Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA

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We describe a new molecular approach to analyzing the genetic diversity of complex microbial populations. This technique is based on the separation of polymerase chain reaction-amplified fragments of genes coding for 16S rRNA, all the same length, by denaturing gradient gel electrophoresis (DGGE). DGGE analysis of different microbial communities demonstrated the presence of up to 10 distinguishable bands in the separation pattern, which were most likely derived from as many different species constituting these populations, and thereby generated a DGGE profile of the populations. We showed that it is possible to identify constituents which represent only 1% of the total population. With an oligonucleotide probe specific for the V3 region of 16S rRNA of sulfate-reducing bacteria, particular DNA fragments from some of the microbial populations could be identified by hybridization analysis. Analysis of the genomic DNA from a bacterial biofilm grown under aerobic conditions suggests that sulfate-reducing bacteria, despite their anaerobicity, were present in this environment. The results we obtained demonstrate that this technique will contribute to our understanding of the genetic diversity of uncharacterized microbial populations.

Only an estimated 20% of the naturally occurring bacteria have been isolated and characterized so far (22). Selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat. Furthermore, many microorganisms are bound to sediment particles and are thus not detected by conventional microscopy.

Molecular biological techniques offer new opportunities for the analysis of the structure and species composition of microbial communities. In particular, sequence variation in rRNA has been exploited for inferring phylogenetic relationships among microorganisms (23) and for designing specific nucleotide probes for the detection of individual microbial taxa in natural habitats (2, 10). These techniques have also been applied to determining the genetic diversity of microbial communities and to identifying several uncultured microorganisms (9, 21). They constitute the cloning of ribosomal copy DNA (21) or polymerase chain reaction (PCR)-amplified ribosomal DNA (rDNA) (9) followed by sequence analysis of the resulting clones.

Here, we present a new approach for directly determining the genetic diversity of complex microbial populations. The procedure is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In denaturing gradient gel electrophoresis (DGGE) (6), DNA fragments of the same length but with different base-pair sequences can be separated.

Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is decreased compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE (12).

This technique has been successfully applied to identifying sequence variations in a number of genes from several different organisms. DGGE can be used for direct analysis of genomic DNA from organisms with genomes of millions of base pairs. This is done by transferring separation patterns to hybridization membranes by capillary blotting with modified gel media (3) or by electroblotting (11, 20) followed by analysis with DNA probes. Alternatively, PCR (17) can be used to selectively amplify the sequence of interest before DGGE is used (4). In a modification of the latter method, GC-rich sequences can be incorporated into one of the primers to modify the melting behavior of the fragment of interest to the extent to which close to 100% of all possible sequence variations can be detected (14, 18).

In this paper, we describe the application of DGGE to the analysis of fragments derived from the variable V3 region of 16S rRNA (16). These fragments were obtained after amplification of 16S rDNA genes from genomic DNA from uncharacterized mixtures of microorganisms. The results demonstrate the presence of up to 10 different rDNA fragments in microbial communities of different origins. By subsequent hybridization analysis with group-specific oligonucleotide probes, particular constituents of the population could be identified. This procedure allows one for the first time to directly identify the presence and relative abundance of different species and thus, to profile microbial populations in both a qualitative and a semiquantitative way.

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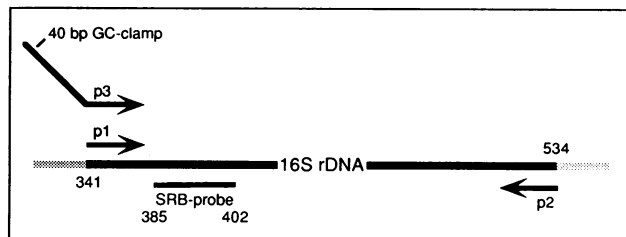† Present address: Max Planck Institute for Marine Microbiology, Fahrenheitstrasse 1, 2800 Bremen 33, Germany.

