed to separate the strains. The two groups revealed 57% genetic relatedness.

In general, ARDRA is a highly useful tool for detecting structural changes in relatively simple microbial communities, but is not the method of choice to measure diversity or to detect specific phylogenetic groups [45, 54].

Automated Ribosomal Intergenic Spacer Analysis

Intergenic transcribed spacer (ITS) regions are located between the 16S and 23S ribosomal genes and display significantly higher heterogeneity in both length and nucleotide sequence compared to the flanking genes. Both types of variations make them suitable for subtyping bacterial strains and closely related species in cases where the fingerprinting of ribosomal gene sequences does not provide sufficient resolution [45, 57]. Automated ribosomal intergenic spacer analysis (ARISA) relies on the variation of species-specific amplicon sizes. A community profile is obtained by electrophoretic separation of fluorescently labeled PCR products and subsequent sensitive laser detection on an automated sequencing system. Fisher and Triplett [27] reported fragment size polymorphisms in the range between 400 and 1,200 bp in a study of the microbial diversity of three freshwater environments in Wisconsin.

Potential problems are the preferential amplification of shorter templates [27] and the fact that because of interoperonic length variation within a single genome [77], a single organism can contribute more than one signal. Jensen et al. [42] reported that around 85% of the species they examined produced two or three peaks. Despite these limitations, ARISA can be very useful for community analysis that requires fine-scale resolution of subtle differences. Automated ribosomal intergenic spacer analysis was successfully used to demonstrate that bacterioplankton communities vary significantly along an estuarine gradient of Moreton Bay, Australia [33] or to establish an "impact order" of individual heavy metals and their combined administration on soil bacterial community structures [101]. In another application, ARISA could assign specific bacterial and fungal community patterns to different soil types [102].

In addition to ITS length determination, amplified ITS regions can be subjected to RFLP analysis (analogous to ARDRA) and/or to DNA sequencing. Both RFLP and sequencing of PCR-amplified ITS 1 regions were applied to distinguish with high resolution different *Pseudomonas stutzeri* strains [31]. Intergenic transcribed spacer/restriction fragment length polymorphism was also found to increase the discrimination of different soft rot *Erwinia* species and their identification compared to ITS/PCR alone [124].

Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) makes use of the powerful resolution of automated sequencing technology and avoids some of the limitations of RFLP analysis (manual labor, low sensitivity, and low genotypic resolution). Marker genes are PCR-amplified using a fluorescent dye attached to the 5′-end of one of the primers so that the products become labeled [5, 12, 54, 62, 63, 88]. Polymerase chain reaction products are subsequently restriction-digested, typically using 4-base cutters. The mixture of restricted PCR products is physically separated using acrylamide sequencing gels or sequencing capillaries. In contrast to ARDRA, only labeled terminal fragments are detected using a laser to produce an electropherogram reducing the complexity of the profiles. The polymorphism is based solely on one parameter, the fragment length. A size standard labeled with a different fluorophore allows the precise assignment of fragment lengths with single base pair resolution.

The fact that the polymorphism is based solely on fragment length allows direct reference to sequence databases such as the rapidly expanding RDP [13, 54, 58, 63]. By performing *in silico* amplification and digestion of the entire 16S rRNA database as a surrogate community [63], RDP can inform researchers about which primer–enzyme combination will be optimal. More importantly, the comparison of experimentally determined fragments with the fragments predicted from cognate 16S rRNA gene sequences in the database may allow phylogenetic assignments of signals or predictions of which organisms might be present in a specific sample [19, 44, 62, 63]. Analysis of independent digests using multiple restriction enzymes increases specificity because an individual terminal restriction fragment (T-RF) may correspond to 15 or more species [44, 63]. It also increases the confidence of ecological interpretation as the use of different restriction enzymes on the same sample results in different fingerprints and can result in different diversity predictions [18, 47].