FIG. 1. Schematic diagram of the rDNA region amplified by PCR in this study. Primers 1 and 2 amplify a fragment of 193 bp, which corresponds to position 341 to position 534 in the 16S rDNA of *E. coli*. Primers 3 and 1 amplify the same region but incorporate a 40-bp GC clamp at its 5′ end at position 341, after which the total size of the fragment increases to 233 bp. The oligonucleotide probe (SRB) used in this study is specific for sulfate-reducing bacteria and corresponds to position 385 to position 402 in the 16S rDNA of *E. coli*.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* DH5α was obtained from N. Goosen (Leiden University, Leiden, The Netherlands). *Desulfovibrio desulfuricans* isolated from the Wadden Sea sediment (The Netherlands) was provided by J. Vosjan (NIOZ, Texel, The Netherlands), and *Desulfovibrio sapovorans* was provided by Simon Bale and Matthew Collins (University of Bristol, Bristol, United Kingdom). *Microcoleus chthonoplastes* and *Thiobacillus thioparus* were isolated from laminated microbial ecosystems (microbial mats) in the Wadden Sea sediments by L. Stal (University of Amsterdam, Amsterdam, The Netherlands), and H. van Gemerden (University of Groningen, Groningen, The Netherlands), respectively. Microbial mat samples obtained from the Slufter sediment on the island of Texel (The Netherlands) were kept alive in the laboratory for several months. Samples 1 and 2 were taken from different depths of the same microbial mat, while sample 3 was taken from another microbial mat specimen. Bacterial biofilms isolated from aerobic and anaerobic wastewater treatment reactors were provided by L. Tijhuis (Delft University of Technology, Delft, The Netherlands).

Nucleic acid extraction. Bacterial genomic DNA was obtained either by (i) freeze-thawing of bacterial cell pellets or (ii) phenol extraction at 55°C and ethanol precipitation. The DNA extracted from the microbial mats and the bacterial biofilms was further purified on a Qiagen column (Diagen, Inc.). These DNA preparations were used as template DNAs in the PCR.

PCR. The variable V3 region of 16S rDNA was enzymatically amplified in the PCR (17) with primers to conserved regions of the 16S rRNA genes (13). The nucleotide sequences of the primers are as follows: primer 1, 5′-C CTACGGGAGGCAGCAG-3′; primer 2, 5′-ATTACCGCG GCTGCTGG-3′; and primer 3, 5′-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3′. Primer 3 contains the same sequence as primer 1 but has at its 5′ end an additional 40-nucleotide GC-rich sequence (GC clamp). A combination of primers 1 and 2 or primers 3 and 2 was used to amplify the 16S rDNA regions in the different bacterial species which correspond to positions 341 to 534 in *E. coli* (Fig. 1). PCR amplification was performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: 250 ng of purified genomic DNA or 1 μl of cell lysate, 50 pmol of each

of the appropriate primers, 200 μmol of each deoxyribonucleoside triphosphate, and 10 μl of 10× PCR buffer (100 mM Tris-HCl [pH 9], 15 mM MgCl, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vol/vol] Triton X-100) were added to a 0.5-ml-volume test tube which was filled up to a volume of 100 μl with sterile Milli-Q water and overlaid with a drop of mineral oil (Sigma Chemicals). The samples were first incubated for 5 min at 94°C to denature the template DNA and subsequently cooled to 80°C, at which point 0.25 U of *Taq* DNA polymerase (SuperTaq; HT Biotechnology, Ltd.) was added. This hot start technique was performed to minimize nonspecific annealing primers to nontarget DNA. The temperature was subsequently lowered to 65°C for 1 min. This temperature, which is 10°C above the expected annealing temperature, was decreased by 1°C every second cycle until a touchdown at 55°C, at which temperature five additional cycles were carried out (5). This procedure reduces the formation of spurious by-products during the amplification process. Primer extension was carried out at 72°C for 3 min. Amplification products were analyzed first by electrophoresis in 1.5% (wt/vol) agarose gels and then by ethidium bromide staining.

DGGE. DGGE was performed with the Bio-Rad Protean II system, essentially as described previously (7, 15). PCR samples were applied directly onto 8% (wt/vol) polyacrylamide gels in 0.5× TAE (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na₂-EDTA) with gradients which were formed with 8% (wt/vol) acrylamide stock solutions (acrylamide-*N*,*N*′-methylenebisacrylamide, 37:1) and which contained 0 and 100% denaturant (7 M urea [GIBCO BRL]) and 40% [vol/vol] formamide (Merck) deionized with AG501-X8 mixed-bed resin (Bio-Rad). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/liter), rinsed for 10 min with Milli-Q water, and photographed with UV transillumination (302 nm) with Cybertech CS 1 equipment.