Species identification by comparison of complex communities with internal and external laboratory databases is computationally challenging, but has become more straightforward through implementation of Web-based automated assignment tools [44]. A prerequisite, however, is the exact determination of fragment lengths. Discrepancies in fragment length between the experimental and predicted (*in silico*) value for a known sequence are often observed [34, 44, 46]. This problem is addressed by using tolerance ranges for length assignment (e.g., ±1 or 2 bases) to allow for matching with database entries. This fact, however, increases the numbers of species associated with each fragment and complicates community predictions given the large number of T-RFs in complex samples. Different fluorescent labels might affect fragment migration in the acrylamide matrix differently, raising problems for length assessment if the size standard and the sample contain incompatible labels. Neufeld and Mohn [81] observed that the fluorophores Cy5 and Cy3 did not visibly alter the migration of PCR products in an acrylamide gel matrix compared to unlabeled PCR products, whereas 6-carboxyfluorescein resulted in retardation. The use of incompatible dye combinations might explain size deviations in capillary-based T-RFLP analysis.

Although T-RFLP allows for highly sensitive detection of labeled DNA fragments and is compatible with high-throughput analyses, one drawback is the need for restriction digestion. Incomplete or nonspecific restriction leads to an overestimation of diversity by increasing the number of fragments. However, restriction efficiency can be tested by including the amplified product from a well-characterized individual sequence in the restriction step [63]. This control PCR product should contain a different fluorescent label from the PCR products being analyzed. The presence of more than one control signal indicates partial digestion.

Overestimation of diversity can also originate from "pseudo-T-RFs" deriving from single-stranded amplicons, which occur during PCR and are thought to form transient double-stranded secondary structures accessible to restriction enzymes [21]. Cutting results in unpredictable fragments dependent on the random presence of restriction sites that are formed by single strands partly annealed in secondary structures. The resulting pseudo-restriction fragments increase the apparent number of T-RFs and have been suspected in a number of studies [22, 23, 41, 83, 95]. Furthermore, the salt required for restriction must be removed to obtain clean runs. Column purification for salt removal is preferable because alcohol precipitation can result in loss of DNA. This time-consuming manual step negates some of the advantages of automation.

Despite its limitations T-RFLP has become a valuable method for rapidly comparing the relationships between bacterial communities in environmental samples and temporal changes. It is a valuable method for comparison of complex communities when high throughput and high sensitivity are required without the need for direct sequence information. An example is the distinction of the human intestinal microfloras from different individuals [43]. In the same study, T-RFLP was also used for monitoring changes resulting from antibiotic treatment and from ingestion of a probiotic product. Dunbar et al. [18] successfully applied the method for differentiating four soil communities representing two pinyon rhizospheres and two between-tree soil environments. The results were consistent with those obtained by analysis of clone libraries. It was concluded, however, that different restriction digests provided variable data as a measure of

relative phylotype diversity and community evenness for these highly complex communities. An insight into the importance of data processing, which is currently the least defined aspect of T-RFLP analysis, was gained by Blackwood et al. [8] and Osborne et al. [89] while they were optimizing data analysis for comparing various soil and sediment samples.

Denaturing Gradient Gel Electrophoresis and Temperature Gradient Gel Electrophoresis

In both denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), small PCR products (around 200–700 bp) are separated on an acrylamide gel having a low to high denaturant gradient [74, 75]. One of the primers carries a 5′-GC rich artificial clamp of around 40 bp [76]. Because PCR fragments analyzed by DGGE and TGGE are generally of the same length (within a few base pairs), they cannot be separated on agarose or nondenaturing acrylamide gels. Whereas a chemical denaturation gradient is applied in DGGE, TGGE uses a temperature gradient. The direction of electrophoresis is perpendicular to the denaturing gradient. Initially, products are separated according to molecular weight, primarily determined by GC content. As they progress through the gel and are subjected to increasingly strong denaturing conditions, PCR products reach a point where strand separation of double-stranded DNA occurs. The melting behavior depends mainly on the length of the product, its GC content, and the nucleotide sequence. The higher the intrinsic stability, the stronger the denaturant condition must be to achieve strand separation. The physical shape of the molecules directly affects their mobility during electrophoresis. Initially, the melting process is only partial, with discrete domains becoming a single strand. Such partially denatured DNA migrates more slowly in the gel compared to the native conformation. As melting progresses, retardation increases. Eventually strand separation stretches over the entire length with the exception of a GC-rich clamp [76]. This clamp has a very high melting point and holds the strands together leading to a "butterfly-shaped" molecule whose migration in the gel is strongly retarded compared to unmelted molecules.

As described above, strand separation can be achieved using the denaturing chemicals formamide (ranging from 0–40%) and urea (ranging from 0–7 M) in the case of DGGE (with a constant gel running temperature of around 60°C), or using a suitable heat gradient in the case of TGGE (which uses no chemical denaturants). Gradients might have to be adjusted to the specific sample for optimal resolution [84]. These methods have the potential to detect differences in the melting behavior of small DNA fragments that theoretically differ by as little as a single base substitution.

The presence of the GC clamp attached to the 5′-end of one of the PCR primers typically tends to lower the PCR amplification efficiency, which is observed as decreased product yields. It also tends to increase the risk of artifact generation in the annealing steps and heteroduplex formation [25, 52, 108]. Low amplification efficiency becomes a problem with some environmental templates that are already difficult to amplify because of the presence of inhibitory substances. If melting points of PCR products are sufficiently high without the GC clamp, the clamp is not essential. This explains why equally good profiling has been observed without using a GC clamp, provided that PCR products have a minimum length and GC content and that gels are not run to the point where complete strand separation is achieved. In this regard it is important to realize that double-stranded DNA melts in discrete domains rather than in a zipper-like fashion. Given sufficiently high melting points of PCR products, a partial denaturation involving individual domains is sufficient for successful separation of a PCR mixture.

The advantages of the method are its affordability for ordinary laboratories and the relative ease in interpreting the results. Also, individual bands of interest (e.g., bands that distinguish communities) can be excised from the gel and identified via sequencing. However, amplification of different phylotypes with similar electrophoretic mobility may result in low DNA sequence quality [15, 111]. Cloning of excised bands results in cleaner sequences compared to direct sequencing, but is significantly more laborious. The use of DGGE/TGGE for screening communities before sequencing can further be limited by the small fragment sizes of PCR products. Sequencing of 300–400 bp might not contain enough information for precise taxonomic classification [90]. Moreover, handling of big gels, problems during PCR (the GC clamp favors primer–dimer formation) [68], and variable gel staining all decrease reproducibility. A problem with using conventional gels for electrophoresis is the significant background staining, which makes it hard to distinguish between background and weak bands originating from less abundant species in the sample. Software used to reduce background artificially introduces a threshold. By converting a smeary image to patterns with sharp bands [2], the final image may no longer reflect the whole community. Moreover, band visualization by traditional staining of gels results in low sensitivity. Another disadvantage of conventional gels is the production of images instead of a digital output in the form of an electropherogram. Semiquantitative analysis is only feasible by cumbersome determination of signal intensities of all bands.

Some limitations can be overcome by working with fluorescently labeled PCR products. The use of fluorescent labels can greatly improve the sensitivity of detection.

Moeseneder et al. [68] reported an increase in sensitivity of around one order of magnitude using a fluorescence label instead of a nucleic acid stain for band detection. Normally the primer, which does not have the 5′-GC-clamp, is labeled.

Fluorescent labels also allow the inclusion of an intralane standard labeled with a different terminal fluorophore [72, 73]. These standards are often needed because gradients in researcher-prepared gels tend to be somewhat variable, creating the need for rigorous normalization to allow gel-to-gel comparisons [24, 81]. Intralane standards facilitate normalization of samples within gels and between different gels [81].

Denaturing gradient gel electrophoresis has been most useful to compare community structural changes in response to a perturbation, for example, a change in the menu of substrates available to the microbial community. Sun et al. [120] used DGGE to document differences in the microbial communities in soil that was either untreated or amended with manure or inorganic fertilizer. Duineveld et al. [17] compared bulk soil and rhizosphere microbial communities to demonstrate enrichment of the latter community by root exudates.