Electroblotting. After DGGE, the gel was allowed to equilibrate in 1× TBE (89 mM Tris-borate [pH 8], 89 mM boric acid, 2 mM Na₂-EDTA) for 15 min. The polyacrylamide gel separation patterns were transferred to a nylon membrane (Hybond-N⁺; Amersham, Amersham, United Kingdom) with an electrotransfer apparatus consisting of two carbon plates mounted in a perspex frame (19). Electrotransfer was performed for about 45 min at a constant amperage of 400 mA (approximately 0.5 mA/cm²). Immediately after being transferred, the membrane was placed for 10 min on a piece of Whatman 3MM filter paper soaked in 0.4 M NaOH–0.6 M NaCl to denature the DNA. It was neutralized by being rinsed twice in a large volume of 2.5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) and was subsequently exposed for 45 s to 302-nm UV light to cross-link the DNA fragments to the membrane.

Hybridization analysis. The membrane was prehybridized for 2 h at 50°C with 50 ml of a solution containing 1% (wt/vol) blocking reagent (Boehringer Mannheim Biochemicals) in 5× SSC–0.1% (wt/vol) sodium dodecyl sulfate (SDS). One hundred nanograms of a ³²P-labelled oligonucleotide probe which is specific for sulfate-reducing bacteria (corresponding to positions 385 to 402 in the 16S rDNA sequence of *E. coli*) was added to the prehybridization solution and incubated overnight at 50°C. The sequence of this probe has been described by Amann et al. (1). After hybridization, the membrane was washed for 30 min at 50°C first with a solution containing 2× SSC–0.1% (wt/vol) SDS and then
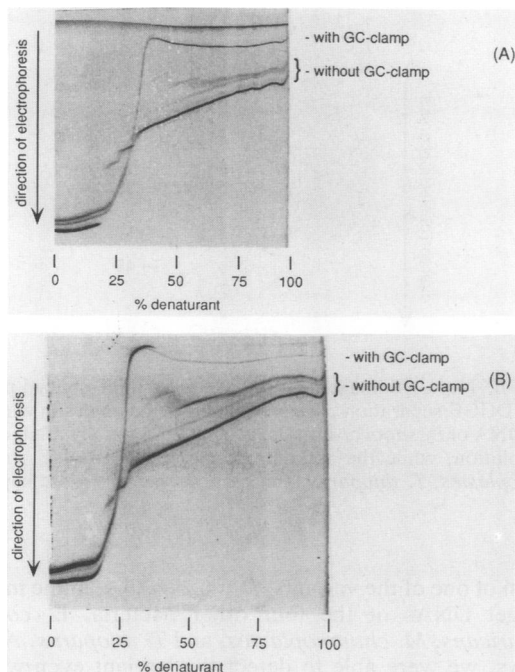
FIG. 2. Negative image of an ethidium bromide-stained perpendicular DGGE separation pattern of PCR-amplified 16S rDNA fragments from *E. coli* (A) and *D. desulfuricans* (B) obtained with primers 1 and 2 or 2 and 3, which introduce a 40-bp GC clamp at the 5' end of the fragments.

with one containing 0.1× SSC–0.1% (wt/vol) SDS. Subsequently, the membrane was sealed in a plastic bag and incubated with Kodak film at −70°C.