Single Strand Conformation Polymorphism

Single strand conformation polymorphism (SSCP) is an electrophoretic method widely used in mutation analysis and has been adapted to the analysis of microbial communities [52]. Like DGGE/TGGE, this genetic profiling method allows separation of PCR products of similar length but with sequence diversity. In contrast to DGGE/TGGE, the separation is based not on double-stranded DNA, but on single-stranded DNA. Strand separation is achieved under denaturing conditions before loading on a nondenaturing acrylamide gel. As an alternative to traditional gels, a capillary array sequencer can be used for automated high-throughput analysis [6].

Under nondenaturing conditions, single-stranded DNA adopts a folded secondary structure. The structure is determined by intramolecular interactions affecting the 3-D conformation, which depends on the nucleotide sequence and the physicochemical environment (e.g., temperature and ionic strength). These secondary structures are used to distinguish between products from different phylotypes even if they have the same molecular weight. Different conformations produce different migration behaviors and mobilities in the gel, enabling the separation of complex mixtures of community DNA [86, 136]. In the case of short PCR products, a single mutation can substantially alter the secondary structure of the single-stranded DNA, thereby leading to different migration velocities and separation in the gel.

Analogous to DGGE or TGGE, individual distinct bands of interest can be isolated and sequenced after extraction from the acrylamide gel. The single-stranded DNA must then be PCR-amplified and cloned into a suitable vector. An advantage compared to DGGE/TGGE and T-RFLP is that no clamped primers or restriction digestions are required. A major limitation of SSCP, however, is the high rate of reannealing of single-stranded DNA during electrophoresis [112]. This is especially critical when loading high DNA concentrations on the gel, which might be required for analysis of high-diversity communities [109]. The band resulting from reannealed strands adds to the two bands produced by the two single strands. Sequences that can adopt multiple coexisting conformations would further increase the number of bands, whereas similar conformations decrease the number of bands [109, 123]. The fact that one species can be represented by multiple peaks complicates the interpretation of results. Reannealing of single strands and heteroduplex formation of strands with similar sequences can be avoided if one of the two primers has a 5′-phosphate group. This modification allows selective removal of the corresponding phosphorylated strand through digestion with lambda exonuclease. This treatment has been shown to reduce concentrations of double-stranded products to below the detection level [109]. Another problem associated with SSCP is that the position of a band in the gel cannot be predicted. Although algorithms for predicting secondary structures of known sequences exist, it is not possible to predict the migration speed (under the given conditions) of the thermodynamically most favored conformation.

Single strand conformation polymorphism was successfully used to show that crenarchaeal consortia associated with rhizospheres from a diverse range of terrestrial plants are distinct from the ones inhabiting the surrounding bulk soils and generally more species-rich [117]. Single strand conformation polymorphism also enabled the study of dynamics and maturation of a biofilm grown on polychlorinated biphenyl (PCB) droplets and the assignment of community diversity to distinct stages of PCB degradation [55].

Denaturing High-performance Liquid Chromatography

Denaturing high-performance liquid chromatography (DHPLC) was originally developed for gene mapping, mutation detection, and identification of clinical isolates [67, 79, 130, 138], but recently also has been applied for analysis of environmental communities. A heterogeneous mixture of 16S PCR products is separated using high-performance liquid chromatography (HPLC) technology rather than an acrylamide matrix. Both temperature and chemical denaturation are used to achieve separation of PCR products of similar size. DNA is injected into an oven-based HPLC cartridge in a solution containing

triethylammonium acetate (TEAA) and acetonitrile. The TEAA dissociates in solution forming the positively charged TEA+ that has both a hydrophobic and a hydrophilic end. The hydrophobic end binds to the hydrophobic beads in the cartridge whereas the positive charge forms ionic bonds to the negatively charged phosphate backbone of double-stranded DNA. The TEA+ molecules thus serve as a bridge to bind DNA to the cartridge material. The strength of DNA binding depends on the fragment length and the content and the position of G and C nucleotides. Differential elution of the bound DNA mixture is achieved by an increase in temperature and an increasing gradient of acetonitrile that weaken the bridging capabilities of the TEA+ ions.