RESULTS

DGGE optimization. To determine the optimal DGGE conditions for characterizing microbial populations, we first analyzed the melting behavior of PCR-amplified 16S rDNA fragments from different species by perpendicular DGGE. Figure 2 shows the melting curves of PCR products from *E. coli* (Fig. 2A) and *D. desulfuricans* (Fig. 2B) obtained with primers 1 and 2 (193 bp) and 3 and 2 (233 bp). At 0% denaturant, two lines are observed as a result of the size differences of the DNA fragments caused by the GC clamp attached to one of the fragments. At a concentration of about 25% denaturant, both fragments display reduced mobilities because of the melting of the melting domain with the lowest melting temperature. Melting of the DNA fragment without the 40-bp GC clamp does not lead to the formation of stable, partially melted molecules but progresses quickly to result in the formation of two single strands, which differ in mobility. When using 30- and 40-bp GC clamps, we observed increased stability of transitional molecules only for fragments carrying the 40-bp clamp (data not shown). Increased stability was not observed with 16S rDNA fragments from both species when the clamp was incorporated into the 3' primer (data not shown). In subsequent experiments, we therefore used only primer 2 (carrying the 40-bp clamp) and primer 3. Figure 3 shows the results of a perpendicular DGGE of a mixture of *E. coli* and *D. desulfuricans* PCR-amplified 16S rDNA fragments. In accordance with our findings in separate experiments, the DNA fragment of *D. desulfuricans*
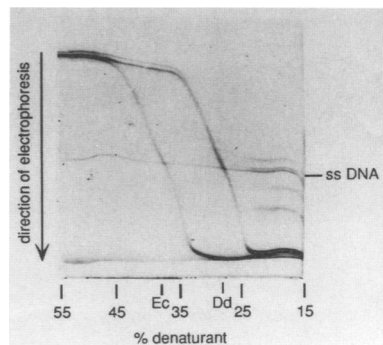

FIG. 3. Negative image of an ethidium bromide-stained perpendicular DGGE separation pattern of a mixture of PCR-amplified 16S rDNA fragments from *E. coli* and *D. desulfuricans*, obtained with primers 2 and 3 (with the 40-bp GC clamp). The transitions in mobility of DNA fragments derived from *D. desulfuricans* and those from *E. coli* are indicated by Dd and Ec, respectively. ss DNA, single-stranded DNA.

became partially melted at about 30% denaturant and showed a steep transition in mobility. At that denaturant concentration, the *E. coli* DNA fragment was still double stranded and did not slow down. It was melted at a higher denaturant concentration of approximately 40%. From the perpendicular gradient analysis, we defined a gradient of 15 to 55% as a starting point to resolve different sequence variants in parallel DGGE gels.

To determine the length of time of electrophoresis for the maximum resolution between two or more DNA fragments, a mixture of *E. coli* and *D. desulfuricans* fragments was applied, after constant time intervals, onto a parallel gel with a linearly increasing gradient of from 15 to 55% denaturant. Figure 4 shows the result of a mixture of *E. coli* and *D. desulfuricans* DNA fragments which were applied to the gel every 10 min for 3 h. For 30 min, both fragments migrated as a single band. After 40 min, the DNA fragment of *D. desulfuricans* was partially melted and was almost completely halted. As expected, the *E. coli* fragment migrated further into the gel and was halted at a higher concentration of denaturants. The denaturant concentration at which the fragments were halted is in good correspondence with the midpoint of inflection observed in perpendicular DGGE
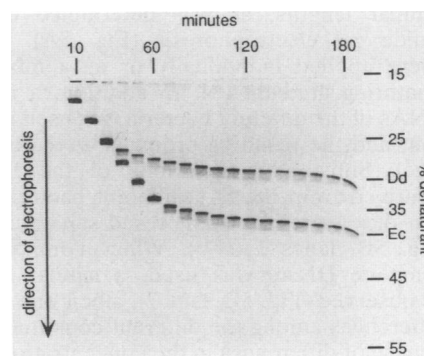