Eluted PCR products pass through a UV detector that records absorbance over time in the form of an electropherogram. Alternatively, fluorescent labels can be attached to PCR products, resulting in substantially higher sensitivity (up to 100 times) in comparison to absorbance at 260 nm, allowing the analysis of less DNA. The high-throughput capacity of the method, short run times, sensitivity, and relative ease of use might render DHPLC a promising profiling method given the necessity of fast and economic community analysis. Fragment recovery can be achieved by adding a fraction collector. Fragments are then available for reamplification, followed by sequencing or T-RFLP phylotyping. Although the best resolution is achieved with PCR product lengths in the range between 150 and 450 bp, fragments up to full-length 16S rDNA amplicons (~1550 bp) can be separated. However, the sensitivity decreases with increasing amplicon length. A major advantage of the method is that (like SSCP) it does not require sample manipulation such as the use of clamped primers or restriction digestion, thereby avoiding the inherent problems with these procedures.

As the method is relatively new and needs proprietary equipment, there are relatively few examples of its application for analysis of natural bacterial communities. More applications to environmental samples will be needed for further validation. Hurtle et al. [40] reported successful identification of 36 out of 39 bacterial isolates in a mixture of a broad spectrum of genera. Denaturing high-performance liquid chromatography was also successfully used for identifying bacteria associated with urinary tract infections from renal transplant recipients [16]. A 100% correlation was found between culture-positive specimens and DHPLC-generated peaks. The method facilitated detection of culture-negative, previously unknown uropathogens. Furthermore, the method has been successfully applied to the analysis of unknown biofilm communities harvested from metal coupons [114]. Technical difficulties were reported in finding an optimal temperature and gradient for elution, especially if the microbial community contains species of widely varying GC contents. Run conditions with unknown samples had to be optimized on a trial and error basis. Barlaan et al. [7] found that the incorporation of a 40-bp CG clamp into the forward primer helped to discriminate genetic differences in defined mixtures containing different marine species™. Primers with a 20-, 10-, or 0-bp CG clamp were less efficient. The optimized conditions were successfully applied to analyze bacterial community composition in an environmental seawater sample. The results correlated with the DGGE pattern obtained from the same samples.

Concluding remarks

Several well-characterized molecular biological techniques can be applied for genetic analysis of microbial communities. The choice of the analysis method depends upon the complexity of the community, the expertise of personnel in the laboratory, the required depth of analysis, availability of instrumentation, and budget and time constraints. Many community profiling techniques are used in conjunction with sequencing. Profiling methods are often used for rapidly screening and comparing communities, but the necessity to phylogenetically identify community members makes sequencing of individual signals or of an entire clone library unavoidable. The trend is typically toward automation, allowing high-throughput analysis, minimal handling, cost effectiveness, and increased reproducibility. In many cases detection of community profiles can be automated using capillary technology instead of acrylamide gels. Compared to a band pattern obtained from conventional gels, the resulting electropherogram can be directly (without scanning and conversion steps) fed into statistical analysis programs to calculate and graphically present relative differences between profiles. Furthermore, the detection of fluorescence provides a significant increase in sensitivity compared to traditional gel staining methods. For example, traditional gel-based DGGE is estimated to detect only community members representing at least 1–2% of the microbial population in an environmental sample [56, 74, 119]. When both DGGE and T-RFLP were used to detect ribotypes in various soil samples, T-RFLP was found to be at least five times more sensitive [123]. This observation might favor the application of T-RFLP on more complex communities with many less abundant species over DGGE/TGGE. Denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis is, however, valuable for communities with a limited number of abundant members. The problem of limited sensitivity in applying DGGE on complex communities might be overcome by limiting the analysis to a specific fraction of the community prior DGGE. Duineveld et al. [17] were able to simplify DGGE profiles from soil communities by including a prior culturing step. Holben et al. [35]

Table 1. Overview of advantages, disadvantages, and main applications of different genotypic profiling methods