FIG. 4. Negative image of an ethidium bromide-stained parallel DGGE separation pattern of a mixture of DNA fragments of *D. desulfuricans* (Dd) and *E. coli* (Ec), obtained with primers 2 and 3, which were loaded every 10 min for a total of 3 h.
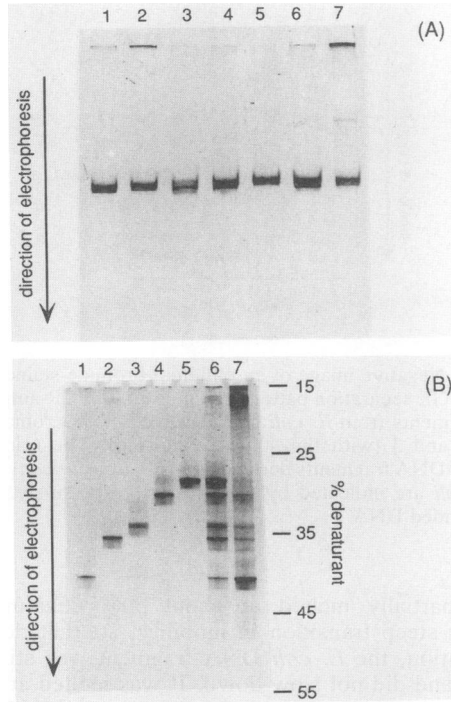
FIG. 5. Neutral polyacrylamide (A) and DGGE (B) analyses of 16S rDNA fragments of different eubacteria obtained after PCR amplification with primers 3 and 2. Both figures show negative images of ethidium bromide-stained separation patterns of *D. sapovorans* (lanes 1), *E. coli* (lanes 2), *M. chthonoplastes* (lanes 3), *T. thioparus* (lanes 4), *D. desulfuricans* (lanes 5), a mixture of these PCR products (lanes 6), and a sample obtained after enzymatic amplification of a mixture of the bacterial genomic DNAs (lanes 7).
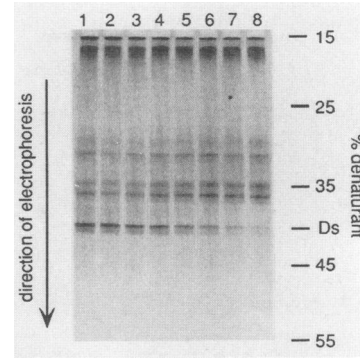

FIG. 6. Negative image of an ethidium bromide-stained perpendicular DGGE separation pattern of eight PCR samples in which the target DNA of *D. sapovorans* (Ds) was twofold serially diluted in the PCR solution, while the amounts of target DNAs of *E. coli*, *M. chthonoplastes*, *T. thioparus*, and *D. desulfuricans* were kept constant.

analysis of the two different 16S amplification products (Fig. 3). After 120 min, the maximum resolution between both DNA fragments was obtained. The fragments did not migrate much further in the gel, even after prolonged electrophoresis. We therefore choose 2.5 h of electrophoresis in a 15 to 55% gradient as the conditions for further experiments.

Analysis of different bacterial species. The conditions described above were then used to separate PCR-amplified rDNA fragments of four proteobacteria (23), i.e., *E. coli*, *D. desulfuricans*, *D. sapovorans*, and *T. thioparus*, and one cyanobacterium, viz., *M. chthonoplastes*. The fragments were of similar lengths, as was determined by neutral polyacrylamide gel electrophoresis (Fig. 5A). The PCR products were applied individually or as a mixture to a parallel denaturing gradient gel. In addition, a mixture of template DNAs of the different bacteria was used in the PCR amplification, and the resulting products were analyzed on the same gel. Substantial separation of the 16S rDNA fragments derived from the five different bacteria was observed when they were electrophoresed separately or as a mixture (Fig. 5B, lanes 1 to 6). When a mixture of the different template DNAs was used, a similar separation pattern was observed (Fig. 5B, lane 7), albeit one with some intensity differences among the different constituents of the mixture because of differences in the concentration of target DNA.

Sensitivity. In order to determine the sensitivity of this assay for detecting sequence variants of the 16S rDNA among complex mixtures of bacteria, we made a serial

dilution of one of the variants, *D. sapovorans*, in the mixture of target DNAs of the four other bacteria, *E. coli*, *D. desulfuricans*, *M. chthonoplastes*, and *T. thioparus*. As Fig. 6 shows, we were able to detect this variant even when it constituted only 1% of the population.

Analysis of microbial populations. We subsequently analyzed samples of complex microbial populations from different kinds of environments, including microbial mats from Wadden Sea sediment and bacterial biofilms obtained from wastewater treatment reactors. DGGE analysis of these microbial communities demonstrated the presence of many distinguishable bands in the separation pattern, most likely derived from as many different bacterial species constituting these populations (Fig. 7A). Although not clearly visible in this figure, similar banding patterns were found for microbial mat samples 1 and 2, which were taken from different depths of the same specimen. DGGE analysis of microbial mat sample 3 showed some bands apart from the bands common to samples 1 and 2 (Fig. 7A). The sample obtained from an aerobic biofilm showed at least 10 distinguishable bands (Fig. 7A, lane 4), while the sample from the anaerobic biofilm showed 8 intensely stained bands. Lane 6 of Fig. 7A shows the DGGE analysis of a mixture of PCR fragments of the five individual bacteria which was applied to the gel as a positive control for the hybridization analysis discussed below.

To analyze the microbial populations for the presence of specific bacteria, the DGGE separation patterns were stained with ethidium bromide, photographed, and subsequently hybridized, after being transferred to a nylon membrane, to an oligonucleotide probe specific for sulfate-reducing bacteria (1). A strong hybridization signal was found with the DNA fragment obtained from *D. desulfuricans*, a well-known sulfate-reducing bacterial species, but not with the DNA fragment obtained from *D. sapovorans* or with those from the other bacteria, i.e., *E. coli*, *M. chthonoplastes*, and *T. thioparus* (Fig. 7B, lane 6), indicating the specificity of the probe. A second, relatively weak hybridization signal was observed with this sample, but it could not be related to one of the ethidium bromide-stained bands in Fig. 7A (lane 6). We obtained no hybridization signal with the DNA fragments from microbial mat samples 1 and 2 (Fig. 7B, lanes 1 and 2) and only a faint signal with microbial mat sample 3 (Fig. 7B, lane 3). With the bacterial biofilm grown under
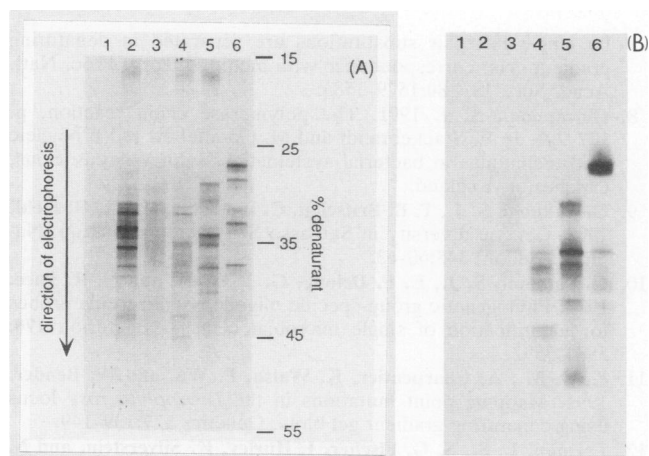
FIG. 7. DGGE analysis of 16S rDNA fragments obtained after enzymatic amplification of genomic DNA from uncharacterized microbial populations and individual bacteria. (A) A negative image of an ethidium bromide-stained parallel DGGE separation pattern of microbial mat samples 1 (lane 1), 2 (lane 2), and 3 (lane 3), a bacterial biofilm grown under aerobic conditions (lane 4), and a bacterial biofilm grown under anaerobic conditions (lane 5) is shown. Lane 6 contains the separation pattern of a mixture of PCR fragments of five individual bacteria, i.e., D. sapovorans, E. coli, M. chthonoplastes, T. thioparus, and D. desulfuricans. This mixture served as a positive control for the hybridization experiment. (B) The results after hybridization analysis of this DGGE separation pattern and hybridization with a oligonucleotide probe specific for sulfate-reducing bacteria are presented.

aerobic conditions, three fragments hybridized (Fig. 7B, lane 4), while at least five strong bands were observed for the bacterial biofilm grown under anaerobic conditions (Fig. 7B, lane 5).

DISCUSSION

Several morphological, biochemical, and genetic characteristics have been used to identify constituents in complex populations of microorganisms. The 16S rDNA sequence divergence of different bacterial species has been exploited as an indicator of diversity. To assess this diversity, PCR amplification of 16S rDNA (13) and cloning and sequence analysis of selected clones have been applied previously (9). Although informative, this approach has a drawback: only qualitative information about the population composition can be obtained, and that only after extensive analysis of large numbers of clones. Species which constitute a low percentage of the population are not readily detectable in this way.