Genotypic methods	*References of applications in this review*
Cloning and sequencing	
Advantages	[116, 125, 126]
High phylogenetic resolution	
Allows species identification/determination of closest phylogenetic neighbor	
Disadvantages	
Although the sequencing can be automated, the cloning is time-consuming	
Suitability	
When phylogenetic assignment is relevant and high-throughput sequencing service is available	
ARDRA	
Advantages	[94, 110, 133]
No special equipment needed	
Disadvantages	
Needs several restrictions for adequate genotypic resolution	
Time- and labor-intensive	
Suitability	
For low resolution comparisons of simple communities or as a screening tool for identification of clones of interest for sequencing	
ARISA	
Advantages	[31, 33, 101, 102, 124]
Allows high resolution of subtle differences	
Compatible with RFLP and sequencing for further downstream analysis	
Disadvantages	
A single organism can contribute more than one signal because of interoperonic length variation	
Suitability	
For high resolution of subtle species differences	
T-RFLP	
Advantages	[18, 43]
High sensitivity	
High throughput and short run times	
Potentially direct phylogenetic assignment of signals	
Allows good between-runs comparability	
Disadvantages	
Incomplete restriction digestion can result in overestimation of diversity	
Multiple restrictions are needed for precise analysis	
Complicated profiles make phylogenetic assignments very challenging	
Restriction digestion can result in pseudo-T-RFs	
Suitability	
The high sensitivity allows application to communities with higher species richness	
The good comparability between runs makes it suitable for study of	
Time courses and for large sample numbers	
DGGE/TGGE	
Advantages	[17, 120]
Bands of interest can be excised from gel for sequencing	
Affordability	
Disadvantages	
Limited sensitivity	
Primer GC clamp decreases yield and favors primer dimers	
Handling of gels needs experience	
Difficult comparability between gels because of gel variability	
Suitability	
For communities with a limited number of abundant members	
SSCP	
Advantages	[55, 117]
No clamped primers or restriction digestion required	
Bands of interest can be excised from gel for sequencing	
Compatible with automated high-throughput analysis	
Disadvantages	
High rate of reannealing of single strands with high DNA concentrations	
Multiple bands per species possible	
Suitability	
When high sensitivity is desired without the need of restriction digestion or GC clamp	

Table 1. (Continued)

Genotypic methods	*References of applications in this review*
DHPLC	
Advantages	[7, 16, 40, 114]
High throughput and short run times	
High sensitivity when using fluorescent labels	
No sample manipulation necessary	
Disadvantages	
Separation parameters have to be optimized for different samples	
Suitability	
Promising for automated and fast analysis after initial optimization, but more validation for its application in microbial ecology is needed	

For more details, refer to text.

fractionated an environmental DNA extract based on GC content before performing DGGE on the various fractions in an attempt to simplify profiles and detect rare community members.

Another advantage of T-RFLP over DGGE/TGGE might be the better comparison of fingerprints from different runs, which is difficult with DGGE because of variations in the denaturing gradient of hand-cast gels. On the other hand, DNA sequence information can be retrieved from DGGE gel, whereas it can only be inferred using T-RFLP. DGGE would probably be a better choice when the goal is to describe previously unknown microbial diversity.

All PCR methods share limitations mainly caused by inefficient or preferential extraction of community DNA [30, 32, 82], varying efficiency of different extraction methods in removing inhibitory substances and in maintaining the integrity of DNA and amplification biases during PCR [66, 127, 128]. Profiles generated by PCR-based methods are a quantitative reflection of the PCR product pool. They are not, however, a quantitative reflection of the original community because of the inherent biases of the end-point PCR methodology. Differences in gene copy number [14], primer specificity [134], and amplification efficiency [53, 98, 104, 121]; sensitivity to template concentration [11]; and the formation of chimeric sequences [49, 131] all lessen the reliability of these methods for quantification of microbial communities [98]. Moreover, the transition from the exponential phase to the plateau phase of the PCR amplification curve occurs earlier for the more abundant species present in the sample, resulting in distortion of community proportions [32, 121]. Less abundant species increasingly "catch up" during later cycles, a phenomenon that can readily be observed using real-time PCR. Awareness of this behavior at least permits some semiquantitative assessment of community population distribution in most cases. Despite these drawbacks, comparative community analysis has evolved into a valuable tool, providing rapid information about the degree of difference between communities from varying environments or communities exposed to different conditions.

Common PCR biases (except gene copy number heterogeneity) can be considerably reduced by using high template concentrations, by performing fewer cycles, and by pooling products from replicate PCR reactions [98]. A problem shared by all methods involving PCR amplification is the frequent occurrence of heteroduplexes between similar sequences, which can result in false signals [52]. Another limitation of DGGE, T-RFLP, and SSCP is that only fragments with a maximum length of about 500 nucleotides can be separated well. In the case of 16S analysis, this limits analysis to no more than three (out of nine) variable regions. This limitation can be overcome by cloning near full-length 16S rDNA PCR products, using nested PCR/DGGE to screen the clone library, and performing DNA sequencing on the clones rather than DNA from excised gel bands [10].

Apart from sensitivity, automation and high-throughput requirements, high resolution with minimal background, and real-time digital output of data will be required for future improvement in the methods. Ideally, community fingerprints should translate directly into taxonomic information using phylogenetic assignment tools (similar to those used in T-RFLP) through the use of the Web-based resources. The same resources that have been established for 16S T-RFLP in the form of the RDP database might be available for ARISA someday. Currently, the sequence databases have far more entries for 16S genes than for ribosomal ITS regions, although the two techniques may otherwise have many advantages in common. Community fingerprint methods are helpful as a comparative tool, but profiles should ideally be related to species composition.

Furthermore, linking community composition, activity and function is a critical issue in microbial ecology. This link will be strengthened by combining genotypic profiling with proteome and the emerging metabolome analysis, which monitor the total protein or the total metabolite composition of a given sample. Whereas genomics only indirectly includes information about the

metabolic potential of a certain community based on the physiological knowledge of its individual members, proteomics and metabolomics would add to the current status of protein expression and metabolic activity at the time point of harvesting. Increasing affordability of these still prohibitively expensive techniques for many laboratories and increasing development of robust and user-friendly instrumentation will foster the application of these approaches, nicely complementing genomics and contributing to more holistic community analysis.

Steps toward linking genomics with activity have already been successfully performed by isotope substrate labeling to better understand metabolic cycling within communities. Stable isotopes are incorporated into DNA of organisms that can metabolize the labeled substrate, allowing its separation from unlabeled DNA by density centrifugation [69, 100, 105]. This helps limit the analysis to members of the community that can actively metabolize the corresponding substrate.

Another approach is the use of mRNA or rRNA transcripts because the transcript level is related to activity. The study of mRNA from functional genes is suitable for providing insight into activity. Relatively recent technical advances like RNA-preserving solutions enable researchers to conserve environmental field samples at room temperature for later laboratory-based RNA extraction and further analysis. Rapid degradation of RNA in dead cells enables researchers to focus on the viable portion of a community. Stable isotope probing can also be applied to RNA, further linking phylogeny to function and activity [59, 60]. Another interesting development in this respect is RNA fingerprinting that uses RNA arbitrarily primed-PCR [4, 113]. The method depends on arbitrary reverse transcription of a subset of a total RNA population using random primers and separation of the resulting cDNA on denaturing polyacrylamide gels after PCR amplification. Sequencing of cloned cDNA adds a detailed structural and functional component to the fingerprints.

Hybridization-based techniques like fluorescence *in situ* hybridization and gene arrays have not been included in this review because they are not true fingerprinting methods. As probes have to be designed beforehand, this approach is normally chosen with extensive knowledge of the community.

The methods described in this manuscript represent the current state of the art in molecular methods for community profile analysis. These methods have advanced from extremely time-consuming approaches used by few to relatively fast and easy techniques, gaining widespread use. It is entirely probable that the next few years will see increased automation, decreased costs, and improved sensitivity. These developments will ultimately lead to an increased ability to elucidate complex community relationships without the biases inherent in traditional culturing.

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