The novel approach that we present here is based on DGGE analysis of 16S rDNA sequences obtained after enzymatic amplification of genomic DNA isolated from complex microbial populations. We optimized this system for the analysis of microbial populations by designing PCR primers and DGGE conditions that result in high-resolution banding patterns. For optimal DGGE separation, incorporation of a 40-bp GC-rich clamp in the 5' primer proved necessary for optimal resolution of the fragments in the denaturing gradient. The banding pattern provided a profile of the populations in that the relative intensity of each band and its position most likely represented the relative abundance of a particular species in the population.

DGGE as a technique for studying microbial diversity is superior to cloning and subsequent sequencing of PCR-amplified rDNA or ribosomal copy DNA fragments. First, it provides an immediate display of the constituents of a population in both a qualitative and a semiquantitative way, and second, it is less time-consuming and laborious. An additional disadvantage of the cloning procedure, viz., sequencing of different clones with the same inserted DNA fragment, is avoided. Restriction enzyme analysis of cloned PCR products before sequencing (8) can circumvent this problem, but this method is time-consuming and allows detection of only a small fraction of the sequence differences. The separation pattern obtained by DGGE analysis can be transferred to hybridization membranes and probed with species- or group-specific oligonucleotides in order to obtain information about the presence of a particular species or a number of different species belonging to a certain group, respectively. For further characterization of the bands observed, DNA fragments can be excised from the DGGE gel, reamplified, and sequenced directly, without the PCR product being cloned first. This makes it a rapid and efficient approach for the analysis of mixed microbial populations from natural environments.

The sensitivity of the detection of 16S rDNA sequence variants by this approach was demonstrated by the number and different intensities of the bands we observed in the DGGE profile of the different populations studied here. In a PCR analysis in which the template DNA of one species, D. sapovorans, was added in decreasing amounts to a mixture of template DNAs of the four other bacteria, we were able to distinguish a specific band in the mixture in which the target DNA of the species composed less than 1% of the total mixture. This indicates that species which are only a minority in the microbial populations can also be detected by this technique.

In the natural microbial populations which we analyzed, i.e., microbial mats and bacterial biofilms, we could discern at least between 5 and 10 different bands for each population, some of which were shared among the different populations. However, bands at identical positions in the DGGE gel are not necessarily derived from the same species. This problem can be addressed by exploiting a particular advantage of DGGE, i.e., using more narrow gradients to provide high-resolution DGGE profiles of particular parts of the original profile. When homoduplex molecules are analyzed on a DGGE gel, as they were here, up to 40% of all possible sequence variants which differ by only a single base pair will be detected (15, 18). If improved resolution is required, this percentage can be increased by performing heteroduplex analysis (12) and two-dimensional electrophoresis (6).

We have applied the DGGE profiling approach described here to addressing the biological problem of genetic diversity of microbial populations and to assessing the presence of sulfate-reducing bacteria in a bacterial biofilm grown under aerobic conditions. Although the sulfate-reducing bacteria are regarded as obligate anaerobes, hybridization signals were obtained with 16S rDNA fragments from this biofilm. This could mean that the probe is not specific only for sulfate-reducing bacteria, which has been suggested by Amann et al. (2), or that sulfate-reducing bacteria might indeed be present in anaerobic microniches in this bacterial biofilm. Sequence analysis of the separated DNA fragments would determine the phylogenetic positions of the inhabitants of the microbial population and would take away this uncertainty.

As DGGE analysis has a high sensitivity for detecting

sequence differences, we can exclude the possibility of the creation of chimeric genes in the course of the PCR (see also reference 9). No bands in addition to those in the separation pattern of a mixture of PCR products of the individual bacteria (Fig. 5B, lane 6) were observed after DGGE analysis of a sample obtained after enzymatic amplification of a mixture of bacterial genomic DNAs (Fig. 5B, lane 7).

The primers we have used in this study are specific for all eubacteria, but other primers can be designed in order to determine the genetic diversity among species from specific eubacterial groups, such as the sulfate-reducing bacteria, or species from other kingdoms, such as the eucaryotes and archaebacteria.

The DGGE profiling method can also be useful for diagnosing the presence and relative abundance of microorganisms, such as bacteria, yeasts, and fungi, in samples obtained from patients suffering from combined infections. It is therefore expected that this approach will contribute to our understanding of the genetic diversity of complex microbial populations.

ACKNOWLEDGMENTS

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